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Imaging Neural Activity in the Primary Somatosensory Cortex Using Thy1-GCaMP6s Transgenic Mice

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TITLE:

Imaging Neural Activity in the Primary Somatosensory Cortex Using *Thy1*-GCaMP6s Transgenic Mice

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KEYWORDS:

GCaMP6s transgenic mice; *In vivo* imaging; Cranial window; Somatosensory cortex; 2-photon imaging; Calcium dynamics

SHORT ABSTRACT:

We describe an experimental procedure for measuring neuronal activity through dual optical windows above bilateral primary somatosensory cortices (S1) in *Thy1*-GCaMP6s transgenic mice using 2-photon (2P) microscopy *in vivo*.

LONG ABSTRACT:

The mammalian brain exhibits marked symmetry across the sagittal plane. However, detailed description of neural dynamics in symmetric brain regions in adult mammalian animals remains

elusive. In this study, we describe an experimental procedure for measuring calcium dynamics through dual optical windows above bilateral primary somatosensory cortices (S1) in *Thy1-GCaMP6s* transgenic mice using 2-photon (2P) microscopy. This method enables recordings and quantifications of neural activity in bilateral mouse brain regions one at a time in the same experiment for a prolonged period *in vivo*. Key aspects of this method, which can be completed within an hour, include minimally invasive surgery procedures for creating dual optical windows, and the use of 2P imaging. Although we only demonstrate the technique in the S1 area, the method can be applied to other regions of the living brain facilitating the elucidation of structural and functional complexities of brain neural networks.

INTRODUCTION

Calcium and its regulation are essential in mediating multiple physiological processes. Monitoring calcium dynamics is useful for understanding normal brain function as well as pathological conditions of brain disorders¹⁻⁸. Imaging GCaMP6s fluorescence is a powerful tool for quantifying spike numbers, timing, and frequency, as well as levels of synaptic input both *in vitro* and *in vivo*⁹⁻¹¹.

The mammalian brain exhibits marked symmetry across the sagittal plane. Although recent neurological research has shed light on the cortical remodeling and neuronal activity triggered by traumatic brain or spinal cord injury^{6,7}, the roles that intact tissue of symmetrically corresponding regions play in recovery are still obscure.

In this study, we describe experimental procedures for creating bilateral symmetric optical windows for *in vivo* imaging. Using these optical windows, we describe the measuring of calcium dynamics from primary somatosensory cortices (S1) in GCaMP6s transgenic mice using 2P microscopy. In the results section, we also show *in vivo* dendritic morphology at different time points in the S1 region in the EGFP transgenic mice using 2P imaging. The observation method regarding the network dynamics in the bilateral S1 areas is but an initial example of how dual open cranial windows will help neuroscientists to probe local and symmetric information processing in the adult mammalian brain in normal conditions or in different disease models *in vivo*.

PROTOCOL

All surgical and animal handling procedures were performed as approved under the Guide for the Care and Use of Laboratory Animals (National Research Council) and the Guidelines of the Indiana University School of Medicine Institutional Animal Care and Use Committee. The schematic illustration of the 2-photon imaging setup is shown in Figure 1.

1. Preparing the animal for imaging

1.1) Place sterile gauze, cotton swabs, and autoclaved surgical tools in the surgery area (Table 1). Turn on a Micro Bead Sterilizer for intersurgery sterilization of surgical tools.

1.2) Anesthetize the mouse (male or female, 1.5-2.5 months, 20-25 g) by intraperitoneal injection (i.p.) of a mixture of ketamine (17.2 mg/mL), xylazine (0.475 mg/mL) and acepromazine (0.238 mg/mL) (6 μ L/g body weight). Wait until the proper depth of anesthesia is reached when the animal ceases to respond to a toe pinch stimulus and has no corneal reflex. During the surgery, give a booster dose of 1/3 the original dose of the anesthetic cocktail as needed to maintain the original anesthetic state.

1.3) Inject buprenorphine subcutaneously (s.c.; 0.05-0.10 mg/kg) as an analgesic agent prior to surgery. Apply protective ointment to the eyes of the animal to prevent corneal drying and cataract formation.

1.4) Place a GCaMP6s transgenic mouse on a heating pad to prevent hypothermia and cover it with a sterile surgical drape. Stabilize the animal's head with a Head Holding Adaptor for Mice.

1.5) Shave the head over most of the scalp with a double-edge razor blade. Clean the surgical area with a sterile alcohol preparation pad followed by povidone-iodine solution.

2. Chronic cranial window preparation

2.1) Make a rectangular cut of the scalp (2 mm x 3 mm) with a pair of scissors on one side (right) of the skull. Push the skin aside with a cotton swab to create an exposure area of >3 mm in diameter (Figure 3A). Remove the connective tissue attached to the skull by gently scraping the skull with a blunt microsurgical blade (Scalpel Blade #10; Figure 3C).

2.2) Mark the S1 area by gently drawing a circle (3 mm in diameter, center coordination: -1.5 mm from the bregma and 2.5 mm from the midline) on the skull using a dental drill (Figure 2B).

2.3) Perform the same procedure on the left skull (Figure 2B).

2.4) Apply a thin layer of Cyanoacrylate Super Glue to the bone to provide a base for dental cement application.

2.5) Under a dissecting microscope, thin down a circular groove in the skull around the S1 area using a high-speed microdrill (Figure 3D). The purpose is to create a smooth edge for placement of an optical window onto it.

2.6) Repeatedly apply artificial cerebrospinal fluid (ACSF, room temperature) to the skull periodically during the thinning process to facilitate drilling and dissipate heat. Perform the drilling intermittently to avoid friction-induced overheating. Suck away the bone debris with a house vacuum line or a vacuum pump.

2.7) After approximately 2/3 depth of the bone is drilled, slowly and carefully thin the remaining 1/3 bone until a circular piece of the skull (bone flap) is completely free from the surrounding skull.

2.8) Slowly and carefully remove the circular bone flap with a pair of # 5/45 forceps and expose the dura (Figure 2C; Figure 3E).

2.9) Keep the exposed brain region moist with ACSF (Figure 3E). Avoid any damage to pial vessels, since hemorrhage will alter cerebral blood flow, accelerate brain swelling, and severely degrade imaging quality.

2.10) Perform the same procedure on the left (contralateral) side of the skull.

2.11) For assembling the optical window, use optical adhesive to glue and cure between coverglass layers one at a time. If necessary, warm the optical window (50 °C for 12 h) in an incubator to enhance the adhesive bonding. The optical window contains 2 portions: the top portion contains a single round cover glass with a diameter of 5 mm and the bottom portion contains 1-3 round cover glasses with a diameter of 3 mm (Figure 2D).

2.12) Store the optical window in ethanol (70%, vol/vol) and rinse it with sterile saline before use. Before optical window installation, check the optical window for imperfections, including an incorrect amount of optical adhesive or inaccurate alignment under a stereoscope.

2.13) Install the optical window over the craniotomy region (Figure 2D). The top portion of the optical window rests on the skull and the bottom portion of the optical window fits within the craniotomized opening and rests on the dura in the presence of CSF (Figure 2D, E).

2.14) Seal the top portion of the optical window edge to the skull with the Cyanoacrylate Super Glue (Figure 2F; Figure 3F).

2.15) When the Cyanoacrylate Super Glue has dried, apply black dental cement (Dentsply) to cover the glass edge. Apply dental cement to all the exposed skull and wound margins to block light (Figure 2G-H; Figure 3B).

2.16) Perform the same procedure on the left side.

2.17) Allow the animal to recover on the heating pad and return the animal to its home cage for recovery under appropriate monitoring. Provide wet food to facilitate chewing and hydration. To reduce pain, administer buprenorphine s.c. (0.05-2.0 mg/kg) every 8-12 h postinjury for 2 d. Allow the mouse to recover for an additional 7–10 d before imaging.

3. Two-photon imaging

3.1) On the day of the experiment, anesthetize the animal with ketamine and xylazine (doses and maintenance of anesthesia as described above). Clean the cranial window with 75% (vol/vol) ethanol.

3.2) Place the mouse under an imaging set-up consisting of a 2P microscope and the Head Holding Adaptor, resting on a heating pad with its head immobilized through ear bars attached to a mouse head holder.

3.3) Image one side of the optical window at a time. To minimize optical aberrations, make sure that the optical window and right skull are oriented perpendicular to the optical axis of the microscope.

3.4) Take an initial picture of the cranial window with bright field illumination at 4x magnification as a reference map for registration with higher magnification 2P angiographies (Figure 4A1).

3.5) Zoom in on the area of interest using 10x magnification (Figure 4A2).

3.6) Select an area of interest in the S1 area in cortical layers I or II (up to 150 μm below the pial surface). If higher magnification is desired, use a 20x objective.

3.7) Search for a field of view with multiple neurons. Acquire images using a 2P microscope (Figure 4B). Determine the exposure time and excitation light level ($\sim 910\text{ nm}$) based on the depth and expression level of GCaMP6s, with weaker signals requiring longer exposure times and concordantly less time resolution. We typically use $\sim 4\text{ }\mu\text{s}$ per pixel exposure times. Acquire images at frame rates of 3-5 Hz at a resolution of 512×512 pixels.

3.8) Start to record the time series. Store coordinates of every region of interest (ROI) relative to the vascular pattern or to fiducial points in low magnification images. Image the same ROI over time. Perform the same procedure to observe calcium dynamics of the S1 area on the left hemisphere. Calculate changes in fluorescence from each ROI.

3.9) Acquire images either individually or as a time-lapse movie (Figure 4C1-C3, D, E).

3.10) Study cortical blood flow dynamics *in vivo* by imaging fluorescent dextran dyes injected into the tail vein of rodents with 2P microscopy. Use vasculatures as landmarks to recognize and image the same ROI in imaging sessions over long periods.

4. Data processing

4.1) Use ImageJ and Origin for image analysis. Import image sequences with ImageJ (Figure 5A-B). For individual images (or t-series movies), select an ROI within an imaged neuron and 2 separate nearby regions for background measurement (Figure 5C). Acquire total intensity with an ROI manager (Figure 5C).

Note: Figure 6 shows representative calcium images. Calcium transient (green) and blood vessel (red) show at different time points in seconds (Figure 6A-D) in a mouse at 7 d after the initial window installation. The z-projection of a 3 min recording period for red and green channels (Figure 6E) or green channel (Figure 6F) is also shown. A z-projection displays a compressed image of all images in the movie from which a rough and circle boundary (encompassing the object neuron of interest in all frames) is manually selected as well as the selection of the 2 independent nearby background regions (Figure 6G).

4.2) From each ROI, calculate the average fluorescence background FB, $FB = (FB1 + FB2) / 2$, and the fluorescence changes of the soma (F) expressed as $\Delta F / F$, $\Delta F / F = (F - FB) / FB$.

4.3) Perform the peak analysis step by step using functions of ImageJ including Baseline Correction, Peak Finding/Determination, Peak Integration, Peak Fitting, and/or Time-Saving Peak Analysis Features.

REPRESENTATIVE RESULTS

Using the bilateral optical window, we obtained results from 2 separate experiments. The first experiment employed EGFP-expressing mice to image dendritic varicosities. Figure 4C1-3 shows examples of images captured at different time points after optical window implantation. Note that the number and location of the dendritic branches and spines are remarkably stable between the 2 views (Figure 4D, E, z-projection).

The second experiment employed *Thy1*-GCaMP6s transgenic mice to measure the intracellular calcium transient, thus illustrating how calcium physiology can be measured within a single neuron *in vivo* (Figure 6). This technique can be used to record and quantify spontaneous activity in populations of layer V pyramidal neurons located at as deep as $>500 \mu m$ below the pia. Average spontaneous responses over time (calculated as the average responses at each time point) can be calculated across numerous trials. The 2P technique has the advantage of detecting activity simultaneously in large or dispersed neuronal populations over an extended period with little mechanical disturbance to the brain as compared to multielectrode recordings.

To generate an acceptable average measurement, 5-10 trials are recommended. The number of trials will be dependent on the spontaneous responses or inherent variability of the responses to a particular stimulation. If all the steps are followed strictly, as described above, a researcher trained in both thinned-skull cranial window technique and *in vivo* 2P calcium imaging should be able to obtain recordings of 100-200 neurons on both sides from 1-2 animals per day. After the peak analysis, a collection of results such as the real location of the peak, the real amplitude, the area, and the full width at half maximum (FWHM) for each peak can be obtained.

Figure 1. Schematic illustration of the components of a cranial optical window for 2-photon (2P) imaging.

Figure 2. Schematic drawing shows key steps for preparing a cranial window.

A) Under a dissecting microscope, remove the skin over the surgical region using a pair of surgical scissors. B) Use a high-speed microdrill to produce a circular groove (3 mm diameter) on the skull over the S1 area until the bone flap within the groove becomes detached from the surrounding bone. C) Slowly remove the bone flap to expose the underlying dura. D) Assemble the optical window with coverglasses with 2 different diameters. The upper coverglass has a larger diameter (5 mm) and the lower coverglasses (usually 1-3) have a smaller diameter (3 mm). The thickness of the coverglasses to be installed will be dependent on the thickness of the skull. E) Place the coverglass onto the circular opening of the skull to create an optical window. The lower portion of the coverglasses should fit within the cranial opening. The bottom of the coverglasses should gently contact the dura. F) Seal the window edges to the skull with cyanoacrylate glue. G) When the cyanoacrylate dries, apply black dental cement lateral to the glue wall. H) Fill the cranial window with ddH₂O and use water-immersion objectives for imaging.

Figure 3. Creation of bilateral windows on the skull.

A) A photographic image shows 2 cranial optical windows on each side of the skull overlying the S1 area. B) High magnification of the boxed region of image (A) showing bilateral optical windows exposing the underlying dura and blood vessels. Scale bar 680 μ m. C) The exposed skull. D) Creation of a circular groove within which a central bone flap is seen. Scale bar 900 μ m. E) After the removal of the bone flap, the cranial opening was filled with artificial cerebrospinal fluid (ACSF). Scale bar 720 μ m. F) Installation of the optical window over the cranial opening and sealing the edge of the window with super glue. The dura and blood vessels are visible through the optical window. Scale bar 600 μ m.

Figure 4. Calcium and dendritic imaging.

A1) Identify regions of interest using blood vessels (BV) as landmarks in *Thy1-GCaMP6s* mice. A2) Identify the same labeled cells (red arrows) over time using the same landmarks and record. B) Representative Z-projection of calcium signals in neurons (red arrows) of the primary somatosensory cortex (S1). Scale bar 10 μ m. C1-3) Visualization of dendrites (green) and a blood vessel (red) in B6.Cg-Tg(*Thy1-YFP*)HJrs/J at different depths in the layer II of the S1 region at 1 d after optical window installation. D) Z-projection of multiple images at the same region at 1 d. E) Z-projection of multiple images at the same region at 7 d after the optical window installation. Note the remarkable stability of the number and location of adult spines and dendritic branches between 1 d and 7 d after optical window installation. Scale bar in C-E 5 μ m.

Figure 5. Calcium signal tracing.

A, B) Examples of imported t-series movies with ImageJ for data analysis of 2 neurons (neuron 1 and neuron 2, respectively). C) A spreadsheet shows data on illumination intensity from region of interest (ROI; within the neuron and 2 separate nearby regions as background measures). D) Calculation of the peak: $\Delta F/F = (F - FB)/FB$, $FB = (FB1 + FB2)/2$ (the average fluorescence background [FB], and the fluorescence changes of the soma [F] expressed as $\Delta F/F$).

Figure 6. Representative calcium images.

A-D) Calcium transient (green) and blood vessels (red) at different time points in seconds in a mouse at 7 d after the optical window installation. Yellow arrow: a relatively less active neuron. White arrow: a spiking neuron. E, F) Z-projection of a 3 min recording period for the red (blood vessels) and green (calcium) channels (E) and green channel only (F). G) Selected neurons and their adjacent background areas are marked. Scale bar 10 μ m.

DISCUSSION

Two-photon imaging enables the simultaneous measurement of the activity of many cells at a reasonable resolution at up to approximately 800 μ m below the brain surface. We integrated 2P and a dual optical window technique with genetically encoded GCaMP6s to observe calcium dynamics in populations of layer V neuronal somata located >500 μ m below the pia. With an open optical window on each side of the skull, the method enables recordings of neural activity across symmetric mammalian brain regions for prolonged and stable calcium imaging *in vivo*. Key aspects of this method include the performance of minimally invasive surgery to create dual open skull optical windows and 2P imaging of neuronal activity in symmetrical S1 regions.

Examining the network activity in bilateral S1 regions is an example of how dual cranial windows could be used to probe local and symmetric information processing in the adult brain *in vivo*. This method also can be used to study the neuronal activity (e.g. using *Thy1*-GCaMP6s mice) and cortical plasticity B6.Cg-Tg(*Thy1*-YFP)HJrs/J from intact tissue of the corresponding symmetric regions in recovery after deafferentation due to a unilateral CNS injury. The method can also be used to examine cortical activity in response to peripheral stimulation strategies such as optogenetic, electrical or chemical stimulations. Although we only demonstrate the technique in the S1 area, this approach could be employed to investigate activities in other symmetric areas in the living brain such as the layer V neurons of the primary motor cortex (M1). Observations on reorganization of brain regions may provide a neural substrate for adaptive motor behavior, which plays a critical role in postinjury recovery of function. It also allows for chronic measurement of the symmetrical activity of genetically identified cells during behavior in head-fixed mice.

Technically, investigators should pay careful attention to the quality and consistency of dual cranial windows. Thinned-skull cranial window technique¹² is highly recommended for the beginners to practice. Spines are calcium compartments because of their morphology and local influx and extrusion mechanisms. In this study, we used B6.Cg-Tg(*Thy1*-YFP)HJrs/J mice as an example to demonstrate dendritic spine dynamics *in vivo* (Figure 4). An open-skull glass window installation may cause high spine turnover and reactive glial responses after surgery¹². In contrast, with the thinned-skull window technique, spines and dendrites can be well preserved.

Choices of dimensions and thicknesses of optical windows will be dependent on the desired field of view, the skull curvature, skull thickness, and the type of imaging¹³. Insufficient pressure of the optical coverglasses on the dura may cause thickening of the dura, regrowth of

the skull, and increased brain motion. In contrast, excessive pressure of the optical coverglasses may impede cortical blood flow.

For optical window assembly, glue and cure additional layers of coverglasses one at a time with optical adhesive and long-wavelength UV light. To help strengthen the adhesive bonding, warm the fabricated window (50 °C for 12 h) in an incubator. Store the optical window coverglass in 70% (vol/vol) ethanol and, before the surgery, rinse it with sterile saline. The optical window needs to be examined for imperfections (such as an incorrect amount of optical adhesive or inaccurate alignment) under a stereoscope before use to avoid failure. If the optical adhesive is too thin, air spaces may be creased, which can cause optical aberrations or cover glass detachment at implantation. In contrast, too much optical adhesive can cause overflow past the edges of the cranial window.

In summary, here we describe an experimental procedure for measuring calcium dynamics or dendritic morphology through cranial windows from 2 symmetric primary somatosensory cortical regions in *Thy1*-GCaMP6s and *Thy1*-YFP transgenic mice, respectively, using 2P microscopy. This technique may greatly facilitate dissecting the complex structural and functional relationship of neural networks.

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DISCLOSURES:

Authors have nothing to disclose.

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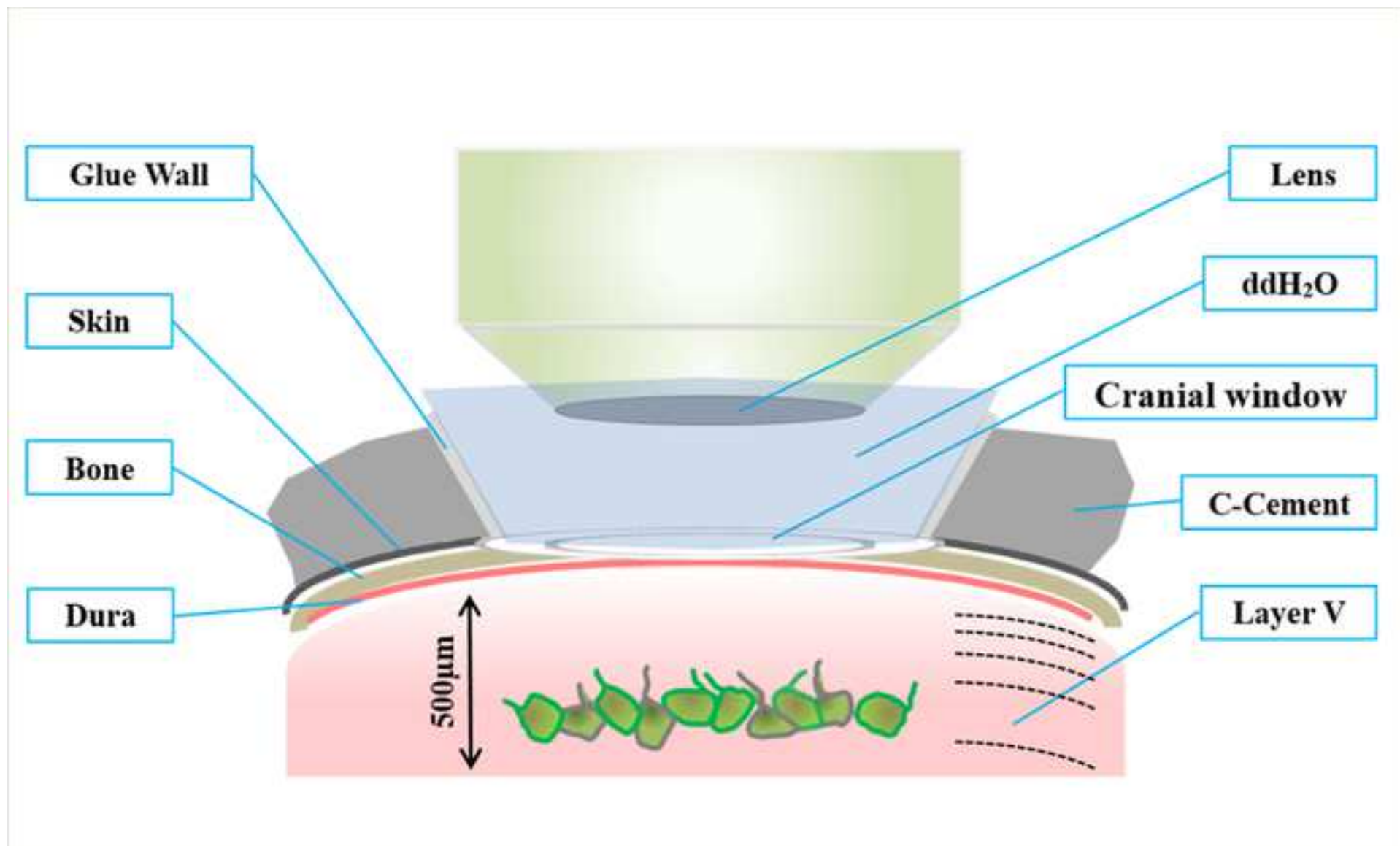


Figure 2

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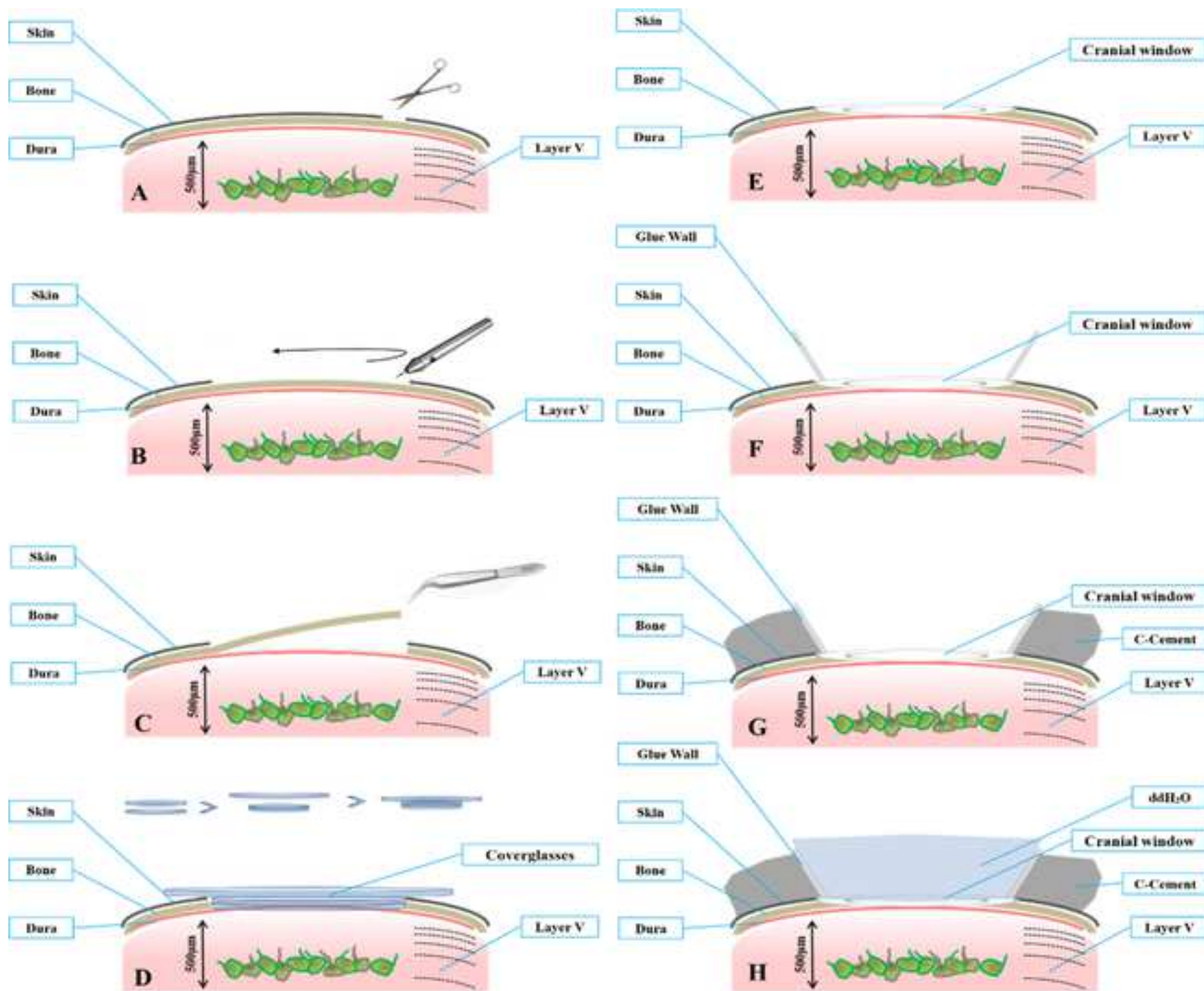


Figure 3

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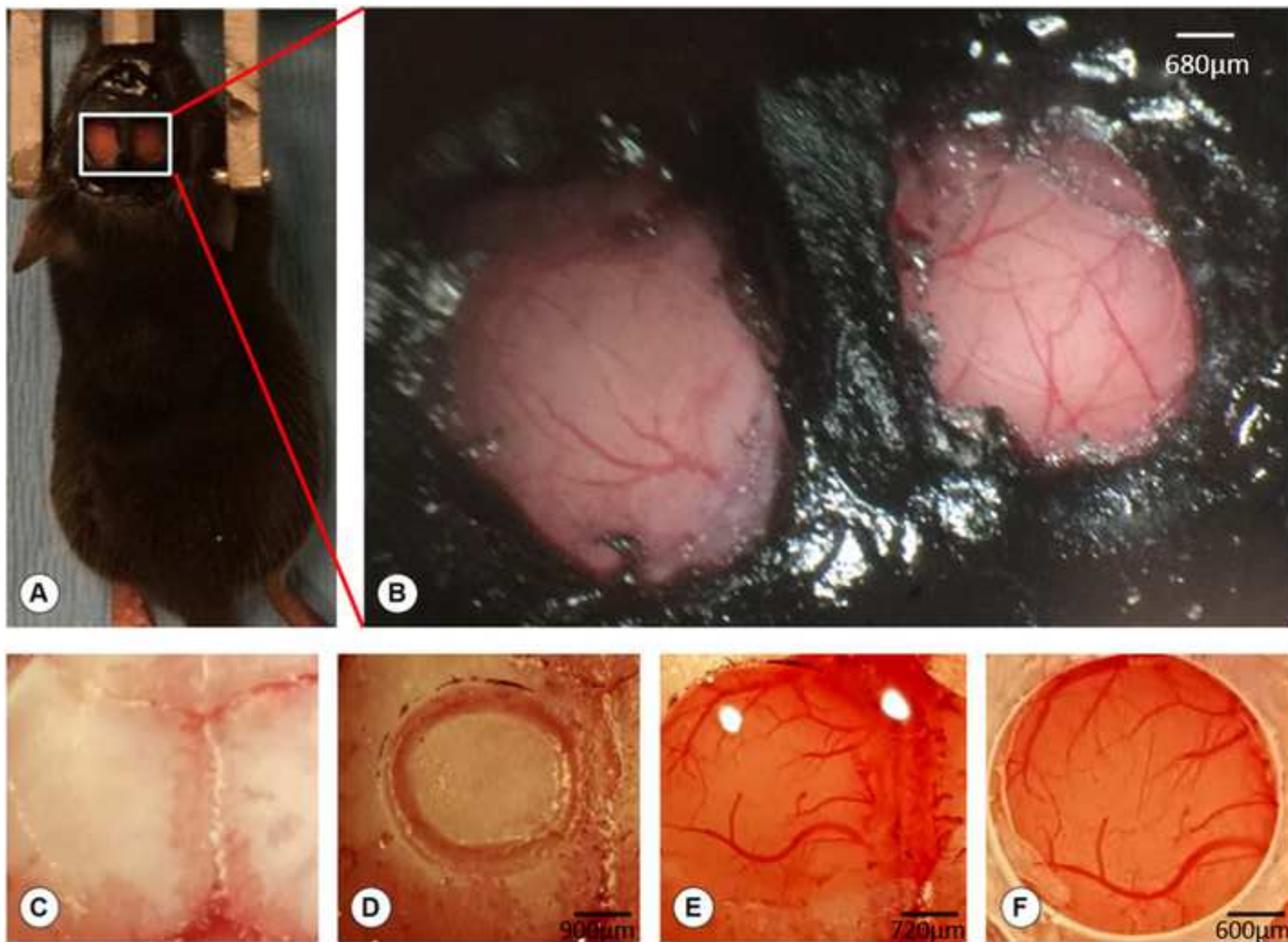


Figure 4

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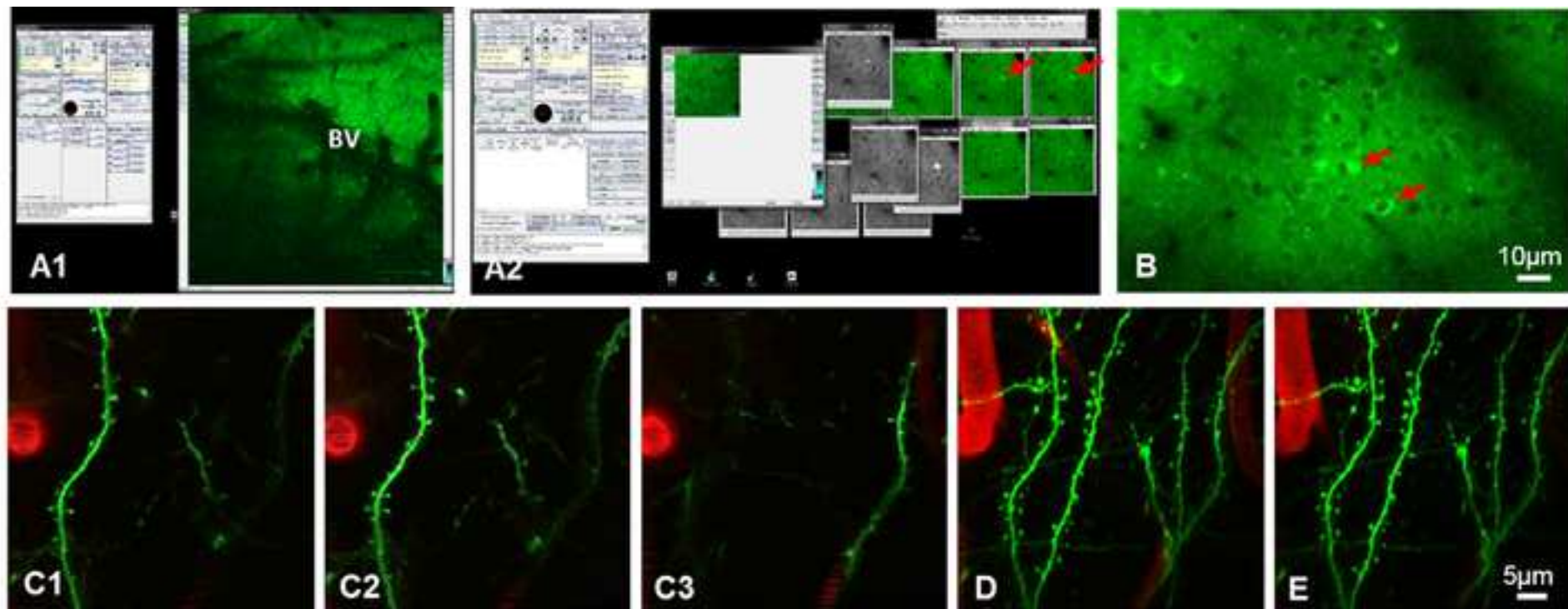
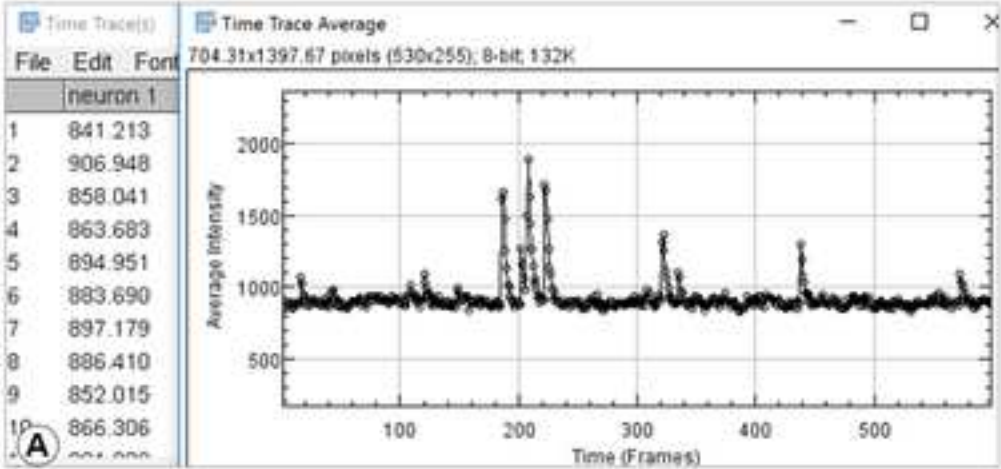


Figure 5

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neuron 1

Frame	time	intensity	FB1	FB2	FB	ΔF
1	3.001	841.213	553.879	519.808	536.8435	0.560901321
2	6.002	906.948	569.333	541.548	555.4405	0.647676724
3	9.003	858.041	572.862	570.441	571.6515	0.500986178
4	12.004	863.683	579.067	563.492	566.2796	0.525188533
5	15.005	894.951	580.208	557.164	568.686	0.573717306
6	18.006	883.690	579.792	545.09	562.441	0.571169243
7	21.007	897.179	572.621	563.785	578.203	0.55166794
8	24.008	886.41	586.562	541.209	564.8855	0.56918526
9	27.009	852.015	565.5	571.887	568.6935	0.459695885
10	30.01	866.306	598.725	570.932	584.8285	0.481299218

C

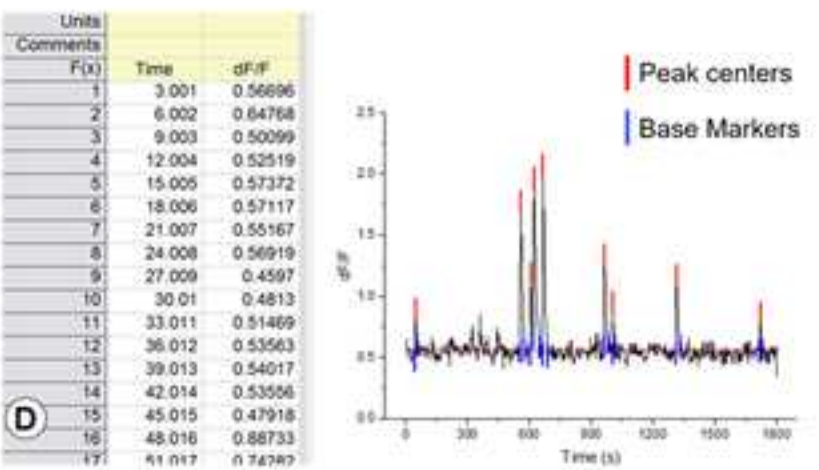
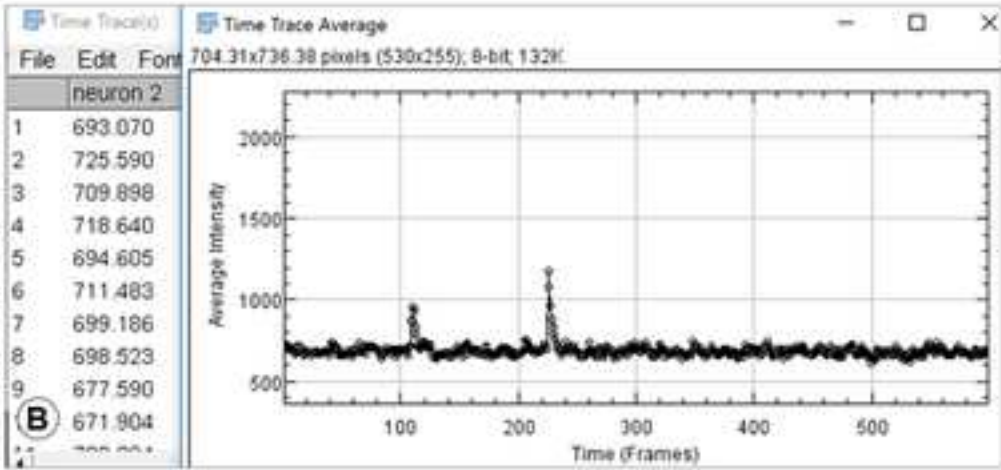
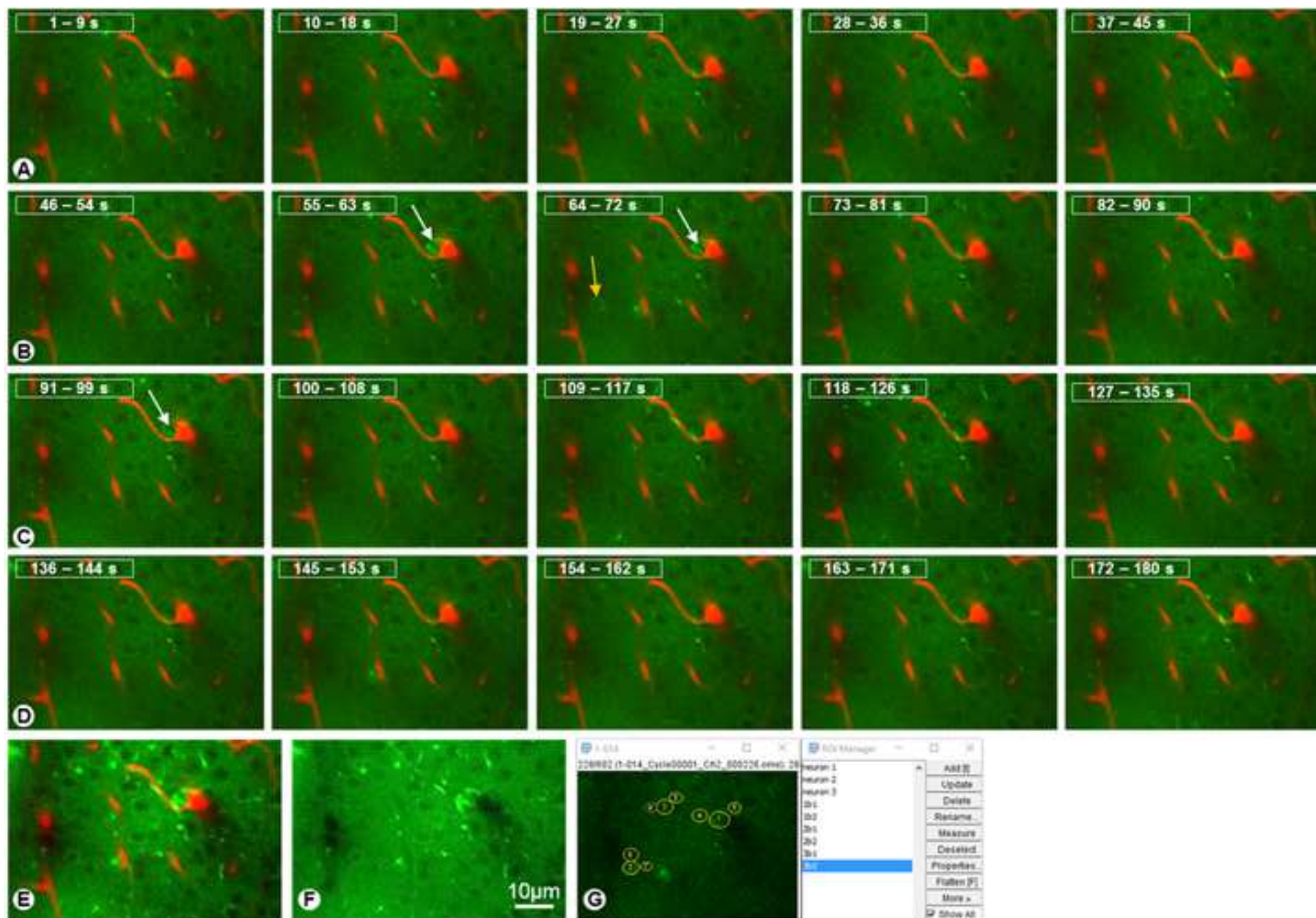


Figure 6

[Click here to download Figure fig 6a.tif](#)



Name	Company	Catalog Number
C57BL/6J-Tg(Thy1-GCaMP6s)GP4.3Dkim/J	Jackson	24275
B6.Cg-Tg(Thy1-EGFP)OJrs/GfngJ.	Jackson	7919
Dumont no. 5 forceps, standard, Dumoxel	Fine Science Tools	11251-30
Dumont no. 5SF forceps	Fine Science Tools	11252-00
Micro Bead Sterilizer	Southern Labware	B1201
Harishige's SG-4N Head Holding Adaptor	Tritech Research	SG-4N
Ideal Micro-Drill Complete Kit	Harvard Apparatus	72-6065
Burrs for Micro Drill	Fine Science Tools	19007-05
Round cover glass, 3 mm	Harvard Biosciences	W4 64-0720
Round cover glass, 5 mm	Harvard Biosciences	W4 64-0700
Norland optical adhesive	Norland Products	7106
Loctite liquid super glue	WB Mason	LOC1647358
Grip cement kit, powder and solvent	Dentsply	675570
Gel foam	Moore Medical	2928
Micro dissecting scissors	Roboz	RS-5841
Artificial cerebrospinal fluid (ASF)	-	-

Comments/Description
Can be any strains made by the genetically-encoded neuronal indicator and effector expressing the green fluorescent calcium indicator, GCaMP, in subsets of excitatory neurons in the cortex.
Can be any strains expressing EGFP in subsets of neurons within specific populations in the cortex; providing a bright, vital Golgi-like stain.
Can be any brand of choice.
Can be any brand of choice.
Can be any brand of choice.
Can be any brand of choice.
Can be any brand of choice.
Can be any brand of choice.
Can be any brand of choice.
Can be any brand of choice.
Can be any brand of choice.
Can be any brand of choice.
Can be any brand of choice. Mix 5 parts of white dental cement powder (Grip Cement) with one part of black Tempera powder paint (to shield from light; some users prefer a 10:1 ratio).
Can be any brand of choice.
Can be any brand of choice.
The ACSF contains 125 mM NaCl, 4.5 mM KCl, 26 mM NaHCO ₃ , 1.25 mM NaH ₂ PO ₄ , 2 mM CaCl ₂ , 1 mM MgCl ₂ and 20 mM glucose. Bubble the ACSF with oxygen (95% oxygen, 5% carbon dioxide) and adjust the pH to 7.4. Prepare fresh ACSF solution before every experiment.



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Title of Article:

Imaging neural activity in the primary somatosensory cortex using Thy1-GCaMP6s transgenic mice

Author(s):

Xiaojing Lin, Tingbao Zhao, WEnhui Xiong, Shaonan Wen, Xiaoming Jin, and Xiao-Ming Xu

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Article Title:	Imaging neural activity in the primary somatosensory cortex using Thy1-GCaMP6s transgenic mice		
Signature:		Date:	2017-07-18

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Responses to Editorial and Reviewers' Comments

Response to the Editorial Comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: The final version of the manuscript has been edited by a medical editor at the authors' Institution.

2. Please abbreviate all journal titles.

Response: Done.

3. Please define all abbreviations before use.

Responses: Done.

4. Please use focused images of uniform size/resolution (at least 300 dpi).

Response: Done.

5. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

Response: Done.

6. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Response: Done.

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Response: Done.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Response: Simplified.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: Added.

10. What is the age/gender of the mouse?

Response: Added in 1.1.

11. Please specify all instruments used throughout.

Response: We have added Table 1 summarizing the instruments.

12. 2.1: What instrument is used for the incision?

Response: Scissors. We have added in the text.

13. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: Protocol 1, 2, and 3.

14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Response: We have modified the steps to follow a logical flow.

Response to Reviewer #1:

Manuscript Summary:

This very informative manuscript provides a very detailed description of an experimental procedure that can be used to chronically assess mouse brain neurons activity through dual open skull windows and 2-photon microscopy.

Major Concerns:

N/A

Minor Concerns:

The following are comments for X Lin, T Zhao, W Xiong, S Wen, X Jin, and X-M Xu about their manuscript titled "Imaging neural activity in the primary somatosensory cortex using Thy1-GCaMP6 transgenic mice".

PROTOCOL

1. Preparing the animal for imaging

1.1) Sentences 1 and 2 (about the ethanol cleaning and the heating pad) appear to be also stated in 1.3 and 1.4 where they are more applicable.

Response: Dropped sentence 1 from 1.1. Moved sentence 2 to the beginning of 1.3.

1.2) Add 'and has no corneal reflex' to sentence 2. Delete 'original' from ...the original anesthetic...' in sentence 3. Delete '...and every 8-12 h postinjury for 2 d...' from sentence 4 as this is stated in 2.13 where it is more applicable.

Response: Thanks! Very helpful.

1.4) Is povidone-iodine also used?

Response: Yes and added.

2. Chronic cranial window preparation

2.1) Refer to Figure 3 as well in sentence 1.

Response: Since Figure 2 is a schematic drawing describing window creation, we prefer not to refer to Figure 3 in sentence 1.

2.5) What size burr (drill bit) is recommended? (Response: Information provided) Is not ACSF applied to the skull not '...the S1 area...' as stated in sentence 3? (Response: Thanks! The sentence has been modified.) What temperature is the ACSF at? (Response: Room temperature, clarified) It is suggested to break up sentence 4 by ending it at '...overheating.'. (Response: Done, thanks!) The next sentence could be started at 'Replace...'. Should '...and...' in sentence 6 be '...to...'? (Response: Changed) Why is Fig. 2C being referred to after the last sentence? (Response: removed)

2.8) Is the right cortex kept moist while the left cortex is exposed?

Response: Yes, a change has been made.

2.9) Is ACSF left between the coverglass and the dura or is this space dried completely? (Response: Yes, added to the sentence.) A picture from the side showing the 2 coverglasses glued and cured together may be helpful. (Response: Difficult to show because the glue is transparent.)

2.10) In sentence 4, should it be (Fig. 2G-H) instead of (Fig. 2G-F)?

Response: Thanks, corrected.

2.11) Is this more applicable in 3.1? How much isoflurane would you recommend? Is this mixed with oxygen? If so, at what flow rate (L/min)?

Response: Isoflurane anesthesia has been moved to 3.1 as suggested.

Figure 2. What is the ddH₂O used for?

Response: To increase the resolution of the microscopy with a water-immersion objectives for imaging.

3. Two-photon imaging

3.2) What manufacturer of a 2P microscope and manipulator would you recommend?

Response: Company information added to the manuscript.

3.8) Delete '...side of the...' in sentence 4?

Response: Deleted.

Figure 4 Legend A2. Would '...structures...' be more appropriate than '...cells...'?

Response: Cells are more appropriate in this manuscript.

4. Data processing

4.4) and Figure 5 Legend D. Do you want these equations to be the same?

Response: The equations are the same now. Thanks!

REPRESENTATIVE RESULTS

Since monitoring calcium dynamics is used as a premise for developing this experimental procedure, should not paragraph 2 be presented before paragraph 1?

Response: We prefer to keep the current order of the paragraphs.

Paragraph 2, Sentence 1: '...single cell...' or '...single neuron...'?

Response: Changed.

Paragraph 2: Is Figure 6 being discussed here?

Response: Fig. 6 has been referenced in the text.

DISCUSSION

Have any differences in results been observed between 2 or 1 brain hemisphere being exposed and having a cranial window?

Response: We have not made a direct comparison in this study. The comparison will be performed in future hypothesis-driven studies.

Paragraph 2: What about studies of sensory dysfunctions and/or pain? Can various peripheral stimulation strategies (for example, optogenetic, electrical, chemical) be used?

Response: Good point; we have added this to the 2nd paragraph of the Discussion.

Paragraph 3, Sentence 4: Should Figure 3 be referred to here? Would this paragraph be more applicable in the PROTOCOL section?

Response: Yes, Figure 3 has been referenced in the text.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

N/A

Major Concerns:

The authors state in several sections (Long Abstract, Introduction and Discussion) that dynamics of neural activity can be viewed in symmetric brain regions, which suggests that 2 regions of the brain can be observed at the same time. It is not clear what the authors mean by this description and maybe it can be re-stated.

Response: In the Long Abstract, we stated “This method enables recordings and quantifications of neural activity in bilateral mouse brain regions one at a time in the same experiment for a prolonged period *in vivo*.”

Minor Concerns:

Line 87 indicates a 40 fold range of dosing for Buprenorphine. Can the authors be more specific for a preferred dose.

Response: In 1.3) we have stated: “Inject buprenorphine subcutaneously (s.c.; 0.05-0.10 mg/kg).”

Line 152 regarding chronic experiments it is not indicated if the skin incision is closed for the 7-10 days between placement of the viewing ports and 2 photon observations.

Response: No suture is needed to close the wound since the skin is replaced by the optical window. The wound margins are covered by dental cement (new 2.15).

Line 292 regarding Figure 1 and Figure 2 - There is a vertical line labeled as 5 um across the cortical layers. This is not correct as indicated in the figures.

Response: Thanks! We have corrected the mislabeling.

Line 326 regarding Figure 6 - What do the arrows in Panels B and C indicate

Response: We have indicated arrows in Figure 6. Thanks again.

Additional Comments to Authors:

N/A