6-21-2024

Protocol for recording neural activity evoked by electrical stimulation in mice using two-photon calcium imaging

Seungbin Park
Purdue University, park1377@purdue.edu

Megan Lipton
Purdue University, liptonm@purdue.edu

Yujiao J. Sun
University College London

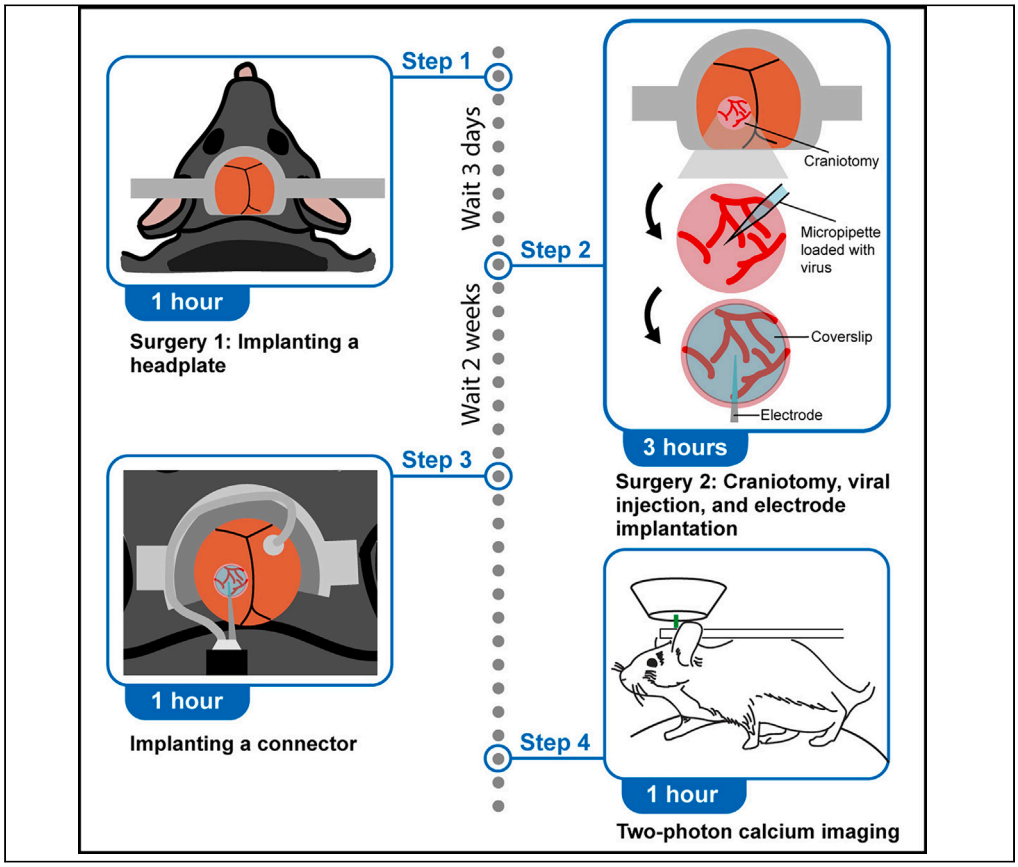
Maria Dadarlat
Purdue University, mdadarla@purdue.edu

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for recording neural activity evoked by electrical stimulation in mice using two-photon calcium imaging



Electrical stimulation provides a clinically viable approach for treating neurological disorders. Here, we present a protocol for recording neural activity evoked by electrical stimulation in mice using two-photon calcium imaging. We detail steps for chronically implanting a head fixation bar, a stimulating electrode, and a glass imaging window. We additionally describe the procedures for viral injections and awake head-fixed recordings.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Seungbin Park, Megan Lipton, Yujiao J. Sun, Maria Dadarlat

mdadarla@purdue.edu

Highlights

Perform chronic surgeries that enable long-term recording of neural activity in mice

Image neural activity evoked by electrical stimulation from an implanted electrode

Image responses evoked in genetically labeled cell types in cortical layer 2/3

Park et al., STAR Protocols 5, 103027
June 21, 2024
https://doi.org/10.1016/j.xpro.2024.103027

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for recording neural activity evoked by electrical stimulation in mice using two-photon calcium imaging

Seungbin Park,[1,3] Megan Lipton,[1] Yujiao J. Sun,[2] and Maria Dadarlat[1,4,*]

[1]Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN 47907, USA

[2]Institute of Ophthalmology, University College London, 11-43 Bath Street, EC1V 9EL London, UK

[3]Technical contact

[4]Lead contact

*Correspondence: mdadarla@purdue.edu
https://doi.org/10.1016/j.xpro.2024.103027

SUMMARY

Electrical stimulation provides a clinically viable approach for treating neurological disorders. Here, we present a protocol for recording neural activity evoked by electrical stimulation in mice using two-photon calcium imaging. We detail steps for chronically implanting a head fixation bar, a stimulating electrode, and a glass imaging window. We additionally describe the procedures for viral injections and awake head-fixed recordings.

For complete details on the use and execution of this protocol, please refer to Dadarlat et al.[1]

BEFORE YOU BEGIN

Electrical stimulation of the brain, including intracortical microstimulation (ICMS), is a direct method to causally link brain activity and behavior. This method has been widely used to study the function and organization of the nervous system.[2–4] Despite its efficacy and long history, it is challenging to clearly understand how ICMS affects neurons[5–9] because electrical artifacts from the electrical stimulation interrupt the recording of exact neural activity evoked by ICMS. Calcium imaging removes this challenge as it is unaffected by electrical artifacts. Two-photon microscopy, in particular, enables both simultaneous recording of a large population of neurons at single-cell resolution[10–12] and long-term chronic recordings,[13] making it ideal to study the effects of stimulation on neural activity.

In consequence of these advantages, insights from experiments combining 2-photon calcium imaging with stimulation have established a basic mechanistic understanding of the impact of ICMS on neural circuits.[1,2,14–18] While many of these studies have been acute,[14–16] more recent chronic work allows for longer-term, clinically-relevant analysis of the effects of stimulation on neural activity.[17,18] Here we present a thorough protocol for performing chronic recording of stimulation-evoked neural activity using two-photon calcium imaging in awake mice.

The following protocol describes the steps for implanting a cranial window and an electrode for simultaneous two-photon imaging and electrical stimulation of cortex in a mouse model. We expect that the protocol could be adapted to other rodent models with only minor modifications. The protocol includes three sequential surgeries: (1) headplate implantation, (2) craniotomy and electrode implantation, and (3) connector implantation. It has been successfully applied in Dadarlat et al.[1] to show how the responses of excitatory and inhibitory neurons to electrical stimulation of mouse primary visual cortex depend on both stimulation amplitude and baseline neural activity levels at the time of stimulation.

Fluorescent protein indicators are necessary for two-photon calcium imaging. They can be introduced either by viral injections or transgenic expression.[19,20] Here, we used transgenic mice to label inhibitory neurons with a red fluorescent marker by crossing transgenic mouse lines Gad2-IRES-Cre with reporter line Ai14 (Jackson labs strains 010802[21] and 007914[22]). Following the craniotomy, these mice were injected with 600 nL total of AAV virus expressing GCaMP6s (AAV1.Syn.GCaMP6s.WPRE.SV40). This virus transfects both excitatory and inhibitory neurons with a fluorescent indicator of calcium concentration. Injections were made into three locations within primary visual cortex at two depths (150 and 300 μm, 100 nL per depth) per location.

Institutional permissions

All procedures were approved by the Institutional Animal Care and Use Committee at Purdue University in accordance with the standards for humane animal care as set by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. You will need to acquire appropriate permissions related to animal experiments from the institutions you are involved.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
AAV1.Syn.GCaMP6s.WPRE.SV40	Provided by Douglas Kim & GENIE Project	Addgene viral prep #100843-AAV1; http://n2t.net/addgene:100843; RRID: Addgene_100843
Chemicals, peptides, and recombinant proteins		
ACSF: D-(+)-Glucose	Sigma-Aldrich	G7021
ACSF: Dulbecco's phosphate-buffered saline, 1× with calcium and magnesium	Corning	21-030-CM
ACSF: magnesium chloride	Sigma-Aldrich	208337
ACSF: potassium chloride	Sigma-Aldrich	P9333
ACSF: sodium bicarbonate	Sigma-Aldrich	S5761
ACSF: sodium chloride	Sigma-Aldrich	S9888
ACSF: sodium phosphate monobasic	Sigma-Aldrich	S8282
Betadine: BETADINE solution 10% povidone-iodine, NDC: 67618-150-09	Avrio Health L.P.	N/A
Dental cement: Clear L-Powder for C&B Metabond	Parkell	S399
Dental cement: quick base for C&B Metabond	Parkell	S398
Dental cement: universal TBB catalyst	Parkell	S371
Dexamethasone: dexamethasone sodium phosphate injection, USP, 4 mg/mL, NDC: 0641-6145-25	Hikma	Covetrus: 070789
Ethanol: ethanol 200 proof	Decon Laboratories	2701
Isoflurane: isoflurane solution, USPNDC: 11695-6777-2	Piramal Critical Care	Covetrus: 029405
Ketamine: ZETAMINE- ketamine hydrochloride injection, 100 mg/mL, NDC 13985-584-10	VetOne	N/A
Mineral oil: Fisher Science Education mineral oil	Fisher Scientific	S25439
Saline: 0.9% sodium chloride irrigation, USP	B. Braun Medical Inc.	R5200-01
Silastic sealants: Kwik-Sil low toxicity silicone adhesive	World Precision Instruments	Kwik-Sil
Silver paint: 842AR Super Shield silver conductive paint	MG Chemicals	842AR-15ML
Xylazine: AnaSed (xylazine injection) 100 mg/mL	Akorn	Covetrus: 061035
Experimental models: Organisms/strains		
Mouse model: male and female offspring of Ai14 x Gad2-IRES-Cre mice, between 3–12 months of age	The Jackson Laboratory	007914, 010802

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
Scanbox	Neurolabware	https://neurolabware.com
Suite2p	Pachitariu et al.[16]	https://github.com/MouseLand/suite2p#readme
Other		
Anesthesia setup: dual procedure circuit	VetEquip	921400
Anesthesia setup: V-1 Tabletop Laboratory Animal Anesthesia System	VetEquip	901806
Autoclave: consolidated sterilizer systems	Consolidated Sterilizer Systems	ADV-PRO
Connector: ED10964-02-ND	Mill-Max Manufacturing Corp.	801-93-002-10-002000
Cotton swabs: cotton tipped wood applicators	Dynarex Corporation	4301
Coverslip: D263 coverglass CS-3R	Warner Instruments	64-0720
Curettes: micro curettes	Fine Science Tools	10081-10
Dental drill: Titan 3 5,000 rpm-LubeFree	Start Dental Instruments	N/A
Dental drill bur: Midwest regular carbide burs	Dentsply Sirona Midwest	386101
Dual ferrule adapter: RN compression fitting 1 mm	HAMILTON	55750-01
Ear bars: dual-sided, non-puncture mouse & neonatal rat ear bars	Stoelting	51649
Electrode: platinum-iridium electrodes PI20030.1A3	MicroProbes for Life Science	PI20030.1A3
Gelatin sponge: SURGIFOAM absorbable gelatin sponge 7 mm	ETHICON	6545663
Glass micropipette: glass capillary GC-1	Narishige	GC-1
Gloves: KIMTECH Purple Nitrile powder-free exam gloves	KIMTECH	55081
Glue for coverslip (Cyanoacrylate): Krazy glue – all purpose super glue	N/A	N/A
Glue for coverslip: Ortho-Jet BCA powder 100 g-REF B1320	Lang Dental Manufacturing Co., Inc.	1320CLR
Headplate	Custom design fabricated by eMachineShop	N/A
Head/headplate holder setup: BE2 - Ø1.25″ studded mechanical component base, 8–32 thread	Thorlabs	BE2
Head/headplate holder setup: CF125 - clamping fork, 1.24″ counterbored slot, universal	Thorlabs	CF125
Head/headplate holder setup: New Standard stereotaxic instruments for rats and mice	Stoelting	51500
Head/headplate holder setup: RA90 - right-angle clamp for Ø1/2″ posts, 3/16″ hex	Thorlabs	RA90
Head/headplate holder setup: TR4 - Ø1/2″ optical post, SS, 8–32 Setscrew, 1/4″-20 tap, L = 4″	Thorlabs	TR4
Heating pad: small animal temperature controller ATC2000	World Precision Instruments	GRO-ATC2000
Hot bead sterilizer: hot bead sterilizer FST 250	Fine Science Tools	18000-45
Kimwipes: KIMTECH Kimwipes delicate task wipes	KIMTECH	34155
Laser: Chameleon Vision II	Coherent	N/A
Oxygen: oxygen medical grade 99.0% 23.3 CF(E)Sz	Indiana Oxygen Co	N/A
Micromanipulator: motorized micromanipulator	Sutter Instrument	MP-285
Micropipette grinder	Narishige	EG-45
Micropipette puller	Narishige	PC-100
Nanoliter pump: UMP3 UltraMicroPump	World Precision Instruments	UMP3
Ophthalmic ointment: Puralube vet ointment sterile ocular lubricant, 3.5 g (1/8 oz)	Dechra	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Surgical forceps: fine forceps	Fine Science Tools	11254-20
Surgical forceps: micro-Adson forceps	Fine Science Tools	11018-12
Surgical microscope: zoom stereo microscope SZ61	Olympus	SZ61
Surgical scissors: hardened fine scissors	Fine Science Tools	14090-11
Two-photon mesoscope	Neurolabware	https://neurolabware.com/
Ultra precise digital just for mouse stereotaxic instrument	Stoelting	51730UD
10 µL syringe: 10 µL, model 1701 syringe, small removable needle, 32 ga, 2 in, point style 3	HAMILTON	1701
Soldering iron: FX-8801 soldering iron handpiece	Hakko	FX8801-02
Soldering iron holder: FH800-03BY, FX-8801 iron holder	Hakko	FH800-03BY
Soldering station: FX-888D digital soldering station	Hakko	FX888D-23BY

MATERIALS AND EQUIPMENT

Lidocaine injection

Reagent	Final concentration	Amount
Lidocaine	2%	1 mL
Saline	0.9%	9 mL
Total	**0.2%**	**10 mL**

Store between 21°C–22°C up to 1 month.

Meloxicam injection

Reagent	Final concentration	Amount
Meloxicam	5 mg/mL	1 mL
Saline	0.9%	9 mL
Total	**0.5 mg/mL**	**10 mL**

Store between 21°C–22°C up to 1 month.

Alternatives: Carprofen (5 mg/kg)

Dental cement

Reagent	Final concentration	Amount
Metabond	N/A	2 scoops
Quick base	N/A	4 drops
Catalyst	N/A	1 drop
Total	**N/A**	**N/A**

The reaction is fast — mix them in a frozen plate and use as soon as possible.

Ketamine injection

Reagent	Final concentration	Amount
Ketamine	100 mg/mL	0.6 mL
Saline	0.9%	2.4 mL
Total	**20 mg/mL**	**3 mL**

Store between 21°C–22°C up to 1 month. Ketamine is a controlled substance and requires special authorization for use and storage.

Xylazine injection		
Reagent	Final concentration	Amount
Xylazine	20 mg/mL	0.1 mL
Saline	0.9%	1.9 mL
Total	**1 mg/mL**	**2 mL**
Store between 21°C–22°C up to 1 month.		

Dexamethasone injection		
Reagent	Final concentration	Amount
Dexamethasone	4 mg/mL	0.1 mL
Saline	0.9%	9.9 mL
Total	**0.04 mg/mL**	**10 mL**
Store between 21°C–22°C up to 1 month.		

Artificial cerebrospinal fluid (ACSF)		
Reagent	Final concentration	Amount
Dulbecco's Phosphate-Buffered Saline	N/A	500 mL
Sodium chloride	N/A	3.48 g
Sodium bicarbonate	N/A	1.1 g
Potassium chloride	N/A	0.09 g
Sodium phosphate monobasic	N/A	0.06 g
Magnesium chloride	N/A	0.06 g
D-(+)-Glucose	N/A	0.9 g
Total	**N/A**	**500 mL**
Store in −20°C freezer.		

AAV1.Syn.GCaMP6s.WPRE.SV40

Use 100 μL virus from Addgene and aliquot into 1 μL portions. No dilution of the virus is needed. Store aliquots in −80°C freezer. Thaw just before use and keep on ice. Discard any excess virus after viral injection.

STEP-BY-STEP METHOD DETAILS

Surgery 1: Implanting a headplate

Timing: 30 min–1 h

Headplate implantation is necessary to fix the head of the mouse while imaging.

1. Pre-surgery preparation.
   a. Wear proper personal protective equipment including lab gown and gloves.
   b. Sterilize all surgical instruments in an autoclave prior to the surgery.

   Note: A hot bead sterilizer may be used between subjects.

   c. Sterilize the surgical area with 70% ethanol.
   d. Maintain sterility of the environment, equipment, and hands with gloves during the entire procedure.
   e. Weigh the animal to calculate the correct dosage of analgesic medication.
   f. Maintain records of surgical procedure as required by institution.
2. Shave the mouse's head between the ears with an electric razor.

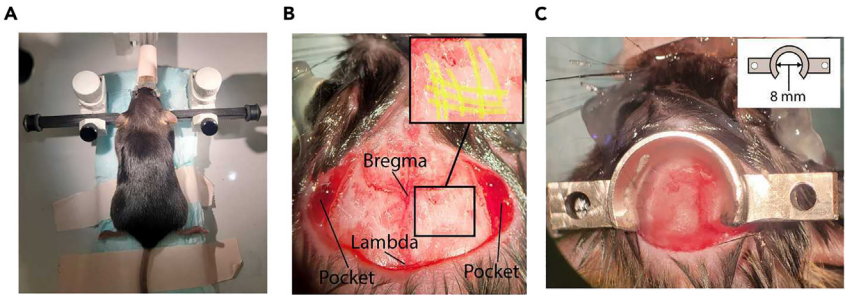


Figure 1. Protocol for implanting a headplate

(A) Head holder setup with a nose cone and a temperature-regulated heating pad. Mouse head is fixed with the ear bars.

(B) Incision, muscle retraction, and scraping. Yellow lines in the inset show scraping in a checkerboard pattern.

(C) Implanted headplate with dental cement. Inset shows a schematic top-down diagram of the headplate.

3. Anesthetize the mouse to areflexia using 2–3% isoflurane in oxygen (flow rate of 1 L/min) in an anesthesia induction chamber.

   Note: Alternatively, Ketamine (90–150 mg/kg) and Xylazine (5–10 mg/kg) can be used. We recommend using isoflurane for shorter surgeries (<1 h); however, ketamine and xylazine is recommended for longer surgeries due to the inflammatory effect that isoflurane has on the brain.

4. Place the mouse in a stereotaxic frame with a temperature-regulated heating pad, setting the temperature to 37.0°C (Figure 1A).
   a. Fix the mouse head with ear bars, targeting the tip of the bars to the ear canals.
   b. Place the nose of the mouse into a tube connected to isoflurane.
   c. Maintain areflexive anesthesia using 1.5–2% isoflurane with oxygen (flow rate 1 L/min) during the procedure.

   ⚠ CRITICAL: Monitor the mouse to ensure that the level of anesthesia is adequate to reduce respiratory rate and prevent reflexive responses to a toe or tail pinch. Adjust the isoflurane level depending on the response of the mouse (troubleshooting 1 and 2).

5. Prepare the mouse for surgery.
   a. Apply ophthalmic ointment to protect the eyes during surgery.
   b. Disinfect the scalp by applying Betadine with cotton swabs. Repeat three times.
6. Inject local anesthetics and analgesics.
   a. Inject 0.03 mL of Lidocaine cutaneously along the planned incision site in the scalp for local analgesia.
   b. Inject Meloxicam (5–10 mg/kg) subcutaneously into the scruff of the animal to prevent pain and inflammation.
   c. For surgeries lasting longer than 30 min, provide the mouse with saline or lactate ringer's solution (5–10 mg/kg) by subcutaneous injection.
7. Use a microscope to view the head throughout the procedure.
8. Cut skin and expose the skull.
   a. Make an incision in the scalp with surgical scissors across a hypothetical midline in the horizontal axis between the ears.
   b. Cut skin and periosteum to expose a 5 mm diameter area of skull, extending from bregma to lambda (Figure 1B).
   c. Clean the skull surface with saline and cotton swabs when it is necessary during the entire procedure.

9. Separate the temporal muscle and tissues attached to either side of the skull using surgical forceps. Push the detached muscle laterally so there are small pockets where the dental cement can adhere (Figure 1B).
10. Thoroughly scrape the skull surface with curettes or forceps in a checkerboard pattern to promote adhesion of glue to the skull (Figure 1B).

Note: Steps 9 and 10 are critical to prevent headplate detachment from the skull (troubleshooting 3). Alternatively, VetBond can be applied to the skull to improve adhesion of the dental cement.

11. Clean the skull with saline and cotton swabs. Then, dry the skull with a Kimwipe.
12. Apply dental cement over the entire exposed skull surface and into the pocket formed by the separation of the temporal muscles on the sides of the skull made in step 9.
13. Attach a titanium headplate centered over the intended site of the cranial window. Wait 5 min for the dental cement to fully dry.

Note: The posterior half of the headplate should be open (e.g. a semi-circular shape) to facilitate electrode implantation (Figure 1C).

14. Transfer the mouse to a closed clean cage or container over a heating pad and leave it until recovery (i.e., mouse is alert and walking). After the animal is fully recovered, return it to its home cage.
15. For two days after surgery, inject analgesia (e.g., 5–10 mg/kg of Meloxicam) every 12–24 h based on any appearance of pain or distress (e.g., lethargy, hunched posture, significant gain or loss of weight, or barbering). Monitor the animal for signs of inflammation or infection (e.g., swelling, bleeding, or abnormal growth in and around surgical site) (troubleshooting 4).

Surgery 2: Craniotomy, viral injection, and electrode implantation

Timing: 2–3 h

In this step, you create a cranial window by removing a piece of skull, inject virus into cortical locations, implant an electrode for electrical stimulation to your targeted area, and cover the cranial window with a cover slip over the cortex and the electrode. The number of viral injections that are performed will determine the spatial extent over which neural responses to stimulation can be imaged. Therefore the number of injections needed will depend on the size of the intended imaging region.

Note: This surgical step is long and technically difficult. We have found that working in teams improves the likelihood of success, where one person creates the craniotomy and does the viral injections and the second person implants the electrode and cranial window.

Note: This surgery may also be split into two steps: viral injection through a burr hole (not described here) followed by electrode implantation in mice with successful viral expression. If desired, the viral injection could be combined with the headplate implant in surgical step 1.

Pre-surgery

16. Wait three days until the implanted headplate is stabilized and the mouse has fully recovered from the headplate surgery.
17. Before performing the craniotomy, prepare for viral injection.
    a. Pull a glass micropipette from a 1 mm inner diameter glass capillary.
    b. Bevel the micropipette tip using a micropipette grinder.

c. Check the micropipette tip under a microscope to ensure it has a 50–80 μm inner diameter tip.
d. Clean the micropipette by forcing air through the micropipette.
e. Fill micropipette with mineral oil by placing non-beveled end of micropipette in a small bowl containing mineral oil.

Note: The micropipette will fill with mineral oil via capillary action in about 5 min.

f. Attach micropipette to a 10 μL syringe using the dual ferrule adapter.
g. Attach micropipette/syringe to nanoliter pump.
h. Digitally expel from the syringe a volume of oil equal to approximately twice the total volume of virus you intend to inject at 20 nL/s.

Note: Expelling this amount will ensure there are no blockages in the micropipette that may cause air bubbles (troubleshooting 5) and that there is sufficient room within the pipette for the virus.

i. Withdraw desired volume of virus into syringe at 20 nL/s and ensure no air bubbles occur (troubleshooting 5).

Surgery

18. Prepare for surgery: perform step 1.
19. Anesthetize the mouse to areflexia using 2%–3% isoflurane with oxygen (1 L/min) in an anesthesia induction chamber.
20. For areflexive anesthesia, inject Ketamine (90–150 mg/kg) and Xylazine (5–10 mg/kg) intraperitoneally.

Note: This surgery can also be conducted under isoflurane anesthesia. However, isoflurane (unlike ketamine) causes cerebral edema. We have found that surgical outcomes improve using Ketamine/Xylazine for anesthesia in this step.

⚠ CRITICAL: During the entire procedure, monitor the mouse to ensure that the level of anesthesia is adequate through respiratory rate and response to a toe or tail pinch. When necessary (usually every hour or before you start to implant the electrode), administer another full or half dose of Ketamine (no Xylazine after the initial induction), depending on the level of anesthesia (troubleshooting 6).

21. Place the mouse on top of a temperature-regulated heating pad set to 37.0°C. Secure the mouse's head by screwing the headplate onto a headplate holder (Figure 2A).

Note: Alternatively, you can secure the mouse's head in a conventional stereotaxic frame.

22. Apply ophthalmic ointment to protect the mouse's eyes.
23. Perform injections.
    a. Inject Meloxicam (5–10 mg/kg) subcutaneously.
    b. Administer Dexamethasone (2.0 mg/kg) to reduce cerebral edema.
    c. For surgeries lasting longer than 30 min, provide the mouse with saline (5–10 mg/kg) by subcutaneous injection.
24. Identify the intended position of craniotomy (i.e., the brain region intended for imaging) using anatomical coordinates. Position a circular glass coverslip on top of the intended craniotomy location and mark four points around the coverslip with a sterile marker (Figure 2B).
25. Use the four reference points made in step 24 to drill four burr holes using a dental drill with a 0.5 mm diameter tip. Use these four burr holes as a reference to drill a circle in the skull.

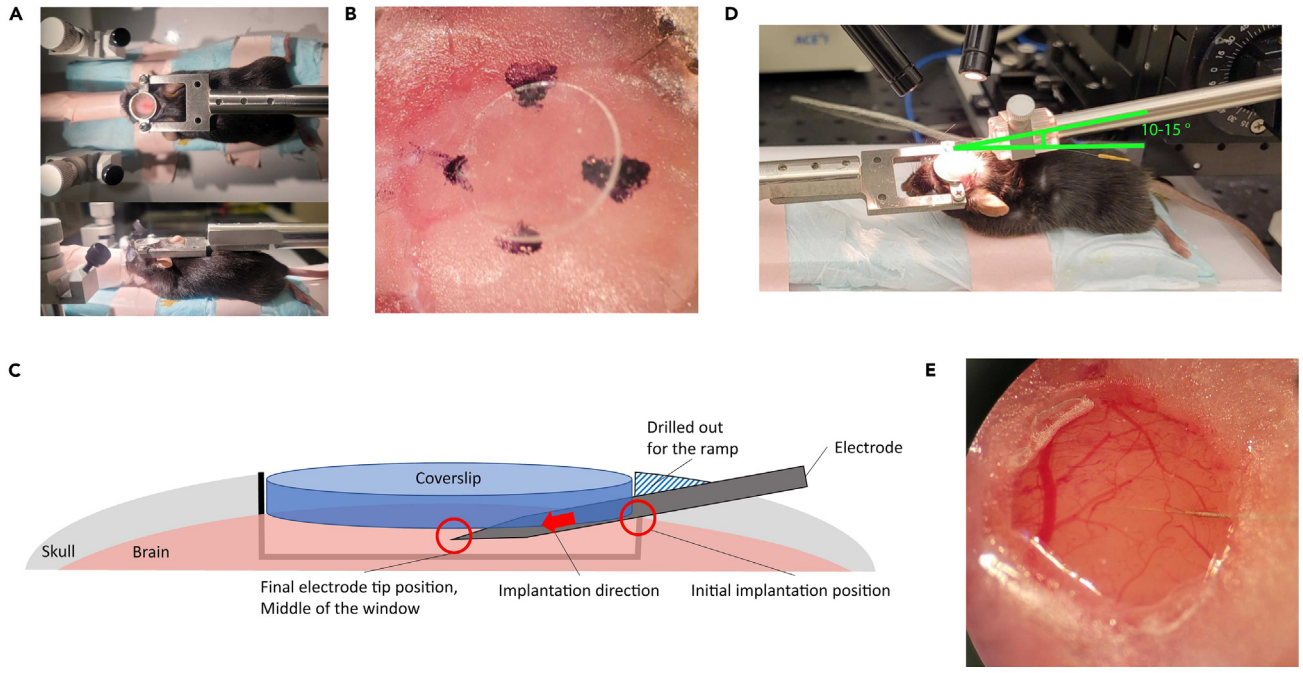


Figure 2. Craniotomy, viral injection, and electrode implantation
(A) Head fixation.
(B) Referential four points for craniotomy.
(C) Diagram for the electrode implantation.
(D) Setup for electrode implantation.
(E) Electrode implanted ~200 μm beneath the cortical surface.

Note: The circle should match the size of the glass coverslip.

Note: Use light pressure while drilling to prevent drilling too far and cause bleeding. To determine how close to the brain you are, regularly apply saline or ACSF as this makes the skull appear more transparent. Saline can be used at the beginning of the surgery, but make sure to use ACSF instead of saline as the skull gets thin.

Note: Pause drilling every 5–10 seconds to irrigate the area with saline or ACSF to prevent the skull and brain from heating up.

⚠ CRITICAL: Be careful not to drill too far through the skull and cause bleeding of the brain (troubleshooting 7). Blood can kill neurons and elicits a strong immune response which will result in poor or occluded images.

26. Before removing the central piece of skull, drill a shallow ramp at the posterior of the craniotomy (Figure 2C).

Note: The shallow ramp is not as deep as the cranial window, but it is a shallower ramp so the electrode can be implanted diagonally.

⚠ CRITICAL: It is critical to make space for the electrode that will be implanted so that the electrode enters the brain at the far edge of the craniotomy, letting the glass window lay flat against the brain.

27. Remove the skull.

a. Use sharp forceps or a bent needle tip to carefully lift and remove the central piece of skull from the craniotomy.
b. Regularly apply ACSF to keep the exposed brain surface moist.

Optional: A piece of absorbable gelatin sponge soaked into ACSF may be used to cover the exposed brain surface.

Note: Leaving the brain surface soaked with ACSF for a few minutes may remove some blood.

28. Perform the viral injections.
    a. Use a micromanipulator to position the micropipette to the target brain area.
    b. Remove gelatin sponge and cover craniotomy with ACSF.
    c. Lower the micropipette until it just touches the cortical surface and zero the z axis of the micromanipulator.
    d. Remove ACSF using a fine-tipped tissue.

    Note: A fine tip can easily be created from a Kimwipe by twisting one corner.

    e. Slowly lower the micropipette until it pierces the brain.

    Note: This is often evident because the brain is initially somewhat compressed while the pipette is lowered and recoils once the pipette pierces the dura and enters the brain.

    f. Adjust the position of the micropipette to target your desired depth (~300 μm below the brain surface).
    g. Inject 100–200 nL of virus at 5 nL/s in the target location. Once liquid is fully injected, wait 2 min without moving the pipette. Then, raise the pipette to 150 μm below the brain surface and repeat.
    h. Carefully inspect the micropipette for bleeding during the infusion (troubleshooting 8).
    i. Withdraw the pipette from the brain. Repeat the procedure at two or more nearby locations if a larger imaging region is desired.

    Note: Waiting two minutes after injecting the full virus volume will ensure the virus does not leak out of the injection site and is therefore fully injected into the desired brain region (troubleshooting 9).

    Note: Pipette insertion can be facilitated by using the bent tip of a fine needle to incise the dura and reduce brain dimpling during insertion.

    Note: After withdrawing the pipette between each injection site, expel a small amount (~5 nL at 1 nL/s) of liquid into the air to ensure there is no blockage in the pipette (troubleshooting 5). Carefully absorb the expelled liquid using the tip of a Kimwipe.

29. Administer another dose of Ketamine if needed following assessment of breathing speed and toe/tail pinches.
30. Secure an electrode onto a micromanipulator and find the optimal initial position of electrode implantation.
    a. To target a depth of 200 μm, place the manipulator at a shallow angle (10–15 degrees; Figure 2D).
    b. Locate the optimal site for electrode insertion by first placing the electrode tip at the center of the craniotomy (a few mm above the cortical surface) and then moving it directly posterior until you reach the edge of the craniotomy (Figure 2C).

⚠ CRITICAL: The point is to adjust the electrode implantation angle to match the angle of the ramp drilled in the previous step, with a goal of having the coverslip lay flat against the surface of the brain (troubleshooting 10). The angle adjustment will depend on the target location for electrode implantation and on the anatomical structure of the mouse. One important consideration is to avoid piercing major blood vessels during implantation. The angle of the micromanipulator is generally set at 10°–15° but it can be adjusted. Adjusting the angle of the mouse's head may be essential for this step to ensure that the electrode is implanted at a depth of ~200 μm in the center of the cranial window. Especially pay attention to the posterior skin to prevent it from bending the electrode during implantation.

31. Lower the electrode by controlling the micromanipulator until it touches the surface of the brain and slowly insert the electrode into the brain to the target depth (Figure 2E).

Note: Good practice is to use coarse mode while piercing the surface of the brain and then switching into fine mode for slower implantation until you reach the desired depth.

32. Gently place the coverslip on the surface of the brain.

⚠ CRITICAL: It is important to ensure that the coverslip contacts the surface of the brain (troubleshooting 10). If there is an empty interspace, tissues may grow and degrade imaging quality. Step 26 is critical for this step (Figure 2C).

Note: Kwik-Sil may be applied between the brain and the coverslip. However, imaging quality is optimal when the coverslip is parallel to the brain surface, which is easiest to achieve by directly placing the coverslip on the brain.

33. Fix the coverslip to the skull by applying cyanoacrylate around the edge of the coverslip.

⚠ CRITICAL: Be particularly careful to prevent the glue running on or underneath the coverslip that obscures the window and eventually disables imaging. When the size of the removed skull fits to the size of the coverslip, the glue does not get under the coverslip. If glue covers the top of the window, it can later be removed by gently rubbing a cotton-tipped applicator soaked in 70% ethanol over the affected area.

Note: A useful tip for carefully sealing the cranial window is to dab some dental cement at a distance of 1 mm from the window and then gently drag a portion of the cement to the edge of the window. This procedure prevents the application of too much glue which could either cover the window or flow onto the brain. The dental cement mixture should also be carefully prepared so that it is viscous enough to not flow underneath the coverslip if carefully applied in this manner.

34. After the glue securing the cranial window dries, secure a small portion of the remaining electrode to the skull by putting dental cement on and around the exposed electrode. Wait for dental cement to fully dry.
35. Use sharp wire cutters to clip the exposed section of the electrode as it exits the dental cement.

Note: Use very sharp wire cutters to avoid pulling out the implanted electrode.

36. Transfer the mouse to a closed clean cage or container over a heating pad and leave it until recovery (i.e., mouse is alert and walking). After the animal is fully recovered, return it to its home cage.

37. For two days after surgery, inject analgesia (e.g., 5–10 mg/kg of Meloxicam) every 12–24 h based on any appearance of pain or distress (e.g., lethargy, hunched posture, significant gain or loss of weight, or barbering). Monitor the animal for signs of inflammation or infection (e.g., swelling, bleeding, or abnormal growth in and around surgical site) (troubleshooting 4).

Implanting a connector

Timing: 1 h

In this step, you implant a connector attached to the implanted electrode so the electrical stimulation can be applied connected to a stimulator. This step might not be necessary if an electrode array is implanted in the previous surgical step or depending on the electrode being used. Skip or adjust the procedure depending on the electrode you choose. Here we describe an example protocol.

Pre-surgery

38. Check the status of the cranial window using a two-photon microscope two weeks after the electrode implantation. Proceed with the connector implantation if the implant is good and neurons are visible.
39. Strip approximately 2 mm from each end of an insulated silver wire and solder one end to one output site on the bottom of a two-pin female connector.

Surgery

40. Prepare for surgery: perform step 1.
41. Anesthetize and head-fix the mouse.
    a. Anesthetize the mouse to areflexia using 2–3% isoflurane in oxygen (flow rate of 1 L/min) in an anesthesia induction chamber.

    Note: Alternatively, Ketamine (90–150 mg/kg) and Xylazine (5–10 mg/kg) can be used. We recommend using isoflurane for shorter surgeries (<1 h); however, ketamine and xylazine is recommended for longer surgeries due to the inflammatory effect that isoflurane has on the brain.

    b. Place the mouse on top of a temperature-regulated heating pad set to 37.0°C. Secure the mouse's head by screwing the headplate onto a headplate holder.

    Note: Alternatively, you can secure the mouse's head in a conventional stereotaxic frame.

    c. Lower isoflurane to 1.5%–2% during surgery. Alternatively administer Ketamine/Xylazine as in step 20.

⚠ CRITICAL: Monitor the mouse to ensure that the level of anesthesia is adequate to reduce respiratory rate and prevent reflexive responses to a toe or tail pinch. Adjust the isoflurane level depending on the response of the mouse (troubleshooting 1 and 2).

42. Apply ophthalmic ointment to protect the mouse's eyes.
43. If needed, expose the end of the electrode by drilling a small portion of dental cement with a dental drill.
44. Make a burr hole in the skull using a dental drill with a 0.5 mm tip on the opposite hemisphere as far as possible from the electrode implantation spot.

    Note: For electrical stimulation of visual cortex, we typically target the ground electrode as anterior as possible.

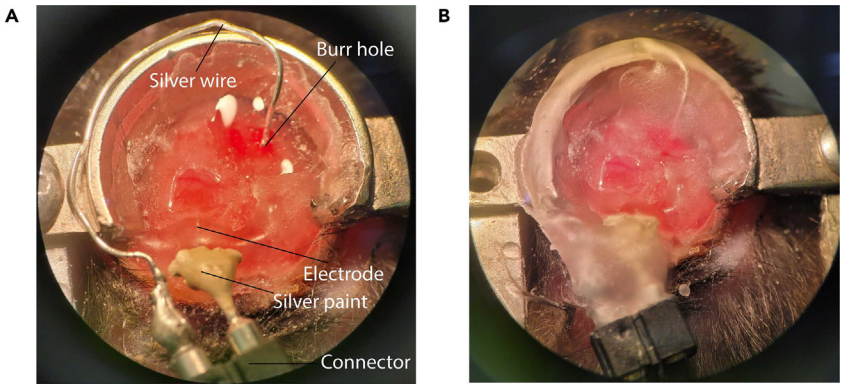


Figure 3. Implanting a connector

(A) Silver wire inserted beneath the skull and secured with Kwik-Sil and dental cement. Connector fixed to the electrode using silver paint.

(B) Connector, electrode, and ground wire secured and covered with dental cement.

45. Fill the burr hole with ACSF and insert the silver wire connected to the connector so that it lies beneath the skull but does not pierce the brain.
46. Fix the inserted silver wire with silastic sealants (e.g., Kwik-Sil) and cover with dental cement (Figure 3A).
47. Fix the connector to the exposed tip of the implanted electrode using silver paint (Figure 3A). Position the electrode at the right position, apply silver paint, and wait for 5 min until the silver paint dries.
48. Cover and secure the connector and electrode with dental cement (Figure 3B).
49. Finally, clean the cranial window with saline and ethanol to remove dirt and glue to improve imaging.
50. Transfer the mouse to a closed clean cage or container over a heating pad and leave it until recovery (i.e., mouse is alert and walking). After the animal is fully recovered, return it to its home cage.
51. Several days after surgery, verify the success of the connector implantation by measuring the impedance of the electrode.

Note: The impedance should be similar to the pre-implantation impedance (troubleshooting 11).

EXPECTED OUTCOMES

This protocol was developed to record neural responses to electrical stimulation in awake, chronically-implanted mice, which mimics clinical applications[23–29] of electrical stimulation. While clinical applications use a multi-channel array, we use a single-channel array in the protocol. Calcium imaging measures fluctuations in calcium concentration within neural cell bodies and other neural processes. Calcium concentration fluctuations reflect changes in neural activity levels. Thus, calcium imaging can be used to compare baseline neural activity prior to electrical stimulation with neural activity induced by electrical stimulation.

Calcium imaging data is obtained in the form of two-dimensional fluorescence images that are collected using a fluorescent microscope such as a two-photon microscope (Figure 4A). In the image shown here, the electrode tip is clearly visible within the center of the imaging frame. For two-photon recordings, a laser with wavelength of 920 nm is used for GCaMP excitation. In our application, neural recordings are conducted at 15.5 Hz at approximately 150–200 μm below the cortical surface (the depth of the electrode tip) in head-fixed mice that freely run on a spherical treadmill.[30] A train of stimuli were applied with various amplitudes once every 10 s for a total of 100 trials. Each stimulus consisted of 25 biphasic cathode-leading pulses delivered at 250 Hz. Electrical stimulation and two

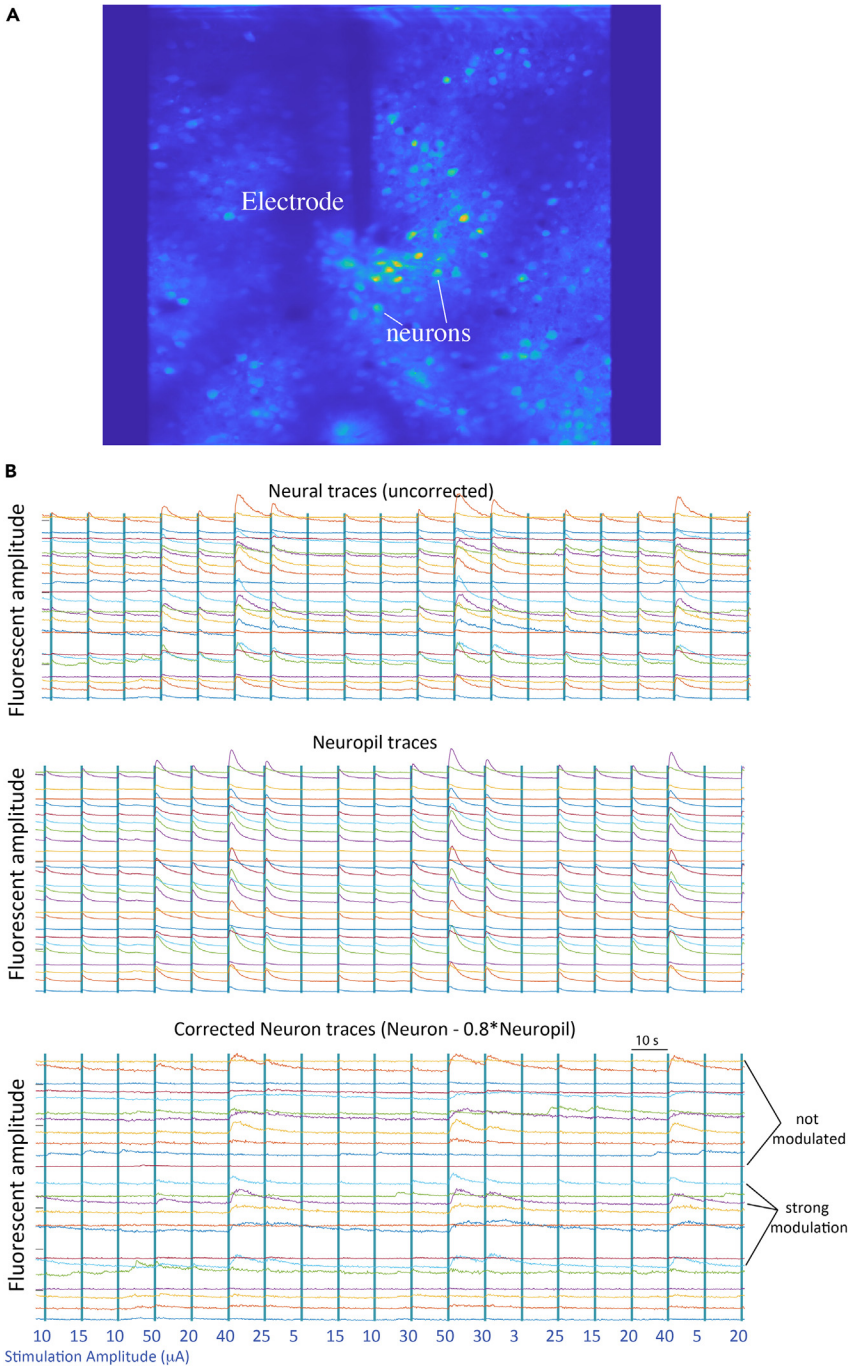


Figure 4. Neural recordings of stimulation-evoked activity

(A) Sample two-photon image of fluorescent neurons and implanted electrode.

(B) Sample neural activity patterns evoked by electrical stimulation at variable stimulation amplitudes are shown during stimulation. Signals (in arbitrary units of fluorescence) show neural traces from detected ROIs (top), neuropil data from an annulus surrounding each ROI (middle), and single-neuron traces following neuropil correction (neural responses minus 0.8*neuropil signal; bottom). Vertical green lines show time point of stimulation, delivered 10 s apart.

photon imaging data are synchronized via TTL pulses sent from the stimulator to the two-photon microscope during stimulation. The imaging frame number at the time of TTL arrival is saved as part of the experimental dataset.

Recorded images were processed by Suite2p to register frames and detect regions of interest (ROI).[31] Custom MATLAB code is used to conduct the subsequent analyses.[32] Given the large, synchronized activation of neuropil following electrical stimulation (Figure 4B, top and middle panels), single neuron responses were estimated by subtracting 0.8 times the neuropil signal from each fluorescent trace from each region of interest with a high (>0.5) probability of being neuron (as defined by Suite2p).

In the neuropil-corrected data (Figure 4B, bottom panel), we could find different groups of neurons: 1) a subset of neurons that are not modulated by the electrical stimulation, 2) a subset of neurons that are strongly modulated by the electrical stimulation, and 3) a subset that have mixed responses. The result shows that the electrical stimulation can elicit strong and synchronized activity in a population of neurons and demonstrates the feasibility of the protocol to record reliable induced neural activity by the electrical stimulation with two-photon calcium imaging. Recording neural activity throughout the stimulation period might lead to a better understanding of the mechanisms driving stimulation-induced neural activity within the cortex.

LIMITATIONS

Even though the protocol is well established and organized, high proficiency of surgical skill is required for a successful procedure. Therefore, the protocol may have high inter-individual variation in success rate and require multiple practice procedures. We found that separating this protocol into three surgeries is easier for the surgeon and has stable outcomes for the animals (thus leading to higher success rates); however, consolidating procedures into fewer surgeries may minimize stress on the animal.

There are several major general limitations of using two-photon calcium imaging to study neural activity evoked by electrical stimulation. First, two-photon imaging has low temporal resolution compared to electrophysiological recordings,[33] which limits the scientific questions that can be addressed with this approach. Second, two-photon imaging is primarily limited to upper layers of cortex.[34,35] Layers 2/3 of cortex have a distinct neural composition and neural connectivity[36] such that recordings of stimulation-evoked activity patterns in these layers may not directly predict activation patterns in deeper layers of cortex. Third, variability in the surgical procedure can affect the performance of the protocol; however, carefully following the detailed steps described in this protocol can reduce heterogeneity across mice.

TROUBLESHOOTING

Problem 1

Mouse anesthetic state under isoflurane anesthesia fluctuates during the surgery (which can be evaluated by observing any movement and reflexive responses to a toe or tail pinch) (Steps 4 and 41).

Potential solution

Increase the level of the isoflurane and observe the mouse by checking reflexive responses to a toe or tail pinch. However, it is not recommended to use extremely high levels of isoflurane. The level of isoflurane will depend on the physiology of individual mice. Additionally, these symptoms may indicate a suboptimal head position that is restricting airflow. Adjust the head position and ear bars and observe the status of the mouse.

Problem 2

Mouse exhibits agonal breathing under isoflurane anesthesia, identifiable by gasping, vocalizations, and large abdominal movements (Steps 4 and 41).

Potential solution

These symptoms show that the isoflurane level is too high. Decrease the level of isoflurane and observe the status of the mouse. Make sure the decreased level is not too low. Additionally, these

symptoms may indicate a suboptimal head position that is restricting airflow. Adjust the head position and ear bars and observe the status of the mouse.

Problem 3

The implanted headplate detaches after surgery (Steps 9 and 10).

Potential solution

If no other procedures have occurred and the protocol permits, the headplate could be re-implanted; however, it is recommended to euthanize the mouse to minimize stress for the animals. To avoid detachment, steps 9 and 10 are critical.

Problem 4

Mouse shows signs of pain or infection after the procedures. This can be observed by irregular behavior, lethargy, hunched posture, grimaced facial expressions, and significant loss/gain of weight (Steps 15 and 37).

Potential solution

Keep observing and evaluating the status of the mouse and provide appropriate medical aids. If the symptoms are too severe or do not improve within a few days, euthanasia is recommended to minimize the pain.

Problem 5

Air bubbles or blockages occur in the micropipette while expelling mineral oil or withdrawing/expelling virus (Steps 17 and 28).

Potential solution

If a blockage occurs (i.e., liquid is not expelling from the micropipette), stretch a Kimwipe flat and carefully pierce the tip of the micropipette through the Kimwipe. If air bubbles are present, expel the liquid in the micropipette until the air bubble is gone.

Problem 6

Mouse wakes up (which can be evaluated by observing any movement and reflexive responses to a toe or tail pinch) under Ketamine anesthesia (Step 20).

Potential solution

Administer additional injections of Ketamine. Wait at least 10 min before giving additional doses as too much Ketamine can kill the animal. As a general rule, give one full dose every 1 h or a half dose every 30 min.

Problem 7

Brain starts to bleed while drilling the craniotomy (Step 25).

Potential solution

Stop drilling and apply ACSF on top of the craniotomy. Let the ACSF sit until the blood clots. Soak up the ACSF with a Kimwipe and repeat if the blood does not clot right away. A gelatin sponge may also be used to soak up the blood. If the bleeding does not stop after 7–10 min, the neurons may be seriously affected; therefore, the animal should be euthanized.

Problem 8

Bleeding during viral infusion (Step 28).

Potential solution

A small amount of bleeding during infusion is not a problem. If it occurs, finish the infusion steps, carefully remove the pipette, and use gelfoam soaked in ACSF to gently remove the blood from

the cortical surface. In contrast, if there is a lot of blood, immediately remove the pipette and cover the cortex with gelfoam. Once the bleeding stops (which make take a few minutes) and the blood is removed, repeat the infusion protocol in a new location.

Problem 9

Virus leaks out of injection site and onto brain surface (Step 28).

Potential solution

The virus may leak out of the injection site if the pipette is removed too early following infusion. If this occurs, carefully position pipette in a nearby location and repeat steps for injection. If the virus leaks out of the brain during infusion, the infusion rate and pressure are likely too high. Maintain the pipette in position, lower the infusion rate, and repeat the infusion protocol.

Problem 10

Electrode implant is at the wrong angle for the cranial window to lie flat against the brain (Steps 30 and 32).

Potential solution

Use a clear silicone elastomer (such as Kwik-Sil) to fill the space. Lay window flat on top of Kwik-Sil.[19] Then, the elevated window can be attached to the skull using either cyanoacrylate or dental cement.

Problem 11

Electrode has an irregular impedance (zero or several MOhm) (Step 51).

Potential solution

If the impedance is zero, there is a short somewhere in the circuit. This is most likely to happen during electrode implantation if the conductive silver paint is not carefully applied. To avoid this issue, make sure to sparsely apply silver paint to connect the electrode to the connector pin. In addition, insulate the ground connector pin before implantation by applying a light layer of cyanoacrylate. Additionally make sure that all bare wires are insulated (e.g., with cyanoacrylate) at the end of the connector implantation. If the impedance is in the MOhm range, the circuit is open. This can happen due to a faulty connection between the electrode and the connector pin (e.g., failing to apply sufficient conductive silver paint) or due to a failure of the ground wire. One alternative to using a ground wire inserted through a burr hole is to implant a ground screw that has been soldered to the ground wire prior to surgery.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maria Dadarlat (mdadarla@purdue.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Seungbin Park (park1377@purdue.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Original data and code have been deposited to Mendeley Data: DOI 10.17632/tzwps2dykh.2.[32]

ACKNOWLEDGMENTS

The work completed in this article was supported by NSF HDR grant 2117997 and the Ralph W. and Grace M. Showalter Research Trust.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, M.D. and Y.J.S.; figures, M.D. and S.P.; manuscript writing, S.P. and M.D.; review and editing, S.P., M.L., and M.D.; animal management, M.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Dadarlat, M.C., Sun, Y.J., and Stryker, M.P. (2024). Activity-dependent recruitment of inhibition and excitation in the awake mammalian cortex during electrical stimulation. Neuron *112*, 821–834.e4. https://doi.org/10.1016/j.neuron.2023.11.022.

2. Histed, M.H., Bonin, V., and Reid, R.C. (2009). Direct Activation of Sparse, Distributed Populations of Cortical Neurons by Electrical Microstimulation. Neuron *63*, 508–522. https://doi.org/10.1016/j.neuron.2009.07.016.

3. Histed, M.H., Ni, A.M., and Maunsell, J.H.R. (2013). Insights into cortical mechanisms of behavior from microstimulation experiments. Prog. Neurobiol. *103*, 115–130. https://doi.org/10.1016/j.pneurobio.2012.01.006.

4. Margalit, S.N., and Slovin, H. (2018). Spatio-temporal characteristics of population responses evoked by microstimulation in the barrel cortex. Sci. Rep. *8*, 13913. https://doi.org/10.1038/s41598-018-32148-0.

5. Stoney, S.D., Thompson, W.D., and Asanuma, H. (1968). Excitation of pyramidal tract cells by intracortical microstimulation: effective extent of stimulating current. J. Neurophysiol. *31*, 659–669. https://doi.org/10.1152/jn.1968.31.5.659.

6. Jankowska, E., Padel, Y., and Tanaka, R. (1975). The mode of activation of pyramidal tract cells by intracortical stimuli. J. Physiol. *249*, 617–636. https://doi.org/10.1113/jphysiol.1975.sp011034.

7. Asanuma, H., Arnold, A., and Zarzecki, P. (1976). Further study on the excitation of pyramidal tract cells by intracortical microstimulation. Exp. Brain Res. *26*, 443–461. https://doi.org/10.1007/BF00238820.

8. Butovas, S., and Schwarz, C. (2003). Spatiotemporal Effects of Microstimulation in Rat Neocortex: A Parametric Study Using Multielectrode Recordings. J. Neurophysiol. *90*, 3024–3039. https://doi.org/10.1152/jn.00245.2003.

9. Hao, Y., Riehle, A., and Brochier, T.G. (2016). Mapping Horizontal Spread of Activity in Monkey Motor Cortex Using Single Pulse Microstimulation. Front. Neural Circuits *10*, 104. https://doi.org/10.3389/fncir.2016.00104.

10. Weisenburger, S., Tejera, F., Demas, J., Chen, B., Manley, J., Sparks, F.T., Martínez Traub, F., Daigle, T., Zeng, H., Losonczy, A., and Vaziri, A. (2019). Volumetric Ca2+ Imaging in the Mouse Brain Using Hybrid Multiplexed Sculpted Light Microscopy. Cell *177*, 1050–1066.e14. https://doi.org/10.1016/j.cell.2019.03.011.

11. Stringer, C., Pachitariu, M., Steinmetz, N., Carandini, M., and Harris, K.D. (2019). High-dimensional geometry of population responses in visual cortex. Nature *571*, 361–365. https://doi.org/10.1038/s41586-019-1346-5.

12. Stringer, C., Pachitariu, M., Steinmetz, N., Reddy, C.B., Carandini, M., and Harris, K.D. (2019). Spontaneous behaviors drive multidimensional, brainwide activity. Science *364*, 255. https://doi.org/10.1126/science.aav7893.

13. Peters, A.J., Chen, S.X., and Komiyama, T. (2014). Emergence of reproducible spatiotemporal activity during motor learning. Nature *510*, 263–267. https://doi.org/10.1038/nature13235.

14. Michelson, N.J., Eles, J.R., Vazquez, A.L., Ludwig, K.A., and Kozai, T.D.Y. (2019). Calcium activation of cortical neurons by continuous electrical stimulation: Frequency dependence, temporal fidelity, and activation density. J. Neurosci. Res. *97*, 620–638. https://doi.org/10.1002/jnr.24370.

15. Eles, J.R., Stieger, K.C., and Kozai, T.D.Y. (2021). The temporal pattern of intracortical microstimulation pulses elicits distinct temporal and spatial recruitment of cortical neuropil and neurons. J. Neural. Eng. *18*, 015001. https://doi.org/10.1088/1741-2552/abc29c.

16. Eles, J.R., and Kozai, T.D.Y. (2020). In vivo imaging of calcium and glutamate responses to intracortical microstimulation reveals distinct temporal responses of the neuropil and somatic compartments in layer II/III neurons. Biomaterials *234*, 119767. https://doi.org/10.1016/j.biomaterials.2020.119767.

17. Suematsu, N., Vazquez, A.L., and Kozai, T.D. (2024). Activation and depression of neural and hemodynamic responses induced by the intracortical microstimulation and visual stimulation in the mouse visual cortex. Preprint at bioRxiv. https://doi.org/10.1101/2024.01.01.573814.

18. Kozai, T.D.Y., Eles, J.R., Vazquez, A.L., and Cui, X.T. (2016). Two-photon imaging of chronically implanted neural electrodes: Sealing methods and new insights. J. Neurosci. Methods *258*, 46–55. https://doi.org/10.1016/j.jneumeth.2015.10.007.

19. Dana, H., Chen, T.-W., Hu, A., Shields, B.C., Guo, C., Looger, L.L., Kim, D.S., and Svoboda, K. (2014). Thy1-GCaMP6 Transgenic Mice for Neuronal Population Imaging In Vivo. PLoS One *9*, e108697. https://doi.org/10.1371/journal.pone.0108697.

20. Daigle, T.L., Madisen, L., Hage, T.A., Valley, M.T., Knoblich, U., Larsen, R.S., Takeno, M.M., Huang, L., Gu, H., Larsen, R., et al. (2018). A Suite of Transgenic Driver and Reporter Mouse Lines with Enhanced Brain-Cell-Type Targeting and Functionality. Cell *174*, 465–480.e22. https://doi.org/10.1016/j.cell.2018.06.035.

21. Taniguchi, H., He, M., Wu, P., Kim, S., Paik, R., Sugino, K., Kvitsiani, D., Fu, Y., Lu, J., Lin, Y., et al. (2011). A Resource of Cre Driver Lines for Genetic Targeting of GABAergic Neurons in Cerebral Cortex. Neuron *71*, 995–1013. https://doi.org/10.1016/j.neuron.2011.07.026.

22. Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. *13*, 133–140. https://doi.org/10.1038/nn.2467.

23. Flesher, S.N., Collinger, J.L., Foldes, S.T., Weiss, J.M., Downey, J.E., Tyler-Kabara, E.C., Bensmaia, S.J., Schwartz, A.B., Boninger, M.L., and Gaunt, R.A. (2016). Intracortical microstimulation of human somatosensory cortex. Sci. Transl. Med. *8*, 361ra141. https://doi.org/10.1126/scitranslmed.aaf8083.

24. Armenta Salas, M., Bashford, L., Kellis, S., Jafari, M., Jo, H., Kramer, D., Shanfield, K., Pejsa, K., Lee, B., Liu, C.Y., and Andersen, R.A. (2018). Proprioceptive and cutaneous sensations in humans elicited by intracortical microstimulation. Elife *7*, e32904. https://doi.org/10.7554/eLife.32904.

25. Hughes, C.L., Flesher, S.N., Weiss, J.M., Boninger, M., Collinger, J.L., and Gaunt, R.A. (2021). Perception of microstimulation frequency in human somatosensory cortex. Elife *10*, e65128. https://doi.org/10.7554/eLife.65128.

26. Fernández, E., Alfaro, A., Soto-Sánchez, C., Gonzalez-Lopez, P., Lozano, A.M., Peña, S., Grima, M.D., Rodil, A., Gómez, B., Chen, X., et al. (2021). Visual percepts evoked with an intracortical 96-channel microelectrode array

inserted in human occipital cortex. J. Clin. Invest. *131*, e151331. https://doi.org/10.1172/JCI151331.

27. Flesher, S.N., Downey, J.E., Weiss, J.M., Hughes, C.L., Herrera, A.J., Tyler-Kabara, E.C., Boninger, M.L., Collinger, J.L., and Gaunt, R.A. (2021). A brain-computer interface that evokes tactile sensations improves robotic arm control. Science *372*, 831–836. https://doi.org/10.1126/science.abd0380.

28. Fifer, M.S., McMullen, D.P., Osborn, L.E., Thomas, T.M., Christie, B., Nickl, R.W., Candrea, D.N., Pohlmeyer, E.A., Thompson, M.C., Anaya, M.A., et al. (2022). Intracortical Somatosensory Stimulation to Elicit Fingertip Sensations in an Individual With Spinal Cord Injury. Neurology *98*, e679–e687. https://doi.org/10.1212/WNL.0000000000013173.

29. Shelchkova, N.D., Downey, J.E., Greenspon, C.M., Okorokova, E.V., Sobinov, A.R., Verbaarschot, C., He, Q., Sponheim, C., Tortolani, A.F., Moore, D.D., et al. (2023). Microstimulation of human somatosensory cortex evokes task-dependent, spatially patterned responses in motor cortex. Nat. Commun. *14*, 7270. https://doi.org/10.1038/s41467-023-43140-2.

30. Dombeck, D.A., Khabbaz, A.N., Collman, F., Adelman, T.L., and Tank, D.W. (2007). Imaging Large-Scale Neural Activity with Cellular Resolution in Awake, Mobile Mice. Neuron *56*, 43–57. https://doi.org/10.1016/j.neuron.2007.08.003.

31. Pachitariu, M., Stringer, C., Schröder, S., Dipoppa, M., Rossi, L. F., Carandini, M., and Harris, K. D. (2016). Suite2p: beyond 10,000 neurons with standard two-photon microscopy. BioRxiv, 061507.

32. Dadarlat, M. (2023). Activity-dependent recruitment of inhibition and excitation in the awake mammalian cortex during electrical stimulation (Mendeley Data). https://doi.org/10.17632/TZWPS2DYKH.2.

33. Siegle, J.H., Ledochowitsch, P., Jia, X., Millman, D.J., Ocker, G.K., Caldejon, S., Casal, L., Cho, A., Denman, D.J., Durand, S., et al. (2021). Reconciling functional differences in populations of neurons recorded with two-photon imaging and electrophysiology. Elife *10*, e69068. https://doi.org/10.7554/eLife.69068.

34. Denk, W., Strickler, J.H., and Webb, W.W. (1990). Two-photon laser scanning fluorescence microscopy. Science *248*, 73–76. https://doi.org/10.1126/science.2321027.

35. Dombeck, D.A., Khabbaz, A.N., Collman, F., Adelman, T.L., and Tank, D.W. (2007). Imaging large-scale neural activity with cellular resolution in awake, mobile mice. Neuron *56*, 43–57. https://doi.org/10.1016/j.neuron.2007.08.003.

36. Petersen, C.C.H., and Crochet, S. (2013). Synaptic computation and sensory processing in neocortical layer 2/3. Neuron *78*, 28–48. https://doi.org/10.1016/j.neuron.2013.03.020.