Chronic imaging of mouse visual cortex using a thinned-skull preparation

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Abstract

In vivo imaging using two-photon laser scanning microscopy (2PLSM) allows the study of living cells and neuronal processes in the intact brain. The technique presented here allows the imaging of the same area of the brain at several time points (chronic imaging) with microscopic resolution allowing the tracking of dendritic spines which are the small structures that represent the majority of postsynaptic excitatory sites in the CNS. The ability to clearly resolve fine cortical structures over several time points has many advantages, specifically in the study of brain plasticity in which morphological changes at synapses and circuit remodeling may help explain underlying mechanisms. In this video and supplementary material, we show a protocol for chronic *in vivo* imaging of the intact brain using a thinned-skull preparation. The thinned-skull preparation is a minimally invasive approach, which avoids potential damage to the dura and/or cortex, thus reducing the onset of an inflammatory response. When this protocol is performed correctly, it is possible to clearly monitor changes in dendritic spine characteristics in the intact brain over a prolonged period of time.

Keywords

thinned-skull; two-photon microscopy; visual cortex; dendrite; imaging

Protocol

1. To visualize neurons in the intact brain using two-photon microscopy, a preparation where neurons are labeled with fluorescent markers is used. In the experiments presented here we use the GFP-M transgenic mouse line which labels layer 5 pyramidal cells[1]. Layer 5 pyramidal cells project dendritic processes into superficial layers, allowing the visualization of dendrites and dendritic spines up to a depth of 300 μm below the level of the pia. An alternate approach is to label cells using viral markers (see Lowery et al., submitted to JOVE)
2. Sterilize the workspace using 70% ethanol and place tools in bead sterilizer for aseptic surgery. Cover operating surface with clean dressing.
3. Anesthetize mice with a Fentanyl cocktail (fentanyl 0.05mg/kg; midazolam 5mg/kg; metatomadin 0.5mg/kg) followed by a quarter dose of Avertin (0.0075 mg/ml) using IACUC approved procedures. Pretreat mice with the analgesic,

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Disclosures
None.

buprenorphine (0.5 mg/kg) administered subcutaneously (SQ). Monitor the level of anesthetic plane by testing reaction to tail/toe pinch.

4. Maintain animal at 37.5 C using heating blanket with attached rectal thermometer.
5. Apply ophthalmic ointment to eyes to prevent the eyes from drying out. Hood eyes with small sheet of paper to protect eyes from light source during surgery.
6. Using scissors, remove hair from the back of head (behind ears) to the eyes.
7. Clean the top of animal's head with sequential application of 70% ethanol, followed by betadine scrub and betadine solution.
8. Make an L-cut incision behind the ears and up along either the right or left side of the midline (depending on which hemisphere will be imaged) to the eyes. Fold the skin over and away from surgery site. Do not remove skin as the skin will be sutured following the imaging session.
9. Using fine forceps gently pull the skin up and away from your area of interest.
10. Gently scrape away the periosteum from the exposed area of the skull with clean forceps or microsurgical blade. Using cotton swab, apply a small amount of 10% Ferric Chloride to the skull to completely dry the periosteum membrane and ensure that it has been removed completely. It is important that the skull is completely dry to ensure proper adhering of the glue (see 11). Using forceps or microsurgical blade, gently scrape away dried periosteum membrane.
11. Apply a thin layer of cyanoacrylate glue around the imaging window of the stainless steel head plate (see Figure 1) and affix the plate to the skull.
12. Using the wooden end of a cotton-swab stick, apply glue to inside seams of the imaging window; making sure to fill all gaps between the imaging plate and curvature of skull.
13. Place a drop of glue accelerator into the window. Quickly wipe away excess glue accelerator with a cotton swab or kim wipe. The glue should be completely solidified before drilling.
14. Begin to thin the skull in the imaging window using a dental drill affixed with a 0.7mm stainless steel burr. Alternate drilling with occasional application of 0.9% sterile saline to avoid generating too much heat on the skull.
15. Thin a 4×4 mm window on the skull using small strokes across the skull surface with the dental drill. Test the thinness of the skull with blunt end forceps. The skull surface will indent slightly with gentle pressure. The optimal skull thickness for imaging is between 10 and 30 μm.
16. When the skull is thin and the window cleaned of all debris, place saline on the skull. Photograph the imaging window with a digital camera mounted to a dissecting scope. Photography and use of the brain vasculature will aid in locating the position to place the imaging plate for subsequent imaging sessions.
17. Transport the animal to the 2-photon rig. The animal should remain on the heating blanket (with rectal probe) during the entire imaging session. Note: while under sedation, the animal's core body temperature will fluctuate greatly in the absence of a heating unit resulting in cortical damage or possibly death.
18. A two-photon microscope with a Mai Tai laser, a 10W solid state pump for maximum power and a modified Olympus Fluoview confocal unit is used. The system is optimized for deep tissue imaging and external detectors are installed as

close to the objective as possible to allow maximal detection of fluorescence from the brain. An Olympus LUMPlan fl/IR 20X/0.95NA objective lens is used. 0.9% saline is used during imaging for objective submersion.

19. Using this microscope, and low digital zoom (x1) identify an area containing brightly labeled neurons.

20. Using a digital camera affixed to the microscope eye piece, take a photograph of the imaging area. Alternatively, sketch out the blood vasculature pattern. This is necessary to return to the same imaging location at a subsequent time point.

21. Obtain a low magnification stack (x20 objective, 512×512 pixel; 5 μm step) through the entire visible extent of the brain (usually spanning ~200 μm) showing the extent of dendritic ramification. Note: this low magnification image will be used to locate the imaging area at subsequent time points as both blood vessels and dendrites maintain their structure in young and adult animals.

22. Using imaging software (Fluoview, v. 5.0, FV300), increase digital magnification 8-fold. Adjust the X, Y coordinates (pan coordinates) if necessary and collect a high magnification stack which will show detailed dendritic morphology including the locations and structure of dendritic spines. Take detailed notes regarding each stack collection, i.e. pan coordinates, the range of collection, the z-step size and the number of steps in your stack. These notes will be used when returning to this dendrite or axon at a subsequent time point.

23. Following imaging, remove the head plate by gently separating plate from skull surface. Using forceps, remove any residual glue remaining on skull and wipe skull surface with 0.9% sterile saline. Suture the skin over the skull together using #6 suturing thread. Wipe area with 70% ethanol. To alleviate pain during post-operative recovery, administer 0.1 mg/kg Buprenorphine analgesic, SQ.

24. Place animal in clean cage under a heat lamp until it wakes up and is mobile. Return animal to original cage.

25. To reimage animal at a later time point (our imaging time points can be anywhere from 2 days to many months apart), remove stitches or reopen the skin on head using aseptic methods as above. Use the digital image of brain vasculature (taken on first surgery date) to locate the original imaging location. Reaffix stainless steel headplate (steps 10–12). Use a microsurgical blade to gently thin away any bone that might have re-grown between imaging time points. If necessary, thin with dental drill. Clear imaging window of debris and apply a drop of 0.9% sterile saline.

26. Carry animal to two-photon microscope and locate original imaging field. Use the digital photograph taken during first imaging session for assistance.

27. Open the Fluoview program and open the original low magnification image. Affix a transparency sheet to the computer screen and sketch the major blood vessel pattern using a transparency pen.

28. Open the Live image and realign the blood vasculature to the sketched pattern on the transparency film.

29. To reimage the 8x zoomed in structures, open up the desired collected stack from the previous imaging session. Sketch the structure (i.e. axonal, dendritic, glial) onto a transparency film, affixed to the computer screen.

30. Zoom in 8x from the Live Image and set the pan coordinates to the first day imaging parameters. Depending on the accuracy of aligning the low magnification

location, it may be necessary to also adjust the angle of the image slightly. Once the desired structure is found, match up the structure to your sketched high magnification pattern. Collect the z-stack with the identical step size and number of z-stack images for subsequent analysis.

31. Repeat steps 29 and 30 until all the high magnification images are re-collected.

32. When imaging is complete, repeat steps 22–24. Re-imaging can be repeated at up to 2 separate time points.

33. Experiments on animals were performed in accordance with the guidelines and regulations set forth by the Vivarium and Division of Laboratory Animal Medicine of the University of Rochester School of Medicine and Dentistry, in full accreditation by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) and are in compliance with state law, federal statute and NIH policy.

Representative Results

In GFP-M expressing mice, a subset of layer 5 pyramidal cells and corresponding dendritic processes can easily be visualized using 2-photon laser scanning microscopy (2PLSM)[1]. Here we demonstrated a thinned-skull preparation in which the skull over the desired imaging location is thinned to ~10–30 μm. Performed correctly, our protocol allowed us to clearly visualize cortical dendrites and spines in the intact visual cortex for acute or chronic analysis (Figure 2).

Discussion

Chronic imaging using two-photon microscopy is becoming an increasingly popular technique to study morphological changes triggered during plasticity[2–5]. Here we demonstrate a thinned-skull preparation to follow identified dendritic spines in the intact mouse brain on different imaging days. In this protocol, the skull is left intact, causing minimal damage to the cortex and resulting in very low levels of neuroinflammation which may alter brain function[6]. This allows the animal to be imaged immediately after the first surgery and then subsequently reimaged days to years later. This is a powerful technique but also has its limitations. The more invasive window preparation which involves a craniotomy and hence associated glial activation, allows repeat imaging of the brain with an arbitrary number of imaging sessions while the cranial window is clear. The thin skull preparation, however, is limited to at most three imaging sessions due to the surgery involved in opening and closing the mouse's skin. Additionally, depth penetration is superior in the cranial window [2]. Careful consideration should therefore be taken when deciding the surgical approach for chronic imaging experiments.

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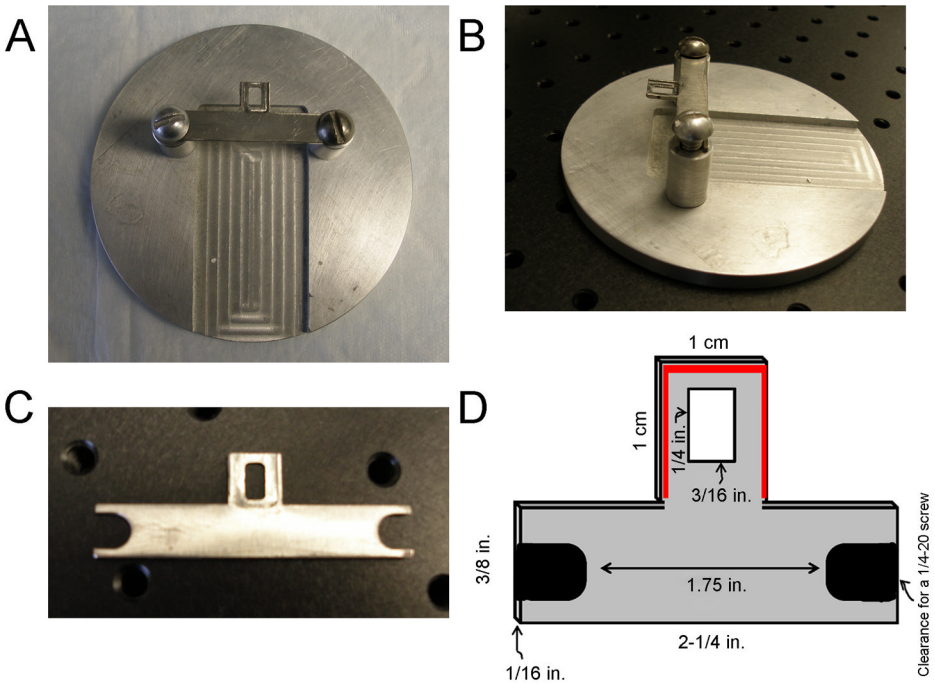

Figure 1. Custom Head and Base Plate

(A) Top view of custom made head and base plate used for thinned-skull surgery preparation. (B) Side view of custom-made head and base plate. (C, D) Detailed schematic and image of head plate.

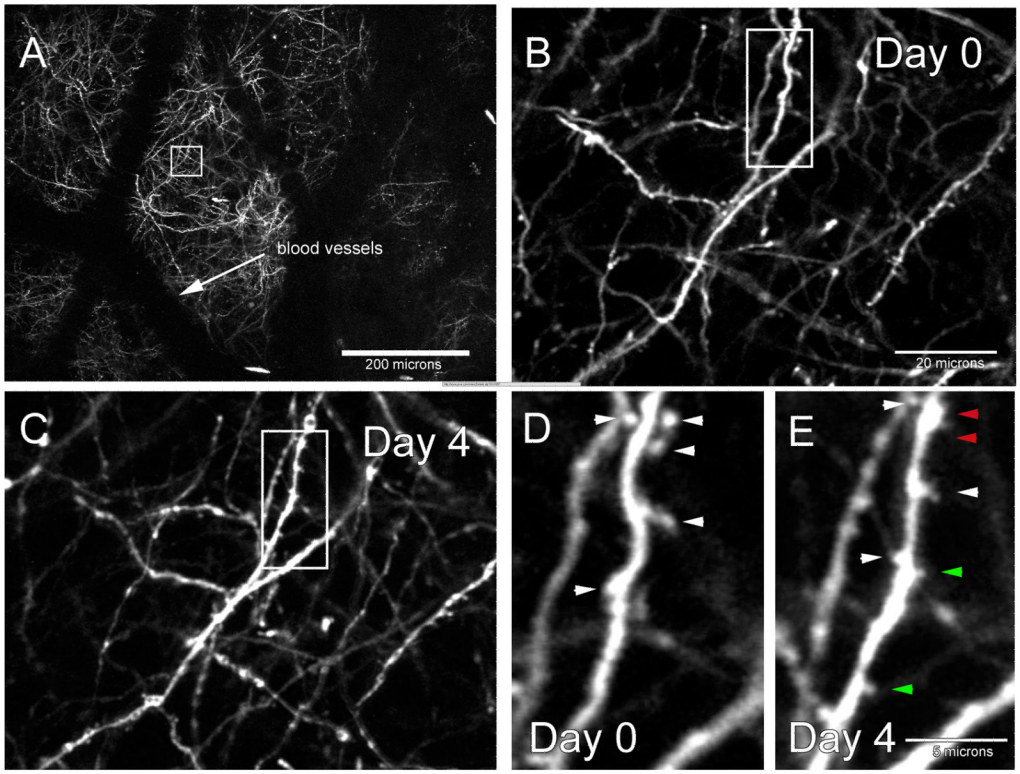

Figure 2. Representative results from 2PLSM following thin skull preparation

(A) Dendrites imaged *in vivo* in the visual cortex of a transgenic GFP-M mouse using two-photon microscopy. Image is a projection of single image obtained every 5 μm from the pia to a final depth of 175 μm. The boxed area in A represents the magnified region in (B) and (C) imaged on day 0 (D0) and four days later (D4). (D, E) Higher magnification of dendritic branch boxed in B and C demonstrating stable spines (white arrow heads), lost/retracting spines (red arrow heads) and new spines (green arrow heads). Scale bars= A. 200 μm, B, C. 20 μm, D, E. 5 μm.

Table 1

Table of Specific Regents and Equipment

Procedure	Reagent	Company	Catalog #	Comments
Fentanyl Cocktail: Anesthesic Cocktail; *IP Dose: 0.0125 ml/g*	Fentanyl Citrate	In-house pharmacy		Final cocktail conc.: 0.05 mg/kg
	Medetomindine Hydrochloride	In-house pharmacy		Final cocktail conc.: 5 mg/kg
	Midazolam HCL	In-house pharmacy		Final cocktail conc.: 0.5 mg/kg
Avertin Cocktail: Anesthetic; *IP Dose: 0.0075 cc/g*	2,2,2-tribromoethanol			Final cocktail conc.: 1%
	2-methyl-2-butanol (Final concentration: 0.775%)			Final cocktail conc.: 0.775%
Body Temp. Regulation	TC-1000 Temp. Control System for Mice	CWE, Inc.	TC-1000 Mouse	
Eye Ointment	Toberadex	In-house pharmacy		
Thin-skull preparation	10% Ferric Chloride	Ricca Chemical, Inc.	3120-32	
	Microsurgical Blade	Sable Industries	S-6400	
	Cyanoacrylate 404,401	Loctite	P/N 46551	
	Cyanoacrylate 401	Loctite	P/N 40140	
	Zip Kicker Glue Accelerator	Pacer Technology	PT-29	
	Micro Drill Steel Burrs 0.7mm tip diameter	Fine Science Tools	19008-07	
	Microtorque Control Box and Tech2000 Handpiece	Ram Products, Inc.	TECH2000ON/OFF	
	#6-0 (0.7 metric) silk suture	Ethicon	K8894H	Taper C-1
Surgical Tools	Eye Dressing Forceps, 10cm, tip width 0.5mm, curved	Fine Science Tools	11152-10	
	Extra Fine Bonn Scissors, 8.5cm, straight tip, cutting edge 13mm	Fine Science Tools	14084-08	
	Standard Pattern Forceps, straight, 2.5mm×1.35mmtip, 12cm	Fine Science Tools	11000-12	