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Review Article

Imaging *in vivo* dynamics of sensory axon responses to CNS injury

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ABSTRACT

Axons in the adult mammalian brain and spinal cord fail to regenerate upon lesion. *In vivo* imaging serves as a tool to investigate the immediate response of axons to injury and how the same injured axons behave over time. Here, we describe the dynamic changes that injured sensory axons undergo and methods of imaging them *in vivo*. First, we explain how sensory axons in the dorsal column of the adult mouse spinal cord respond to axotomy. Then, we highlight practical considerations for implementing two-photon based *in vivo* imaging of these axons. Finally, we describe future directions for this technique, including the possibility of *in vivo* imaging of subcellular dynamics within the axon.

1. Introduction

Axons in the adult mammalian central nervous system (CNS) usually do not regenerate after injury (Hilton and Bradke, 2017). Consequently, functional deficits persist in individuals after spinal cord injury (SCI). Worldwide, around three million people have sustained SCIs with an incidence rate of around 180,000 per year (Lee et al., 2014). Improving the lives of individuals with SCI has motivated scientists to understand why adult CNS axons fail to regrow and to search for neuroregenerative strategies. Regeneration in the adult CNS fails due to two reasons: first, the intrinsic growth capacity of the adult neuron is diminished (Curcio and Bradke, 2018). Second, inhibitory factors are expressed in the adult CNS, including those associated with myelin and the extracellular matrix (Bradbury et al., 2002; Schwab and Strittmatter, 2014).

In vivo imaging has emerged as an essential technique to understand the biology of axon regeneration failure and the potential effects of treatments. One major advantage of this technique compared to histological analysis is the possibility to follow injured axons at multiple time points. Therefore, the danger to misinterpret spared axons for regenerating ones or *vice versa* is reduced (Laskowski and Bradke, 2013; Steward and Willenberg, 2017). In the past, wide-field and two-photon microscopy and acute as well as chronic preparations of mice have been used to image injured axons in the spinal cord (Davalos et al., 2008; Erturk et al., 2007; Farrar et al., 2012).

Here, we review the methodology of *in vivo* imaging axons in the adult mouse spinal cord, with a focus on dorsal column sensory axons (Fig. 1A). We discuss recent findings gleaned from this model system that demonstrate the dynamic nature of degeneration and regeneration following axotomy. We describe different injury models used and their suitability for two-photon *in vivo* imaging. Moreover, we report our current techniques used to image axon dynamics after injury. Then, we review pitfalls and problems encountered during two-photon imaging of axons in the CNS and suggest solutions. In the end, we highlight future opportunities in the *in vivo* imaging field.

2. Anatomy of dorsal column sensory axons and DRG

Most studies that conduct *in vivo* imaging of axons in the adult mouse spinal cord focus on dorsal column sensory axons. These axons are located superficially, within the first 300 µm, and are the most optically accessible axonal pathway. Dorsal column sensory axons originate from neurons in the dorsal root ganglia (DRG) lateral to the spinal cord (Watson and Kayalioglu, 2009). DRG or primary sensory neurons are pseudo-unipolar in morphology, with each neuron projecting a single axon that bifurcates close to the cell body, sending one axonal process peripherally and the other centrally (Willis and Coggeshall, 2004). The two main populations of DRG dorsal column sensory neurons are mechanosensory and proprioceptive in function (Niu et al., 2013). We and other researchers focus on the axons emanating from neurons in the lumbar DRG L3–5, which are relatively large and project their peripheral axons *via* the sciatic nerve in the mouse (Rigaud et al., 2008). The surgical accessibility of the sciatic nerve facilitates tracing these axons by application of a trans-ganglionic tracer in the sciatic nerve.

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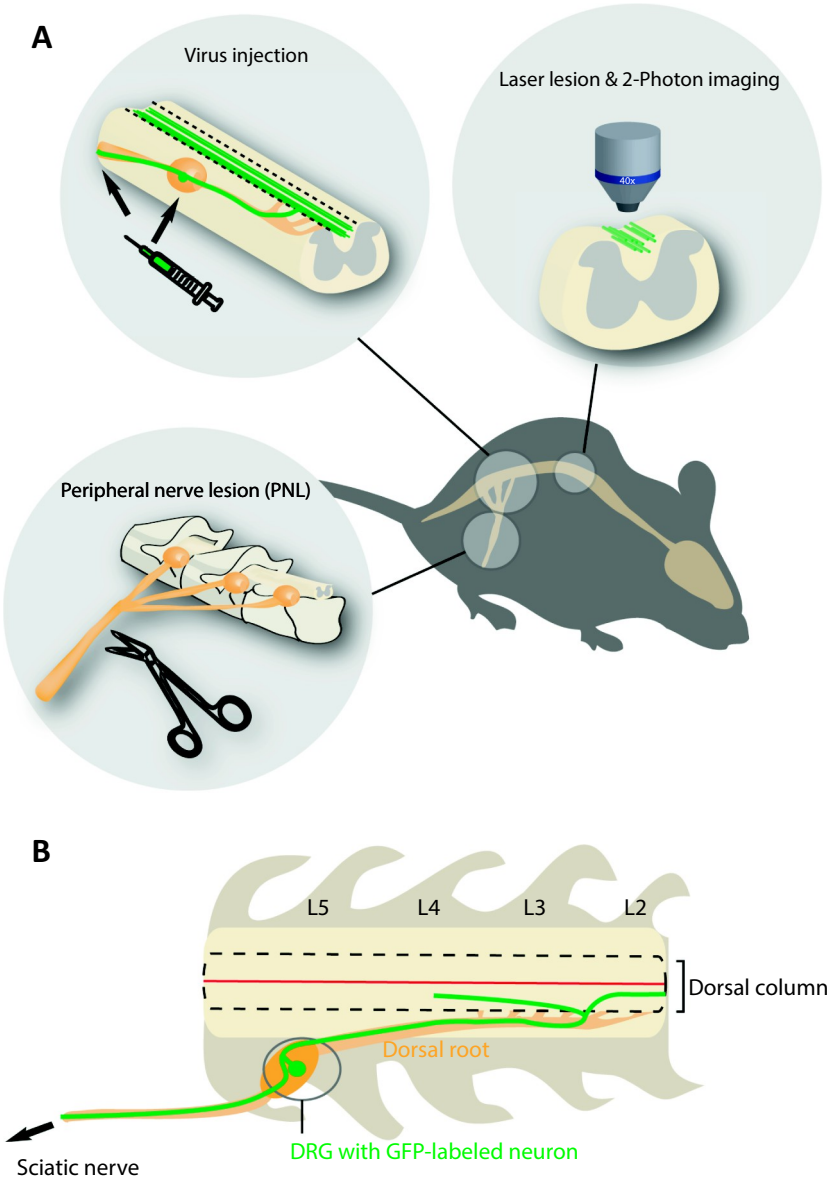

Fig. 1. Imaging DRG sensory axons *in vivo* and their anatomy. (A) Central axons of sensory neurons of the dorsal root ganglia (DRG) are a widely used model for *in vivo* imaging. They are simple to trace by virus injection, set into a pro-regenerative mode by peripheral axotomy and dorsally positioned in the spinal cord for convenient two-photon imaging. (B) Anatomy of lumbar DRG neurons projecting peripherally *via* the sciatic nerve and centrally into the dorsal column. DRG (orange) neuron somata are located lateral to the spinal cord within the DRG. Mechanosensory and proprioceptive neurons of the lumbar DRG 3–5 send their peripheral axon into the sciatic nerve and their central branch into the spinal cord. After entering the spinal cord, they branch with one ascending and one descending branch within the dorsal column (dashed box). Within the spinal cord they project parallel to the dorsal vein (red). Here we illustrate a representative neuron in L5 DRG with its central and peripheral branch (green).

3. The response of sensory axons to injury

Injury to sensory axons in the spinal cord triggers a dynamic response. In this regard, *in vivo* imaging is crucial to understand the time frame of events and when and how interventions can alter this response. In general, the axonal response to injury can be divided into acute, subacute and chronic phases. These phases have been described by *in vivo* imaging studies combined with histological analyses.

3.1. Acute (0 to 24 h post-injury)

The rupturing of the axon's membrane immediately triggers an acute intrinsic response to injury largely studied in the large neurons of the invertebrate *Aplysia californica* (Erez et al., 2007; Sahly et al., 2006; Spira et al., 2003; Ziv and Spira, 1995). *In vivo* studies in the mouse showed that by 10 to 20 min after axotomy, axon tips proximal and distal to the site of injury fragment and degenerate several hundred micrometers in a process termed “axonal dieback” (Kerschensteiner et al., 2005). Acute axonal dieback is induced by the elevation of intracellular calcium (George et al., 1995). Calcium induces microtubule and actin depolymerisation, resulting in retraction in cultured DRG neurons. *In vivo* studies have confirmed degeneration processes in the distal and proximal axon tip after lesion (Kerschensteiner et al., 2005). By 24 h, the distal fragment of the sensory axon begins to undergo Wallerian degeneration. Conversely, the proximal axon forms a distal swelling. Histological analysis revealed that these swellings are comprised of disorganized microtubules and an accumulation of vesicles and organelles termed a retraction bulb (Erturk et al., 2007). For the axon to regenerate, a retraction bulb needs to transform back into a growth cone-like structure with organized microtubules (Bradke et al., 2012).

3.2. Subacute (24 h to 7 days post-injury)

In the subsequent days after injury, sensory axons continue to dieback from the injury site. Both *in vitro* culture and *in vivo* imaging studies have demonstrated the role of macrophages in this process (Busch et al., 2009; Evans et al., 2014). Phagocytic macrophages invade the lesion site and mediate subacute axonal dieback. Blood-derived immune cells, including macrophages and CNS-resident microglia, are latent at the lesion site. However, with an *in vivo* imaging approach, Evans and colleagues showed that the secondary axonal damage is due

to blood-derived macrophages and not microglia. This damage results from direct physical contact of those macrophages with dystrophic axon tips which then thin and dieback.

3.3. Chronic (after 7 days post-injury)

For a long time the view that injured axons in the white matter either retract or degenerate has persisted (Ramon y Cajal, 1928). This view changed when it was shown that chronically, dystrophic retraction bulbs remain at the proximal end of injured axons (Erturk et al., 2007). Remarkably, in the injured human spinal cord, retraction bulbs have been observed in the vicinity of the lesion site years after injury (Ruschel et al., 2015). Interestingly, sensory axon tips are often co-localized with NG2-positive cells, where they persist for a long time. Some evidence from histological analyses suggests that injured axons form synaptic-like structures with NG2-positive cells (Filous et al., 2014). Moreover, regeneration failure at the dorsal root entry zone (DREZ) is partly due to induction of presynaptic differentiation (Di Maio et al., 2011). Here, the authors saw through *in vivo* imaging of axonal dynamics that when encountering the DREZ, axons penetrated further into the CNS instead of turning around. Altogether, a model where axon tips are entrapped by NG2-positives cells which leads to regeneration failure has emerged in the past decade. It is consistent with an emerging concept that adult axons fail to regenerate because they are in a synaptogenic mode (Tedeschi et al., 2016).

4. Sensory axon regeneration

While most sensory axons fail to regrow, a proportion of them can regenerate small distances after CNS injury in the absence of treatment (Erturk et al., 2011). Sensory axons regenerate in lesion models with minimal scarring and without treatment as early as 6 to 24 h after injury (Kerschensteiner et al., 2005). Interestingly, the regenerative response changes considerably depending on the position of axonal injury relative to branch points (Lorenzana et al., 2015). The highest regenerative response is observed when the axon is injured proximal to the main branch point in the spinal cord. Conversely, injury to individual axonal branches distal to bifurcation is followed by a relatively meager regenerative response. However, dual injury to both distal axonal processes stimulates a robust regenerative response. This implies that an intact axonal process suppresses regrowth of an injured process of the same neuron. Consistent with this hypothesis, inhibition of the calcium channel subunit Alpha2delta2 enhances regeneration, suggesting that synaptic transmission *per se* is an inhibitor of regeneration (Tedeschi et al., 2016). This hypothesis remains to be tested.

The ability of central axons to regenerate without treatment also depends on neuronal identity. In the adult mouse brain, ~55% of cortical layer 6-derived axons regenerate after laser-induced lesion (Canty et al., 2013). Conversely, only ~20% of cortical layer 2/3/5-derived axons regenerate after the same injury. Moreover, serotonergic axons have an unusual capacity to regenerate after injury (Jin et al., 2016). Unlike cortical axons or sensory axons, regenerating serotonergic axons are able to recapitulate the layer-specific distribution and morphological features of uninjured axons. The intrinsic mechanisms underlying the ability of serotonergic axons to regenerate so robustly are unclear. Unlike glutamatergic axons, serotonergic neurons release their transmitters extrasynaptically *via* volume transmission (Trueta and De-Miguel, 2012). Given that glutamatergic axons regrow so poorly (Geoffroy et al., 2016), it is tempting to speculate that differences in signal transmission underlie the contrasting regenerative abilities of these neuronal subpopulations.

Axons of the peripheral nervous system encounter a growth-promoting environment after lesion compared to the growth-inhibitory environment of the CNS. This, combined with the intrinsic growth capacity of PNS axons, leads to functional regeneration after injury (Huebner and Strittmatter, 2010). As seen in the CNS the distal part of the injured axon undergoes Wallerian degeneration, giving space for the proximal part to regenerate and re-innervate its target. DRG peripheral nerve axons initiate growth within 2 days after lesion. The speed of growth in the PNS is reduced compared to axons lesioned in a scar-free environment *in vitro* (Bareyre et al., 2011; Chierzi et al., 2005).

A more robust growth response of central axons is seen if DRG neurons are experimentally set into a regenerative state. If the peripheral branches of a DRG are injured prior to lesion of the central branches, central axons can regenerate (Neumann and Woolf, 1999; Richardson and Issa, 1984). This so-called conditioning effect has been used extensively to study similarities and differences between axon growth during development and regeneration after injury (Tedeschi et al., 2016; Ylera et al., 2009). In summary, the conditioning paradigm is very useful to understand aspects of regeneration and degeneration. Nonetheless, from a clinical perspective the conditioning effect is irrelevant. Thus, other experimental strategies have been developed to promote regeneration.

Manipulating the axon and the axon environment have proven successful in boosting regeneration and functional recovery. As we already discussed, the two major impediments after central lesion are the poor intrinsic growth capacity of adult neurons in the CNS and the inhibitory lesion scar that forms (Cregg et al., 2014; Erturk and Bradke, 2013). The field has focused on enhancing axon regeneration by different strategies (Bradbury and Carter, 2011; Burnside and Bradbury, 2014; Eva et al., 2012; Galtrey and Fawcett, 2007; Geoffroy and Zheng, 2014; Schwab et al., 2014; Silver et al., 2014; Venkatesh and Blackmore, 2017). In this review, we mainly focus on *in vivo* imaging studies that contributed to the understanding of axon regeneration and therefore cannot mention all the important work done in the field.

In vivo imaging studies have been mainly used to investigate promoting intrinsic growth with pharmaceutical interventions (Bradbury et al., 2002; He and Jin, 2016; Hellal et al., 2011; Ruschel et al., 2015). For example, altering the cytoskeleton by stabilizing microtubules with Taxol or Epothilone B/D, or inhibition of the voltage-gated calcium channel subunit Alpha2delta2 with pregablin, enhances regeneration after injury (Ruschel and Bradke, 2018; Sandner et al., 2018; Tedeschi et al., 2016). These studies have utilized *in vivo* imaging to understand the direct effect of drugs on axon regeneration. Microtubule stabilizing drugs, for example, prevent axonal dieback and retraction bulb formation and enhance regeneration. Furthermore, the fibrotic scar is reduced upon such treatment. Whether macrophage-mediated dieback specifically is also inhibited remains to be tested. In the future, this could be answered with a more detailed *in vivo* imaging study visualizing not only axons but also other cell types. Additionally, imaging of microtubule or actin dynamics *in vivo* could help us to understand how the intrinsic growth program after injury is regulated (Kleele et al., 2014). Finally, the effect of potential treatments, for example with pregabalin, on growth cone dynamics can be dissected in more detail.

5. *In vivo* imaging of axon dynamics in the spinal cord

Some of the pioneers of imaging axon regeneration in the spinal cord are M. Kerschensteiner and colleagues (Kerschensteiner et al., 2005). They have shown how to image simple axonal dynamics after injury with a basic wide-field microscopy set up. This method has been used in several other studies investigating axon outgrowth after pre- and post-conditioning, retraction bulb formation and degeneration (Erturk et al., 2007; Ylera et al., 2009). Yet, to image with high temporal and spatial resolution, wide-field microscopy is not sufficient.

Recently, more sophisticated imaging techniques including two-photon imaging have been used to image regenerating axons in the spinal cord. Two-photon imaging has several advantages compared to wide-field microscopy. First, two-photon imaging permits higher spatial and temporal resolution relative to wide-field microscopy. It also allows deeper tissue penetration and evokes lower photo-toxicity, improving conditions for *in vivo* imaging. Finally, a two-photon microscope allows

lesions of single axons with laser pulses, minimizing inhibitory scar formation and thus facilitating investigating the intrinsic response of axons to injury (Lorenzana et al., 2015; Ylera et al., 2009). In *C. elegans*, laser lesions are widely used for screening of pro- regenerative or degenerative genes (Byrne and Hammarlund, 2017; Chen et al., 2011).

5.1. Chronic versus acute imaging

In vivo dynamics in the spinal cord can be imaged in an acute or chronic manner. Previously, chronic imaging has been performed in an acute preparation at different time points after injury. For example, phase-specificity for the transcription factor STAT3 after lesion has been shown by chronic imaging (Bareyre et al., 2011). However, preparing the mouse acutely for imaging at every imaging time-point is stressful for the mouse due to the need for repetitive surgical interventions. Therefore, the spinal window has been developed by different labs to perform chronic imaging (Farrar et al., 2012; Fenrich et al., 2012; Figley et al., 2013; Tedeschi et al., 2016). This technique is especially useful to image the same axons over weeks and, potentially, up to several months after installation. A spinal window has been used to show that pharmacological intervention with methylpredinisolone after spinal cord injury has a direct effect on axonal dieback, blood-flow and calcium influx (Tang et al., 2015). However, for the acute phase and for some time points after injury, the acute preparation and imaging of the mouse has advantages. Furthermore, the choice of imaging might depend on the injury model chosen.

5.2. Injury models

Most studies of regeneration in the CNS have used retinal ganglion cells of the optic nerve or descending or ascending tracts in the spinal cord as the model system (Hilton et al., 2013; Sun et al., 2011; Tuszynski and Steward, 2012). Different injury models of varying severity can impact spinally projecting neurons. Arguably, the most relevant model for clinical translation is the contusion injury in which the spinal cord is bruised by a discrete and defined impact. This kind of injury shows a similar pathology as seen after some spinal cord injuries in humans and is therefore considered more physiological. It includes the formation of a cyst, which develops after injury in rats and in primates (James et al., 2011). The dorsal column lesion, by which the spinal cord substructure is transected with a micro-knife, is commonly used to investigate sensory axon regeneration. Performing this injury, the dura is disrupted which should be taken into account. However, if the injury is performed correctly, sprouting by spared fibers can be excluded by confirming that there is an absence of tracer signal in the brain stem (Lu et al., 2004). The contusion injury might be the most reproducible one. Nonetheless, it is difficult to control for spared fibers and to distinguish between sprouting and regeneration.

The least invasive model is the laser lesion. Performing a laser lesion causes minimal scaring in the brain and in the spinal cord of mammals (Canty et al., 2013; Ylera et al., 2009). After traumatic CNS injury, different cell types, including macrophages and astrocytes, invade the injury site. Those cells define the lesion site by building a fibrotic and astrocytic scar secreting inhibitory factors and presumably also building a physical barrier for regenerating axons (Cregg et al., 2014). When axons are lesioned with a highly localized laser, the extrinsic injury response is minimized (Ylera et al., 2009). Thus, the intrinsic response can be studied in more detail. Laser induced lesions have been used previously in chronic injury models of DRG neurons. Using such minimally invasive lesions, it was shown that even chronically, injured sensory axons are able to regenerate if subsequently conditioned in the absence of a scar (Ylera et al., 2009). Furthermore, the precision of the laser lesion made it possible to dissect the importance of injury location along the axon for regeneration (Lorenzana et al., 2015). Canty and colleagues specifically ablated single axons in the brain of mice to study degeneration and regeneration (Canty et al., 2013). Using this injury model, they found that some axons extend at the speed of peripheral nerve regeneration. Although most axons failed to regenerate, synaptic density at the remaining branch was restored. These very elegant studies highlight the potential of two-photon microcopy studying injury by ablating single axons with the two-photon laser and subsequent chronic imaging. In summary, ablating single axons with a laser can be used to study regeneration with minimal scarring.

6. Practical aspects of *in vivo* imaging

In this part, we will discuss the practical aspects of *in vivo* imaging. In short, we will explain how we visualize axons, how the animals are prepared for imaging and which microscope we use.

The axons in the dorsal column are visualized in the commonly used thy1-GFP-M mouse line which expresses green fluorescent protein (GFP) in a subset of neurons under the thy1 promotor (Feng et al., 2000). In this mouse line, the expression of the fluorescent molecule is specific for neurons (Vidal et al., 1990). Different lines express the fluorescent label in a different pattern. For *in vivo* imaging, the lines in which GFP is only expressed in a small percentage of neurons are suitable to facilitate imaging of single axons. These mice can be directly imaged with a basic wide-field microscope set up (Kerschensteiner et al., 2005).

Alternatively, axons can be labeled by injecting adeno-associated virus (AAV) expressing enhanced GFP (eGFP) into the sciatic nerve or directly into the DRG to transduce DRG neurons. Labeled axons are visualized in the spinal cord from one week post-injection. Sciatic nerve injection is a relatively minimally invasive surgical procedure and mice recover very fast. However, all neurons with axons in the sciatic nerve, including motor axons of the ventral root, might be transduced. Injection of virus into the DRG is more invasive, yet sensory neurons are labeled more specifically. In our lab, we use either thy1-GFP or AAV expressing eGFP-injected mice for structural *in vivo* imaging (Fig. 1A).

All our imaging is done in anesthetized mice. To anesthetize the mouse, we use a mixture of ketamine and xylazine (KX). This mixture is commonly used in mouse and rat surgeries (Flecknell, 1993) and is preferable compared to using isoflurane, which has a vasodilating effect and thus results in more bleeding (Lenzarini et al., 2016). Another advantage is that KX is applied intraperitoneally so that there is no need for space-consuming anesthesia equipment.

Finally, to image axons in the spinal cord we use a two-photon microscope. Our two-photon Zeiss LSM7MP microscope is equipped with an Insight X3 laser. This laser is tunable up to 1300 nm, with a higher power output than conventional two-photon lasers. Thus, excitation and imaging in the red spectrum is more feasible. Emitted fluorescence is detected by either two BiG (Binary Gallium arsenide phosphide) detectors or ultrasensitive nosepiece detectors equipped with exchangeable filter sets. Thus, multiple fluorophores can be visualized concurrently.

6.1. Acute imaging

To image axons acutely in the spinal cord, the mouse is anesthetized and prepared for sterile surgery. A small incision is made above the desired spinal cord region. As a reference point, we use the apex of the vertebral curvature, which corresponds to the vertebral level T12. Then, two small incisions are made along the axis of the spinal cord on both sides of the dorsal processes. A laminectomy is carried out to expose the spinal cord underneath vertebra T12 (Fig. 2C). This corresponds to the spinal cord segments L2–3 (Harrison et al., 2013). The spinal cord is stabilized with spinal cord holders STS-A from Narishige (Fig. 2A). These holders are fixed on a custom-made 3D printed insert for the microscope stage. The mouse is clamped at the vertebrae rostral and caudal to the laminectomy and elevated slightly above a heating pad to maintain its body temperature (Fig. 2B). Additionally, the tail is clamped to improve stability.

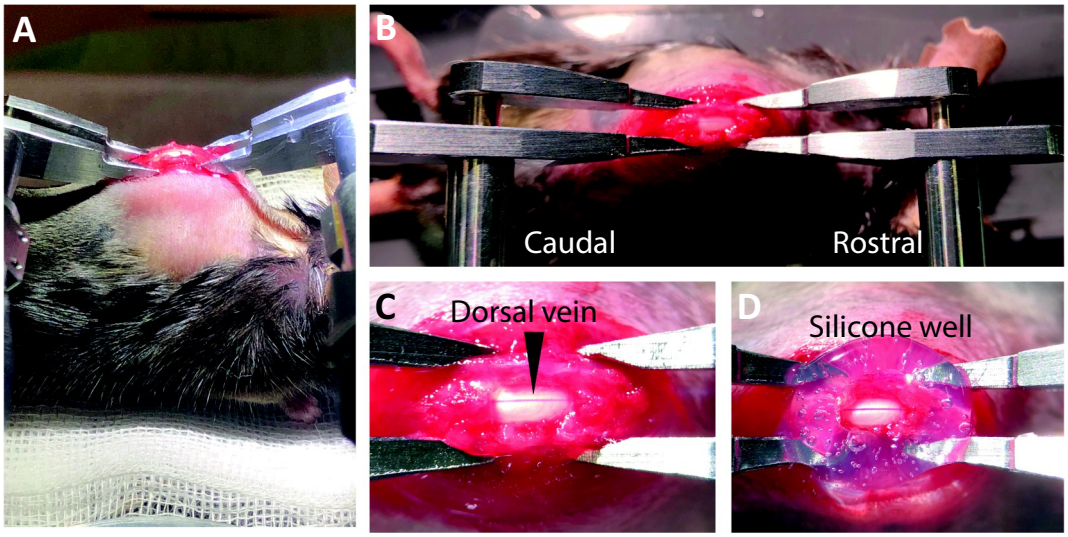

Fig. 2. Acute imaging set up for dorsal column sensory neurons at spinal level T12.
The anesthetized mouse is clamped with spinal cord holders from Narishige at the vertebrae caudal and rostral to the laminectomy side at T12 (A and B, top view). After cleaning and preparing the clamped spinal cord (C) a silicone well is formed around the laminectomy and filled with ACSF (D).

To allow direct immersion with the water objective without the need for a coverslip, a small silicone well is made (Fig. 2D). The well is filled with pre-warmed artificial cerebrospinal fluid (ACSF) to maintain physiological conditions. To obtain a stable well without any leakage, it is crucial to perform surgery precisely and cleanly. The initial incision in the skin above the spinal cord should be minimal. The removal of muscles and connective tissue should be focused to the vertebrae of interest. Before making the well, bleeding has to be stopped and the tissue should be dried as much as possible with cotton sticks. The exposed spinal cord is then protected with a piece of cellulose sponge soaked in sterile saline. Then, the well is created with a silicone elastomer. The elastomer is applied around the laminectomy site in the shape of a ring. This has to be done slowly in order for the silicone elastomer to cure in the desired place. After several minutes, the silicone elastomer cures. The cellulose sponge to protect the spinal cord is taken off and the well is filled with pre-warmed ACSF (Fig. 1C).

Once the surgery is completed, the mouse is placed under the two-photon microscope. KX is re-administered every hour with half the initial dose. The axons in the spinal cord are then ready for imaging. After acquiring all needed images, the muscles are sutured above the spinal cord and the skin clipped with wound clips. Then, the mouse is placed in a recovery cage to wake up.

6.2. Laser injury

To study the dynamics of single axons *in vivo* we use the laser axotomy (Ylera et al., 2009). Axons in the dorsal column are visualized and an overview image is taken (Fig. 3A, B). Using a Zeiss set-up allows localized scanning of only one spot in the field of view (Fig. 3C). With the pixel dwell time we calculate the duration the selected spot is scanned. Usually 30,000 to 50,000 scanning cycles of the selected spot resulting in 2 to 4 s of scanning time at the lowest scanning speed are sufficient to cut the desired axon. For scanning the spot, the gain of the detectors is set to 0 and the laser power is increased 5 to 7 times of the scanning power used to visualize the axons. To simplify the method, the same laser wavelength as for imaging GPF labeled axons, 920 nm, is used. After scanning the spot, the lesion is visualized in the conventional frame-scanning mode. Successful lesioning depends on the objective used, the power of the laser and the depth in which the axon is located in the spinal cord. We observe disrupted tissue immediately after lesion with high auto-fluorescence (Fig. 3D). The rupture of the axon is confirmed after imaging a z-stack. The parameters used always lead to immediate dieback of the axon of a few micrometers (Fig. 3D).

6.3. Chronic imaging—Spinal cord window

Spinal windows facilitate repeated imaging of axons within the same mouse. With implanted windows, the spinal cord can be theoretically imaged for up to one year without damaging the spinal cord (Fenrich et al., 2012). The limiting factor here is scarring or bleeding that restricts the visibility of the axons in the spinal cord. In our lab, we use a custom-made window as seen first in a study investigating developmental changes that limit regeneration (Fig. 4A)(Tedeschi et al., 2016). The implantation procedure is straightforward and similar to methods that have been described in detail previously (Farrar et al., 2012). Before performing a laminectomy, the vertebra at T12 is clamped on both lateral sides with two metal bars. The bars are fixed with dental acrylic and cyanoacrylate and then a laminectomy is performed as described above. Finally, the two bars are connected by a top plate. At the first time point, the selected injury is performed. A 4 mm cover glass is placed on top after applying silicone elastomer on the spinal cord to fill the gap between the spinal cord and the coverslip. The window is sealed rostrally and caudally and the skin is glued to the edges of the window (Fig. 4C1–6). The mouse is then ready to image under the two-photon set up (Fig. 4D). After imaging, the mouse is left to recover as described above. At the next imaging time point, the mouse is anesthetized with KX again and fixed in the holder. Two-photon imaging can be performed as needed at multiple time-points.

6.4. Stabilization of animals for in vivo imaging

One of the biggest obstacles in imaging *in vivo* is movement of the animal. Even under anesthesia, movement of the animal is one major challenge during *in vivo* imaging. The spinal cord of a C57/Bl6 mouse consists of 34 vertebrae (Harrison et al., 2013). The vertebrae are connected by soft tissue and protect the spinal cord (Gianino et al., 1996). This segmentation of the spinal cord leads to a high degree of flexibility. Therefore, movement due to breathing and pulse is not abolished by stabilizing single vertebrae as described above. The greater the resolution required, the more movement disturbs image acquisition. In response to these issues, several groups have assessed different anesthesia methods or ventilation systems to help minimize movement artifacts. Davalos and colleagues suggest using a mixture of ketamine, xylazine and azepromazine, which induces less pronounced

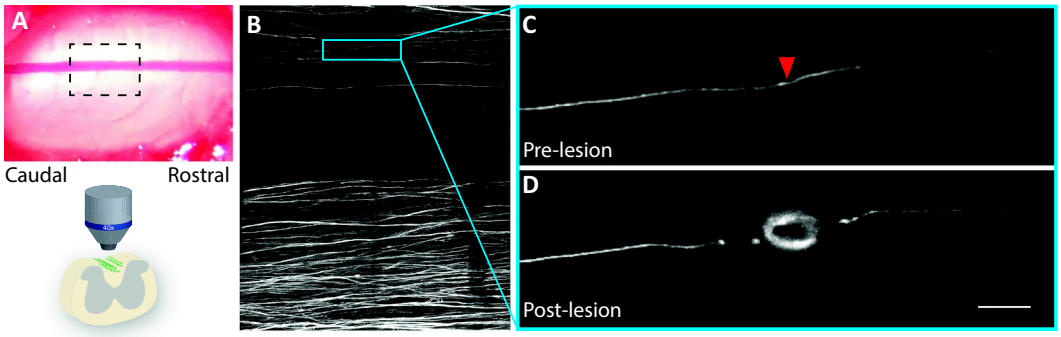

Fig. 3. Laser lesion of a single axon in the dorsal column.
(A) Photograph of the laminectomy side at T12. Dashed box indicates area imaged with the two-photon microscope in B. (B) Maximum intensity projection of GFP-labeled dorsal column sensory axons (B). Magnification of blue box in B before (C) and after laser lesion (D). Red arrowhead in C indicates side for laser lesion. Scale bar, 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

breathing (Davalos et al., 2008). In our hands, we have not observed a difference between this anesthetic cocktail and KX anesthesia.

6.5. Signal intensity

Another major consideration during *in vivo* imaging is signal intensity and, more generally, the signal-to-noise ratio. Auto-fluorescence of tissue as well as light scattering weaken the signal of labeled axons. A high signal-to-noise ratio is critical for imaging small structures or subcellular proteins. To reduce auto-fluorescence, improvements could be made by using fluorophores in the red or far-red spectrum, which immensely reduces background (Renier et al., 2014). Thus, lasers capable of high power stimulation of red and far-red fluorophores are advantageous.

To improve the signal of small structures it is worthwhile to remove the dura. This has to be done for other applications as well, for example when applying intravital dyes (Romanelli et al., 2013). In general, as mentioned above, the injection of an AAV1 carrying a payload that consists of a CMV-GFP expression cassette is an effective alternative to the use of genetically labeled mouse lines (Tedeschi et al., 2016). This virus leads to high signal intensity in transduced neurons by at least one-week post-injection.

7. Future directions

Axons of DRG neurons in the spinal cord serve as a great model to study regeneration. Their superficial position in the spinal cord makes them easily accessible. Furthermore, the conditioning paradigm makes them a reproducible model to assess the mechanisms that drive axon regeneration. Whether axons display dynamic features such as growth and pruning or whether they are relatively static is important to consider for growth-promoting interventions (Lorenzana et al., 2015). Furthermore, *in vivo* imaging can identify the mode in which regenerating axons grow, in an elongating or branched manner.

In vivo two-photon imaging combined with lesions of single axons and targeted genetic manipulations can be used to dissect in more detail the role of intrinsic factors. For example, in a seminal study, STAT3 was demonstrated to have a phase-specific role in axon regeneration. By overexpressing STAT3 in DRG neurons, Bareyre and colleagues demonstrated that the molecule was essential for early initiation of axon regeneration but did not promote growth at later stages after injury (Bareyre et al., 2011). Novel approaches to introducing genes into DRG neurons, such as through *in vivo* electroporation, will facilitate future studies that assess how different genes instruct or restrain axon growth (Saijilafu et al., 2011).

Innovations that enhance imaging depth will facilitate a broader range of *in vivo* imaging spinal cord studies. To date, two-photon imaging has not been sufficient to visualize spinal axons deeper than those

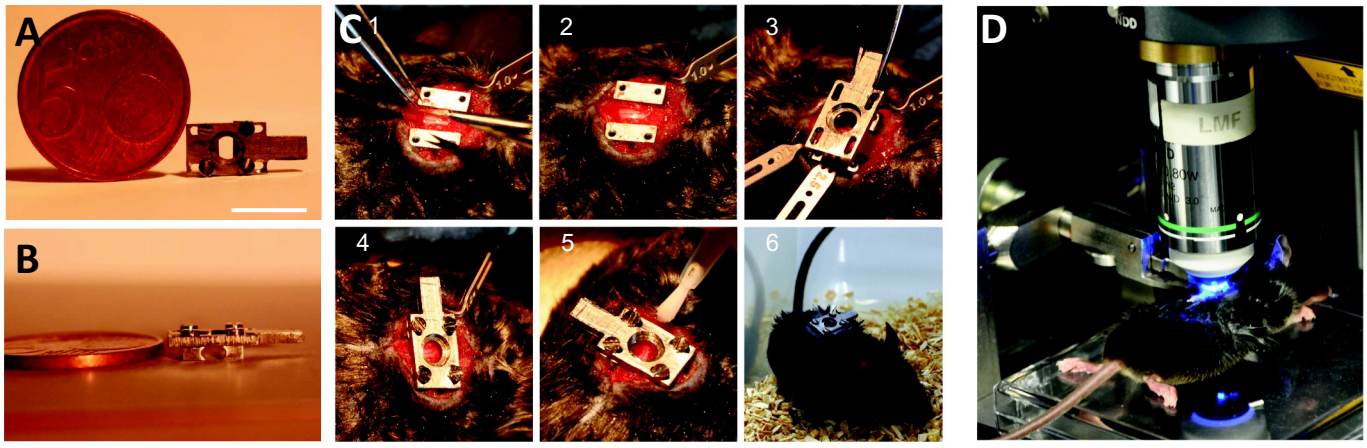

Fig. 4. Implantation of the spinal cord window.
(A, B) A custom made spinal window for implantation and chronic *in vivo* imaging. (C) Steps (1–6) of implanting the spinal cord chamber. (C1 and 2) Fixing two metal bars lateral to the spinal cord at T12. (D) Set up of the anesthetized mouse under the two-photon. Scale bar, 200 µm.
(Adapted from Tedeschi et al., 2016.)

found in the superficial dorsal column due to scattering of light by central myelin. If enhanced imaging depth was possible, one tantalizing prospect for future investigations would be *in vivo* imaging of alternative spinal pathways such as serotonergic axons or the corticospinal tract (Hilton et al., 2016). The use of prisms might enable scientist in the future to look at axons laying deeper in the spinal cord (Low et al., 2014). Moreover, three-photon-imaging might conceivably permit scientists to view live axons deep within the spinal cord (Cheng et al., 2016).

Methods that enhance imaging resolution are also needed in order to image subcellular dynamics. A recent study utilizing two-photon stimulated emission depletion (STED) microscopy led to high resolution imaging of dendritic spines in the mouse cortex (Pfeiffer et al., 2018). This could be an interesting approach to investigate cytoskeletal dynamics within the live spinal cord. For example, Wegner and colleagues recently imaged neuronal actin dynamics using STED microscopy (Wegner et al., 2017). The limiting factor here is the depth at which two-photon STED can be used. Microtubule dynamics have been imaged by tagging microtubule plus end binding proteins with fluorophores utilizing two-photon microscopy (Kleele et al., 2014). Investigating the cytoskeleton after injury *in vivo* to further enhance the understanding of growth cone formation and axon growth after injury will be an aim in the future.

Finally, *in vivo* imaging will likely provide fruitful insight into how neuronal activity and calcium signaling influence CNS axon regeneration. Although calcium imaging is routinely done in the brain (Grienberger and Konnerth, 2012), most calcium indicators are often localized to the somatodendritic compartments of neurons and do not diffuse into distal axons. Recently, a novel approach has been developed to target calcium indicators towards axons (Broussard et al., 2018). This approach will likely facilitate investigating how calcium changes in the axon during degeneration and regeneration.

Even if regeneration is achieved by axons in the CNS, the question remains whether these axons integrate back into the system and transmit the right signals. Imaging of regenerating single axons together with imaging of neuronal activity could in the future elucidate whether and how those axons integrate back into a functional circuit. To investigate the influence of neuronal activity, one possibly would be to combine *in vivo* imaging of dorsal column sensory axons with designer receptors exclusively activated by designer drugs (DREADDs). These receptors permit longitudinal excitation and silencing of neuronal activity (Roth, 2016). Thus, their activation in live axons might prove instructive in helping to determine how neuronal activity influences the dynamics of injured axons.

Overall, *in vivo* imaging has made great advances in the past decade from wide-field to now routinely utilized two-photon imaging. The latter technique holds great promise in the future to understand processes leading to degeneration and regeneration in more detail.

Conflict of interest

None.

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