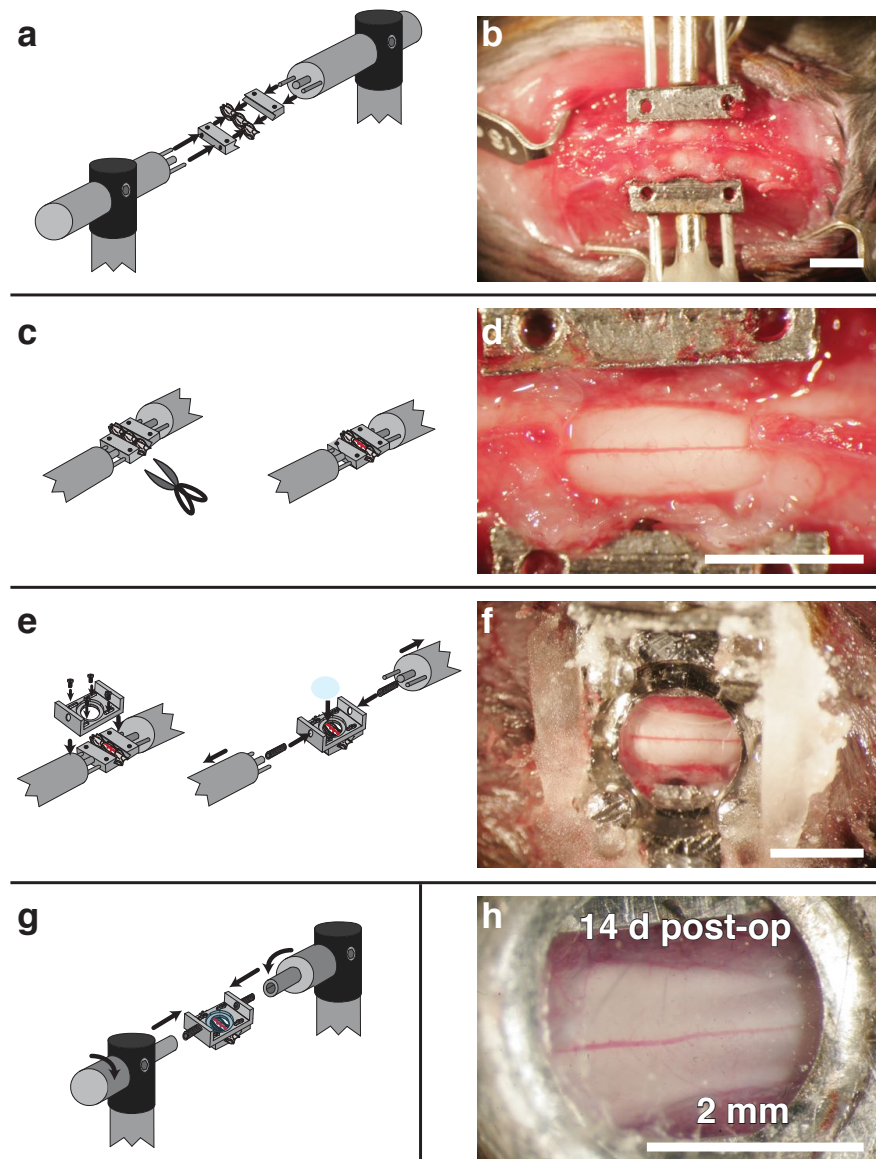
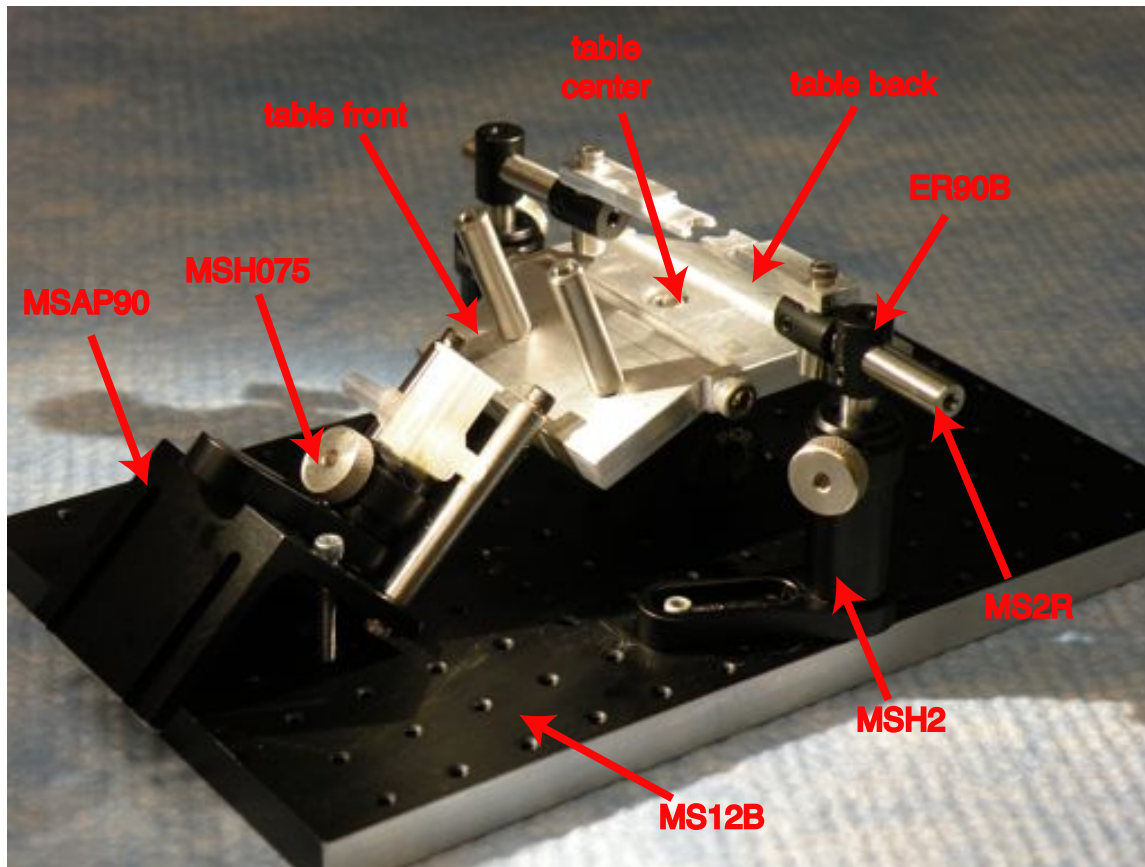


Supplementary Figure 1



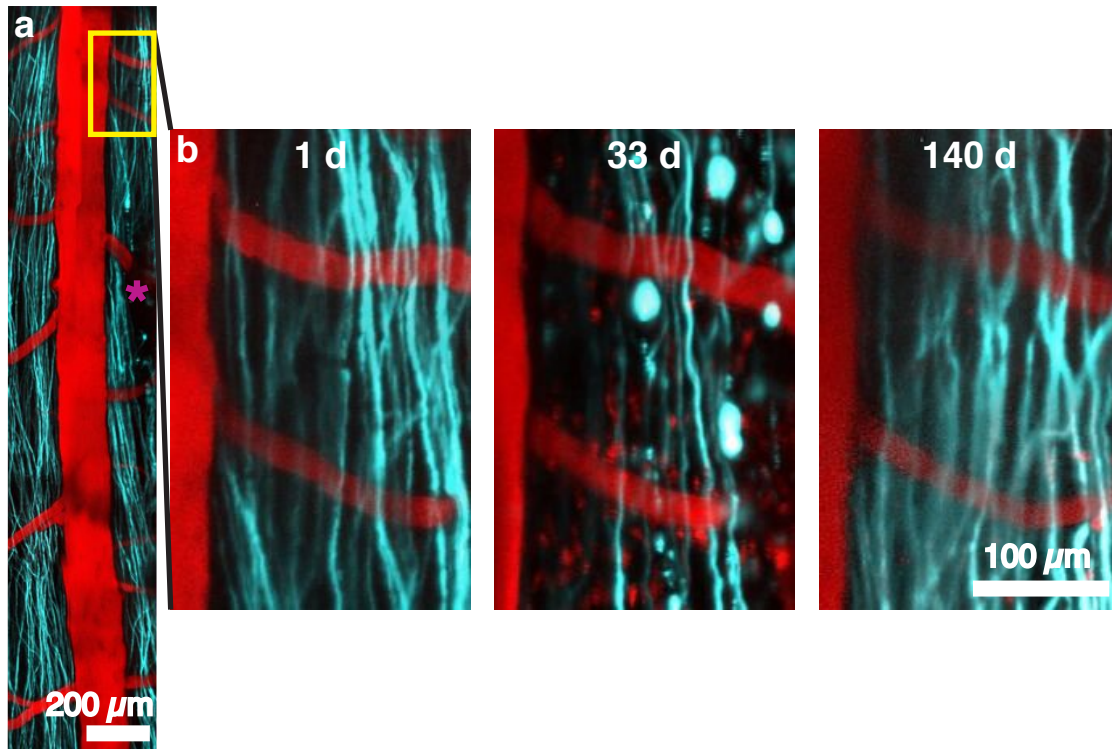
Supplementary Figure 1: A metallic spinal chamber implant was mounted via a custom delivery system onto the vertebral column and provided long-term optical access to the spinal cord. The exposed vertebrae (T10-T12) were clamped by notched metal bars attached to holder posts (a,b) after soft muscle had been retracted. Vanna scissors were used to complete a dorsal laminectomy (c,d) on the central vertebra (T11). A top plate was secured using four screws, maintaining the clamping pressure (e). Silicone elastomer was placed over the cord and the chamber was sealed with glass, while set screws were inserted into the wings of the top plate (e,f). Holder posts were removed (e) and the skin was sealed using a combination of cyanoacrylate adhesive and dental acrylic cement (f). To image, mice were anesthetized and the spine was locally immobilized by securing the implant to holder posts via the set screws (g). Optical access was possible for several weeks or more (h).

Supplementary Figure 2



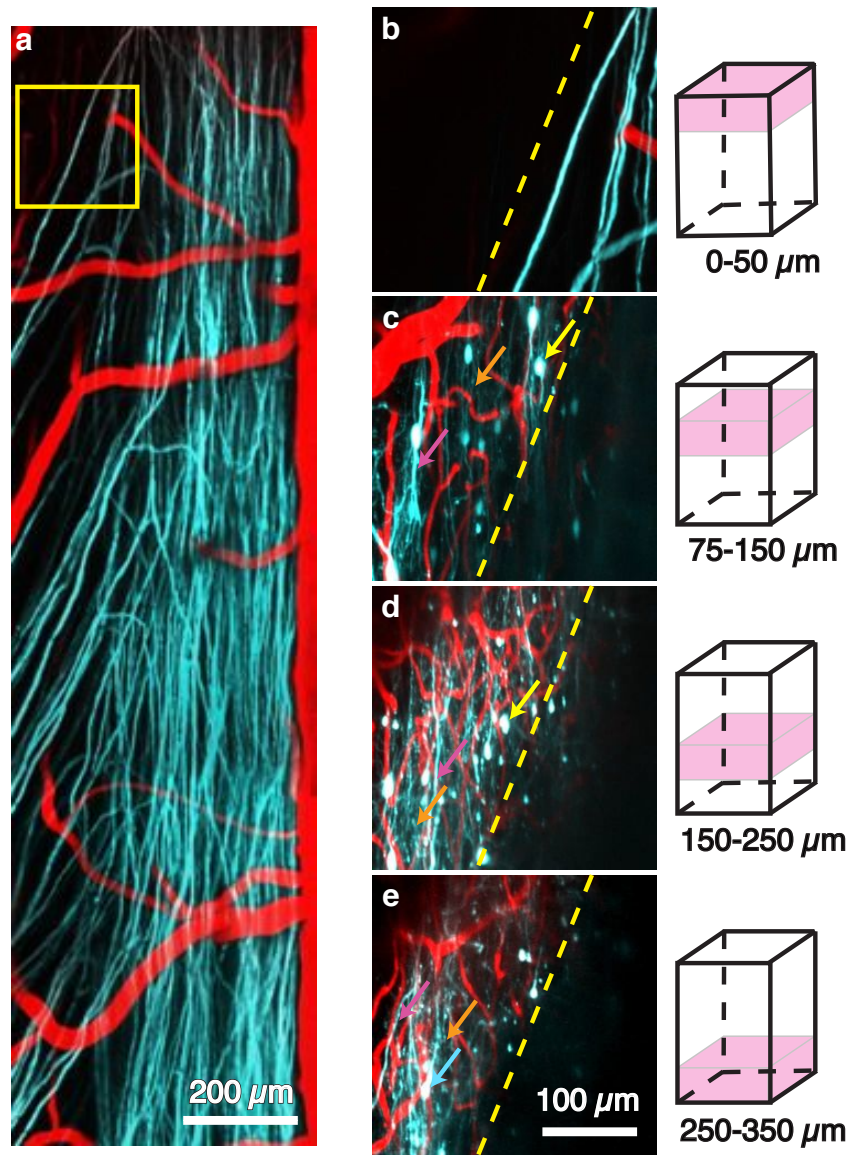
Supplementary Figure 2: A custom surgery table allows for both surgery and imaging procedures. A procedure table –composed of rotary wings and a center part–designed for surgery and imaging is readily built with relatively inexpensive optics parts. Parts as labeled are available from Thor Labs under the following part numbers: $\varnothing 6$ mm posts (MS2R) and post-holders (MSH2), right-angle post adapters (ER90B), post-holder for nose-cone (MSH075), angle bracket (MSAP90), $\varnothing 1/2$ ” post (TR2) and post-holder (PH1.5) (below the surgery table), and breadboard (MS12B). The posts that screw to the set screws in the top plate of the imaging chamber, as illustrated in Fig. 1g, mount to the ER90B right-angle adaptors. Shown mounted here is a vertebral clamp for acute imaging where a chronic chamber is not required. Optionally, posts can be added to the table front to prevent sliding during surgery.

Supplementary Figure 3



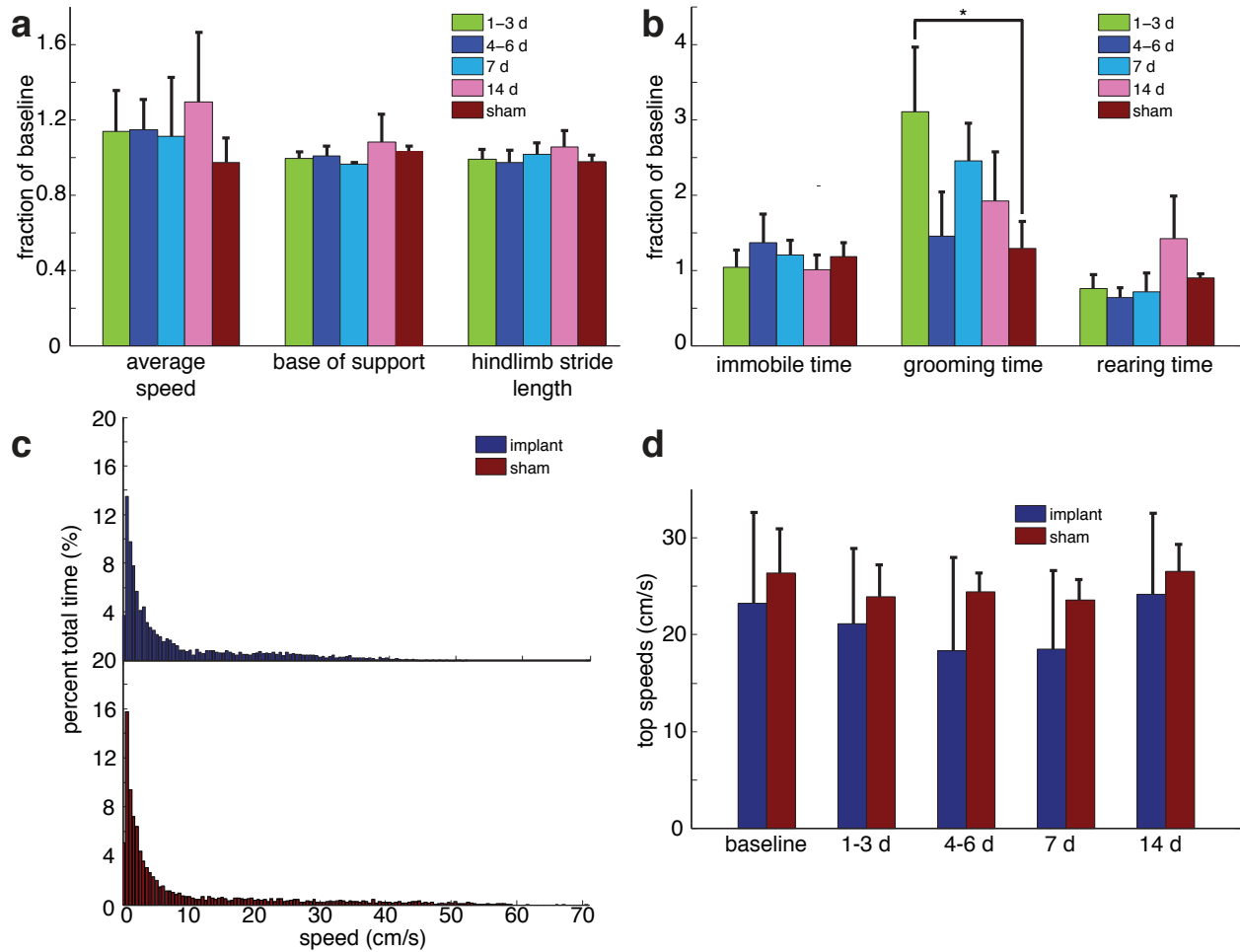
Supplementary Figure 3: Imaging with individual axonal resolution is possible out to as many as 140 days post-surgery. In one animal considered, multiple imaging sessions were possible out to as long as 140 days. An injury induced during surgery was evident one day after the surgery (**a**, mauve asterisk) and a region rostral to the injury (**a**, yellow box) remained sufficiently clear so as to image individual axons. Axonal degeneration progressed rostrally over time (**b**), with little change on one day post-surgery, severe degeneration in the selected region by day 33, and only spared axons present at day 140.

Supplementary Figure 4



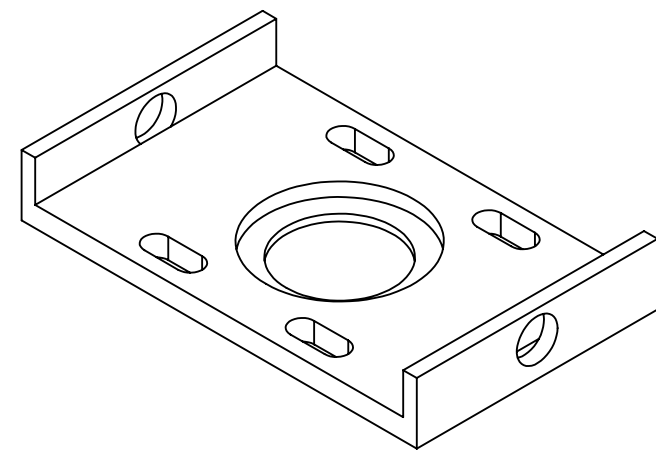
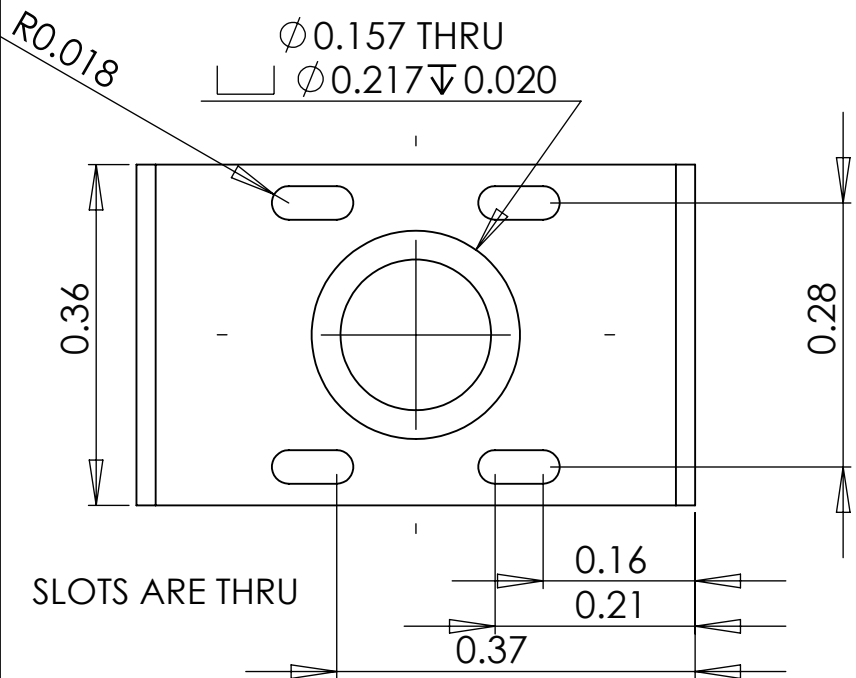
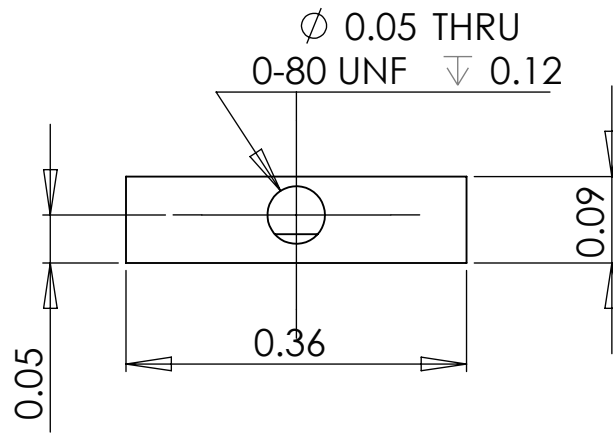
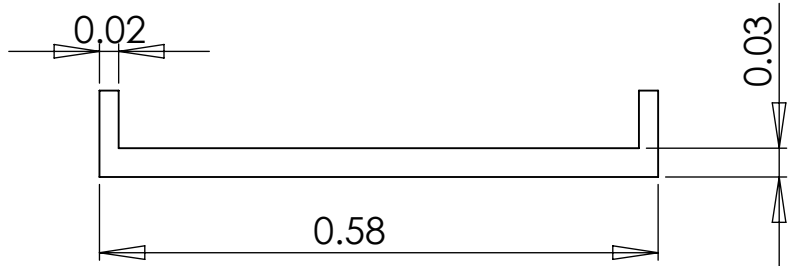
Supplementary Figure 4: Anatomically myelin-poor regions of the spinal cord enable deep-tissue imaging. Regions of the dorsolateral spinal cord between dorsal roots (a, yellow box) exhibit regions deficient of densely myelinated axons (YFP, teal). In these regions, imaging of neuron cell bodies (yellow arrows), dendrites (orange arrows), axons (mauve arrows), and vasculature (Texas Red dextran, red) in the dorsal horn is possible up to several hundred micrometers below the surface (b-e). Image projections are taken at and over the depths given by the illustrations (right). The dotted line marks the stark change in contrast between areas underlying myelinated superficial axons and regions of sparse axon density.

Supplementary Figure 5



Supplementary Figure 5: Implanted mice do not show significant changes in mobility or motor function compared to sham controls. Measurements of ink footprints and videography of implanted and control mice running down an enclosed track were used to determine stride length, base of support, and average speed. Measurements were taken from multiple post-operative timepoints as well as from sham-operated controls ($n = 3$ mice per group; consecutive daily measurements made in each group), with results expressed as a fraction of the preoperative within-subject baseline (a). No significant differences were seen between groups in any category. Open field testing also was used to measure cumulative rearing time, grooming time, and time spent immobile during a 5-minute test. Grooming time was significantly higher in implanted mice 1-3 days post-operatively than in controls ($P = 0.0069$; $n = 3$ mice per group); otherwise, implanted mice did not differ from controls (b). Top speed was characterized from the speed histogram (c) as the mean of the speeds above the 75th percentile. Top speeds were compared both pre-operatively and post-operatively (d) and no significant differences were seen across groups. Error bars denote the standard deviation.

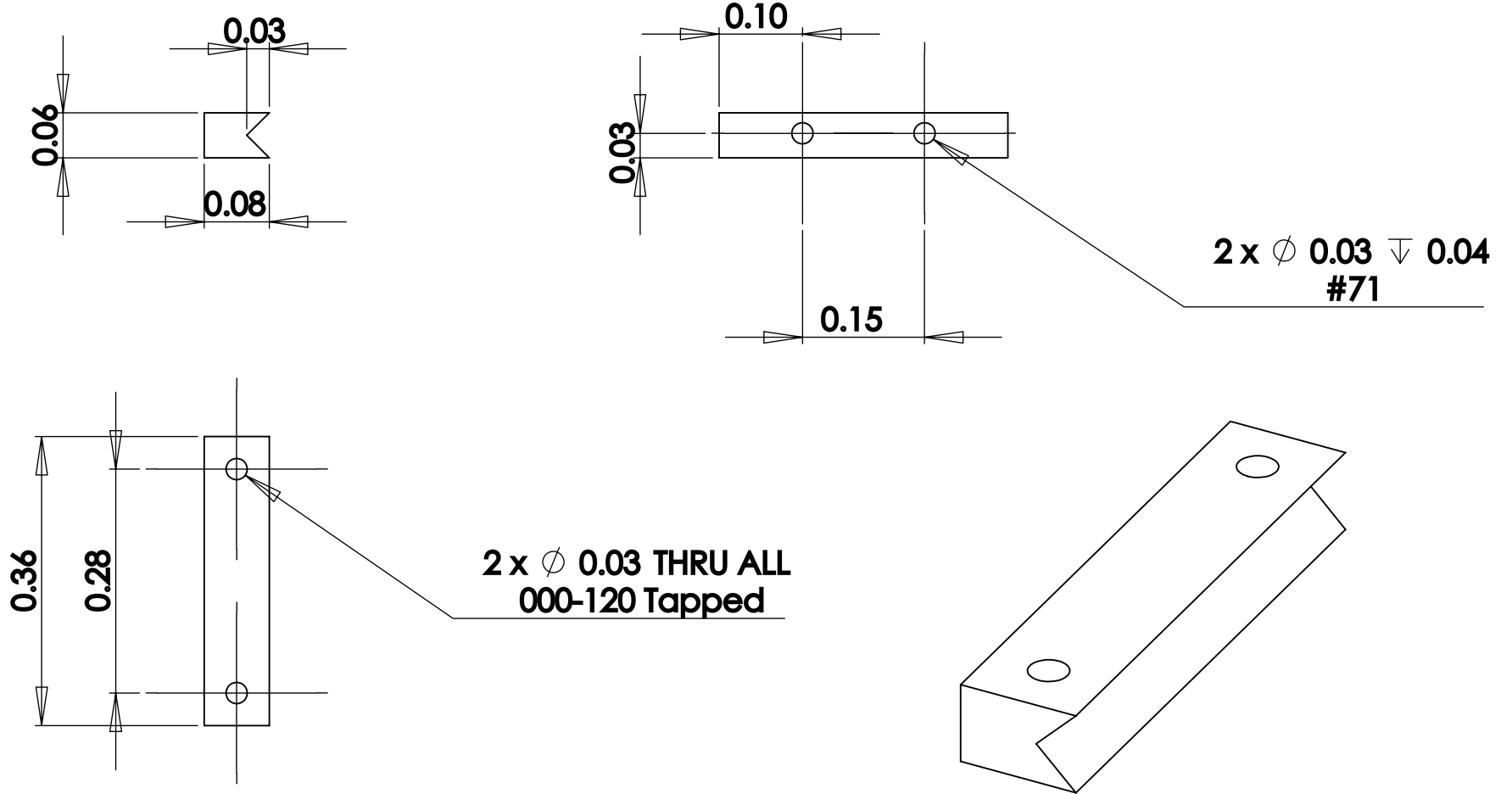
Supplementary Note 1



SCALE 5:1

PART: CHAMBER TOP PLATE
QUANTITY: 1
MATERIAL: 316 STAINLESS STEEL

Supplementary Note 2



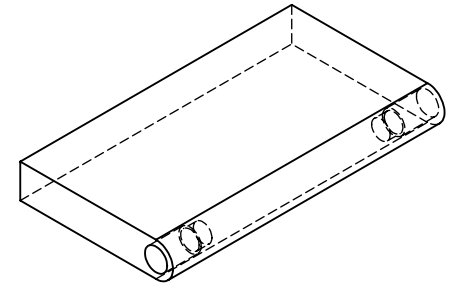
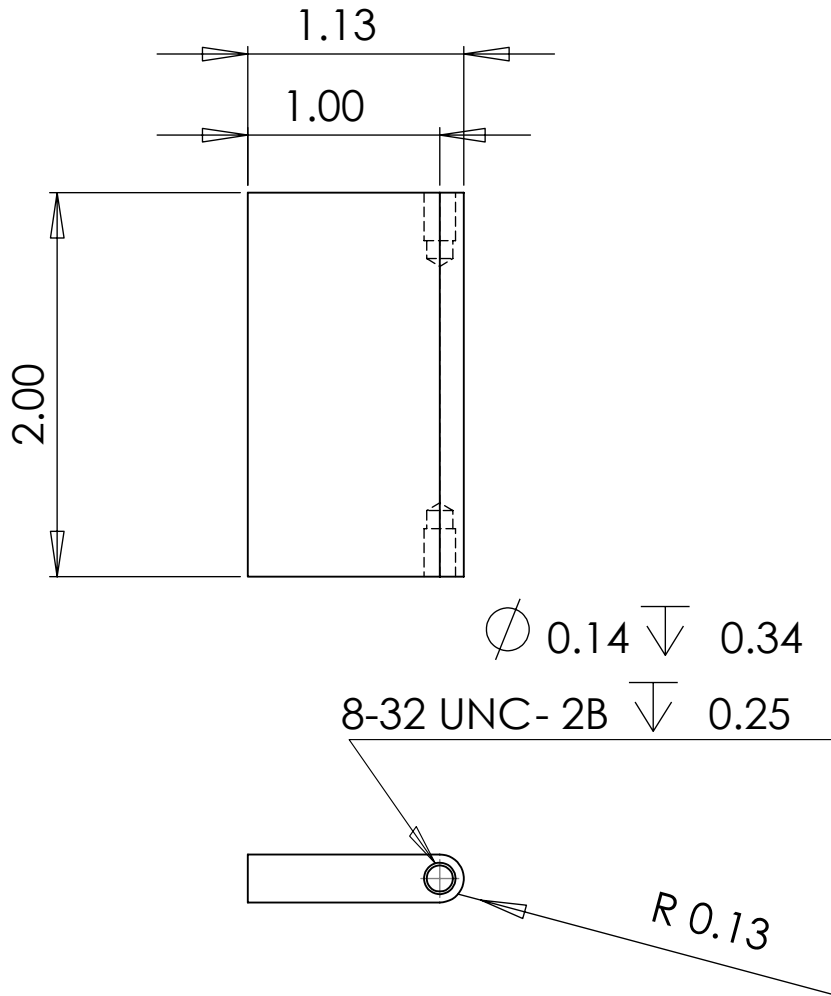
SCALE: 5:1

PART: CHAMBER SIDE BAR

QUANTITY: 2

MATERIAL: MAGNETIC STAINLESS STEEL (E.G. 430)

Supplementary Note 3



SCALE: 1:1

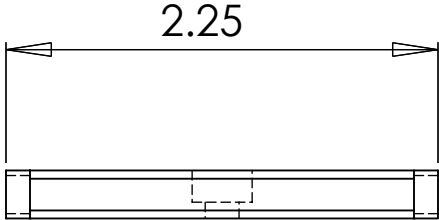
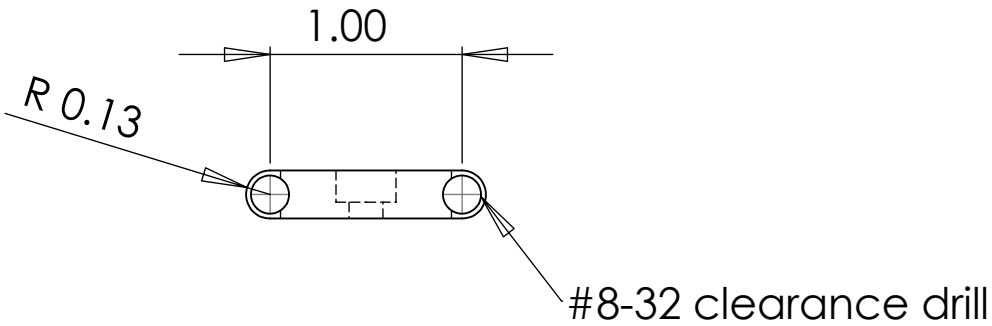
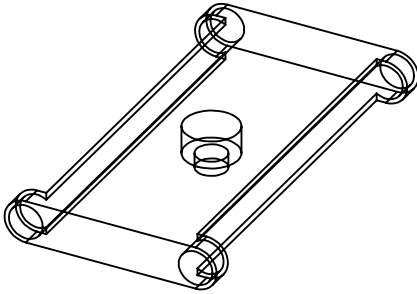
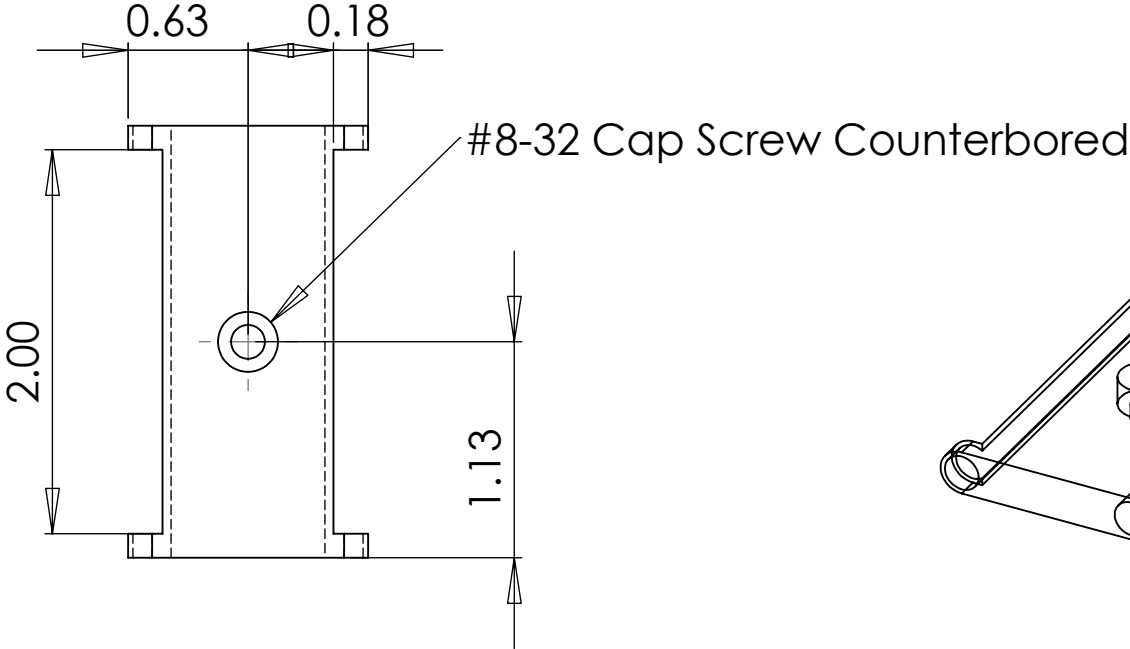
PART: TABLE FRONT/BACK

QUANTITY: 2

MATERIAL: ALUMINUM

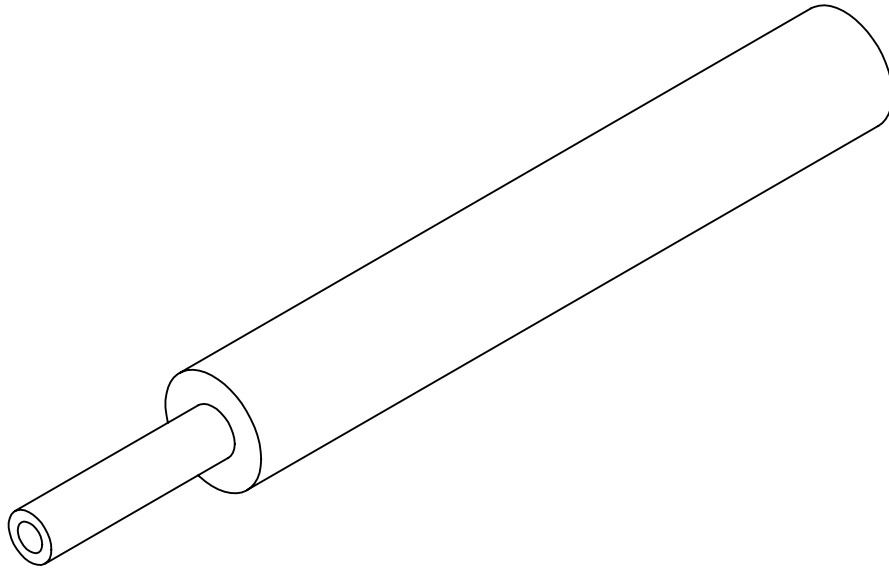
REV

Supplementary Note 4

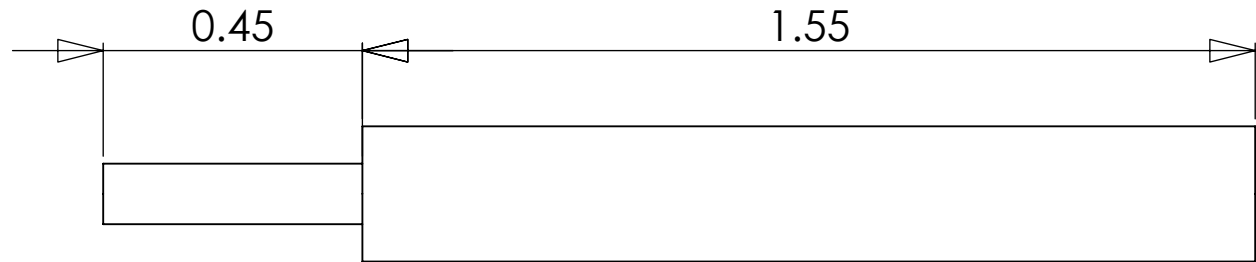
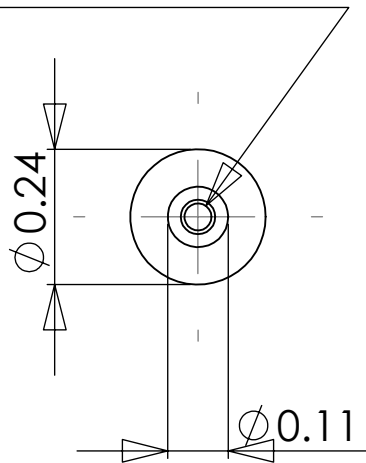


SCALE: 1:1
PART: TABLE CENTER
QUANTITY: 1
MATERIAL: ALUMINUM

Supplementary Note 5



$\phi 0.05 \downarrow 0.26$
0-80 UNF - 2B $\downarrow 0.20$



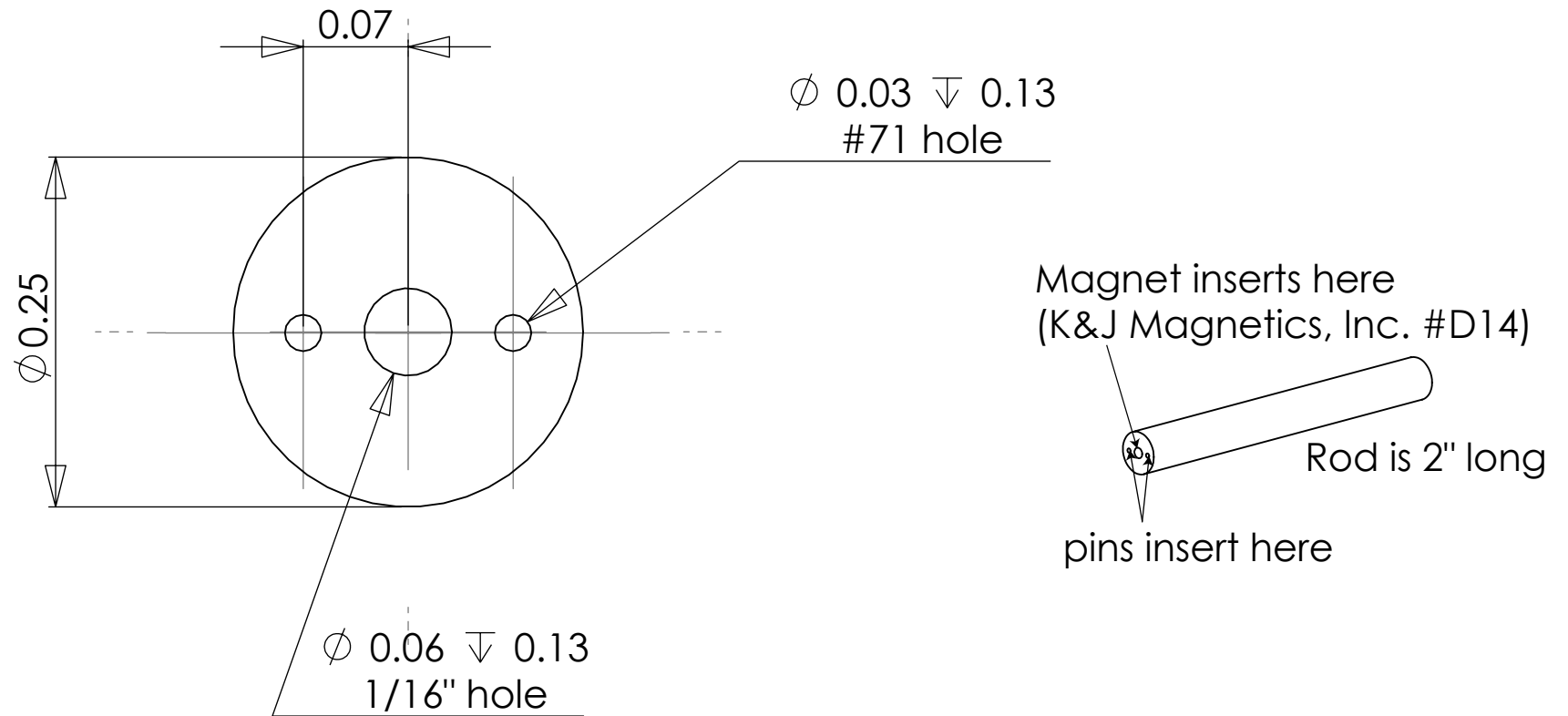
SCALE: 3:1

PART: CHAMBER HOLDER FOR IMAGING

QUANTITY: 2

MATERIAL: MODIFIED THOR LABS POST (STAINLESS STEEL)

Supplementary Note 6



SCALE: 8:1

PART: BAR HOLDER FOR SURGERY

QUANTITY: 2

MATERIAL: MODIFIED THOR LABS POST (STAINLESS STEEL)

Supplementary Protocol

1. A mouse is anesthetized on 5 % isoflurane mixed with 100 % oxygen in an induction chamber.
2. Once initial anesthesia has been achieved, the isoflurane is reduced to ~1.5-2 % for the duration of the procedure.
3. The mouse receives glycopyrrolate (0.05 mg per 100-g mouse), an anticholinergic, via intramuscular injection into the hindlimbs.
4. The mouse is transferred to a shaving/washing station away from the sterile surgery field where the relevant portions are shaved and receive three alternating washes of iodine and 70 % ethanol applied by cotton applicator.
5. The mouse receives 0.1 mL of 0.125 % bupivacaine, a local anesthetic, subcutaneously near the peak of the thoracic curvature. Between the ears or anywhere distal to the surgery site, 1 mL per 100-g mouse of 5 % glucose in saline, 0.25 mL per 100-g mouse of 0.5 mg/mL ketoprofen, and 0.2 mL per 100-g mouse of 0.1 mg/mL dexamethasone are administered by subcutaneous injection.
6. The mouse is transferred to custom spinal surgery table (Supplementary Fig. 3) with a feedback-controlled heating blanket overlaid with sterile drapes. All surgical tools should be sterilized by autoclaving or other suitable sterilization technique.
7. A small (~1 cm incision) is made with a #10 scalpel in the skin near the thoracic arch, and fascia connecting the skin to the underlying muscle is gently pulled away using forceps.
8. Retractors are used to hold back the skin, creating an approximately ~1" x 1.5" exposed field.

9. After finding T11, T10 is firmly grasped through the muscle using forceps, and a scalpel is used in combination with a bone scraper to remove the muscle overlying T10-T12. Sterile cotton applicators are used to control bleeding as well as to gently remove tissue adhered to the bone.
10. Using curved scissors and forceps, tendons are severed from the transverse processes of vertebrae T10-T12.
11. Tissue lateral to the vertebral column is severed and retracted from the bone deep and wide enough to allow insertion of the side bars, but taking care not to injure the dorsal roots. Bleeding is controlled with sterile cotton applicators. **Critical Step:** To avoid necrosis, tissue must be trimmed smooth, especially along the transverse process of the vertebrae.
12. Side bars are delivered on custom 3-pronged post-holders (bar holder) and fuse the 3 vertebrae by clamping. The compression is held by tightening set screws on the right-angle post adapter.
13. **Critical Step:** It is essential that all soft tissue be effectively removed from T11. This can be achieved by drying the bone with cotton applicators and/or by applying a modest amount of cyanoacrylate glue and removing it after hardening, effectively providing a means of tissue debridement.
14. Vanna scissors are inserted just into the epidural space on either lateral aspect of the dorsal lamina of T11. If the bone is sufficiently dry, the initial cut should cause the bone to crack along its length.
15. The process is repeated for the contralateral side, and any rostral or caudal soft tissue is gently cut away while pulling the dorsal lamina away from the spinal cord with forceps.

Bleeding is controlled at the edges of the laminectomy using sterile cotton applicators. Blood is cleared from the spinal cord by flushing the exposed cord with artificial cerebrospinal fluid (ACSF). **Critical Step:** During the removal of the bone, part of the periosteum may detach and cover the spinal cord. This tissue should be delicately removed with a dural hook or small needle. Care should be taken to distinguish the periosteum from the dura mater.

16. Once the initial lamina is removed, carefully trim back the bone on the transverse processes to more fully expose the spinal cord. The surface of the bone may be sealed by carefully applying dental cement to both the bone surface and the side bars.
17. Once bleeding has been controlled and the spinal cord cleanly exposed, the top plate is positioned on top of the side bars and centered over the laminectomy.
18. Four #000-120 screws can then be partially inserted, starting with diagonally opposite slots. Once the final position is satisfactorily achieved, these screws may be tightened.
19. Kwik Sil elastomer is injected into the laminectomy site, taking care to avoid air bubbles.
20. Immediately after injection, a #0 cover slip is inserted to seal the laminectomy, gently applying pressure to squeeze the silicone into the rest of the chamber.
21. Approximately 10-20 minutes should be given to allow the silicone to set.
22. The silicone and chamber are sealed at the rostral and caudal edges by applying small amounts of dental acrylic and/or superglue to the chamber, silicone, and the exposed bone of T10 and T12.
23. The set screws on the right-angle adapters are loosened and the custom-delivery system is gently removed from the side bars.

24. Retractors are removed, and the skin is pulled up around the edges of the implant and secured with cyanoacrylate glue.
25. #0-80 set screws are inserted into the wings of the top plate.
26. Dental acrylic is applied on top of the skin at the edges of the implant and used to fill the slots in the top plate, providing a good seal on the chamber.
27. A second injection of 0.1 mL of 0.125 % bupivacaine is administered around the implant and the animal is transferred to a clean cage on a heat pad to recover.

Step-by-Step Imaging Procedure

1. The mouse is anesthetized on 5 % isoflurane mixed with 100 % oxygen in an induction chamber.
2. Once anesthesia has been achieved, the isoflurane is reduced to ~1.5-2 % for the duration of imaging.
3. The mouse receives glycopyrrolate (0.05 mg per 100-g mouse), an anticholinergic, via intramuscular injection into the hindlimbs.
4. The mouse is transferred to the custom surgery table (Supplementary Figure 3) with a feedback-controlled heating pad.
5. Tapped posts (chamber holders) are inserted into the right-angle adapter and twisted onto the set screws on the wings of the chamber.
6. Set screws on the right-angle adapter are tightened to prevent the chamber from moving during imaging.

7. The posts are raised in the post-holders to elevate the thorax from the surgery table, thereby allowing for free expansion of the chest upon inspiration without introducing motion artifact.

Set screws on the post-holders prevent up-down motion of the posts.

8. Vasculature is labeled by intravascular injection of a fluorescent dextran by retro-orbital injection. Briefly, a syringe is inserted under the eyeball with the bevel towards the nasal septum while dye is slowly ejected.

9. Multiphoton microscopy is performed for up to several hours.

10. The tapped posts (chamber holders) are unscrewed and the mouse is allowed to recover.

Trouble Shooting

Problem	Possible Reason	Solution
1. Chamber detaches from spinal cord post-surgically	<ul style="list-style-type: none"> • insufficient clamping pressure • insufficient gluing 	<ul style="list-style-type: none"> • ensure appropriate pressure applied and that set screws in right-angle adapters are tight • apply glues more liberally at the rostral and caudal edges of the implant
2. Profuse bleeding in peripheral tissue during surgery.	<ul style="list-style-type: none"> • severing of major artery 	<ul style="list-style-type: none"> • cauterize bleed with electro- or thermal cautery • apply pressure to bleed until stopped with cotton applicator
3. Air bubbles in silicone under window.	<ul style="list-style-type: none"> • air in mixer tip • air entering through edges of chamber 	<ul style="list-style-type: none"> • ensure that air has been evacuated from the mixer tip prior to filling chamber • apply glues more liberally at the edge of the chamber to prevent silicone from pulling away from tissue • ensure that silicone has opportunity to set prior to disturbing the chamber
4. Blood in window post-surgery.	<ul style="list-style-type: none"> • bleeding in peripheral tissue entering under silicone • bleeding from vertebral body • bleeding from the spinal cord itself 	<ul style="list-style-type: none"> • determine the source of bleeding by removing the silicone, and if necessary, replace it. • apply glues more liberally at the rostral and caudal edges of the implant to seal out any peripheral fluid from entering • ensure that the bone has stopped bleeding before applying silicone • carefully applying a thin layer of superglue to the bone can help reduce bone bleeds

Problem	Possible Reason	Solution
5. Rapid (few days) loss of image contrast	<ul style="list-style-type: none"> • fibrous tissue growth 	<ul style="list-style-type: none"> • trim back more of transverse processes and seal with dental acrylic • reduce space for fibrous tissue to grow by minimizing the space between the glass and spinal cord by appropriate leveling of the implant
6. Motion artifact complicates imaging.	<ul style="list-style-type: none"> • chamber is loose • chamber is not securely held • mouse is insufficiently elevated • spinal cord moving within vertebral column 	<ul style="list-style-type: none"> • ensure appropriate pressure is applied and that set screws in right-angle adapters are tight • apply glues more liberally at the rostral and caudal edges of the implant • ensure all set screws are tightly fastened • elevate mouse so that chest can expand freely upon inspiration

Supplementary Note 7: Equipment and Settings

Image(s): Figure 1a,d

Acquisition device: Canon DS12601 Digital Camera

Lens: Canon Macro Lens (EF 100 mm)

Photo Processing: image brightening, uniform linear contrast adjustment

Image(s): Figure 1c

Acquisition device: Canon DS12601 Digital Camera

Lens: Leica MZ12.5 Stereomicroscope

Photo Processing: image brightening, uniform linear contrast adjustment

Image(s): Figure 2a

Objective Lens: Zeiss 20x water immersion (NA = 1.0)

Original bit depth: 16 bit/color

Fluorophore(s): YFP, Texas Red

Excitation Wavelength: 1040 nm

Bandpass Emission Filters: 517/65 nm, 645/65

Dichroic: 600-nm long-pass

x-y resolution: 1.11 $\mu\text{m}/\text{pix}$

z increment: 1 $\mu\text{m}/\text{frame}$

z-projection type: standard deviation

Filtering: none

Image(s): Figure 2b

Objective Lens: Zeiss 20x water immersion (NA = 1.0)

Original bit depth: 16 bit

Excitation Wavelength: 1040 nm

Fluorophore(s): YFP

Bandpass Emission Filters: 517/65 nm

Dichroic: 600-nm long-pass

x-y resolution: 0.59 $\mu\text{m}/\text{pix}$
z increment: 0.5 $\mu\text{m}/\text{frame}$
z-projection type: standard deviation
Filtering: bandpass filtering (0-80 pixel)

Image(s): Figure 3a,b,c (insets)
Microscope: Olympus BX41
Objective Lens: Olympus UPlan Fl 20x/0.5
Original bit depth: 8 bit
Excitation Source: Halogen lamp
Bandpass Emission Filters: standard GFP filter cube
Field of View: 880 μm
Image processing: image cropping

Image(s): Figure 3c
Microscope: Olympus BX41
Objective Lens: Olympus UPlan Fl 4x/0.13
Original bit depth: 8 bit
Type: Bright field image
Field of View: 4.2 mm/panel
Image processing: image stitching (4 panels), image cropping

Image(s): Figure 4a-c
Objective Lens: Zeiss 20x water immersion (NA = 1.0)
Original bit depth: 16 bit/color
Fluorophore(s): YFP, GFP
Excitation Wavelength: 920 nm and 1040 nm
Bandpass Emission Filters: 517/65 nm, 550/50
Dichroic: 560-nm long-pass
x-y resolution: 0.59 $\mu\text{m}/\text{pix}$
z increment: 1 $\mu\text{m}/\text{frame}$

z-projection type: average

Filtering: linear unmixing

Image(s): Figure 5a

Objective Lens: Olympus 4x (NA = 0.28)

Original bit depth: 16 bit/color

Fluorophore(s): YFP, Texas Red

Excitation Wavelength: 1040 nm

Bandpass Emission Filters: 517/65 nm, 645/65

Dichroic: 600-nm long-pass

x-y resolution: 2.78 $\mu\text{m}/\text{pix}$

z increment: 5 $\mu\text{m}/\text{frame}$

z-projection type: standard deviation

Filtering: linear unmixing, image stitching (3 panels)

Image(s): Figure 5b

Objective Lens: Zeiss 20x water immersion (NA = 1.0)

Original bit depth: 16 bit

Fluorophore(s): YFP

Excitation Wavelength: 1040 nm

Bandpass Emission Filters: 517/65 nm, 645/65

Dichroic: 600-nm long-pass

x-y resolution: 0.59 $\mu\text{m}/\text{pix}$

z increment: 1 $\mu\text{m}/\text{frame}$

z-projection type: standard deviation

Filtering: bandpass filtering (0-80 pixel)

Image(s): Supplementary Figure 1b,d,f,h

Acquisition device: Canon DS12601 Digital Camera

Lens: Leica MZ12.5 Stereomicroscope

Photo Processing: image brightening, uniform linear contrast adjustment

Image(s): Supplementary Figure 2

Acquisition device: Nikon P80 Digital Camera

Photo Processing: image brightening, uniform linear contrast adjustment

Image(s): Supplementary Figure 3

Objective Lens: Olympus 40x water immersion (NA = 0.8)

Original bit depth: 16 bit/color

Fluorophore(s): YFP, Texas Red

Excitation Wavelength: 1040 nm

Bandpass Emission Filters: 517/65 nm, 645/65

Dichroic: 600-nm long-pass

x-y resolution: 0.59 $\mu\text{m}/\text{pix}$

z increment: 1 $\mu\text{m}/\text{frame}$

z-projection type: standard deviation

Filtering: none

Image(s): Supplementary Figure 4

Objective Lens: Olympus 40x water immersion (NA = 0.8)

Original bit depth: 16 bit/color

Fluorophore(s): YFP, Texas Red

Excitation Wavelength: 1040 nm

Bandpass Emission Filters: 517/65 nm, 645/65

Dichroic: 600-nm long-pass

x-y resolution: 0.59 $\mu\text{m}/\text{pix}$

z increment: 0.5 $\mu\text{m}/\text{frame}$

z-projection type: standard deviation

Filtering: none