Chapter 17

Optogenetic Approaches for Mesoscopic Brain Mapping

Michael Kyweriga and Majid H. Mohajerani

Abstract

Recent advances in identifying genetically unique neuronal proteins has revolutionized the study of brain circuitry. Researchers are now able to insert specific light-sensitive proteins (opsins) into a wide range of specific cell types via viral injections or by breeding transgenic mice. These opsins enable the activation, inhibition, or modulation of neuronal activity with millisecond control within distinct brain regions defined by genetic markers. Here we present a useful guide to implement this technique into any lab. We first review the materials needed and practical considerations and provide in-depth instructions for acute surgeries in mice. We conclude with all-optical mapping techniques for simultaneous recording and manipulation of population activity of many neurons in vivo by combining arbitrary point optogenetic stimulation and regional voltage-sensitive dye imaging. It is our intent to make these methods available to anyone wishing to use them.

Key words Optogenetics, Virus, Transgenic, Mouse, Neuron, Circuit, Brain, Channelrhodopsin (ChR2), Voltage-sensitive dyes (VSD)

1 Introduction

One of the major goals in neuroscience is to map the structural and functional connectivity of the brain. Many neuroscientists believe that this work will lead to important discoveries that will in turn lead to new treatments of neurological disorders and diseases ranging from stroke to autism [1, 2]. The advent of optogenetics has revolutionized neuroscience by enabling the use of light to interrogate genetically identified neurons within neural circuits using opsins, light-activated proteins [3]. Importantly, such control of neurons is reversible and operates on sub-millisecond timescales. With the technique scientists can activate or silence specific classes of neurons to test specific hypotheses. This work can be performed in awake behaving animals or in brain tissue slices to examine short- and long-range functional connections between neurons. These opsin proteins can be inserted into neurons via viral vectors, electrophoresis, or by breeding transgenic animals [1, 2, 4, 5]. These mapping experiments are typically performed in conjunction

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with either electrophysiology or imaging methods depending on the specific research question. In addition to using optogenetics to perturb neural circuits, it can also be used as a reporter. For example, channelrhodopsin can be inserted into small inhibitory neurons and pulses of blue light will invoke spiking in those cells. This method enables the identification of these small, often quiescent, cells for blind in vivo whole-cell or juxtacellular recordings to investigate their role in large-scale mapping experiments [6, 7].

The selectivity of genetically identified cells has led to many new discoveries, such as the role of specific types of inhibitory and excitatory neurons in the cortical minicolumn [8–10]. To further add to the specificity of spatially restricted areas of the circuits of interest, modern lasers, directed by galvanized mirrors can focus beams of light with ~50 μm precision into target tissues, reaching cortical layers 2/3 [11]. For even greater spatial precision, two-photon optogenetic activation of red-shifted opsins can allow for activation and recording of many individual cells simultaneously [12].

The two primary classes of optogenetic opsins are those that cause neurons to depolarize (e.g., Channelrhodopsin-IIa; ChR2) or hyperpolarize (e.g., Archaerhodopsin). ChR2-type proteins can be used to activate neurons that have an excitatory or modulatory role in brain circuits. If the intent is to temporarily ablate specific brain regions, ChR2 can be placed into inhibitory neurons (e.g. Parvalbumin-positive basket cells) or Archaerhodopsin can be inserted into excitatory or modulatory cells. While ChR2 and Archaerhodopsin are two of the most popular opsins, there are many light-activated proteins available for brain mapping experiments (please *see* [13] to review many of the available opsins). These designer proteins are continuously upgraded to enable control with many different wavelengths of light.

Here we describe in vivo optogenetic functional mapping procedures combining optogenetic stimulation with regional voltage-sensitive dye imaging in transgenic mice to assess intrahemispheric and interhemispheric functional relationships. Any transgenic animal that expresses optogenetic opsins within subsets of cortical neurons could be used. Although optogenetic opsins are expressed in axons of passage and some of the transgenic animals might exhibit some variability in expression levels across the brain, these transgenic mice provide advantages over multiple viral injections due to incomplete sampling and potential for tissue damage at each injection site. To monitor and map intracortical population activity, we use organic voltage-sensitive dyes that offer the ability to monitor activity over large spatial scales (up to 50 mm^2) and with millisecond time resolution [14–16].

The combination of optogenetics and imaging to map functional circuits in vivo is paving the way for new insights in neuronal functioning. One of the strongest advantages of the methods described here is the activation of nonprimary sensory areas. This can enable investigations of network activity and functional maps

following the stimulation of secondary or even higher level association areas. In the future, development of novel opsins for optogenetic stimulation and genetically encoded activity sensors [17, 18] may allow for longitudinal and simultaneous stimulation and imaging of more than one class of cell, within the same animal, by using opsins activated with different colors light. Such methodological approaches could be used to deduce functional relationships between cortical areas and large-scale circuit organization in various mouse models of human disease, to study the recovery after injury such as stroke or traumatic brain injury.

2 Materials

2.1 Experiment Setup

Once the experimental goals are planned, obtain the required animals with opsins expressed in genetically identified cell classes. Transgenic mice and rats can be purchased from commercial vendors and bred to into standing colonies. For experiments involving other animal species or nontransgenic mice and rats, viral vectors can be purchased for specific experiments. Some researchers elect to use transgenic Cre mice and then inject specific opsins into small spatial regions of the brain. Please note that some transgenic lines of mice are frozen down and require reconstitution of the line which can take up to 2 months.

2.1.1 Illumination Sources

Solid-state blue (473 nm) or yellow (589 nm) laser to activate optogenetic opsins in our transgenic mouse lines, and a 627 nm red LED to excite the VSD.

2.1.2 Light Filtering

1. VSD fluorescence excitation (620–640 nm) and bandpass emission (673–703 nm) filters.
2. Dichroic mirror (590 dcxr). This separates the red LED for VSD and blue or yellow laser for optogenetic stimulation.
3. Neutral density filters to assist with reducing saturation of LEDs.

2.1.3 Voltage-Sensitive Dye

1. RH1691 [19].
2. Syringe filter, 0.2 μm (Pall Acrodisc Supor Membrane; *see* **Note 6**).

2.1.4 VSD Data Collection

1. CCD camera.
2. EPIX E4 or E8 frame grabber with XCAP 3.8 imaging software.
3. 50 and 35 mm front-to-front video lenses.

2.1.5 Photostimulation

1. PCI-6229 M Series Data Acquisition Board (DAQ; National Instruments, Austin, TX, USA).

2. Windows XP computer (*see* **Note 8**).
3. Ephus software [20] (*see* **Note 8**).
4. Galvanometer scan mirrors.
5. Laser power meter (Thorlab, NJ).

2.1.6 Common Lab Supplies

1. Analytic scale.
2. Magnetic stir plate and stir bar.
3. pH meter or strips.
4. Vacuum pump or line.
5. Vortex.
6. Ultrasonic cleaner.
7. Large beaker (>1 L).
8. Graduated cylinder.
9. Vacuum filter.
10. Syringe filter, 0.45 μm (Pall Acrodisc Supor Membrane).
11. Weigh boats/paper.
12. 50 mL screw top tubes.
13. 1.7 mL Eppendorf tubes.
14. Large syringe (>25 mL).

2.2 Surgical Station

2.2.1 Surgical Tools and Equipment

1. Scalpel blade handle.
2. Fine-tipped scissors.
3. Fine tweezers.
4. Tissue grabber forceps.
5. Cauterizer (*see* **Note 3**).
6. Tools for setting up and adjusting hardware (e.g., Screwdrivers, needle-nose pliers).
7. Clippers to shave mouse.
8. Thermodynamic heat blanket with rectal probe.
9. Surgical drill; pneumatic or battery operated.
10. Dissecting scope with at least 3× magnification power.
11. Light source to illuminate surgical site.

2.2.2 Surgical Supplies

1. Kim wipes.
2. Labeling tape (*see* **Note 12**).
3. Cotton swabs.
4. Artificial tears.
5. Chlorhexidine.
6. Ethanol 70 %.
7. Gelfoam.

8. 2–3 cm needle (25–30 G).
9. 16 G needle.
10. 1 cc syringes.
11. #11 scalpel blades.
12. Transfer pipettes.
13. Bone wax (*see* **Note 12**).
14. Sterile saline, ~50 mL (*see* **Note 5**).
15. Sterile brain buffer, ~100 mL (*see* **Note 5**).
16. Lubrication for rectal probe.
17. Drill burs, FG ¼ (Midwest Carbide Burs).
18. Super glue.
19. Agarose (Type-III, Sigma).
20. PE10 tubing, ~8–12 cm.
21. Straight edge razors.
22. Microscope coverslip glass to cover the agar and produce a uniform imaging surface (may need to be cut to size).

2.2.3 Isoflurane Delivery

1. Oxygen tank and regulator.
2. Isoflurane vaporizer.
3. Induction chamber.
4. Tubing to connect isoflurane vaporizer to induction chamber and nose cone.
5. Charcoal canister to collect isoflurane.
6. Exhaust snorkel for escaped isoflurane.

2.2.4 Surgical Stage

1. Isoflurane nose cone assembly.
2. Head plate (we can provide specific details upon request) and supports (*see* **Note 1**).
3. Dental cement powder.
4. Jet accelerant.
5. 12 well porcelain plate and stir sticks.
6. Acetone.

2.2.5 Surgical Drugs

1. Isoflurane.
2. Oxygen.
3. Dexamethasone (to reduce cerebral edema).
4. Lidocaine with Epinephrine (to reduce surgical pain and blood loss).
5. Atropine.
6. Euthansol (to euthanize animal upon completion of experiment).

2.2.6 Reagents

1. Sterile brain buffer: 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 (*see* **Note 5**).
2. Sterile glucose in brain buffer: 0.5 mM glucose in brain buffer.

3 Methods

3.1 Lab Setup Before Beginning Experiments

Incorporating optogenetic techniques into a research lab requires a fair amount of strategic planning. One of the first steps is to determine which animal species will be used as this will dictate whether transgenic animals or viral injections are needed. Our lab primarily uses transgenic mice, due to readily available mouse lines from commercial sources and the ease of breeding a colony to have mice available for daily use. We also combine our transgenic mouse lines with viral vectors for spatial control of optogenetic expression. This is useful for experiments requiring tight spatial control when illumination areas are larger than desired.

The present protocol focuses specifically on adding optogenetics and VSD imaging to a standing lab. For labs seeking to add other recording techniques in addition to imaging, such as electrophysiology, many reviews and book chapters are already available regarding the setup of these techniques. The three primary light sources of stimulating opsins are lasers, LEDs, and arc lamps. All of these have their own inherent advantages and limitations (for review, *see* [5]). The light source can then be directed onto the cortex either through fiber optic cables or with a network of galvanized mirrors. In our lab we used laser stimulation controlled with scanning galvanized mirrors [1, 14].

Proper setup of imaging equipment will lead to easier data acquisition during experiments. It is far easier to setup and adjust the physical illumination before data acquisition than to try to correct poor images after recording. LEDs are an inexpensive way to illuminate brain tissue. When the LED is first turned on, the intensity may fluctuate and decay for a short time until it is fully warmed up and reaches a stable level. Be sure to test your LEDs. There is also a need to balance sufficient lighting with over-saturation. When the current delivered to the LED is too low, it can cause fluctuations of the light intensity, however currents needed for stable illumination may cause saturation. To solve this problem we use inexpensive neutral density filters. To ensure uniform illumination of the cortex, we use two LEDs on posts positioned approximately 10–15 cm from the brain. Note that the frame rate of the camera and pixel bin size will dictate the amount of light needed to adequately illuminate the brain.

Below we will discuss how to properly secure the mouse skull to a head plate (*see* **Note 1**). This will add to mechanical stability,

critical for stable images. To add further stability, many imaging experiments are performed on anti-vibration air tables.

3.2 Surgery Introduction

Successful imaging experiments are highly dependent upon well-done surgeries with undamaged brain tissue. Based on our experience, mice survive surgeries best when they are treated gently. Reduce all unnecessary tissue damage and trauma. This lessens the need for excessive use of anesthetics and lowers the risk of large inflammatory responses. Ensure that the animal's body temperature remains at 37 °C from induction of anesthesia and throughout the entity of the experiment (*see* **Note 10**). Throughout the experiment we keep our saline and buffers warmed to 37 °C to ensure we aren't causing undue shock on the animal.

3.3 Presurgical Procedures

1. Ensure all materials and supplies are on hand (*see* Subheading 2) and that all surgical tools have been thoroughly cleaned and disinfected.
2. Collect the correct animal and place in transport cage. Record in lab notebook: animal ID number, weight, gender, date of birth, genetic strain as well as other information required by your institution, such as its litter, cage, room, and protocol numbers.
3. Ensure water in beaker with brain buffer/saline tubes is warmed to 37 °C.
4. Ensure surgical area is ready and prep yourself for surgery.
5. Place mouse into isoflurane induction chamber and lock lid. Set isoflurane to 3–5 % and slowly turn oxygen flow rate up to 0.5–1 L/min. Wait 2–3 min for mouse to fall asleep and ensure it is areflexic to painful foot pinches.
6. Inject Dexamethasone (8 mg/kg, IP) to reduce the risk of cerebral edema.
7. Inject glucose in brain buffer (10–15 mL/kg, IP) to hydrate the mouse. Inject again after the mouse urinates, about once every 1–2 h.
8. Shave off all hair around surgical site. If the mouse begins to wake up (whisking/voluntary movements) place back into induction chamber. Use labeling tape to remove loose fur/hair from the shaving site and to clean up the shaving station (*see* **Note 12**). This will assist in reducing contaminates from the surgical site.

3.4 Surgical Procedures

1. Place mouse onto surgical table and fit into nose cone. Set isoflurane to 0.5–1.5 % (adjust as needed) and oxygen flow rate to 0.5 L/min (*see* **Note 9**). Check the mouse's respiration rate and ensure it is areflexic throughout entirety of experiment, about every 5 min.

2. Use lubrication to insert rectal thermometer 1 cm and tape it down. Ensure the body temperature is 37 °C (*see* **Note 10**).
3. Moisten eyes with artificial tear lubricant to prevent corneal desiccation.
4. To conveniently deliver meds and hydration fluids we use a catheter inserted into the right intraperitoneal space, which reduces the need for moving the animal when giving injections. Cut a piece of PE10 tube to 8–12 cm in length. Use a marker to make a mark ~1.5 cm from end. Insert the marked side of the tubing into a 16 G needle. Put mouse on its back/left side to expose its right abdomen. Insert the needle into the right intraperitoneal space. You should feel two "pops," the first through the skin and the second through the peritoneum. While holding the needle still, carefully insert the tube into the abdomen (about 1 cm). Pinch the tube through the skin and gently remove the needle. The tube is properly placed when the marked end of the tube is at the surface of the skin. Super glue in place and wait until dry before returning mouse back into prone position (*see* **Note 2**).
5. For pain relief and reducing blood loss, we use 7 mg/kg Lidocaine with Epinephrine SQ, over surgical site. Wait ~ 5 min for drug to diffuse into tissue, then ensure the mouse is areflexic by pinching the skin over the surgical site with forceps, first lightly, then firmly. If the mouse responds, increase the isoflurane until it becomes areflexic (*see* **Note 4**).
6. Scrub surgical site with Chlorhexidine. Begin in the middle of the site and work your way outwards. Repeat three times (*see* **Note 4**).
7. Scrub surgical site with 70 % Ethanol. Begin in the middle of the site and work your way outwards. Repeat three times (*see* **Note 4**).
8. Once the mouse is areflexic to pain, begin removing the skin and muscle tissue over the region of interest for your experiments. Note skull landmarks such as the midsagittal suture, lambda, bregma, or the squamosal bone. Every 3–5 min moisten the skull with brain buffer. This will prevent the dura from adhering to the skull. Stop any noticeable bleeding with gel foam or use the cauterizer for significant hemorrhaging (*see* **Note 3**).
9. In Fig. 1, we show a bilateral hemisphere craniotomy spanning across the midline, from lambda to 3 mm anterior of bregma. Attach head plate to posts and position mouse underneath (Fig. 1a). This takes patience and practice. Use gauze and whatever is needed to position the mouse. The skull is rounded so ensure that the region of interest is centered and level. This creates a large craniotomy window approximately 7 mm^2 [14]. Ensure skull landmarks are present and take a photo (Fig. 1b), which will assist with reconstructing cortical maps with skull landmarks (*see* **Note 11**).

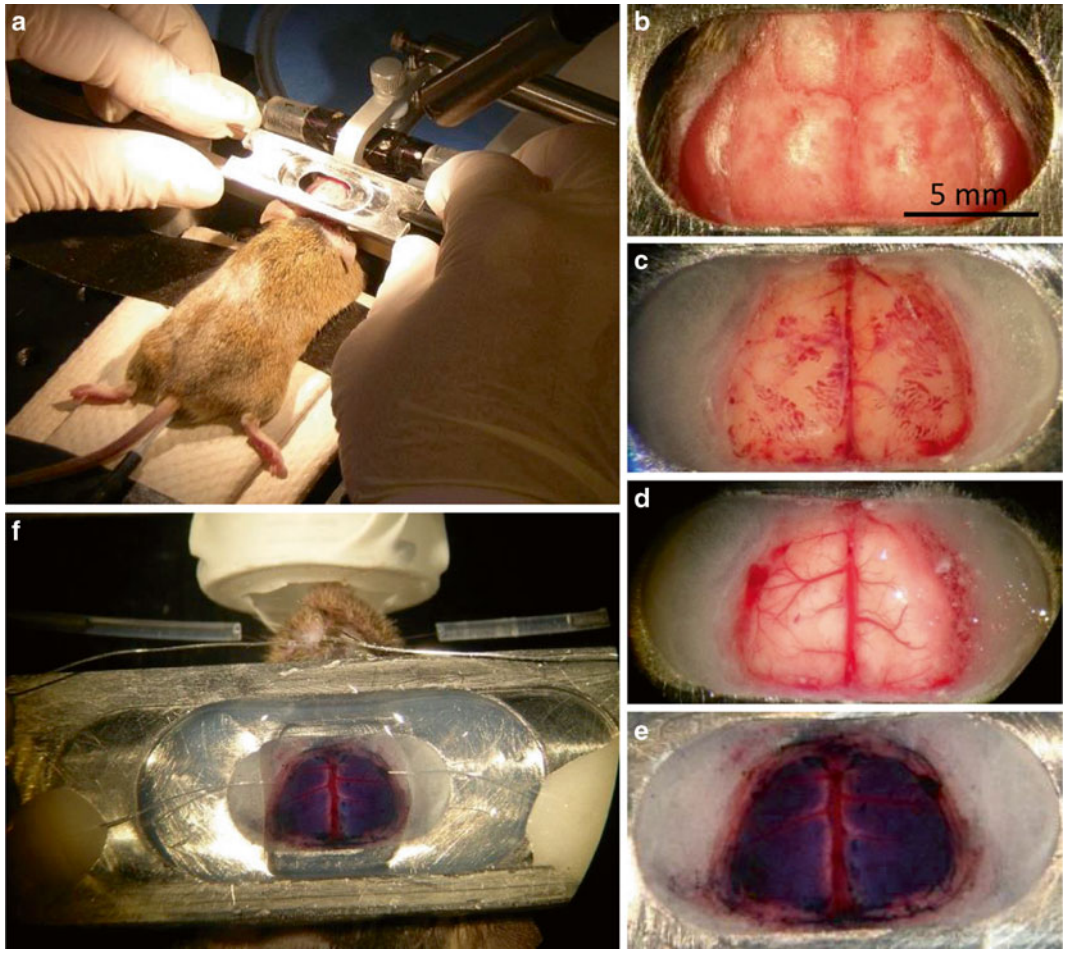

Fig. 1 Surgical set up for a VSD experiment in a ChR2 mouse with a large bilateral craniotomy. (**a**) Following surgical removal of soft tissues, the head plate is positioned over the skull (*see* **Notes 1** and **11**). (**b**) Close up view of the position of the skull underneath the head plate. Note the central suture and bregma are clearly visible. (**c**) Dental cement is added to the edges to secure the head plate to the skull while also serving as a well for the voltage-sensitive dye. Following craniotomy the dura is exposed. (**d**) Completed durotomy, note the brain tissue appears much cleaner than in **c**. (**e**) VSD staining is complete. Note the *deep purple color*. (**f**) Agar and coverslip protect the brain

10. Use super glue around the edges of craniotomy site. The glue should lightly secure the head plate to the skull in the midline anterior and posterior regions (*see* **Note 7**).
11. To reduce cortical pulsations a cisternal drain can help. Once the super glue is dry, remove tissue from top of skull to foramen cisterna. Expose the dura and pierce with a needle or new #11 scalpel. When done correctly, there should be a flash of clear cerebrospinal fluid (CSF). Place a small moistened piece of gauze over the drain to facilitate draining of CSF.

12. Mix dental cement and jet accelerant. Use a razor to cut a cotton swabs at a ~45° angle to make a stir-stick/applicator. Pour the powder into one of the wells in the 12-well porcelain plate, add jet liquid (about 1:1 ratio), and stir well. It will be quite runny at first, but will rapidly harden.
13. Add cement all around the craniotomy site, ensuring that landmarks are fully visible. Keep adding to build the well. This takes patience and many coats. You may need to mix up more cement. Once the well is built, allow to dry for 5–30 min. Ensure it is completely solid before continuing. Before the cement dries use a stir-stick to gently scrape it away from landmarks, such as the central suture or bregma.
14. Figure 1c shows the complete craniotomy and the well made from dental cement. Use the drill to carefully remove excess cement as needed and begin the craniotomy. Ensure the drill burr is sharp and replace as needed during surgery. Since the mouse skull is only about 1 mm thick, carefully remove a few hundred microns of bone at a time. Take extreme care to not punch the drill through the skull, which can cause extensive brain damage or hemorrhaging if large dural vessels are damaged. To remove debris from the well, carefully squirt brain buffer or saline on one side and use another transfer pipette to remove it. For stubborn pieces, carefully remove with fine-tipped forceps. While drilling through the skull, every few minutes stop and gently press on the skull piece that will eventually be removed. Only press down 50–100 μm; when sections are fully drilled the skull will depress into the brain. The drilling is complete when the piece to be removed is no longer attached and is floating on the dura. Be sure to distinguish between free moving skull and skull that has been loosened from poor adhesion to the dental cement. Fill the cranial well with brain buffer, this will assist in removing the skull from the dura (*see* **Note 5**). With extreme care gently remove the skull from the dura with fine-tipped forceps or a fine-tipped probe. The skull may see-saw, and lifting one end of skull may cause the other end to press into the brain. This is usually a symptom of incomplete drilling. Use a new #11 scalpel blade and gently cut the bone where the drilling is incomplete. Once the skull is removed stop any bleeding with gel foam. Note the condition of the dura. It is possible that it may have been removed with the skull.
15. If a durotomy is desired, use fine-tipped forceps or a microprobe and tiny spring scissors to remove the dura (Fig. 1d). Take extreme care to not damage the brain or blood vessels.
16. Again remove any debris by flushing with brain buffer using extreme care.
17. Fill the well with brain buffer.

3.5 Voltage-Sensitive Dye Preparation

We use the dye RH1691 that can be purchased in 10 mg bottles. We divide this into 20, 0.5 mg aliquots, which is enough for one experiment. Since the dye is light sensitive, protect it from light at all times.

1. The ratio of dye to brain buffer depends on experiment. For preps with a durotomy use 0.6 mg of dye:1 mL brain buffer. For preps with the dura intact use 1 mg of dye::1 mL brain buffer.
2. Mix solution with a vortex for 10 min on high.
3. Place tube in an ultrasonic cleaner for 5 min, ensuring the tube is immersed in the water. This can help to further dissolve the dye.
4. Let rest for 10–20 min, then centrifuge for 1 min at 12,000 rpm (g-force = 13,523).
5. Filter with 0.2 μm Pall Acrodisc filter (*see* **Note 6**).

3.6 Staining the Brain with Voltage-Sensitive Dye

1. Make a dam out of bone wax at the edges of the craniotomy to minimize dye needed. The bone wax may leave a film in the buffer, rinse and replace the brain buffer as needed.
2. Immediately before adding the dye, carefully remove all brain buffer. Twist the end of a Kim wipe to a fine point and under the dissecting scope carefully remove all traces of brain buffer from the edges of the craniotomy. This will greatly assist the staining of the entire brain, otherwise microfluidics will prevent the staining of the edges of the craniotomy.
3. Apply to brain and allow to soak for 60–120 min until the brain is deep purple. Use a 200 μL pipetter to gently mix the dye every 10–20 min. Ensure the brain is inundated at all times and add more as needed since it may evaporate or leak.
4. Rinse off with brain buffer and then fill well with brain buffer (Fig. 1e).

3.7 Protect the Brain and Reduce Movement Artifacts with Agar

1. Mix 200 mg of agar in 15 mL brain buffer (1.3 %) in a small beaker.
2. Bring to boil in the microwave and ensure there is no undissolved agar.
3. Cool while continuously stirring and apply over brain once the agar is <42 °C. Ensure there is no debris, bubbles, or undissolved agar as this can reduce the quality of the images by scattering light.
4. Immediately after applying agar, gently place glass coverslip. Place one end down first and slowly lower to force out air bubbles. When done correctly, the glass should rest on the bottom layer of the head plate (Fig. 1f).
5. Wait a few min for agar to set, then place a couple of pieces of gel foam at the corners of the agar and drop brain buffer every ~10 min to prevent the agar from desiccating.

3.8 Mapping

1. Figure 2a depicts our imaging set up. We illuminate the brain with two red LEDs (620–640 nm excitation filter) positioned ~10 cm from the brain. On laser stimulation trials, a 473 nm laser is positioned over cortical regions of interest via galvanometer scan mirrors before passing through a dichroic mirror to the brain. Light emitted from the brain surface passes through a bandpass filter (673–703 nm) and is recorded with a CCD camera.
2. To image VSD activity, focus the camera into the brain until the blood vessels are blurry, a depth of approximately 500 μm.
3. Each trial should begin with 300–500 ms of illumination with the red LED, followed by laser stimulation of the region of interest or physical stimulation of the sensory system. The pre-trial recording period allows for a baseline to compare the VSD fluorescence before and after stimulation. The red LED illumination should continue for 1–2 s after stimulation, with 10 s between trials to ensure the brain does not adapt or habituate to the stimulation (this may vary depending on the specific experiment). In Fig. 2b we show cortical activity following visual stimuli and laser stimulation of the primary visual cortex. We find that 20 trials are usually sufficient to obtain robust stimulus-evoked responses.
4. To arbitrary optogenetically stimulate neurons, we use a 473 or 589 nm diode pumper solid-state lasers. We position the laser beam on the cortex using Ephus software, which control galvanometer scan mirrors, via analog output voltage from the DAQ. The Ephus program controls the overall timing of individual stimulation trials with TTL triggers to XCAP.
5. Set the laser output to 5 mW using a laser power meter.
6. Position laser over region of interest. This can be accomplished by controlling galvanized scanning mirrors [1]. Figure 2c shows an example of an optical stimulation grid. Each blue circle represents a site of optogenetic laser stimulation. Five examples of cortical responses to optogenetic stimulation are shown on the right.
7. Previous work in our lab has demonstrated that 1 ms, 5 mW laser pulses are sufficient to evoke robust firing from ChR2 expressing neurons [1].

4 Notes

1. While there are many ways to immobilize the animal's head, we find that using a head plate attached/sealed to skull with dental cement is the best option. This is because ear bars or customized bite bar/nose cone assemblies require the building of a well for the VSD staining procedure. The head plate performs both functions simultaneously.

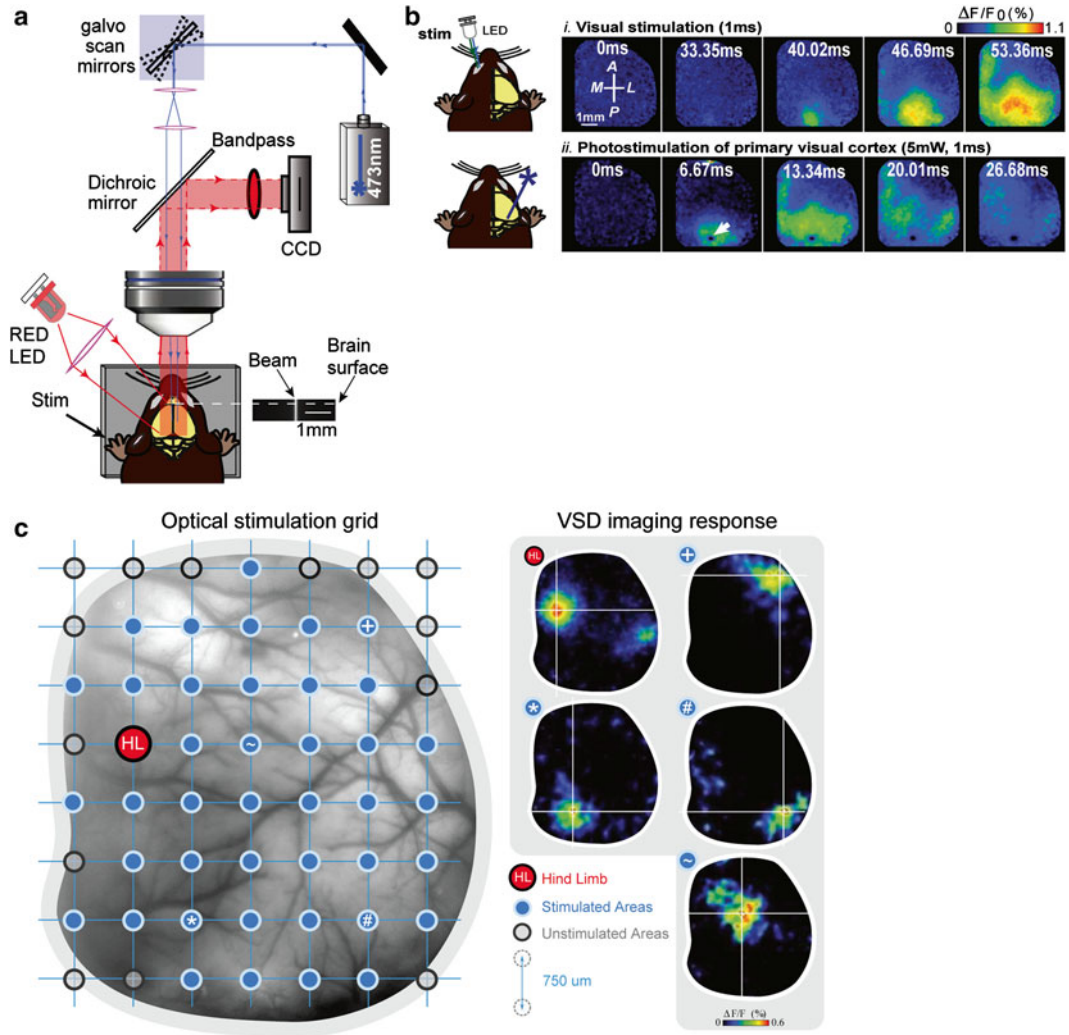

Fig. 2 Mapping interhemispheric and intrahemispheric connectivity using ChR2 stimulation and VSD imaging. (**a**) Experimental set up for simultaneous ChR2-photoactivation and VSD imaging. Galvanometer scan mirrors position a 473 nm laser at specific cortical locations while VSD fluorescence is monitored in epi-fluorescence mode. An image of laser beam demonstrates that it is relatively collimated. (**b**) Example of VSD responses in a unilateral craniotomy preparation during visual stimulation of the contralateral (*left*) eye (i), or direct photostimulation (*white arrow*) of the right V1 (ii). (**c**) Optical stimulation grid (*left*) and VSD imaging responses (*right*). Stimulated areas are denoted by *blue circles* spaced 750 μm. *Special symbols* superimposed over *blue circles* highlight responses shown on the *right*. Figure 2a and b reproduced from Lim et al., 2012, Figs. 1a and 2d, respectively, with permission from Frontiers in Neural Circuits

2. When placing the catheter, we find that some combinations of marker ink and glue cause an exothermic reaction that can melt the tubing and prevent injections. Be sure to practice this procedure before performing it on an animal.
3. We use a cauterizer to assist with stopping bleeding during surgery. Since surgical cauterizers are expensive, we instead

use a fly-tying cauterizer, typically used for making fly fishing lures. It is an excellent alternative for a fraction of the price.

4. Shaving the surgical site can cause small cuts in the skin that may cause a painful response when applying alcohol during the surgical prep. Be sure to wait for the lidocaine to fully infuse before proceeding.
5. Always use normal saline or isotonic brain buffer (290–300 mOsm, 7.2–7.4 pH) on exposed tissue; never use pure water as it is extremely hypotonic and destructive to cells.
6. Through much trial and error we found that the Pall Acrodisc 0.2 μm filter must be used to filter the dye, as other filter brands somehow prevent the dye from functioning properly.
7. For easy and controlled application of super glue, we remove the plunger from a 1 cc syringe to add about 0.5 mL. Replace the plunger to apply very small quantities.
8. The free open-source Ephus software platform requires Windows XP, although future updates are scheduled to work with Windows 7.
9. If the mouse makes raspy/gurgling/clicking sounds, we find that turning down the isoflurane usually solves the problem. Atropine can also help (1–2 mg/kg SQ) by reducing tracheal secretions. Note that atropine is fast acting and is quickly metabolized. Therefore, preventative doses at the beginning of each procedure are unlikely to prevent mucus buildup later in the experiment.
10. If maintaining the mouse at 37 °C becomes difficult, ensure the tail is on the heat blanket and drape an insulated blanket over their body.
11. We strongly advise taking photos before and after the craniotomy. As an inexpensive alternative, modern cell phone cameras often have excellent resolution. With practice the camera can be positioned by hand over the eyepiece of the dissecting scope.
12. Bone wax and tape are extremely useful disposable tools during surgery as well as setting up and during the experiment. We use small pea-sized blobs of bone wax and/or labeling tape to hold things down. Both of these can be used for temporary or long-term solutions.

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