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Laminectomy and spinal cord window implantation in the mouse

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Cover Letter

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30 July 30, 2018

Dr. Alisha DSouza
Senior Review Editor
JoVE
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Cambridge, MA 02140

JoVE58330
"Laminectomy and spinal cord window implantation in the mouse"

Dear Dr. DSouza,

I am submitting the revised manuscript JoVE58330 "Laminectomy and spinal cord window implantation in the mouse" by Pietruczyk et al., for consideration in JoVE. We thank you and the reviewers for providing opportunities to improve our manuscript. The revised manuscript contains significant changes that address reviewers' concerns. I briefly outline below some of the major changes we have implemented and provide a detailed response in the Response to Reviewers document.

We have substantially revised each section of the manuscript to address the concerns raised by our reviewers. 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 have included supplementary files users can download to customize and to print backplates. 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. In light

of the extensive revisions, we have not used the “track changes” feature on the revised manuscript.

We provide here a response to the Editorial Comments. We also provide a detailed point-by-point response to the reviewers’ concerns regarding the original manuscript in the Reviewers Response document. We hope that the revised manuscript addresses the majority of the reviewers’ concerns and would be of interest for publication in *JoVE*. I look forward to hearing from you in the near future.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please provide an email address for each author.
Email addresses have been provided for each author.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
We corrected the use of Mg/ml to mg/mL on page 2 line 2 and minutes to min on page 2 line 8-9.

4. Please include a space between all numbers and their corresponding units: 15 mM, 37 °C, 60 s; etc.
We have carefully proofread and made corrections to the formatting as noted, including on page 1 line 42-44.

5. Please use subscripts in chemical formulae to indicate the number of atoms, e.g., H2O.
We added subscripts in chemical formulae.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have made corrections to avoid the use of personal pronouns, including on page 1 line 22-29, page 4 line 30-31.

7. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Vetbond, Kent Scientific SomnoSuite, Leica M205, Leica MC170, etc.
We have removed all commercial language from manuscript as noted.

8. 1.1: What is used to warm aCSF? Please specify.
We corrected text on page 1 line 31 to specify a water bath is used to warm aCSF.

9. 2.3: Please specify where ophthalmic ointment is applied.
We corrected text on page 3 line 20 to specify where to apply ophthalmic ointment (to the eye).

10. 4.7: Please add more details to this step. Alternatively, add references to published material specifying how to perform the protocol action.
We have added a section titled “Imaging Preparation” in which we add more details.

11. References: Please do not abbreviate journal titles.
We have utilized the JoVE Style Output in Endnote, downloaded and updated July 31, 2017. However, we find that the output continues to abbreviate the journal titles. Per our previous correspondence, we are grateful for your offer to permit us to submit the manuscript with the references in the abbreviated journal title format.

With sincere regards,

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TITLE:

Laminectomy and Spinal Cord Window Implantation in the Mouse

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KEYWORDS:

Spinal cord, laminectomy, mouse, two-photon, cranial window, blood-brain barrier

SHORT ABSTRACT

This protocol describes implantation of a glass window onto the spinal cord of a mouse to facilitate visualization by intravital microscopy.

LONG ABSTRACT:

This protocol describes a method for spinal cord laminectomy and glass window implantation for *in vivo* imaging of the mouse spinal cord. An integrated digital vaporizer is utilized to achieve a stable plane of anesthesia at a low-flow rate of isoflurane. A single vertebral spine is removed, and a commercially available cover-glass is overlaid on a thin agarose bed. A 3D-printed plastic backplate is then affixed to the adjacent vertebral spines using tissue adhesive and dental cement. A stabilization platform is used to reduce motion artifact from respiration and heartbeat. This rapid and clamp-free method is well-suited for acute multi-photon fluorescence microscopy. Representative data are included for an application of this technique to two-photon microscopy of the spinal cord vasculature in transgenic mice expressing eGFP:Claudin-5 — a tight junction protein.

INTRODUCTION:

Transgenic animal models expressing fluorescent proteins, when combined with intravital microscopy, provide a powerful platform for addressing biology and pathophysiology. To apply these techniques to the spinal cord, specialized protocols are required to prepare the spinal cord for imaging. One such strategy is to conduct a laminectomy and spinal cord window implantation. The key features of an ideal laminectomy protocol for microscopy include preservation of native

tissue structure and function, stability of the imaging field, quick processing time, and reproducibility of results. A particular challenge is to stabilize the imaging field against the motion induced by respiration and heartbeat. Multiple *ex vivo* and *in vivo* strategies have been reported to achieve these goals[1-5]. Most *in vivo* methods involve clamping the sides of the spinal column[2,4] and is often followed by implanting a rigid metal apparatus[3,4] for stability during surgery and downstream imaging applications. Clamping the spinal column can potentially compromise blood flow and induce blood-brain barrier (BBB) protein remodeling.

The purpose of this method is to make the intact spinal cord available for optical imaging in the living mouse while minimizing the invasiveness of the protocol and improving outcomes. We describe a single laminectomy and cover-glass implantation procedure paired with a minimally invasive oval plastic 3D-printed backplate that still achieves robust mechanical stability. The backplate is directly adhered to the anterior and posterior vertebral spines with dental cement. The backplate is equipped with lateral extension arms with screw holes that rigidly attach to the microscope stage via a metal arm. This effectively anchors the intact anterior and posterior vertebra to the microscope stage, providing mechanical resistance to the motion artifact that would otherwise be introduced by respiration and heartbeat. The method has been optimized for laminectomy of a single vertebra at thoracic level 12, omitting the clamps utilized in alternative strategies for stability during *in vivo* imaging. The procedure is rapid, taking approximately 30 min per mouse.

This protocol can be used to study disease mechanisms of the BBB. The BBB is a dynamic microvascular system comprised of endothelial cells, vascular smooth muscle, pericytes, and astrocyte foot processes that provide a highly selective environment for the central nervous system (CNS). Representative data depict the application of this protocol in transgenic mice engineered to express enhanced green fluorescent protein (eGFP):Claudin-5, a BBB tight junction protein. The provided backplate printing files can also be customized for alternative applications.

PROTOCOL:

All experiments follow the University of Illinois, Chicago Institutional Animal Care and Use Committee protocols.

1. **Reagent Preparation**

1.1. Prepare artificial cerebral spinal fluid (aCSF) to contain 125 mM NaCl, 5 mM KCl, 10 mM Glucose, 10 mM HEPES, 2 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$ in ddH_2O. Sterile filter and freeze in individual-use aliquots. Warm aCSF in a water bath to 39 °C before use.

1.2. Warm low melting-point agarose (2%) in aCSF until fully dissolved in a water bath set to 65 °C. During the laminectomy, cool the melted agarose aliquot to 39 °C in a water bath, so that it can ready at close to physiologic temperature for step 5.2.

Note: The agarose solution can be stored at -20 °C in single-use aliquots.

1.3. Prepare sterile 50 mg/mL carprofen in bacteriostatic water. Store at 4 °C.

1.4. Clean cover-glasses with 70% ethanol, three washes of ddH_2O, and store dry in a dust-free container.

2. Backplate 3D Printing

2.1. Use 3D CAD software is used to create a model to the dimensions shown in **Figure 1**. The interior is an ellipse widest at the bottom surface with respect to the printer and cut with a lofted cut to a smaller ellipse forming a lumen at the opposite surface. Two projecting arms with holes to accept screws extend laterally, for attachment to the backplate holding fork. From this 3D structure, create a triangulated 3D mesh file (.STL file).

Note: See **Figure 1B–D** and **Supplementary Files 1 and 2**.

2.2. Upload the triangulated 3D mesh file to a 3D printer.

2.3. Print backplates using a 0.4 mm hot-end nozzle and a 0.2 mm layer height. Select nozzle temperature of 205 °C, bed temperature of 45 °C, and printing speed of 45 mm/s.

2.4. Assess the resultant 3D printed backplates visually for structural integrity (**Figure 1E**); gross structural failure (absent lumen, collapsed wall) indicates printing defects (**Figure 1F**).

3. Surgical Preparation

3.1. Preheat the heating pad.

3.2. Load isoflurane into the delivery syringe while working in a chemical fume hood. Attach the delivery syringe to the isoflurane unit.

3.3. Select an 8–12-week-old mouse. Weigh the animal. Induce anesthesia using 2% isoflurane in an induction chamber. Inject carprofen at 5 mg/kg subcutaneously.

3.4. Position the nose-cone and deliver isoflurane at 2% with a flow rate of 150 mL/min for maintenance of a surgical plane of anesthesia (**Figure 2A–E**). Wrap the heating pad with a disposable absorbent pad for ease of cleanup.

3.5. Position an animal on the heating pad at the surgical station and install the nose cone. Lubricate the thermometer with petroleum jelly and insert it 5 mm into the rectum. Tape the thermometer probe to the tail for stability. Apply ophthalmic ointment to eyes of the mouse.

3.6. To maintain hydration, apply 200 µL of lactated Ringer's solution by subcutaneous injection every 30 min until termination of the experiment.

3.7. Spray the dorsum with 70% ethanol, remove fur with clippers, and clean the site with povidone-iodine.

4. Laminectomy

4.1. Position the animal between the ear bars; these maintain the head position of the mouse with respect to the nose-cone.

4.2. Confirm that animal is deeply anesthetized as assessed by lack of interdigital pinch reflex and steady respiratory pattern.

4.3. Make a 1.5 cm rostral-caudal incision at midline over the lower thoracic/upper lumbar region using #11 blade (**Figure 2E**). Separate the skin by grasping it with blunt toothed forceps and/or gloved fingers. Use forceps to separate and peel back any remaining transparent connective tissue underneath the skin. The superficial musculature should now be exposed; displace this with a foam surgical spear.

4.4. Use foam surgical spears (or a curette) to clear away the remaining, deeper musculature of the target vertebra (thoracic 12). To create a seat for the backplate, also clear away muscle from the posterior aspect of thoracic 11, and the anterior aspect of thoracic 13. Control any bleeding by applying gentle pressure with a surgical spear or use a minimal pulse with a cautery gun. Continue to remove the remaining muscle away from the tendons using forceps

4.5. Once muscles are removed, carefully detach the tendons by cutting with forceps. There should be plenty of space for visualizing and manipulating the cord when this step is complete. Check that the dura matter of the inter-vertebral space, the semi-transparent laminar bone, the central superficial blood vessel underneath the bone, and anterior radiating artery are now clearly visible.

4.5. Wet the region with warm aCSF. Use the microdrill to repetitively thin the laminar bone using straight strokes parallel to the long axis of the spinal cord (**Figure 2F**, **Figure 3**). If desired, utilize a gliding stage to rotate the surgical platform for enhanced ergonomic comfort (for example, a right-handed operator may rotate the surgical platform counterclockwise for the drilling step).

Note: The gliding stage used is constructed of an upper aluminum plate that slides ±15 mm with respect to the fixed base plate.

4.6. Gently grasp the superficial spinous process with forceps and lift the vertebra; the bone should lift away easily. If there is resistance, repeat bone thinning with the drill and if necessary use iris scissors, being careful to aim the scissor tips upward to avoid damaging the tissue.

Note: In order to maintain the dura intact, it is essential not to tug on the bone.

4.7. Use #4 forceps to clear away any bone shards. Use a surgical spear to apply gentle steady pressure to control any bleeding. Rinse tissue with warm aCSF. Do not allow the tissue to dry out.

5. Cover-glass Implantation

5.1. Gently apply a 3 mm borosilicate cover-glass to the exposed cord.

5.2. Ensure agarose is cooled to 39 °C. Using a small spatula, apply warm 2% agarose/aCSF to the edge of the cover-glass and allow capillary action to draw it under the surface.

Note: At temperatures below 39 °C, the agarose may start to gel. If this occurs, rewarm using a water bath or microwave. Some operators prefer to first apply one drop of agarose and lay the cover-glass on top.

5.3. Apply tissue adhesive to the exposed bony articular processes of the intact adjacent vertebra at thoracic level 11 vertebral spine and thoracic level 13 vertebral spine. Apply additional tissue adhesive in a ring around the laminectomy site, over the adjacent tendon and transverse process.

Note: Tissue adhesive is required for proper adherence of the dental cement in the subsequent steps. The articular processes form a natural seat on which the backplate can rest stably (**Figure 3**). Adhesion to the articular processes will form the strongest points of attachment.

5.4. Mix dental cement with accelerant in a porcelain mixing tray. Utilize a small spatula to transfer dental cement onto the tissue adhesive layer. Use dental cement to adhere the backplate to the surgical field, centered over the window. Allow 10 minutes for the dental cement to cure.

Note: The firm adhesion of the backplate to the anterior and posterior articular processes provides the fundamental structural stability of the implant.

5.5. Use additional dental cement to fill in the interior base of the backplate, and the underside of the backplate : tissue interface.

Note: The extra application of dental cement improves adherence and reduces risk of leakage of microscope objective immersion fluid (saline) out the bottom of the backplate.

5.6. Advance the forked backplate holder to the appropriate position over the window. Secure the backplate into the backplate holder with screws.

Note: This protocol utilized a custom-machined backplate holder (**Figure 2G–H**).

5.7. Apply saline to the backplate to test for leakage. If any fluid leaks, dry the area and apply more dental cement.

6. Imaging Preparation

6.1. Transfer the animal on the surgical platform to the optical table.

Note: Our surgical platform, backplate holder, and isoflurane nose-cone holder can be transported between surgical and two-photon imaging stations as one unit, while applying continuous isoflurane anesthesia (**Figure 2D**,**H**). Similar units can be assembled from holding forks, beams, and supportive pillar posts obtainable from commercial sources (*e.g*. ThorLabs). For intravital microscopy, there should be at least 11 inches of clearance between the microscope objective and the optical table to accommodate the height of the surgical platform.

6.2. Affix the surgical platform to the optical table using a stainless-steel mounting post and counterbored clamping fork.

6.3. Apply fresh saline into the well of the backplate. Lower a water-immersion lens into the well.

6.4. Use transmitted or epifluorescence light to identify the area of interest and focus. Switch to laser scanning mode and perform *in vivo* imaging according to the appropriate two-photon laser excitation wavelength, dichroics and bandpass filters for the fluorophores present in the tissue[6].

REPRESENTATIVE RESULTS:

Implanted glass windows and intravital two-photon microscopy provides a useful tool for assessing dynamic changes in CNS proteins. The functional integrity of the BBB is influenced by the expression, subcellular localization, and turnover rates of tight junction proteins[7]. Previous studies have demonstrated that tight junction proteins undergo rapid and dynamic remodeling at steady state[8]. The currently described laminectomy and glass window preparation has been used in transgenic eGFP:Claudin-5 mice[9], which bear fluorescent tight junction proteins, to assess BBB tight junction remodeling in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis[10]. In the representative data, imaging of eGFP:Claudin-5 was achieved with a two-photon microscope with 920 nm excitation, a 40× infrared objective (0.8 NA), and a green fluorescence emission filter (**Figure 4**). Optical stacks were sampled at 2 µm axial steps to 100 µm beneath the dural surface. Data depict visualization of the fluorescently labeled junctions throughout a vascular plexus. Single optical slices and Z-projection images are included (**Figure 4**). The clear delineation of tight junction structures in the Z-projection (**Figure 4B**) indicates that minimal X–Y image displacement is produced after successful laminectomy, window placement, and backplate implantation.

FIGURE AND TABLE LEGENDS:

Figure 1. Custom printed backplate stabilization device. A) Orthogonal backplate views. **B–D)** Triangulated mesh models of dorsal and ventral surfaces of the backplate. **E)** Correctly printed backplate. **F)** Incorrectly printed backplate. See **Supplementary Files 1** and **2**.

Figure 2. Surgical station for laminectomy. A) Isoflurane anesthesia delivery system utilizing an integrated digital vaporizer including (i) a touch screen display for controlling anesthesia, (ii) control dials, (iii) inputs for optional add-on physiology modules, (iv) anesthesia concentration adjustment knob, (v) syringe pump pusher block, (vi) syringe with isoflurane, (vii) integrated digital vaporizer, (viii) inspiration tubing, (ix) inspiration tubing for induction chamber, (x) induction chamber, (xi) inspiration tubing for nose-cone, (xii) expiration tubing for nose-cone, (xiii) expiration tubing for induction chamber. **B)** Instruments used in the laminectomy include (i) feedback controlled heating unit, (ii) K-coupled rectal thermometer probe, (iii) flexible silicone heating pad, (iv) #11 blade, (v) #5 forceps, (vi) toothed titanium forceps, (vii) titanium iris scissors, (viii) bone microdrill, (ix) cautery gun, (x) 3D printed backplate, (xi) absorbent foam surgical spears, (xii) ceramic mixing tray for acrylic resin, (xiii) acrylic resin and accelerant, (xiii) tissue adhesive, (xiv) ophthalmic lubricant, (xv) 3 mm cover-glass. **C)** Stereomicroscope and surgical platform. During surgery, the surgical platform sits on a gliding stage (silver and black round base on microscope stage). **D)** Custom surgical platform. The isoflurane nose-cone holder is adjustable in the Y- and Z-axes to accommodate small and large mice. Ear bars stabilize the head with respect to the nose-cone. The brass fork is adjustable in X-, Y-, and Z-axes to for positioning over spinal cord laminectomy or cranial opening. The fork is mechanically anchored to the surgical platform to provide optimal stabilization of the imaging field during surgery and downstream applications including two-photon intravital microscopy. During surgery, the surgical platform is mounted on a gliding stage. **E)** Example of a mouse positioned with nose-cone, ear-bars, and rectal thermometer probe. **F)** Example of a surgical field after laminectomy. A bone drill is used to thin and remove residual vertebral bone. Scale bar is 2 mm. **G)** Oval backplate and glass window implanted onto the exposed spinal cord. **H)** Mouse positioned in the surgical station after completed laminectomy.

Figure 3. Schematic depiction of anatomic placement of the spinal cord window. A) Schematic depiction of the superior view of a lower thoracic vertebral body, spinous process, and spinal cord (sc) segment. A dotted circle depicts the seat of the articular process, the main support point for backplate adhesion. **B)** Schematic depiction of the superior view of the spinal cord window. The target vertebral spine (here, T12) has been removed. A thin layer of agarose overlays the spinal cord. A coverglass rests on top of the agarose. Tissue adhesive is applied over the transverse processes (and, not shown here, on the exposed articular process of the adjacent, intact vertebral spines). Dental cement overlays the tissue adhesive. The backplate adheres to the tissue cement, resting on the transverse processes (shown) and the articular process of the adjacent, intact vertebral spines (not shown in this panel). An additional thin layer of dental cement is applied on the interior of the backplate rim. The backplate is depicted in a cutaway view to visualize the cover-glass. **C)** Schematic depiction of the lateral view of the spinal cord window. The target vertebral spine (here, T12) has been removed. A thin layer of agarose overlays the spinal cord. A cover-glass rests on top of the agarose. Tissue adhesive is applied over the exposed articular process of the adjacent, intact T11 and T13 vertebral spines. Dental cement overlays the tissue adhesive. The backplate adheres to the tissue cement, resting on the transverse processes and the articular process of the adjacent, intact vertebral spines (shown). The backplate is depicted in a cutaway view; in a true lateral view the agarose and cover-glass would be obscured by the lateral wall of the backplate. Anatomic structures are based on detailed

magnetic resonance imaging of the C57Bl/6 spinal column conducted by Harrison and colleagues [11].

Figure 4. Tight junction microstructure visualized by eGFP:Claudin-5 in the mouse spinal cord by intravital two-photon microscopy. A) Single optical section taken at 30 µm beneath the dural surface in a healthy eGFP:Claudin-5 mouse. Red arrow depicts an eGFP:Claudin-5 tight junction segment extending perpendicular to the longitudinal tight junction axis. Scale bar represents 5 µm. Inset: scale bar represents 10 µm. **B)** Z-projection of the vascular network extending 100 µm beneath the dural surface of the healthy mouse spinal cord. The optical stack was sampled at 2 µm axial step size and includes the slice from panel A. No image alignment has been performed. Sharp delineation of the junctional structures in the Z-projection demonstrates minimal image displacement between consecutive frames. **C)** Representative subset of optical slices taken at 10 µm intervals from the resultant Z-stack.

DISCUSSION:

The method described here allows for stable imaging of the spinal cord in mice through a glass window. This method has been applied to assess BBB remodeling in transgenic eGFP:Claudin5+/- mice that express a fluorescent BBB tight junction protein, but it could be applied equally well for studies of any fluorescent proteins or cells in the spinal cord.

Multiple methods for laminectomy and spinal cord stabilization have been developed. All protocols address stabilizing the spinal cord during imaging and window implementation for visual access to the structure of interest. The number of vertebra removed and the degree of invasiveness of the available protocols vary (*e.g.,* components glued on the surface of superficial bone, as in the present protocol, *versus* embedded more deeply). Davalos and Akassoglou[2] developed a laminectomy method using removable clamps on each side of the spinal column and one clamp at the base of the tail of the mouse in order to stabilize the spinal cord. This innovative strategy to suspend the animal relieved some of the thoracic displacement caused by the movement of the lungs expanding against the surgical table. To create a well for containing immersion fluid for a water-immersion microscopic lens, a rim of gelatin seal (*e.g.* Gelseal) was made around the spinal cord and filled with aCSF. The seal rim could be disrupted during imaging, but could also be easily wiped away at the end of the session to enable wound closure and subsequent reimaging. This method has been widely adopted[12,13]. Other groups have developed alternative stabilization methods. Fenrich *et al.* [4] hand-prepared modified paper clips as a way to secure the spinal column. These modified paper clips were secured in the lateral vertebral pedicles with cyanoacrylate glue and maintained as permanently implanted handles for a removable external clip and external holding fork for excellent motion stability. Cupido and colleagues have presented variations on the aforementioned methods with incorporation of agarose overlaid onto the cord[2,4,12]. Farrar and Schaffer[3] developed a quadrilateral metal stabilizer that could allow for the implementation of a glass window onto three vertebrae rather than only one. This method also allowed the spinal cord to be attached with screws to a larger bridge stabilizer during imaging to reduce potential motion. A miniaturized one-photon microscope implanted directly onto the laminectomy imaging chamber has also been developed for *in vivo* recording in freely moving mice at the one-photon level, but is not yet readily available

to most laboratories[13]. In a different approach, Weinger *et al.*[1] dissected an entire spinal cord and embedded it in agarose for *ex vivo* imaging, which allows for unsurpassable motion stability and access to the ventral spinal cord, but abrogates blood flow. Some limitations of these developments include lengthy surgical time[4], possible disruption of the gelatin seal rim[2], the need to customize the cover-glass dimensions to fit the desired area of spinal cord[4], manual modification of paper clips[4,12], relatively invasive surgical techniques[12,14], and air bubbles forming when using the silicon elastomer[3,4].

We have developed an alternative method that offers several advantages. This protocol has been optimized to reduce the amount of time spent during surgery. Whereas some surgical protocols require longer procedure times ranging from an hour and a half[4] to an hour[3]; once mastered, this laminectomy method can be performed in approximately 30 min. Reducing the time spent in surgery can decrease physiologic stress to the mouse, and facilitate higher-throughput experimentation. This protocol removes a single vertebra, and incorporates superficial adhesion of the stabilization device making it less invasive than some comparable protocols[4,5,12,14]. Like the method of Figley *et al.*, by utilizing a plastic implant this protocol offers compatibility with acoustic imaging [5].

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[10,12] or silicone polymers[3-5]. 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 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 the development of the current protocol omitted either the agarose layer or the overlying cover-glass, and found that the consequent light scatter reduced available depth of imaging.

A differentiating feature of this protocol is the incorporation of a 3D printed backplate and supportive backplate fork holder. After the laminectomy and window implantation, the preparation is stabilized by the addition of an 3D-printed oval backplate that is fixed in place with dental cement. The backplate serves two functions: first, it provides structural support and stabilization of the spinal cord, and second, it creates a lip to hold fluid for immersion objectives for microscopy. In prototypes of this setup, commercially available pillar posts, adaptors, and holding forks were used; we have recently switched to custom machined parts as depicted here. In either case, the essential feature is to provide structural rigor to stabilize the imaging field against the perturbations in space and time induced by heartbeat and respiration. Although the body of the animal is resting loosely on the heating pad, the spinal cord and its imaging field are slightly suspended from the holding fork, which also decreases respiratory displacement. The plastic substrate offers slight flexibility to accommodate tension from screwing into the plate holder. The black plastic color used for printing reflects minimal light into the fluorescence field. Through these methods, we successfully generate image stacks that can be used without *post*

hoc alignment adjustment. Furthermore, the 3D backplate described herein is inexpensive to produce, costing only pennies in material for each print, once the printer is purchased. Furthermore, the costs of 3D printers have fallen in the recent years. The 3D printed backplate structural files (See **Supplementary Files 1** and **2**) published with this protocol can be readily modified to accommodate individual laboratory needs. We designed the long dimension of the backplate to accommodate the intervertebral space created by removal of thoracic 12 vertebral spine, which overlies the lumbar 2/3 spinal cord segments[11]. To apply this technique to a different vertebral section, the accompanying CAD files can be modified.

This protocol utilizes a commercially available low-flow anesthesia system that deploys a digital integrated direct injection vaporizer as an alternative to the traditional passive vaporizer. The major feature of the low-flow unit is the reduced operator exposure to isoflurane, a substantial health benefit. The low-flow anesthesia unit also offers cost savings due to the reduced consumption of isoflurane and utilization of room air instead of compressed gas. In the present study, 2% isoflurane delivered by integrated digital vaporizer at 150 mL/min, together with feedback-controlled thermal support, achieved a stable plane of anesthesia and appropriate maintenance of core body temperature. Consistent with this, published comparisons of digital integrated vaporizers and traditional vaporizers have also concluded that the digital integrated vaporizer yields a stable plane of anesthesia and good preservation of core body temperature, heart rate, respiratory rate, and recovery while utilizing less isoflurane[15,16].

A non-steroidal anti-inflammatory drug (NSAID) such as carprofen can be administered Pre-operatively as a supplementary analgesic. Over the course of few hours, NSAIDs inhibit inflammatory cytokine transcription and interstitial edema; multi-day administration attenuates severity of neuroinflammatory diseases including experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis[17,18]. Particularly in the study of neuroinflammatory disease, the beneficial effects of carprofen analgesia must be carefully weighed against disease modifying effects when determining analgesia and anesthesia for an experiment in close coordination with appropriate regulatory boards.

A limitation of this method is that it is not readily amenable to repeated imaging sessions across multiple days. The main reason is that the backplate structure is too large to close the skin over. Therefore, there is a risk that a mouse would dislodge the backplate upon waking from anesthesia. If repeated imaging was essential, there are several strategies that could be deployed, including reducing the size of the backplate, or changing the mount. As with any surgical procedure, there is a learning curve for operators. Close coordination with institutional animal care offices and review boards is required.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

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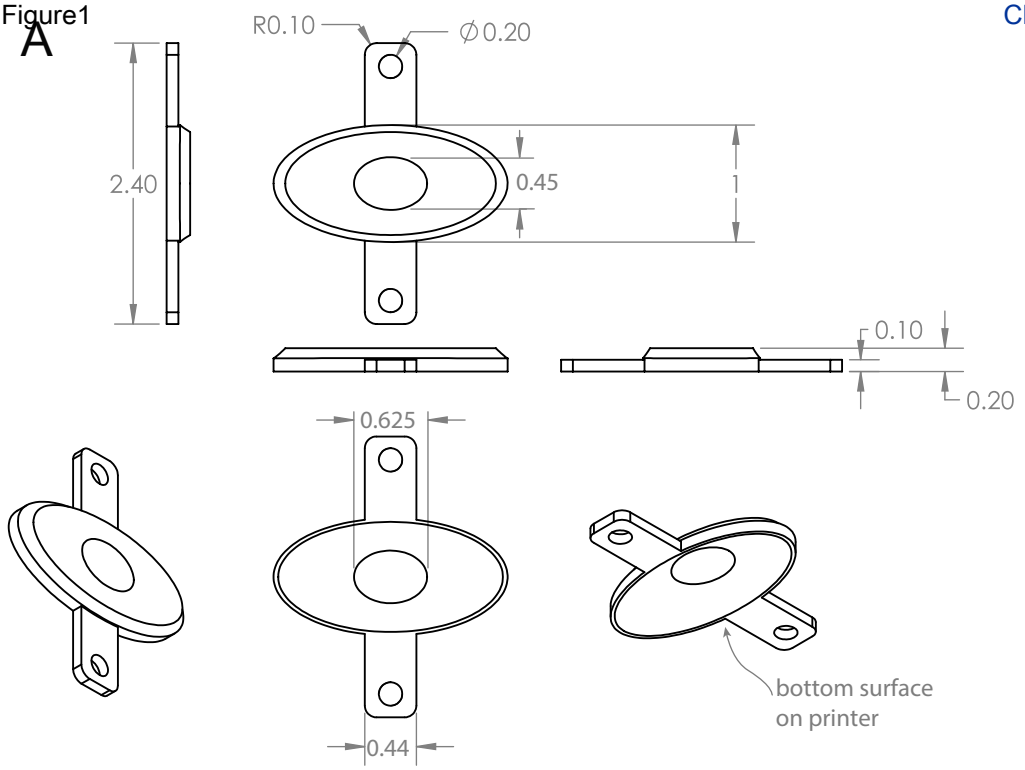

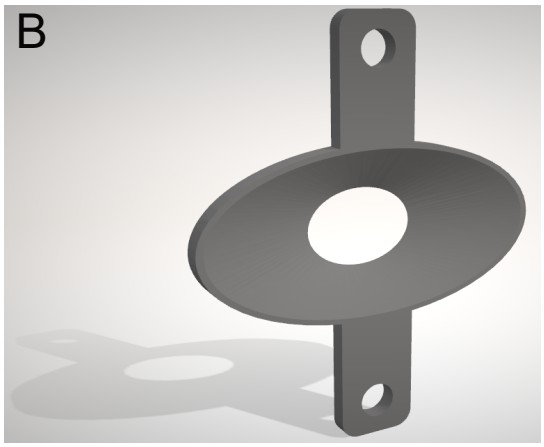

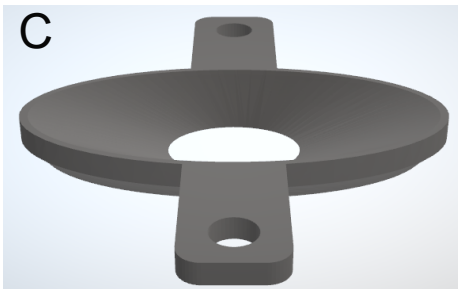

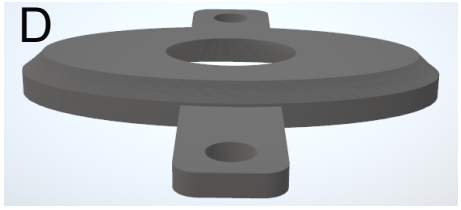

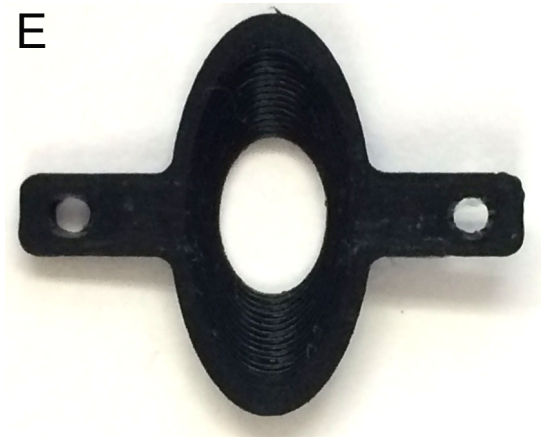

Figure2

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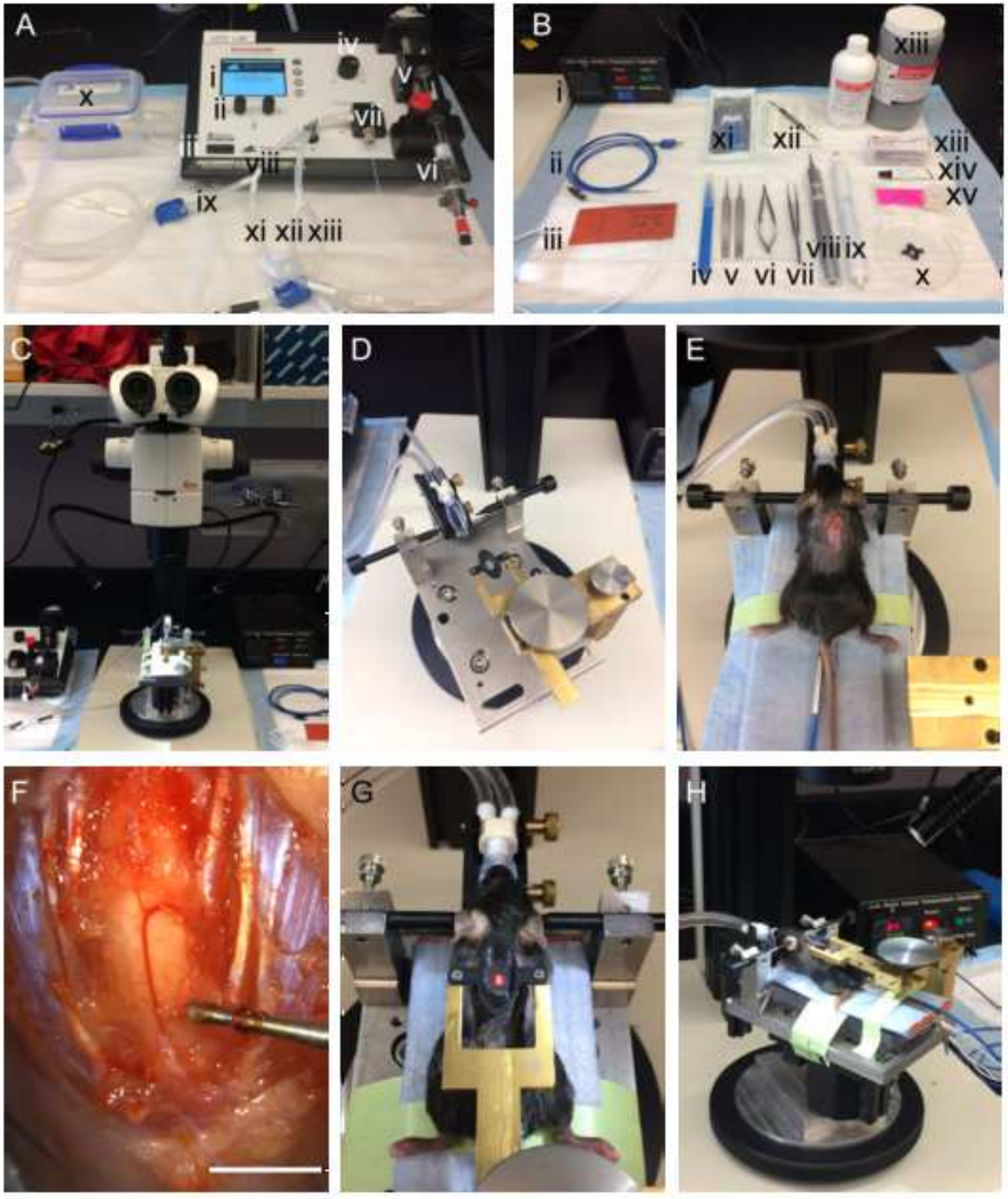

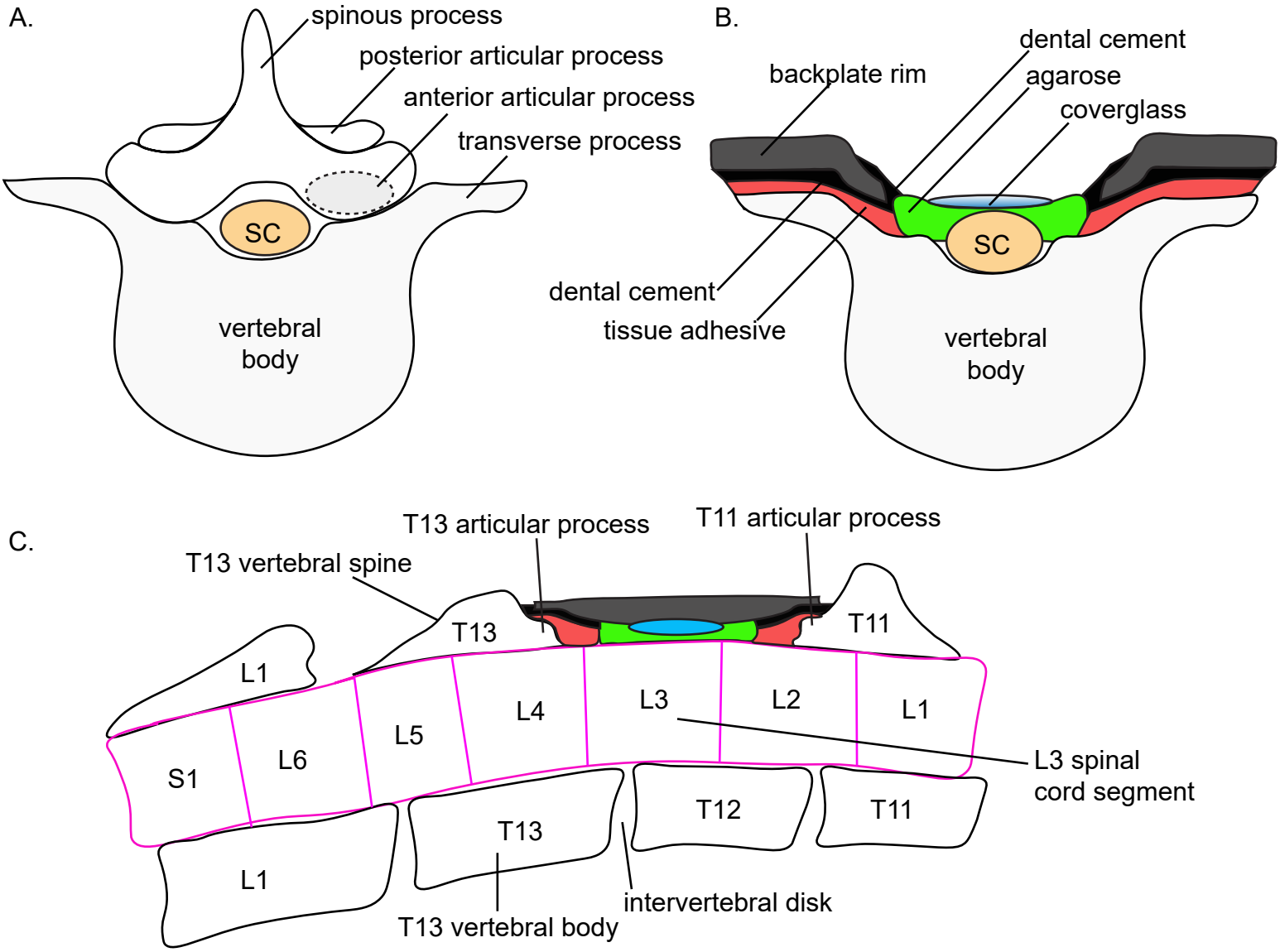

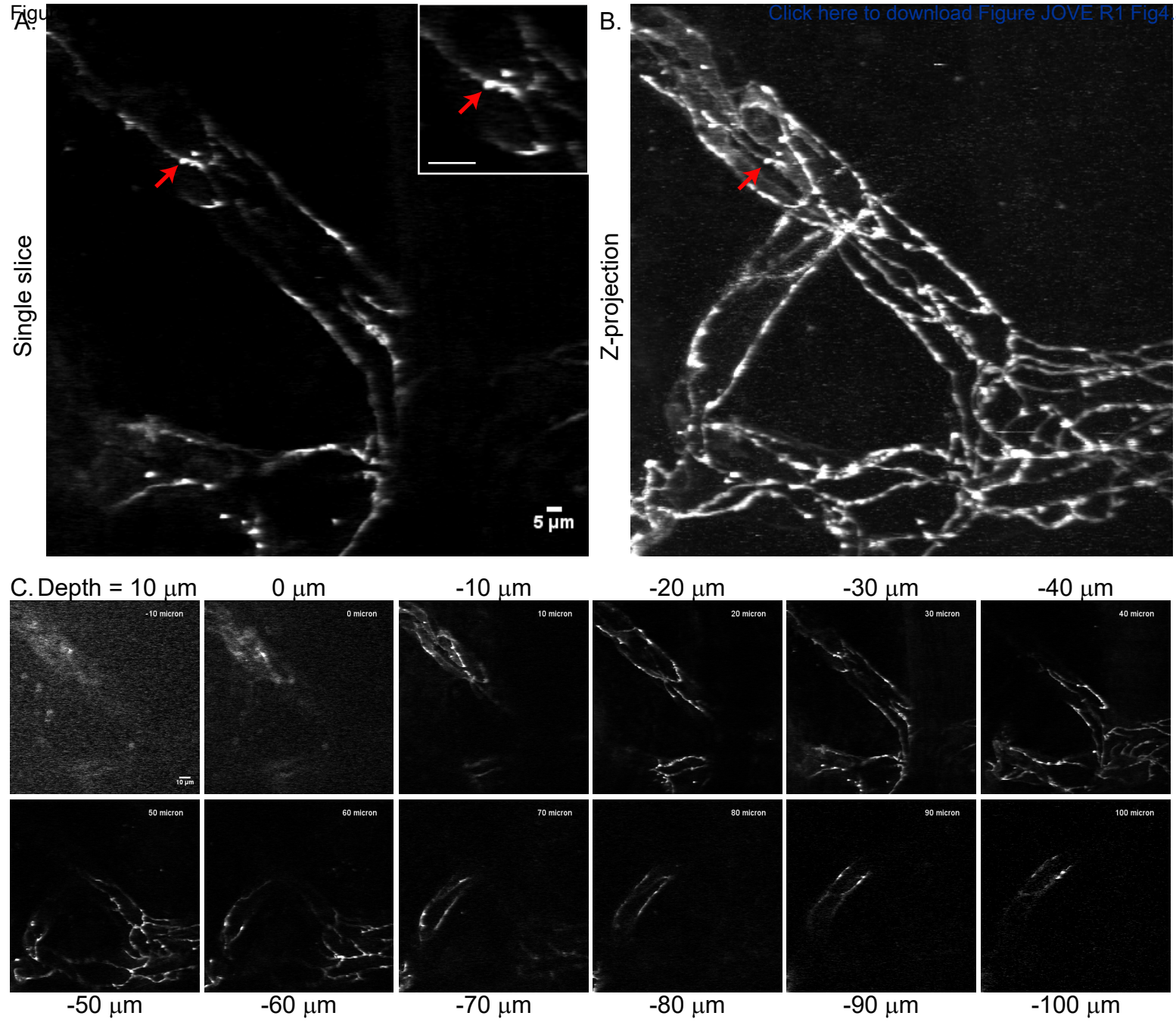

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3D printer	Raise3D	Pro2	For printing backplates
PLA 3D printing filament	Inland	PLA+-175-B	Black plastic 3D printing material
3D CAD software	Dassault Systemes	Solidworks software	used to design 3D shapes
3D printer software	Raise3D	Ideamaker software	software used to interface with the 3D printer
3D printed oval backplate	custom		Stabilizing imaging field
Surgical dissecting microscope	Leica	M205 C	Equipped with Leica FusionOptics, Planapo 0.63x M-series objective, and gliding stage
Microscope camera	Leica	MC170	HD color camera for visualizing surgical field
Gliding stage	Leica	10446301	The gliding stage is constructed of two metal plates. The base plate is fixed. The upper plate slides on greased interface to allow rotational and linear movement.
Surgical station and stabilization fork	Whale Manufactoring	custom	Laminectomy
SomnoSuite low-flow isoflurane delivery unit	Kent Scientific	SS-01	Surgical anesthesia administration with integrated digitial vaporizer
Stainless steel 1.5 inch mounting post	ThorLabs	P50/M	For mounting surgical station onto optical table for two-photon imaging
Counterbored Clamping Fork for 1.5" mounting Post	ThorLabs	PF175	For stabilizing surgical station mount onto optical table for two-photon imaging

Ideal bone microdrill	Harvard apparatus	72-6065	Thinning bone for laminectomy
Water bath	Fisher Scientific	15-462-10	Warming saline
Cautery gun	FST	18010-00	Cauterizing minor bleeds
Heating pad	Benchmark	BF11222	1.9” x 4.5” silicone heater with 20” Teflon leads, 10W, 5V
K type thermocoupled rectal probe	Physitemp	RET3	Measuring mouse body temperature
petroleum jelly	Sigma	8009-03-8	Lubricating rectal probe
Feedback-regulated thermal controller	custom	NA	Commercially available alternatives include the Physitemp TCAT series
PVA Surgical eye spears	Beaver-visitec international	40400-8	Absorbing blood
Electric trimmer	Wahl	41590-0438	Trimming mouse fur
Blade, #11	FST	14002-14	Surgical tool
Forceps, #5	FST	11254-20	Surgical tool
Forceps, #4	FST	14002-14	Surgical tool
Titatnium toothed forceps	WPI	555047FT	Surgical tool
Titanium Iris scissors	WPI	555562S	Surgical tool
Vetbond tissue adhesive	3M	084-1469SB	Preparing tissue surface for dental acrylic
Ceramic mixing tray	Jack Richeson	420716	Mixing dental acrylic agent with accelerant
Orthojet dental acrylic	Lang Dental	1520BLK, 1503BLK	Permanently bonding backplate to tissue
Small round cover glass, #1 thickness, 3 mm	Harvard apparatus	64-0720	optical window
NaCl	Fisher Scientific	7647-14-5	For aCSF
KCl	Fisher Scientific	7447-40-7	For aCSF
Glucose	Fisher Scientific	50-99-7	For aCSF
HEPES	Sigma	7365-45-9	For aCSF
MgCl2·6H2O	Fisher Scientific	7791-18-6	For aCSF
$CaCl_2 \cdot 2H_2O$	Fisher Scientific	10035-04-8	For aCSF
Carprofen	Rimadyl	QM01AE91	Analgesia
Bacteriostatic water	Henry Schein	2587428	Diluent for carprofen
Isoflurane	Henry Schein	11695-6776-2	Anesthesia
Lactated ringer solution	Baxter	0338-0117-04	Hydration for mouse

Agarose High EEO	Sigma	A9793	gel point 34-37 degrees C
Opthalmic lubricating ointment	Akwa Tears	68788-0697	Prevent corneal drying
MOM Two-Photon Microscope	Sutter		

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30 July 30, 2018

JoVE58330
"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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Supplemental Coding Files

Supplementary File 1 - STL for 3D printing.STL