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Thinned-skulled Cranial Window Preparation (Mice)

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[Abstract] Imaging structural plasticity or activity of neurons in the brain circuit will facilitate understanding the neural mechanisms underlying animal behavior. Here we describe a modified procedure, the polished and reinforced thinned-skull cranial window preparation, by which we can image dendrites and spines in mouse layer I cortex for weeks (Zhang *et al*., 2016). By this method, we also imaged the glioma initiation in the mouse cortex for two weeks in previous work (Zhang *et al*., 2012), which included the photographs and video for reference.
Keywords: Neuroscience, Mouse brain, *in vivo* imaging, Cranial window, Structural plasticity

[Background] Three cranial window procedures are currently available for *in vivo* imaging, open-skull cranial window (Trachtenberg *et al.*, 2002), thinned-skull cranial window (Yang *et al.*, 2010) and polished and reinforced thinned-skull cranial window (Drew *et al*., 2010). Each protocol has both advantages and disadvantages. Open-skull has best optical imaging quality, unlimited repetitive imaging times and large field of view, but needs to wait for 2 weeks to recover from surgery and also has the inflammation and glia activation issue; Thinned-skull protocol has minimal disturbance from inflammation and glia activation on the brain, but has limited repetitive imaging times, only 2-5 times for a cranial window, small field of view(< 300 μm in diameter); Polished and reinforced thinned-skull method allows unlimited repetitive imaging, large field view(< 3 mm in diameter) and minimal disturbance, but the optical imaging quality decreases over time because of light diffusion and absorption at the interface with regenerated bone. Researcher may choose an appropriate protocol according to specific study.

Materials and Reagents

1. Small paper towel
2. Miniature blade (Surgistar, catalog number: 6900)
3. Cotton swabs
4. #0 cover glass (3 mm diameter) (Warner Instruments)
5. Custom-made silicone whip (see Drew *et al*., 2010 supplemental)
6. Sodium chloride injection (USP)
7. Cyanoacrylate glue (Loctite)
8. Mouse, Thy1-YFPH, 6~12 weeks, male

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Note: Younger mouse has faster bone regeneration, which may lead to shorter time window for high quality images.

9. Ketamine (diluted to 10 mg/ml)
10. Xylazine (diluted to 1.5 mg/ml)
11. Carprofen (diluted to 0.05 mg/ml)
12. Artificial tears ointment (Rugby Laboratories)
13. Saline
14. 7.5% Betadine
15. 70% alcohol
16. 2% lidocaine
17. Diamond paste (3.5 micron diamond pastes) (Wicked Edge, catalog number: WE0535SP)
18. Tin oxide (Lortone, catalog number: 591-038)
19. Liner Bond 2V (KURARAY, catalog number: 1921-KA)
20. Isofluorane (0.2%, 0.4 L/min)

Equipment

1. Heating blanket
2. Sterile glass beaker
3. Scissors
4. Thumb forceps
5. Small sterile drape or platform
6. Custom-made headpiece holder
7. High-speed micro drill (CellPoint Scientific, model: Ideal Micro Drill Kit)
8. 0.5 mm-diameter diamond drill bit (Widget Supply, catalog number: D-CM13)
9. Hex nut (Small Parts, catalog number: HNX-0090-C)
10. Dissecting microscope

Software

1. ImageJ

Procedure

Notes: Work area preparations:

1. *The aseptic surgical field is the disinfected skin and exposed surgical wound on the dorsal skull.*
2. *The aseptic surgical field is situated on the CLEAN benchtop that is covered with fresh, absorbent paper:*
   a. *The surgery stage with a heating blanket on its base to maintain a 37 °C body temperature.*

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A small paper towel is placed over the mouse's neck and back to cover fur;

b. *A sterile instrument resting area, e.g., sterile glass beaker to suspend sterile instrument tips above the clean surgical area.*

1. Weigh the mouse and anesthetize by intraperitoneally (i.p.) injecting ketamine (100 mg/kg) and xylazine (15 mg/kg) at appropriate volume. Carprofen (0.5 mg/kg, s.c.) is used for analgesia. Usually, the surgery will last for 90 min, if the duration of ketamine/xylazine anesthesia needs to be prolonged, ketamine alone, at half of the induction dose (50 mg/kg ketamine) or ketamine with xylazine at one-quarter of the induction dose (25 mg/kg ketamine; ~3 mg/kg xylazine) can be given.
2. Shave the hair from the dorsal head and extend the furless area caudally to the first cervical vertebrae, making sure to leave lateral margins large enough to prevent hair from entering the incision. Apply ophthalmic ointment to eyes to keep the eyes moist during surgery. Disinfect the exposed head skin with 7.5% Betadine and 70% alcohol.
3. Full anesthesia is confirmed by absence of a response to a toe pinch and is also monitored frequently during the surgery.
4. Inject a small volume of 2% lidocaine under the surface of the dorsal scalp to provide additional local analgesia, and then remove a round (along the skull edge and beside the eyes ~1 cm diameter) section of skin from the dorsal scalp sufficient to expose bregma, lambda and the sutures. Use fine forceps and miniature blade to peel and scrape periosteum tissue from the skull.
5. Use wet cotton swab (dipped in saline) to clean wound and dry cotton swab to absorb extra solution on the skull. Apply a thin layer of cyanoacrylate tissue adhesive along the edges of the skin excision to bond the skin to skull. Wait for 5 min to let the tissue adhesive get dry.
6. Outline the cortical area of interest with a marker. The interest area should not be over skull suture lines, to avoid damage to underlying blood vessels, and it's also hard to get good thinned cranial window above sutures because of the heterogeneous bone around sutures.
7. Treat the skull with the primer and then with the bonding agent equipped in the dental cement kit. Wait for about 1 min until the bonding agent gets dry. Then cover the skull with a thin layer of dental cement (~0.2 mm) except the interest area, making a shallow bowl around the target area which can hold a little solution facilitating subsequent polishing step. To stabilize the mouse head for subsequent surgery and imaging, we embed a sterile hex nut into the dental cement at a distance from the interest area, saving space for imaging objective. After the preparations are all done, treat the dental cement for about 1 min.

   Note: One of modifications of our procedure from the original one (Drew et al., 2010) is the use of light-sensitive dental cement. It has strong bonding and also gives us enough time to prepare the window around the interest area without rush.
8. Mount the mouse on the stereotaxic stage by screwing a headpiece holder onto the hex nut on the mouse head.
9. The mouse is ready for the cranial window thinning. Apply one drop of saline onto the target

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area on the skull, and then hold a high-speed micro drill equipped with 0.5 mm-diameter diamond drill bit to thin the skull under a dissecting microscope. Keep the hand steady and thin evenly in one direction, keep the skull moist to avoid the thermal injury to the underlying brain tissue caused by high speed drill bit. Saline absorbs heat and also helps soften the bone.

10. The mouse skull comprises two thin layers of compact bone, sandwiching a thick layer of spongy bone. Remove the external layer of compact bone and most of the spongy bone quickly with the drill. Make sure the bone is wet.
11. After removing the majority of the spongy bone, dry the skull a little bit, you can see the remaining cavities within the spongy bone under the dissecting microscope, indicating that thinning is approaching the internal compact bone layer. At this stage, skull thickness should still be more than 50 μm. Continue the skull thinning carefully until no cavities can be seen, the skull thickness will be ~20 μm, then start the polishing procedure, two polishing steps are in the same way, and the volume ratio of diamond paste to saline or tin oxide to saline is about 1:1. Less or more diamond paste (tin oxide) results in low viscosity or high viscosity, which will cause low polishing quality.
12. Apply one drop of saline, then add diamond paste (6-15 μm diameter), stir with custom-made silicone whip to get turbid suspension, continue the stirring for approximately 10 min. The diamond-paste polishing serves two purposes. First, it will further thin the skull without applying pressure on the thinned skull, thereby avoiding accidental damage to the cranial window. Second, it serves as an initial polishing step for the thinned skull before the fine-grained tin oxide is used for further skull polishing.
13. Following the diamond-paste polishing, the thinned skull is polished with tin oxide same as step 12 for another 10 min. Once the skull is polished, the tin oxide is flushed away with saline until the thinned skull appears clean.
14. Get the skull dry, then apply one drop of clear cyanoacrylate glue on the skull, put the #0 cover glass on the glue, press the glass slightly for ~1 min to make sure the layer of cyanoacrylate glue between the thinned skull and the coverslip as thin as possible. Wait for about 5-10 min to make sure the glue gets dry.
15. After surgery, inject subcutaneously 1 ml warm sterile saline and provide with supplemental heat to maintain body temperature until fully recovered from anesthesia.
16. Return the mouse to their home cage, administer ibuprofen in water (2-4 mg/ml) as analgesic, and monitor daily for the first three postsurgical days.
17. For imaging, mouse is anesthetized with isofluorane on the custom stereotactic frame under the microscope. The cranial window can be used for longitudinal *in vivo* imaging for weeks.

Data analysis

For image analysis and processing, software ImageJ is recommended. For the dendritic bleb analysis in our previous work (Zhang *et al.*, 2016), we first set up the criteria to identify ‘blebs’, bleb

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morphology is ‘beads on a string’, which is totally different from regular dendritic spine; the bleb size is above 2 μm^2 (area). Then use the ImageJ function of Analyze Particles to quantify the bleb number. For detailed procedures, please see ImageJ manual.

Notes

Steady hand during thinning is the key for a perfect cranial window, which needs a lot of practice. We also set up a custom-constructed three-axis motorized translation stage (unpublished data) to thin the skull automatically, which is also good except that the cranial window is smaller because the skull surface is not flat.

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