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"Laminectomy and spinal cord window implantation in the mouse"

Response to Reviewers:

We thank the reviewers for their feedback our manuscript. We are gratified that the reviewers were enthusiastic about our methodology and found our protocol to be useful. The suggestions of the reviewers have been very helpful in revising our manuscript. We have revised the title, abstract, introduction, methods, and discussion to focus on the advantages and disadvantages of our protocol relative to other laminectomy and window implantation protocols, and have deemphasized the application to study of the blood-brain barrier. We have greatly expanded the details of the protocol section to enhance clarity. We added several paragraphs to the discussion highlighting advantages and limitations of our protocol relative to other protocols in the literature. We added two new figures, one depicting the dimensions and construction of the backplate (Figure 1), and one depicting anatomic representation of the backplate implantation site (Figure 3). We substantially revised figure 1 (now figure 2) to better depict the tools and configuration of the surgical setup. We replaced the previous representative results figure with a new figure (Figure 4), that incorporates a series of single optical sections as well as a Z-projection, designed to convey more information about the imaging field with respect to tissue surface landmarks and the depth of imaging that can be achieved with the currently described laminectomy and spinal cord window preparation. Below, we provide a point-by-point response to the reviewer's concerns regarding the original manuscript.

Reviewer #1:
Manuscript Summary:
The authors have presented a nice modification of widely used surgeries for spinal cord intravital imaging. The described method uses a convenient 3D printed well for containing immersion fluid and a less invasive and easier to use method of stabilization to account for movement that may also be easier to learn.

Major Concerns:
The major innovation in this work is the 3D printed backplate for animals, but instructions on producing this (type of printer, type of plastic, exact dimensions, etc) are missing. Perhaps the authors could make available a file that could be used directly for printing, as well as the

directions for doing this? This should also be added to the video, along with instructions for troubleshooting some common printing problems and how to tell if these have occurred.

1.1 We have added to the manuscript details regarding the use of the 3D printer to prepare backplates with PLA plastic. We have included as supplementary material the file we wrote to create our 3D backplate as well as the product printing file ready for upload to a printer. We also have included a new figure (Figure 1) to depict the backplate dimensions, triangulated mesh three-dimensional structural projections, and photographic examples of successful and unsuccessful prints.

Minor Concerns:
Please discuss how agar affects imaging quality/depth of possible imaging over time. Please address potential heat damage with too hot agarose, and specify a temperature. Is leaving the spinal cord open for short-term imaging an option?

1.2 We have added a paragraph to the discussion addressing these points. To avoid light scattering during intravital microscopy that could be caused by the differences between the refractive indices of air, water, and tissue, most protocols overlay an optically transparent substrate over the exposed spinal cord. Common substrates include high purity, low-melting temperature agarose or silicone polymers. Agarose offers the advantage of ease of use, with minimal bubble formation, and is appropriate for acute imaging sessions. To protect the tissue from heat damage, it is convenient to heat the agarose to beyond the melting point (e.g. 65 °C) at the start of the protocol and then allow it to cool to ~39 °C in a water bath during the laminectomy, so it can be ready at the appropriate time for application to the exposed spinal cord. For chronic imaging, silicone polymers are more resistant to dehydration. Pilot trials during development of the present protocol omitted either the agarose layer or the overlying coverglass, and found that the consequent light scatter reduced available depth of imaging, but this has not been tested in a rigorous way. Leaving the spinal cord open for short-term imaging is an option for imaging superficial structures but not deeper structures; the described combination of agarose with coverglass achieves a superior signal-to-noise ratio.

We also have not rigorously tested the temperature at which hot agarose would compromise tissue physiology. We predict that temperatures above 55 °C would cause scalding. We apply agarose at approximately 39 °C because this is the closest to physiological temperature that doesn't cause the agarose to gel.

When making the video, make sure to thoroughly discuss/address troubleshooting of the laminectomy procedure with dental drill and scissors.

1.3 The reviewer is correct that the removal of bone is a challenging step. We will highlight this in the video.

Carprofen is an anti-inflammatory, please discuss its use in inflammatory models such as EAE, reference as able, or state as a limitation.

1.4 We agree with the reviewer that NSAIDs are expected to modulate the inflammatory process and therefore potentially complicate analysis of inflammatory disease models. We have included in the discussion several studies addressing effects of NSAIDs in EAE and acknowledge this as a limitation. Most such studies document neuroprotective effects over the course of weeks; few address acute changes. In principal, it may not be necessary to administer NSAIDs for acute, terminal procedures. However, in all cases, investigators must work in close coordination with appropriate regulatory boards when conducting animal studies. At present time, our IACUC requires us to utilize NSAIDs, so we have not performed a side-by-side comparison of imaging outcomes in the presence versus absence of NSAIDs.

Is the plastic backplate attached primarily to bone with dental cement, or muscle, or connective tissue? Since this is the main point of stabilization for imaging, discuss how this is able to adequately compensate for breathing and heartbeat and how much residual movement is left. Does having the head in ear bars help with this?

1.5 We thank the reviewer for pointing out the lack of clarity on this point in the original manuscript. We have substantially rewritten the protocol to expand and clarify (see for example sections 5.4 and 5.5), and added a new figure (Figure 3) in which the placement of the backplate is depicted in reference to anatomically accurate renditions of the spinal column. Briefly, when T12 is removed, the strong points of backplate attachment are to the bony posterior articular process of the T11 vertebral spine, and to the anterior articular process of the T13 vertebral spine. The attachment is made via tissue adhesive and dental cement. Auxiliary support is from attachment at the transverse processes. We also include in representative results and in Figure 4 evidence in support of adequate stabilization and minimal residual movement. We also have indicated (section 4.1) that the main function of the earbars is to restrict motion of the head with respect to the isoflurane nosecone. Without the earbars, there is the possibility that the operator might inadvertently displace the mouse head from the nosecone.

Make sure equipment list is complete - with current format this isn't readable and looks like a lot might be missing.

1.7 We have expanded the equipment list.

Reviewer #2:

Manuscript Summary:

The authors present a protocol a window preparation of the mouse spinal cord optimized for intravital microscopy of the blood-brain barrier. The introduced protocol is not a real novel window preparation of the lumbar spinal cord, but it seems to have several advantages e.g. the

short preparation time (30 minutes) and the clamp-free window.

Major Concerns:
At the same time the introduced methodology also has significant limitations due to the necessity e.g. of 3D printes supports, which are not easily accessible to every laboratory. The publication should thus include mention on how such supports could be made available to a broader scienfitic community. Also the manuscript needs a more thorough comparison of the preparation introduced here to other spinal cord window preparations. The latter is essential to understand the suitability of this preparation for specific experiments.

2.1 We hope that 3D printing technology will be utilized by many laboratories in the near future, especially now that several models are available for less than $1000. To facilitate adoption of our backplate model by other laboratories, we have included as supplementary material the file we wrote to create our 3D backplate as well as the product printing file ready for upload to a printer. We also have included a new figure (Figure 1) to depict the backplate dimensions, three-dimensional structure, and examples of successful and unsuccessful prints.

We also have substantially expanded the discussion to include a more thorough comparison of the preparation used here to other spinal cord window preparations. Please see page 6 line 22 – page 7 line 22.

Also, videos need to be included into the manuscript to prove the stability of the window preparation over the time period of scanning, which is essential if one aims to follow the junctional signal as a landmark.

2.2 We have included a new representative results figure (Figure 4), which depicts individual optical slices and a maximal-intensity Z projection. Z-projection images highlight stability; any instability of the imaging field results in displacement in X-Y visible in the Z-projection. In incorrectly prepared implantations, motion instability results in duplicated biological structures in the Z-projection. In Figure 4B, the eGFP:Claudin-5 tight junctions are visualized as thin, clear, bright linear segments that do not repeat.

Figure 1A: the entire equipment visible to the left of the Leica microscope is not properly addressed in the text. A closer look and a detailed figure legend (e.g as in Figure 1B) needs to be provided.

2.3 We have replaced Figure 1A to depict a closer view of the SomnoSuite anesthesia system in which all the components are numbered and described individually in the legend.

It is mentioned on the figure legend of Fig. 1A, that the authors used a SomnoSuite anesthesia system that is obviously a safer anesthesia unit compared to the other traditional units but is not explained very well. Also the authors simply mentioned 2% isoflurane has been used for anesthesia, but adjustment of other parameters e.g. ventilation rate (stroke/min) or volume/min

are omitted.

2.4 We have amended the protocol to indicate flow rate (150ml/min)(protocol step 3.4). We also have added a paragraph to the discussion describing the advantages of the lower rate of isoflurane delivered by the SomnoSuite integrated digital vaporizer (page 9 line 14-24).

Fig.1E would benefit from presence of the heating pad and of the rectal probe in the correct positions. Even if these are described in the text, a visual display of the final preparation (even without animal) would once more highlight the importance of these measures for in vivo animal experiments.

2.5 In the revised Figure 2, panel 1E and 1H show the correct position of the heating pad and rectal probe.

Fig.2 lacks the scale bar. The depth of imaging and any other adjustments of the two-photon microscope for the acquisition of these images were not mentioned in the figure legend. Information on the two-photon microscope and the objectives used need to be included - it is not clear if the figure shows a capillary.As the described method has been optimized specifically for BBBand junctional imaging, the reader would strongly benefit from 1) a lower-magnification overview showing where exactly the image was acquired (position of the imaged junctions in a 3d context, compared to meninges and bigger vascular landmarks), and 2) a higher magnification inlet showing more convincingly the re-arrangement of the structure indicated here by white/yellow arrows.

2.6 We have created a new figure (Figure 4) depicting representative data of tight junction imaging in the eGFP:Claudin-5 mouse spinal cord, including an inset to show an example of a tight junction protrusion. We have added description of the methodological details of image acquisition, scale bar, depth annotation, Z-projection, and a series of representative single optical slices. The series of representative single optical slices are used to illustrate depth of the structures of interest with respect to the dura (depicted at 0-10 microns) to provide a structural landmark. Because we image with a 40x objective using a relatively small 204.8 square micron ROI, we have not included in a single image multiple different anatomic landmarks. We also have modified the manuscript title, abstract, introduction, and discussion to place emphasis on the laminectomy, window implantation, and backplate stabilization described in this protocol, and de-emphasized BBB remodeling. We believe that this shift should address the common concern raised by reviewers 2 and 3.

Reviewer #3:
Manuscript Summary:
This manuscript describes a minor variation in previously described methods for spinal cord imaging in living mice, in an effort to limit the impact of spinal stabilization on blood flow.

Major Concerns:

Although the differentiating aspect of the proposed approach is the use of a custom printed plastic chamber and the use of dental cement to stabilize it on the adjacent vertebrae, the manuscript lacks a thorough description of these steps, so its potential usefulness in its current form is limited. Section 4 therefore needs a far better description of the procedure with more detail on each step and how, when, exactly where each component is positioned and held in place.A representative schematic showing these steps and their anatomical relation to the spinal column would also help.

3.1 We thank the reviewer for highlighting the lack of clarity in the original manuscript. Please see comment 1.5 above, including description of the expanded protocol steps and the new representative schematic figure.

The 3D printed component should also be better described and the design file should be provided to facilitate the proposed method's implementation by the community.

3.2 Please see response 1.1 above, in which we describe inclusion of supplementary design files and a new figure depicting dimensions and views of the backplate.

The title states this is a method for "imaging of the blood brain barrier." This is misleading and inaccurate. This is simply a variation of a spinal cord imaging method that might offer an easier way to stabilize the spinal column than previously described protocols. Prior methods can and have been successfully used to image changes in BBB integrity which can be accomplished as the authors also acknowledge by properly controlling the clamping pressure. Similarly, using a coverslip directly on top of the exposed cord and compressing it with the plastic chamber that is immobilized with dental cement might also limit natural tissue behavior in and around the cord that can limit blood flow and hence BBB properties. To make the argument that this approach is superior for imaging subtle vascular/BBB properties the authors would need to directly compare properly performed prior methods against their approach and demonstrate how it performs significantly better. Alternatively, they can simply describe their method for what it can offer, which currently is an improvement on prior protocols, which can be easier for inexperienced users to learn and routinely perform to accomplish the required spinal stabilization.

3.3 In response to the reviewer's concerns, we have modified the manuscript title, abstract, introduction, and discussion to place emphasis on the laminectomy, window implantation, and backplate stabilization described in this protocol, and de-emphasized BBB remodeling. We believe that this shift better focuses attention on the methodological advantages of the current protocol, and should address common concerns raised by reviewers 2 and 3.

The table listing the used tools and reagents is incomplete.

3.4 We have expanded the Materials table.

Minor Concerns:

The language needs editorial work. Also, some parts of the protocol use expressions that are too casual for a scientific text like " thin the bone to nothing", "if you didn't do this already", if you do a good job" should be replaced with actual instructions in the form of a protocol that lists what should be done when.

3.5 We have carefully proofread and changed casual text with scientific language and more informative instructions.

What is the "gliding stage" and how is it engineered?

3.6 We have added to section 4.5 and to the materials list a description of the gliding stage. The gliding stage is constructed of two metal plates. The base plate is fixed to the microscope stage. The upper plate slides on greased interface to allow rotation around the center, as well as movement forward/back and side/side.

Items in fig 1 are not all visible. tools listed there should correspond to items on the list of reagents and tools required.

3.7 We updated and expanded the reagents and material list. In the revised Figure 2, we also added a closer view of the anesthesia unit and detailed description of its component parts.

None of the previously published methods actually requires "clamping the spinal cord" clamping occurs on the sides of the spinal column, not the cord itself.

3.8 We thank the reviewer for highlighting this inaccuracy in the original manuscript. We updated page 1 line 8 and page 1 line 10 to state that sides of spinal column are clamped.

What is a freehand laminectomy procedure?

3.9 We have omitted the aforementioned imprecise language, and instead included a greater degree of detail in all of the protocol steps, particularly protocol sections 4, 5, and 6.

What is the purpose of using Vetbond? What does this mean: " Dental cement adheres poorly to untreated tissue"?

3.10 We have added detail to the protocol (section 5.4) to indicate that Vetbond tissue adhesive is required for proper adherence of dental cement in subsequent steps. We have determined through experience that direct application of dental cement to bone (or to muscle or tendon) results in a weak bond. In contrast, application of Vetbond tissue adhesive to bone (or to muscle or tendon), followed by application of dental cement to the Vetbond-coated surface, results in a strong bond.

We hope that the reviewers will find our responses satisfactory to their concerns.

With sincere regards,

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