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## Video Article

# Two-photon Imaging of Cellular Dynamics in the Mouse Spinal Cord

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## Abstract

Two-photon (2P) microscopy is utilized to reveal cellular dynamics and interactions deep within living, intact tissues. Here, we present a method for live-cell imaging in the murine spinal cord. This technique is uniquely suited to analyze neural precursor cell (NPC) dynamics following transplantation into spinal cords undergoing neuroinflammatory demyelinating disorders. NPCs migrate to sites of axonal damage, proliferate, differentiate into oligodendrocytes, and participate in direct remyelination. NPCs are thereby a promising therapeutic treatment to ameliorate chronic demyelinating diseases. Because transplanted NPCs migrate to the damaged areas on the ventral side of the spinal cord, traditional intravital 2P imaging is impossible, and only information on static interactions was previously available using histochemical staining approaches. Although this method was generated to image transplanted NPCs in the ventral spinal cord, it can be applied to numerous studies of transplanted and endogenous cells throughout the entire spinal cord. In this article, we demonstrate the preparation and imaging of a spinal cord with enhanced yellow fluorescent protein-expressing axons and enhanced green fluorescent protein-expressing transplanted NPCs.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/52580/>

## Introduction

Mouse models of demyelination, including experimental autoimmune encephalomyelitis (EAE) and intracranial infection with neuroadapted mouse hepatitis virus (MHV), are excellent tools to study molecular pathways and cellular interactions associated with disease. They have led to and supported the effectiveness of FDA approved pharmaceutical therapies, mainly targeting cessation of autoimmunity and inflammation<sup>1</sup>. However, once endogenous remyelination has failed, the currently approved therapies do not effectively repair demyelinated lesions in the central nervous system. Therefore, repair-focused therapies at this stage of disease are critical for the alleviation of chronic symptoms and improvement of quality of life. Recently, neural precursor cells (NPCs) have come to the forefront as a potential regenerative therapeutic modality to target areas of inflammation and demyelination. Several studies have highlighted the ability of NPCs to induce endogenous remyelination and participate directly in remyelination<sup>2-8</sup>. Because NPCs are involved in direct remyelination, it is imperative to understand their kinetics and interactions with endogenous cells following transplantation. After transplantation, NPCs migrate ventrally to areas of white matter damage, then rostrally and caudally relative to the transplant site<sup>5,9</sup>. The kinetics of migration differ in response to environmental cues; NPCs transplanted into a non-damaged spinal cord have greater velocities than NPCs transplanted into a damaged spinal cord<sup>6</sup>. After a migratory period, transferred NPCs proliferate extensively, at a higher rate in a damaged spinal cord relative to an intact spinal cord<sup>6</sup>. Finally, the majority of NPCs differentiate into oligodendrocytes and initiate direct remyelination<sup>4,6,9</sup>.

The demyelinated lesion is complex and can include a diverse population of cells at various stages of activation. For example, an active multiple sclerosis (MS) lesion may include a significant population of activated T cells, M1 microglia and M1 macrophages, but a chronic silent MS lesion may be comprised primarily of reactive astrocytes with few inflammatory cells<sup>10-13</sup>. Because of the diversity of effector cells, two-photon (2P) imaging in mouse models of demyelination is an extremely useful tool to help understand local cellular interactions within the lesion. In MS and many extensively used MS research models, the majority of lesions are located on the ventral side of the spinal cord, a region inaccessible to intravital 2P imaging due to lesion depth and the high lipid content of the spinal cord. To circumvent these issues and study cell-cell interactions within lesions along the ventral spinal cord we have developed a simple *ex vivo* 2P imaging preparation<sup>6</sup>.

This study follows up a previous methods publication, which showed the procedure for transplanting enhanced green fluorescent protein (eGFP)-expressing NPCs into the spinal cord of mice following JHMV strain of MHV-induced demyelination<sup>14</sup>. Five week old mice are infected with JHMV and transplanted with eGFP-NPCs at thoracic level 9 on day 14 post-infection. The protocol presented here provides detailed steps on how to extract the spinal cord, make an *ex vivo* agarose preparation, and image transplanted eGFP-NPC interactions with enhanced yellow fluorescent

protein (eYFP)-expressing axons. Mice expressing eYFP under the neuronal-specific Thy1 promoter were used in this procedure<sup>15</sup>. Only some of the axons express eYFP, making it useful for imaging individual axons. Here we show spinal cords removed at 7 days post-transplant; however, spinal cords can be extracted at any time point following transplantation. While we show interactions of NPCs with damaged axons, our protocol can be used in combination with genetic fluorescent markers of other cell types to investigate a multitude of cellular interactions occurring throughout the mouse spinal cord.

## Protocol

NOTE: Ethics Statement: The protocol for animal handling was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Irvine, protocol #2010-2943.

### 1. Removal of Spinal Cord

1. Place paper towels wetted with ~100% liquid isoflurane, USP in euthanizing chamber and place dry paper towels on top. Place mouse in chamber on top of dry paper towels so mouse is not touching the isoflurane and make sure the chamber is covered. Wait at least one minute after cessation of breathing to ensure that the mouse is euthanized.
2. Perform a spinal transection of the neck to ensure the mouse is euthanized. Do not perform a cervical dislocation as this could damage the spinal cord.
3. Spray the mouse with 70% ethanol to wet the hair.
4. Use sharp Fine scissors to remove the hair and skin from the back of the mouse to expose the spinal column from approximately cervical lamina 1 (C1) to sacral lamina 4 (S4).
5. Using a scalpel with a #10 blade, make incisions to the left and right of the spinal column to separate it from muscle and fat. This will make removal of the spinal cord much easier.
6. optional: a curved Luer rongeurs can be used to scoop away additional flesh.
7. While holding the top of the spinal vertebrae with serrated Graefe forceps, insert titanium curved Vannas scissors with the curved side up into the exposed spinal column at C1, being careful not to touch the spinal cord.
8. Slide the titanium curved Vannas scissors all the way to the right and make one small cut. A small crunch sound should be heard and felt as the scissors cut the vertebrae. Repeat this cut on the far left side of the cord. Be careful not to go too quickly or make too large of a cut as this will risk damaging the cord with the scissors.
9. The single lamina should be able to be lifted with the forceps once the sides of the vertebrae are cut. Repeat this step for each lamina until S4, continuing to hold and pull back the spinal cord vertebrae.  
NOTE: the vertebrae above the transplant site will likely come off once it is cut down to the transplant site. Continue the procedure starting just below the transplant site.
10. optional: lay the vertebrae down to see where the transplant site is and measure and cut ~20 mm rostral and caudal to the transplant site.  
NOTE: When transplanting NPCs more will not be needed as the transplanted NPCs generally do not migrate farther than 15 mm. The amount of spinal cord needed will be dependent on experiment and migration characteristics of cell type transplanted. Making rostral and caudal cuts that are equidistant to the transplant site will enable the transplant site to be located more easily during imaging.
11. Using a scalpel with a #11 blade carefully cut the ganglia on the right and left of the ventral side of the spinal cord starting at the rostral end. This will enable the spinal cord to be removed from the ventral vertebrae more easily.  
NOTE: Done quickly (steps 1.7-1.11), the spinal cord will not dry out. However, 1x PBS can be administered to the cord to keep it moist.
12. Invert the mouse and hold the mouse up so the spinal cord is facing down. Carefully peel out the spinal cord using closed serrated Graefe forceps. Without nicking the spinal cord, carefully cut any remaining ganglia to allow the spinal cord to be lifted out in a single intact piece.
13. Make sure the spinal cord is in RPMI-1640 and place it on ice for transport to the 2P microscope.

### 2. Preparation of Spinal Cord for Imaging

1. Embedding spinal cord in agarose
  1. Keep the isolated spinal cord in chilled RPMI-1640 on ice prior to imaging.
  2. Weigh out agarose (low gelling temperature) and prepare a 5% solution in 5 ml of 1x PBS. Microwave the 5% agarose solution for 15 sec to dissolve the agarose and let the solution cool to 37 °C.
  3. Prepare the spinal cord by placing it on a sheet of Parafilm with the ventral side facing up.
  4. Pipet approximately 5 ml of 5% agarose solution over the ventral side of the spinal cord. Let the agarose solidify by cooling to room temperature. Full solidification takes approximately 5 min.
  5. Apply a light coat of tissue adhesive to a 22 mm square cover slip.
  6. Invert the embedded spinal cord, placing the ventral side on Parafilm. The dorsal side will now be facing up. Adhere the cover slip to the dorsal side, and submerge the agarose embedded spinal cord/cover slip preparation in RPMI to solidify the adhesive. Remove excess solidified agarose using a razor blade.
2. Mounting the spinal cord on the microscope stage
  1. Apply petroleum jelly to the bottom of the cover slip. This will stabilize the preparation during perfusion.
  2. Place the spinal cord preparation in an imaging well on the microscope stage with the ventral side facing up toward the 25X dipping objective. The custom-built imaging well is approximately 20 mm deep, 50 mm long, and 50 mm across.
  3. Image while superfusing the preparation with warmed (37 °C), oxygenated (95:5 oxygen:carbon dioxide-carbogen) RPMI-1640 medium without serum.
    1. Pre-warm media to 37 °C in the water bath and connect it to the tubing pump by inserting the tubing into the media bottle.

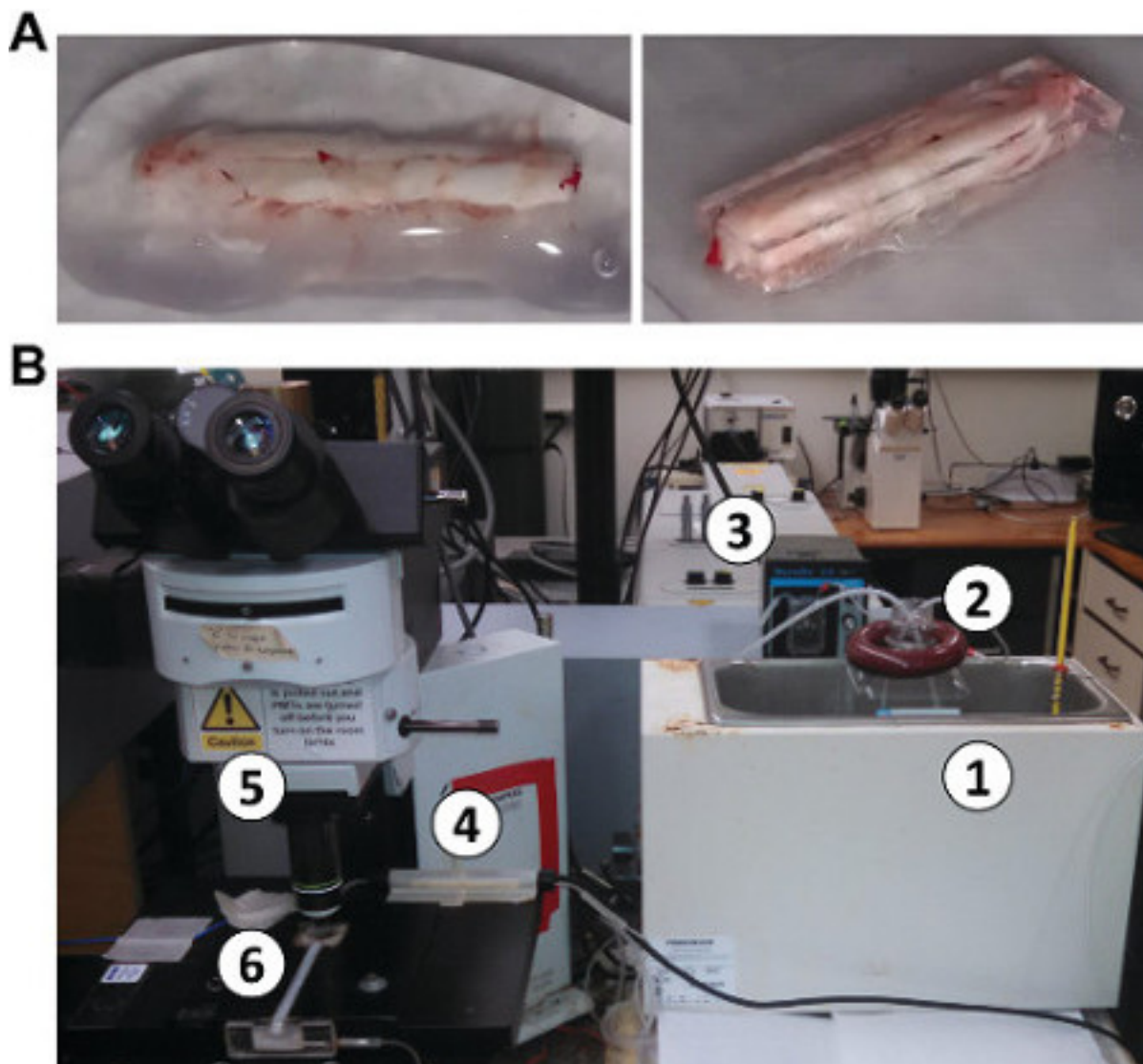
2. Retain the media temperature at 37 °C a heater device at the connection of the tubing to the chamber. Superfuse media through well at 3 ml/min using tubing connected to the tubing pump (**Figure 1B**).

### 3. 2P Imaging of Ventral Spinal Cord

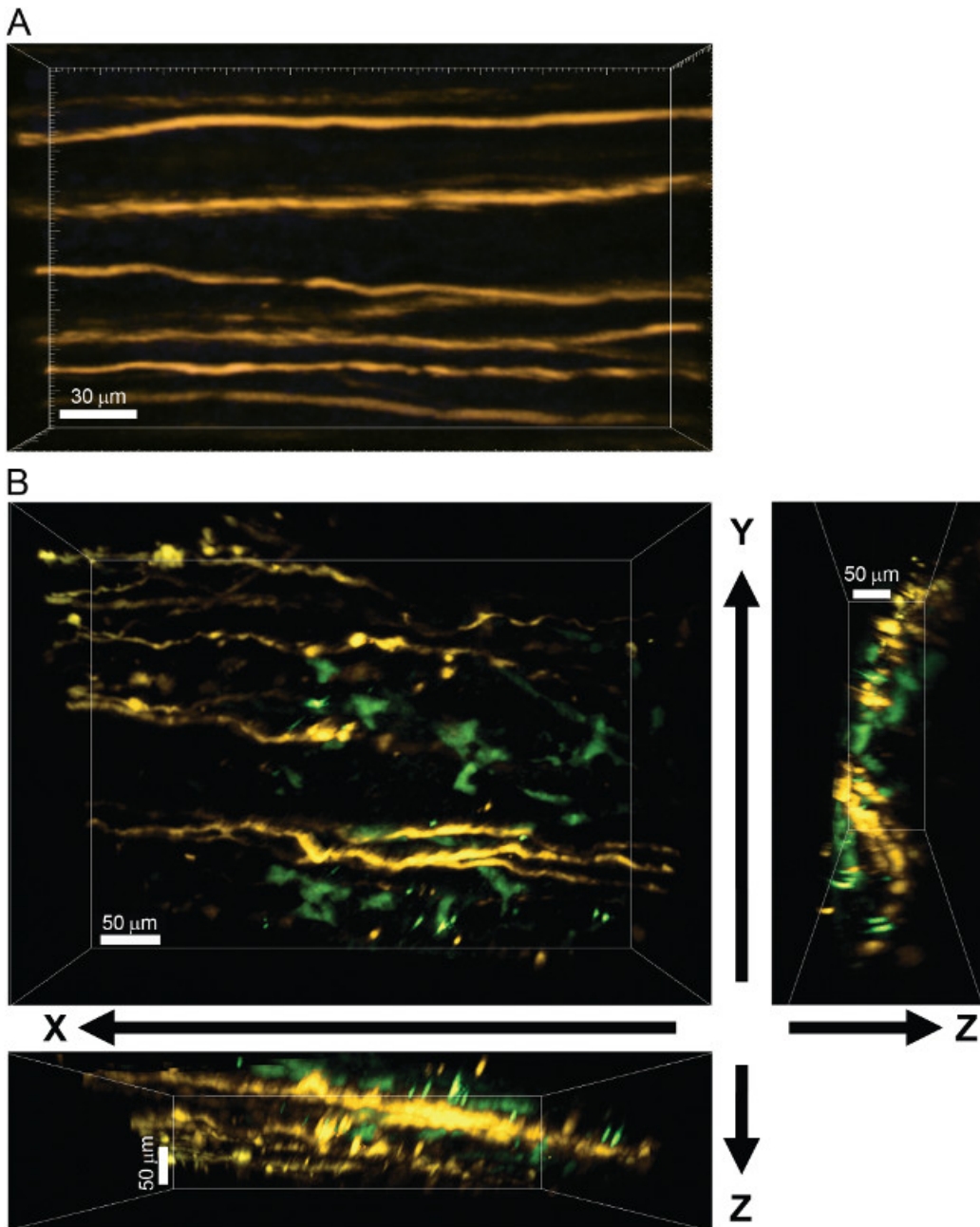
1. Microscope Setup
  1. Acquire images from Thy1-eYFP spinal cords using a Chameleon Ultra Ti: Sapphire laser tuned to 900 nm. Attenuate laser power at the specimen to <5% to ensure minimal phototoxicity<sup>16</sup>. Maintain temperature by perfusing oxygen-perfused RPMI-1640 at a constant 37 °C using a single inline solution heater to ensure stable imaging, and to prevent tissue drift and cellular damage.
  2. To separate eGFP and eYFP fluorescent signal, place a 520 nm single-edge dichroic and a 560 nm single-edge dichroic beam splitter in series to separate 2P emission into three channels, purposely splitting the green emission to enhance visibility.  
NOTE: These channel are referred to as blue-green (emission <520 nm), green-yellow (520–560 nm), and red (emission >560 nm). Photomultiplier tubes detect emitted light in each channel.
2. Locating imaging areas of interest
  1. Using the eyepiece and a bright field light source, focus the dipping objective at the ventral edge of the spinal cord to set a reference point.
  2. Make sure ambient light source is off and switch to 2P excitation by opening the laser shutter.
  3. If available, when searching tissue for areas of interest, use a lower resolution setting, higher volume without digital zoom, and higher scan rate than when acquiring final images. Typical settings with this system when searching tissue for a region of interest are: resolution: 256 pixels; volume: x = 600  $\mu$ m, y = 600  $\mu$ m, z = 0-300  $\mu$ m.
  4. Observe the eYFP axons near the ventral edge of the spinal cord. Second harmonic signal from collagen (blue) will be brightest at the spinal cord tissue edge. Locate the eYFP axons just dorsal to the second harmonic signal from the collagen and eYFP signal is visualized in the green-yellow channel.
  5. Locate the transplant site in the longitudinal center of the spinal cord preparation<sup>14</sup>. For mice 1 day following eGFP-NPC transplantation, locate the transplant site by focusing the beam path in the z plane deep into the tissue. The transplant site will vary slightly between animals but is generally located ~200  $\mu$ m from the ventral edge. Observe the clusters of eGFP-NPCs in the white matter tracts, closer to the ventral and lateral edges in mice after day 1 post-transplantation.
3. Acquiring final images
  1. Acquire image resolution of 512 pixels; volume: x = 270  $\mu$ m, y = 212  $\mu$ m, and z = 100  $\mu$ m using Slidebook 6 software. Compile z-stacks by acquiring sequential focal planes in 2.5  $\mu$ m increments.
  2. Perform a bidirectional scan with proper interlace offset to ensure rapid quantification of any migrating objects in the spinal cord. Ensure that the ideal acquisition frame rate to determine cellular velocity within the spinal cord is approximately 1 frame/sec.  
NOTE: Imaging settings can be changed to individual user preferences, such as acquisition frame rate, imaging resolution and imaging volumes. Larger imaging volumes will generally take longer to acquire at a given resolution.
  3. Analyze, crop, smooth, and pseudocolor Slidebook image files with image analysis software such as Bitplane Imaris 7.7. Produce final time-lapse videos by combing consecutive imaging volumes using an automated process in Slidebook and Imaris.

### Representative Results

While the explanted spinal cord imaging protocol can be used to visualize any fluorescence within the spinal cord, our representative results demonstrate eGFP-NPC interactions with eYFP-axons. First, we show the embedded ventral spinal cord preparation in **Figure 1A**. Next, we show the 2P microscope setup and key components in **Figure 1B**. **Figure 2** demonstrates eGFP and eYFP fluorescence in a single z-stack within the ventral spinal cord. Acquisition of consecutive z-stacks can be compiled to produce time-lapse videos to analyze real-time cellular dynamics within the intact tissue. Using a 520 nm single-edge dichroic and a 560 nm single-edge dichroic beam splitter, as noted in the protocol, can separate eGFP and eYFP signal. Individual channels can be pseudocolored green and yellow using imaging software.



**Figure 1: Spinal cord and microscope setup.** (A) A spinal cord embedded in a 5% agarose gel (left) and mounted on a coverslip following removal of excess agarose (right). (B) An image of the microscope setup with key components labeled. 1. Water bath set at 37 °C. 2. Pre-warmed RPMI-1640. 3. C/L variable-speed tubing Pump. 4. Single inline solution heater. 5. Dipping objective. 6. Digital thermometer. [Please click here to view a larger version of this figure.](#)



**Figure 2: Example 2P image acquired inside the ventral spinal cord.** 3D reconstructions of the ventral side of an uninfected, non-damaged (A) and JHMV-infected, demyelinated (B) spinal cord from a Thy1-eYFP mouse following transplantation with eGFP-labeled NPCs. Fluorescently-labeled axons are pseudocolored yellow and NPCs are pseudocolored green. Image resolution: 512 pixels; image volume: (A)  $x = 239 \mu\text{m}$ ,  $y = 259 \mu\text{m}$ ,  $z = 65 \mu\text{m}$  constructed using 26 z-stacks spaced  $2.5 \mu\text{m}$  apart and (B)  $x = 497 \mu\text{m}$ ,  $y = 389 \mu\text{m}$ ,  $z = 127.5 \mu\text{m}$  constructed using 51 z-stacks spaced  $2.5 \mu\text{m}$  apart.

## Discussion

Real-time 2P imaging of intact tissue is required to investigate NPC kinetics and interactions following transplantation into the demyelinated mouse spinal cord. Intravital 2P imaging is commonly used to determine cellular dynamics on the dorsal side of the spinal cord in living mice, and has been used to study dorsal demyelination in demyelinating disease<sup>17-19</sup>. However, because transplanted NPCs migrate to the ventral white matter, which lies too deep to image in situ using 2P microscopy, an *ex vivo* preparation is necessary. This methodology has been used in our lab to determine the motility and remyelination kinetics of NPCs transplanted into the damaged and non-damaged spinal cord<sup>6</sup>. *Ex vivo* preparations enable scanning of spinal cords not only longitudinally but also transversely from the ventral side<sup>6,20</sup>. This allows for analysis of differences in cell movement (speed and direction) and interactions at the transplant site, adjacent to the transplant site, and at various distances from the transplant site<sup>6</sup>. Whereas we initially devised this preparation to image transplanted eGFP-NPCs on the ventral side of the spinal cord, it can be used to image any fluorescently labeled or dyed cell from the ventral, dorsal, or lateral sides by altering the orientation the spinal cord is mounted. Benefits of this preparation over intravital imaging include allowing ventral side imaging and lacking the need for anesthetization and

survival surgery, which is still needed with the new intravital preparations using a 'glass window' setup that allow for multiple imaging sessions without repeated surgeries<sup>19,21</sup>. It should be noted that one limitation of this procedure compared to the 'glass window' approach in combination with intravital imaging is the inability to obtain multiple time points from a single mouse, since the spinal cord is removed and the mouse is euthanized.

2P imaging is ideally suited for resolving the dynamics of single cells within intact tissues<sup>22,23</sup>. 2P excitation utilizes near-infrared photons that allow for extended periods of deep tissue imaging with minimal phototoxicity. 2P excitation occurs upon the simultaneous absorption of two photons by a fluorophore. The high concentration of photons necessary for 2P excitation is achieved by spatio-temporal compression of the 2P laser output at a single focal plane. This restricts fluorescent excitation to the focal point, further minimizing phototoxicity in spinal cord regions outside of the focal plane. Near-infrared photons also have reduced scattering, enabling imaging up to approximately 300  $\mu\text{m}$  from the ventral side of the spinal cord<sup>16</sup>.

As with any new technique, however, 2P imaging of the explanted spinal cord has limitations that must be kept in mind. This imaging protocol utilizes a 520 nm dichroic mirror that enables separation of eGFP and eYFP fluorescence by diverting eGFP emission into the fluorescent channel typically reserved for blue light. Second-harmonic generation created by collagen in the spinal cord also appears in the blue channel, but is readily distinguished from eGFP-labeled cells which are significantly brighter in this preparation, and have a portion of their emission identifiable in the green-yellow channel as collagen does not. This technique of channel blending and separation of different portions of an emission spectra using specific dichroics is often useful for visual or automated identification of individual fluorophores with close or nearly overlapping emission spectra. Phototoxicity is another limitation of this 2P imaging protocol. Cells within a single imaging volume are sensitive to phototoxicity; therefore, experimentalist should be keenly aware of signs of phototoxicity. Phototoxicity is typically first evident in the reduction or lack of cell motility, followed by photobleaching and/or morphological changes in local tissues. Several factors can damage the tissue leading to phototoxicity or rendering it unsuitable for live-cell imaging. It is critical to monitor the laser power and temperature and oxygenation of the perfused RPMI-1640. It is also imperative to ensure proper removal of the spinal cord from the mouse without excessive stretching or compressing of the spinal cord and without cutting the spinal cord with the blades. If handled properly, the explanted spinal cord preparation will remain viable for up to ten hours, as determined by robust cellular motility within the ventral spinal cord with no diminution at ten hours post-extraction. A variety of extracted tissue is commonly imaged for long lengths of time and considered viable<sup>23-26</sup>. Therefore, quantification of cellular dynamics in multiple regions within a single spinal cord is possible. Finally, it should be noted that the vast majority of cells that are not fluorescently labeled will not be visualized by this preparation (unless naturally autofluorescent), and it is important to acknowledge that labeled cells are interacting with a complex environment of other unlabeled, seemingly invisible endogenous cells and structural elements.

## Disclosures

The authors have no competing interests.

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