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Review Article

Imaging spinal cord activity in behaving animals

Nicholas A. Nelson[a,b,1], Xiang Wang[a,1], Daniela Cook[a,1], Erin M. Carey[a], Axel Nimmerjahn[a,*]

[a] Waitt Advanced Biophotonics Center, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA
[b] Biological Sciences Graduate Program, University of California, San Diego, La Jolla, CA 92037, USA

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ABSTRACT

The spinal cord is the primary neurological link between the brain and peripheral organs. How important it is in everyday life is apparent in patients with spinal cord injury or motoneuron disease, who have dramatically reduced musculoskeletal control or capacity to sense their environment. Despite its crucial role in sensory and motor processing little is known about the cellular and molecular signaling events that underlie spinal cord function under naturalistic conditions. While genetic, electrophysiological, pharmacological, and circuit tracing studies have revealed important roles for different molecularly defined neurons, these approaches insufficiently describe the moment-to-moment neuronal and non-neuronal activity patterns that underlie sensory-guided motor behaviors in health and disease. The recent development of imaging methods for real-time interrogation of cellular activity in the spinal cord of behaving mice has removed longstanding technical obstacles to spinal cord research and allowed new insight into how different cell types encode sensory information from mechanoreceptors and nociceptors in the skin. Here, we review the current state-of-the-art in interrogating cellular and microcircuit function in the spinal cord of behaving mammals and discuss current opportunities and technological challenges.

1. Introduction

Deciphering the relationship between animal behavior and cellular activity in the central nervous system (CNS) remains one of the major challenges in neuroscience. Traditionally, electrophysiological approaches have been used to sparsely sample from electrically excitable cells at millisecond-scale temporal resolution. This has led to the discovery of important behaviorally related phenomena such as place, grid, and head-direction cells in the brain and central pattern generator (CPG) neurons in the spinal cord (Grillner, 2006; Moser et al., 2017). While new electrophysiological probes now allow high-density recordings within and across brain regions (Steinmetz et al., 2018), these approaches are invasive, do not allow identification of molecularly defined cell types or their precise position, only sample from electrically active cells, and only over relatively short periods of time due to their sensitivity to tissue drift, glial scar formation, or probe degradation (Jorfi et al., 2015).

In contrast, optical imaging in combination with appropriate labeling techniques allows comprehensive sampling from dense networks of electrically excitable and non-excitable cells (genetically or otherwise defined such as by their connectivity pattern) and their cellular compartments (e.g., axonal boutons, dendritic spines, or astrocytic microdomains) with high spatial and temporal resolutions (up to sub-μm and milliseconds, respectively), over extended periods of time (up to months), and in the presence of considerable (μm-scale) tissue movement. Theoretically, any tissue region can be accessed and imaged through implantation of micro-optics. However, this approach can be more invasive than competing techniques and therefore requires careful consideration of its impact on microcircuit function and behavior. To date, due to the availability of high-performance calcium indicators, most imaging studies have focused on measuring intracellular changes in calcium ion concentration, a proxy of neuronal action potential firing. Calcium imaging has led to new insights into how defined neurons in cortical and deep brain regions of either head-restrained or freely moving animals encode, store, or modify different types of afferent or efferent information (Hamel et al., 2015) as well as the discovery of unanticipated forms of behaviorally related glial cell excitation (Bazargani and Attwell, 2016; Nimmerjahn, 2009; Nimmerjahn and Bergles, 2015).

Despite these advances in brain imaging, optical recordings from the spinal cord, the primary neurological link between the brain and peripheral organs, has for a long time been limited to anesthetized animals. This is in part due to the spinal cord's greater and more irregular tissue movement, which impairs optical measurements (Nimmerjahn et al.,

* Corresponding author.
E-mail address: animmerj@salk.edu (A. Nimmerjahn).
[1] Co-first authors.

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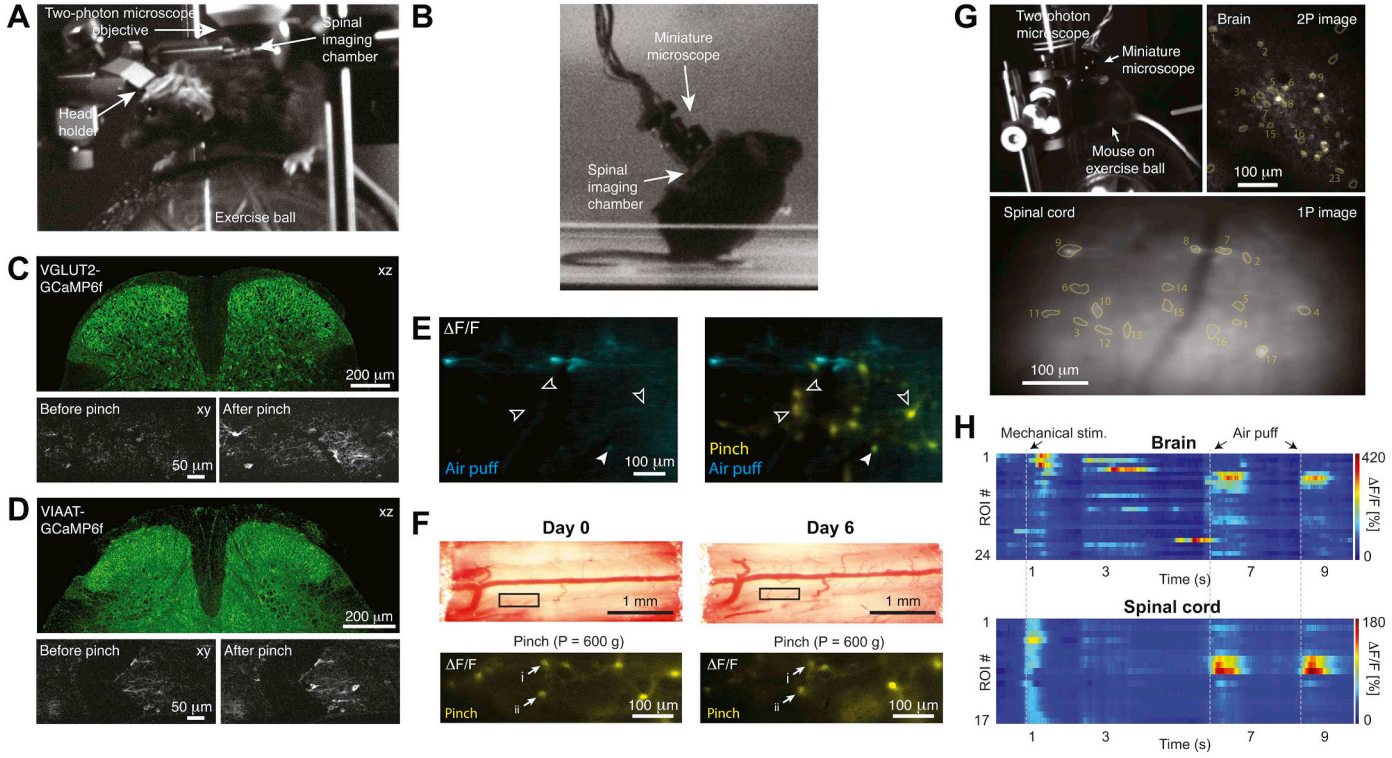


Fig. 1. Current approaches and opportunities. (**A**) Imaging in focally restrained animals with conventional microscopes offers tight control over sensory input and precise readout of motor responses. The image shows a head- and vertebra-restrained mouse on an exercise ball. Two-photon imaging during cutaneous (e.g., tail pinch) stimulation allows real-time readout of sensory- and motor-evoked cellular activity. (**B**) Imaging in unrestrained animals with wearable miniature microscopes allows use of well-validated sensory and motor tests that require a broad range of animal movement. The image shows a mouse with a 2.5-g miniature microscope on a linear track. (**C-D**) Use of transgenic mouse lines (and viral vectors) allows measurement of sensory-evoked calcium activity in genetically defined neurons. (**C**) Coronal spinal cord section showing GCaMP6-positive excitatory neurons in a Vglut2-Cre x Ai95D transgenic mouse (*top*). *Bottom*, two-photon images showing pinch-evoked calcium activity. (**D**) Coronal spinal cord section showing GCaMP6-positive inhibitory neurons in a Viaat-Cre x Ai95D transgenic mouse (*top*). *Bottom*, two-photon images showing pinch-evoked calcium activity. (**E**) Sensory-evoked calcium activity in AAV9-CaMKII-GCaMP6-transduced excitatory neurons in the spinal dorsal horn of an awake unrestrained mouse imaged with miniaturized one-photon microscopy. Air puff (blue) and pinch (yellow) applied to the animal's proximal tail activates partially overlapping cell ensembles (filled arrowhead). (**F**) Wearable miniature microscopes allow repeated measurement of sensory-evoked calcium spiking from the same neurons across days. *Top*, dorsal view of lumbar spinal cord blood vessels as seen through an implanted optical window 0 (left) and 6 days (right) after laminectomy. *Bottom*, responsive neurons in the black boxed region (two somata indicated). (**G-H**) Combined two- and miniaturized one-photon microscopy allows simultaneous readout of sensory-evoked activity in brain and spinal cord of behaving mice. (**G**) *Top left*, image of a head-restrained mouse on an exercise ball. *Top right*, two-photon image showing responsive GCaMP6-expressing neurons in primary somatosensory cortex. *Bottom*, one-photon image showing concomitantly active GCaMP6-expressing neurons in the spinal dorsal horn. (**H**) *Top*, calcium spiking in 24 regions of interest (ROIs) from cortical neurons in response to two cutaneous stimuli (pinch and air puff). *Bottom*, calcium spiking in 17 ROIs from spinal dorsal horn neurons in response to the same stimuli. ROIs are indicated in G by yellow outlines and numbers. Arrows and dashed vertical lines indicate type and onset of stimuli. (A-B) and (E-F) adapted from (Sekiguchi et al., 2016) with permission from Nature Publishing Group.

2009; Sekiguchi et al., 2016). Because anesthesia alters cellular activity and precludes animal behavior, key aspects of spinal cord sensory and motor processing remain poorly understood. New imaging approaches now allow stable measurement of cellular activity and molecular signaling events within spinal cord microcircuits in behaving mice (Cheng et al., 2016; Sekiguchi et al., 2016). In this review, we will take a look at current state-of-the-art imaging techniques for optically interrogating cellular activity in behaving mammals and discuss what the future may hold, with particular focus on current technological opportunities and challenges.

2. Approaches for high-resolution imaging in the spinal cord of behaving animals

To date, two complimentary approaches have demonstrated optical interrogation of cellular activity in the spinal cord of behaving mice (Fig. 1A-B) (Cheng et al., 2016; Sekiguchi et al., 2016). Both of these approaches have strengths and weaknesses (Table 1), and hence the biological question at hand determines which approach is better suited.

2.1. Imaging in focally restrained animals with conventional one- and multi-photon microscopes

If tight control over the animal's sensory environment, precise readout of its motor response, minimally invasive access to deep tissue regions, or multi-modal interrogation (e.g., combined imaging and electrophysiology, or cellular-resolution optical manipulation) is required the use of focal restraint has been the method of choice (Kerr and Nimmerjahn, 2012). In conjunction with conventional one- and multi-photon microscopes this approach offers the ability to precisely relate sensory stimuli, cellular activity, and animal behavior. In densely labeled tissue, where mixing of signals from neighboring neurons, neuropil, or out-of-focus illumination is a potential concern, multi-photon microscopes have an edge over their one-photon counterparts. They offer optical sectioning and large depth penetration (up to ~500 µm with current three-photon microscopes (Cheng et al., 2016)) (Fig. 2C), and facilitate concomitant readout of subcellular structure and function (i.e., visualization of the anatomical organization of information processing) in health and disease. However, optical sectioning can also be a disadvantage, particularly in tissues in which axial tissue movement

Table 1
Typical parameters of technologies for cellular-level activity imaging in behaving mammals.

Imaging in focally restrained animals with conventional microscopes

Technology	*Behavioral constraint*	*Lateral res.*	*Axial res.*	*Imaging depth in spinal cord*[a]	*Field of view (FOV)*[b]	*Frame rate*[c]	*Multiplex imaging capability*	*All-optical interrogation capability*	*Notes*	*Reference(s)*
One-photon microscopy	Stationary	0.4–2 μm	1–3 μm	? (≤80 μm*)	600–1000 μm (up to 3 mm)	5–50 fps[d]	Yes	Yes	–	[1-3] (not yet applied to spinal cord)
Two-photon microscopy (standard)	Stationary	0.3 μm	1 μm	150–200 μm	250–700 μm	1–30 fps	Yes	Yes	Sensitive to tissue movement	[4] (see also [5–11])
Two-photon microscopy (widefield)	Stationary	0.6–1.2 μm	4–16 μm	? (≤200 μm*)	3.5–5 mm (up to 10 mm)	< 1 fps[e]	Yes	Not yet demonstrated	Sensitive to tissue movement	[12–15] (not yet applied to spinal cord)
Three-photon microscopy	Stationary	0.8–1.0 μm	2.8–4.4 μm	Up to 500 μm	200–250 μm	8–8.5 fps	Yes	Not yet demonstrated	Sensitive to tissue movement	[16] (see also [17–19])

Imaging in unrestrained animals with wearable miniature microscopes

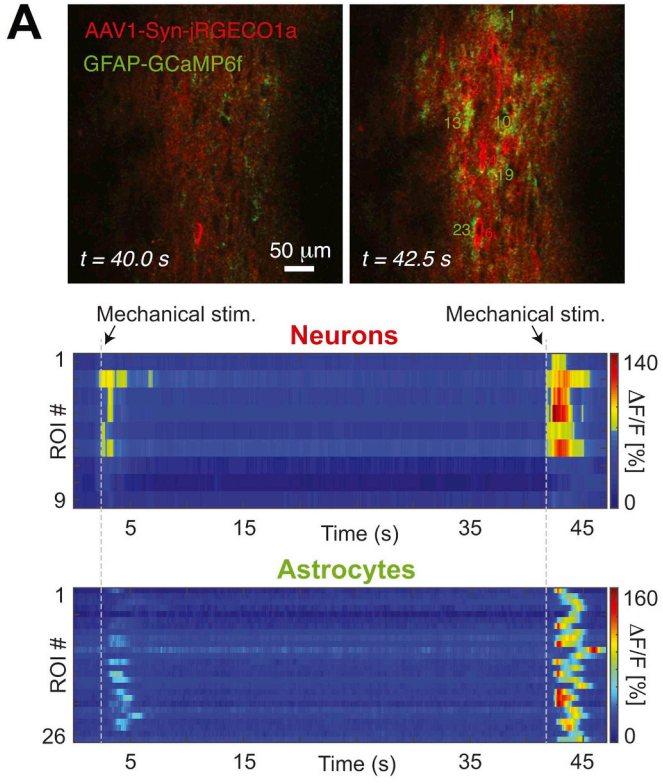
Technology	*Behavioral constraint*	*Lateral res.*	*Axial res.*	*Imaging depth in spinal cord*[a]	*Field of view (FOV)*[b]	*Frame rate*[c]	*Multiplex imaging capability*	*All-optical interrogation capability*	*Notes*	*Reference(s)*
One-photon microscopy	Tethered	2.5–6 μm	10–18 μm	60–80 μm	240–1100 μm	10–60 fps[f]	Yes (limited)	Yes (limited)	Weight: 1.1–3 g	[4] (see also [20–26])
	Untethered	? (2.5–6 μm*)	? (10–18 μm*)	? (≤80 μm*)	550–800 μm	20–30 fps	No	No	Weight: 4–5 g; battery rec. duration: 15–60 min	[27–28] (not yet applied to spinal cord)
One-photon microscopy (widefield)	Tethered	14 μm	? (≥18 μm*)	? (≤80 μm*)	4–7.8 mm	15–30 fps	No	No	Weight: 33 g	[29] (not yet applied to spinal cord)
Two-photon microscopy	Tethered	0.6-3 μm	3.4-10 μm	? (≤150 μm*)	≤300 μm	1-15 fps[g]	Yes	No	Weight: 2.2-5.5 grams[h]; sensitive to tissue movement	[30-36] (not yet applied to spinal cord)

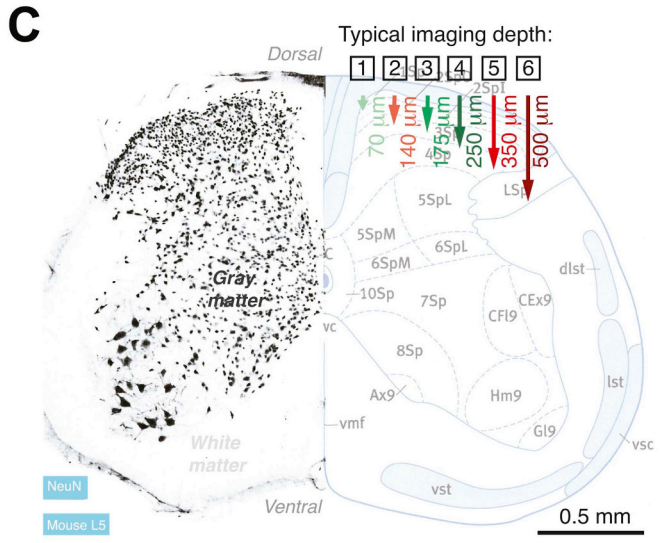
[1] (Prevedel et al., 2014), [2] (Ghitani et al., 2017), [3] (Bouchard et al., 2015), [4] (Sekiguchi et al., 2016), [5] (Johannssen and Helmchen, 2010), [6] (Laffray et al., 2011), [7] (Cartarozzi et al., 2018), [8] (Yoshihara et al., 2018), [9] (Nishida et al., 2014), [10] (Cirillo et al., 2015), [11] (Ran et al., 2016), [12] (Chen et al., 2016), [13] (Sofroniew et al., 2016), [14] (Stirman et al., 2016), [15] (Tsai et al., 2015), [16] (Cheng et al., 2016), [17] (Ouzounov et al., 2017), [18] (Guesmi et al., 2018), [19] (Horton et al., 2013), [20] (Flusberg et al., 2008), [21] (Ghosh et al., 2011), [22] (Cai et al., 2016), [23] (Jacob et al., 2018), [24] (Szabo et al., 2014), [25] (Zhang et al., 2019), [26] (Glas et al., 2019), [27] (Shuman et al., 2018), [28] (Liberti III et al., 2017), [29] (Scott et al., 2018), [30] (Sawinski et al., 2009), [31] (Engelbrecht et al., 2008), [32] (Göbel et al., 2004), [33] (Helmchen et al., 2001), [34] (Ozbay et al., 2018), [35] (Piyawattanametha et al., 2009), [36] (Zong et al., 2017), [37] (Skocek et al., 2018).

[a] Depends on thickness and reflective properties of overlying white matter.
[b] Depends on objective lens.
[c] Depends on image sensor/scanner, FOV, and binning/pixel resolution.
[d] Up to 40 fps volume rate with SCAPE [3], and up to 50 fps with light-field microscopy [1].
[e] Higher frame rates possible with smaller FOVs (e.g., 7 fps for a 1.8 mm FOV; [12]).
[f] Up to 16 fps volume rate with miniature light-field microscopy [37].
[g] Up to 40 fps with smaller FOVs [36].
[h] As low as 0.6 g for ultra-compact designs [31].
* Estimated value for the spinal cord.

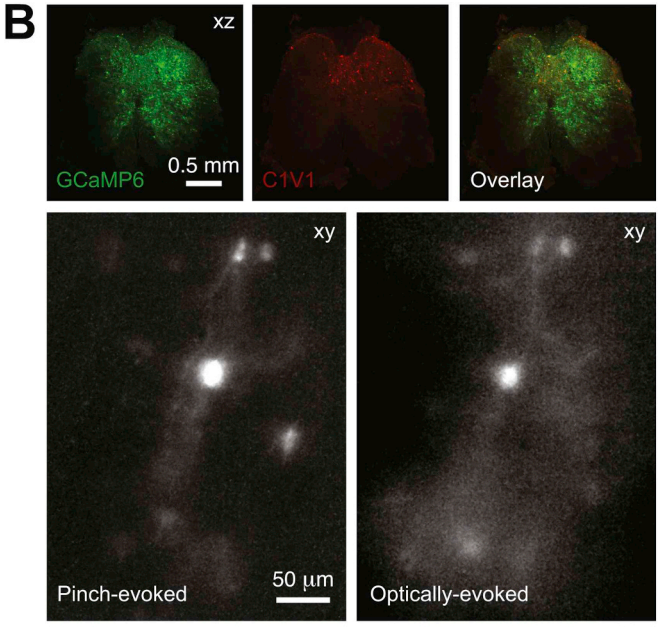
cannot be tightly controlled during image acquisition, though adaptive focus control offers one potential solution to this problem (Laffray et al., 2011).

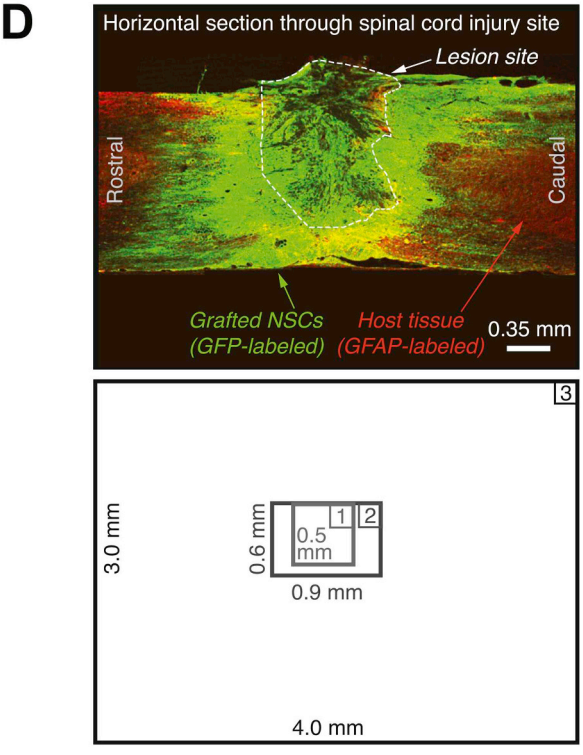
While surgical procedures for stable imaging in the brain of behaving animals are now well established and continue to provide significant contributions to our understanding of brain function, interrogating the spinal cord presents unique technical and behavioral challenges. The spinal cord exists within an anatomically dynamic region of the body, exacerbating the animals' ability to exert significant forces on the implanted optical window during restraint. The magnitude and frequency of such forces can be influenced by a variety of factors (e.g., gender, age, or strain of the animal). Additionally, recurrent forces (e.g., due to repeated restraint) can progressively destabilize the surgical preparation. As a result, imaging may be associated with considerable motion artifacts (up to tens of microns along the spinal cord (Sekiguchi et al., 2016)) that can be difficult to correct





1 One-photon imaging of GCaMP6 @ 480 nm
2 One-photon imaging of jRGECO1a @ 560 nm
3 Two-photon imaging of GCaMP6 @ 920 nm
4 Three-photon imaging of GCaMP6 @ 1,300 nm
5 Two-photon imaging of jRGECO1a @ 1,070 nm
6 Three-photon imaging of RCaMP1h @ 1,700 nm





Typical FOV, lateral resolution and frame rate:

1 0.5mm x 0.5mm, 0.3 µm, 1-30 fps (two-photon (2P) imaging)
2 0.6mm x 0.9mm, 2.5-6 µm, 30-45 fps (one-photon imaging)
3 3.0mm x 4.0mm, 0.6-1.2 µm, 0.1-1 fps (widefield 2P imaging)

(caption on next page)

Fig. 2. Select challenges. (**A**) Multiplex imaging in behaving animals promises to allow direct measurement of the cellular and molecular interactions that underlie tissue physiology and pathology. While multi-color imaging over a wide wavelength range (450–650 nm) remains challenging in unrestrained animals, multi-photon imaging is readily adaptable to different indicator combinations. *Top*, example two-photon fluorescence images from a time-lapse recording in the mouse lumbar dorsal horn, showing jRGECO1a-expressing neurons and GCaMP6-positive astrocytes. *Bottom*, calcium transients in 9 neuronal and 26 astrocyte regions of interest (ROIs) in response to two cutaneous pinch stimuli of different amplitude. Select ROIs are indicated. Arrows and dashed vertical lines indicate type and onset of stimuli. (**B**) The ability to perform all-optical interrogation in unrestrained animals promises to shed light onto how activity patterns in different cell types relate to one another and are causally linked to behavior. New miniature microscopes have the potential to perform simultaneous imaging and optogenetic manipulation. *Top*, coronal spinal cord section showing AAV-mediated co-expression of GCaMP6 (left) and the excitatory opsin C1V1 (center). The overlay is shown on the right. *Bottom*, pinch- (left) and optically evoked (right) calcium excitation in dorsal horn neurons. (**C**) Current imaging approaches provide optical access up to lamina V, as illustrated in this schematic. Implantation of micro-optics has been successfully used in the brain to extend imaging depth but remains unexplored in the spinal cord. (**D**) Standard two- and miniaturized one-photon microscopes permit high-speed, cellular-resolution activity measurements across modest FOVs in behaving animals. New widefield multi-photon microscopes now enable cellular-resolution recordings across millimeter-sized FOVs, potentially facilitating more comprehensive study of how sensory or motor information is normally encoded in the spinal cord at cellular and population levels. Additionally, they will facilitate study of disease conditions and treatment strategies, such as neural stem cell transplantation for improved functional recovery after spinal cord injury (top image). However, frame rates of these microscopes are currently low, complicating motion correction and signal extraction. (C) and (D) adapted from (Sengul et al., 2012) and (Lu et al., 2014) with permission from Academic Press and Elsevier, respectively.

post hoc. Measures to reduce motion artifacts may include the gradual habituation of the animal to recording conditions before an optical window implant is made, use of a silicone elastomer to dampen tissue movement (though care must be taken not to inhibit blood flow or cause cell death due to tissue compression), or renewal of the optical window when motion artifacts become too severe to efficiently correct offline. Another drawback of using focally restrained animals in sensorimotor research is that this approach limits the animal's behavioral repertoire and prevents use of established behavioral tests to assess sensory and motor function. Adapting the animal to the focally restrained environment can be difficult and time-consuming. Even after training, animals may fail to fully recapitulate real-world behavior. This is because freely moving animals use sensory and proprioceptive feedback loops to gauge their own movements and movement decisions with respect to their environment. The full activity of such feedback loops is difficult to replicate even with the most elaborate virtual reality setups (Ravassard et al., 2013; Rowland et al., 2011). Nevertheless, for somatosensory information accessible to focally restrained mice, cellular and molecular mechanisms can be studied effectively.

2.2. Imaging in unrestrained animals with wearable miniature microscopes

Miniaturized one- and two-photon microscopes allow imaging in unrestrained animals. These devices can be repeatedly utilized without significant adverse effects on animal behavior. One of the key advantages of wearable microscopes is that they allow for a broad range of animal movement and are therefore compatible with most of the behavioral assays and experimental designs that are already widely deployed and validated across neuroscience research institutions and in the pharmaceutical industry (e.g., sensory and motor assays such as the von Frey filament, Hargreaves, or kinematic weight bearing tests). While there is a trade-off in terms of experimental control over how the animal interacts with its environment, animals can make use of important sensory input, such as from the vestibular system. Miniature one-photon microscopes allow cellular-resolution imaging across large fields of view (FOVs) (typically $\geq 600 \times 900\,\mu m^2$) and with high frame rates (≥ 30 Hz at full resolution) (Cai et al., 2016; Flusberg et al., 2008; Ghosh et al., 2011; Jacob et al., 2018; Liberti III et al., 2017; Sekiguchi et al., 2016; Shuman et al., 2018; Szabo et al., 2014; Zhang et al., 2019). Their extended depth of field makes them less susceptible to axial tissue movement, though as mentioned above this can be a drawback for signal separation in densely labeled tissue. Depending on tissue labeling, imaging is generally restricted to superficial tissue regions (e.g., lamina I and II in the spinal cord, and layer 2/3 in the cortex). In contrast, wearable two-photon microscopes can offer larger optical depth penetration and higher lateral and axial resolutions (Engelbrecht et al., 2008; Göbel et al., 2004; Helmchen et al., 2001; Ozbay et al., 2018; Piyawattanametha et al., 2009; Sawinski et al., 2009; Zong et al., 2017). However, these devices tend to be heavier, have more rigid tethers, considerably smaller FOV ($\leq 300\,\mu m$), and lower frame rates (typically 1–15 fps, but up to 25–40 fps with smaller FOVs). The latter makes them less suitable for imaging in the spinal cord, which requires high frame rates in order to minimize artifacts due to rapid and non-uniform tissue movement during behavior.

Compared to tabletop microscopes, miniaturized devices provide lower optical depth penetration and spatial resolution. Additionally, given the short working distance of their miniature objectives, and the overall size and weight constraints dictated by what the animal can carry, the ability to perform multi-modal interrogations is limited. Nevertheless, miniature microscopes enable study of sensory and motor processes at cell, circuit, and system levels in ways not possible with tabletop microscopes.

3. Biological insights revealed by imaging spinal cord activity

3.1. Sensory evoked microcircuit activity under anesthetized conditions

Imaging experiments under anesthetized conditions have provided important insight into how dorsal horn neurons encode sensory information from mechanoreceptors and nociceptors in the skin. For example, using neonatal intraperitoneal injection of Cre-dependent, GCaMP3-expressing AAV8 into reporter mice, Vrontou et al. demonstrated that massage-like stroking of hairy skin, but not noxious punctate mechanical stimulation activates subsets of MRGPRB4-positive C fibers in spinal segments L1-L4, while pinching but not stroking activated subsets of MRGPRD-positive C fibers. Johannssen et al. showed that brief (~200 ms) mechanical stimulation (pinch) of the ipsilateral hindpaw evokes calcium transients in subsets (~10%) of dorsal horn neurons in L1, stained by multi-cell bolus loading of the synthetic cell permeant calcium indicator Oregon Green 488 BAPTA (OGB)-1 AM (Johannssen and Helmchen, 2010). Using in utero electroporation of CAG promoter-driven YC-Nano50 Nishida et al. demonstrated that pinch, brush, and heat activate partially overlapping subsets of dorsal horn neurons in L1 (Nishida et al., 2014), while multi-cell bolus loading of OGB-1 AM allowed Ran et al. to show that heat- and cold-responsive dorsal horn neurons in L4 encode absolute temperatures and relative temperature drops, respectively (Ran et al., 2016). Finally, Ikeda et al. showed that noxious stimuli (intraplantar capsaicin or formalin injection) evoke prolonged calcium increases in OGB-1 AM labeled lamina I neurons in L4-L5 (Ikeda et al., 2006).

3.2. Sensory evoked microcircuit activity under naturalistic conditions

A recent study demonstrated feasibility of obtaining repeated, cellular resolution optical measurements under awake conditions (Fig. 1A-B, E-F). Using AAV9-CaMKII-mediated expression of GCaMP6f in excitatory neurons of the mouse lumbar dorsal horn (spinal segments L4-L5), Sekiguchi et al. showed that distinct stimuli (pinch, air puff, and

grooming) applied to the same cutaneous region (proximal tail) activate overlapping ensembles of spinal lamina I/II neurons in unrestrained behaving mice (Fig. 1E). For a given stimulus (pinch), individual neurons were found to encode stimulus type, amplitude, and duration. Additionally, stimulus amplitude was encoded at the population level, with larger neuronal ensembles being activated by more intense stimuli. In the absence of sensory input calcium spiking was sparse and infrequent, and unlike in the cortex only modestly influenced by behavioral state (awake resting or running) (Fig. 1G-H). The potential effect of motor activity on sensory processing (Seki and Fetz, 2012; Seki et al., 2003) was not investigated, though.

Apart from neuronal spiking, cutaneous stimuli also evoked calcium transients in AAV5-GfaABC1D-GCaMP6f labeled dorsal horn astrocytes (Sekiguchi et al., 2016). Innocuous mechanical stimuli (tail pinch) led to calcium excitation primarily in astrocyte microdomains, the fine glial processes near neuronal synapses. Because miniature one-photon microscopes did not provide sufficient spatial resolution, these transients were measured with two-photon microscopy in focally restrained animals. Microdomain transients appeared largely unsynchronized in the two-dimensional optical sections, and their frequency but not amplitude or duration correlated with stimulus intensity. In contrast, noxious stimuli led to widespread (millimeter-wide) coordinated astrocyte excitation that involved somata, major processes, and microdomains. Calcium transients in a given astrocyte compartment were longer in duration and larger in amplitude under noxious compared to innocuous stimulus conditions, suggesting that astrocytes can show modulation of their calcium transient amplitude and duration in addition to their frequency. Notably, AAV9-CaMKII-GCaMP6f-transduced neurons showed a gradual rather than a step function-like transition in their response to innocuous and noxious stimuli, suggesting that distinct types of neural activity or sets of neurotransmitters may underlie the different forms of astrocyte excitation.

3.3. The impact of general anesthesia on microcircuit activity

Could similar results have been obtained under general anesthesia? To address this question, Sekiguchi et al. compared individual cells' spontaneous and sensory-evoked calcium transients under awake conditions with those in the same animal under anesthesia. Their findings indicate that isoflurane anesthesia, which is known to mediate antinociception (Kingery et al., 2002), increases the sensory response threshold of excitatory neurons and suppresses their spontaneous calcium spiking. Similarly, astrocytes showed reduced frequency of innocuous stimulus-evoked and spontaneous microdomain transients. Large-scale coordinated responses to noxious pinch were almost completely blocked (Sekiguchi et al., 2016). A recent study has corroborated this finding by showing that noxious pinch fails to evoke coordinated astrocyte excitation under ketamine-xylazine anesthesia, while astrocytes still responded to a noxious chemical stimulus (formalin injection) with coordinated large-scale excitation (Yoshihara et al., 2018). How similar or different this formalin-induced astrocyte response may look like under awake conditions remains to be determined. In summary, general anesthesia powerfully suppresses spontaneous, sensory and motor behavior evoked calcium activity in dorsal horn neurons and astrocytes, emphasizing the need for further studies in behaving animals.

4. Opportunities

4.1. Determining the role of molecularly defined neurons in spinal microcircuits

To date, most of our knowledge about the role of different molecularly defined cell types in processing of distinct types of information in different regions of the spinal cord mainly comes from genetic, electrophysiological, pharmacological, and circuit tracing studies. While extremely powerful, these approaches provide little information about the time varying activity patterns that underlie the moment-to-moment processing of sensory or motor information during behavior. Targeted cell ablation, for example, typically takes days and often extends beyond or fails to completely cover the target region (e.g., defined vertebral segments), leading to compensatory changes in microcircuit function and connectivity. Opto- and chemogenetic approaches allow rapid interrogation of microcircuit activity but can be associated with acute or chronic off-target effects (Nimmerjahn and Bergles, 2015; Otchy et al., 2015). Electrophysiological methods permit measurement of fast sub- and supra-threshold electrical activity from sensory and motor neurons in the spinal cord of behaving mice and monkeys (Confais et al., 2017; Hadzipasic et al., 2016; Seki and Fetz, 2012; Seki et al., 2003), but inherently miss the calcium-based excitation of non-neuronal cells. Activity-based histological markers, such as c-Fos, pERK or CaMPARI, do not permit readout of the rapid (millisecond to second), single-trial activity changes that control sensory-guided behaviors (Wang et al., 2019).

In contrast, optical imaging allows stable and repeated (up to months) activity measurements from dense networks of electrically and chemically excitable cells, including neurons and astrocytes, with high spatial and temporal resolutions. This ability to perform repeated measurements is particularly important for study of slow study, such as sensory-guided motor learning or disease progression, which benefits from longitudinal tracking and unique identification of cells. Yet, most imaging studies to date have used fluorescent labeling approaches to measure calcium spiking in relatively broad classes of dorsal horn neurons in the lumbar spinal cord and only over short periods of time. For example, Ikeda et al., Johannssen et al. and Ran et al. used multicell bolus loading of OGB-1 AM, which results in labeling of both neurons and astrocytes (Ikeda et al., 2006; Johannssen and Helmchen, 2010; Ran et al., 2016). Nishida et al. used in utero electroporation of CAG promoter-driven YC-Nano50, which labels a diverse subset of excitatory and inhibitory neurons (Nishida et al., 2014), and Sekiguchi et al. used AAV9-CaMKII-mediated expression of GCaMP6f, which labels a variety of excitatory dorsal horn neurons (Sekiguchi et al., 2016). One potential concern with recordings from broad classes of neurons is that integrating imaging data with previous genetic studies and circuit models, which are built around molecularly defined neurons, is difficult. Additional work is therefore needed to shed light onto how calcium activity in genetically defined neurons relates to activation of specific sensory modalities and sensorimotor integration. Many transgenic Cre driver mouse lines and viral vectors are now available to address this question (Daigle et al., 2018), though some of these still await thorough characterization in the spinal cord. Gene expression studies also continue to refine our knowledge about the true extent of excitatory and inhibitory neuron subtypes in different parts of the spinal cord (Bikoff et al., 2016; Gabitto et al., 2016; Häring et al., 2018; Molofsky et al., 2014; Sathyamurthy et al., 2018). Different colored calcium indicators (Akerboom et al., 2013; Inoue et al., 2019) in combination with transgenic and viral vector-based approaches allow multiplex measurement from distinct neuronal populations, though care must be taken to avoid indicator overexpression, imaging-induced phototoxicity, or viral vector-mediated inflammatory responses (Kohro et al., 2015; Ortinski et al., 2010; Steinmetz et al., 2017; Tufail et al., 2017). Likewise, while even single-color voltage imaging in live rodents remains challenging today (Bando et al., 2019), new genetically encoded sensors for neurotransmitters, -modulators, and -peptides (Feng et al., 2019; Marvin et al., 2018a; Marvin et al., 2018b; Patriarchi et al., 2018; Sun et al., 2018) are beginning to allow direct and high-resolution measurement of how neuromodulatory inputs, such as from noradrenergic, serotonergic, or dopaminergic projection fibers, influence local microcircuit activity. This provides an opportunity to elucidate the contribution that supraspinal pathways make to modulating cellular communication in the spinal cord under naturalistic conditions. While multiplex imaging is relatively straightforward to implement on

conventional microscopes, development of novel miniature one-photon microscopes with color-corrected optics will likely be necessary for multiplex interrogations over a wide wavelength range (450–650 nm) in unrestrained animals.

The ability to perform multimodal interrogations in the spinal cord of behaving animals will be particularly transformative for dissecting the cellular and molecular basis of disease states, and their dependence on biological variables such as gender or genotype. For example, a fine balance exists in the activity between different types of excitatory and inhibitory neurons in the spinal dorsal horn, and disruption of this excitation-inhibition balance has been implicated in pain and itch (Braz et al., 2014; Koch et al., 2018). Both multi-photon and miniaturized one-photon microscopy permit minimally invasive measurements from lamina I and II (Fig. 1C-F), the primary termination zone of nociceptive primary afferents (Koch et al., 2018; Todd, 2010). In combination with opto- or chemogenetic tools (Christensen et al., 2016), this allows direct interrogation of cellular activity patterns and molecular signaling pathways that underlie hyperalgesia or allodynia. Study of deeper regions up to lamina V to interrogate, for example, the activity of different types of neurons in the low-threshold mechanoreceptor recipient zone (Abraira and Ginty, 2013; Koch et al., 2018), can be achieved with multi-photon microscopy (Cheng et al., 2016; Sekiguchi et al., 2016). Importantly, both imaging modalities also permit long-term measurements in animal models of chronic human disease, such as in established chemical (e.g., formalin) or surgical assays (e.g., partial sciatic nerve ligation, spinal cord injury) of inflammatory or neuropathic pain, or mouse mutants for chronic itch. This promises to shed light onto how activity changes in molecularly defined neurons contribute to the initiation or maintenance of pathological states, as well as potential mechanistic differences between them. As mentioned above, wearable microscopes have the particular advantage of being compatible with well-validated sensory and motor tests. Combining virus-mediated, opto-, and chemogenetic manipulations with imaging therefore provides exciting opportunities for uncovering mechanistic relationships between the activity in defined subsets of neurons and behavior. Nevertheless, to develop a more complete understanding of complex phenomena such as pain, which includes sensory-discriminative and affective-emotional dimensions, concomitant monitoring of multiple CNS regions and signaling pathways may be necessary in the future. In principle, such measurements across functionally connected brain and spinal cord regions in behaving mice are already feasible using, for example, fiber photometry (Kim et al., 2016; Sych et al., 2019) or a combination of above-mentioned microscopy techniques (Fig. 1G-H). While the latter approach is restricted to fewer recording sites, the former is limited to bulk activity measurements.

4.2. Defining the role of neuron-astrocyte communication in spinal neural networks

Neuronal activity depends on bi-directional communication with non-neuronal cells which, amongst others, control metabolic supply, extracellular ion homeostasis, neurotransmitter concentrations, and synaptic plasticity. Given their close contact with neuronal synapses and blood vessels, and their ubiquitous expression of neurotransmitter receptors and transporters, astrocytes play particularly important roles in regulating neuronal excitability, plasticity and microcircuit function (Bazargani and Attwell, 2016; Nimmerjahn and Bergles, 2015; Oliveira et al., 2015). Yet, current circuit models of sensory and motor processing in the spinal cord tend to neglect neuron-astrocyte communication (e.g., its role in gain modulation) – in part due to our incomplete knowledge about astrocyte excitation, its spatiotemporal and molecular relationship to neuronal spiking, and the signaling cascades induced under naturalistic conditions. To date, two-photon imaging in the lumbar spinal cord of anesthetized and awake mice has shown that dorsal horn astrocytes, loaded with synthetic or genetically encoded calcium indicators, respond to innocuous sensory stimuli with localized microdomain transients (Cirillo et al., 2012; Sekiguchi et al., 2016). While the frequency of these transients appears to correlate with the levels of ongoing synaptic activity, how spinal astrocytes differentiate between, integrate, and modulate the activity in distinct neuronal populations, as has been shown in the brain (Deemyad et al., 2018; Mariotti et al., 2018; Martin et al., 2015; Matos et al., 2018; Perea et al., 2014), is largely unknown. Opto- or chemogenetic methods to perturb the activity in molecularly defined neurons, or knockdown of specific receptor pathways on astrocytes may help resolve these questions.

Noxious mechanical (pinch) and chemical stimuli (formalin) have been shown to evoke coordinated calcium activity in astrocytes across large (millimeter-scale) regions of the superficial dorsal horn, suggesting that this form of excitation depends on the activation of specific afferent inputs (e.g., C and Aδ fibers, which innervate superficial laminae and transmit nociceptive information). While acute mechanical stimuli (pinch) evoked brief phasic (around three seconds-long) astrocyte responses, formalin injection led to long-lasting (around five minutes-long) intracellular calcium elevation. The extent to which these large-scale events are mechanistically similar or different, and how they potentially regulate local excitatory or inhibitory activity in the spinal dorsal horn remains to be determined. Future studies will also need to determine how descending neuromodulatory projections influence neuron-astrocyte communication in the spinal cord. Norepinephrine, for example, has been shown to play major roles in large-scale and slow (around 10s-long) calcium excitation of cortical astrocytes in response to voluntary or forced locomotion (Ding et al., 2013; Paukert et al., 2014), though running did not evoke coordinated transients in dorsal horn astrocytes (Sekiguchi et al., 2016).

Despite these apparent differences between gray matter astrocytes in spinal cord and brain, one conserved function appears to be the modulation of neural circuit activity. For example, Shiratori-Hayashi et al. showed in two mouse models of chronic itch (atopic and contact dermatitis) that scratching-induced skin lesions coincide with upregulation of signal transducer and activator of transcription 3 (STAT3) in dorsal horn astrocytes (Shiratori-Hayashi et al., 2015). STAT3-dependent release of lipocalin-2 (LCN2) by reactive astrocytes was shown to sensitize an itch processing network that involved gastrin-releasing peptide receptor (GRPR)-expressing dorsal horn neurons. Inhibition of astrocytic STAT3 prevented excessive scratching and worsening skin lesions. However, whether TRPV1-positive C fiber activation in these models evokes coordinated calcium excitation in dorsal horn astrocytes, and conversely whether large-scale calcium transients in above-mentioned pain models involves release of LCN2 remains unknown. Nevertheless, what seems clear is that astrocytes in the spinal cord can either release neuroactive substances, such as ATP, or modulate their transporter expression or activity in a calcium-dependent manner in response to neurotransmitters and neuropeptides (Bardoni et al., 2010; Carlsen and Perrier, 2014; Christensen et al., 2018; Cirillo et al., 2015; Werry et al., 2006), and this can have a behavioral effect. Inhibition of calcium-dependent release of ATP from astrocytes in dnSNARE mice, for example, has been shown to reduce acute but not chronic mechanical pain thresholds (Foley et al., 2011). Optogenetic activation of astrocytes induced pain hypersensitivity in a reversible and time-dependent manner, potentially through ATP-mediated disinhibition of spinal projection neurons (Nam et al., 2016). Release of cytokines and chemokines from astrocytes (e.g., through Cx43 hemichannels), as well as other astrocyte-derived signals such as thrombospondins, has been shown to contribute to the initiation or maintenance of chronic pain (Chen et al., 2014; Chen et al., 2012; Ji et al., 2016; Kim et al., 2012; Kronschläger et al., 2016; Liu et al., 2016). Likewise, in the ventral spinal cord, astrocytes have been shown to functionally interact with specific motoneurons, and their dysregulation can affect motoneuron survival, function, and circuit organization (Bruijn et al., 1997; Kelley et al., 2018; Molofsky et al., 2014). Again, the potential role of astrocyte calcium signaling in this process remains to be elucidated. Work in the brainstem suggests that astrocytes can influence neuronal rhythm-

ogenesis through calcium-dependent release of soluble factors, such as ATP and S100β (Gourine et al., 2010; Morquette et al., 2015). Similar signaling pathways, either alone or in conjunction with neuromodulator (e.g., dopamine) signaling, may regulate central pattern generator (CPG) function in the spinal cord (Acton et al., 2018; Carlsen and Perrier, 2014).

In summary, while it is clear that neuron-astrocyte communication plays important roles in regulating spinal microcircuits, the activity patterns and signaling pathways that underlie this bi-directional communication remain incompletely understood. High-resolution, multiplex imaging in behaving animals in combination with cell type-specific genetic manipulations provides an opportunity to fill this knowledge gap (Fig. 2A). In particular, it may help uncover how astrocytes integrate or discriminate between the different afferent, local, and descending inputs. Additionally, imaging in established animal models of disease promises to reveal new non-neuronal targets for the development of novel drugs, or the effects of existing treatments on aberrant activity (e.g., the acute and chronic effects of opioids on glial and neuronal network excitation) (Drdla et al., 2009; Grace et al., 2014; Ji et al., 2016). While animal models may never offer a perfect mirror of the complex manifestations seen in humans, the ability to home directly in on affected cellular circuits and monitor their activity live and longitudinally will likely prove invaluable for the development of more effective treatments that get to the root of a particular sensory or motor disorder.

5. Challenges

5.1. Technological challenges

The development of imaging methods for real-time measurement of defined dorsal horn cell populations in behaving mice has removed major obstacles in the study of spinal sensory processing under naturalistic conditions. At the same time, several technical hurdles remain that hamper deeper understanding of important aspects of normal and aberrant spinal cord function. One such impediment is the presently limited ability to perform multiplex imaging or all-optical interrogation in unrestrained animals, which would aid in elucidating how activity patterns in different cell types relate to one another and are causally linked to behavior. Current miniaturized microscopes that use high-numerical aperture (NA) gradient index (GRIN) lenses typically offer color correction over only a narrow spectral range (tens rather than hundreds of nm) and suffer from inherent on- and off-axis aberrations. Use of commercial conventional lenses can overcome some of these issues while keeping hardware costs low, but this typically restricts imaging to surface tissues given the size of corresponding objective lenses. Some custom and commercially available miniature microscopes based on either GRIN or conventional lenses now allow two-channel imaging and the potential to perform simultaneous optogenetic stimulation with or without electronic focusing (Fig. 2B) (Owen et al., 2018; Stamatakis et al., 2018). However, to make full use of the available color palette of fluorescent indicators, including new near-infrared calcium indicators for deeper imaging (Qian et al., 2019), and to further improve image resolution, sharpness, and contrast, development of custom-designed miniature compound optics will be necessary. Custom optics will also allow implementation of longer working distance (WD) objectives that facilitate combined optical and electrical recordings (e.g., to relate optical to electrical spiking activity), or imaging through implanted optics, such as microprisms (Andermann et al., 2013). Cellular-resolution voltage imaging in behaving mammals will likely remain challenging for a while. Apart from the necessary improvements of the current generation of voltage sensors, considerable efforts in hardware development will be required (e.g., integration of faster and more sensitive image sensors). Compared to calcium measurements, voltage imaging would provide more precise estimation of spike timing and number, particularly from fast-spiking cells.

First-generation hardware solutions for wire-free imaging in unrestrained animals already exist (Liberti III et al., 2017; Shuman et al., 2018). These battery powered devices save data either directly to a removable memory card on the microscope or transmit the data to a wireless receiver. The capacity of the small batteries currently limits recording duration to approximately 15–60 min. While solutions for wireless power transmission also exist, they are not yet suited for integration into miniature microscopes (e.g., due to weight, power density, or heat dissipation concerns). Wire-free microscopes are heavier than their wired counterparts (about twice as heavy or 4–5 g total weight). Nevertheless, they can allow for more naturalistic behavior, improved behavioral performance, and use of larger test arenas.

Another technical challenge involves functional imaging in deep, particularly ventral spinal cord regions of behaving mice (Fig. 2C). Spinal gray matter is surrounded by thick and dense layers of myelinated axons. Myelin is highly reflective over a broad wavelength range (Kwon et al., 2017; Schain et al., 2014; Yaroslavsky et al., 2002). As a result, imaging of green fluorescent calcium indicator-labeled cells is limited to laminae I and II (or around 60–80 μm depth) with miniaturized one-photon microscopes (Sekiguchi et al., 2016), up to lamina IV with two-photon microscopes (or 150–200 μm depth) (Johannssen and Helmchen, 2010), and up to lamina V with three-photon microscopes (or ~500 μm depth from the dorsal surface of the spinal cord) (Cheng et al., 2016). However, because three-photon microscopy typically relies on low repetition rate lasers with currently limited output power, image frame rates tend to be low, making post-acquisition correction of motion artifacts difficult particularly at greater tissue depths where frame averaging is needed to achieve sufficient signal-to-noise ratio (Guesmi et al., 2018; Ouzounov et al., 2017; Weisenburger et al., 2019). Adaptive focus control and ratiometric imaging can be used with some efficacy to reduce axial movement artifacts (Johannssen and Helmchen, 2010; Laffray et al., 2011). However, behavior-induced within-frame distortions due to tissue warping remain a concern for spinal cord imaging (and electrophysiology). In particular, animals implanted with a dorsal optical window tend to show different degrees of tissue displacement in lateral compared to central vein areas during behavior, leading to non-linear distortions within a given imaging field.

Imaging depth may be extended using red-shifted calcium indicators (Dana et al., 2016; Qian et al., 2019). Nevertheless, this approach is unlikely going to provide access to all ventral spinal cord regions (Fig. 2C). An approach that has recently demonstrated acute imaging of motoneurons and surrounding glia in anesthetized mice involved the use of a ventrolateral laminectomy (Cartarozzi et al., 2018). This approach has opened the door to imaging of ventral spinal cord circuits, but in its current form seems incompatible with imaging in behaving animals as it required fixing the animal in an angled support, removing the dura mater, and displacing the ventral and dorsal roots. Additionally, imaging approaches based on intracellular changes in calcium ion concentration, while suited to determine behavior-dependent recruitment of molecularly defined neurons, may be too slow for capturing the millisecond dynamics of sensorimotor signaling during movement (Confais et al., 2017; Hadzipasic et al., 2016).

Implantation of micro-optics, such as GRIN lenses or microprisms, is another option. This approach has been used successfully in the brain of behaving mice and enabled weeks- to months-long neuronal calcium activity measurements in regions as deep as the basolateral amygdala and lateral hypothalamus (Hamel et al., 2015). In principle, this approach should be applicable to the spinal cord. If successful, it could enable measurement of how rhythmic and non-rhythmic motor movements engage different classes of spinal neurons (e.g., V0, V1, V2a, V2b, V3 premotor interneurons), how their activity relates to muscle activation and kinematic measures such as speed in quantitative and standardized motor assays (e.g., treadmill running, hindlimb reflex), and what the phase relationship is between the different types of neurons. However, given the narrow lateral extent of the spinal gray

matter, particularly in mice, even the smallest-diameter GRIN lenses (with equally small FOV) would be expected to cause significant disruption of sensorimotor circuitry. In contrast, implantation of reflective glass microprisms, which do not require en face tissue compression or removal, can be less invasive while allowing high-speed imaging across laminae (Andermann et al., 2013). Nevertheless, given the comparatively large and non-uniform movements of the spinal cord during animal behavior, the feasibility of this approach and its impact on spinal microcircuits remains to be determined. Use of micro-optics and coherent fiber bundles may also be useful in gaining access to sensory and motor circuits in the cervical spinal cord, which due to its short-length, small-diameter vertebrae and large neck muscles makes spinal plate implantation and access by large-diameter optics difficult.

Another important challenge involves the FOV of typical two- and miniaturized one-photon microscopes (Fig. 2D). Behavior involves activation of spinal sensory or motor circuits of considerable (millimeter-scale) extent. For example, afferent fibers of the sciatic nerve, which innervate most of the leg's skin, transmit sensory information to dorsal horn neurons located between spinal segments L4-S3, and corresponding efferent fibers transmit ventral motor neuron signals to flexor and extensor muscles in the leg. Conventional microscopes offer FOVs of typically 250–700 μm in diameter. This corresponds to only a fraction of the length of one spinal segment (e.g., in mice the rostra-caudal extent of cervical and lumbar spinal vertebrae is 0.6–1 mm and 1.2–1.5 mm, respectively) (Harrison et al., 2013). While sequential measurements from different regions along the spinal cord are certainly feasible in order to map out the activity patterns across the network, achieving repeatable population responses across trials can be more challenging. In the brain, cellular-resolution two-photon imaging across an ultra-large (up to 10 mm) FOV has recently been demonstrated (Chen et al., 2016; Sofroniew et al., 2016; Stirman et al., 2016; Tsai et al., 2015). This approach should be readily applicable to the spinal cord, but at present suffers from relatively low frame rates (< 1 Hz for the largest FOV and highest resolution). Higher frame and volume rates can be achieved with one-photon imaging. A recently developed head-mounted widefield macroscope, for example, has enabled video-rate imaging across millimeter-sized FOVs ($7.8 \times 4\,mm^2$) (Scott et al., 2018). Lateral resolution was limited to 14 μm and, due to the microscope's considerable weight (33 g), imaging had to be performed in rats. In contrast, light-field microscopy permits high-speed volumetric imaging in unrestrained mice and has been shown to allow measurement of neuronal calcium spiking within a $700 \times 600 \times 360\,\mu m^3$ volume at 16 Hz in the brain (Skocek et al., 2018). At present, this technique requires extensive postprocessing, preventing real-time feedback during experiments, and is limited to around 6 μm lateral and 15 μm axial resolution. The latter complicates signal separation in densely labeled tissue, but sparse and spatially localized labeling (e.g., using H2B-driven nuclear expression) can alleviate this issue.

In summary, further technological innovations are needed to enable more comprehensive measurement of cellular activity in the spinal cord of behaving mammals. Such dense functional recordings, in combination with genetic, pharmacological and anatomical methods, have the potential to provide a detailed mechanistic description of the cellular basis of behavior, complementing studies in lower model organisms, such as larval zebrafish (Vanwalleghem et al., 2018), which due to its optical transparency has allowed study of spinal mechanosensory circuits not or only partially accessible in mammals (Fetcho and O'Malley, 1995; Knafo et al., 2017; Miyazawa et al., 2018; Warp et al., 2012). Corresponding findings will therefore be important in guiding similar studies in higher model organisms. Nevertheless, important physiological differences exist between spinal circuits in developing zebrafish and adult mammals (e.g., with regard to spinal cord regeneration after injury (Vajn et al., 2013)). Exactly how findings from these different model organisms relate to one another remains to be determined.

5.2. Data analysis challenges

A prerequisite for being able to decipher the relationship between animal behavior and cellular activity is that individual cells can be identified and registered through space and time post-acquisition. While standard software can track the same cells from frame to frame when they stay nearly fixed within an image plane or volume, this becomes more complicated when animal movements cause nonlinear deformations between neighboring cells, or when cells show significant movement from volume to volume (e.g., in long-term recordings from injured tissue; Fig. 2D). Manual annotation or semi-automated tracking followed by manual proofreading represents a possible solution to this issue. However, this approach is laborious and can introduce a severe bottleneck in the data analysis pipeline. New algorithms allow non-rigid motion correction of both one- and two-photon image data (Lu et al., 2018; Pachitariu et al., 2016), including machine learning approaches to teach a computer to recognize cells on the basis of the local constellation of neighboring cells (Nguyen et al., 2017). Nevertheless, performance of these algorithms across data sets remains variable and depends on proper pre-processing of the user-dependent image data, particularly for one-photon recordings.

Following motion correction, the next challenge involves signal extraction. Several open-access software packages allow semi-automated detection of neuronal spikes in one- and multi-photon calcium imaging data (Pnevmatikakis, 2018; Stringer and Pachitariu, 2019). While most of these algorithms involve extensive offline processing, a few permit real-time spike estimation (Giovannucci et al., 2017; Zhang et al., 2018b). The latter will be particularly helpful in implementing closed-loop interrogation of spinal circuits during behavior (e.g., in animals with spinal cord injury or chronic pain). Fewer options exist for efficient and unbiased quantification of astrocyte calcium transients (Agarwal et al., 2017; Srinivasan et al., 2015; Wang et al., 2018). Additionally, given the quite distinct spatiotemporal dynamics of calcium transients in astrocytes and neurons, software that performs well on both, or allows their parallel processing, is currently lacking. Similar considerations apply to multiplex recordings that involve neurotransmitter and -modulator indicator signals.

Even with fully extracted cellular dynamics, understanding how calcium signals relate to animal behavior, particularly in freely moving animals, poses unique challenges. Every trial or experiment can generate unique cellular and behavioral activities. Stochastic changes in behavioral state or experimental conditions can create inherent variability across datasets, complicating extraction of general cellular mechanisms. Additionally, outwardly identical behavioral states can differ in internal state (e.g., neuromodulator signaling), potentially leading to differences in how animals or local microcircuits respond to sensory input. One approach to deal with this intrinsic variability is to simply acquire so much data that similar trials can be clustered and compared. An alternative approach is to turn to computational modeling (e.g., of hindlimb circuit function and organization) and machine learning (e.g., for animal pose estimation) (Arac et al., 2019; Mathis et al., 2018; Pereira et al., 2019).

In summary, the increased ability to perform multiplex measurements in behaving mice calls for new integrated software tools that allows efficient extraction and correlation of signals from different signal sources.

6. Outlook and conclusions

Novel imaging approaches, in combination with new labeling, genetic and circuit tracing techniques, now for the first time enable direct measurement of how sensory modalities and motor programs are encoded within molecularly defined cell types under naturalistic conditions. This provides unique opportunities to broaden our knowledge about the computational logic of spinal cord circuits at molecular, cellular, and circuit levels. Their application to animal models of

human disease promises to uncover cellular and molecular mechanisms that underlie onset or progression of pathophysiological changes, such as those responsible for the transition from acute to chronic pain. Identification of such mechanisms will aid in the development of new or improved treatment strategies. Additionally, imaging can be used to assess the efficacy of pharmacological (e.g., opioids, gabapentinoids) and non-pharmacological treatments (e.g., physical therapy, neurostimulation), both short- and long-term, as a function of different biological variables (e.g., genotype, gender, age) and diverse medical conditions (e.g., spinal cord injury, chronic pain). While several technological challenges still remain, the opportunities that lie ahead are truly exciting!

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Author contributions

A.N. wrote the manuscript and prepared the figures and table. N.N. and X.W. contributed to layout and content. D.C. provided images for Figs. 1-2, and X.W. for Fig. 1. All authors reviewed and edited the manuscript.

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