Video Article

A Thin-skull Window Technique for Chronic Two-photon *In vivo* Imaging of Murine Microglia in Models of Neuroinflammation

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Abstract

Traditionally in neuroscience, *in vivo* two photon imaging of the murine central nervous system has either involved the use of open-skull[1,2] or thinned-skull [3] preparations. While the open-skull technique is very versatile, it is not optimal for studying microglia because it is invasive and can cause microglial activation. Even though the thinned-skull approach is minimally invasive, the repeated re-thinning of skull required for chronic imaging increases the risks of tissue injury and microglial activation and allows for a limited number of imaging sessions. Here we present a chronic thin-skull window method for monitoring murine microglia *in vivo* over an extended period of time using two-photon microscopy. We demonstrate how to prepare a stable, accessible, thinned-skull cortical window (TSCW) with an apposed glass coverslip that remains translucent over the course of three weeks of intermittent observation. This TSCW preparation is far more immunologically inert with respect to microglial activation than open craniotomy or repeated skull thinning and allows an arbitrary number of imaging sessions during a time period of weeks. We prepare TSCW in CX_3CR_1 GFP/+ mice [4] to visualize microglia with enhanced green fluorescent protein to ≤150 μm beneath the pial surface. We also show that this preparation can be used in conjunction with stereotactic brain injections of the HIV-1 neurotoxic protein Tat, adjacent to the TSCW, which is capable of inducing durable microgliosis. Therefore, this method is extremely useful for examining changes in microglial morphology and motility over time in the living brain in models of HIV Associated Neurocognitive Disorder (HAND) and other neurodegenerative diseases with a neuroinflammatory component.

Video Link

The video component of this article can be found at https://www.jove.com/video/2059/

Protocol

1. Preparing the Animal for Imaging

1. In our experiments, we use adult CX_3CR_1GFP/+ mice that express GFP in mononuclear cell lineages, including microglia.[4] Different strains of mice can be used to meet the needs of a specific project.
2. Anesthetize the mouse with an intraperitoneal injection of a three drug cocktail consisting of 0.05 mg/kg Fentanyl, 5.0 mg/kg Midazolam, and 0.5 mg/kg Medetomidine.[5] Surgical preparation for TSCW starts after the animal no longer responds to painful stimuli, such as a tail pinch. Throughout the surgery and imaging, we keep the animal on a heating pad to prevent post-anesthesia hypothermia. If necessary, a booster dose of one-third the original dose of the anesthetic cocktail can be given to restore the original anesthetic plane.
3. Cover the animal's eyes with a protective ophthalmic ointment to keep the eyes moist during anesthesia, which suppresses the animal's blink reflex. Remove the hair from the scalp of the animal using a razor, shears, scissors, or chemical methods. Disinfect the scalp using a 10% povidone-iodine solution and 70% ethanol. All surgical instruments need to be autoclaved, sterilized with a glass-bead sterilizer, or disinfected with 70% ethanol.
4. Make a midline incision of the scalp using small scissors, starting 4-5 mm caudal to the skull and advancing forward to the front of the eyes. It is important that this incision be long enough that the skin will not interfere with the gluing of the head plate that is used to stabilize the mouse head during two-photon imaging (Figure 1). Identify the area to be thinned under a dissecting scope. Avoid areas of interest directly located over cranial sutures, as the skull is less stable in these areas and underlying large vessels and meninges will interfere with imaging.
5. Apply 100% ethanol followed by 10% solution of ferric chloride with a sterile cotton swab to dry the membranes on top of the skull and scrape them away using fine tweezers or a razor blade. Failure to remove the membranes results in unstable head plate attachment.
6. Place a thin layer of the Permabond 910 glue around the edges of the viewing window underneath the head plate. Place the head plate coated with glue over the area of interest on the animal's skull with light pressure (Figure 1). It is important that the head plate is only glued to the bone of the skull. Any skin or membranes that are caught between the head plate and the skull will decrease the stability of the bond.

Apply a small amount of acrylate around the viewing window using a syringe to instantly bond the glue. Finally, apply a small amount of Loctite 454 to the edges of the viewing window to prevent leaks.

7. Screw the head plate to the animal holder (Figure 1) and check the stability of the head plate under a dissection scope by lightly probing with a pair of forceps. There should be no movement of the skull relative to the head plate. Place a drop of saline on the viewing window to ensure there are no leaks.

2. Preparing the Thinned Skull Cortical Window

1. In preparation for thinning the skull, dry the skull using a combination of sterile cotton swabs and compressed air. We initially thin the skull with a sterile IRF 007 drill bit in a Microtorque II drill set at 4000 rpm. Very gently, begin thinning a 2-2.5 mm diameter circular area of the skull. Use only light sweeping motions nearly parallel to the skull; use no direct downward pressure. Stop drilling every 20-30 seconds to remove bone dust using the compressed air. These breaks in drilling allow the skull to cool so there is no heat-induced damage to the underlying brain tissue.
    a. As the drilling progresses, notice the transition to the moister spongy bone layer (i.e. the "diploe"). Once this layer is reached, exercise extra caution with the drill.
2. Occasionally check the thinness of the skull by placing saline over the thinned area and viewing under the dissecting scope. Saline will further enable heat dissipation. As the skull is thinned, smaller vasculature becomes visible.
3. Use a sterile dental microblade to achieve the final thinning under saline, as the microblade provides much more tactile feedback about the stability of the skull. This allows for much thinner preparations than with the drill alone. From our experience, the optimal skull thickness is 10-30 µm.
    a. It is important to check the thinness of the skull under epifluorescence multiple times during the first few attempts at making a thin skull window. The sharpness and depth of visible microglia and vasculature give a good indication as to when the window is ready for imaging.
4. Once the window is ready, glue a custom-made rectangular piece of #0 coverglass with a dimension of less than 2 mm on each side over the window. Using a 1.0 mm diameter glass pipette, place a small drop of cyanoacrylate glue on the thinned skull area and carefully lower down the small piece of coverslip, then press gently against the skull to squeeze out excess amount of the glue. It is important to avoid bubble formation between the glass and the glue since trapped air will cause the area underneath to become optically opaque. The cyanoacrylate glue is used because it remains transparent when dry and also prevents bone and membrane regrowth keeping the skull under the window thin and translucent. If there is any glue on top of the cover glass, use the microblade to carefully remove it once the cover glass is in place and the glue is dry.
5. An analgesic (for example, Buprenorphine 2 mg/kg sub-cutaneously) is administered immediately after the surgery and re-administered at any signs of pain, including reluctance to move, eat or drink, weight loss, salivation, piloerection, respiratory sounds, etc.

3. Two-photon Imaging

1. In preparation for two-photon imaging, cover the TSCW with saline and locate the area of interest under epifluorescence (Figure 2). To enable subsequent imaging of the area, take a picture using a photographic camera.
2. For two-photon imaging, we use a custom-made two-photon microscope[6] with a Ti:Sapphire laser tuned to 920 nm. Fluorescence is detected by using a photomultiplier tube in whole-field detection mode and a 580/180 emission filter. A 20X water-immersion lens (0.95 N.A.) is used throughout the imaging session. The maximum output of the laser power at the objective is set between 50 and 65 mW.
3. Select an area of interest in cortical layers I or II (up to 150 µm below the pial surface). To facilitate re-imaging, take pictures of the same field of view at 1X, 2X, and 3X digital zoom. Blood vessels can serve as landmarks to easily find the same field of view during subsequent imaging sessions. Under a zoom of 3X, multiple z-stacks with 80-90 Z-steps in each stack and a 0.69 µm step can be acquired every 5 minutes for up to 30 minutes, which enables a good sampling of microglial morphology and behavior over time (Figure 3). Between acquisitions of z-stacks, one should verify the level of saline over the TSCW, as well as the animal's depth of anesthesia.

4. Injection of the HIV-1 Neurotoxin, Tat

1. First, silanize the inner lining of a 35 gauge needle and 10 µL Microvolume syringe to prevent Tat deposition. Withdraw the silanizing solution (Sigmacote) until it fills both the needle and syringe. Allow the solution to sit for 5 minutes at room temperature. Empty the solution from the syringe, remove the plunger, and allow the syringe and needle to dry completely. Finally, wash the syringe and needle thoroughly with deionized water.
2. Perform a small craniotomy 0.5 mm in diameter either 3 mm rostral or lateral to the TSCW where two-photon images have been obtained, using a smaller drill bit and carefully thinning the skull until a pair of forceps with sharp tips can remove the thinned layer of bone away easily. We use a 10 µL Microvolume syringe fitted with 35 gauge needle controlled by an Ultramicropump III syringe pump mounted on a 3-axis micromanipulator and after lining up the needle tip to the craniotomy, it is carefully advanced to a depth of 700 to 900 µm below the pial surface where 3 µL Tat_{1-72} (or other pro-inflammatory agents) or saline (or control vehicle) is delivered at 80 nL/min.

5. Animal Housing

1. If the animal is to be imaged multiple times in a single day, cover the open area of the skull with a mixture of opthalmic ointment and Vaseline to prevent discomfort or damage to the skull in between imaging sessions. Disconnect the support bridge from the small head-plate (see Figure 1B) and place the animal in a warmed cage with easily accessible food and water. All animals are to be housed singly after surgery at all times. Once the imaging sessions for an animal are complete for a given day, carefully remove the entire head-plate and suture the skin

back over the skull with #6 or smaller suture. Again, house the animals singly with easily accessible food and water between experimental days. Check the animals daily for any signs of stress, pain, or infection.

6. Representative Results

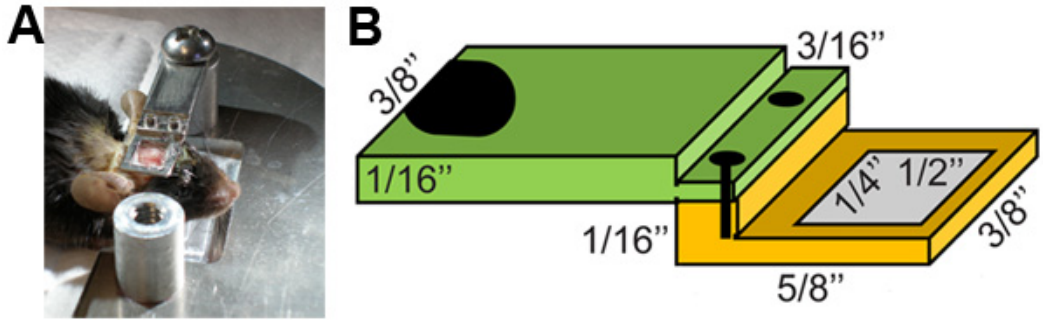

Figure 1. Stabilization of animal for surgery and two-photon imaging (A) and head plate design (B).

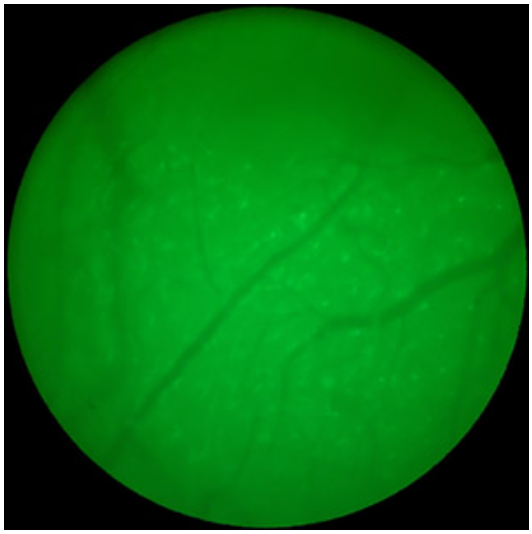

Figure 2. GFP-labeled microglia visualized with epifluorescence.

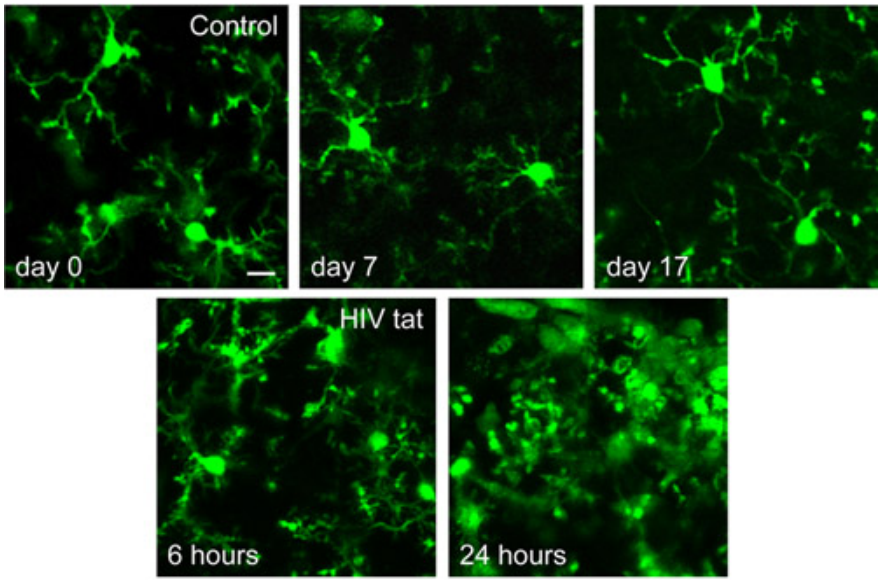

Figure 3. GFP-labeled microglia visualized with two-photon imaging after implantation of the TSCW, approximately 50 µm below the pial surface. The single two-photon sections taken 15-30 minutes after implantation of the TSCW (day 0), as well as 7 and 17 days later,

demonstrate that the windows stays clear while microglia remain inactivated in absence of inflammatory insults. The z-projections taken 6 and 24 hours post-injection of the HIV neurotoxin tat exhibit impressive morphologic changes of activated microglia. Scale bar: 10 µm.

Discussion

There is growing consensus that the actual substrate for HIV-1 associated neurocognitive deficits (HAND) is destruction of synaptic architecture, presumably caused by pro-inflammatory mediators released from HIV-1 infected mononuclear phagocytes. Microglial activation, multinucleated giant cells, and gliosis due to astrocytic hypertrophy are considered nonspecific hallmarks of HIV-1 infection and inflammation in the CNS [7]. In contrast, the severity of premortem neurologic disease has been correlated with loss of synaptic complexity, as well as macrophage burden in the CNS [8,9,10,11]. However, dynamic interactions between microglia, peripheral infiltrating macrophages, and neurons in patients with HAND simply cannot be modeled using conventional static processing obtained from conventional immunocytochemical studies. Recent studies from our laboratories have suggested that at least some of these interactions between peripheral and central mononuclear cells and neurons can be modeled in part by stereotactic injection of the HIV-1 protein Tat_{1-72} into brain parenchyma (Lu, Marker, Tremblay, Qi, and Gelbard, unpublished data). We therefore decided to apply *in vivo* two photon imaging to examine the interplay between monocyte-derived macrophages, brain resident microglia and neurons, in a tractable small animal model for neuroAIDS. While open-skull or thinned-skull preparations afford a single examination of cortical architecture for a brief period of time (hours), investigators interested in the time course of subacute events cannot use these preparations without artifactually activating microglia/macrophage due to surgical manipulation of the calvarial window. Here we demonstrate a simple way of circumventing these limitation by gluing a tiny piece of glass coverslip over the thinned skull area preventing the calvarium from regenerating in the TSCW as well as maintaining translucency for ≥3 weeks. We further demonstrate that this technique allows us to monitor the behavior of peripheral monocytes entering cerebral microvasculature as well as brain-resident microglia in response to stereotactic injection of HIV Tat_{1-72} in the cortex of mice heterozygous for CX_3CR_1/GFP (to identify cells of mononuclear lineage). This technique can easily be adapted to use on mice with different genetic backgrounds; for example, we routinely use heterozygous CX_3CR_1/GFP X Thy-1 YFP [12] mice to investigate interactions between monocyte-derived macrophages, brain resident microglia and neurons in *in vivo* models of neuroinflammation relevant to HAND. Our TSCW preparation allows investigators to study cell-cell interactions between the periphery and CNS that may occur over periods of weeks, in which the animal can be repeatedly imaged before and after experimental treatment. Thus, each animal can serve as its own control. One caveat for this TSCW model is that the target cells under investigation need to be either genetically engineered to express enhanced fluorescent proteins (eXFP) or be labeled with a fluorescent dye without mechanical breach of the calvarium; and that eXFP expression must be robust enough to allow cellular architecture to be visualized after repeated imaging sessions over a period of weeks.

Disclosures

No conflicts of interest declared.

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