**Responses to Reviewer Comments:**

**Editorial comments:**  
  
1) Since this manuscript has been requested for priority filming please address the editorial and peer review comments at your earliest.   
  
2) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.   
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
The use of two photon microscopy for intravital imaging is a growing technique that offers many benefits to observing changes in cell to cell interactions in normal or traumatized tissues. The present study attempts to illustrate a distinction between intrinsic micro glial cells and invading macrophages after spinal cord injury. There are only minor issues with the text however the images and accompanying Figure Legends provide little detail about what is presented and the overall quality of the images is low. Specifically there should be higher magnification images of cell-blood vessel interaction presented in Figure 2 D, some indication of the same region being examined in Figure 1D and E, an indication of how retracting axons in 1E are distinguished from those in 1D and some more information about the route of migration taken by the CX3CR1 cells in Figure 3B. At least indicate the start point for the cells observed. This will require some change of images and improved text.

> We appreciate Reviewer 1’s comments. We have now included higher resolution images of cells in contact with the blood vessels in revised Figure 2, showing the close proximity between these cells and the blood vessels. These changes can now be found in Figure 2 H-J. The start position and direction of the cellular movement are now included in Figure 3 C. The tracks are displayed using color-coding based on time, thus allowing for the direction and the relative time of movement to be visualized. We have also addressed these changes in the text.

For minor points in the text,  
1) the second sentence of the abstract is a run on of thoughts.

> We have addressed this in the text.

2) page 7 paragraph 2, it is not possible to see cells adhering to the inside of blood vessels

> We have changed the wording to state that the cells are seen within the blood vessels, not necessarily adherent to the vessels (page 9). We have also updated Figures 2 H-J to address this point.

3) 4.13 add the volume of fluorescent dye used for intravenous injection

> We have now addressed this in the text (step 4.11).

4) page 8 paragraph 1, if the stability of the spinal cord is critical then more details should be provided to assist the reader in knowing what to look for for stability and how to achieve it.  
  
> We have now addressed this on page 11 of the text.

**Reviewer #2:**   
*Manuscript Summary:*   
This protocol explores the use of bone marrow chimera mice to explore microglia and macrophage responses after a compression model of spinal cord injury with two-photon laser microscopy. All of these concepts are of particular importance in the research fields and would be of particular interest for a video protocol. The manuscript is also well written and the protocol is clear and easy to follow. There are a few minor concerns that are described in more detail below.  
  
*Major Concerns:*  
None.  
  
*Minor Concerns:*  
1.Some of the wording is awkward, such as the second sentence of the abstract or the second sentence of the introduction. Careful proof-reading of the manuscript is recommended.

> We have made changes in the text to address these errors, and fixed multiple minor errors throughout the manuscript text.

2.The authors state that "Recently, functional distinctions between microglia and macrophages in the CNS are beginning to be discerned…". As many of the references provided for this statement are at least 10 years old, this does not seem to be 'recent' or a 'beginning' and the wording should be reconsidered.

> We agree with the reviewer, and the wording has been changed in the text to better reflect the chronology of the citations.

3.The authors state that contusion models often spare the dorsal columns, which is inaccurate. To the best of this reviewer's understanding, most contusion models actually target the dorsal columns more than any other region. The authors may wish to reconsider this statement.

> Although it is a common impression that the contusion model targets dorsal columns, it is not actually true. Although in the contusion model the impact device does come in direct contact first with the dorsal columns, the dorsal column tissue actually moves away during the impact and is often spared, especially on the very dorsal portion of the cord. In fact, the majority of tissue damage occurs in the central cord, possibly including the most ventral portion of the dorsal columns. The lesion then extends laterally into the surrounding white matter during secondary injury. Indeed, myelinated axons with relatively normal morphology are only found in the dorsal columns of contused animals, except in cases of severe contusion injuries (please consult the following reference: *Ek CJ, Habgood MD, Callaway JK, Dennis R, Dziegielewska KM, et al. (2010) Spatio-Temporal Progression of Grey and White Matter Damage Following Contusion Injury in Rat Spinal Cord. PLoS ONE 5(8): e12021. doi:10.1371/journal.pone.0012021)*

We have now changed the wording in the text to make this statement clearer.

4.A statement indicating how the correct or target vertebra was identified should be included.  
  
> We have added this statement to step 3.8.

5.Statements about temperature maintenance during surgical approaches should be included in all surgical protocols.

> This statement was originally included in step 4.12. We have now added additional language to step 3.4.

6.A dosage/volume for fluorescent dye injection may be useful in step 4.11.

> We have now added this information in step 4.11.

7.The recommended breathing rate varies throughout the protocol, from 60 - 100 breaths/minute. This should be kept constant throughout the protocol, unless there is a rationale for the change?

> We thank the reviewer for noticing this error. We have now standardized the rates to 60-100 bpm throughout the text.   
  
8.In step 4.13, the yellow fluorescence should be an indication of axons rather than neurons.

> We did not mention yellow fluorescence in step 4.13. We have now changed the wording in the introduction regarding the yellow fluorescence.

9.In figure 1, please indicate the identity of the green fluorescent cells.  
  
> We have now added the labels in the figure.

10.In figure 1, the authors at first state that the lesion grows over time and then later state that the lesion has similar dimensions over time. Please clarify.  
  
> We meant to indicate that the lesion increases in size over the first week following trauma, and then the lesion size stabilizes thereafter. We have now clarified this point in the figure legend.

11.In figure 1, the authors include reference #51, but it is unclear what this reference is in support of. Please clarify.  
  
> We apologize for this typo. This reference is now removed.

12.In figure 2, the experimental design in the image is reversed from that in the legend.

> We thank the reviewer for noticing this error, and we have now fixed the error in the figure legend.

13.In the discussion, the authors refer to shielding of the brain to protect microglia from irradiation induced damage or number reduction. However, the focus of this manuscript is the spinal cord, so it would be more appropriate to discuss the impact of irradiation on spinal cord microglia and how that might alter the outcomes of this study.

> We appreciate the point the reviewer is making. We discussed irradiating the brain since our study did not shield the spinal cord. Other studies have shielded the brain during irradiation and then investigate changes in the spinal cord. Presumably, some of the changes observed in the spinal cord are due to irradiation effects in the brain. We have changed the text to make this point clearer. Shielding the spinal cord separately would be technically challenging due to its position in the center of the body. See reference 53.  
  
**Reviewer #3:**   
In this paper the authors present a simple injury model that allows the observation and quantification of cell movement within a spinal cord lesion and quantification of axonal position relative to the lesion. This is a nice technique and the authors provide clear and easy to follow protocols to their procedures. A few issues should be addressed to improve the clarity and presentation of the work.  
  
-The authors should clearly label the figure panels. For example, indicate what is stained in green and yellow and include arrow heads/asterisks to indicate observations reported in the text e.g. indicate on the figure the axons at the caudal end that have retracted and axons at the rostral end undergoing Wallerian degeneration (lines 391-393).

> We have now clearly labeled microglia, macrophages, blood vessels and axons in the figures. In addition, we have labeled, by solid and open arrows, portions of the axons undergoing either the wallerian degeneration or the retraction bulbs. We have also made corresponding changes in the figure legends.  
  
-Figure 2B and 2C: indicate/label cell clusters to improve clarity of the FACS data e.g. colour coding of cell clusters would be informative.

> We have now highlight the relevant region of the flow plot and have also indicated such in the legend.  
  
-Fig 2D-G: Provide more detail and better labelling in this figure. It is not clear how the authors conclude that CX3CR+GFP cells adhere to the inside of blood vessels after injury in 2G (line 415-416).

> We have added Figure 2 H-J to show this more clearly.

-Line 462-462: it would be helpful to indicate the dorsal root axons in the figure.

> We have now marked the axons with arrows in both Figures 1 and 2.  
  
-Line 464: also indicate "large and amoeboid shaped cell bodies" in the image and provide more description on what criteria were used to identify this distinct morphology.

> We have now marked cells with arrows in the figure, and have added more descriptions in the figure legend. We realize the morphologic description of activated microglia can be subjective, and for these incidental observations, no specific calculation / quantitation of cell morphology were made.  
  
-It would strengthen the manuscript if the authors included slowed down movie frames so that the reader can identify an example of a moving cell; the authors could also indicate the track that would be quantified from these images.

> We have now provided a movie with a slower frame rate. We have also included an inset showing a cell moving along a single track.

-The authors claim that "This method…provides a quantifiable lesion to assay the degree of axonal dieback in the dorsal columns when coupled with complementary fixed tissue analysis". They should either demonstrate this by showing how axon die back can be assayed, or remove this claim.

> We have now highlighted and clarified the fact that quantifying the degree of axonal dieback in the dorsal column using fixed tissue analyses have commonly been shown in the literature but is not directly relevant to the current protocol.

-Remove "dynamic" from the title and tone down the claim throughout the manuscript that this study shows "dynamic interactions between microglia/macrophages and axons". This is a nice methodology paper with a thorough description of procedures, but it does not provide new data on how inflammatory cells interact dynamically with axons.

> We have now removed “dynamic” from the title and shortened the discussion regarding this point. We have also clarified our point that the discussion surrounding the “dynamic” nature of microglia / macrophages interaction with axons was in reference to a published paper (Evans et al. Exp. Neurol. 2014; 254:109).

Minor:  
A number of sentences in the manuscript do not make sense and/or are poorly phrased; a thorough proof read and grammatical check should be performed. A few examples:

> We have now made changes to the abstract, and have corrected wording and typographical errors throughout the text.

Abstract: Intravital twophoton microscopy allows for the study of macrophages and microglia in the traumatic spinal cord lesion in the living animal using two-photon microscopy can be performed in adult animals with a traumatic injury to the dorsal column.  
  
Line 63-64: "…increasing its relevance as an anatomical site of which to focus efforts on improving feasible experimental approach"  
  
Line 61-62: "Investigations of these cellular interactions in the intact spinal cord are of particular interest since this is the only easily accessible CNS white matter". What does this mean? How can imaging inflammatory events in the "intact" white matter be of any use?

> Our statement was intended to point out the fact that long-distance axons within a white matter tract cannot be imaged in the cortex intravitally. The spinal cord is therefore the easiest area in which to access these tracts for the study of diseases involving the white matter. The word “intact” was meant to refer to the animal, not the spinal cord. We have now clarified this in the text.

Over-use of the word "traumatic" in long abstract and throughout the manuscript; this description is not commonly applied to a dorsal column crush injury, but for more severe contusion-type injuries.  
  
Even more so, the "pin-prick" injury is not a model of traumatic spinal cord injury (line 108).

> We have taken steps to reduce the use of the word, “traumatic”.