

## Review Article

# Intravital imaging of immune cells and their interactions with other cell types in the spinal cord: Experiments with multicolored moving cells

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## ABSTRACT

Intravital imaging of the immune system is a powerful technique for studying biology of the immune response in the spinal cord using a variety of disease models ranging from traumatic injury to autoimmune disorders. Here, we will discuss specific technical aspects as well as many intriguing biological phenomena that have been revealed with the use of intravital imaging for investigation of the immune system in the spinal cord. We will discuss surgical techniques for exposing and stabilizing the spine that are critical for obtaining images, visualizing immune and CNS cells with genetically expressed fluorescent proteins, fluorescent labeling techniques and briefly discuss some of the challenges of image analysis.

## 1. Introduction

Intravital imaging of immune cell movement in the spinal cord has presented a number of specific challenges but has resulted in a greater understanding of immune cell interactions within the nervous system. Watching cellular movement in the spinal cord has provided compelling evidence for the role of the immune system in a variety of disorders. Immune mechanisms play a large role in evolving spinal cord pathology, whether it be from traumatic injury or disease processes such as multiple sclerosis or amyotrophic lateral sclerosis. A full, intact immune response requires: (1) signaling at the primary site; (2) signaling at secondary lymphoid organs such as the spleen, lymph nodes and bone marrow and; (3) immune cell movement throughout the body to the site of inflammation via the vascular system. Models that use explanted tissue or slices are intriguing but limit the full dynamics and complexity of the immune response. Tissue within an explant can be maintained at close to physiologic conditions, but the amount of trauma the tissue has sustained as well as the absence of intact circulatory and lymphatic systems make their use for immune applications limited. To this end, intravital imaging utilizing intact animals with fluorescently labeled cell populations to document the movements and cell-to-cell interactions of immune cells within the nervous system has provided spatiotemporal data that cannot be obtained by any other method. (Fig. 1)

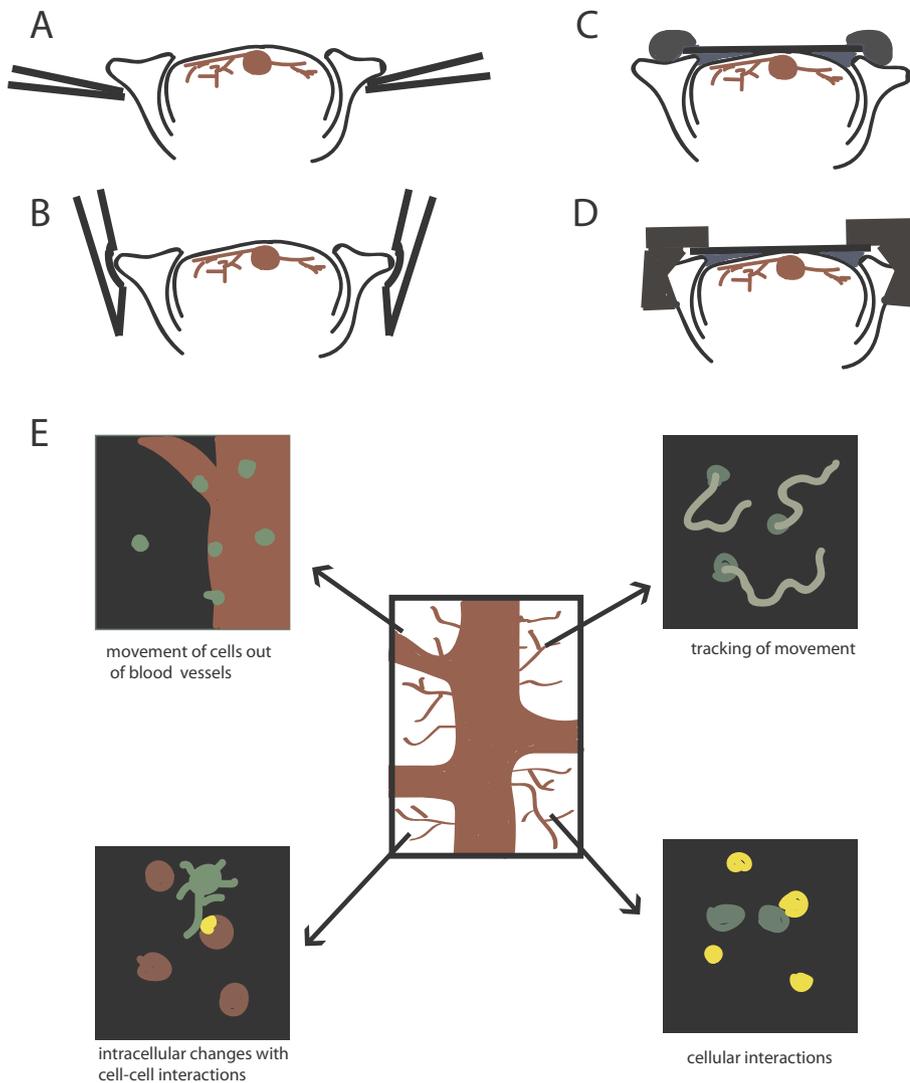
Here, we will discuss some technical hurdles specific to investigation of the immune system in the spinal cord and some of the unique situations that may arise when planning intravital imaging experiments

involving the immune system. These include methods of surgically exposing the spinal column to allow light penetration and then maintaining this exposure for the duration of imaging. This also requires stabilizing the tissue with one of a variety of fixation devices to minimize movement from breathing and cardiac function. The need to expose and stabilize tissue has yielded an assortment of methods including acute surgical procedures, chronically implanted glass windows and imaging chambers. A variety of techniques have been successfully utilized to image axonal movement after traumatic injury and blood vessel biology in several models but have proven particularly challenging in studying immune system dynamics due to the inflammatory reaction to implanted devices and to surgical procedures themselves. Beyond surgical techniques, complicated multi-color labeling schemes need to be carefully developed for each experiment, most often utilizing combinations of fluorescent labeling strategies to identify specific cellular subgroups with multiple distinguishable fluorophores to look at cellular interactions and potentially functional molecular markers.

Labeling strategies to identify and differentiate multiple structures simultaneously typically involve combinations of fluorescent labeling techniques including transgenic animals with fluorescent proteins expressed under cell type specific promoters, exogenous fluorescent dyes, cell transfer from either a genetically labeled animal or labeled cell culture as well as functional reporters such as calcium dyes. These methods can also be used alongside traditional techniques such as neuronal tract tracing and fluorescently labeled antibody staining. Often multiple strategies in concert are required to answer complex and interesting questions about cellular interactions. Hopefully, this

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**Fig. 1.** Examples of surgeries/types of windows and some common questions addressed with two photon imaging.

A: example of flat clamps used to stabilize the spinal cord from below the lateral processes, similar to the Stoelting spinal cord adapter. B: Example of shaped clamps that fit over lateral spinal processes. C: example of a simple spinal window held in place with cyanoacrylate glue (purple) and dental acrylic (gray). The space between the glass is filled with a polymer such as agarose or KwikSil. D: Example of a spinal window imaging chamber with machined clamps around the lateral vertebral processes and above the glass that are held together with a screw to keep the glass in place above the spinal column. E: examples of immune cell related questions often addressed with intravital imaging.

technical discussion will provide a basic level of understanding of the complex experimental design needed to obtain useful information about the immune system in the spinal cord using live two-photon imaging and can guide initial discussions about experimental design.

## 2. Surgical techniques for exposure of the spinal cord and stabilization for imaging

Achieving adequate exposure and stability of the spinal column to allow for imaging with minimal movement artifact is critical to the success of intravital imaging of the spinal cord. This requires a single or multi-level dorsal laminectomy for more widespread imaging. However, if limiting imaging to the intervertebral spaces is acceptable, imaging is possible by removing part of the ligamentous tissue between the bones and imaging through this space (Kim et al., 2010). The spinal cord can then either be imaged transiently or a glass window can be implanted for chronic studies. Imaging of immune processes requires special consideration and there are pros and cons for acute versus glass window imaging techniques as they both cause varying degrees of extra tissue damage and inflammation requiring additional thought about controls.

### 2.1. Acute surgical procedures

Acute surgical procedures to expose spinal cord tissue for imaging

can be very useful due to their simplicity and lack of need for long term anti-inflammatory medication but are limited by the number of time-points that can be collected and increasing difficulty of surgery with repetition due to the development of scar tissue from the surgical approach and repeated exposure of the dura. These can easily be used regardless of the vertebral level of imaging - acute preparations can be performed in the cervical, thoracic and lumbar spinal cord with only small adjustments in technique and positioning to allow for the correct angles for imaging and stabilization of the animal. Acute surgical procedures to image the dorsal portion of the spinal cord, including the dorsal columns and the dorsal roots, start with a skin incision at the spinal level of interest. This is followed by incision of the external oblique muscle to expose the dorsal processes of the vertebrae and then by removal of the paraspinal muscle. Exposure of a region of the spinal cord can be established either by removing the intervertebral disk or by a laminectomy to remove the dorsal portion of the spinal column. Bleeding from the posterior branch of the lumbar artery during laminectomy can be staunched but requires some patience. Bleeding can be stopped with pressure and time or cauterization but can remain a challenge throughout an imaging session. Severe bleeding can jeopardize an experiment completely if blood continues to obscure the imaging field.

Movement artifact must be directly addressed, especially in acute preparations without the stabilization of a permanently implanted window with either metal or acrylic supports used to hold the window

in place. Breathing and heartbeat result in periodic movement artifact, examples of which have been published in protocols for spinal cord imaging (Farrar et al., 2012) that prevent stable scanning of a single image or tracking of structures over time. Most techniques focus on reducing this artifact by holding the tissue still with a variety of clamps and other mechanisms that stabilize and isolate the entire spinal column without touching the neural tissue of the spinal cord aside from normal extension of the animal. Both self-made and commercial apparatuses have been used in successful experiments. Home-made devices for spinal cord stabilization can work well. As an example, a standard double-edged razor blade can be used, using the laser cut holes intended for attachment of the razor to a handle for attachment to the lateral processes of the vertebrae by bending the attachment points and using these as a spring-clamp around the lateral processes (Kim et al., 2010). Another example of a simple, low-profile stabilizing mechanism involves paperclips bent into a loop that can be adhered to the bones above and below the laminectomy (Fenrich et al., 2013a,b). Commercial spinal cord stabilizers are also available that clamp around the lateral processes of the vertebrae above and below the imaging site (Narishige Group, Japan) or versions that use tines that hook underneath the spinal cord (Harvard Apparatus, USA). While these devices are easiest to use in the lower thoracic spinal cord, minor modifications in placement of the stabilizing clamps and surgical techniques to work around the ribs in the cervical regions and the pelvis in lower lumbar regions are typically easy to make. All of these have been successful at reducing movement artifact during imaging, although some degree of heartbeat artifact is unavoidable.

In some cases short-term glass coverslips are placed on top of the spinal tissue (Farrar et al., 2012), which can make preserving a moist environment simpler and provide extra protection for the spinal column during imaging. Acute imaging sessions can be repeated every 3–4 days generally for a maximum of 4 times. In this situation, the open spinal cord is usually covered by a protective material such as gelfoam (Evans et al., 2014) or a gel dura substitute (Kerschensteiner et al., 2005) before the skin is sutured and is easily removed before repeat imaging sessions. However, sterile technique is paramount as the risk of infection increases each time the spinal cord is exposed.

Acute imaging techniques using a variety of these strategies have been used in several models, and we will discuss a few examples here. Open preparations were initially used to look at microglial responses in a variety of pathologies. For example, the response of microglia in EAE and clustering of microglia in perivascular areas was described and then the same model was used to look at the ability of serum proteins such as fibrinogen to trigger microglial activation (Davalos et al., 2012). This led to experiments to study the consequences of diminishing microglial activation via pharmacologic methods. Open techniques facilitated the application of drugs and a simple model that only involved a single surgery (Davalos et al., 2012). In a different pathological model, the response of microglia to methylene blue in ALS, showed that methylene blue inhibits microglial reaction to a laser injury (Dibaj et al., 2012). Interactions between motor-neurons and glia as well as responses of microglia to ATP and calcium signaling were also described using open surgical models that allowed for easy access for induction of drugs (Cartarozzi et al., 2018). These techniques have also been used in traumatic injury models to look at monocytic reaction to injury. They allow for easier control of bleeding than an injury immediately covered by a glass window and can avoid steroid treatment (Evans et al., 2014). These are only a few examples of the data obtained with open surgical preparations that have allowed for interesting experimental interventions. Many more permutations have been used and are available for future experiments.

## 2.2. Considerations specific to cervical and ventral portions of the spinal cord

Imaging of the cervical spinal cord allows for the investigation of

additional questions, such as comparing movement of immune cells at different spinal cord levels in EAE and other diseases, or imaging of neural afferents from the upper portion of the body. The cervical spinal cord, due to the proximity of the lungs and ribs, is more difficult to stabilize and typically requires intubation and ventilation of the animal. Imaging is synchronized with mechanical respiration using the ventilator to predict position of the tissue during the respiratory cycle (Haghighyegh Jahromi et al., 2017). Mechanical ventilation has been used to minimize artifact from breathing (Kerschensteiner et al., 2005; Dibaj et al., 2010) and can be advantageous to provide consistent blood oxygenation and management of acidity levels during long periods of anesthesia for time lapse imaging.

The dorsal aspect of the spinal cord is convex in shape, so the space between the window and the spinal column is often filled with agarose, silicone (Dombeck et al., 2007) or another substrate such as Kwik-sil (2) to prevent: (a) shifting; (b) the accumulation of immune cells; (c) formation of scar tissue, and; (d) bone regrowth under the glass window. In other cases the glass window is clamped tightly against the dorsal columns utilizing Kwik-sil plus the lateral processes of the vertebrae to minimize the space and keep the chamber and glass coverslip in place (Farrar et al., 2012). These windows are implanted in an area that is usually under constant flexion and so the implant impedes movements significantly more than cranial windows.

The dorsal portion of the spinal cord is the simplest area to image but limits the utility of the technique to pathophysiology involving only the dorsal columns and the medial parts of the dorsal roots. Access to gray matter in the spinal cord is much more difficult, as is accessing long tract pain/temperature and motor pathways. Acute surgical preparations to approach some of these more difficult regions are becoming more common. One such preparation allows for imaging of motor neurons using a wire and acrylic device to turn the exposed spinal cord to the side and retract tissue away from the imaging site (Kerschensteiner et al., 2005). In this case, a retrograde tracer, known to collect in areas with motor neurons, is injected and then imaged with this preparation to confirm that the imaging area is directly accessing motor neurons (Cartarozzi et al., 2018). Flat glass coverslip windows are commonly used and provide protection for the spinal cord. Flexible film such as PDMS may prove very useful in eliminating empty space between the spinal cord and the coverslip thus decreasing the risk of bone regrowth and immune cell accumulation in this space while also allowing for more variation in shape and placement of the imaging window. The flexible film may also permit easier injection of drugs, dyes and cells into the spinal cord or imaging field and perhaps for increasing access to more lateral portions of the cord (Heo et al., 2016).

## 2.3. Specific considerations with chronic window implantation and the immune system

Chronic spinal windows involve a small glass coverslip implanted directly above the dorsal columns of the spinal cord which is affixed to the surrounding bone using a tissue compatible material such as dental acrylic (Lang dental) and cyanoacrylate glue. These techniques are very powerful, commonly used (Davalos et al., 2008; Davalos and Akassoglou, 2012; Farrar et al., 2012; Fenrich et al., 2012; Fenrich et al., 2013a,b; Figley et al., 2013; Haghighyegh Jahromi et al., 2017) and are reviewed in this issue. Use of a spinal window is by its very nature a foreign body implanted along the surgically exposed spinal cord. While chronic window implants are especially useful with longer time courses, they do require some additional thought when used in immunology applications. While acute surgical procedures are also inflammatory and require thought in immunology models, they generate more immediate tissue trauma without a permanent foreign body. Stabilization of the tissues with chronic window implantation is mechanically different from that with acute surgical procedures and typically relies on attachment of the vertebra to the window housing.

Pharmacologic management of inflammation due to the window

implantation allows for higher imaging quality with increased clarity, increased tolerance of the implants and decreased swelling of the nervous tissue itself. The steroid, Dexamethasone, is administered for its anti-inflammatory properties for the first few days after window implantation. This is standard practice in both cranial windows and spinal cord windows, but dexamethasone is a potent immunomodulator used in the clinical treatment of spinal cord injury and in autoimmune central nervous system diseases, although clinical outcomes from treatment are not strikingly therapeutic when used alone (Bracken et al., 1992). The use of anti-inflammatories is often needed for successful surgery and have been shown to normalize the appearance of microglia by a week after implantation (Farrar et al., 2012). Although steroids are stopped within the first few days after implantation, there is a high possibility of affecting the disease process under investigation if treatment is given during the course of disease. Need for steroid administration and the course of treatment should be carefully discussed and controls considered, as well as the timing of induction of disease in relation to this short steroid course. These issues should be considered and either controlled for or timed to avoid the most active disease processes.

Of interest, all surgical procedures and window implantation surgeries require the administration of pain medications, and these also need to be considered. Acute surgical imaging can be performed under anesthesia with analgesia provided after experiments, but chronic windows allow for initial administration of pain medications without subsequent chronic dosing during the period of experimentation. Carprofen, a common veterinary NSAID, is the most often used pain medication, and also has some anti-inflammatory effects, often described in traumatic brain injury studies (Thau-Zuchman et al., 2012). Buprenorphine, an opiate, is also commonly used and has fewer immunologic complications and good pain control, and also has relatively well known pharmacology (Santiago et al., 2009) but will not assist with window clearing and is also more difficult to work with due to regulatory agency concerns with opiates.

The impact of a spinal window implant on the quality of intravital imaging was assessed using a Thy-1 CFP, LysM EGFP, CD11c EYFP triple transgenic mouse with rhodamine or quantum dots to visualize blood vessels (Fenrich et al., 2012). Thy-1 CFP was used to identify axons (Feng et al., 2000), LysM to identify a group of macrophages, monocytes and granulocytes (Faust et al., 2000). CD11c is used to identify dendritic cells, although it is also expressed in a subset of B and T cells (Lindquist et al., 2004). LysM<sup>+</sup> and CD11c<sup>+</sup> cells were followed over time in a window with no added pathology as a control. Cellular infiltration peaked at 8 days after implantation for CD11c<sup>+</sup> cells and 6 days after implantation for LysM<sup>+</sup> cells. Quantum dots were not taken up by perivascular cells, whereas both a basal level of phagocytosis and increased phagocytosis with inflammation could be visualized when using rhodamine. Dexamethasone and rimadyl, both anti-inflammatory medications - were administered prior to and directly after spinal window implantation. However, cellular infiltration was still observed up to 8 days post implantation (Fenrich et al., 2012). While this cellular immune response to the window is predictable and can be controlled, it must be considered in the same way that an immune response must be considered in acute surgical models. Even the baseline normal level of phagocytosis of vascular dyes by perivascular cells and cells underneath the glass window can be enough to impair imaging of deeper tissues. If immune cells are fluorescently labeled and accumulate in the space between the dura and the implanted glass this can lead to limited imaging depth and can obscure the pathology of interest occurring in the underlying spinal cord parenchyma.

### 3. Fluorescent labeling strategies specific for imaging of the immune system

Fluorescent labeling of multiple cellular groups has been essential for research involving immune cell function by allowing for

identification, tracking and observation of cell-to-cell interactions that are critical to the function of the immune system. Some of the commonly imaged cell groups involve neurons, astrocytes, microglia, oligodendrocytes, macrophages, neutrophils and T-cells using combinations of genetic strategies, cell dyes and cell transplantation and lineage tracing methods. (Fig. 2)

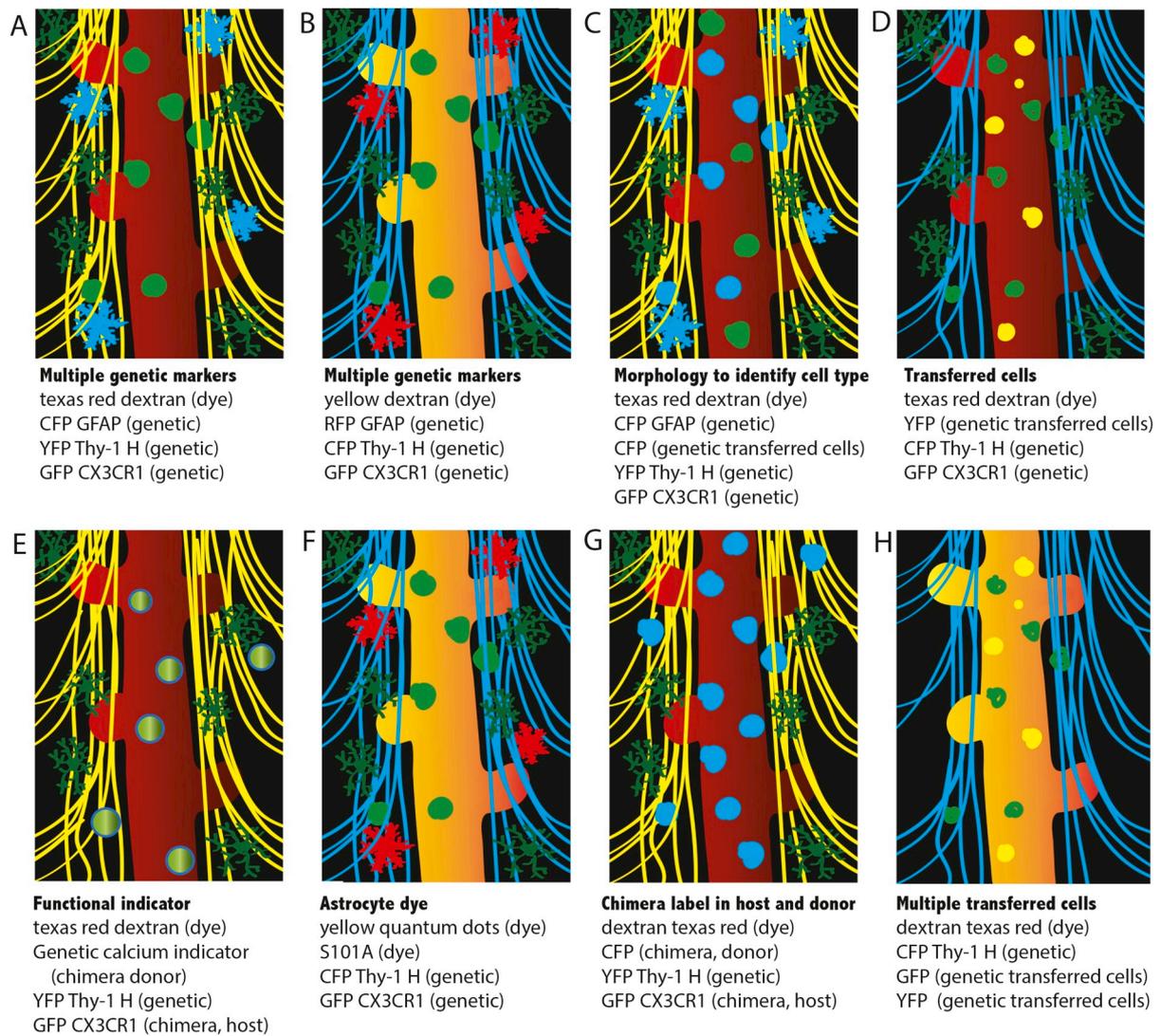
#### 3.1. Neurons

Neurons were the first cells to be imaged in the spinal cord with a genetically induced cell filling fluorescent dye (Kerschensteiner et al., 2005). The beautiful, bright labeling obtained in the Thy-1 mouse lines available in multiple colors and with multiple subsets of labeled neurons was critical for the advancement of these techniques (Feng et al., 2000). These lines have been particularly useful due to varying percentages of cells expressing fluorescent proteins allowing for identification of single cells and tracing of specific axons. For the purpose of spinal cord imaging, the Thy-1 YFP H line has been very useful with 30% of the axons in the dorsal columns made visible with brilliant YFP labeling. Thy-1 GFP M and Thy-1 CFP mice have similar subsets of neurons labeled in the dorsal columns and are very useful in multiplex fluorescent studies. These mice in various combinations are used in most spinal cord imaging applications, looking at everything from the dynamics of the cut ends of axons in regenerating dorsal roots (Lorenzana et al., 2015) to axonal damage after contusion injury (Horiuchi et al., 2015) to dieback after a laser induced injury. Thy-1 mice are commonly used as landmarks for other studies and positive controls for practice surgeries and setting up imaging because of their bright, easy to image fluorescence.

Traditional techniques in neuroscience, such as tract tracing with fluorescent markers can be useful, especially for identifying specific groups of neurons. In 2018, Cartarozzi used a sciatic nerve tracer to identify their imaging site as a motor pathway (Cartarozzi et al., 2018). A pH sensitive sciatic nerve tracing dye has been used to identify portions of axons that have been incorporated into lysosomes in macrophages following a dorsal column crush injury (Evans et al., 2014). Use of dextran tracers from the sciatic nerve can label the ends of damaged axons in spinal cord injury as the dye accumulates in the most proximal portion of damaged axons (Busch et al., 2009).

#### 3.2. Microglia and myeloid cells

Microglia and myeloid cells, including macrophages, are phagocytic cells and part of the innate immune system. They are known to play key roles in healthy CNS biology as the cellular component of the innate immune system in the CNS (Li and Barres, 2018) and are also involved in many forms of pathology. Identifying microglia and macrophages by cell surface markers has remained a challenge, but there are several easily available mouse strains with genetically expressed fluorescent proteins in microglia and macrophages that have made these cells easy to study with intravital imaging. CX3CR1, the fractalkine receptor, is highly expressed on microglia and on a subset of macrophages and has been particularly useful for cell identification with the availability of CX3CR1-GFP transgenic knock-in mice that have provided a platform for studying the biology of CX3CR1 and the fractalkine signaling pathway (Jung et al., 2000). CX3CR1 labeled cells have lent themselves particularly well to intravital imaging with beautiful, detailed, bright green cellular morphology. The CX3CR1 transgenic mouse line can be readily crossed with other transgenic mice and is a good model for the study of multiple diseases. However, there is a caveat that CX3CR1, the fractalkine receptor, is expressed at lower levels than in normal animals, or is absent in the homozygous CX3CR1<sup>GFP/GFP</sup> mouse. Studies using CX3CR1<sup>+/GFP</sup> and CX3CR1<sup>GFP/GFP</sup> mice have been useful to interrogate the immune response to disease in the spinal cord, which ranges from short-term imaging of microglia in response to small sterile laser injuries (Davalos et al., 2005) to cellular level studies looking at



**Fig. 2.** A few examples of possible imaging schemes illustrating a few of the many possible combinations of cells that can be visualized with multiple fluorescent labeling techniques.

**A:** Dextran Texas red labeled blood vessels in a triple transgenic mouse with GFP CX3CR1 microglia and circulating cells, CFP GFAP astrocytes and YFP Thy-1 dorsal column axons. **B:** Yellow quantum dot labeled blood vessels in a triple transgenic mouse with GFP CX3CR1 microglia and circulating cells, RFP GFAP astrocytes and CFP Thy-1 dorsal column axons. **C:** Dextran Texas red labeled blood vessels in a triple transgenic mouse with GFP CX3CR1 microglia and circulating cells, CFP GFAP astrocytes, YFP Thy-1 dorsal column axons. Adoptively transferred CFP labeled immune cells introduced by IV injection, to illustrate the possibility of distinguishing cell types by morphology. While using morphology to distinguish cell types is generally not advisable since unusual looking cells or cells in close proximity cannot be distinguished (for example at the bottom of the panel), it is included here because it is often discussed when color combinations become limited. **D:** Dextran Texas red labeled blood vessels, triple transgenic mouse with GFP CX3CR1 microglia and circulating cells, CFP GFAP astrocytes and YFP Thy-1 dorsal column axons. Ubiquitin YFP adoptively transferred cells in circulation. **E:** Dextran Texas red labeled blood vessels, chimeric double transgenic mouse with GFP CX3CR1 microglia and YFP Thy-1 dorsal column axons as the host and a genetically labeled calcium indicator mouse as the donor, replacing GFP circulating macrophages from the CX3CR1 mouse with those expressing a calcium indicator. **F:** Yellow quantum dot labeled blood vessels. Double transgenic mouse with GFP CX3CR1 microglia and circulating cells, CFP Thy-1 dorsal column axons. Astrocytes labeled with SR101. **G:** Dextran Texas red labeled blood vessels. Chimera with the host double transgenic mouse with GFP CX3CR1 microglia and YFP Thy-1 dorsal column axons and the chimera donor bone marrow derived cells labeled with CFP. **H:** Dextran Texas red labeled blood vessels. Single transgenic mouse with CFP Thy-1 dorsal column axons. Circulating adoptively transferred CX3CR1 GFP positive cells and adoptively transferred YFP positive cells isolated from an ubiquitin YFP mouse.

the interactions of microglia with T cells in EAE models (Davalos et al., 2012). CX3CR1<sup>+/GFP</sup> and CX3CR1<sup>GFP/GFP</sup> mice have also been useful for investigations of interactions between macrophages and axons in traumatic injury (Evans et al., 2014), interactions of microglia with blood vessels (Barkauskas et al., 2013) and studies showing that CX3CR1<sup>GFP/GFP</sup> mice have improved repair via sprouting and synaptogenesis in comparison to heterozygous mice (Freria et al., 2017). Different methods have been used to distinguish between the several types of cells that express CX3CR1. The two most common CX3CR1 positive cell types are microglia and infiltrating macrophages. Microglia, the

resident immune cells in the CNS, are developmentally derived from the yolk sac during embryology (Ginhoux et al., 2010) whereas macrophages develop in the adult bone marrow and gain access to the CNS via the circulation. The first technique used to separate these two cell types involves CX3CR1 GFP transgenic mice crossed with CCR2 RFP mice in order to distinguish between a subset of infiltrating macrophages and resident microglia (Jax: 032127). CCR2 is expressed in a subset of peripheral monocytes, T-cells and NK cells but not microglia. Since CX3CR1 is expressed in microglia as well as a population of peripheral monocytes, double positive CX3CR1/CCR2 cells can be used

to track movement of this population of peripheral macrophages (Saederup et al., 2010; Mizutani et al., 2012) and separate them from microglial populations. This model was useful in determining whether resident microglia or peripheral monocytes were the major cause of axonal damage in EAE, identifying infiltrating macrophages in the cell accumulation in the CNS, and also identifying a role for the CCR2 receptor in trafficking of these cells (Saederup et al., 2010). While the CX3CR1/CCR2 double transgenic animal allows a certain amount of specificity in distinguishing CNS from peripheral cells, it comes with the caveat that the relevant cell surface proteins have been replaced by fluorescent proteins that may be phenotypically different than their normal counterparts (Mizutani et al., 2012). Loss of CX3CR1 and CCR2 show changes in phenotype and pathology in EAE (Huang et al., 2006), spinal cord injury (Ma et al., 2002; Donnelly et al., 2011; Freria et al., 2017) and LPS toxicity (Corona et al., 2010). Double labeled CX3CR1/CCR2 positive cells are derived from bone marrow and not microglia, but do not necessarily identify cells as peripheral - there are still populations of singly labeled CX3CR1 and CCR2 labeled cells in the periphery (Saederup et al., 2010), making this model imperfect for distinguishing microglial origin cells from those with bone marrow origins.

Phenotypic plasticity of macrophages in inflammation and disease is temporally modulated. While consensus on classification of macrophage phenotypes is still evolving, the general scheme of M1 versus M2 is commonly used, although debated. The defining feature of M1 macrophages is a destructive inflammatory state, expressing iNOS. The defining feature of M2 macrophages is an anti-inflammatory repair state, commonly expressing arginase. There is well known overlap between these categories and gradients of phenotypic activity, potentially with other functions. Regardless, a general consensus is that arginase is a defining feature of M2 wound healing phenotype macrophages and iNOS is a defining feature of M1 inflammatory macrophages and has been recently used in the EAE model to investigate the time course of inflammation and resolution of disease. It was found that the initial stages of EAE are dominated by iNOS expressing phagocytes and as the disease progresses the cells co-express arginase and iNOS, whereas during lesion resolution, the phagocytes predominantly express arginase (Locatelli et al., 2018). It was also found that macrophages in the meninges express arginase whereas macrophages in the parenchyma predominantly express iNOS.

The Csf1 receptor is expressed only by microglia in the CNS and, thus, fluorescent reporter mice can be considered as an alternative to Cx3CR1-GFP mice. MacBlue is a Csf1R promoter driven mouse line that expresses CFP (Jax #026051) but recently, a Csf1R-mApple fluorescent mouse has been developed which allows these animals to be bred to existing GFP reporter mice (Hawley et al., 2018). The Mafia mouse also uses Csf1R to drive GFP expression with the additional feature that macrophages undergo apoptosis when the dimerizing compound AP20187 is administered providing a way to transiently deplete the mice of macrophages genetically (Burnett et al., 2004). This genetically induced apoptosis is useful as a complement to a commonly used depletion method utilizing chlodronate liposomes that release an apoptotic compound into phagocytic cells. Chlodronate liposomes are effective at phagocytic cell depletion and do not require a transgenic animal giving them broad applicability, but utilize the phagocytic properties of cells instead of the Csf1R gene to selectively deplete a population of cells which include both macrophages but also dendritic cells (Van Rooijen and Sanders, 1994).

### 3.3. Neutrophils

Neutrophils have been imaged intensely because they are highly mobile and are the first responders to acute injury. However, they have been shown to play a role beyond their innate functions and can activate CD8 T cells (Lim et al., 2015). LysM is expressed specifically in macrophages and granulocytic neutrophils, with highest expression in

neutrophils. Mice were created by replacement of one copy of LysM with GFP, yielding bright fluorescence in these two cell types (Faust et al., 2000). These mice have been used in intravital microscopy extensively to monitor neutrophils and to a lesser extent macrophages. In a pin-prick SCI model, LysM<sup>+</sup> cells rapidly infiltrate the injury site and are associated with axonal destruction (Fenrich et al., 2013a,b). To be able to multiplex myeloid reporters it was important to develop reporter mice that produce a range of fluorescent proteins other than GFP. To address this need, Ly6G Catchup tdTomato has been developed to specifically track neutrophils (Hasenberg et al., 2015).

### 3.4. Astrocytes

Historically, staining of astrocytes with SR101 has provided a simple, easy method to identify astrocytes and their complicated morphology, including their finest processes and end feet. After intracranial injection, the dye is taken up by astrocytes and diffuses through gap junctions. Its use is limited by the need to directly inject dye into the tissues (Nimmerjahn et al., 2004), and has the potential to induce seizures and abnormal neuronal activity (Rasmussen et al., 2016). Although with some questionable cellular specificity, SR101 has been useful as an adjunct cellular marker while investigating calcium signaling in astrocytes in the process of identifying cells that had been patched and filled with other dyes and to identify regions within tissues (Johannssen and Helmchen, 2010). Some of the genetic fluorescent constructs for labeling astrocytes have been used in spinal cord imaging while others have so far only been utilized in the cortex (Tian et al., 2006). ECFP under the GFAP promoter has been used in a triple transgenic mouse to image neurons, microglia and astrocytes simultaneously in the stabilized lumbar spinal cord (Nadrigny et al., 2017). The same GFAP-EGFP animal has also been used in another paper describing techniques for visualizing the ventral spinal cord, also highlighting the potential for imaging of multiple cell types. A variety of genetically encoded fluorescent proteins including AMCyan1, ASRed2 and mRFP1 have also been expressed in astrocytes, all derived from fluorescent proteins from corals, and giving more spectral options for double-labeling of cell types with GFP or YFP expressing neurons (Hirrlinger et al., 2005). While these transgenic mice have been used for imaging in the cortex along with other dyes, they have not yet been used extensively in the spinal cord. The complexity of imaging schemes in the spinal cord has generally lagged behind that in the cortex, and the availability of transgenic fluorescent astrocyte animals are likely to be useful as the complexity of imaging experiments in the spinal cord increases. Each of these constructs will also have different applications. The transgenic astrocyte labelled mice have differing resolution of cell processes with some that can reveal the thinnest processes of astrocytes when compared to GFAP immunostaining and some that will not clearly label these fine processes, but regardless, will prove useful for the identification of astrocytes in combination with other fluorescent labels.

### 3.5. Oligodendrocytes

Oligodendrocytes, which myelinate axons and function to increase signal transmission efficiency are interesting in the context of immunology since they are a major target of the immune response in multiple sclerosis and are disrupted in traumatic injury. Being relatively (but, interestingly, not completely) immobile (Dutta et al., 2018), they are less commonly the subject of intravital imaging applications. Oligodendrocytes labeled with DSRed1 expressed under the proteolipid protein promoter, show the complex architecture of myelinating cells with a large cell body and processes wrapping around multiple axonal structures (Hirrlinger et al., 2005). In explanted spinal cords, two-photon imaging has been used to watch oligodendrocyte precursor cells responding to demyelinating lesions, looking at their dynamic interactions with the demyelinated axons (Greenberg et al., 2014) and could also be used in live animal preparations.

### 3.6. T-cells

T-cells are a heterogeneous population comprised of several sub-populations with distinct functions: (1) cytotoxic CD8<sup>+</sup> T cells; (2) T helper CD4<sup>+</sup> T cells of which an important subset are T regulatory cells; and (3)  $\gamma\delta$  T cells. Due to this, T-cells are commonly identified in intravital imaging in the central nervous system either by derivation from fluorescently labeled donor animals or by loading of dyes into cultured cells before transfer to the host animal. T-cell imaging outside of the CNS is its own field of study, focusing on cellular interactions and the formation of cellular contacts and the so-called immunologic synapses (Germain et al., 2006). For immunologic synapse formation to occur, the dendritic cell - T cell interaction needs to be prolonged and thus the contact time has become a measure of activation for T cells. Interaction times can be easily measured with intravital imaging (Mempel et al., 2004). T-cell labeled mice have been used extensively to look at cellular extravasation from blood vessels in the CNS. For example, using an adoptively transferred CD4 T-cell derived from a 2D2 GFP mouse, T-cell arrest, rolling and extravasation from blood vessels was readily visualized (Haghighyegh Jahromi et al., 2017). Crossing 2D2 mice with ubiquitin CFP mice has yielded a genetically expressed fluorescent cell that can then be used for adoptive transfer, demonstrating T-cell infiltration into EAE lesions at sites with leaky blood vessels (Barkauskas et al., 2013). Although less specific for T-cell subtypes than adoptive transfer models, CXCR6<sup>GFP/GFP</sup> mice have CXCR6 replaced with GFP and have been used as a marker for either NKT or T cells depending on their location in the body. CXCR6 labeled mice have been used as a T-cell marker within the CNS where it was found that T cells move linearly through the dorsal columns in a model of EAE (Kim et al., 2010).

The microenvironmental conditions and cellular interactions that result in T cell activation has been a major point of investigation for T cell biologists. The links between T cell activation and intracellular molecular changes with the number and duration of cell-cell contacts *in vivo* has been a fascinating area of research which has illuminated the nuanced and diverse functions of the T cell subsets in different disease states. This is sometimes done with calcium imaging (which will be addressed later) but translocation of other functional proteins has also yielded useful information. A T cell activation reporter construct utilized H2B RFP as a cell nucleus marker and Nuclear Factor of Activated T Cells (NFAT) GFP fusion protein as a signaling reporter molecule that translocates from the cytoplasm to the nucleus upon activation. Use of NFAT-GFP mice provided evidence that the cell-cell interaction did indeed result in the predicted cell signaling and T cell activation (Marangoni et al., 2013).

### 3.7. Mouse lines with multiple genetically encoded fluorescent proteins

Transgenic mice derived from breeding multiple fluorescent protein expressing lines together can often be useful to determine cell-cell interactions between cell populations. For example, "PrismPlus" mice have oligodendrocytes expressing CFP under the myelin basic protein promoter, microglia expressing GFP under the CX3CR1 promoter, neurons expressing YFP under the synaptosome associated protein (Snap25L10a), and astrocytes expressing DsRed under the AldH1 promoter (Gaire et al., 2018). The PrismPlus mouse has been used to determine the reactivity of cells to neural electrode implants. It was found that the microglial population increases as astrocytes ensheath the electrodes, whereas neurons and oligodendrocytes remain largely unaltered (Gaire et al., 2018). Due to the inclusion of the major neural cell subsets, this mouse line could be used in the spine for an array of pathologies and spinal injury models.

Multiple transgenic mouse lines have been developed by the time-consuming process of cross-breeding several fluorescently labeled mouse models. The Debarbieux lab has used a triple transgenic with Thy-1 CFP, LysM EGFP and CD11c EYFP in both an EAE and a pin-prick spinal cord injury model (Fenrich et al., 2013a,b; Caravagna et al.,

2018). However, distinguishing specific immune populations in these animals using only intravital imaging is challenging- LysM and CD11c are both expressed on only subsets of cells in different activation states and only label between 50 and 80% of the total immune cells in the tissue, requiring additional methods such as flow cytometry and traditional immunostaining to identify the remainder of cells and the specific myeloid subsets to which these cells belong (Caravagna et al., 2018). Intravital imaging has proven very useful for showing the movement, localization and interaction of immune cells with blood vessels and neurons as well as the time course of infiltration of cells into developing EAE lesions and interactions within the CNS parenchyma (Caravagna et al., 2018). In a pin-prick lesion in the spinal cord in the same triple-transgenic mouse, imaging again revealed the time course of myeloid cell infiltration into the lesion and interactions with dying axons (Fenrich et al., 2013a,b).

### 3.8. Cre-inducible fluorescent markers

Cre-lox recombinase expressing mice provide some flexibility to express an array of fluorescent proteins in specific cell populations, labeling only cells in which cre is expressed either under a specific promoter or by viral expression or other methods, drastically increasing options for fluorescent protein expression. Tamoxifen inducible cre adds the option for activating genetically encoded fluorescence non-invasively with drug administration. For example, the Cx3CR1 inducible Cre mouse (Jax # 21160) can be used to activate a fluorescent protein such as YFP or calcium indicator in microglia only in cells expressing cre, as opposed to the Cx3CR1<sup>GFP/GFP</sup> mouse, where this knock in is constitutively active. Similarly, CD11c Cre can be used to view specific activated microglia and dendritic cells (Jax # 008068); Csfr1 Cre can be used to label tissue resident macrophages (Jax # 029206); Ncr1 Cre mice can be used to track NK cells (Jax # 022739) and FoxP3 GFP hCre mice can be used to view T-reg cells (Jax 023161). Finally, CD4 Cre mice are especially useful because all T cells express Cre since they express both CD4 and CD8 during thymic selection (Jax 022071). However, there is a tamoxifen inducible Cre recombinase under the control of CD4 which allows Cre to be expressed specifically in peripheral CD4 T cells (Jax 022356).

### 3.9. Calcium imaging

Although commonly used to visualize calcium signaling with axon potentials in neurons in the cortex, calcium imaging can be useful when looking at immune cells as a second messenger indicator of signaling upon receptor binding or cellular responses to disease or trauma. The development of Forster resonance energy transfer (FRET) constructs that have changes in conformation and fluorescence emission in the presence of calcium yielded a revolutionary functional reporter since calcium concentration fluctuations and signaling are vital for so many intracellular signaling processes. Calcium concentration reporter constructs have been well summarized in many reviews, including Takaharu Okada in 2016 (Okada et al., 2016), but have continued to evolve, especially expanding the options for genetically encoded fluorescent calcium signaling molecules. Intravital imaging requires a bright calcium related signal to overcome light scattering from neighboring cells, limiting fluorophores with low quantum yield to comparison to *in vitro* experiments. We will mention a few calcium sensors that have been useful in intravital imaging of immune cells.

Genetically encoded calcium sensors allow longer imaging sessions than synthetic dyes that must be injected into spinal cord tissues and then introduced into cells by opening the cell membrane via electroporation or chemical methods, or directly introduced by cell filling techniques with patch clamp techniques. Many of the newer genetically expressed calcium imaging methods rely on FRET for detection of calcium induced conformational changes. For these dyes to recognize calcium, a calcium-dependent molecular interaction results in a

conformational change, closing the distance between the donor fluorophore and the acceptor fluorophore and allowing FRET to occur. FRET based indicators have the benefit of a baseline labeled cell that can be used to identify and track the motile immune cell then the FRET signal from a calcium bound conformation at a different wavelength is detected as a functional readout. The greatest drawback of the calcium indicators is photobleaching and potential damage to the cells due to the need to image at high scan rates to capture the fast temporal nature of calcium flux. GCaMP6f has become the calcium reporter of choice for neuronal imaging because of the fast calcium flux response due to a circular form of GFP which has a calmodulin and linked M13-peptide that changes confirmation with calcium binding, causing a baseline fluorescence to increase upon calcium binding (Chen et al., 2013). Lck-GCaMP6f allows membrane associated expression of the calcium indicator in tamoxifen inducible Cre expressing astrocytes under the Aldh1 promoter (Srinivasan et al., 2016). “Twitch” calcium sensors, the most commonly used being Twitch-1 and Twitch 2b, are also FRET-based calcium sensors relying on the c-terminal domain of Opsanus troponin C. They have a large dynamic range, quick response properties and were initially validated in both the central nervous system and in lymph nodes (Thestrup et al., 2014). These Twitch and FRET based calcium sensors have been used in combination with imaging of T-cells in EAE in the spinal cord to identify the anatomic location of T-cell activation within the CNS parenchyma (Kyrtasous et al., 2017).

Lck-GCaMP6f allows membrane associated expression of the calcium indicator in tamoxifen inducible Cre expressing astrocytes under the Aldh1 promoter (Srinivasan et al., 2016). In lymphocytes and other immune cells, FRET based calcium signaling biosensors such as Twitch-1 or other fluorescent variants such as Twitch-2b (Thestrup et al., 2014) and YC3.60 (Nagai et al., 2004) have been used. Twitch-2b was expressed in vivo in microglia via a microRNA-9-regulated lentiviral vector which showed that a calcium wave can be observed in neighboring microglia upon laser damage (Brawek et al., 2017). Twitch-1 has been used to look at calcium signaling in T-cells in EAE as they enter into the central nervous system (Kyrtasous et al., 2017). FRET-based genetically encoded calcium indicators have been used in EAE. EAE was induced by transfer of in vitro MOG specific T cells that express a calcium reporter Twitch-1 via retroviral transduction (Mues et al., 2013). Spinal multi-photon imaging of Twitch-1 T cells during onset and at the peak of EAE showed that although there were calcium bursts that indicate T cell activation, the T cells did not slow down or dwell (Kyrtasous et al., 2017).

### 3.10. Blood vessel dyes

Fluorescent dyes injected into the vasculature provide a flexible and easy to manage system for blood vessel imaging, and intravascularly injected dyes are used nearly universally to provide landmarks. Fluorescent dye conjugated dextrans have very low toxicity, are inexpensive, easy to obtain and provide a wide range of colors with bright, specific fluorescence for imaging (Invitrogen, Fisher, etc). In addition to simple labeling of blood vessels, different sizes of dextran can be used to investigate permeability of the blood vessels (Egawa et al., 2013). Dextran dyes are excreted renally within several hours allowing for dye clearance with repeated injections at a later time and can in fact be used for evaluating renal function (Hall et al., 2011). Dye-filled blood vessels can be used to evaluate flow through normal blood vessels (Shih et al., 2012) and change in flow between vessels after an insult such as a blood vessel occlusion simulating a stroke in the spinal cord (Farrar et al., 2015) or in the cortex (Shih et al., 2013), the technical details of which are outside the scope of this review. Two colors of dextran can also be used, one after the other, for blood vessel imaging before and after an event such as a spinal cord injury (Chen et al., 2017). Repeated imaging of blood vessels over time can provide great insight into biological processes such as angiogenesis and the blood vessel response to a mechanical spinal cord injury (Dray et al.,

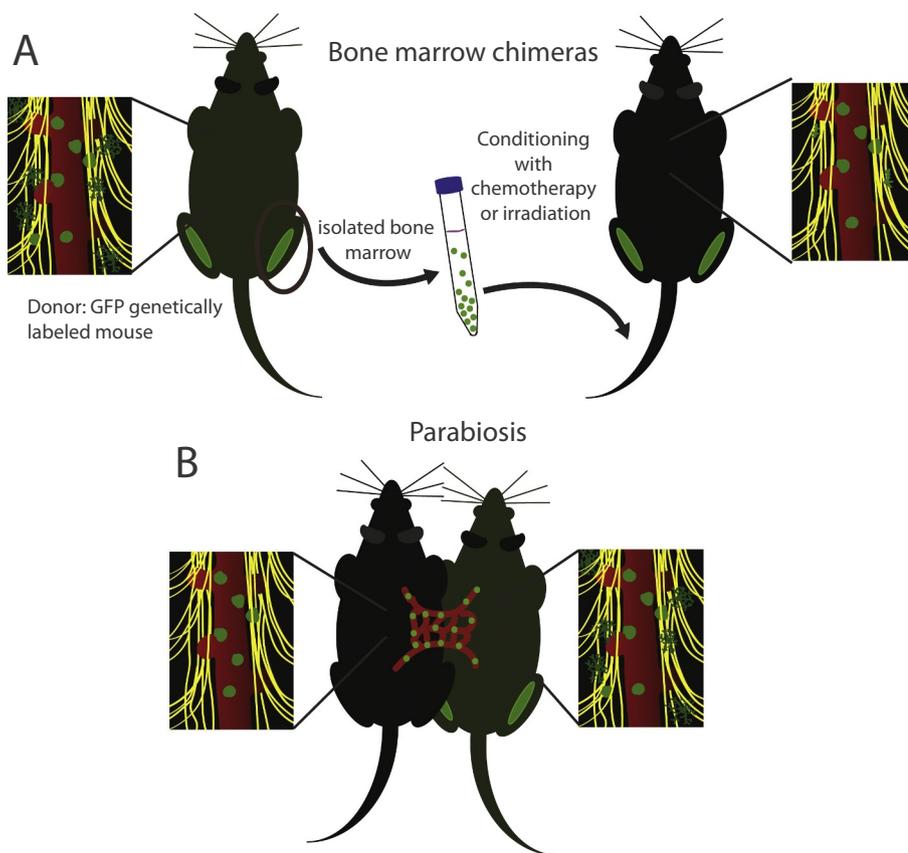
2009) or in looking at the change in size of blood vessels after spinal cord injury treated with various pharmacological agents (Tang et al., 2015). Labeling blood vessels in an EAE model allows imaging of T-cell movement along the lumen of vessels and timing of speed of rolling and crawling and eventually arrest along the blood vessel wall (Barkauskas et al., 2013; Haghayegh Jahromi et al., 2017). When dextran particles extravasate during imaging, they can be phagocytosed by macrophages and will maintain fluorescence for longer periods up to days after initial uptake, which can be useful for long term identification of phagocytic cells (Barkauskas et al., 2015). Quantum dots provide another option for blood vessel labeling (Larson et al., 2003) with less chance of extravasation due to their larger size and can provide a wide variety of colors (Wang et al., 2018). Quantum dots have been used for labeling blood vessels as landmarks (Fenrich et al., 2012), much as dextrans have. Fluorescently labeled lectins are also an option for visualizing blood vessels by binding to glycoproteins on the basal side of endothelial cells (Di Giovanna et al., 2018). Lectins provide specific staining for endothelial cells that will continue to be visible in fixed tissues, but may have some cross-reactivity also labeling microglia (Hiero-Bujalance et al., 2018). In addition to intravenous injectable dyes, mice with genetically labeled endothelial cells are also available and provide another option that does not involve injected dyes or particles. Animals with genetically labeled endovascular cells have very stable labeling of vascular endothelial structures over time, but they do require breeding with another genetically labeled animal to obtain multiple-labeling, often with a Cre-expressing vascular marker that must then be crossed to a fluorescent reporter line. Even with the challenges of using these mouse lines, these are very useful, especially when the vascular endothelium of angiogenesis is of prime interest. The many genetic models available for fluorescent labeling of vasculature have also been well organized in a comprehensive review from Andy Shih's lab (Hartmann et al., 2015). For our purposes, an example is a transgenic mouse with Tie2-GFP-labeled endothelium (Jax mouse 003658) (Motoike et al., 2000) that has proven useful for imaging angiogenesis in related disease models and has been used in imaging in the cortex, associating neuronal activity with angiogenesis during the critical period (Whiteus et al., 2014) and angiogenesis with hypoxia, also in the cortex (Masamoto et al., 2014), as well as reaction of the endothelium to clot formation (Grutzendler et al., 2014). The Tie2-GFP line has not yet been used in the spinal cord and is not in the standard B6 background, introducing some confounds when crossed with other mouse lines. While there are also other potential mouse lines that could be used for genetic labeling of endothelium for imaging, these likely have more sparse labeling or have not yet been used for intravital imaging, even in the cortex.

### 3.11. Cell membranes and lineage tracing

Understanding of the location of specific cell populations in the context of the microenvironment can provide tremendous insights into a specific pathology. Thus, mTmG reporter mice were developed where all cells express tdTomato localized to the membrane, but in cells that express Cre recombinase, EGFP is localized to the membrane (Jax #007576) allowing identification and lineage tracing if needed with a cell-membrane specific fluorophore (Muzumdar et al., 2007). Cell membrane labeling allows researchers to account for cell-cell interactions while tracking a specific subset of interest, gaining information on cells that would otherwise be missed because they do not express a fluorescent protein. PDGFR $\alpha$ -CreER: mT/mG mice were generated to show that oligodendrocytes were created even three months after a spinal cord injury in mice, remyelinating axons near the injury (Hesp et al., 2015).

### 3.12. Photoconvertible proteins

Photoconvertible fluorescent proteins are activated by UV light, not multiphoton excitation, and monomeric forms such as Dendra2



**Fig. 3.** Bone marrow chimeras/parabiosis for the identification of immune cells.

**A:** Schematic of a traditional bone marrow chimera with a CX3CR1 GFP donor mouse and a Thy-1 YFP recipient mouse. Bone marrow is isolated from the long bones of the donor CX3CR1 GFP mouse, washed and then injected into a host conditioned with either chemotherapy or irradiation. These methods will cause some infiltration of bone marrow derived cells into the parenchyma of the spinal cord, although at much lower numbers and with somewhat different morphology than in the donor animal, shown in the inserts. The inserts show a schematic of potential imaging after a dorsal laminectomy. In these examples, the donor CX3CR1 GFP/Thy-1 YFP double transgenic mouse has axons labeled in yellow and microglia and peripheral macrophages labeled in green. The recipient Thy-1 YFP mouse has axons labeled in yellow and bone marrow derived CX3CR1 + cells labeled in green, showing primarily circulating cells and a small number of ramified but smaller than normal GFP + cells in the parenchyma. **B:** Example of a parabiotic experiment with both a CX3CR1 GFP positive animal and a wild type animal. Circulating cells are transferred between both animals. Since the host animal does not require conditioning, there is no infiltration of the blood derived cells into the healthy CNS parenchyma in these animals. The inserts here show potential imaging after a dorsal laminectomy from a normal CX3CR1 GFP/Thy-1 YFP double transgenic mouse as the donor mouse and a Thy-1 YFP mouse with only circulating CX3CR1 GFP cells derived from the donor.

(Gurskaya et al., 2006) and mEOS (McKinney et al., 2009) can be used for super resolution microscopy to determine protein localization within cells. Labeling of the soma and projections of an individual neuron with UV light has been performed *in vivo* to describe the movements of proteins into the dendrites and, additionally, sections of brain have been photoconverted in a live mouse and then brain slice imaging has been performed tracing the projections from the activated area. Photoconvertible proteins can theoretically be useful in determining how cells move from a localized lesion site through the CNS or from the leptomeninges into the parenchyma or vice versa (Louveau et al., 2018). Kaede (Ando et al., 2002) and KikGR (Habuchi et al., 2008) mice are useful for two photon microscopy because of bright emission fluorescence and a new variant of Kikume which has been recently developed that can be photoconverted and then post-conversion proteins identified by multiphoton excitation in zebrafish and potentially in mice (Ando et al., 2002; Habuchi et al., 2008).

### 3.13. Transplantation – bone marrow chimeras and parabiosis

Techniques typically associated with immunology such as bone marrow transplantation and parabiosis are useful for distinguishing cells that are formed during development and have low turnover compared with those that are derived from the bone marrow as an adult (Tashima et al., 2016). Bone marrow transplantation and parabiosis techniques are particularly useful for investigating pathologies in the CNS, since the primary immune cell population in healthy tissue is microglia, which are derived from the yolk sac early in development (Ginhoux et al., 2010). Microglia can, thus, be distinguished from circulating monocytes, neutrophils and lymphocytes. While these techniques are not without caveats, in appropriate circumstances they can be very useful. (Fig. 3)

Bone marrow chimeras have been traditionally used to discriminate between cell types based on developmental lineage. Targeting cells that

arise from the bone marrow but later on distribute within the rest of the body is achieved by ablating the bone marrow in the adult and then replacing it with complete bone marrow isolated from an animal with a genetic trait different than that of the host, typically a fluorescent protein or congenic surface protein. Traditional chimeras require bone marrow ablation with either irradiation or chemotherapy, such as busulfan. Caution should be used with both irradiation and chemotherapy induced bone marrow ablation since these can cause transient opening of the blood brain barrier and potentially allow infiltration of bone marrow progenitors before reconstitution of the bone marrow occurs. Effects of radiation in the brain have been mitigated by lead shielding of the head during irradiation leading to lower rates of peripheral cell infiltration into the spinal cord (Shechter et al., 2009; Shechter et al., 2013). Directly shielding the spinal cord is more difficult than shielding the head due to the shape of the spine. A study looking at microglial response to peripheral nerve injury also included some needed information about the dose effect of radiation used to form chimeras on CD11b positive cell infiltration into the spinal cord, showing a clear correlation between dose of radiation and cell infiltration in controls and in animals with peripheral nerve injury (Tashima et al., 2016). The percentage of chimeric cells in the bone marrow after transplantation is decreased with lower radiation doses and split doses, but these differences in overall cell engraftment are not nearly enough to account for the differences in cell infiltration into the CNS. This work indicates that, at the very least, there should be thought put into using either lower or split-dose radiation techniques to ablate bone marrow for chimeras and considering using head and or spinal cord shielding during irradiation. It is difficult to measure the full effect of irradiation on blood brain barrier breakdown and cell infiltration in many different spinal cord and CNS disease models, introducing even more uncertainty into these models. Busulfan conditioning, in which the chemotherapy drug is used to ablate the bone marrow in the host animal, resulted in significantly less bone marrow derived cells accumulating in the lumbar spinal cord

compared to irradiation in a model of amyotrophic lateral sclerosis. Fewer bone marrow cells accumulating in the uninjured CNS after bone marrow transplantation in mice suggests that busulfan is effective at bone marrow ablation with less damage to the blood brain barrier (Lewis et al., 2013). Protocols for chimera production are well established, such as in a protocol using CX3CR1 GFP mice from Astrid Cardona's lab (Garcia et al., 2013), although any genetically labeled animal can be used as either the recipient or donor. Chimeras have been used with intravital imaging to identify populations of blood derived CX3CR1 cells and to observe their interactions with axons after spinal cord injury (Evans et al., 2014).

Parabiosis, in which the circulations of two genetically distinct animals are surgically attached, allows for complete mixing of circulating blood and can be used to identify blood derived cells expressing a fluorescent protein without introduction of immature bone marrow cells into the circulation. These models do not require bone marrow ablation in the host animal to observe circulating cells in the host, although radiation of the host animal can be done to increase rates of chimerism. Parabiosis models avoid both the non-physiological flood of bone marrow progenitor cells into the circulation that occurs during the production of a chimeric mouse, and leakiness of the blood brain barrier and potential excess of infiltrating cells in response to inflammation due to irradiation. In an EAE model, parabiosis was used very effectively to show that circulating monocytes are drivers of the destructive stage of EAE that results in motor deficits and, also, that they are a cell type that does not convert into microglia but, instead, recede back into the circulation (Ajami et al., 2011). In addition to providing a strategy for delivering blood derived cells that are genetically labeled to another animal, parabiotic animals can be used to deliver functionally different cells to an animal for treatment and modification of disease processes. For example, parabiotic models have been used to give blood derived from younger animals to older ones to study effects of aging in the central nervous system (Ruckh et al., 2012). While useful, parabiosis has not been used extensively with intravital imaging, likely due to the technical issues surrounding combining two surgically challenging techniques.

### 3.14. Adoptive cell transfer and vital dyes

Isolation of a specific population of cells from either a donor animal or cell culture and then transferred into a host animal for imaging is an easily performed technique, that while not physiologic, has a lot of advantages. This gives another level of control for cell type identification since FACS and immunohistochemical and functional assays can be performed on cells before transfer. It also provides more flexibility in labeling of cells for imaging, allowing for genetically induced fluorescent protein expression from the donor animal, electroporation of fluorescent protein constructs and simple incubation with vital dyes. Dyes that can be introduced into cells or used to label the cell membrane simply by incubating isolated or cultured cells with dye are simple ways to identify transferred cells. Adoptive cell transfer has been particularly useful in EAE models, where it can be used for both cell identification and as an intrinsic part of disease induction. In this case, transfer of T-cells from a MOG immunized GFP positive animal can be used to induce EAE in the host animal. Transfer of labeled T-cells has been used in the cortex in EAE to observe movement of labeled MOG specific T-cells (Bartholomäus et al., 2009). Dye-based labeling techniques have been reviewed many times and commercial catalogues provide comprehensive lists of these dyes, although labeling concentrations are not always optimized for multiphoton imaging. A nice list of common probes and their excitation/emission spectra for two photon imaging are found in a review by Michael Cahalan et al. (Cahalan et al., 2002). We will give some examples here. CFSE has been used in flow cytometry to identify cells that have divided. Fluorescence intensity halves with each cellular division, providing a powerful way of looking at the number of cycles of cell division, but also results in loss

of fluorescence with each division which limits imaging after several cycles. SNARF is a pH indicator with a very bright yellow-orange fluorescence at the normal intracellular pH, making it a useful dye for cellular imaging. It also generates a spectral shift in high pH environments. It is easily loaded into cells with low concentration DMSO (Pescic et al., 2013). These dyes can be powerful in looking at single cell activation when combined with calcium indicators and provide a lot of flexibility in color selection when used with genetically expressed fluorescent proteins.

### 3.15. Virally induced vital dyes

Viral vectors have been injected in the cortex to transduce genetic constructs that express in neurons at the injection site. This technique has been used extensively in neuroanatomy and optogenetics allowing the expression of proteins for mapping of circuits and control of ion channels, respectively. Retrovirus, lentivirus, adenovirus, rabies virus and alpha-herpes virus are used in imaging as retrograde, anterograde, and trans-synaptic tracers. Although there are examples of static, in vitro and intravital cortex imaging, this technique has not yet been used for intravital spinal imaging, but due to its possibilities merits mention. Employment of virally induced fluorescent markers in intravital spine imaging has been hampered in part due to the technical difficulty of imaging in the spine and the scientific questions at hand given that two photon microscopy is generally able to image mostly sensory neurons, and the ease of use of the Thy-1 reporter mouse strains and so far little use of tract tracing.

Sindbis viral vectors are popular for high resolution in vivo imaging of cortical neurons. However, the neurons survive no longer than 5–10 days after infection and thus are not useful for long term imaging of neurons and calls into question the physiologic state of the neuron (Ehrensgruber et al., 1999). Retroviruses are single-stranded RNA viruses that must be introduced into proliferating neuronal precursors, thus useful for developmental studies but useless for long term studies in adults. Additionally, studies show that expression from the integrated virus is silenced in embryonic neuronal precursors (Gaiano et al., 1999). Lentiviral vectors are an attractive alternative as these viruses allow for stable integration of the virally transferred gene into the host genome regardless of their state of division and it accepts larger foreign DNA fragments (up to 10 kb) than other virus types allowing innovative constructs to interrogate neural function though expression levels can be low (Naldini et al., 1996) (Löw et al., 2010). Lentiviral vectors are also resistant to stem cell specific gene silencing in various types of stem cells as compared to retroviral vectors (Lois et al., 2002). Adenovirus infects both cycling precursors and post-mitotic neurons, however, there are immune responses caused by the endogenous genes in the vectors which can adversely affect the results of experiments (Yoon et al., 1996). Adenovirus also infects cells non-specifically, thus at the injection site neurons, astrocytes, oligodendrocytes and microglia will all be labeled (Akli et al., 1993). The solution to the specificity problems with retroviruses and adenoviruses was to create a recombinant vector that only included the machinery for viral replication, packaging, and insertion into the host genome, thus recombinant adeno-associated virus (rAAV) vectors were created (Xiao et al., 1997). rAAV vectors can be engineered to specifically infect neurons or other neural cell subtypes, do not induce an immune response, and integrate into the genome, resulting in long-term fluorescence expression (Peel et al., 1997), (Snyder et al., 2011). The utility of rAAV vectors has expanded by taking advantage of Cre-lox recombination technology. Here, one introduces Cre expression into the vector and injects it into a mouse that expresses loxP stop sites around a transcriptional stop cassette preceding a fluorescent reporter gene sequence, thus allowing the expression of a fluorescent protein in a specific neural cell subtype that is also infected by the virus (Harris et al., 2012). Exploiting the nature of neurotropic viruses, such as rabies and alpha-herpes virus, neuroanatomists have been successful in tracing

and visualizing functionally connected neurons (Smith et al., 2000; Ohara et al., 2009). Rabies, pseudorabies and alpha-herpes virus vectors are double-stranded DNA replication-competent viruses that can spread from an infected neuron to its postsynaptic partner, and recently have been engineered to reduce toxicity which allows longitudinal multiphoton imaging for 4 months or possibly longer (Enquist et al., 1998; Chatterjee et al., 2018). In the last five years, the Allen Brain Institute has assembled a group of minimally toxic AAV vectors with a range of fluorescent proteins and channel rhodopsins expressed under the human synapsin 1 and CAG promoters along with cre-dependent drivers and the FLP-FRT recombination system (Madisen et al., 2015). Viruses from this library, along with several new lines have then been used to investigate the connectome in the cortex (Oh et al., 2014). These vectors are available from Addgene, and although these methods have not been used in the spinal cord for intravital imaging, they will open up many new labeling combinations, especially when combined with other methods.

### 3.16. Anesthetics

Methods of sedation to maintain animal comfort and avoid movement during imaging can be critical. As a counterpoint, awake imaging while animals are performing tasks such as running on a ball are more technically challenging, but can also yield very useful information about calcium signaling in axons during movement (Blum and Zuo, 2013; Sekiguchi et al., 2016). Anesthetics are typically used due to the technical challenges of awake imaging, especially in the spinal cord with multiple bony articulations in contrast to the skull, which lends itself more easily to awake imaging. Anesthetics can have effects on normal immunology, with reports of primarily small anti-inflammatory effects and with some potential toxic effects, and many reviews on this subject are available, primarily in humans in the setting of operative anesthesia, although also in mice (Schneemilch, Schilling et al., 2004; Stollings et al., 2016; Yuki and Eckenhoff, 2016; Cruz et al., 2017). Anesthesia in intravital imaging is also well reviewed (Germain et al., 2006; Alieva et al., 2014). As a brief summary, either inhaled or injected anesthetics are typically used, and they each have distinct benefits and concerns. Isoflurane provides an inhaled anesthetic that is easy to titrate, simple, quick on and quick off anesthetic that can be used over extended periods of time, making it the usual first consideration for anesthesia for imaging, although this requires a nose-cone and close monitoring to maintain appropriate depth of anesthesia over time. Avertin (tribromoethanol) is another option that can be given with an intraperitoneal injection. Avertin is also relatively quick acting with a short half-life, necessitating either multiple injections or placement of an intraperitoneal catheter for either a slow infusion or intermittent bolus injections (Papaioannou and Fox, 1993; Weiss and Zimmermann, 1999). Since Avertin is stored in the fat, it does have the potential to build up over time making longer term anesthesia difficult. It has been used with good effect for imaging of immune cell movement in the lymph node (Miller et al., 2002). Ketamine and Xylazine is another commonly used anesthetic combination (Alieva et al., 2014). This is frequently used with good results, but does have concerns with frequent re-dosing and concern for respiratory depression (Alieva et al., 2014). Paralytics are typically not used to avoid intubation and need for respiratory support, but if animals are to be intubated to coordinate imaging with breathing, paralytics can be useful.

### 3.17. A few technical aspects of intravital imaging in relation to immunology

While the technical aspects of two photon intravital imaging have been reviewed extensively elsewhere, it seems pertinent to discuss a few common themes here that pertain specifically to imaging of the immune system. Two-photon microscopy is performed primarily by scanning the tissue in a linear pattern to create single images and then

creating z-stacks from multiple scans. Since the time-scale of movement for immune cells is relatively fast on the time scale of seconds to minutes, imaging for immune applications is often performed several times each minute at a high rate of scan rates as technically possible. Imaging of intracellular calcium signaling occurs on a much shorter time scale necessitating either small imaging field size to accommodate faster scanning or the use of resonance scanning techniques (Piyawattanametha et al., 2006).

Acquiring both the surgical and microscopy skill sets required to master intravital imaging in mice is a process that can take months. The time to collect the data set from the animals can range from days to months. However, the time and care required for post-acquisition image processing, algorithm development, data management and data analysis is sometimes overlooked while designing experiments, although these exercises are critical to obtaining statistically and biologically relevant results. Many of the models discussed here utilize multi-colored imaging - primarily CFP, GFP, YFP and RFP in addition to other dyes. These conditions with multiple labels are powerful in looking at cellular interactions and multiple types of cell movement in the same animal, but they also introduce concerns in separating the emission spectra of each of these dyes to reliably separate one labeled cell type from another. While the mathematics and specifics of spectral imaging and linear unmixing are outside the scope of this review, obtaining the cleanest images possible is vital to obtaining useful data, especially with the high-dimensional models discussed here.

As a brief overview, choosing dyes that have the least possible overlap will make post-processing and deconvolution less troublesome. There are many commercial online applications to assist with this, and although these are not specific for two photon imaging and some focus on flow cytometry, they provide a good basic starting point for looking at potential color combinations and a pictorial representation of the emission and excitation spectra for these dyes in one-photon imaging. Specific excitation and emission spectra for two photon imaging at this point must be researched separately, and can also vary based on the application in which imaging is used. Although some data is available (Ricard et al., 2018), such spectra should be obtained experimentally in each new set-up. With this caveat, ThermoFisher and Invitrogen's spectrviewers can be helpful for predicting reasonable fluorophore choices. The University of Arizona also has a large database that can be helpful (<http://www.spectra.arizona.edu/>). In our experience, before starting final imaging for experiments, taking preliminary images with small variations in excitation and looking at the resulting emission spectra for overlap can be useful in determining the best excitation wavelength for continued imaging. Emission filter choice is also critical, occasionally eliminating the need for further post-processing. Filter arrangements can include preliminary filters and then secondary filters, eliminating the regions of the spectrum in which the most overlap between emission spectra occurs, with the caveat that this also decreases the amount of light to reach the detector, requiring brighter fluorophores or less restrictive filters and reliance on mathematical processes to separate colors. Occasionally, there can be almost complete overlap between more similar fluorescent probes such as GFY and YFP necessitating changes in experimental design. Even with careful experimental design and practical evaluation of overlap, it is inevitable that there will continue to be some overlap between fluorophores, necessitating further mathematical deconvolution. If needed, spectral imaging can be helpful. Spectral imaging requires acquisition of an emission spectrum during imaging sessions, adding another dimension to the images. While this can be a very powerful technique, it requires more complex instrumentation, longer scan times likely increasing photobleaching and making more frequent time-lapse image acquisition challenging. Varying the excitation wavelength and obtaining images over this variation and then using this data to distinguish between fluorophores is also possible, but has many of the same caveats. Linear unmixing is often used, and in very simplified terms involves comparing the expected excitation spectra for each of the fluorophores involved to

the emission spectra obtained from imaging multiple dyes and using a mathematical matrix to predict the contributions of each dye involved that would be needed to produce the experimental spectra. These techniques and others have been extensively reviewed and only a few references are included here (Lansford et al., 2001; Dickinson et al., 2003; Zimmermann et al., 2003; Garini et al., 2006). Zeiss, Leica and as well as other commercial microscopy companies provide basic tutorials on understanding the basics of these techniques. Even after optimizing images in post-acquisition processing, image processing of cell motility within intravital imaging datasets to extract quantifiable metrics from the dynamic images requires massive amounts of computation and can require months of analysis. Advanced algorithms are required for both detecting individual cells in 3D in multiple channels and correctly tracking the same cell over time. Both commercial and custom software are used for cell tracking, and we will only briefly mention a few commercial or easily available options here for simplicity. Code and assistance using custom software programs are typically included in supplemental information to the studies in which they are used. ImageJ is a free-ware program created at NIH which has readily-available cell tracking protocols that can be easily customized and can serve as a platform for powerful custom statistical methods, for example, tracking (Tinevez et al., 2017). Workflows built with open source packages include ImageJ/Fiji (Schindelin et al., 2012), Icy (de Chaumont et al., 2012), CellProfiler (Carpenter et al., 2006) or CellCognition (Held et al., 2010) and can complement the use of commercial tools, e.g. Imaris (Bitplane), Amira (FEI), MetaMorph (Molecular Devices), or Volocity (PerkinElmer). While these have not yet been used extensively in two photon imaging, there are available cell-tracking plug-ins that can be found on the ImageJ plugins website such as CellTrack and MTrack2, and are also often included in supplemental information for the papers where they are used. For example, a nice description of some of the available methods of cell tracking and theory behind cell tracking is included along with a description of a new sequential Monte-Carlo based method of tracking cell movement in lymph nodes imaged with two photon microscopy in a methods paper (Olivieri et al., 2011). More commonly used, Imaris (BitPlane, UK) and Volocity (Quorum Technologies, UK) are commercial products that very accurately identify and track cells in intravital datasets. The Imaris cell tracking module is commonly used, for example, tracking CX3CR1 positive cells (Evans et al., 2014), as is the Volocity tracking software, for example tracking CXCR6 positive cells in EAE (Kim et al., 2010). In addition, these commercial programs simply allow post-processing stabilization of the image by registering immobile cells to a fixed location in the image over time. Attempts to stabilize imaging can greatly decrease the movement artifacts in the dynamic imaging dataset caused by breathing and heart rate, thus enhancing the ability of the researcher to faithfully analyze the movement of the cells of interest within the field of view. Once the individual tracks have been identified for each cell, many more metrics can be analyzed, for example, the number and duration of interactions with other cells, the regions that are resulting in cellular accumulation, and functional readouts such as calcium flux. Borrowing from intravital imaging in the lymph node, statistical methods describing random walks, swarms and clusters (Miller et al., 2002; Germain et al., 2006; Liou et al., 2012; Lämmermann et al., 2013) may also be useful, although rarely used in CNS imaging. The movement metrics analyzed are determined by the scientific question proposed and, therefore, these programs allow researchers to develop their own algorithms that work within these programs which has led to programming communities where algorithms can be shared. An image processing obstacle, when compared to cranial window imaging, is that the image of the spine collected within rectangular field of view will generally be convex, in the same shape as the dorsal column in the spinal cord, whereas in a cranial window, the cortex will generally be flat and fill the field of view. The convex shape of the spinal cord increases the total volume needed to encompass the curved shape of the tissue, although imaging empty space around the spinal cord and make stabilization more

difficult. Location of cells is easily measured as a function of distance from the dura, which then becomes computationally harder when the dura is curved requiring either other landmarks or more complicated mathematics for quantification. Suffice to say, image analysis of intravital datasets can be as complex and even more time consuming than data acquisition, although analysis can be made simpler with thoughtful experimental design and acquisition of high quality images.

#### 4. Conclusions

Intravital imaging is beginning to yield exciting discoveries about immune cell responses and their interactions with the many other cell types in the spinal cord. The immune system presents specific challenges for intravital imaging beyond that with other models, requiring additional thought and planning when deciding on methods for exposure of the spinal tissue for imaging. Experimental design with multi-color labeling of immune cells, as well as endogenous components of the immune system, presents interesting and exciting possibilities for the successful use of intravital imaging to investigate the complexities of the immune response in spinal cord disease.

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#### Declaration of Competing Interests

The authors have no competing financial interests to disclose.

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