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Review

In vivo imaging
A dynamic imaging approach to study spinal cord regeneration

Claudia J. Laskowski, Frank Bradke *

Deutsches Zentrum für Neurodegenerative Erkrankungen e.V., Axon Growth and Regeneration Group, Ludwig-Erhard-Allee 2, 53175 Bonn, Germany

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ABSTRACT

Upon spinal cord injury, severed axons and the surrounding tissue undergo a series of pathological changes, including retraction of proximal axon ends, degeneration of distal axon ends and formation of a dense fibrotic scar that inhibits regenerative axonal growth. Until recently it was technically challenging to study these dynamic events in the mammalian central nervous system. Here, we describe and discuss the recently established genetic tract tracing approach of *in vivo* imaging. This technique allows studying acute pathological events following a spinal cord lesion. In addition, the novel development of chronic spinal cord preparations such as the implanted spinal chamber now also enables long-term imaging studies. Hence, *in vivo* imaging allows the direct observation of acute and chronic dynamic degenerative and regenerative events of individual neurons after traumatic injury in the living animal.

Contents

Introduction

The wiring and complex connections of neuronal pathways within the mammalian central nervous system (CNS) have long been the subject of intense investigations in spinal cord research. Neuronal tracing techniques constitute indispensable tools to study the development and trajectories of axonal tracts, as well as the regeneration potential of axons after traumatic injury. Spinal cord injury (SCI) leads to a cascade of diverse pathological events: While proximal axon ends retract and do not regenerate upon injury, distal axon ends undergo a process called Wallerian degeneration (reviewed in Waller, 1850; Coleman and Freeman, 2010). At the same time meningeal fibroblasts and astroglia, as well as diverse immune cells infiltrate the lesion site and lead to restructuring of the injured tissue and formation of a dense scar (reviewed in Silver and Miller, 2004). The sequence and timing of pathological as well as regenerative events can be most reliably studied in the natural environment of the affected neurons. Hence, progress in understanding the underlying pathobiology of these complex events also depends on our ability to image individual neurons.

Classical tracing approaches are useful applications for examining anatomical relationships between various brain regions and characterizing the architecture of arborizations and synaptology of axon terminals. However, there are some limitations in using these approaches for the assessment of axonal regeneration after SCI (reviewed by Kobbert et al., 2000; Raju and Smith, 2006; Schofield, 2008): (1) Classical tracing

* Corresponding author. Fax: +49 228 43302 689.
E-mail address: frank.bradke@dzne.de (F. Bradke).

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techniques are generally "static" end-point experiments, where each animal provides only one piece of data. The adequate interpretation and analysis of the data end-points must be deduced by comparing the individual static images. (2) The tracer application requires complex surgery and extensive expertise from the scientist, potentially introducing new variables and complicating subsequent interpretation of the data. (3) The tracing of specific neuronal pathways may be incomplete or erroneous. For example, variability in amount and location of dye injection can lead to variations in the number of labeled fibers (Bareyre et al., 2005; Joosten et al., 1987; Steward et al., 2004). Some common tracers such as cholera toxin subunit B (CTB) and biotinylated dextran amines (BDA) can erroneously be taken up by fibers of passage, which have been damaged as a consequence of the dye injection (Brandt and Apkarian, 1992; Reiner et al., 2000). (4) Finally, using classical tracing approaches it is technically more demanding to label and follow single nerve fibers. Even though classical tracing techniques as well as reductive approaches, such as cell culture studies have provided useful insights into understanding axonal pathobiology after SCI, the necessity for the development of more innovative and dynamic imaging approaches *in vivo* was inevitable.

As one of the first examples of *in vivo* imaging, the genetic tract tracing technique developed by Kerschensteiner et al. (2005) allows the *in situ* observation and analysis of lesioned axons at the single cell level in their natural habitat. A single structure is followed over time and multiple data points are obtained from one animal. Hence, interpretation of data is easier to infer, since the pathological events may directly be observed while they are happening in the living animal. Therefore, *in vivo* imaging enables direct observation of dynamic degenerative and regenerative processes after traumatic injury and constitutes a unique tool to unravel the events underlying spinal cord pathology. In the future, *in vivo* imaging will help to gain insight into how neurons in the nervous system change in relation to behavioral adaptations, experience and especially pathological events such as SCI.

In this review we aim to describe and discuss this dynamic imaging technique. First, we delineate how a basic *in vivo* imaging experiment is implemented in practice. Second, we discuss the strengths of *in vivo* imaging and illustrate the necessity for its development in addition to conventional tracing approaches. We then address the current limitations and caveats of *in vivo* imaging. Finally, we suggest potential future applications of this technique.

The experimental procedure

In this section, we delineate the principal steps to perform an *in vivo* imaging experiment. This description is important for understanding the main strengths and limitations of this technique addressed in the respective sections of this review. A basic setup for *in vivo* imaging is depicted in Fig. 1. However, the setup is versatile and can be easily modified and customized to meet the particular researcher's needs.

Among the most useful mouse models for *in vivo* imaging are transgenic mice expressing fluorescent proteins under the control of the promoter Thy1 (Feng et al., 2000). Thy1 is an immunoglobulin superfamily member expressed by projection neurons throughout the nervous system, as well as by several non-neuronal cell types, including thymocytes (Gordon et al., 1987; Morris, 1985). Deletion of a particular intron selectively inhibits expression in non-neuronal tissue (Vidal et al., 1990) and makes Thy1 a neuron-specific promoter. Taking this to their advantage, Sanes and colleagues generated 25 independent transgenic lines expressing at least one of four fluorescent proteins termed XFPs (yellow [YFP], green [GFP], red [RFP] and cyan [CFP] fluorescent protein) in the nervous system in a unique pattern (Feng et al., 2000). The diversity of the labeled neuronal subsets in the obtained lines was attributed to differences in the integration pattern and/or copy number of the neuron-specific XFP transgene. Among the lines generated, "low-expressing" lines such as GFP-M and GFP-S are most suited for *in vivo* imaging. In these lines, GFP expression is only driven in a small percentage (1–10%) of sensory dorsal root ganglion (DRG) neurons. Hence, single axons at the surface of the surgically exposed dorsal column can be directly observed after SCI using simple wide-field epifluorescence microscopy (Fig. 2) (Kerschensteiner et al., 2005). These genetically traced axons are superior to surgically or virally delivered fluorescent tracers, because the fluorescent proteins are produced continuously, there is no undesired immunological response and cell-type specificity is stable over generations.

In vivo imaging is usually performed on adult mice (8–16 weeks). After induction of anesthesia, the mice are kept on a heating pad to maintain a constant body temperature. Efficient ventilation, anesthesia

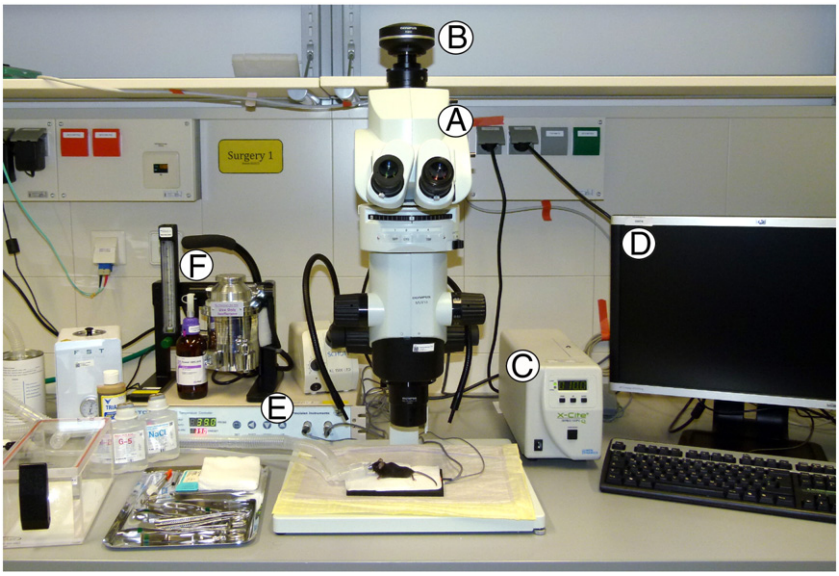

Fig. 1. The basic experimental setup for *in vivo* imaging. The imaging setup consists of (A) an upright wide-field epifluorescence microscope (MVX10 Macroview, Olympus), (B) a black and white CCD camera (XM-10, Olympus), (C) a computer controlled fluorescence microscope light source (X-Cite 120PC Q) and (D) the corresponding digital imaging software (Cellsens®, Olympus). (E) A temperature controller and heating pad are required to monitor the animal's body temperature (ATC 1000, World Precision Instruments). (F) A table top laboratory animal anesthesia system (VetEquip) is used to induce and maintain the anesthesia.

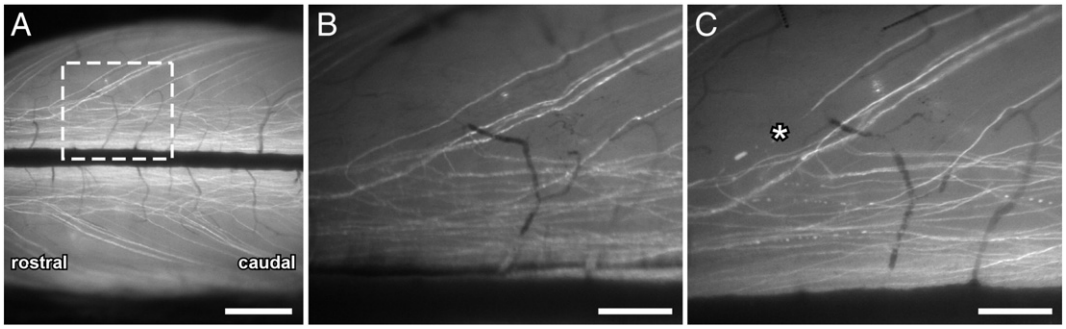

Fig. 2. Dorsal column of a Thy1-GFP mouse exposed by laminectomy of the lumbar vertebra 1. (A) Low magnification view of fluorescent dorsal root ganglion axons entering the dorsal column and then running in parallel to the surface of the spinal cord. Blood vessels appear black. (B, C) Higher magnification views of (A). The dorsal roots of single sensory axons bifurcate and enter the dorsal column. One axon was imaged (B) before and (C) 4 h after transection (asterisk). Scale bars, (A) 500 μm and (B, C) 200 μm.

and analgesia are monitored constantly. Next, the lamina is removed at lumbar level 1 (L1) of the spinal cord to expose the dorsal column, *i.e.* the central axons of dorsal root ganglion (DRG) neurons that have their peripheral axons bundled within the sciatic nerve. Alternatively, a laminectomy may be performed at any other spinal cord level (cervical, thoracic or lumbar) provided that the spinal cord is even in this region. In other words, a laminectomy within the rostrocaudal curvature of the mouse spinal cord should be avoided. To obtain the optimal fluorescence signal the layer of dura mater covering the spinal cord should be removed at the site of laminectomy. A needle or fine iridectomy scissors may then be used to carefully transect single or groups of superficial axons in the dorsal columns on either side of the prominent dorsal vein (Fig. 2). Due to the occasional presence of minor blood vessels at the lesion site, transection can lead to small bleedings. However, these bleedings can generally be stopped with cotton tipped applicators and do not alter the regeneration behavior of the axons (Erturk et al., 2007; Laskowski and Bradke, unpublished results). At this point, a potential pharmacological substance can be directly administered onto the spinal cord (Erturk et al., 2007) or an osmotic pump can be implanted intrathecally (*e.g.* White et al., 2008). Before and after induction of an injury (and treatment) the transected axons are imaged. Most dorsal column axons, which originate from the DRGs, run in parallel to the dorsal spinal cord surface. The superficial fraction of sensory axons can thus easily be imaged using conventional wide-field epifluorescence microscopy and deep tissue penetration microscopy is not required. However, the use of two-photon laser scanning microscopy does have clear benefits, including reduced phototoxicity, deeper tissue penetration and greater spatial resolution than conventional wide-field epifluorescence microscopy (reviewed in Denk et al., 1990). Alternatively, the two-photon laser beam can be used to micro-ablate single axons. In this approach the dura mater does not have to be removed and the small lesion will only lead to minor scarring (Ylera et al., 2009). Furthermore, if performing a real-time imaging experiment (as opposed to repetitive imaging sessions), it is critical to maintain physiological temperatures and pH-levels in the environment of the superficial axons being imaged. This is possible by continuously superfusing the spinal cord with a physiological saline solution or artificial cerebrospinal fluid (aCSF) pre-warmed to 35–37 °C. After imaging, the lamina is closed with surrounding muscle tissue and the skin stapled until the next imaging time point. Post-surgery, the animals are revived and kept in their home cages on a heating pad for some days. They are further supplemented with physiological saline or glucose solution to prevent dehydration and treated with antibiotics and analgesics for the following week.

Strengths of *in vivo* imaging

In vivo imaging is a dynamic imaging approach, in which a single structure, for instance a severed axon, can be followed over time. Dynamic approaches can be subdivided into two categories: continuous real-time imaging and repetitive imaging. While the former approach is most suited to study acute pathological events directly after SCI with high temporal resolution, the latter approach allows the analysis of changes that extend over a longer time frame (>1 day). Compared to static techniques, both dynamic approaches allow easier interpretation of data and reconstruction of the main pathological steps, because the key events may be directly observed while they are happening in the living animal. This is most important, since SCI is not a fully synchronized and reproducible event and will not show the identical time course in every single animal. Hence, adequate temporal resolution as given by *in vivo* imaging will contribute to clarifying mechanistic aspects and causative relationships in SCI.

A fundamental advantage of *in vivo* imaging over conventional tracing approaches is the direct visualization and confirmation of axonal transection (Fig. 3). It is possible to unequivocally differentiate between axonal fibers and sprouts that regenerated from transected axons and fibers that were spared by the original lesion. In addition, the evaluation of therapeutic interventions becomes more direct and reliable using *in vivo* imaging. For instance, it is possible to distinguish between drug effects, that protect the axon and thus inhibit degeneration and/or that increase regeneration directly. Both mechanisms may eventually result in enhanced regeneration across the lesion site (Fig. 4). In static imaging approaches, only the final outcome becomes apparent: regeneration of axonal fibers across the lesion site. The mechanism of the treatment remains elusive.

In general, *in vivo* imaging is most suited to examine acute effects taking place within the first days after SCI. Long-term observations are technically more demanding due to the gradual formation of a fibrotic scar at the site of the laminectomy. Therefore, the recent advent of novel strategies that permit the monitoring of regeneration over weeks and months will be of great importance in spinal cord research. Dray et al. (2009) imaged the spinal cord for up to 4 months by protecting the spinal cord with a thick layer of agarose prior to resuturing the animal. Similarly, in a second study (Di Maio et al., 2011) the laminectomy area was tightly covered by applying a piece of thin synthetic matrix membrane over the exposed cord and dura after each imaging time-point. In addition, the membrane was stabilized with a layer of artificial dura that covered the entire laminectomy site. Hence, scarring accumulated on the membrane rather than on the dura surface. As a result, the authors identified and followed single dorsal root axons over time-periods of up to 140 days.

While these recent advances allow imaging the spinal cord over longer time-points, the described approaches are still limited by the necessity for repeated surgical procedures to regain optical access to the relevant tissue under investigation. Repeated surgeries increase the risk of infection and inflammation, may cause additional tissue damage and lastly, may cause pain and distress to the animal. Inevitably, these factors also rule out the possibility of using these animals in behavioral

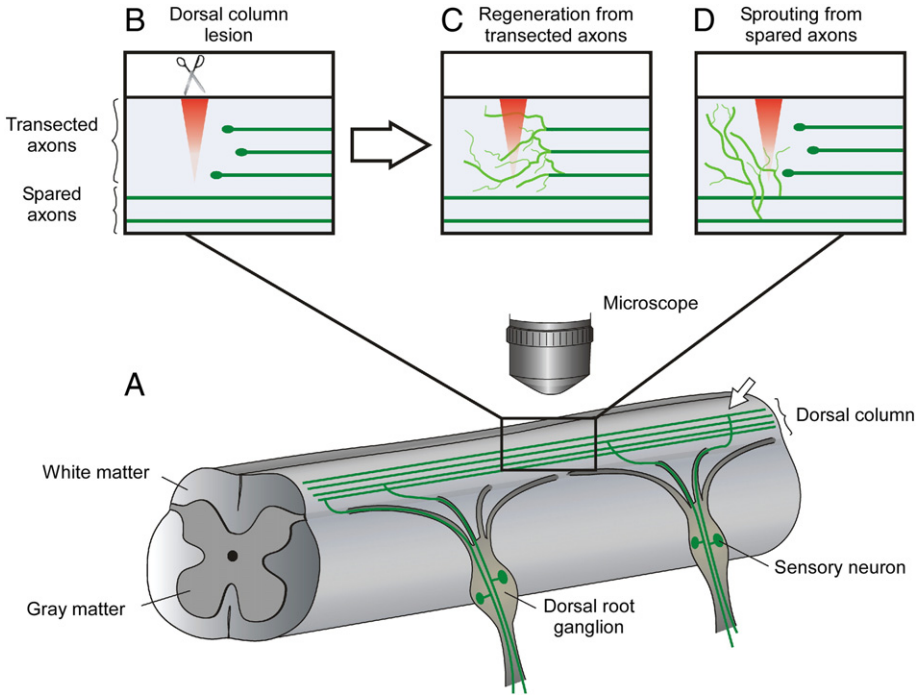

Fig. 3. *In vivo* imaging allows direct visualization and confirmation of axonal transection. (A) Schematic illustration of a Thy1-GFP mouse spinal cord. Sensory axons can be identified by entry into the spinal cord through a dorsal root and branching (arrow) into a descending and an ascending branch. The axons then run in parallel to the spinal cord surface and can be imaged using conventional wide-field epifluorescence microscopy. (B) Dorsal column lesion generally results in severing as well as sparing of fibers. Transected fluorescent sensory axons can be unequivocally distinguished from spared fibers using dynamic imaging approaches such as *in vivo* imaging. As opposed to static end-point imaging approaches, *in vivo* imaging also allows to discriminate between (C) regenerative outgrowth from transected axons and (D) compensatory sprouting from spared fibers.

studies. To date, cranial window preparations that provide continuous optical access to the rodent brain are already widely used in the field (*e.g.* Holtmaat et al., 2009). However, development of chronic glass windows for the spinal cord is complicated by the fact that the spinal cord – compared to the cranium – is not rigid and needs to remain flexible. At the same time, chronic spinal cord implants need to preserve the mechanical stability of the spine, while minimizing motion artifacts due to breathing and heartbeat.

In spite of these associated difficulties, two independent research groups recently developed chronic preparations for the spinal cord (Farrar et al., 2012; Fenrich et al., 2012). Both manuscripts include comprehensive protocols and illustrations of the surgical procedure.

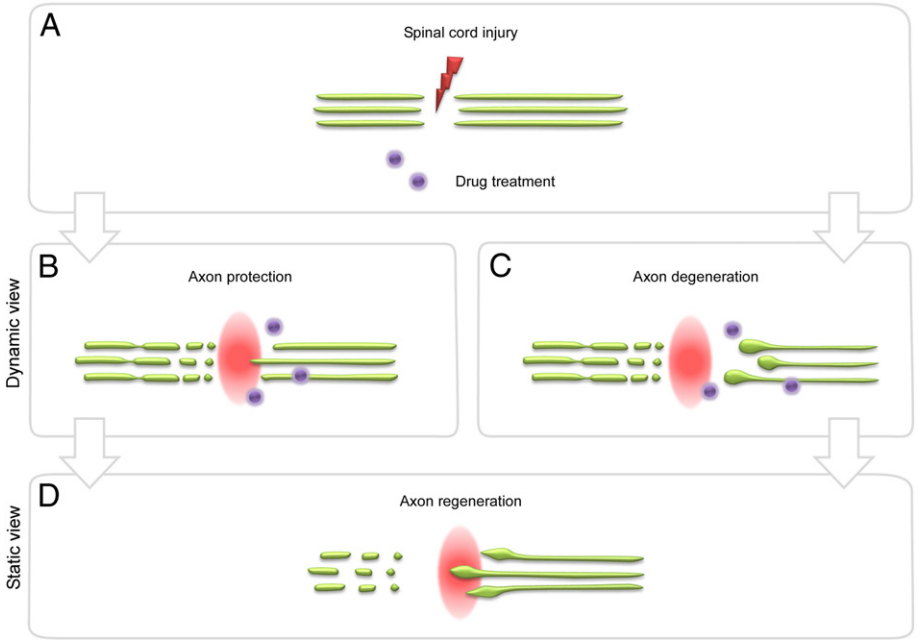

Fig. 4. *In vivo* imaging is a dynamic approach to study axonal regeneration. (A) Direct visualization of axonal transection and subsequent repetitive imaging enable investigation of the axonal pathobiology before and after treatment. Information about the therapeutic mechanisms can thus be inferred using *in vivo* imaging: (B) A particular treatment may either protect transected nerve fibers and thus decrease axonal degeneration in the first place or (C) it can positively act on axonal endbulbs after antecedent degeneration events. Both mechanisms may potentially result in enhanced regeneration (D). In static imaging approaches, by contrast, only the final outcome (D) is apparent: regeneration of axonal fibers across the lesion site. The causative mechanism of the treatment (B and C) remains elusive.

In short, in the first approach by Farrar et al., muscle tissue and tendons were carefully removed from the dorsal laminae of the thoracic vertebrae T11 through T13. The three vertebrae were then fused by clamping them on either side with small metal bars. Subsequently, a dorsal laminectomy was performed at thoracic level 12 without removal of the dura mater. The bone was then sealed using a combination of cyanoacrylate and dental acrylic. Next, a top plate was attached to the metal bars. A silicone elastomer was applied over the spinal cord, and the chamber sealed by a glass coverslip on top. Finally, the skin was glued to the base of the top metal-plate. During imaging sessions, the spines were stabilized and the mice elevated off the surgical table to minimize artifacts caused by breathing of the animal. For weeks after injury, the animals did not show any apparent signs of spinal or vertebral damage and behaved normally as assessed by two independent tests of locomotor function. In addition, the axon morphology was stable for periods of up to 8 weeks of imaging. Solely, a mild inflammation at the site of laminectomy was observed. In comparison to a laser injury of the spinal cord though, this inflammatory response is many orders of magnitude smaller and should not interfere with regeneration studies. However, in spite of leaving the dura intact, the authors reported that the image contrast and lateral resolution diminished over time owing to a gradual fibrous growth over the surface of the spinal cord. Due to this fibrosis, duration of imaging times between individual animals varied from as little as 5 days to as many as 140 days with 13 imaging sessions. In animals where fibrous growth was dense, axons could not be resolved appropriately at any depth. In those animals with minimal fibrosis (approximately 50% of all animals) axons were imaged for more than 5 weeks and imaging depth was limited to 30–50 μm using two-photon laser scanning microscopy. In occasionally myelin-poor regions between adjacent dorsal roots however, imaging was possible up to 300 μm in depth, allowing imaging of regions well into the dorsal horn. Despite the apparent advantages, the approach by Farrar et al. requires sophisticated custom made implantation materials and surgical apparatus. Hence, a second group (Fenrich et al., 2012) developed an effective, low cost and high throughput method for implantation of glass windows into the spinal cord. In short, the muscles covering the spinous and transverse processes of the thoracic vertebra 12 (T12) through lumbar vertebra 2 (L2) were resected and the animals suspended from a spinal-fork stereotaxic apparatus. Prior to surgery standard staples were shaped, sterilized and placed to fit closely along the sides of the pedicles of the exposed vertebrae. The staples were then glued into place with cyanoacrylate and served as anchoring points for the glass window setup. A laminectomy was performed without damage to the dura mater. A line of liquid silicone elastomer was applied to the surface of the spinal cord, and a glass window was immediately placed on top so that the glass rested on the edges of the bone and was flat relative to the spinal cord. Then cyanoacrylate was applied to the surrounding bone and muscle. Finally, a layer of dental cement was applied over the cyanoacrylate to stabilize the setup. For imaging with two-photon laser scanning microscopy, animals were connected to a clamp by the embedded paperclip. Using this approach, the mice were highly mobile after surgery, showed no signs of distress and continued to gain weight throughout the experiment. Furthermore, the implanted glass window *per se* improved overall image quality by reducing the rostrocaudal curvature of the spinal cord and the amplitude of respiratory and cardiac movement artifacts. Despite the gradual formation of a thin vascular tissue layer between the window and the spinal cord, the authors reported that high imaging quality was preserved throughout the experiment duration. Fenrich et al. imaged the spinal cord at sub-cellular resolution for up to 350 days, and 22 imaging sessions. Moreover, using this approach the success rate was raised to 86% at two weeks after implantation and to 77% at five weeks after implantation.

In summary both approaches require only a single surgery, and enable high imaging frequency and long imaging duration over time. In addition, the spinal cord implants do not interfere with normal behavior, such as locomotion, grooming and rearing of the animals. This provides the great opportunity to study not only the disease dynamics and putative responses to therapeutic agents at the cellular level, but also the direct assessment and correlation of the potential behavioral outcome in the animals.

In addition to the development of these novel long-term imaging strategies it is possible to complement *in vivo* imaging with classical post-mortem analyses. At any time point after SCI, the animals can be perfused and prepared for immunohistochemistry. This is feasible since the fluorescence signal of the GFP positive axonal structures is maintained both in live and paraformaldehyde-fixed tissue (Feng et al., 2000). Thereby, the severed axons and the local environment in which the observed axonal changes took place can be reexamined up to months after dynamic imaging. In addition, the observed dynamic changes may be correlated with ultrastructural changes by processing the tissue for electron microscopy (*e.g.* Di Maio et al., 2011; Erturk et al., 2007).

Limitations of *in vivo* imaging

Despite its numerous advantages, *in vivo* imaging is a fairly novel approach with diverse procedural limitations. The introduction of further technical refinements as well as the continuous development of modern microscopy approaches will steadily improve this technique and finally overcome the major drawbacks. Until then, some limitations which are inherent to *in vivo* imaging as well as limitations common to both *in vivo* imaging and classical tracing techniques need to be considered while interpreting data.

One general limitation of using mice as model system for SCI is their inherent regeneration capability and their small size in comparison to bigger or more slowly regenerating species (reviewed in Griffin et al., 2010). These characteristics inevitably limit the spatial and temporal resolution in regeneration studies using mice as model system and particular care needs to be dedicated to the correct placement of the lesion and timing of consecutive outgrowth measurements. Additionally, strain diversity is likely to have substantial effects on the regeneration behavior of mice (*e.g.* Basso et al., 2006). The differing regenerative and behavioral responses to SCI suggest that inherent genetic factors influence recovery and need to be considered in studies with inbred or genetically engineered mouse strains.

A prerequisite for performing a reliable *in vivo* imaging experiment is extensive expertise of the experimenter in spinal cord surgery and imaging to minimize investigator-induced effects. Prolonged anesthesia of the animal typical for real-time experiments, the surgical procedure itself, phototoxicity and the risk of infection during imaging sessions constitute potential stressors for the nervous system. Moreover, *in vivo* imaging is complicated by anatomical variations and absence of reliable and recurrent anatomical reference points between individual animals. This makes it challenging to consistently identify and place the lesion at the same location of the spinal cord in different animals. In addition, the extent of the lesion (depth and width of the injury) needs to be standardized to minimize variability in the results.

In addition to the described investigator-induced changes, interpretive pitfalls can also affect the outcome and reliability of an *in vivo* imaging study. Most importantly, skewing of geometrical relationships of the tissue due to changes in orientation of the animal complicates the finding and imaging of the same region of the spinal cord during consecutive imaging sessions. Finding stable landmarks, first for imaging and later for reliably quantifying axonal outgrowth, thus poses a challenge for *in vivo* imaging. Most suitable for this purpose are prominent blood vessels or easily identifiable axonal branches and trajectories. However, spinal cord tissue gradually changes over time following a traumatic lesion. Often, next to formation of a scar, smaller blood vessels are degraded and new blood vessels appear. Changes in the labeling due to bleaching of the fluorescence signal, degeneration or regenerative growth of axonal structures may also affect the appearance of the tissue under observation. Finally, alterations caused by an animal's breathing, pulse or muscle contractions during imaging can also lead to image distortion and therefore

artifacts in the data. These temporal changes necessitate the development of a registration method to align image sequences for later identification and quantification of axonal outgrowth. Until then, measurements of regenerative axonal outgrowth using *in vivo* imaging may contain slight variability in the data.

A further drawback of *in vivo* imaging is its limited applicability in diverse SCI paradigms and imaging of specific tracts. To date, *in vivo* imaging is most convenient to study the regenerative behavior of sensory axons running within 30–50 μm in depth of the most superficial layer of the dorsal columns. At best, two-photon laser scanning microscopy can increase the imaging depth to a couple of hundred μm in depth. This provides that the fibrous growth over the exposed spinal cord is minimal and that the amount of light-scattering myelin is relatively low. Moreover, to obtain adequate image quality and resolution over a considerable amount of time it is important that the lesions performed are relatively small, ideally affecting only few axonal fibers. If larger lesions like contusion, transection or hemisection are performed, massive bleeding and tissue damage will occur. In the case of a hemisection or transection the dura is usually removed. Therefore the bleeding may be controlled and the tissue cleared for imaging by superfusing the site of laminectomy with artificial CSF or saline solution. As a result, *in vivo* imaging is applicable even for larger lesion paradigms, bearing in mind that only the superficial layers of the dorsal column may be imaged. However, in a more clinically relevant injury model like contusion the dura mater is generally left intact. Therefore, blood, intraparenchymal cells and axonal debris will spread under the dura and make imaging virtually impossible. Also problematic for *in vivo* imaging is the gradual formation of a fibrotic scar after large lesions. While in the acute phase after a large lesion imaging is still feasible, at later time points (>3 days) the resolution and image quality will decrease to a point where imaging is no longer possible. In addition, in large lesion paradigms the inflammatory and immune response will be considerably higher than in small lesions and needs to be taken into account. In conclusion, large lesion paradigms are probably most suited for *in vivo* imaging in studies with intent to analyze the effect of the immune response on axonal regeneration, as well as for studies that require dynamic data from *in vivo* imaging to be supplemented with data from deeper tissue layers, such as the corticospinal tract (CST) for example.

Lastly, the choice of the transgenic mouse line needs to be reconsidered in large lesions. Given the small amount of fibers likely to regenerate after large lesions, the use of mouse lines such as YFP.H or GFP.H – which express a fluorophore in up to 80% of their sensory fibers – may be more appropriate. However, if too many axons are labeled, it will be increasingly difficult to follow the trajectories of single degenerating or regenerating fibers. To this end, the development of novel clearing and subsequent three-dimensional (3D) imaging techniques of fixed spinal cord tissue could be helpful (Erturk et al., 2012). They enable the visualization of trajectories of regenerating axons throughout the entire depth of the spinal cord by rendering the tissue transparent, without the need for time intense tissue sectioning. Thus, clearing and 3D-imaging of deeper layers of spinal cord tissue could supplement dynamic *in vivo* imaging data and contribute to assessing the success of experimental therapies in SCI.

Perspectives

To this point, regeneration studies of the mammalian CNS have been dominated by static end-point measurements. New model systems of spinal cord regeneration will increasingly complement these established models. In particular, dynamic imaging approaches like *in vivo* imaging, that are adapted to observe and assess pathological events following SCI while they are occurring in the living animal will be of great value. Most importantly, *in vivo* imaging may now be applied to monitor injured axons for extended periods of time (weeks to months) by employing advanced techniques such as multi-photon microscopy and chronic preparations like the spinal chamber or spinal cord glass window (Farrar et al., 2012; Fenrich et al., 2012).

In addition, manipulation of gene expression in the nervous system of a living animal will be important to unravel the role of specific genes in spinal cord regeneration. So far, inducible knockout approaches using the Cre recombinase and acute virus-based gene delivery have been successfully used to modulate gene expression. However, these methods are expensive and involve labor-intensive processes. Moreover, viral particles may have undesired effects on the immune system of the host, which could potentially affect the experimental results and interpretation of data. Hence, one of the most exciting novel tools to manipulate gene expression in mice with high spatiotemporal control is *in vivo* electroporation of adult sensory neurons (Saijilafu et al., 2011). Using this novel approach the authors electroporated fluorescent constructs into sensory neurons and showed that the transfected genes start to be expressed within a few hours after electroporation. In addition, different constructs were electroporated into two independent dorsal root ganglions (DRGs) of the same animal. This allows both control and experimental groups to be included in the same animal. Hence, the throughput is increased, due to the reduced number of animals needed in a study and the easy availability of expression constructs or siRNA/shRNA on the market. Finally, the expression level of fluorescent proteins in single nerve fibers is high enough to use this technique for *in vivo* imaging (Laskowski and Bradke, unpublished results). Taken together, this method will serve as a powerful tool to genetically dissect axon regeneration in the living animal.

With the advent of conditional technology, genetic axonal labeling and *in vivo* imaging of injured axons, mouse models continue to have a prominent role in dissecting the complex molecular cascades following SCI. Nonetheless, the development of rat models that express genetically encoded indicators may turn out to be of even greater importance, since histology and immunohistochemistry have shown significant differences in the SCI neuropathology between the two species. While mice show accumulation of a dense fibrous connective tissue that fills the lesion site (Inman and Steward, 2003), SCI in rats results in large cystic cavities that extend rostral and caudal to the initial injury site (*e.g.* Stokes and Jakeman, 2002). The greater physiological similarity of rat and human SCI pathology and the larger size of rats in comparison to mouse models allow for more reliable manipulations and use in a variety of complex surgical paradigms such as SCI (Jakeman et al., 2001). However, the lack of tools available to manipulate the rat genome had slowed the use of this animal model so far. Foremost, the introduction of a novel Thy1-GFP rat model whose axons constitutively express GFP and which was originally introduced to study facial nerve regeneration (Magill et al., 2010), will prove to be a powerful translational tool for diverse SCI paradigms. In addition, it will be intriguing to see whether *in vivo* DRG electroporation and subsequent *in vivo* imaging of the rat spinal cord might also be feasible.

Together, the recent advances in genetically encoded indicators and microscopy techniques allow capturing key neuronal events as they take place in their native habitat. Thus, dynamic imaging approaches like *in vivo* imaging provide a powerful testing ground for results from molecular investigations and classical tracing approaches.

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