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A large lateral craniotomy procedure for mesoscale wide-field optical imaging of brain activity

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Abstract:	The craniotomy is a commonly performed procedure to expose the brain for in vivo experiments. In mouse research, most labs utilize a small craniotomy, typically 3 x 3 mm. This protocol introduces a method for creating a substantially larger 7 mm x 6 mm cranial window exposing most of a cerebral hemisphere over the mouse temporal and parietal cortices (e.g., bregma 2.5 to - 4.5 mm, lateral 0 - 6 mm). To perform this surgery, the head must be tilted approximately 30 degrees and a majority of the temporal muscle must be retracted. Due to the large amount of bone removal, this procedure is intended only for acute experiments with the animal anesthetized throughout the surgery. The main advantage of this innovative large lateral cranial window is to provide simultaneous access to both medial and lateral areas of the cortex. This large unilateral cranial window can be used to study the neural dynamics between cells, as well as between different cortical areas by combining multi-electrode electrophysiological recordings, imaging of neuronal activity (e.g., intrinsic or extrinsic imaging), and optogenetic stimulation. Additionally, this large craniotomy also exposes a large area of cortical blood vessels, allowing for direct manipulation of the lateral cortical vasculature.
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TITLE:
A large lateral craniotomy procedure for mesoscale wide-field optical imaging of brain activity

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Neuroscience, craniotomy, cranial window, imaging, mouse, middle cerebral artery, cortical activity, brain

SHORT ABSTRACT:
This protocol presents a method for creating a large unilateral craniotomy over the temporal and parietal regions of the mouse cerebral cortex. This is especially useful for real time imaging over an expansive area of a cortical hemisphere.

LONG ABSTRACT:
The craniotomy is a commonly performed procedure to expose the brain for *in vivo* experiments. In mouse research, most labs utilize a small craniotomy, typically 3 mm x 3 mm. This protocol introduces a method for creating a substantially larger 7 mm x 6 mm cranial window exposing most of a cerebral hemisphere over the mouse temporal and parietal cortices (*e.g.*, bregma 2.5-4.5 mm, lateral 0-6 mm). To perform this surgery, the head must be tilted approximately 30° and a majority of the temporal muscle must be retracted. Due to the large amount of bone removal, this procedure is intended only for acute experiments with the animal anesthetized throughout the surgery.

The main advantage of this innovative large lateral cranial window is to provide simultaneous access to both medial and lateral areas of the cortex. This large unilateral cranial window can be used to study the neural dynamics between cells, as well as between different cortical areas by combining multi-electrode electrophysiological recordings, imaging of neuronal activity (*e.g.*, intrinsic or extrinsic imaging), and optogenetic stimulation. Additionally, this large craniotomy also exposes a large area of cortical blood vessels, allowing for direct manipulation of the lateral cortical vasculature.

INTRODUCTION:

The craniotomy is a standard procedure used by neuroscientists to reveal a portion of the brain. Since the dawn of electrophysiology, the craniotomy has allowed unprecedented breakthroughs in the field of neuroscience. Dense mapping of the cerebral cortex with electrodes has led to experiments testing hypotheses and theories based on these maps. We have recently entered a new era where the craniotomy is being utilized for *in vivo* imaging of cortical blood flow[1-3] and neurovascular architecture[4], enabling real time visualization of cortical activity within the exposed areas[5-7]. Although many studies use craniotomies combined with *in vivo* optical imaging techniques to study the structure and function of cortical neurons, glia, and cortical vasculature[8,9], further investigations are limited by small areas of exposed cortex (but see[10]).

The purpose of this protocol is to provide a method for creating a large lateral craniotomy, exposing the cerebral cortex from the midline to the squamosal bone, and extending beyond bregma and lambda. This large craniotomy enables simultaneous viewing of the association cortices (retrosplenial, cingulate, and parietal), primary and secondary motor, somatosensory, visual, and the auditory cortex. This method has been previously coupled with voltage sensitive dye imaging (VSDI) to investigate how multiple cortical areas interact with one another during spontaneous and stimulus-induced cortical activity[5,11,12]. The most challenging aspects of this procedure include positioning the head of the animal, fixing the head plate, and avoiding hemorrhage while separating the temporal muscle from the parietal bone. Care must also be taken during the drilling and skull removal processes as the skull curves at an oblique angle.

PROTOCOL:

The following protocol follows the University of Lethbridge Animal Care Committee (ACC) guidelines, and is conducted in accordance with the standards of the Canadian Council on Animal Care (CCAC).

1. Preparation

1.1. For prolonged study periods, autoclave all opened surgical supplies and ensure that sterility is maintained throughout the surgery. If multiple surgeries are required, autoclave between surgeries. Otherwise, use tool cleaning solutions, bead sterilizers, and isopropyl alcohol to clean surgical tools.

1.2. Place the mouse in an induction chamber and anesthetize with 3 – 4% isoflurane. Follow with 1.0 – 2.0% isoflurane for maintenance during the surgery. Further reduce to as low as 0.6% during imaging, provided proper anesthesia is maintained and the mouse remains areflexic to painful stimuli.

Note: Prolonged use of isoflurane can cause dehydration.

1.2.1. Use subcutaneous injections of saline, 0.1 mL per 10 g body weight, every 1 – 2 h. When adequately hydrated, the mouse will urinate once every 1 – 2 h.

1.3. Closely monitor the mouse to ensure consistent anesthesia throughout surgery and imaging. Do not leave the mouse unattended and take care that it never regains consciousness.

1.4. Transfer the mouse to the head-holder set-up and place on a thermo-regulating heating pad set to 37 °C (Figure 1A). Secure the upper teeth in a teeth holder.

1.5. Apply ophthalmic ointment to reduce corneal drying.

1.6. Provide local anesthetic by injecting 0.03 mL of lidocaine (2% epinephrine) subcutaneously over the craniotomy site. Wait 3 – 5 min for the drug to be absorbed into the tissue.

1.7. To reduce cerebral edema, inject (4 mg/kg) of dexamethasone (5 mg/mL) intramuscularly.

1.8. Rotate the mouse's head towards the left approximately 30° to expose the right lateral side of the head and secure the mouse's head with the blunt end of ear bars (Figure 1A).

1.9. Wipe the surgical area with cotton swabs dipped in 4% chlorhexidine (3 times) and 70% alcohol (3 times). Use each cotton swab only once.

1.10. Ensure there is plenty of brain buffer on hand (at least 50 mL). Here, use a solution comprised of 134 mM sodium chloride, 5.4 mM potassium, 1 mM magnesium chloride hexahydrate, 1.8 mM calcium chloride dihydrate, and 5 mM HEPES sodium, pH balanced to 7.4 with 5 M hydrogen chloride.

2. Removing the skin and retracting the muscle from the skull

2.1. Perform nearly all of these procedures while viewing the skull under a dissecting microscope (*e.g.*, 0.7 – 4.5x power, depending on the situation).

2.2. Lift up the skin 1 mm left of the midline (just behind the ear) with forceps and make a small horizontal incision with surgical scissors.

2.3. Insert the scissors into the incision, and cut 1 cm rostrally.

2.4. At the initial incision point, make a 5 – 6 mm lateral cut towards the right ear, and then cut towards the rostral end of the previous incision (step 2.3). Ensure that the widest part of the exposed area is at least 7 mm.

2.5. Cut the skin around the right ear, near the right eye. Expose the right side of the skull and temporal muscle. Trim the skin further if the surgical area needs to be extended.

2.6. Fix the skin around the incision by putting a few drops of butyl cyanoacrylate glue between the skull and the skin.

2.7. Using a cotton swab, rub the surface of the skull in a circular motion to remove the periosteum from the skull. Ensure that none remains and that the skull is completely dry. The skull will quickly air dry after application of the gel foam soaked in brain buffer, if more drying is needed, use cotton tip swabs. This step is crucial for head plate fixation (Figure 1B).

Note: If there is periosteum left on the skull, or if the skull is not dry before gluing on the head plate, it will likely detach. If this happens, gently remove the head plate and start over. Bleeding may occur during this process; allow a few minutes for it to clot, and then gently remove. This process is not recommended to be repeated more than twice.

2.8. Using spring scissors and forceps, separate the temporal muscle from the skull; cut and retract the muscle downwards until reaching the squamosal bone (Figure 1C). Be careful not to damage the superficial temporal vein that runs along the level of the squamosal bone near the eye, otherwise hemorrhaging might occur.

2.9. Control bleeding with gel foam pre-soaked in brain buffer, and drop butyl cyanoacrylate glue onto the bleeding site. For serious hemorrhaging use a heat cauterizer.

3. Craniotomy

Note: The surgeon must remain diligent during removal of the skull and dura to avoid unnecessary complications. Troubleshooting steps are included should complications arise.

3.1. Put two drops of ethyl cyanoacrylate glue on either side of the head plate[13], and glue the head plate over the craniotomy area (Figure 1D & 2A).

3.2. Mark the location of bregma either with a fine tip marker or cut a small triangular piece tape and point a corner at bregma (Figure 2A).

3.3. Fill the opening space between the skull and head plate with dental cement leaving only the craniotomy area exposed. Wait for the dental cement to dry and harden, typically 5 – 10 min (Figure 2B).

3.3.1. Once the cement is set, briefly fill the well with brain buffer and allow to soak for 3 – 5 min. Use a rolled tissue to remove brain buffer before drilling (Figure 2C).

3.4. Outline the surgical area by lightly scoring the surface of the skull with a dental drill. Use a pneumatic drill (set to maximum of 20 PSI), with a FG ¼ burr, and controlled with a variable speed foot pedal.

3.5. Gently trace the drill along the original scoring to deepen it, ensuring the drill does not penetrate through the skull into the brain (Figure 2D). Take turns every few minutes between drilling and dabbing the skull surface with moistened gel foam. This will reduce heating and drying of the skull from mechanical friction and prolonged exposure.

Note: The skull will quickly air dry after application of the wet gel foam. If more drying is needed, use cotton tip swabs.

Caution: The skull is uneven in thickness. For example, the parietal-temporal ridge is the thickest area, while skull regions near the midline and squamosal landmarks are relatively thin.

3.6. During drilling, periodically check for buckling of the skull by gently pressing on it with forceps or the non-moving drill bit. When the bone begins to buckle, stop drilling and immerse the entire window in brain buffer.

Note: If blood rushes out of an area, it may suggest that the dura has been damaged. If this is the case, place a semi-wet gel foam over the area and try to soak up the blood while gently applying pressure to the gel foam with a cotton tip swab.

3.7. Wait for at least 5 min before skull removal to soften the bone and to reduce the chance of the dura sticking to the bone, making the skull removal process easier.

3.8. Perform the skull removal process while the skull is submerged in brain buffer.

Note: If a portion of the skull remains stubbornly attached, a #11 scalpel blade can be used to gently score the skull. Take extreme care to not puncture the blade through the skull and into the brain.

3.9. Beginning from the bottom, gently pry the loose skull from the dura using forceps.

Note: If a small amount of bleeding occurs during the skull removal process, remove the buffer with a transfer pipette or syringe, and then replace with new buffer.

3.10. Once the bone is loose and "floating" on the dura, firmly grip the bone with forceps and lift the bone from the dura. Keep the bone horizontal, otherwise the opposite edge may sink into the tissue.

3.11. To control bleeding, roll the corner of a tissue into a point and remove most of the buffer from the cranial well. Quickly apply gel foam, pre-soaked in buffer, to the bleeding area while adding very light pressure with a cotton tip swab.

Note: The bleeding usually comes from the edge of the bone or the surface of the dura; both cases are normal and bleeding will quickly stop if no major blood vessels are damaged. If bleeding continues, blood may fill the entire window, forming a clot sheet over the imaging area.

3.11.1. To remove the clot sheet, carefully pick up pieces of clotted blood from the imaging area while leaving the blood clot intact around where the source of the bleeding was. Take care to not remove the blood clot from the bleed source as this may cause even more blood loss. Irrigate the surface of the brain with brain buffer to wash away any blood.

3.11.2. Take care to avoid touching the delicate brain tissue or adding foreign material to the brain; repeat until bleeding has stopped, for approximately 2 – 5 min (Figure 2E).

Note: At this point, the craniotomy is ready for preparing the cranial window (see step 5). If needed, remove the dura before implanting the cranial window (see step 4).

4. Dura Removal

Note: Dura removal requires extreme care and may take over 15 min.

4.1. Draw away excess buffer from the craniotomy. While maintaining a moist surface, grab a small piece of dura with forceps and make a small incision using spring scissors.

4.2. Using forceps, grasp the opening of the incision, lift the dura, and snip off the dura piece by piece with spring scissors.

4.3. Drop more brain buffer on the brain surface to float the dura and help it separate from the brain. Continue until all dura is removed from the cranial window site. When performed correctly the brain will appear to be very clean with distinct blood vessels and no blemishes (compare Figure 2E with 2F).

Caution: Some areas of the dura are attached to small arterioles on the surface of the brain (*e.g.*, near the midline proximal to the parietal association area), and removal of such can rupture the arteriole. In such cases it may be better to leave a small piece of dura intact over top of the arteriole. The VSD may not penetrate that small area, but this is preferred to having major bleeding.

4.4. Fix the brain surface in agarose as soon as possible to minimize movement from pulsation and to prevent further swelling (see step 5).

5. Preparing Cranial Window

5.1. Prepare 1.3% agarose by heating 200 mg of agarose powder dissolved in 15 mL of brain buffer.

5.2. Place a thermometer in the hot agarose and cool the agarose down to just above solidifying temperature (~ 40 °C).

Note: Running cool water over the outside of the agarose container may speed the cooling process. Gently stir continuously to ensure no bubbles or particulates are present.

5.3. Draw up the agarose with a transfer pipette and drop the agarose directly on the brain. Quickly place the cover slip over the surface and fasten the cover slip with agarose drops on the corners.

6. Euthanasia

Note: In our experience, this procedure takes experienced surgeons at least 3 – 4 practice surgeries to attain greater than 90% success rate. Less experienced surgeons may require even more practice. During the craniotomy or durotomy, the brain may sustain damage, such as if the drill punches through the bone into the brain. Some minor damage at the edge of craniotomy may be permissible. However, if the brain does not look “clean” with bright red undamaged

blood vessels and white cortex, the experiment may need to be terminated. Examples of poor preparations include those with dead blood vessels, or when the cortex is marked with torn or damaged blood vessels. If any of these signs are present, the experiment will unlikely yield high quality data.

6.1. Give an intraperitoneal injection of sodium pentobarbital at 100 mg/kg. Ideally, if the needle is inserted into the liver, death will be very rapid (< 1 min).

6.2. If perfusion is required, verify that the animal is deeply anesthetized before proceeding (see step 1.2).

6.3. Alternatively, if perfusion is not required, wait for a minimum of 5 min and then verify that the mouse is dead. Confirm the absence of respiration, heartbeat, as well as a lack of pain withdrawal and corneal reflexes. Also observe for pale blue/white coloring of extremities and darkening of the blood vessels over the cortex.

REPRESENTATIVE RESULTS:

To study the interactions between cortical areas within a single hemisphere, we used a large craniotomy extending across the sagittal sinus and 5 – 6 mm lateral. This cranial window included primary (motor, somatosensory, visual, auditory), secondary (motor, visual), and association (retrosplenial, cingulate, parietal association) cortices of right brain hemispheres (Figure 3A). For this work we used voltage sensitive dye (VSD) imaging, which reflects changes in the membrane potential[3]. This protocol would also be useful for other extrinsic (e.g., calcium[14] and glutamate[15] imaging) or intrinsic imaging experiments. When stimulating the hindlimb, forelimb, whiskers, visual, or auditory system of lightly anesthetized mice using urethane, we observed consensus patterns of cortical depolarization (Figure 3B). Consistent with previous studies[5,16-19], we found that brief tactile stimulation of C2 barrel cortex led to activation of primary somatosensory areas, as well as "islands" of responses within functionally related areas. For example, the primary motor cortex (M1) or secondary representation of somatosensory cortex (S2; Figure 3Bi). A single 1 ms tone pip (25 kHz) stimulation led to the activation of primary auditory cortex (A1) approximately 20 ms after auditory stimulation (Figure 3Bii). Over the next few milliseconds, the depolarization spread across the auditory cortex and passed to neighboring secondary somatosensory cortex. Approximately 25 ms after tone onset, a secondary cortical depolarization would emerge, located 1.0 ± 0.2 mm medial and 1.9 ± 0.1 mm posterior relative to bregma (n = 9 mice). This is approximately the location of the parietal association area (ptA). The VSD signal then propagated to the midline area where other cortical association areas are located including retrosplenial (RS) and cingulate cortex (CG). Therefore, auditory stimulation led to the activation of two separate focal areas, from which traveling waves of VSD depolarization spread to a larger area within midline cortex. Focal stimulation of the contralateral eye with a 1 ms green LED pulse, led to activation of the primary visual cortex within 40 ms (Figure 3Bv). This primary activation of the visual cortex was followed by: (1) Spatial expansion of VSD depolarization into neighboring areas located medially, laterally, and anterior to the initial activated area; (2) Depolarization of a second medial cortical region approximately 50 ms after stimulation (n = 8 experiments) located along sagittal suture. This was similar to sensory stimulations of forelimb (Figure 3Biii), hindlimb (Figure 3Biv), C2 whisker, or audition. Evoked VSDI responses from sensory stimulation of the forelimb, hindlimb or audition initially activated

the respective primary sensory cortices, followed by an anisotropic spread of activity, as well as midline activation of cortex around 20 – 40 ms after stimulation. This result was similar to responses from visual and whisker stimulation. The propagation of sensory-evoked activity along these midline routes and frequent activation of same regions by spontaneous activity[6] may suggest that these regions are the central hub of the connection core of the mouse cortex, in which sensory information may integrate with spontaneous cortical activity.

Figure 1. Surgical setup and preparation.
(A) Mouse head is shaved, cleansed, rotated approximately 30° for lateral exposure, and secured with the blunt end of ear bars. Isoflurane anesthetic is delivered via a nose piece and teeth holder. (B) Close up showing skin and periosteum removed from the parietal skull plate, the temporal muscle is untouched. (C) The temporal muscle is removed exposing the temporal plate and squamosal bone, note the superficial vein is undamaged. (D) Prior to fixation, the head plate is positioned into the correct location with wax.

Figure 2. Step-by-step surgical procedure.
(A) The head-plate is attached to the skull with cyanoacrylic glue at the anterior and posterior locations. Note the location of bregma (black piece of triangular tape). (B) The cranial window is prepared by applying thickened dental cement between the head-plate and skull. Note that bregma and the squamosal landmarks remain visible. (C) Following drying of cement, brain buffer is added to soften the skull and to prevent adhesion of the dura. A rolled tissue will help removing brain buffer prior to drilling. (D) The edges of the craniotomy have been scored. Note the vasculature is more easily seen through the thinned bone near the dental cement edges. (E) The parietal and temporal skull plates have been removed and the dura is visible. Note blemishes of blood on the dura from minor bleeding, which is normal. Careful examination under 2 – 4 x magnification will reveal two layers of blood vessels, one in the dura and the other in the pia. (F) The dura is removed revealing a pristine cortex. Pial vasculature is bright red with no blemishes present. Note the stray pieces of white colored dura at the edges of the cranial window. In this example there was a minor dural bleed at the posterior portion of the cranial window, which quickly clotted.

Figure 3. Unique and consensus activation patterns during multiple forms of sensory stimulation.
(A) Schematic of the unilateral craniotomy showing the imaged cortical regions. (B) Photomicrograph of the wide unilateral craniotomy with bregma indicated by a white circle in each image. Patterns of cortical activation are shown in a mouse anesthetized with isoflurane (0.5%) after (i) stimulation (stim) of the contralateral C2 whisker, (ii) auditory stimulation, (iii) contralateral forelimb stimulation, (iv) contralateral hindlimb stimulation and (v) visual stimulation of the contralateral eye with a light-emitting diode (LED). There was midline activation after all forms of sensory stimulation (white arrows) at 10 – 25 ms after primary sensory cortex activation. The responses are the mean of 20 trials. The image second from the left in the second row (ii) indicates the anterior (A), posterior (P), medial (M) and lateral (L) directions. Modified with permission from Mohajerani, *et al.,* 2013.

DISCUSSION:

This innovative protocol for a large cranial window enables simultaneous imaging over the temporal and parietal areas of the cerebral cortex. Combined with optical imaging, it can help to reveal neural dynamics within cortical areas during spontaneous and stimulus-induced activity. This expansive craniotomy also exposes a large extension of the cortical vasculature network, including the proximal end of the middle cerebral artery (MCA), enabling *in vivo* imaging of blood flow and direct manipulation of lateral vessels for ischemic models. This technique will be of great use for recently developed lines of mice expressing voltage and calcium indicator proteins[20]. These mice offer the practical advantage of bypassing the need for incubating voltage sensitive dyes on the cortex. These extrinsic dyes take time to adequately penetrate the brain tissue (~60 – 90 min) and are limited by their mild toxicity. Large craniotomies have also been previously utilized to study the developing rat brain with VSDI[11]. Newborn rats have a much larger head and is comparable in size with adult mice. This affords researchers with a unique opportunity to study developmental problems in neuroscience, albeit not with transgenic mice.

The main limitations of this method are the inability for chronic experiments. The curvature of the skull makes the drilling process more challenging and time consuming than smaller craniotomies. For this large craniotomy, it is vital to position the head with the central suture and squamosal landmarks to be parallel to the focusing plane of the lens. While some distortion of the brain is expected from the curvature of the brain, these are overcome by focusing into the superficial layers of the cortex. This problem is further alleviated by obtaining numerous repetitions of stimulation and averaging. In summary, our large craniotomy technique is widely applicable for the study of current problems in neurobiology.

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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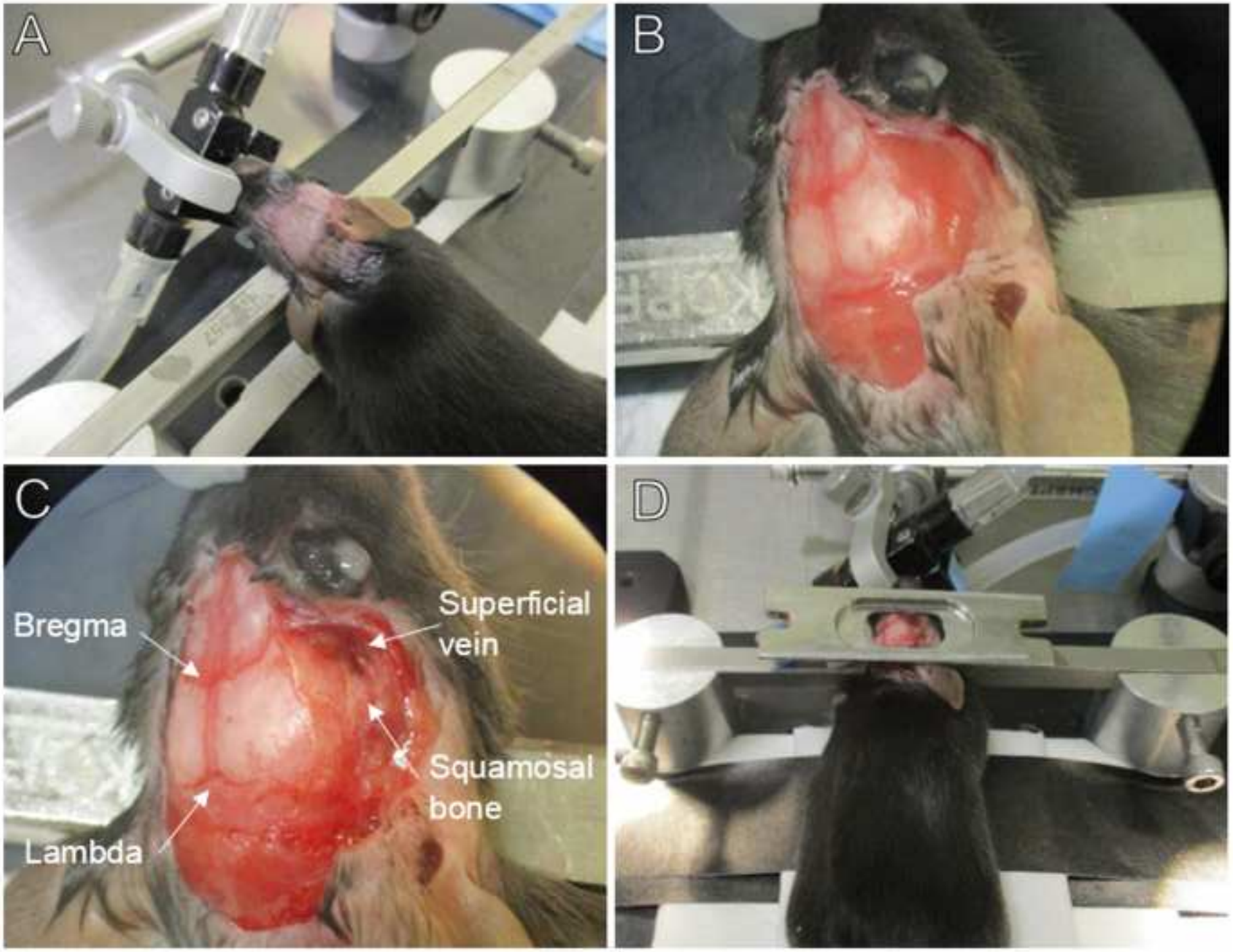

Figure 2

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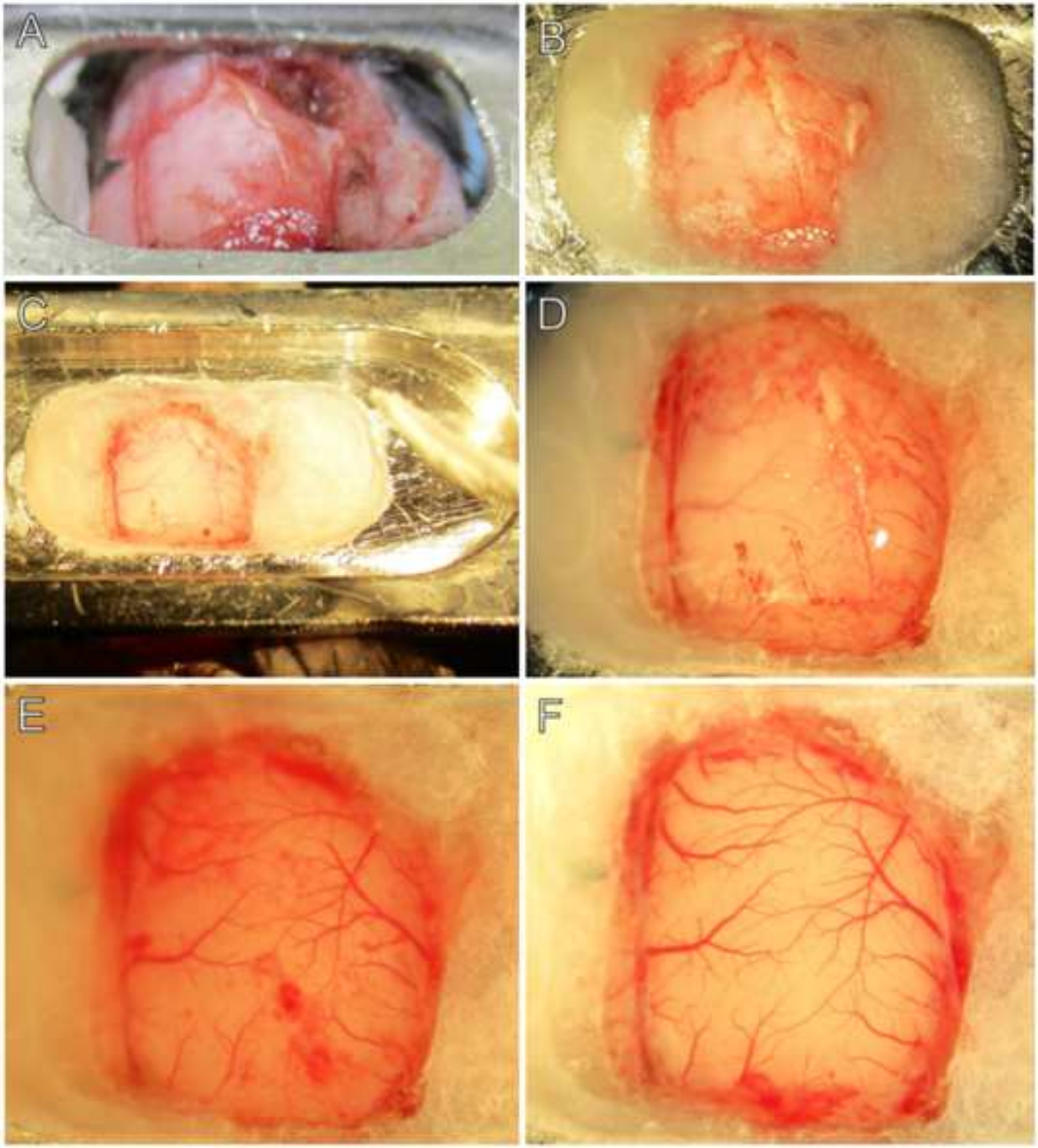

Figure 3

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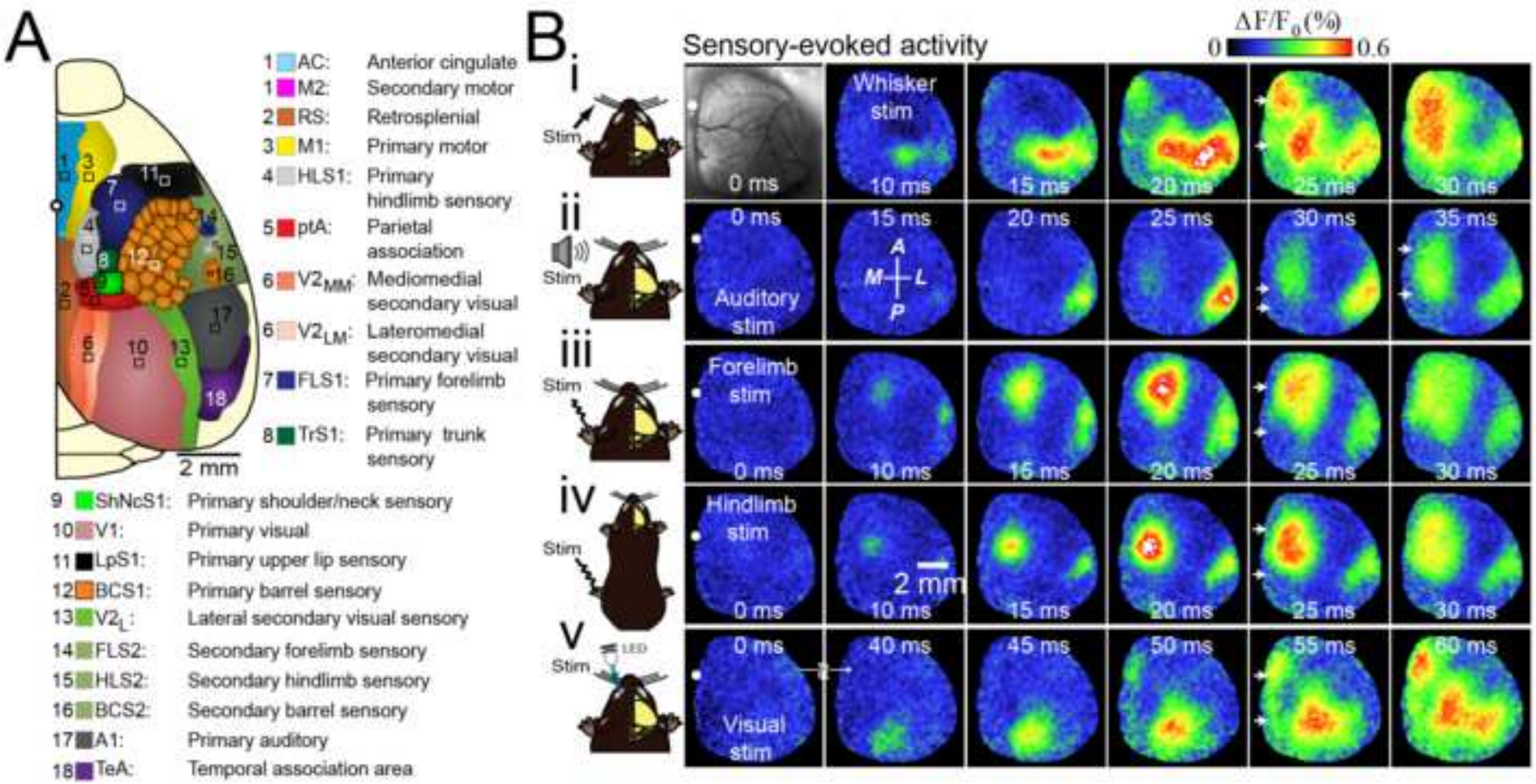

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment Table of Specific Materials_14Oct2014.xlsx

Name of Material/ Equipment
Heating Pad
Fine Scissors
Forceps
Spring scissors
Jet tooth shade powder
Jet tooth shade liquid
Drill Heads - Carbide Burs FG 1/4 389
Agarose Powder
Gelfoam
Isoflurane
Lidocaine 2% Epinephrine
Dexamethazone 5 mg/mL
Butyl cyanoacrylate glue (VetBond)

Company	Catalog Number
FHC	40-90-2
Fine Science Tools	14058-09
Fine Science Tools	11251-35
Fine Science Tools	15000-00, 15000-10
LANG Dental	Jet Tooth Shade Powder
LANG Dental	Jet Tooth Shade Liquid
Midwest Dental	385201
Sigma-Aldrich	A9793
Sinclair Dental Canada	Pfizer Gelfoam
Western Drug Distribution Centre Ltd	124125
Western Drug Distribution Centre Ltd	125299
Western Drug Distribution Centre Ltd	125231
Western Drug Distribution Centre Ltd	12612

Comments/Description

2 or more pairs are recommended
1 pair should be designated for dura removal
to be mixed with the Jet Liquid
to be mixed wihth the Jet Powder

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Author(s): Sunny Wang, Richard Kline, Majid Mahrajerani

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Institution: University of Lethbridge

Article Title: Making an extended lateral craniotomy for in vivo wide field optical imaging

Signature: [signature] Date: Aug 26, 2014

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MS # (internal use):

01 Nov 2016

Dr. Sephorah Zaman
Science Editor, JoVE

Dear Editor,

We are delighted by the positive response to our manuscript MS#: JoVE52642 entitled "A large lateral craniotomy procedure for mesoscale wide-field optical imaging of brain activity" by Michael Kyweriga, Jianjun Sun, Sunny Wang, Richard Kline, and Majid H. Mohajerani. We have now revised the manuscript along the lines suggested by the Reviewers. We thank the Reviewers and Editor for their constructive comments, which we feel, have led to a much stronger manuscript. We describe our response to each of the Reviewer/Editor's points below and we feel have been able to address all of their concerns/suggestions. We outline the reviewer's comments in italic black below, our answers in red and edits to the manuscript in blue font. The previous authors Wang and Kline have left my lab. Therefore, in order to complete the manuscript, I enlisted help from Drs Kyweriga and Sun to complete the report. The present authors have made substantial changes to the text in an effort to clarify our meaning, reduce redundancies, add details, and remove inaccuracies. We have tried to highlight all of these changes to the best of our ability.

On behalf of the authors,

Majid H. Mohajerani Ph.D.

University of Lethbridge

Editorial comments:

1) *All of your previous revisions have been incorporated into the most recent version of the manuscript. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions.*

2) *The instruction to administer anesthesia should occur before the instruction to monitor mouse for consistent anesthesia. Therefore, editor moved step 1.3 to before step 1.2. Please maintain this change moving forward.*

   We are fine with suggested changes.

3) *The length of your protocol section following revisions exceeded our 3 page limit for filmable content. Editor added highlighting to the protocol section to indicate steps that will be filmed. JoVE is unable to film steps involving anesthesia and euthanasia therefore steps 1.2, 1.3 and section 6 were omitted from the highlighting.*

4) *Prior to peer review, the highlighted portion of your protocol is close to our 2.75 page highlighting limit. If, in response to peer review, additional details are added to the protocol, please adjust the highlighting to identify a total of 2.75 pages of protocol text (which includes sub-headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. The highlighting should include complete statements and not portions of sentences. See JoVE's instructions for authors for more clarification.*

   We did adjust the highlighting in yellow to identify a total of 2.75 pages of protocol text.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This paper by Kyweriga et al. documents a lateral craniotomy procedure that is useful for in vivo imaging in mice. The authors describe the surgical steps taken to perform the craniotomy. At present, not enough detail is included in the figures in particular to make this a user-friendly protocol.

We agree. We've greatly expanded Figures 1 & 2 and added comments and Notes. See below for more details.

Major Concerns:

1. *A troubleshooting section should be included that deals with quality control, how to assess if things have gone wrong and to what extent they have gone wrong, and when to decide the prep is unusable. Documentation of percent successful surgeries to expect would be useful. Troubleshooting other steps, including if there is leakage at the head plate, bleeding at various stages of the surgery including dura removal versus initial drilling, would be useful.*

We have now added multiple notes to sections 1-5 to help with troubleshooting.

(Page 3, line 41) Note: Prolonged use of isoflurane can cause dehydration. We recommend subcutaneous injections of saline, 0.1 mL per 10 g body weight, every 1 – 2 hrs. When adequately hydrated, the mouse will urinate once every 1 – 2 hours.

(Page 4, line 16): Rotate the mouse's head towards the left approximately 30° to expose the right lateral side of the head and secure the mouse's head with the blunt end of ear bars (Figure 1A).

(Page 5, line 6): Note: If there is periosteum left on the skull, or if the skull is not dry before gluing on the head plate, the plate will likely detach. If this happens, gently remove the head plate and start over. Bleeding may occur during this process, allow a few minutes for it to clot, then gently remove. This process is not recommended to be repeated more than twice.

(Page 5, line 21) Note: The surgeon must remain diligent during removal of the skull and dura to avoid unnecessary complications. Troubleshooting steps are included should complications arise.

(Page 5, line 34) Note: Once the cement is set, briefly fill the well with brain buffer and allow to soak for 3 – 5 minutes. Use a rolled Kim wipe to remove brain buffer before drilling (Figure 2C).

(Page 6, line 1): Note: The skull will quickly air dry after application of the wet gel foam, if more drying is needed, use cotton tip swabs.

Caution: The skull is uneven in thickness. For example, the parietal-temporal ridge is the thickest area, while skull regions near the midline and squamosal landmarks are relatively thin.

(Page 6, line 11): If blood rushes out of an area, it may suggest that the dura has been damaged; if this is the case, place a semi-wet gel foam over the area and try to soak up the blood while gently applying pressure to the gel foam with a cotton tip swab

(Page 6, line 20): If a portion of the skull remains stubbornly attached, a #11 scalpel blade can be used to gently score the skull. Take extreme care to not puncture the blade through the skull and into the brain.

(Page 6, line 26): If a small amount of bleeding occurs during the skull removal process, remove the buffer with a transfer pipette or syringe, then replace with new buffer.

(Page 6, line 37): Note: The bleeding usually comes from the edge of the bone or the surface of the dura; both cases are normal and bleeding will quickly stop if no major blood vessels are damaged. If bleeding continues, blood may fill the entire window, forming a clot sheet over the imaging area. To remove the clot sheet, carefully pick up pieces of clotted blood from the imaging area while leaving the blood clot intact around where the source of the bleeding was. Take care to not remove the blood clot from the bleed source as this may cause even more blood loss. Irrigate the surface of the brain with brain buffer to wash away any blood. Take care to avoid touching the delicate brain tissue or adding foreign material to the brain; repeat until bleeding has stopped, for approximately 2 – 5 min (Figure 2E).

(Page 7, line 6): Note: Dura removal requires extreme care, and may take over 15 min.

(Page 7, line 19): Caution: Some areas of the dura are attached to small arterioles on the surface of the brain (e.g., near the midline proximal to the parietal association area), and removal of such can rupture the arteriole. In such cases it

may be better to leave a small piece of dura intact over top of the arteriole. The VSD may not penetrate that small area, but this is preferred to having major bleeding.

(Page 7, line 25): Note: The brain surface should be fixed in agarose as soon as possible to minimize movement from pulsation and to prevent further swelling (see step 5).

(Page 7, line 36): Note: Running cool water over the outside of the agarose container may speed the cooling process. Gently stir continuously to ensure no bubbles or particulates are present.

We added a new bullet point to section 6 discussing surgical endpoints (how to decide when to terminate the experiment). We also added the number of practice surgeries required and the expected success rate once trained (Page 7, line 45):

In our experience, this procedure takes experienced surgeons at least 3 – 4 practice surgeries to attain greater than 90% success rate. Less experienced surgeons may require even more practice.

6. *The figures need a great deal more detail. As presented, it is not possible for the reader to understand how the craniotomy is performed. Pictures of each step of the process documented in the text, including skull removal and dura removal, should be included. As it stands, the single picture of the intact skull before drilling does little to show the craniotomy procedure, which is the main point of the protocol. I would recommend combining figures 1 and 2, and including a new figure 2 that demonstrates all the steps with detailed pictures.*

We appreciate the reviewer's concern and have completely overhauled figures 1 and 2 with step-by-step photos.

Minor Concerns:

6. *The long abstract should include a statement about what methods are unique to this preparation rather than a standard craniotomy.*

We have revised the long abstract to highlight difficulties due to the size of the craniotomy, retraction of the temporal muscle, and head tilt (Page 2, line 8):

This protocol introduces a method for creating a substantially larger 7 mm x 6 mm cranial window exposing most of a cerebral hemisphere over the mouse temporal and parietal cortices (e.g., 5ergma 2.5 to – 4.5 mm, lateral 0 – 6 mm). To perform this surgery, the head must be tilted approximately 30 degrees and a majority of the temporal muscle must be retracted. Due to the large amount of bone removal, this procedure is intended only for acute experiments with the animal anesthetized throughout the surgery.

6. Line 103, Is it clear that dexamethasone reduces swelling acutely? Some labs administer 24 h prior to surgery.

As dexamethasone is injected at the beginning of surgery, and the surgeries are roughly 4 hours long, with an additional 4 hours of imaging, using dexamethasone does help with swelling in our experiments.

3. Line 179. For how long is the brain irrigated?

We revised the manuscript and added the following section (Page 6, line 42):

> Irrigate the surface of the brain with brain buffer to wash away any blood. Take care to avoid touching the delicate brain tissue or adding foreign material to the brain; repeat until bleeding has stopped, for approximately 2 – 5 minutes (Figure 2E).

4. Line 162. More details needed on skull removal process. What if bleeding occurs at various stages (see comment about figures).

We now added various Notes to the skull removal process to help with troubleshooting. (See Major concerns 1)

5. Line 193. How long does it usually take to remove dura?

Added the below to the dura removal process (Page 7, line 6):

> Note: Dura removal requires extreme care, and may take over 15 min.

6. Discussion could include treatment of the issue of different focal depths introduced by brain curvature. How is this accounted for? What is the NA of the lens used and is there any reason to believe medial cortex is in focus and lateral cortex is out of focus, for example?

We now have added the following to the discussion (Page 9, line 27):

> The main limitations of this method are the inability for chronic experiments. The curvature of the skull also makes the drilling process more challenging and time consuming than smaller craniotomies. For this large craniotomy it is vital to position the head with the central suture and squamosal landmarks to be parallel to the focusing plane of the lens. While some distortion of the brain is expected from the curvature of the brain, these are overcome by focusing into the superficial layers of the brain. This problem is further alleviated by obtaining numerous repetitions of stimulation and averaging. In summary, our large craniotomy technique is widely applicable for the study of current problems in neurobiology.

Reviewer #2:

Manuscript Summary:

The manuscript describes how to conduct an extended lateral craniotomy. I would suggest to accept this proposal after minor revisions.

Major Concerns:

N/A

Minor Concerns:

Abstract
1. A craniotomy is the opening of the skull and is not connected to any brain region or imaging or electrophysiology. Even our ancestors many thousand years ago conducted craniotomies … but did not image for sure ... Please correct.

We now have made minor corrections to rephrase and clarify our meaning of the abstract.

2. Maybe this technique could also be used for cerebellar cortex or include olfactory bulb or both hemispheres? Please comment.

While it is possible to image the cerebellar cortex and olfactory bulbs, our current head plate setup limits our imaging range. To do a bilateral window of this size would require a different protocol as there would be problems such as cross suture drilling, curved bilateral bone removal (would have to be done quickly to reduce bilateral swelling), dams for dental cement placement (as there is no bone to place the dental cement on, on either side), as well as an imaging setup which enables a 180 degree arc-rotation.

3. This procedure should only be used for experiments in anesthetized animals. The animal should never wake up. Please state clearly already in the abstract.

We have added the below to the abstract (Page 2, line 12):

> Due to the large amount of bone removal, this procedure is intended only for acute experiments with the animal anesthetized throughout surgery.

Introduction
Please also cite a CC Petersen paper where they expose very wide brain areas.

We now have cited Aronoff et al., 2012.

Protocol
4. If isoflurane is used for longer periods saline should be injected subcutaneously to avoid dehydration. Please add.

We now have added the following text (Page 3, line 41):

Note: Prolonged use of isoflurane can cause dehydration. We recommend subcutaneous injections of saline, 0.1 mL per 10 g body weight, every 1 – 2 h. When adequately hydrated, the mouse will urinate once every 1 – 2 h.

5. Hair removal cream can be a very good alternative addition to get rid of hair. Maybe add.

Hair removal cream is not recommended by our veterinary staff due to previous irritation issues and we are thus not experienced with it.

6. Many other procedures suggest to use dexamethasone and carprofen (rimadyl). If you have experience please comment on carprofen.

Carprofen is not required due to this being an acute surgery. Our institution uses Metacam as our main NSAID (for Cox inhibition) with Dexamethasone for chronic surgeries, as such, we do not use carprofen.

7. How do you dry the skull? Please add.

The buffer evaporates quite quickly on the skull (within 30 seconds), so nothing in particular needs to be done. If the skull needs to dry faster, we use our dental drill as it has an air outtake. We have added the following text (Page 6, line 1):

Note: The skull will quickly air dry after application of the wet gel foam, if more drying is needed, use cotton tip swaps.

8. In recovery surgeries the cutting of muscles should be always avoided. Why do you not just separate the tendons from the bone? Just a suggestion ... there would be no bleeding and much less pain especially if experiments are done in lightly anesthetized animals.

Transferring this from an acute to chronic surgery may be considered at a later date, but even with just separation of the tendons, the animal will still be overly distressed to maintain proper experimental control in awake animals.

9. During the drilling the surface should be kept wet because of heating and not drying. Please correct.

We also want to stop any drying from happening as the dura has a tendency to stick to a dried skull. We now have added the underlined section in the text (Page 5, line 42):

3.5 Gently trace the drill along the original scoring to deepen it, ensuring the drill does not penetrate through the skull into the brain (Figure 2D). Take turns every few minutes between drilling and dabbing the skull surface with moistened gel foam. This will reduce heating and drying of the skull from mechanical friction and prolonged exposure.

10. What drill speed do you use? What dental drill do you use? What do you do if you break through the bone? Is the bone thickness the same around the craniotomy?

There is no set drilling speed as we use a pneumatic drill at 20 maximum PSI with a foot pedal. A higher drilling speed will drill through the skull faster but have a higher risk of causing damage whilst a slower speed will decrease the chance of over drilling into the dura, but take a lot longer. We added the following parenthetical to step 3.4 (Page 5, line 39):

> Note: A pneumatic drill (set to maximum of 20 PSI), with a FG ¼ burr, and controlled with a variable speed foot pedal should suffice.

What dental drill do you use?

Any stock dental drill which fits the Midwest Regular Carbide Burs FG ¼ will do (see above).

What do you do if you break through the bone?

The bone should never be broken through. Nevertheless we added the below for troubleshooting (Page 6, line 12):

> Note: If blood rushes out of an area, it may suggest that the dura has been damaged; if this is the case, place a semi-wet gel foam over the area and try to soak up the blood while gently applying pressure to the gel foam with a cotton tip swab.

We have added the following text to section 6 (Page 8, line 1):

> During the craniotomy or durotomy, the brain may sustain damage, such as if the drill punches through the bone into the brain.

Is the bone thickness the same around the craniotomy?

The bone does not have even thickness. We now have added the below to 3.5. (Page 6, line 4):

> Caution: The skull is uneven in thickness. For example, the parietal-temporal ridge is the thickest area, while skull regions near the midline and squamosal landmarks are relatively thin.

11. Do you need cracks in the bone before stopping with drilling? What typical magnification are you using during this process?

The skull should never "crack" as it is not that brittle. Make sure to moisten the brain often. We use 0.7 – 4.5 x magnification. We have added the following text to section 2.1 (Page 4, line 29):

> Nearly all of these procedures should be performed while viewing the skull under a dissecting microscope (We use an Olympus SZ61 with 0.7 – 4.5 x power, depending on the situation).

12. What instruments do you use for prying the skull loose from the dura?

We use forceps for prying the skull loose from the dura. The following text is added to 3.10 (Page 6, line 29):

> Once the bone is loose and "floating" on the dura, firmly grip the bone with forceps and lift the bone from the dura. Keep the bone horizontal, otherwise the opposite edge may sink into the tissue.

13. When irrigating the brain, add that the brain should not be pressed.

We now have added the following text to the manuscript (Page 6, line 42):

> Irrigate the surface of the brain with brain buffer to wash away any blood. Take care to avoid touching the delicate brain tissue or adding foreign material to the brain; repeat until bleeding has stopped, for approximately 2 – 5 min (Figure 2E).

14. I guess the brain is pulsating after opening the skull. If yes, please mention.

No significant pulsation is visible, although the brain will rise after dura removal, it is quickly fixed with agarose after exposure to minimize movement.

15. After removal of the dura, the brain pulsation should be really serious. Please mention.

There should be no problem with pulsation if the surgeon moves quickly. Added the below for urgency's sake (Page 7, line 25):

> Note: The brain surface should be fixed in agarose as soon as possible to minimize movement from pulsation and to prevent further swelling (see step 5).

16. line 216: should read "... has passed away."

We rephrased section 6.4 as follows (Page 8, line 15):

> 6.3 If perfusion is not required, wait for a minimum of 5 minutes then verify that the mouse is dead: confirm the absence of respiration, heartbeat, as well as a lack of pain withdrawal and corneal reflexes. Also observe for pale blue/white coloring of extremities and darkening of the blood vessels over the cortex.

Representative Results:

17. line 232: should read "... led to the ..."

Added the following section to the text (Page 8, line 41):

> Therefore, auditory stimulation led to the activation of two separate focal areas, from which traveling waves of VSD depolarization spread to a larger area within midline cortex.

18. line 234: should read "... and passed ..."

Added the following section to the manuscript (Page 8, line 35):

> Over the next few milliseconds, the depolarization spread across the auditory cortex and passed to neighboring secondary somatosensory cortex.

Discussion:
19. In the limitations clearly state (again) that this procedure should be not used for awake experiments.

We have clearly stated in the abstract that this procedure should be only used in an anesthetized animal.

20. I guess the movement artifacts are too large for two-photon microscopy. Please comment on this somewhere in the discussion.

Two-photon microscopy can be certainly used (see Sofroniew et al., Elife 2016), although that require certain surgical and imaging consideration.

21. Can this craniotomy be used for intrinsic imaging?

Absolutely! We added this technique to the long abstract, introduction, and discussion.

22. How long do you typically use this craniotomy for imaging? What is the limit? Do you do any special things during imaging to maintain the preparation?

We can image the neural activity up to 5 to 6 hours, as long as the animal's vital signs are well maintained and it shows no sign of distress.

Figures:
23. Please add reference for Figure 3A.

We have added the reference.

24. Please specify your microscope.

We have added this information to section 2.1 (Page 4, line 29).

Reviewer #3:

Manuscript Summary:

This manuscript describes a surgical method to make a large craniotomy over one cranial hemisphere in mice. This method is useful for terminal in vivo imaging of large areas of cortex. Previously this method was used in conjunction with voltage sensitive dye imaging.

Major Concerns:

I have no major concerns. The described methods do not stray too far from other windowing methods and therefore do not raise any concerns.

Minor Concerns:

Many investigators have stopped using ear bars in mice due to the difficulty of placing ear bars and the potential for fracturing cranial bones. The alternative approach is to do the initial approach without the head fixed, glue on the plate, and then fix the plate to a post for drilling the craniotomy. This alternative approach should be considered, but making this change is not required, and surely the method described will work, especially when done carefully.

We mention in section 1.8 that we use the blunt end of the ear bars to assist in positioning the head (Page 4, line 17):

> 1.8. Rotate the mouse's head towards the left approximately 30° to expose the right lateral side of the head and secure the mouse's head with the blunt end of ear bars (Figure 1A).

Please describe the brain buffer solution. Perhaps you can provide a reference to an artificial CSF solution.

We have added section 1.10 to detail the brain buffer we use:

> Ensure there is plenty of brain buffer on hand (at least 50 mL). Our solution is comprised of 134 mM sodium chloride, 5.4 mM potassium, 1 mM magnesium chloride hexahydrate, 1.8 mM calcium chloride dihydrate, and 5 mM HEPES sodium, pH balanced with 5 M hydrogen chloride.

Should reference 5 be cited for figure 3a? Are any permissions required?

We have properly cited that figure (modified with permission from Mohajerani et al., 2013).

Please explain why you should wait 5 minutes before removing the skull in section 3.5.

From our experience, the skull becomes softer after immersing in buffer for a while, making the bone removal process much easier. This step is also crucial to make sure that the dura does not stick to the skull during removal as the dura is more likely to stick to a dried skull. We have added the following text to the manuscript (Page 6, line 15):

> 3.7. Wait for at least 5 min before skull removal to soften the bone and to reduce the chance of the dura sticking to the bone, making the skull removal process easier.

Additional Comments to Authors:

This craniotomy method could potentially be used with broad field calcium imaging, intrinsic optical signal imaging, and other methods. The authors should consider adding a paragraph in the discussion about other imaging methods that would be well paired with this cranial windowing method.

Thank you for the suggestion, we have added these ideas to our discussion.

Reviewer #4:

Manuscript Summary:

The article provides a technique for an extended cranial window covering the medial and lateral aspects of the sensory-motor cortices. The authors used this preparation to explore the propagation of spontaneous and evoked activity between distant areas using voltage sensitive dye imaging

Major Concerns:

The illustration of the resulting craniotomy in the figures is scarce (only a thumbnail in figure 3). A close up on the cranial window to be able to visualize finer features of the cortex such as blood vessels is missing. A full figure dedicated to the illustration of the window preparation should be added.

Other reviewers had the same suggestions. We have created two new figures 1 & 2 with multiple photos documenting the many details of the surgery.

Minor Concerns:

The craniotomy performed here is very large. How much can the edema be minimized? In the case where the dura is removed, is dexamethasone sufficient to avoid the edema?

The brain will swell after dura removal regardless of what is done. We have added the following text to address the urgency (Page 7, line 25):

> Note: The brain surface should be fixed in agarose as soon as possible to minimize movement from pulsation and to prevent further swelling (see step 5).

Please discuss the feasibility of the equivalent surgery in a rat and its applicability for two-photon imaging. Is the stability of the window strongly affected by the size of the opening?

We have not tried this in rats, but it could be feasible. There would be increased bleeding, but the actual craniotomy process might become easier since it is unlikely to overdrill as they have thicker and stronger skull.

The description of the result of the voltage sensitive dye imaging could be shortened; more troubleshooting details about the surgery itself should be added instead.

We have adjusted the discussion to meet this suggestion.