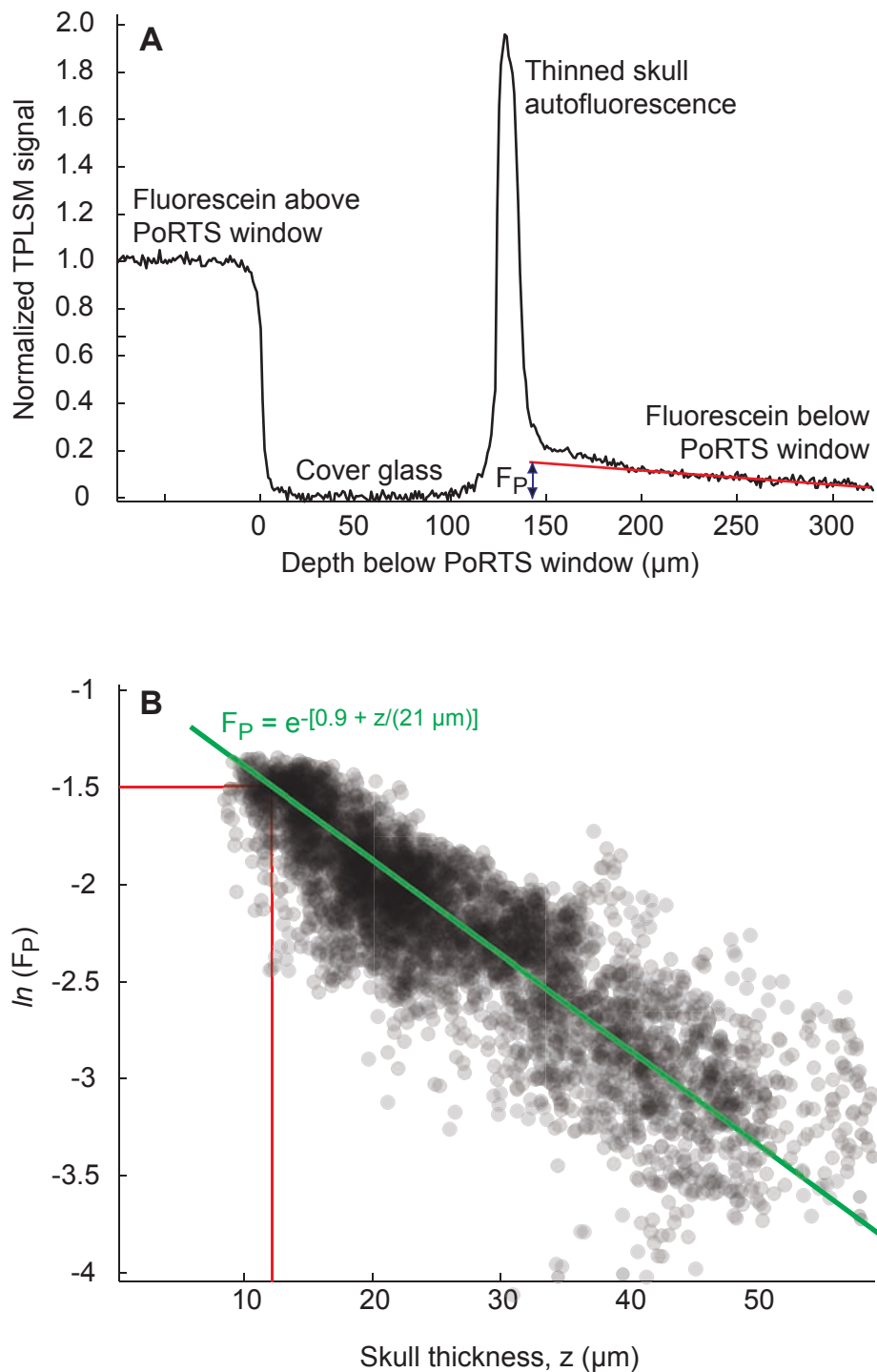


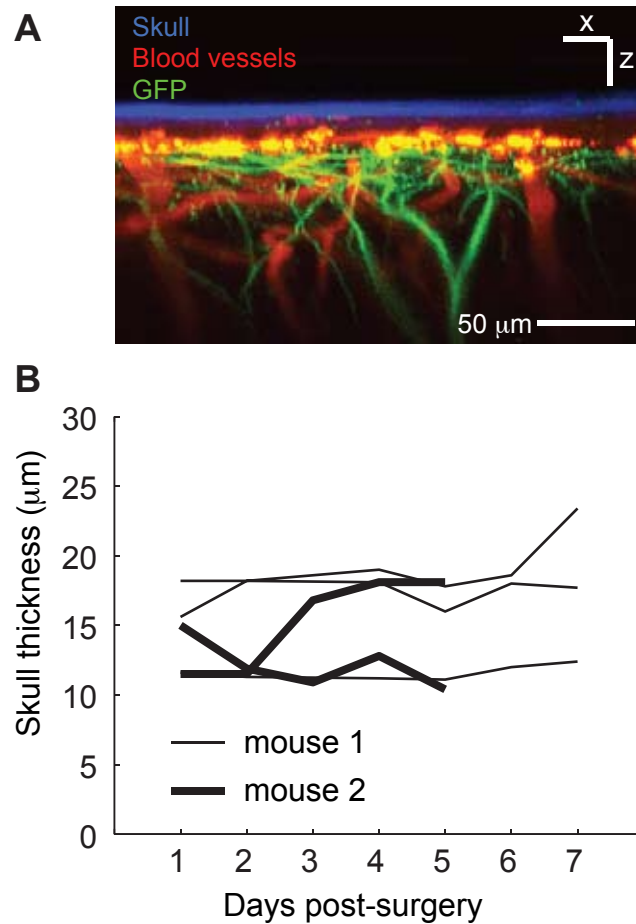
Chronic optical access through a polished and reinforced thinned skull

Patrick J Drew, Andy Y Shih, Jonathan D Driscoll, Per Magne Knutsen, Pablo Blinder, Dimitrios Davalos, Katerina Akassoglou, Philbert S Tsai & David Kleinfeld

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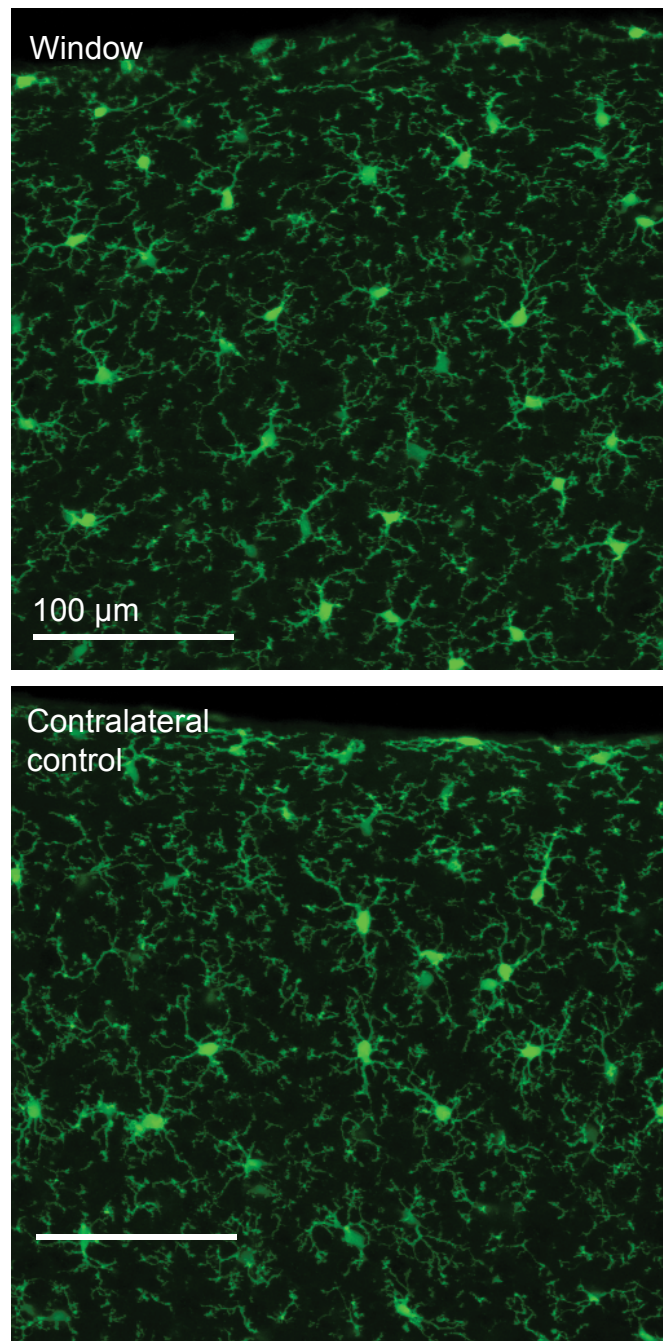


Supplementary Figure 1. Scattering losses in TPLSM as a result of a thin skull. (A) Axial trace of two photon excited fluorescence obtained through an excised PoRTS window. The window was placed in a bath of fluorescein and imaged as a function of depth at constant laser power with a 40x (0.8NA) water dipping objective. The thinned skull becomes strongly stained by the fluorescent bath. **(B)** Scattering losses as a function of skull thickness. A shallow wedge of thinned skull was measured as in panel A, but without a cover glass attached. The green line is a linear fit; red crosshairs represent the characteristics of the typical thinned skull used in the PoRTS window. The reduced two photon efficiency encompasses loss of incident photons from scattering by the bone, reduced excitation efficiency due to aberrations, and scattering loss of fluorescent photons by the bone.

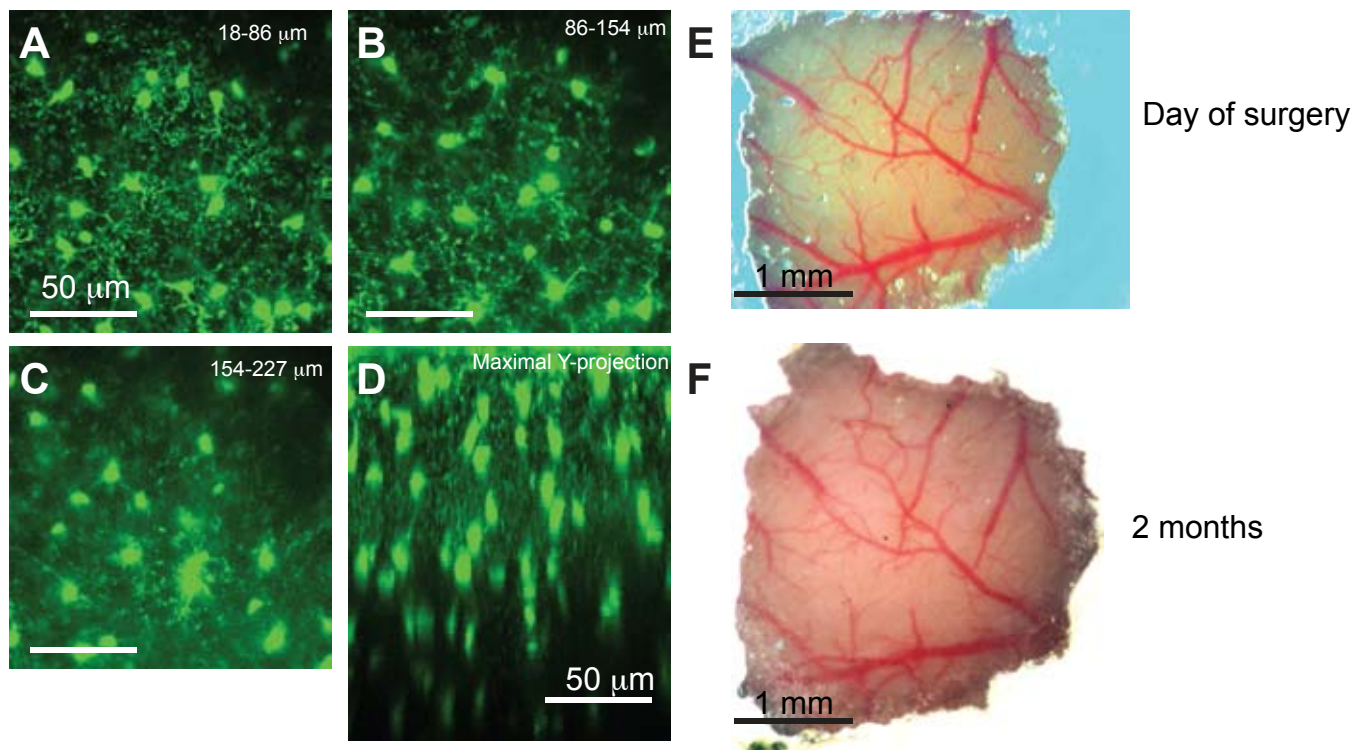


Supplementary Figure 2. Estimate of the thickness of the thin skull for the first week after surgery.

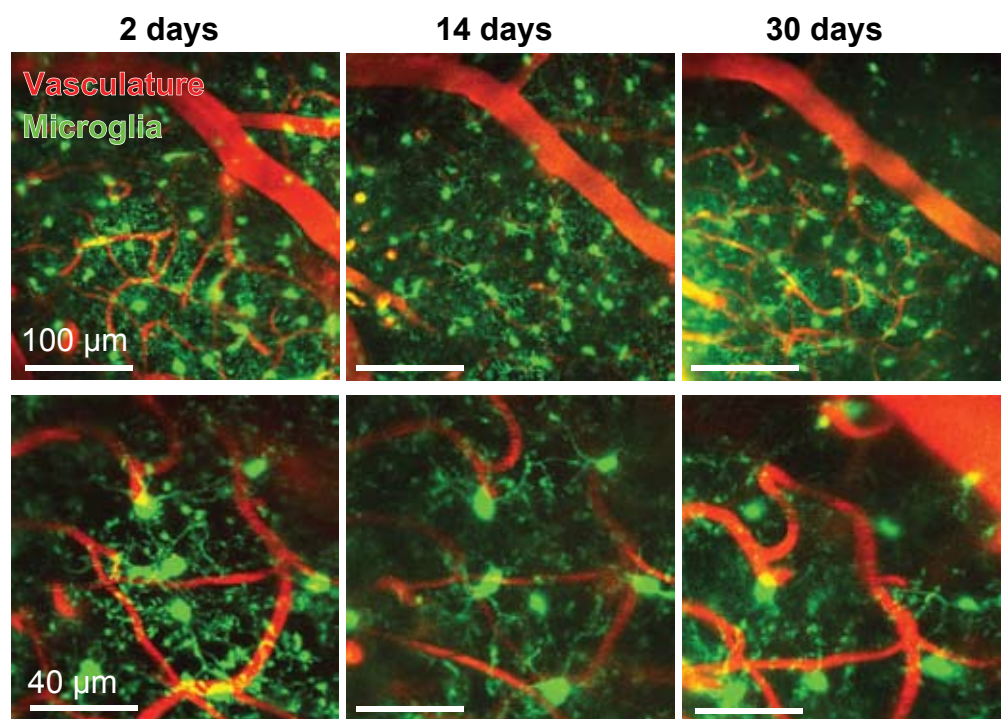
(A) Maximal side projection of a TPLSM z-stack through a PoRTS window two-days after window implantation. Green corresponds to EGFP driven by a thy-1 promoter in a subset of neurons, red corresponds to Texas-red dextran labeled vasculature, and blue corresponds to the second-harmonic signal from the skull (2 animals). **(B)** Plot of skull thickness over time from two longitudinally imaged mice, with two and three imaging locations each following PoRTS window implantation showing lack of bone regrowth.



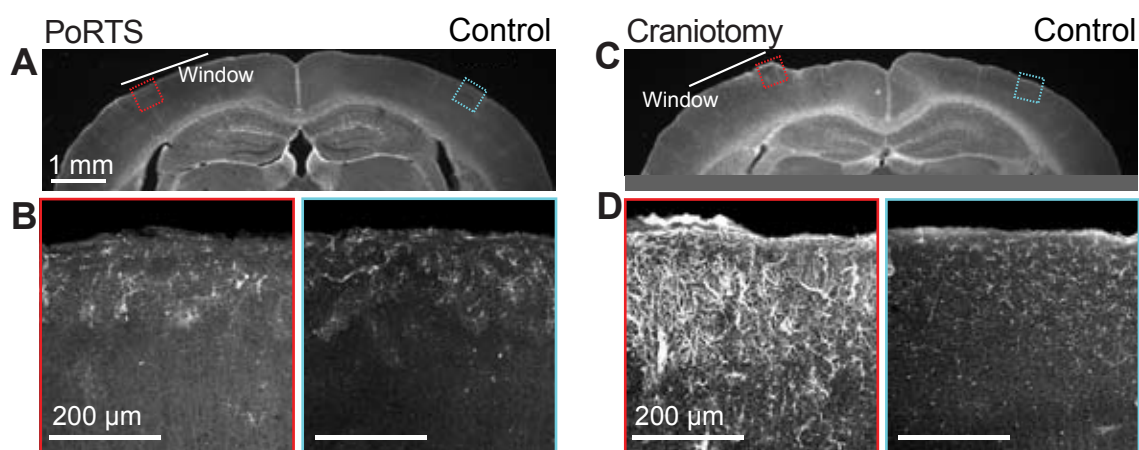
Supplementary Figure 3. Histological images of microglia under a PoRTS window. Confocal images of microglia from Cx3cr1eGFP^{+/−} mouse under polished and reinforced window (top) and from contralateral somatosensory cortex (bottom) of same section two days after window implantation (3 animals). The microglia under the PoRTS window show normal morphology.



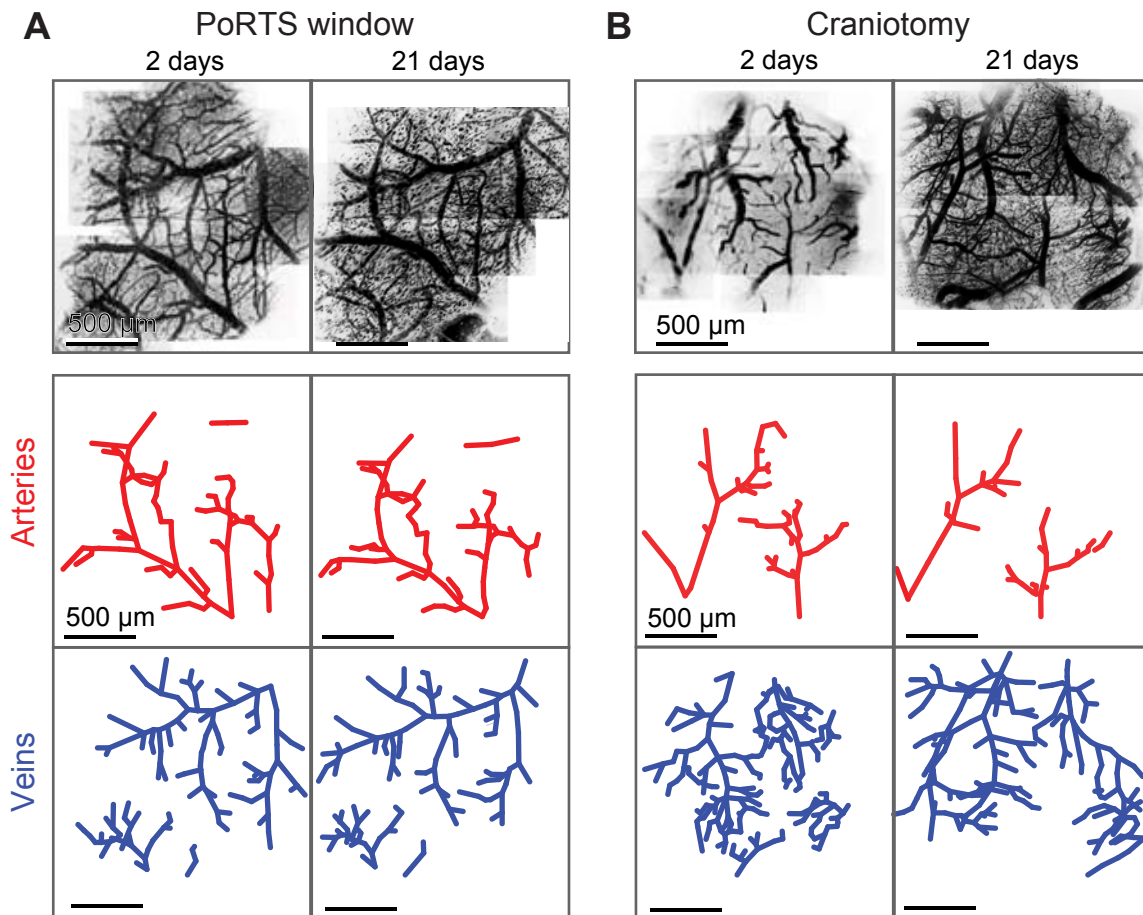
Supplementary Figure 4. Chronic imaging of EGFP labeled microglia through a PoRTS window. Maximal TPLSM z-projections of stack taken through a two month old PoRTS window show that the window retains enough clarity to image microglial processes over one hundred micrometers below the surface. Average laser power from varied with depth from 25 – 170 mW and a 3.9 μs dwell time per pixel was used. No frame averaging was performed. **(A-C)** Maximal projections along the z-axis for 15 – 86 μm below the surface (panel A), 86 – 154 μm (panel B), and 154 – 227 μm (panel C). **(D)** Maximal projection along the y-axis. **(E,F)** Wide field views of the surface vasculature from the same animal on the day of surgery (panel E) and two months later (panel F).



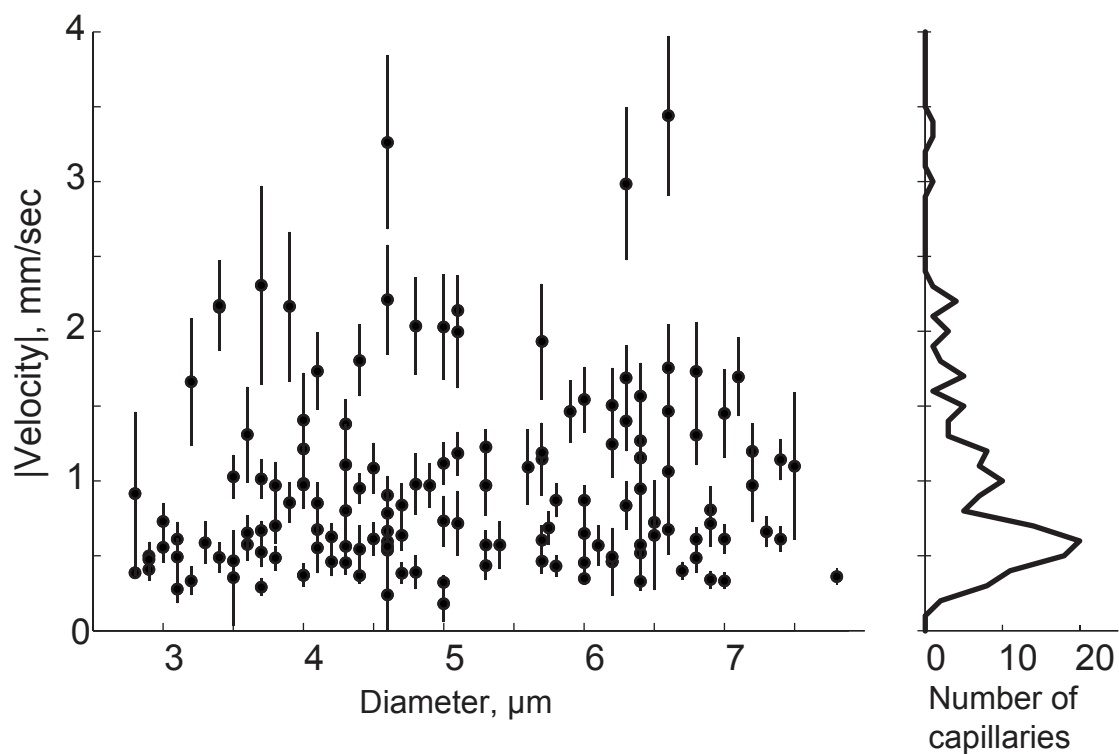
Supplementary Figure 5. Simultaneous chronic imaging of EGFP labeled microglia and labeled vasculature through a PoRTS window. Top row, maximal projection of top 100 μm of cortex under polished window of Cx3cr1eGFP $^{+/-}$ mouse two days, two weeks and one month after procedure. The vasculature is labeled with Texas-red dextran; EGFP labeled microglia are green. Bottom row, high magnification of the same site as in top row, with a maximal projections along the z-axis of the upper 37 μm (2 animals).



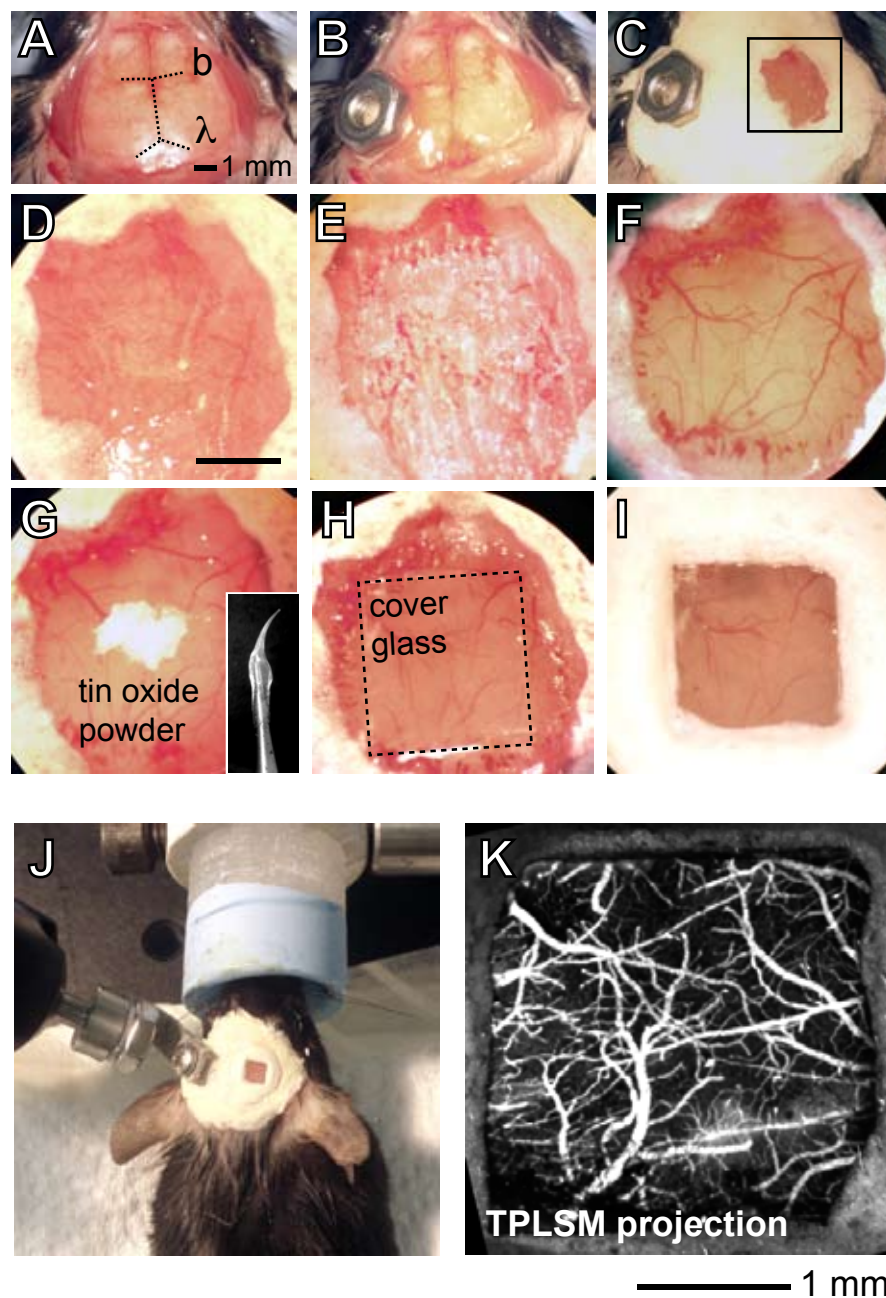
Supplementary Figure 6. Glial fibrillary acidic protein expression under a PoRTS window and under a craniotomy. **(A)** Widefield image of section of brain stained for GFAP two weeks after PoRTS window implantation (3 animals). White line shows extent of PoRTS window on left. Red and blue squares denote regions enlarged in panel B. **(B)** Confocal image of GFAP staining underneath PoRTS window (left) and control contralateral hemisphere (right) showing similar basal levels of GFAP expression. **(C)** Widefield image of section of brain stained for GFAP two weeks after craniotomy procedure (3 animals). White line shows extent of craniotomy window on left. Red and blue squares denote regions enlarged in panel D. **(D)** Confocal image of GFAP underneath craniotomy (left) and control contralateral hemisphere (right) showing increased levels of GFAP staining under the cranial window



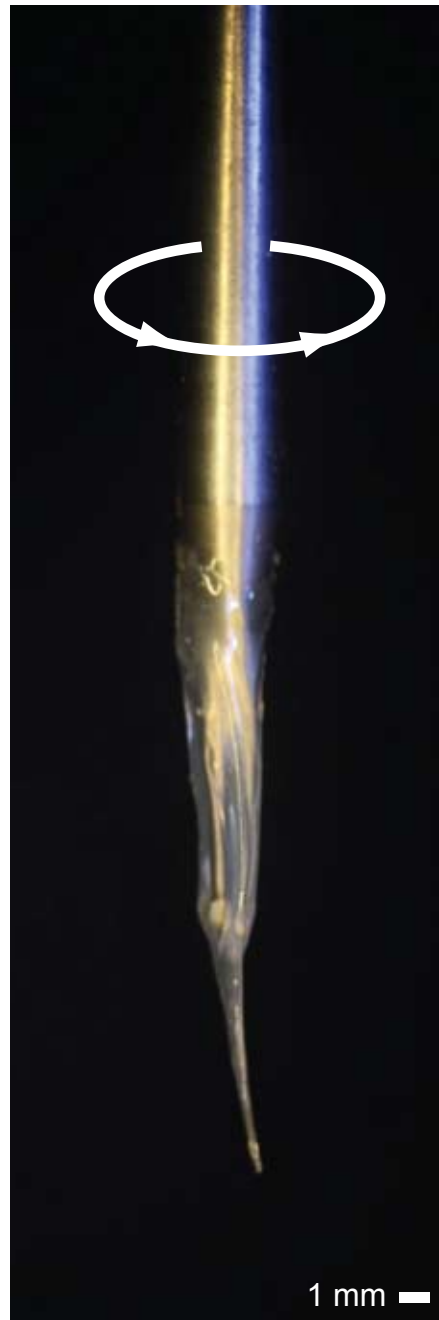
Supplementary Figure 7. The pial vasculature below a PoRTS window is stable. Images were taken with a 10x objective. (A) Montages of maximal projections of Texas red-conjugated- dextran labeled vasculature through a PoRTS window, obtained two days and two weeks after surgery, along with traces of arteries and veins. The contrast has been inverted in the montages (2 animals). **(B)** Montages of maximal projections of Texas red-dextran labeled vasculature in an animal with a craniotomy, obtained two days and two weeks after surgery (2 animals). Note that the structure of the surface arteries and veins shows alterations in topology



Supplementary Figure 8. Average red blood cell velocity through microvessels in cortex of the awake mouse. Left, the distribution of the average velocities of red blood cells that move in capillaries through larger microvessels measured in awake animals, plotted as a function of vessel diameter. Error bars show standard deviations, circles show means (159 vessels from 17 animals). The graph on the right is cumulative across all vessels sizes showing the large range in spontaneous velocity.



Supplementary Figure 9. Procedure for forming a PoRTS window. (A) Remove the scalp and scrape the periosteum from the skull surface. The location of suture lines are shown (dotted lines), as well as the location of bregma (b) and lambda (λ). (B) Secure a small nut to the lateral aspect of the skull using cyanoacrylate glue. Seal the backside of the nut with tape, to ensure that glue does not enter the threads. Apply glue to entire skull surface to help the dental cement adhere to the skull. (C) Apply dental cement to the entire surface of the skull, except the region of the windows. Build a thick layer of cement around the nut to strengthen the attachment. (D-F), Slowly thin the skull within the window region using a $\frac{1}{2}$ mm drill burr. Thin through the trabecular layer of the bone (panel E), which contains vasculature. Bleeding can be controlled by flushing with mACSF. Thin the skull evenly throughout the window until the pial vasculature is visible after application of mACSF. Flush the window regularly with mACSF to reduce heat buildup. (G) Polish the thinned skull using a slurry of fine-grained grit, such as tin oxide. Agitate the slurry for five minutes with a silicone whip (inset). (H) Apply a thin layer of cyanoacrylate glue to the window and carefully lay a pre-cut piece of #0 cover glass onto the glue. (I) Seal the cover glass and glue with dental cement. (J) After the window is complete, cut the skin to fit along the edges of the head cap. Apply dental cement all along the edges of the skin to protect it. The head of the mouse is secured by screwing a bolt, attached to the imaging apparatus, to the nut; this mount is stable for anesthetized preparations. (K) Maximal projection of TPLSM image of the vasculature of the entire PoRTS window after intravenous injection of FITC-dextran dye; collected with a 5x objective.



Supplementary Figure 10. The polishing whip tool. Tool is made by dipping drill bit into aquarium sealant, rapidly withdrawing it, and allowing it to cure. The whip is used to agitate the slurry of polishing grit and mACSF.

Supplementary Note

Surgical Methods

We focus on details of the surgical technique to generate a PoRTS window.

Equipment

- High speed drill, either (K.1070 Micromotor Kit; Foredom) or air powered dental drill (Midwest Quiet-Air)
- Diamond glass-cutter
- Dissecting microscope
- Electrical shaver (Series 8900, Wahl)
- Extra sharp forceps (11255-20; Fine Science Tools)
- Heat pad, feedback regulated (40-90-8D, 40-90-5D-02, and 40-90-2-07; FHC)
- Hemostats (13013-14; Fine Science Tools)
- Isoflurane vaporizer (Ohmeda IsoTec 4)
- Periosteal elevator (RS-8820; Roboz)
- Screwdriver, miniature
- Surgical scissors (14010-15; Fine Science Tools)

Reagents

- Betadine surgical scrub (Purdue Products)
- Isoflurane (AHN3637; Baxter Healthcare Corp.)
- Lactated Ringer's (Baxter Healthcare)
- Lidocaine solution, 2 % (v/v) (1503430; Henry Schein)
- Modified artificial cerebral spinal fluid (mACSF), free of carbonate and phosphate (Kleinfeld and Delany, 1996) (125 mM NaCl, 10 mM glucose, 10 mM HEPES, 3.1 mM CaCl₂, 1.3 mM MgCl₂, pH 7.4)
- Ophthalmic ointment (009614; Major Pharmaceuticals)

Disposables

- Drill bits, 0.5 mm steel burr (19007-05; Fine Science Tools) or Carbide burrs, 1/2 mm and 1/4 mm tip sizes (1003995TV and 1007205TV; Henry Schein Inc.)
- Cottoned-tipped applicator (23-400-100; Fisher Scientific)
- Cover Glass, no. 0 thickness (72198-10; Electron Microscopy Sciences)

- Cyanoacrylate glue (no. 31428 H04308; ND Industries)
- Dental acrylic (Grip Cement; Dentsply)
- Kimwipes (Kimberly Clark Professional; Roswell)
- Scalpel blades
- Threaded nut (#00-90; Small Parts Inc.)
- Silicone aquarium sealant (Perfecto Manufacturing)
- Surgical gauze (1212; Dukal Corp.)
- Tin oxide powder (eqt-tinox; Mama's Minerals)

Preparation

- Induce anesthesia. Ensure surgical plane of anesthesia by checking for lack of toe pinch reflex. Shave the scalp with an electrical razor.
- Apply opthalmic ointment to eyes to retain moisture.
- Maintain body temperature at 37°C using a feedback regulated rectal probe and heat pad.
- Clean the scalp with Betadine scrub, followed by swabbing with 70 % isopropanol.
- Inject lidocaine subcutaneously for local analgesia. Do not exceed 7 mg/kg total dose, subcutaneous or intra-incisional. Check for lack of toe pinch reflex to ensure an adequate level of anesthesia.
- Inject lactated Ringer's solution intraperitoneally, 3 mL per kg rodent every 2 hours to maintain body fluids and energy requirements.

Mounting a head bolt

- Remove the scalp over the entire dorsal skull surface (**Supplementary Fig. 9A**). Use a periosteal elevator to remove the thin periosteum from the surface of the skull.
- Apply a thin layer of cyanoacrylate glue to the cleaned skull surface.
- Attach a connector to secure the animal to the imaging apparatus. For anesthetized animals, a #00 nut secured with a small dab of cyanoacrylate glue followed by coverage in dental cement is sufficient (**Supplementary Fig. 9B**). This is later secured to the imaging setup using a bolt. For awake animal studies, a small lightweight head frame or a hexagonal pin is used.
- Cover the rest of the skull surface with a thin layer of dental cement, except the location of the window (**Supplementary Fig. 9C**). Ensure that all exposed edges of

the skin are covered by cement.

- Stabilize the mouse in a head mount.

Generating a PoRTS window

- Using the drill, thin a 3 mm by 3 mm section over the appropriate part of cortex. Only thin for a few seconds at a time and constantly wet the skull with mACSF to prevent heating. This will require thinning through the vasculature of the skull, which may bleed, but can be controlled by flushing with mACSF (**Supplementary Fig. 9D,E**). The final thickness of the skull should be ~10 to 15 μm , with the pial vessels clearly visible through wet bone (**Supplementary Fig. 9F**).
- Polish the window region with tin oxide powder (**Supplemental Fig. 9G**), or a series of increasingly fine grits. For this, agitate a slurry of grit and mACSF with a drill bit that had been dipped in silicone aquarium sealant and withdrawn, leaving a tapered whip (**Supplementary Fig. 10**).
- Flush away the tin oxide powder from the window using mACSF and allow to dry.
- Apply a small dab of cyanoacrylate glue to the window using the wooden tip of a broken cotton-tipped applicator.
- Place a precut #0 cover glass atop the glue. Excess cyanoacrylate cement can be removed from the glass with a scalpel (**Supplementary Fig. 9H**).
- Seal the edges of the cover glass with dental cement and form a well to hold water for the dipping lens (**Supplementary Fig. 9I**).
- Stabilize the animal, using the frame as a head support (**Supplementary Fig. 9J**), for imaging (**Supplementary Fig. 9K**).