Published in final edited form as:

Nat Protoc. 2010 February ; 5(2): 201–208. doi:10.1038/nprot.2009.222.

Thinned-skull cranial window technique for long-term imaging of the cortex in live mice

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Abstract

Imaging neurons, glia and vasculature in the living brain has become an important experimental tool for understanding how the brain works. Here we describe in detail a protocol for imaging cortical structures at high optical resolution through a thinned-skull cranial window in live mice using two-photon laser scanning microscopy (TPLSM). Surgery can be performed within 30–45 min and images can be acquired immediately thereafter. The procedure can be repeated multiple times allowing longitudinal imaging of the cortex over intervals ranging from days to years. Imaging through a thinned-skull cranial window avoids exposure of the meninges and the cortex, thus providing a minimally invasive approach for studying structural and functional changes of cells under normal and pathological conditions in the living brain.

INTRODUCTION

Transcranial two-photon laser scanning microscopy (TPLSM) imaging of the cortex in living mice is a minimally invasive method, which allows repeated imaging of brain cells and vasculature at high optical resolution over intervals ranging from seconds to years^{1–17}. By creating a thinned-skull cranial window with skull thickness ~20 μm , it is possible to image fluorescently labeled structures located upto 300–400 μm within the cortex^{1,2,6,10}. In the past several years, this transcranial imaging approach has been used to study the development and plasticity of synaptic connections^{2–4,6,7,10,11,17,18}, neuronal network activity⁵, cerebral vasculature⁸, amyloid plaques^{6,19,20} and the structure and function of microglial cells in the living intact cortex^{1,13,14}.

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Note: Supplementary information is available via the HTML version of this article.

AUTHOR CONTRIBUTIONS All authors contributed to the development/improvement of the thinned-skull imaging technique and the manuscript preparation. G.Y. wrote the initial draft and made the figures. F.P. obtained the movie of imaging through a thinned-skull preparation. C.P. conducted microglia-imaging experiment. J.G. and W.G. were responsible for the initial development of the thinned-skull imaging approach. G.Y. and F.P. improved the technique.

Published online at <http://www.natureprotocols.com/>.

Technical limitations and controls for imaging through thinned-skull windows

The limitations of the thinned-skull technique are mainly related to the fact that skull thickness is critical for image quality and achieving the optimal thickness in a consistent way requires significant surgical practice. Although a thinned-skull window approach has been used in various studies, such as intrinsic optical imaging of functional organization within rat barrel cortex²¹, TPLSM imaging of the cerebral vasculature⁸ and amyloid plaques¹⁹, these studies were focused on imaging much larger structures than synaptic connections (dendritic spines, filopodia and axonal boutons) and did not require the skull to be exceedingly thin^{8,19}. A non-uniform skull thickness may cause significant spherical aberrations, resulting in decreased two-photon excitation and distortion of fluorescent structures located deep inside the cortex²². We have found that the skull thickness should be $< 25 \mu\text{m}$ in order to obtain high-resolution images of synapses^{2,7,10}. Conversely, over-thinning the skull beyond $15 \mu\text{m}$ carries the risk of mild cortical trauma resulting from pushing the skull downwards with the drill bit or microsurgical blade, leading to mild cortical inflammation. In such cases, the thinning process generally leads to the activation of microglia, as manifested by their amoeboid morphologies, and is often associated with mild neuronal injury, as manifested by axonal and dendritic blebbing, and the eventual disappearance of fluorescent structures. Injury of this type can be prevented by carefully using the microsurgical blade at an angle during the thinning procedure and avoiding pushing the skull against the cortical surface. Furthermore, although multiple imaging sessions within the first few days after the initial surgery can be easily carried out and require only minimal removal of debris over the thinned cranium, repeated imaging over intervals greater than 2–3 d requires skull re-thinning, which could be challenging for an inexperienced operator. It is often difficult to carry out more than five imaging sessions in the same animal, as the optical properties of the skull may gradually deteriorate with repeated thinning.

To control for the quality of images acquired through thinned-skull windows, it is important to measure the thickness of the skull by imaging it with TPLSM to ensure that the thinned region is $\sim 20 \mu\text{m}$ and relatively even in thickness. It is also important to ensure that no surgery-induced cortical injury has occurred, as indicated by axonal and dendritic blebbing or glial activation, in the superficial cortical layer under the thinned-skull regions. Supplementary Movie 1 shows an imaging stack from a successful preparation covering the thinned skull ($\sim 20 \mu\text{m}$), meningeal layers and neuronal structures within the first $100 \mu\text{m}$ under the pial surface. The finest structures, such as dendritic filopodia, should be clearly seen under the thinned-skull window. Images of the superficial cortical layer (the first $50 \mu\text{m}$ under the pial membrane) obtained with a thinned-skull preparation should have nearly the same signal to noise ratio as those obtained with the same laser power and imaging settings using a craniotomy approach².

Comparison of thinned-skull versus open-skull windows for *in vivo* imaging

In addition to the thinned-skull preparation, the cortex can also be visualized through open-skull windows after performing a craniotomy and replacement of the skull by a cover glass with or without a layer of agarose on top of the dural layer^{23–31}. In Table 1, we compared and contrasted the results from the studies of postsynaptic dendritic spine plasticity using

thinned-skull and open-skull windows in the past several years. It is clear that these two methods have generated drastically different results when measuring the turnover rates of dendritic spines in the cortical excitatory pyramidal neurons^{7,10,11,23,24,26,28}. This discrepancy has led to contradictory views on the degree of structural synaptic plasticity in the adult brain and how long-term information might be stored in synaptic circuits^{2,26,32–34}. Although the precise reasons responsible for the differential degree of spine plasticity measured using these two preparations remain to be determined, it is evident that skull removal can induce a significant inflammatory reaction and could, thus, confound efforts to elucidate changes of neurons and glia in the intact brain^{7,20,31}. In contrast, the thinned-skull window approach not only causes minimal perturbation but also allows imaging of the cortex immediately after surgery as opposed to the open-skull method, in which optimal imaging quality is achieved many days after craniotomy^{2,10,11,24,26}. Therefore, we believe that the thinned-skull window approach should be used for a majority of experiments requiring *in vivo* TPLSM imaging of the living cortex, whereas the open-skull window approach may be used in those experiments in which it is necessary to image a large portion of the cortical surface or bathe the cortex with pharmacological agents.

Applications of thin-skull windows for *in vivo* imaging

In the past several years, TPLSM imaging through a thinned-skull window has become an important tool for studying structural and functional changes in the living cortex^{1–14}. With the availability of a large number of fluorescent reporters to probe the structure and function of the brain^{35,36}, we envision that the transcranial TPLSM imaging will greatly expand our present understandings of how the neural circuits are assembled and modified throughout life and how glial and other cells function in the living brain.

MATERIALS

REAGENTS

- *Experimental animals*: Transgenic mice expressing fluorescent proteins in the cytoplasm of cortical neurons (e.g., thy1-YFP line) or CNS resident microglia (e.g., CX3CR1-EGFP line)^{37,38} ! **CAUTION** Animal breeding and handling should comply with relevant institutional and national animal care guidelines.
- Ketamine (Fort Dodge)
- Xylazine (Shenandoah)
- Sterile alcohol prep pad (Fisherbrand, cat. no. 06-669-62)
- Sterile lubricant eye ointment (DEL Pharmaceuticals)
- Cyanoacrylate glue (Fisher Scientific, cat. no. 11-999-24)
- 6-0 Silk suture (LOOK, cat. no. M552830)
- NaCl (Sigma, cat. no. S9888)
- NaHCO₃ (Sigma, cat. no. 401676)
- KCl (Sigma, cat. no. P9333)

- NaH_2PO_4 (Sigma, cat. no. S8282)
- MgCl_2 (Sigma, cat. no. 449172)
- Glucose (Sigma, cat. no. G7528)
- CaCl_2 (FisherBiotech, cat. no. 996862-36)
- Dextran Rhodamine B MW 70,000 (Molecular Probes, cat. no. D1841) 10 mg ml^{-1} in sterile saline.

EQUIPMENT

- Head immobilization device: Custom built plate and skull holder (Fig. 1a and Supplementary Fig. 1)
 - A 14 cm $\times$ 10 cm $\times$ 0.1 cm aluminum plate.
 - Two 18 mm $\times$ 18 mm $\times$ 18 mm aluminum blocks.
 - Two ¼ inch screws and two spacers.
 - Three or four conventional double-edge shaving blades.
 - Custom built plate: Glue the two aluminum blocks to the plate. The blocks are placed about 2 cm from one of the short sides of the plate and 3 cm from each other. A hole with internal thread was drilled on each block to accommodate the ¼ inch screw.
 - Skull holder: Glue 3 or 4 shaving blades to each other.
- High-speed micro drill (Fine Science Tools, cat. no. 18000-17)
 - ▲ **CRITICAL** High-speed operation of the drill is critical for consistent results. Slow drill speeds may result in nicking of the skull or uneven thinning. Make sure that the drill is adequately charged and in good working order.
- Micro drill steel burrs (Fine Science Tools, cat. no. 19007-05 or 19007-07)
 - ▲ **CRITICAL** The drill bur should be frequently replaced and should not be used if it becomes heavily contaminated with debris or if it is performing poorly.
- Double-edge shaving blades (CAMB Machine Knives International LLC, cat. no. CMK169S)
- Microsurgical blades (Surgistar, cat. no. 6900) ▲ **CRITICAL** The size, dimensions and quality of the microsurgical blade are important for achieving maximum thinning without depression of the skull. Use a new blade each time for optimal skull thinning.
- Dissecting stereomicroscope (Olympus, BX50WI)
- CCD camera (Olympus, U-CMAD3): although the CCD camera is attached to a stereomicroscope in our setup, it is advantageous to have it directly equipped on the TPLSM microscope platform if possible.

- TPLSM microscope: We have used either a Bio-Rad 2001 multi-photon microscope or a custom-built multi-photon microscope equipped with a mode-locked laser system. For both systems, the laser system (Tsunami and Millennia Xs, Spectra Physics) is tunable from 690 to 1000 nm wavelength with 80 MHz pulse repeat and < 100 fs pulse width. The laser-scanning system is coupled to an upright fluorescence microscope. For the custom-built unit, an Olympus laser scanning system is used and detectors (photomultiplier tubes) are placed close to the objective to facilitate the signal detection. It is worth mentioning that standardized microscope objective holders generally do not provide a positioning accuracy sufficient for co-centering different objective lenses at micron precision. However, some microscope producers provide objective holders including spring loaded adjustable micropositioners, providing the means to co-center the objective lenses. Such objective holders are useful when switching from one objective to another for imaging.
- Water-immersion objective (e.g., Olympus $\times 60$; NA 1.1, Olympus)
 - ▲ **CRITICAL** Although many $\times 40/\times 60$ water immersion long working distance objectives are suitable for TPLSM imaging, the decisive factor for resolution purposes is the numerical aperture. Any objective used for TPLSM should be assembled from lenses with special coatings, which do not absorb or reflect a large fraction of the incident laser light. The users should choose the objectives based on their experimental needs and check with microscope producers for their newest development of lenses for TPLSM imaging. For example, Olympus renders a $\times 25$ -water immersion lens at 1.05 NA, which is specially developed for the purpose of TPLSM and useful for *in vivo* imaging. We generally use Olympus $\times 60$, NA 1.1 objective for *in vivo* imaging of dendritic spines. **CAUTION** Keep the objectives immersed in water after imaging and clean them periodically with 100% ethanol.

Image acquisition software (Fluoview 5.0 or Lasersharp 2000)

REAGENT SETUP

Ketamine–xylazine mixture (KX)—20 mg ml⁻¹ of ketamine and 3 mg ml⁻¹ of xylazine in 0.9% NaCl solution; store the solution at room temperature (20 °C). Properly stored KX solution is stable for up to 1 year.

Artificial cerebrospinal fluid (ACSF)—119 mM NaCl, 26.2 mM NaHCO₃, 2.5 mM KCl, 1 mM NaH₂PO₄, 1.3 mM MgCl₂ and 10 mM glucose; gas with 5% CO₂/95% O₂ for 10–15 min, then add 2.5 mM CaCl₂. Filter sterilize with a 0.22 μ m filter apparatus and store at 4 °C. ACSF is stable for 3–4 weeks.

If overt contamination (solution becomes cloudy) or precipitation is apparent, then discard and prepare fresh ACSF.

PROCEDURE

Thinned-skull preparation ● TIMING 30–45 min

1| Anesthetize mice by intraperitoneal injection ($5\text{--}6\text{ }\mu\text{l g}^{-1}$ body weight) of KX. Wait for 5–10 min. Place the mouse on a cotton pad after a surgical level of anesthesia has been reached. A heating pad may be inserted under the cotton pad to maintain a body temperature of $\sim 37\text{ }^{\circ}\text{C}$.

! CAUTION All animal experiments related to surgery and imaging should comply with relevant institutional and national animal care guidelines.

▲ CRITICAL STEP Continuously monitor the depth of anesthesia by testing the animal's reflexes (e.g., pinching the animal's foot with a blunt pair of forceps and checking for the absence of the eye blinking reflex) during the surgery and inject more KX when necessary.

2| Thoroughly shave the hair over most of the scalp with a double edge razor blade. Remove the residual hair and clean the scalp with sterile alcohol prep pad, then perform a midline scalp incision, which extends approximately from the neck region (between the ears) to the frontal portion of the head (between the eyes). Carefully disrupt the fascia located between the scalp and the underlying muscle and skull with a pair of spring scissors, taking care not to sever blood vessels.

3| Lubricate both eyes with a drop of eye ointment. If microglial imaging is being carried out, then inject $100\text{ }\mu\text{l}$ of rhodamine dextran solution retro-orbitally for vessel labeling before the application of eye ointment or alternatively inject it through the tail vein.

! CAUTION Dehydration of eye tissue can cause permanent damage to the eyes.

4| Localize the brain area to be imaged based on stereotactic coordinates and mark it with a pen.

5| Remove the connective tissue attached to the skull over the area to be imaged by gently scraping the skull with a blunt microsurgical blade. Local anesthetics may be added to the skull surface to minimize pain associated with drilling of the periosteum.

! CAUTION If local anesthetics are added, it is important to examine the effect of anesthetics on the brain structure (e.g., spine and filopodia dynamics), as it may confound the results of experiments.

6| Place a small amount of cyanoacrylate glue around the edges of the internal opening of the skull holder and press the holder against the skull. Make sure that the area to be imaged is exposed in the center of the internal opening (Fig. 1a).

▲ CRITICAL STEP Skull immobilization, which is in part determined by the quality of the skull holder to skull bonding, is very important to reduce movement artifacts during imaging.

7| Gently pull the loose skin up to the edges of the internal opening of the skull holder and apply a small amount of glue to the edge of skin to adhere it to the skull.

▲ CRITICAL STEP Gluing the skin around the internal opening of the skull holder and the skull helps to hold the ACSF in place during imaging when using a water immersion lens.

8| Wait for ~5 min until the skull holder is well glued to the skull. Attach the skull holder to the skull immobilization device by gently inserting the lateral edges of the skull holder between the aluminum blocks and washers/screws of the skull immobilization device. Tighten the screws to completely immobilize the skull holder (Fig. 1a). Wash the opening of the skull holder a few times with ACSF to remove the remnants of non-polymerized glue. This helps prevent the glue from contaminating the microscope objectives.

9| Immerse the skull with a drop of ACSF (close to body temperature, 36–37 °C). Use a high-speed micro-drill to thin a circular area of skull (typically ~0.5–1 mm in diameter; Fig. 1b) over the region of interest under a dissecting microscope. Carry out drilling intermittently during the thinning procedure to avoid friction induced overheating. ACSF helps soften the bone and absorbs heat. Replace the ACSF periodically and wash away the bone debris.

▲ CRITICAL STEP Do not thin a large region (>1.5 mm) to a thin layer (< 50 µm) as it is difficult to exercise fine control of the drilling process and may cause damage to the underlying cortex.

10| The mouse skull consists of two thin layers of compact bone, sandwiching a thick layer of spongy bone. The spongy bone contains tiny cavities arranged in concentric circles and multiple canaliculi that carry blood vessels. Remove the external layer of compact bone and most of the spongy bone with the drill. Some bleeding from the blood vessels running through the spongy bone may occur during the thinning process. This bleeding will usually stop spontaneously within a few minutes.

11| After removing the majority of the spongy bone, remaining concentric cavities within the bone can usually be seen under the dissecting microscope, indicating that drilling is approaching the internal compact bone layer. At this stage, skull thickness should still be more than 50 µm. Continue skull-thinning with a microsurgical blade to obtain a very thin (~20 µm) and smooth preparation (~200 µm in diameter) (Fig. 1b). During the thinning process, repeatedly examine the preparation with a conventional fluorescence microscope until the dendrites and spines in the area of interest can be clearly visualized (Supplementary Fig. 2).

▲ CRITICAL STEP Hold the microsurgical blade at ~45° during thinning and take great care not to push the skull downwards against the brain surface or to break through the bone, as minor brain trauma or bleeding may potentially cause inflammation and disruption of neuronal structures. The thickness of the skull can be directly measured by imaging the skull auto-fluorescence with the TPLSM microscope. Periodic measurement of the skull thickness during thinning may help the novice user in preventing skull over-thinning.

! CAUTION It is important not to thin a large area (>300 µm in diameter) to < 15 µm in thickness, as it may cause disruption of neuronal structures and activation of immune

cells as indicated by neurite blebbing, microglia process retraction and growth of epidural connective tissues, which greatly reduce the quality of subsequent images over days and may confound experimental results.

Mapping the imaging area for future relocation ● TIMING 5 min

12| In order to identify the same imaged area at a later time point, take a high quality picture of the brain vasculature with a CCD camera attached to a stereo dissecting microscope (Fig. 2a) or directly to the TPLSM setup.

13| Carefully move the prepared mouse with the entire skull immobilization device to the TPLSM microscope stage. Select a properly thinned area for imaging under a fluorescence microscope and carefully mark the selected area on the CCD brain vasculature map by observing the pattern of blood vessels adjacent to it (Fig. 2a).

TPLSM imaging of neuronal or glial structures ● TIMING 15 – 45 min

14| Tune the TPLSM to the appropriate wavelength (e.g., 920 nm for YFP and 890 nm for GFP). When possible, use high numerical aperture water-immersion objectives (e.g., $\times 60$, 1.1 NA) to acquire images.

! CAUTION Mouse ACSF should be used at all times during imaging for objective immersion. If there is a sudden deterioration of imaging quality, check that the lens remains fully immersed in ACSF. Gradual leakage of ACSF is usually due to inadequate gluing of the scalp around the skull holder openings (Step 6).

15| For neuronal imaging, obtain a low magnification stack of fluorescently labeled neuronal processes (e.g., $\times 60$ objective; $200\ \mu\text{m} \times 200\ \mu\text{m}$; 512×512 pixel; $2\ \mu\text{m}$ step), which serves as a higher resolution map for accurate relocation of the same region at later time points in conjunction with the CCD brain vasculature map (Fig. 2b). If microglial imaging is being performed, then take a low-magnification ($\times 10$ air objective) stack of the rhodamine–dextran labeled vasculature and mark the selected area before switching to $\times 40/\times 60$ objective, and make sure that objectives ($\times 10$ and $\times 40/\times 60$) are co-centered.

? TROUBLESHOOTING

16| Without changing the position of the stage, take high-magnification images (e.g., $66.7\ \mu\text{m} \times 66.7\ \mu\text{m}$; $512\ \text{pixel} \times 512\ \text{pixel}$; $0.75\ \mu\text{m}$ step; Fig. 2c) from the same area. The stack is typically taken within $\sim 100\ \mu\text{m}$ below the pial surface for spine imaging (Supplementary Movie 1) and within $\sim 200\ \mu\text{m}$ for microglia imaging.

! CAUTION We typically use laser intensities in the range of 10 to 30 mW (measured at the sample) to minimize phototoxicity.

? TROUBLESHOOTING

Recovery ● TIMING >1 h

17| Following imaging, detach the head immobilization device from the skull by gently pushing the skull away from the skull holder. When removed, the head immobilization

device should take with it any remaining glue and be separated from the skin. If this does not occur, gently remove any remaining dried glue on the skull and/or detach the skin from the skull holder with a pair of forceps. Suture the scalp with 6-0 silk and leave the mouse in a separate cage until it is fully mobile. After recovery, return the mouse to its original housing cage until the next viewing.

■ **PAUSE POINT** Depending on the design of the experiment, re-imaging can be obtained hours to years after the first view.

Re-imaging ● TIMING 1 – 2 h

18| Repeat Steps 1–8. Find the thinned region based on the brain vasculature map (CCD for dendrite imaging and/or $\times 10$ two-photon angiography for microglia). If the second view is within 1 week after the first view, then carefully remove the connective tissue that has re-grown on top of the thinned region using a microsurgical blade and check the image quality with the TPLSM microscope. If the image quality is poor (blurry features, high background fluorescence or significantly reduced depth of penetration), use a microsurgical blade to carefully shave the skull (as in Step 11) until a clear image can be obtained. If the second view is more than a week after the first view, repeat Steps 9–11.

▲ **CRITICAL STEP** The bone in the thinned area can re-grow substantially if the time interval between imaging sessions is longer than a week. The newly grown bone layer is optically less transparent for TPLSM imaging, so it is generally necessary to shave the bone slightly thinner than the previous imaging session.

? TROUBLESHOOTING

19| Find the previously imaged region under the fluorescence microscope. Align the region according to the low-magnification map under TPLSM, and then zoom in to high magnification to further align it. After the region is precisely aligned with the first view, take images as in Step 16.

Multiple-session imaging ● TIMING 1 – 2 h

20| For multiple imaging sessions, repeat Steps 18 and 19.

▲ **CRITICAL STEP** Although we have successfully imaged the same region with high optical clarity up to five times, the number and quality of imaging sessions is dependent upon the quality of the initial surgery, the intra-session interval and the experience of the operator. Although thinning the skull to the extremes ($\sim 15 \mu\text{m}$) during the first surgery may provide excellent clarity in the initial imaging session, it will make repeated imaging sessions more difficult because of an increased propensity for compensatory bone re-growth. We recommend minimizing the perturbation to the system by limiting the number of imaging sessions and by thinning the skull only as much as necessary to gain an image clear enough to observe the desired structures.

● TIMING

Steps 1–11, Thinned-skull preparation: 30–45 min

Steps 12 and 13, Mapping the imaging area for future relocation: 5 min

Steps 14–16, TPLSM imaging of neuronal or glial structures: 15–45 min

Step 17, Recovery: >1 h

Steps 18 and 19, Re-imaging: 1–2 h

Step 20, Multiple session imaging: 1–2 h

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

ANTICIPATED RESULTS

With YFP-expressing mice this method has been used successfully to image individual dendritic spines and axonal varicosities in various brain areas including visual, somatosensory, motor and frontal cortices over intervals of upto 19 months^{2,10}. Figure 2d,e shows examples of images obtained over 3-d interval in mice at 4 months of age. Note the remarkable stability of the number and location of adult spines and axonal varicosities between the two views (Fig. 2d,e). Most adult spines persist even over a 19-month interval (Fig. 2f,g). Because of the non-invasive nature of the thinned-skull technique, this method is also suitable for brain immune cell imaging (e.g., microglia cells), which are highly sensitive to the environment and respond immediately after perturbation^{1,7}. Figure 3 shows images of GFP-expressing microglia cells in the cortex over 1 d with minimal changes of their ramified shape under a normal physiological state.

Concluding remarks

Studies in the past several years have demonstrated that the transcranial TPLSM imaging approach is minimally invasive and technically reliable, allowing studies of detailed changes of neurons and glia over time in the living intact cortex. On the other hand, open-skull preparations involve skull removal and implantation of a glass window, which can cause significant neuroinflammation and induce artifactual changes in the cortex^{7,20,31}. Indeed, it has been found that large open-skull preparations (~5 mm in diameter) induce significant activation of microglia and astrocytes, initial dendritic spine loss after surgery and a dramatic increase in spine turnover⁷. These findings have been replicated by a more recent study (see Table 1 for details) even though the authors of this study claimed the opposite and suggested that open-skull preparation is excellent for chronic imaging if surgeries are done properly³¹. It is worth mentioning that potent immunomodulators such as dexamethasone are frequently used following open-skull preparations to reduce inflammation and achieve optimal imaging properties^{23,24,26–30}. These substances may have a significant impact on brain physiology, further confounding the studies of brain structure and function. For these reasons, we think that the claim that open-skull preparations are excellent for chronic imaging of cortical structures is assumptive and whether the results generated by such a preparation represent physiological conditions remains to be determined. We suggest that transcranial TPLSM imaging should be the method of choice for studying cortical structure and function in the living brain except for experiments that cannot be easily performed

without removing the skull. In such cases, it is important to interpret the data carefully in the context of the confounding factors associated with open-skull preparations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institutes of Health to W.-B.G. and J.G. as well as an Ellison Medical Foundation/AFAR Postdoctoral Fellowship to G.Y.

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Figure 1. Schematic diagram of thinned-skull preparation. **(a)** A head immobilization device including a custom built plate and a skull holder is used for reducing movement artifacts during imaging. The skull holder is glued on the mouse skull and tightened on the aluminum blocks of the custom built plate. The region of interest is exposed in the center. **(b)** A circular area of skull (typically ~0.5–1 mm in diameter, marked with blue circle) over the region of interest is shaved. The thinnest region (marked with pink circle) for two-photon laser scanning microscopy (TPLSM) imaging is ~20 μm in thickness and ~200 μm in diameter. All experiments using animals were carried out under the institutional and national guidelines.

Figure 2.

Long-term transcranial two-photon laser scanning microscopy (TPLSM) imaging of fine neuronal structures. **(a)** CCD camera view of the brain vasculature under the thinned skull. The cortical vasculature can be clearly seen through the thinned skull. The vasculature pattern remains stable over months to years and can be used as a landmark to relocate the imaged region at subsequent time points. Arrow indicates the region where subsequent TPLSM images were obtained. **(b)** Two dimensional projection of a 3D stack of dendritic branches and axons in the primary visual cortex ($\times 60$, $\sim 0.39 \mu\text{m pixel}^{-1}$). The stack was 50 μm deep (2 μm step size). The boxed region was then imaged at higher magnification. **(c)** High power 2D projection of a 3D stack ($\times 60$, $\sim 0.13 \mu\text{m pixel}^{-1}$, 10 μm reconstructed, 0.70 μm step size) reveals clear neuronal structures including axonal varicosities, dendritic shafts and dendritic protrusions. **(d,e)** Axonal and dendritic branches from two animals imaged 3-d apart show the same spines and boutons at the same locations (adapted from reference 2). **(f,g)** Dendritic branches imaged over 19 months apart. The arrows indicate spines that are eliminated in the second view. The arrowheads indicate spines that are formed in the second view. Note that most spines in **f** persist in **g** (adapted from reference 10). Two-dimensional projections of three-dimensional image stacks containing dendritic segments of interest were used for **d–g**. Scale bar: 50 μm (**a**), 5 μm (**b,c**), 1 μm (**e**) and 2 μm (**g**). All experiments using animals were carried out under the institutional and national guidelines.

Figure 3.

Transcranial two-photon laser scanning microscopy (TPLSM) imaging of enhanced green fluorescent protein (EGFP) labeled microglia and cortical vasculature. **(a,b)** Two-dimensional projections of a 3D z-stack from the visual cortex ($\times 40$, digital zoom = 1) from a mouse harboring a single copy of the CX3CR1-EGFP allele driving EGFP expression in a subset of myeloid cells including CNS-resident parenchymal microglia imaged 24 h apart. The cortical vasculature has been labeled in red by intravenous injection of a rhodamine–dextran conjugate solution. The stacks are 40 μm in depth (1 μm step size) and are representative of a section of cortex spanning 40–80 μm below the dural surface. EGFP-labeled microglia retain their characteristic highly branched morphology indicative of a resting state. Scale bar: 20 μm . All experiments using animals were carried out under institutional and national guidelines.

TABLE 1Comparison of *in vivo* imaging through thinned-skull and open-skull windows.

	Thinned-skull windows	Open-skull windows
Immediate chronic imaging after surgery	Yes ^{2,7,10,11,17}	No (there is a ~1–2 weeks of opaque period after surgery) ^{23,24,26,28,29,31,39}
Number of imaging sessions	Unlimited within 2 d; up to 5 for chronic imaging with intervals of days to years	Theoretically unlimited
Maximum imaging interval	>2 years	Several weeks to months (often terminated by skull regrowth and dura thickening)
Window size	~0.2 mm diameter ^{2,7,10,11,17}	~2–5 mm diameter ^{23,24,26,28,29,31,39}
Requirement of systemic anti-inflammatory and antibiotic medication administration	No ^{2,7,10,11,17}	Yes ^{23,24,26,28,29,31,39}
Astrocyte activation	Not detectable if the skull thickness is >20 μm ^{7,20}	Prolonged and extensive ^{7,20,31}
Microglial activation	Not detectable if the skull thickness is >20 μm ^{7,20}	Rapid and extensive (5-mm diameter window size) or moderate (smaller window size) ^{7,20,31}
Net spine loss in adult animals within the first 10 d after surgery	No ^{2,7,10}	Yes, by some groups ^{7,31} , suggesting that the degree of spine loss may depend on the size of the cranial window
Spine turnover in adult barrel and visual cortices	~5% over 1 month ^{2,7,10,11}	Up to 30–50% over 1 month ^{23,24,26,28,29,31}
Variability of spine turnover between preparations	Very small ^{2,7,10,11,17}	Very large ^{7,23,24,26,28,29,31}
Adult spine turnover after brief sensory deprivation	No ¹¹	Significant change ^{23,26,28}

TABLE 2

Troubleshooting table.

Step	Problem	Possible reason	Solution
15,16	Artificial movements of neuronal structures during imaging	Animal wakes up Attachment between the skull and the skull holder is loose	Anesthetize the animal again Detach the skull holder and glue it to the skull again. No obvious movements should be observed when the skull holder is tightly glued to the skull
	Skull holder does not hold artificial cerebrospinal fluid (ACSF) during imaging	The internal opening of the skull holder is not sealed by the surrounding skin	Dry the skin, then pull it around the skull holder and glue the edge of the skin to the skull
16,18	Poor visibility	Skull is thick	If the skull is too thick, carefully use drill or microsurgical blade to shave more and check again
		Skull is thin but uneven or marred by the drill bit or blunt microsurgical blades	Unevenly thinned or marred skull is a result of improper thinning. It may be difficult to correct once part of the bone is already exceedingly thin. Use another animal for the experiment
		Bleeding	If there is bleeding from the bone, wait until it stops spontaneously and wash with ACSF Bleeding underneath the bone indicates that the brain has been pushed or compromised during the thinning procedure. Use another animal for the experiment
	Neurite blebbing or microglial process retraction	Pushing the skull downwards against the brain surface during the thinning Over-thinning of the skull Breakage of the bone	Use another animal for the experiment. Neurite blebbing indicates the disruption of neuronal structures and is concurrent with high spine turnover. Microglial process retraction is indicative of inflammatory response