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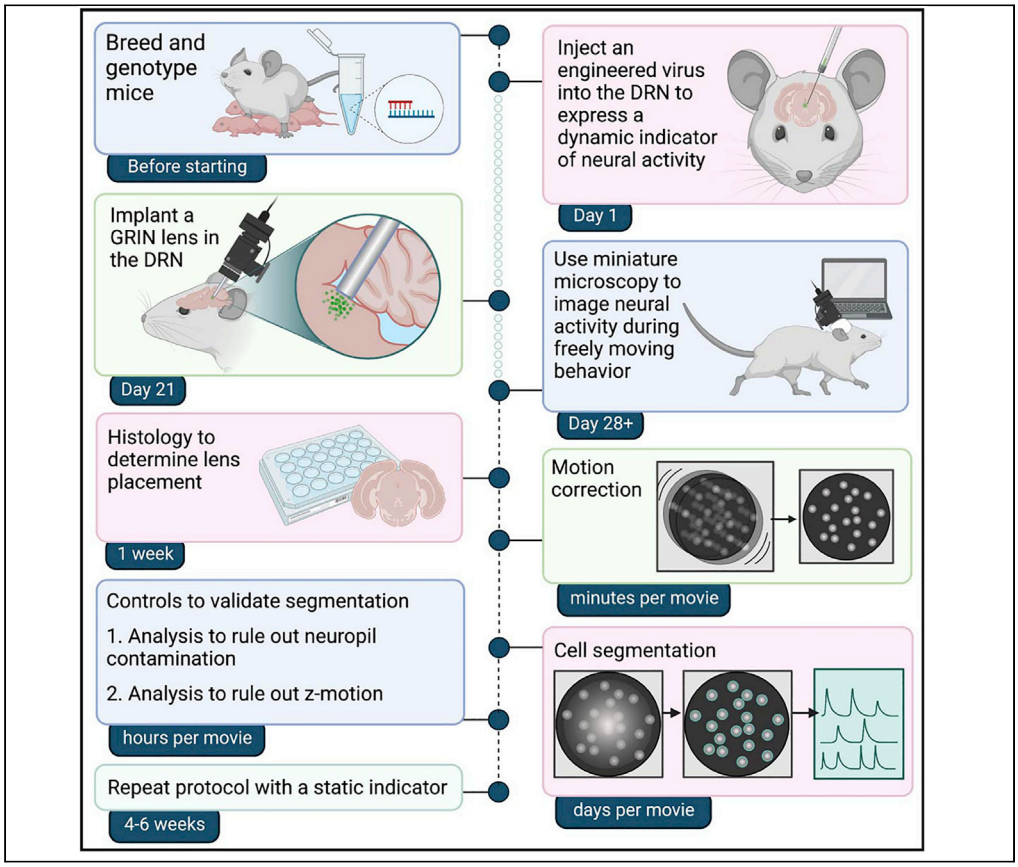
Protocol for *in vivo* imaging and analysis of brainstem neuronal activity in the dorsal raphe nucleus of freely behaving mice



In vivo brainstem imaging with miniature microscopy has been challenging due to surgical difficulty, high motion, and correlated activity between neurons. Here, we present a protocol for brainstem imaging in freely moving mice using the dorsal raphe nucleus as an example. We describe surgical procedures to inject a virus encoding GCaMP6m and securely implant a GRIN lens in the brainstem. We then detail motion correction and cell segmentation from the data to parse single-cell activity from correlated networks.

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

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Highlights

Expression of GCaMP in serotonergic neurons of the mouse dorsal raphe nucleus (DRN)

Implantation of a GRIN lens in the mouse DRN for imaging in freely moving mice

Motion correction by background subtraction and cross-correlations to a reference frame

Cell segmentation and data validation from areas with high motion and correlated activity

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SUMMARY

***In vivo* brainstem imaging with miniature microscopy has been challenging due to surgical difficulty, high motion, and correlated activity between neurons. Here, we present a protocol for brainstem imaging in freely moving mice using the dorsal raphe nucleus as an example. We describe surgical procedures to inject a virus encoding GCaMP6m and securely implant a GRIN lens in the brainstem. We then detail motion correction and cell segmentation from the data to parse single-cell activity from correlated networks.**

For complete details on the use and execution of this protocol, please refer to Paquelet et al. (2022).[1]

BEFORE YOU BEGIN

This protocol includes details specific to imaging DRN serotonergic neurons ($DRN^{5\text{-}HT}$) using the virally-expressed calcium indicator GCaMP6m in male Sert-Cre mice.[2,3] However, the experimental approach can be adapted for use in other rodent species by adjusting stereotactic coordinates, and for the imaging of different cell populations using different mouse lines and viral constructs. The analytical methods can be used on calcium imaging data from any brain area with highly correlated activity between neurons or significant brain motion.

Institutional permissions

Researchers must acquire permissions from the institutions that regulate their use of live vertebrate animals. Our procedures were conducted in accordance with the U.S. NIH Guide for the Care and Use of Laboratory Animals and the New York State Psychiatric Institute Institutional Animal Care and Use Committees at Columbia University.

Mate and genotype mice

Timing: 8–10 weeks

1. Mate mice according to the husbandry instructions from the source laboratory or vendor. For Sert-Cre mice, see these instructions from The Jackson Laboratory.

⚠ CRITICAL: Because Sert-Cre is a knock-in transgenic line, heterozygotes have only one copy of the gene encoding the serotonin transporter. Avoid homozygotes by mating a heterozygote with either a homozygote or an appropriate wild-type control (see the linked instructions).

2. Identify mice expressing Cre recombinase using your preferred genotyping method.

Note: For the use of this protocol in Paquelet et al.,[1] mice were first assigned an identification number using an ear punch. Then, the tip of each mouse's tail was harvested and DNA was extracted using Proteinase K and the DirectPCR Lysis Reagent according to the manufacturer's instructions. Samples were genotyped using EconoTaq PLUS GREEN using the protocol described here. Primers were chosen to identify both Cre and a positive control as described here by The Jackson Laboratory.

Prepare materials

Timing: 30 min

3. Aliquot all viruses into PCR tubes and store them in a −80°C freezer. We recommend an aliquot size of 2–4 μL.
4. Prepare medicated sucralose gels for the postoperative delivery of carprofen.
   a. Dilute carprofen in water to a final concentration of 0.5 mg/mL. The addition of food coloring is recommended to ensure even mixing of the drug into the gel.
   b. Add 2.4 mL of this solution to each sucralose gel according to the manufacturer's instructions.

Note: This protocol will require one gel per cage following viral injection, and one gel per mouse following lens implantation. Gels can be stored for up to 2 weeks at 4°C.

5. Sterilize surgical tools using the method approved in your institutional animal use protocol.
6. Prepare phosphate-buffered saline solution (PBS) by diluting 10× PBS in purified water. This protocol will require up to 100 mL of PBS per mouse.
7. Sterilize (e.g., autoclave) at least 5 mL of PBS per mouse for use during surgeries.
8. Prepare the buffer used to dilute viruses by adding the following to 476 mL ultrapure distilled water: 14 mL of 5 M NaCl, 10 mL of 1 M Tris pH8.0, and 50 μL of 100× Pluronic F-68.

Note: Sterile PBS is an acceptable alternative to this buffer.

Prepare for data processing

Timing: 1–2 h

9. Download the Inscopix Data Processing Software (IDPS), FIJI (ImageJ), MATLAB, CNMF-E, CaITracer3beta, and the code deposited with this protocol. Links are provided in the key resources table.

Note: Complete copies of both CNMF-E and CaITracer3beta are also available within the code deposited with this protocol. These are static copies downloaded in 2021 and 2018, respectively. They have been confirmed to be compatible with our code in Matlab 2020a and 2021b.

10. In MATLAB, make sure the code deposited with this protocol is on your path.
11. Run the `cnmfe_setup` script to prepare to run CNMF-E.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
AAVDJ-DIO-EF1a-GCaMP6m	Stanford	Cat#GVVC-AAV-092
AAV5-flex-GFP	UNC Gene Therapies	Cat#AV4531
Chemicals, peptides, and recombinant proteins		
Isoflurane	Patterson Veterinary	Cat#1404322005
Sucrose	Sigma-Aldrich	Cat#S7903-5KG
Sodium azide	Sigma-Aldrich	Cat#S2002-100G
Rimadyl (carprofen), injectable	Patterson Veterinary	Cat#07-844-7425
Ethanol (200 proof anhydrous)	Decon Labs	Cat#V1001TP
Betadine	ThermoFisher Scientific	Cat#19-027136
5 M NaCl	Invitrogen	Cat#AM9760G
1 M Tris-HCl pH8.0	Invitrogen	Cat#15568025
Pluronic F-68 (100×)	Gibco	Cat#24040032
UltraPure Distilled Water	Invitrogen	Cat#10977023
Paraformaldehyde	Electron Microscopy Sciences	Cat#15714-S
Proteinase K	Sigma-Aldrich	Cat#03115828001
Ketamine, injectable	McKesson	Cat#485956
Experimental models: Organisms/strains		
Mouse: wild type controls: C57BL/6J (female, 8–12 weeks old)	Jackson Laboratory (jax.org)	Cat#000664; RRID: SCR_004633
Mouse: Sert-Cre: B6.129(Cg)-*Slc6a4*$^{tm1(cre)Xz}$/J (male, 8–12 weeks old)	Jackson Laboratory (jax.org)	Cat#014554; RRID: IMSR_JAX:014554
Oligonucleotides		
oIMR1084 (transgene, forward)	Integrated DNA Technologies	GCG GTC TGG CAG TAA AAA CTA TC
oIMR1085 (transgene, reverse)	Integrated DNA Technologies	GTG AAA CAG CAT TGC TGT CAC TT
oIMR7338 (internal positive control, forward)	Integrated DNA Technologies	CTA GGC CAC AGA ATT GAA AGA TCT
oIMR7339 (internal positive control, reverse)	Integrated DNA Technologies	GTA GGT GGA AAT TCT AGC ATC ATC C
Software and algorithms		
nVista Data Acquisition Software	Inscopix	www.inscopix.com
Inscopix Data Processing Software	Inscopix	www.inscopix.com
MATLAB	Mathworks	https://www.mathworks.com/products/matlab/;
CNMF-E	Zhou et al.[4] Lab of Liam Paninski	https://github.com/zhoupc/CNMF_E
CalTracer3beta	Lab of Rafael Yuste	http://www.columbia.edu/cu/biology/faculty/yuste/methods
FIJI (ImageJ)	National Institutes of Health Schindelin et al.[5]	https://imagej.nih.gov/ij/
Custom MATLAB code	This paper	github.com/Kang-Miller-Labs/DRN-Imaging-STAR-Protocols; Zenodo: https://doi.org/10.5281/zenodo.7497993
Other		
Hardened fine scissors (8.5 cm)	Fine Science Tools	Cat#14090-09
Curved forceps (Dumont #7b)	Fine Science Tools	Cat#11270-20
Straight forceps (Dumont #3c)	Fine Science Tools	Cat#11231-20
Tweezers (Dumont #5, Biological, Dumostar)	Electron Microscopy Sciences	Cat#0209-5-PO
Hemostats (Halsted-Mosquito, Curved)	Fine Science Tools	Cat#13009-12
Student scalpel handle #3	Fine Science Tools	Cat#91003-12
Scalpel blades (#10)	Fine Science Tools	Cat#10010-00
5-0 vicryl violet 18″ C-3 cutting sutures	AD Surgical	Cat#S-G518R13
Shaver (Wahl Mini Figura Pet Trimmer)	Amazon	Cat#B00LM334KC
Q-tips (small cotton-tipped applicators)	Thermo Fisher Scientific	Cat#23-400-119
SUGI absorption spears	Fine Science Tools	Cat#18105-01
Sterile latex surgical gloves	AD Surgical	Cat#A520-LTX70

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ideal Micro-Drill	Roboz Surgical Instrument Co.	Cat#6300A
Burr for Micro-Drill (0.5 mm tip diameter)	Fine Science Tools	Cat#19007-05
SomnoSuite Low-Flow Anesthesia System with heating pad	Kent Scientific	SomnoSuite
Stereotax with digital display	David Kopf Instruments	Model 940
Mouse gas anesthesia head holder	David Kopf Instruments	Model 923-B
UltraMicroPump with Micro4 controller	World Precision Instruments	UMP3
Dual gooseneck illuminator (150 W)	Stoelting	Cat#59259
MediGel Sucralose	ClearH_2O	Cat#74-02-5022
McCormick culinary blue food color	Amazon	Cat#B008OGD2RK
Puralube sterile ocular lubricant	Chewy	Cat#83592
Phosphate-buffered saline (PBS, 10×)	Bio Basic	Cat# PD8117
Tuberculin syringes (1 mL)	BD	Cat#309659
Hypodermic needles (27G × 0.5 in)	BD	Cat#305109
Vetbond tissue adhesive	3M Healthcare	Cat#084-1469SB
Topicaine 5	Amazon	Cat#B001CY9XW4
Kwik stop styptic powder	Miracle Care	Cat#423594
PCR tubes	SSIbio	Cat#3240-00S
Hamilton syringe (10 μL)	World Precision Instruments	NANOFIL
33 g beveled NanoFil needle	World Precision Instruments	Cat#NF33BV-2
Parafilm M	Parafilm	Cat#PM996
Toothpicks	Amazon	Cat# B01N753BQ3
Dental cement (Radiopaque L-powder for C&B Metabond)	Parkell Inc.	Cat#S396
"B" Quick Base for C&B Metabond	Parkell Inc.	Cat#S398
"C" Universal TBB Catalyst	Parkell Inc.	Cat#S371
Weigh boats	Heathrow Scientific	Cat#14251B
Surgifoam gelatin sponge	Thermo Fisher Scientific	Cat# NC0451961
Pan head, stainless steel machine screw (000-120 × 1/16")	Antrin Miniature Specialties	Cat#AMS120/1P
GRIN lens (ProView Integrated Lens), 0.5 × 6.1 mm	Inscopix	Cat#1050-004415
Miniature microscope (nVista3.0 system)	Inscopix	nVista3.0
Baseplate cover	Inscopix	Cat#1050-004639
Commutator	Inscopix	Cat#1000-005088
Kwik-Sil Silicone adhesive	World Precision Instruments	KWIK-SIL
Lens paper	Fisher Scientific	Cat#11-996
Cryostat sectioning blades	Leica	Cat#14035838926
24-well plates	Corning	Cat#353226
Vectashield antifade mounting medium	Vector Laboratory	Cat#H-1000
Superfrost plus microscope slides	Thermo Fisher Scientific	Cat#12-550-15
Rectangular cover glass	Thermo Fisher Scientific	Cat#12-543-DP
Nail polish	Electron Microscopy Sciences	Cat#72180
Embedding molds	Polysciences	Cat#18646A-1
Tissue-Tek O.C.T. Compound	Sakura	Cat#4583
Mouse Brain Atlas in Stereotaxic Coordinates	Academic Press	Franklin and Paxinos[6]
EconoTaq Green PCR mix	Lucigen	Cat#30033-1
DirectPCR Lysis Reagent (mouse tail)	Viagen Biotech	Cat#102-T
Quick-load 100 bp DNA Ladder	New England Biolabs	Cat#N0467S
Semimicro spatula	Fisher Scientific	Cat#14-374
Decapitation scissors	World Precision Instruments	Cat#501225
Perfusion pump	Traceable	Cat#3386
Skin marker	Fine Science Tools	Cat#18000-30
Stereomicroscope (3.5×–90× articulating microscope)	AmScope	Cat#SM-7TZ-FRL
Fluorescence microscope	Carl Zeiss	Cat#1016-758
Cryostat	Leica	Cat#CM3050 S

MATERIALS AND EQUIPMENT

General surgical tools

All materials and equipment referenced in this protocol are listed in the key resources table, save basic laboratory equipment including a PCR machine, water bath, sterilization equipment, e.g., an autoclave and bead sterilizer, lab tape, Styrofoam bucket, a centrifuge that can hold PCR tubes, a −80°C freezer, pipettes and corresponding pipette tips, glassware, tubes, and a computer. This section contains important information about the materials and equipment required for this protocol. This information is organized by the procedure for which the materials are required. Unless otherwise noted, all materials and equipment can be substituted for equivalent products from different vendors.

Both the viral injection and lens implantation will require basic surgical tools, including fine scissors, curved forceps, straight forceps, hemostats, sutures, drill bits, a fur trimmer, Q-tips, scalpel blades, a scalpel handle, SUGI absorption spears, tweezers, and sterile surgical gloves.

⚠ CRITICAL: All surgical tools that are not labeled sterile and for single use only should be sterilized prior to use and in between multiple surgeries on the same day according to your institutional animal use protocol.

Equipment used for all surgeries includes the Micro-Drill, isoflurane anesthesia system with heating pad, stereotax, gooseneck lamp, and a stereomicroscope with an articulating arm.

Alternatives: The SomnoSuite anesthesia system can be substituted for a standard isoflurane vaporizer (e.g., Parkland Scientific, Cat#PS5000V) with an oxygen tank and waste canister. In this case, it would also be necessary to have a separate heading pad, e.g., a T/pump warm water recirculator (Stryker, Kent Scientific, Cat#TP-700).

⚠ CRITICAL: It is important that the stereomicroscope used for lens implantation has an articulating arm such that the mouse can be viewed from the side during the lowering of the lens (step 29).

Surgeries also require mouse healthcare items, including the sucralose hydration gel, injectable carprofen, food coloring for the mixing of carprofen into the gel, ocular lubricant, ethanol, betadine, sterile PBS, injection syringes and needles, Vetbond, styptic powder, and Topicaine.

Alternatives: Instead of delivering postoperative carprofen using a Sucralose MediGel, mice can be injected once daily with carprofen in sterile PBS at a dose of 5 mg/kg.

Viral injection surgeries specifically require a Styrofoam bucket with ice for the transportation of the virus, P10 pipette and tips, a Hamilton syringe equipped with a 33G NanoFil needle, parafilm, and an injector (UltraMicroPump) with a digital controller. Prior to use, the virus is stored in PCR tubes, spun down in a centrifuge, and diluted using sterile PBS or dilution buffer using the following recipe. When prepared using sterile procedures, this buffer does not need to be autoclaved.

Virus dilution buffer

Reagent	Final concentration	Amount
5 M NaCl	140 mM	14 mL
1 M Tris pH8.0	20 mM	10 mL
Pluronic F-68 (100× = 10%)	0.001%	50 μL
UltraPure distilled water	N/A	476 mL

Store at room temperature (68–74°F) indefinitely.

Lens implantation requires toothpicks, dental cement (powder, base, catalyst, and accessories to scoop and mix the cement), Surgifoam, a weigh boat, sterile PBS, head screws with a compatible screwdriver, a lens, miniature microscope, Inscopix implantation hardware, baseplate cover, nVista imaging software, a computer, lens paper, and styptic powder. If using Proview lenses instead of Integrated lenses, implantation also requires separate baseplates and silicone adhesive.

Alternatives: Imaging GCaMP-expressing cells in the DRN can be accomplished using an nVista, nVoke, or nVue scope from Inscopix. Equivalent miniscopes from other suppliers have not been tested but should be compatible with this protocol.

Imaging experiments will require a computer and relevant behavioral apparatuses. The use of a commutator is recommended to prevent cable tangling during imaging experiments. The Inscopix commutator is compatible with nVista3.0, nVoke2.0, and nVue miniscopes.

Histology to determine lens placement is preceded by cardiac perfusion, which requires ketamine as an anesthetic, fine scissors, decapitation scissors, a spatula, hypodermic needles, a perfusion pump, PBS, and paraformaldehyde (PFA). Brain freezing, sectioning, and mounting requires PFA, PBS, embedding molds, O.C.T., a cryostat, cryostat blades, well plates, Vectashield mounting medium, glass slides and coverslips, nail polish, a fluorescence microscope, and a mouse brain atlas in stereotaxic coordinates. 5% sucrose and 0.05% sodium azide in PBS can optionally be used as a preservative for floating brain sections.

⚠ CRITICAL: Paraformaldehyde is a hazardous irritant of the skin, eyes, and lungs, and a probable carcinogen. Be careful to avoid contact by wearing PPE and avoid inhalation by working in a chemical fume hood.

⚠ CRITICAL: Use a different set of surgical tools for survival surgeries and terminal surgeries because of the use of PFA.

Alternatives: Brains may be sectioned on a vibratome or microtome with appropriate modifications to this protocol.

Finally, data processing and analysis requires the software listed in the key resources table and a relatively powerful computer.

⚠ CRITICAL: To run IDPS and CNMF-E, we recommend at least 64 GB of RAM, 1 TB on a solid-state drive, and a 3 GHz processor.

STEP-BY-STEP METHOD DETAILS

Viral injection

Timing: 30 min setup + 30 min per mouse

The first aim of this protocol is to optically access DRN neurons expressing a fluorescent indicator of neural activity (Figure 1). This is achieved in two surgeries, a viral injection and lens implantation. In this first step, the calcium indicator GCaMP6m is virally expressed in serotonergic neurons of the dorsal raphe nucleus (DRN). These methods follow standard practice for viral injections in the mouse brain, except for that the virus must be injected at an angle to avoid a confluence of blood vessels in the vertical path above the DRN.

1. The morning of the viral injection surgeries, assemble the required materials.

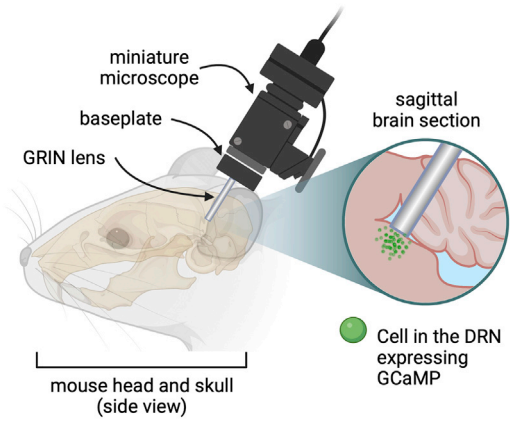

Figure 1. *In vivo* imaging of the mouse DRN using miniature microscopy

A GRIN lens is implanted through a craniotomy in the mouse skull at a posterior angle to avoid major sources of bleeding in the vertical path above the DRN. Cells in the DRN express an indicator of neural activity, e.g., the calcium indicator GCaMP. The baseplate allows for the attachment of a miniature microscope during imaging sessions.

a. Prepare a 1% dilution of 50 mg/mL carprofen in sterile PBS for perioperative analgesia. This surgery will require approximately 0.25–0.3 mL per mouse for a final dose of 5 mg/kg.
b. Retrieve and dilute the virus.
   i. Fill a Styrofoam bucket with ice.
   ii. Remove aliquots of the virus from the −80°C freezer and allow them to thaw in the in the ice bucket for 5 min. You will need approximately 1 μL per mouse.
   iii. Centrifuge the tubes containing the virus for several seconds to ensure the entire volume is collected at the bottom of each tube.
   iv. If using more than one aliquot, combine aliquots into a single PCR tube using a P10 pipette and the corresponding 10 μL pipette tips.
   v. Dilute the virus with the virus dilution buffer.

⚠ CRITICAL: The dilution ratio will depend on the concentration of viral particles, which is reported by the supplier. The final concentration should be approximately 2×10^{12} vg/mL to avoid toxicity at higher concentrations.

2. Prepare the surgical equipment.
   a. Load a fresh drill bit into the micro drill.
   b. Turn on the micro drill, light source, digital stereotax display, and the digital controller for the injector.
   c. Attach the injector to the stereotax and load the Hamilton syringe onto the injector.
   d. Adjust the stereotax arm to have a lateral angle of 22° relative to the vertical axis (see Figure 2).
   e. Using the knobs on the stereotax, move the syringe in all dimensions to ensure the tip of the needle can reach the approximate injection coordinates.

Note: If using the right arm, it may be necessary to rotate it counterclockwise for injection left of the midline, and vice versa.

⚠ CRITICAL: Ensure the stereotax arm and injector are secure at the angle of 22°. The weight of the injector can cause the arm to slowly fall to a greater angle. This would interfere with both the leveling of the head (step 14) and the accuracy of the injection.

3. At the surgery station, lay out the required surgical tools: scissors, curved forceps, straight forceps, hemostat, sutures, drill bits, Q-tips, shaver, scalpel, scalpel blade, SUGI spears, 70% ethanol, betadine, eye lubricant, 1 mL syringe, 1 syringe needle per mouse, Vetbond, Topicaine, and a Hamilton syringe loaded with a 33-gauge NanoFil needle.

Note: For the use and care of Hamilton syringes, consult the manufacturer's instructions.

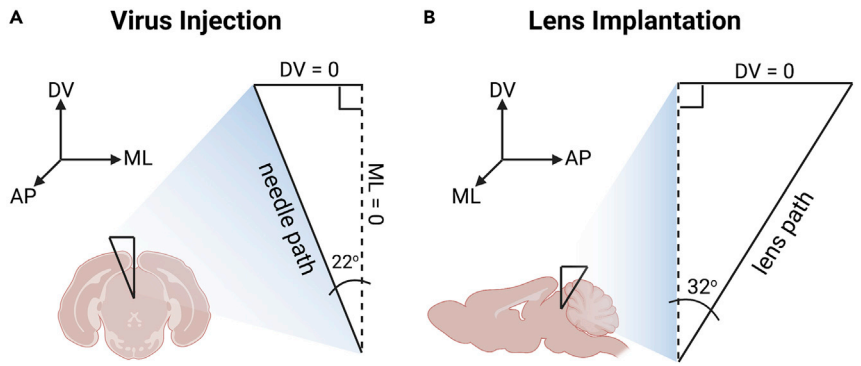

Figure 2. Angles of virus injection and lens implantation

Both the needle used for viral injection and the GRIN lens are inserted at angles to avoid major sources of bleeding in the vertical path above the DRN.

(A) The virus is injected at a lateral angle of 22 degrees. The needle should be angled relative to the vertical path above the DRN and be parallel to the coronal plane.

(B) The lens is implanted at a posterior angle of 32 degrees. The lens should be angled relative to the vertical path above the DRN and be parallel to the sagittal plane. AP, anteroposterior; DV, dorsoventral; ML, mediolateral.

4. Load the virus into the syringe.
   a. Slide one of the ear bars inwards, towards the middle of the stereotax.

   Note: The ear bar is a convenient surface, but any flat surface reachable by the injection needle is acceptable.

   b. Wrap the ear bar with parafilm.
   c. Using the P10 pipette, deliver a drop of up to 10 μL of the diluted virus onto the parafilm.
   d. Move the tip of the needle into the middle of the drop. To do this accurately may require the use of the stereoscope.
   e. Withdraw the entire drop into the Hamilton syringe using the digital controller.

   Note: Note that the volume marking at the meniscus is often less than the volume pipetted due to evaporation during step 4 and the dead space in the needle itself.

   f. Pull up the needle using the stereotax knobs and swing the stereotax arm to the side.

   ⚠ CRITICAL: Throughout the surgery, be careful not to bump the needle. If this happens, you can discard the bent needle and replace it with another without ejecting or withdrawing the virus. Remember to prime the needle following replacement as in step 16.c.

5. Ensure the heating pad is on, and warm enough to provide thermostasis (37°C).
6. Induce anesthesia with 3% isoflurane delivered via the SomnoSuite system at 500 mL/min.
7. When the mouse is fully anesthetized, divert the isoflurane to the nose cone of the stereotax, reduce anesthesia to 1.5%, and adjust the oxygen flow rate as required. For the SomnoSuite system, the flow rate should now be approximately 80 mL/min.
8. Place the mouse in the stereotax.
   a. Open the mouse's mouth with curved forceps.
   b. Fit the front teeth (upper incisors) into the hole on the mouse holder.

   ⚠ CRITICAL: The bar that goes into the mouse's mouth can push the tongue back and obstruct the mouse's airway. Be sure the tongue is not pushed back. You may even grab the tongue with your forceps when placing the mouse in the stereotax.

c. Move the mask into place and tighten.

⚠ CRITICAL: It is essential to monitor the level of anesthesia of the mouse throughout the surgery. If the mouse's breathing changes, they appear to awaken, or respond to a toe pinch, slowly and gradually adjust the percentage and/or flow rate of isoflurane until breathing normalizes and the pain response is absent.

d. Position ear bars so that the head is stable.

9. Immediately after placing the mouse in the stereotax, lubricate its eyes with ophthalmic ointment.
10. Inject the mouse subcutaneously with 0.25–0.3 mL of the carprofen solution prepared in step 1.a.

Note: For particularly large or small mice, the volume of the injection should be adjusted to achieve a dose of 5 mg/kg.

11. Shave the fur from the mouse's head.
12. Sterilize the skin with Q-tips soaked in betadine and 70% ethanol. Move the Q-tips in a spiral from the center to the edge of the shaved area. Alternate the solutions for a total of 3 cycles.
13. Expose the skull.
    a. View the mouse's head through the stereoscope.
    b. Using straight forceps, pinch a small patch of shaved skin at the base of the head.
    c. Using scissors, make an approximately 1-cm incision along the midline to expose the skull.

⚠ CRITICAL: The incision must expose both bregma and lambda and make space for the burr hole which will be drilled several millimeters lateral to lambda. Avoid extending the incision past bregma, as suturing near the eyes can be difficult and interfere with the mouse's ability to close its eyes.

    d. Using two fresh Q-tips, clear the fascia from the surgical field by pulling it in opposite directions until it tears.
14. Level the head.
    a. Move the injection needle back into place so that the 22° rotation of the stereotax arm is perpendicular to the mouse (see Figure 2).

Note: Alternatively, the stereotax arm can be kept upright for this step. If so, be sure to zero out the digital controller with the needle at bregma *after* angling the arm before moving on to step 15.

    b. Move the needle so it just barely touches bregma.
    c. Zero out the digital controller.
    d. Raise the needle and move it back to lambda.
    e. Lower the needle so that the tip just barely touches lambda.
    f. If the z-coordinate reads less than -0.05 or more than 0.05 mm, lower or raise the head bar of the stereotax, respectively, and repeat this step.

⚠ CRITICAL: When adjusting head position, take care not to let the mouse fall out of the ear bars and avoid trauma to the face by loosening the nose cone before raising or lowering it.

15. Drill the craniotomy.
    a. Move the needle so that the tip just touches the skull at the anteroposterior and mediolateral injection coordinates (-4.5 AP, 1.12 ML).

Note: The mediolateral injection coordinate will be either positive or negative depending on which way the stereotax arm is rotated.

b. This is where you will drill the burr hole for the injection, so identify and remember landmarks on the skull relative to the tip of the needle. Alternatively, you can mark the spot on the skull with a surgical skin marker.
c. Move the needle out of the way to make room for the drill.
d. Drill a small burr hole (approximately 0.5 mm in diameter) using the Micro-Drill at the coordinates.

Note: If the dura is still apparent after drilling, it can be removed gently using a 27G needle in a circular motion.

e. Stem any bleeding by quickly absorbing blood using a SUGI spear. If necessary, the hole can be further cleaned with Q-tips and sterile saline.

16. Inject the virus.
    a. Move the needle back to the AP and ML coordinates.
    b. Make sure the needle will be able to pass through the hole to the brain when lowered. If not, move the needle out of the way and expand the hole using the drill.
    c. Prime the needle.
        i. Set the digital controller of the injector to inject 60 nL at 30 nL per second.
        ii. With the needle still raised above the skull, repeatedly "inject" this volume until you see a drop of liquid form on the tip of the needle.

Note: Especially before the first injection after filling or replacing the needle, the volume of air that needs to be ejected can be relatively substantial.

        iii. Use a Q-tip to absorb this drop before lowering the needle into the brain.
    d. Lower the needle into the brain to a dorsoventral coordinate of -3.2 mm.
    e. Inject 400 nL of virus at 100 nL per minute.
    f. Wait 5 min after the injection finishes.
    g. Raise the needle up to a dorsoventral coordinate of -3.1, and again inject 400 nL of virus at 100 nL/min.
    h. Wait 10 min after the injection finishes before pulling the needle out of the brain.

Note: A total volume of 800 nL of our diluted virus (2×10^{12} vg/mL, AAVDJ-DIO-EF1a-GCaMP6m, Stanford GVVC) was required to achieve spread throughout the entire DRN and did not appear toxic. The total volume of this injection may need to be optimized for use with a different virus.

17. Suture the two sides of the incision together using, for example, interrupted or locking stitches, and apply Topicaine.
18. Holding each side together with forceps, apply a small amount of Vetbond along the length of the incision for extra security.
19. Place the mouse in a recovery cage for 30 min before returning it to its home cage. The recovery cage is simply an empty cage with bedding and water only.
20. For 1 week post-operatively, keep a CPF-treated MediGel in the mouse's cage for analgesia.

⚠ **Pause point:** Wait 3 weeks between the viral injection and lens implantation to allow for viral expression and therefore visualization of neurons during implantation.

Note: Although it is possible to inject the virus and implant the lens in the same surgery, we do not recommend this. Injecting the virus in advance of implanting the lens allows for more accurate targeting by visualization of cells and/or fluorescence during implantation. Additionally, lenses in animals without cells or fluorescence can be retracted, cleaned, and used for the next animal.

GRIN lens implantation

Timing: 30 min setup + 1 h per mouse

The implantation of a GRIN lens for miniature microscopy is adapted from previously published protocols.[7] The typical method calls for the lens to be implanted vertically. To image the DRN, the lens must instead be implanted from a posterior angle (see Figure 4) because the vertical path above the DRN contains a confluence of major blood vessels. Posterior implantation then requires the severing of several muscles attached to the skull to allow space for the building of the headcap.

21. Prepare materials and equipment.
    a. Prepare Surgifoam by soaking small (1–2 mm) chunks in sterile PBS in a weigh boat.
    b. Attach the Proview system to the stereotax arm and load the lens according to the manufacturer's instructions. Swing the arm to the side.
    c. Prepare all other equipment as described in steps 2 and 3, excluding the injector and its digital controller, and including the miniature screws.
22. Repeat steps 5–13 for preparing the mouse for brain surgery.

⚠ CRITICAL: Make sure the incision and clearance of fascia goes far enough back to see the muscles that attach to the posterior lambdoid suture (see Figure 3).

23. Sever the trapezius and occipitalis muscles attached to the posterior lambdoid suture as depicted in Figure 3.

Note: Bleeding may occur. Stem the bleeding with styptic powder and/or surgical foam placed between the severed muscle and skull.

24. Place the head screws.
    a. Drill two small holes (approximately 0.5 mm in diameter) in the parietal bones of the skull as depicted in Figure 3.

Note: The coordinates of these holes should be approximately 1–2 mm anterior to lambda and 2 mm lateral to the midline. If the holes are small enough, i.e., 0.5 mm in diameter, the screws should not fall through and will be sturdy when screwed in. Otherwise, you can drill only partially through the skull and only penetrate using the screws.

    b. Screw a miniature screw into each hole.
    c. Once the screws are stable enough to stand without being held up by the screwdriver, screw them 1.5 turns further.

⚠ CRITICAL: Visually assess the protruding length of the screws compared to their total length throughout this step to ensure they are not deep enough to touch the brain.

25. Use the lens to level the skull as in step 14. Be careful that the lens does not touch the skull.

26. Build the first part of the head cap.

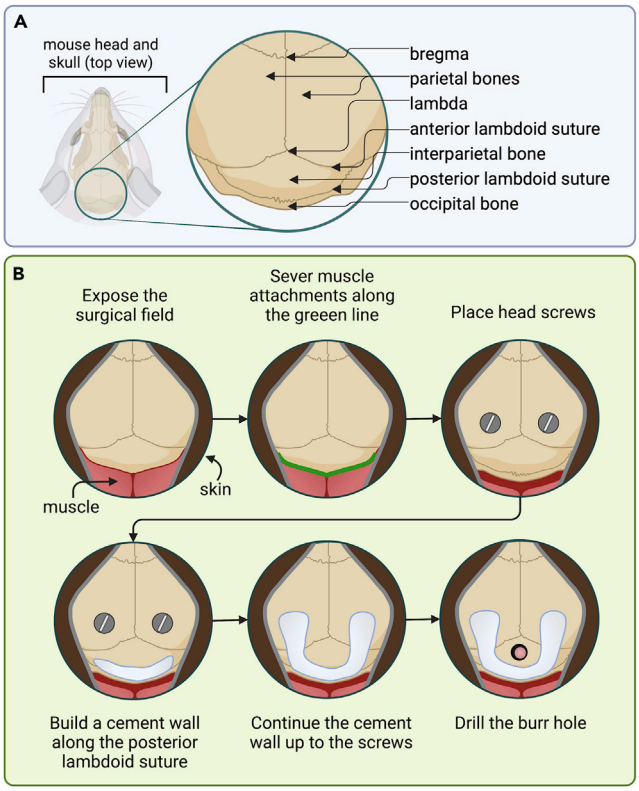

Figure 3. Preparing the surgical field for DRN lens implantation

(A) Diagram of the mouse skull from above. The landmarks that are visible during surgery are labeled.

(B) Steps to prepare the skull for lens implantation and building of a stable headcap.

a. Ensure that the skull is dry using Q-tips or lightly blowing on it with a compressed air canister.

b. Mix cement according to the manufacturer's instructions. An appropriate amount of cement can be prepared by combining one scoop of Metabond powder, 5 drops of base, and one drop of catalyst.

Note: The cement is initially liquid and solidifies over a period of several minutes. For this step, the cement should be viscous and applied carefully to avoid seepage, especially onto the severed muscles.

c. Create a cement cup as illustrated in Figure 3.
   i. Start by applying the cement along the posterior lambdoid suture.
   ii. Extend the cement wall to the screws. Ensure that the cement forms a continuous wall that contacts the skull along its entire length.
   iii. Wait approximately 10 min for the cement to dry before proceeding.

⚠ CRITICAL: The height of the walls of the cement cup should be approximately 1–2 mm. This is most important for the posterior wall. If the wall is too high, it will interfere with the lowering of the lens. If this occurs, ensure the cement is completely dried, and then use the Micro-Drill to file down the obstruction. Then repeat step 25 to ensure the skull is still level.

27. Adjust the stereotax arm holding the lens so that it is angled posteriorly by 32° (Figure 2).

⚠ CRITICAL: Make sure the stereotax arm is stable at 32°. If the angle increases even slightly due to the weight of the arm, the lens will not hit the target.

28. Locate the implantation site and drill the craniotomy.
    a. Move the stereotax arm so that the lens is at bregma. To do this, imagine a line from the center of the bottom of the lens projecting towards bregma.
    b. Zero out the digital controller and move the lens back to -6.53 mm AP and 0 mm ML.
    c. Visually identify and remember any landmarks around the target. Alternatively, mark the skull with a skin marker at these coordinates.
    d. Move the lens up and to the side to make room for your drill.
    e. Drill a craniotomy of approximately 1 mm in diameter in this location.

⚠ CRITICAL: The hole needs to be wide enough for the lens to fit, and long enough for the lens to be lowered at an angle.

    f. If the dura is still visible through the hole, remove it using a 27G needle in a circular motion.

⚠ CRITICAL: Before implanting, make sure the lens and implantation site are free of debris. Lens paper can be used to clean the surface of the lens. If there is bleeding from the craniotomy, absorb it with a SUGI spear. Sterile saline and Q-tips can then be used to clear the implantation site of any debris.

Note: It is not necessary to aspirate, cut, or pierce the tissue along the intended lens tract prior to implantation.

29. Implant the lens.
    a. Move the lens back to the coordinates (-6.53 mm AP and 0 mm ML) and begin to lower it into the hole.
    b. Turn on the imaging LED using the imaging software. For details, see the manufacturer's instructions.
    c. Once the lens enters the brain, proceed slowly by advancing the lens by 0.1 mm, pausing 5 s, drawing the lens up by 0.05 mm, and then pausing for 5 s.

Note: If new bleeding from the craniotomy begins during the lowering of the lens, soak up the blood with a SUGI spear.

    d. Proceed until you see in-focus cells as in Figure 4.

Note: In some cases, you will not see cells during implantation, but will see fluorescence during implantation and cells 1–2 weeks later during imaging experiments. In this case, stop advancing the lens when you see fluorescence and are near the typical DV coordinates. The final DV coordinate in our hands is approximately -3.8 mm. If there are no cells or fluorescence in the field of view, consult Troubleshooting problem 1.

Note: If there are no cells or fluorescence in the field of view, the lens can be reused by retracting it and cleaning it gently with ethanol and lens paper. In this case, either euthanize the mouse or close the incision and provide post-operative care as described in steps 34–36.

    e. Once you reach the coordinates, take a snapshot of the field of view, and turn off the imaging LED.
30. Finish the head cap.
    a. With the lens and microscope still in place, mix cement as in step 26.b.

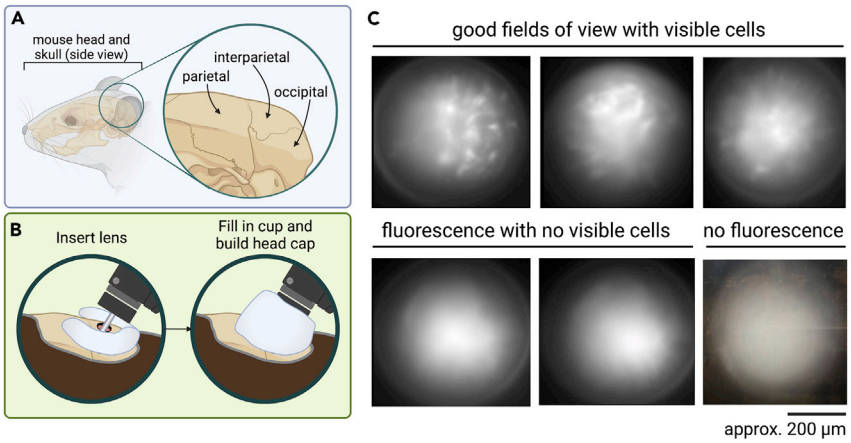

Figure 4. Lowering the lens into the DRN and assessing the field of view

(A) Anatomy of the mouse skull relevant to lens implantation.

(B) Lowering of the lens into the craniotomy and final head cap. This diagram is specific to the use of an Integrated lens. When implanting a Proview lens, the baseplate is substituted with an adapter, which should not be cemented.

(C) Example fields of view during implantation (step 29) or baseplating (step 40). Note that a total lack of fluorescence has a distinctly different tone. Scale bar = 200 µm.

b. Fill in the cement cup using toothpicks to reach the correct location. The cement should be liquid enough to fill the cup but not seep into the craniotomy.
c. Continue building as much of the headcap as you can until you run out of cement, maximizing contact with the implant.
d. Wait 15–20 min, or until the cement is completely dry.

⚠ CRITICAL: Do not get cement on any components other than the implant, especially the scope, implantation equipment, and the hole for the screw that secures the scope in the baseplate or holder.

31. Retract the scope while holding the head cap steady with forceps.
32. If necessary, mix a new batch of cement as in step 26.b and further build up the head cap.
33. If using Proview lenses instead of Integrated lenses, mix a small amount of Kwik-Sil and, using a toothpick, fill the bowl around the top of the lens to protect it. If using an integrated lens, attach the baseplate cover and secure with a screw.
34. Suture the incision to close the wound around the headcap and apply Topicaine. You may use Vetbond to secure sutures, but do not glue the skin or sutures to the headcap itself.
35. Place the mouse in a recovery cage for 30 min before returning it to its home cage. The recovery cage is simply an empty cage with bedding and water only.
36. For 1 week post-operatively, keep a CPF-treated MediGel in the mouse's cage for analgesia.
37. If necessary, singly house the mouse for the rest of the experiments.

Note: Some mice can be housed together with headcaps on, while others will remove each other's head caps. If group housing is important for your experiments, you may test the viability of group housing with a small cohort.

Pause point: If using Integrated lenses, wait 1–2 weeks for the mouse to recover before beginning imaging experiments. If using Proview lenses, wait 1 week and then perform the baseplating step described below.

Baseplating

Timing: 20–30 min per mouse

When using Proview lenses, the baseplate must be aligned with the lens and cemented in place during a separate procedure. The following steps describe this procedure. Note that this section does not apply to those using Integrated lenses.

38. Induce anesthesia, place the mouse in the stereotax, and lubricate the eyes as in steps 5–9.
39. Remove the rubber mold from the top of the lens using straight forceps.
40. Lower the baseplate and attached scope at a posterior angle of 32 degrees until the field of view is acceptable as described in step 29.d and shown in Figure 4.

Note: If there are no cells in the field of view, consult Troubleshooting problem 1.

41. Mix cement and apply with toothpicks to attach the head cap to the baseplate.
42. When the cement is dry, raise the scope as described in step 31.

Pause point: Begin imaging experiments as early as 2 days after baseplating. If during this period, the field of view is lost, consult Troubleshooting problem 2. If the head cap detaches, consult Troubleshooting problem 3.

Imaging

Timing: 1–2 weeks

Imaging experiments should be conducted according to the miniscope manufacturer's instructions. For imaging in the DRN using the calcium indicator GCaMP6m, we recommend an LED power of up to 1.8 mW/mm^2, a frame rate of 20 Hz, and a maximum imaging duration of 30 min per session. To prevent cable tangling, the use of the Inscopix commutator is recommended. To avoid losing the field of view, we recommend imaging on consecutive days and limiting the length of the experiment to 1–2 weeks. However, in several animals we have observed normal activity and a consistent field of view for up to 4 weeks.

Histology

Timing: 1.5 weeks

This step is necessary to determine the placement of the lens in the brain. The tissue fixation step is like other histology protocols, except for the prolonged fixation of the head with the implant still in place. Considerations for determining lens placement are also standard, save special considerations for determining the imaging plane of an angled implant using coronal brain sections.

43. Harvest brains.
    a. Once behavioral experiments are completed, perfuse mice transcardially with 4% paraformaldehyde (PFA) in PBS.[8] This step requires a perfusion pump, fine scissors, decapitation scissors, needles, ketamine, paraformaldehyde, PBS, a spatula, and curved hemostats.
    b. Decapitate the mouse with decapitation scissors and place the entire head with lens and head cap still attached into 4% PFA and store at 4°C for 72 h.

⚠ CRITICAL: The lens should remain in place during the additional fixation period following cardiac perfusion. This modification of standard mouse brain harvesting is recommended

to strengthen the lens tract enough to be distinguishable from the nearby fourth ventricle after sectioning.

Pause point: Allow heads to incubate in PFA for 72 h.

c. Remove the head cap by gripping the base with hemostats and pulling upwards, in the direction parallel to the lens.
d. Dissect brains and place them in 30% sucrose in PBS at 4°C.

Pause point: Wait until the brains sink before proceeding. This usually takes 2–3 days.

44. Freeze and section brains.
    a. Place each brain in an embedding mold and cover in OCT.

⚠ CRITICAL: Make sure that the brain is oriented so that it lays flat on the bottom of the mold, the olfactory bulbs are nearly touching one side of the mold, which should be labeled 'Front', and that the anteroposterior axis is perpendicular to this front wall.

    b. Freeze the embedded brain by placing it in a −80°C freezer for at least 30 min.
    c. Place the embedded brain in the cryostat 20–30 min before sectioning so that its temperature equilibrates. The temperature of the cryostat should be between −18°C and −20°C.
    d. Slice the brain in 30–50 μm sections.
        i. Unless otherwise required for your experiment, discard sections until just before the DRN.
        ii. Then, collect sections serially – one per well in a 24-well plate with PBS – until the end of the DRN.

Note: To prevent degradation of the tissue and GCaMP fluorescence, proceed to the next step within 7–10 days. Alternatively, sections can be stored for up to 3 months at 4°C in PBS with 5% sucrose and 0.05% sodium azide.

Note: To reduce the required number of well plates, you can 'start over' on the same plate when you reach the last well. The ordering of sections is preserved using anatomical landmarks.

45. Determine lens placement.
    a. Mount floating sections serially on Superfrost Plus microscope slides.
    b. Allow the slides to dry for 20–30 min at room temperature (68–74°F) in a slide box or slide storage folder. Ensure that the slides are not exposed to light while they dry.
    c. Pipette approximately 100 μL of Vectashield mounting medium onto the center of the slide and mount the coverslip. Lower the coverslip slowly to avoid bubbles.
    d. Visualize sections using a fluorescence microscope.

Note: Because the lens was implanted at an angle relative to the coronal sections, you will see a progression from the anterior edge of the lens to the center of the bottom of the lens where the tract will be lowest and widest, to the posterior edge of the lens, which will be deeper but thinner, and successive oval tracts that are more and more dorsal.

Note: To facilitate the following step, you may take a picture or pictures of the section(s) at low magnification (2.5–5×) using a camera attached to the microscope and a computer with imaging software.

    e. Determine the coordinates of the lens location using a mouse brain atlas.

Note: Typically, the lens location is recorded as the coordinates at the center of the bottom of the lens; however, note that the imaging plane spans from the anterior to posterior edge of the lens tract.

Motion correction

Timing: 5–10 min per movie

Motion of the field of view during calcium imaging is a significant barrier to extracting single-cell activity data, and motion in the brainstem is much greater than in other areas. Because of this, popular algorithms, including the one employed by the Inscopix Data Processing Software, often fail to correct for motion in DRN imaging data. Fortunately, brainstem neurons typically have baseline activity, and therefore baseline fluorescence, in every frame of the movie. This facilitates a simple motion correction algorithm based on cross-correlations between each frame and a reference. The movie is first filtered and processed to crudely subtract background fluorescence, thus highlighting cells. Then, cross-correlations of each frame to a representative reference frame are calculated and each frame is offset to maximize this value. By default, the reference frame is the first frame of the movie. If the first frame is known to not be representative, the reference frame can be adjusted according to the instructions in the second paragraph of Troubleshooting problem 4.

Sample data can be downloaded here. This movie is a temporally downsampled clip of a representative movie recorded during repeated footshocks.[1]

46. Pre-process the movie in the Inscopix Data Processing Software application (IDPS). In the same step, you will spatially downsample the movie by a factor of 4 for final dimensions of approximately 250 × 250 pixels and crop the movie to the field of view. It should be a circle inscribed in a square.

    Optional: Spatially bandpass the movie in IDPS.

    Note: Spatial bandpassing is unnecessary but harmless at this stage. Both the motion correction and cell segmentation algorithms incorporate forms of spatial bandpassing. It is, however, convenient for visualization of the data before processing.

47. Export either the cropped raw movie, or the cropped and spatially bandpassed movie, as a TIFF.
48. Motion correct the movie in MATLAB.
    a. If your computer has multiple processing units, run the code using parallel processing as follows. To run the code using single-stream processing, replace `'par'` with `'nonpar'`.

```
>> [mcmovie,mcfiltered,offsets] = mctophat('par');
```

    Note: The code calls several utility functions from the NoRMCorre method of motion correction[9] in order to load the movie into MATLAB. These functions allow you to quickly load video files when prompted.

    Note: `mcmovie` is the motion-corrected movie, saved as a TIFF stack in the same folder as the raw movie was chosen from. `mcfiltered` is the motion-corrected, top hat filtered movie, which will be saved in the same folder. This output is for quality control purposes only and should not be used as the input to CNMF-E. `offsets` is the array of pixel values by which

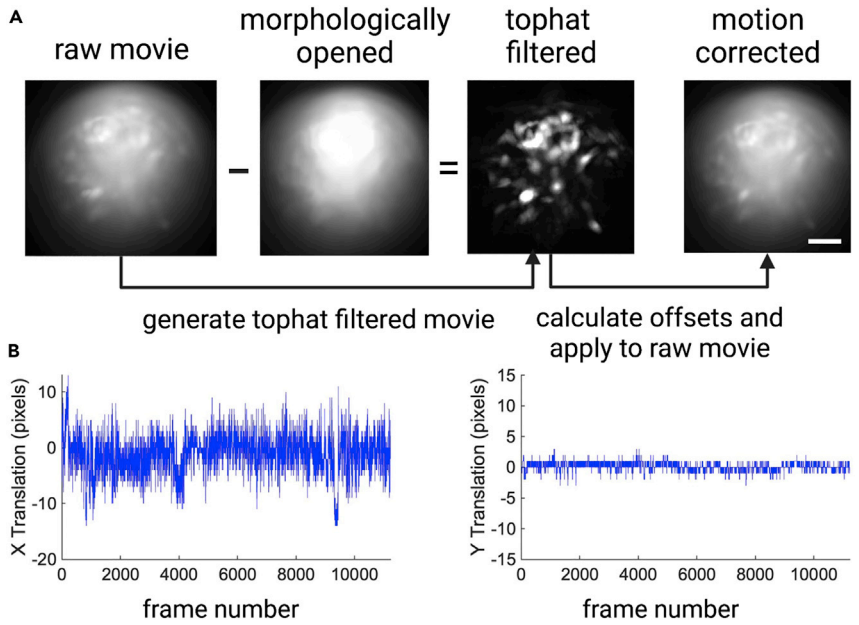

Figure 5. Customized motion correction for 1-photon imaging data from the brainstem

(A) Steps included in the motion correction algorithm described in step 48. Scale bar = 100 μm.

(B) Offsets from a representative movie demonstrating approximately ±10 pixels in one dimension (left) and ±1 pixel in the other (right).

each frame was adjusted to align to the reference frame. The function will also save a figure for visualization of these offsets over the course of the movie, as in Figure 5B.

b. Open the saved movie file (`mcmovie`) in ImageJ.

c. Confirm proper motion correction.

Note: See troubleshooting problem 4 and Troubleshooting problem 5 of this protocol for common problems and solutions to poor motion correction.

d. Save the movie using ImageJ, overwriting the `mcmovie` file.

Note: This is necessary because missing metadata from the motion correction step makes the raw file incompatible with CNMF-E.

Cell segmentation

Timing: 4–6 h per movie

Constrained non-negative matrix factorization for microendoscopic data (CNMF-E) is a common algorithm used to parse calcium imaging movies into single-unit activity traces. For complete details of the this method, see Zhou et al.[4] Correlated activity and residual motion in movies from the brainstem can cause two issues with this method of segmentation: multiple units interpreted as a single unit (cell merging), and single units interpreted as multiple units (cell splitting). The methods described below address both issues by combining CNMF-E background subtraction with manual ROI selection.

49. Subtract background with CNMF-E.

a. Initialize CNMF-E as previously described.[4]

Note: We recommend running CNMF-E using the program's `demo_endoscope.m` script. The code deposited with this protocol includes a copy of this script with parameters set for use with the sample data. Note that the sample data must first be motion corrected before running CNMF-E. For DRN movies, we suggest using single-stream processing (to reduce the incidence of cell splitting); an optimized maximum diameter of cells in the range of 15–25 pixels; a Gaussian kernel width of one third of this value; and a minimum local correlation and peak-to-noise ratio of at least 0.9 and 12, respectively. To reduce cell-splitting, we additionally use a merge threshold of [0.6 0.6 0], which requires higher spatial correlation, but slightly lower temporal correlation, than the default settings. Note that this initialization step involves initialization, two iterations of CNMF-E without manual intervention, and applying the results to the full resolution movie if necessary (`demo_endoscope` lines 1–170). Depending on the computer's hardware, this step will take 20–40 min for a standard movie with 10,000–20,000 frames.

Note: Depending on the movie, these parameters may need to be adjusted. The maximum diameter of cells will depend on the size of cells in the movie. The Gaussian kernel width is optimally one third of this value. For data from the DRN, the minimum correlation is set relatively high, and the peak-to-noise ratio relatively low. The higher the minimum correlation, the fewer non-cell units will be detected by the program. A lower peak-to-noise ratio allows for the detection of cells with a relatively low signal-to-noise ratio. Other parameters, e.g., merge thresholds and deconvolution parameters, are less important when using this protocol, because CNMF-E is used only for background subtraction and not compiling the final set of cells.

b. During manual intervention (`demo_endoscope` lines 172–173), split merged cells and trim large components to achieve units comparable to those shown in Figure 6A.

Note: CNMF-E commonly misinterprets the lens edge as cells. Be mindful of the approximate location of the lens edge in the field of view and delete units that are either on or outside of it.

c. Run a third and final iteration of CNMF-E (`demo_endoscope` lines 174–194). This will take 10–20 min depending on the computer's hardware.

Optional: To improve the quality of the image used for ROI selection, repeat step b.

Note: Steps after line 195 of the `demo_endoscope` script are optional for this protocol.

d. Save the results by entering the command below.

```
>> save_cnmfe
```

Note: The key files saved are a standard deviation projection of the background-subtracted, denoised movie, to be used for ROI selection, and the background-subtracted movie (`Ysignal`), to be used for generating traces.

Note: Although Inscopix Data Processing Software now implements CNMF-E, it does not provide the necessary level of manual intervention or the required outputs to be compatible with the following steps. For this protocol, CNMF-E must therefore be run natively in MATLAB.

50. Select ROIs using CaTracer.
    a. Open CaTracer by entering `caltracer3beta` into MATLAB.
    b. Under Image, select 'Open'.

c. Select the standard deviation projection of the deconvolved movie that was saved in step 49.d.
d. Select 'Okay' to the next pop-up window. Because there is only one image, this value is irrelevant.
e. Select 'Next'.
f. Enter an approximate cell diameter. This is only for automatic detection, which will be deleted in favor of manual drawing.
g. Click 'Detect'. Then select 'Delete' near the bottom right of the GUI and click on ROIs to delete them. Press escape to exit delete mode.
h. Now select 'Add' and begin drawing ROIs. To confirm each ROI, double click inside it. Examples are shown in Figure 6B.

Note: It is helpful to identify cells by adjusting the brightness and contrast of the image.

i. Under the 'Export' tab, select 'Export contours to file', and save the contours to your computer.

51. Generate traces.
    a. Load the contours saved in CaITracer into your MATLAB workspace. By default, this variable should be called `CONTS`.
    b. Apply the contours to the background-subtracted movie to generate raw traces using the following code.

```
>> Ysignal = loadmovie;

>> traces = generate_traces(Ysignal,CONTS);
```

    c. In the pop-up window, select the background-subtracted movie, here defined as `Ysignal`. Each value in the timeseries for a trace will be the average fluorescence within the ROI.
    d. Z-score traces to account for differences in average brightness between cells. Use the following code.

```
>> traces = zscore_traces(traces);
```

52. To generate spatial footprints that are compatible with cell registration software for longitudinal tracking of single cells,[10] run the following code. In the pop-up window, select the denoised movie from CNMF-E, here defined as `Yac`.

```
>> Yac = loadmovie;

>> [] = roi2footprint(Yac,CONTS);
```

53. To manually check trace quality and discard components with motion artifacts, use the following code.

```
>> [traces_sorted,contours_sorted,ind_del] = sort_cells(traces,CONTS,frame_rate);
```

Note: The outputs are the same traces and contours without the discarded cells. `ind_del` is a logical vector indicating which components were discarded from the original dataset.

Validate dataset

Timing: 6–8 weeks

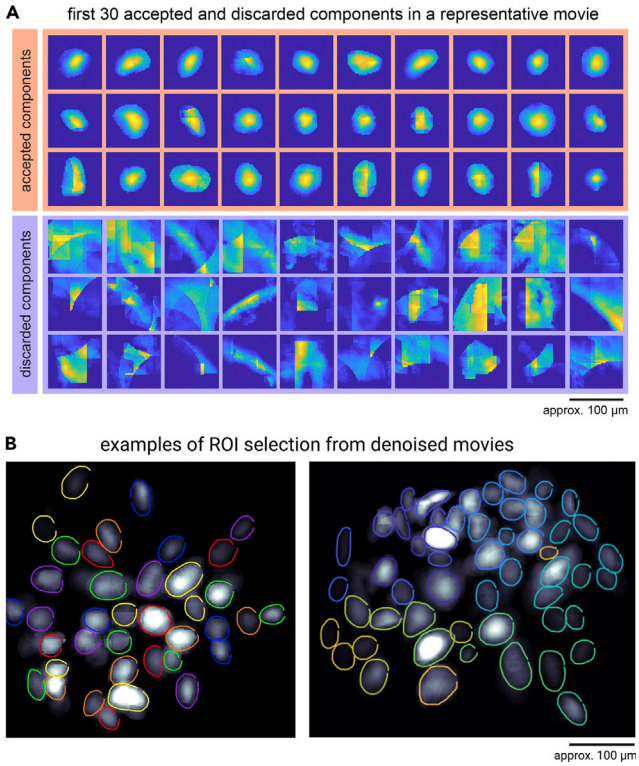

Figure 6. Cell segmentation methods for DRN calcium imaging data

(A) The first 30 accepted and rejected components after initialization and the first iteration of CNMF-E. Note that the cells have been split and trimmed to each contain one cell body without processes or local background fluorescence. Scale bar = 100 μm.

(B) Example ROIs selected using CaITracer. The ROIs are selected from standard deviation projections of denoised movies after background subtraction with CNMF-E. Scale bar = 100 μm.

Due to correlated activity between cells and the likelihood of z-motion, data should be validated using analytical and/or experimental controls. Artificially correlated activity can arise from neuropil contamination, insufficient background subtraction, or motion in the z-plane that causes cells to get brighter or dimmer as they all move closer or further away from the lens, respectively. The following control analyses are designed to determine whether neuropil contamination or z-motion contributes to correlated activity, and whether z-motion contributes to activity in response to behavior.

Note that the time required for this section varies widely by step. Steps 54 and 55 require 2–4 h. Step 56 requires 6–8 weeks, but can be completed concurrently with the experiments detailed in previous sections.

54. Determine whether neuropil contamination contributes to correlated activity.
    a. Repeat steps 50 and 51, but instead of tracing cells, select ROIs with the size and shape of cells in the area between cells, i.e., the neuropil.

Note: This can be done in a randomly chosen subset of movies instead of across the entire dataset.

    b. Determine whether the pairwise correlation between neuropil ROIs is significant. The script `neuropil_ROI_analysis.m` provides code for each of the steps below.
        i. First compute pairwise correlation values between traces in each movie.

ii. Repeat for neuropil ROIs in the same movies.
iii. Then, compute the chance level of pairwise correlation by shuffling the traces in time and iteratively calculating correlation values.
iv. Finally, compare the distributions using two-sample Kolmogorov-Smirnov (K-S) tests.

Note: These tests are performed using the native MATLAB function `kstest2`, for which the equations are given in their documentation. If cells in the dataset are highly correlated, the distribution from real cells should skew significantly greater than chance. The distribution of neuropil ROIs, however, should be similar to chance, as demonstrated by either an insignificant p-value or a relatively small D-statistic.

c. Calculate the pairwise distances between cells and check for a correlation between distance and activity using the script `correlation_vs_distance_analysis.m`. If activity is more correlated between neighboring cells, this may indicate that local background fluorescence or neuropil has not been sufficiently subtracted by CNMF-E.

55. Determine the effect of z-motion on observed activity.

Note: Because z-motion is theoretically subtracted during CNMF-E, the traces used for this analysis will be generated from the raw movie, after crudely subtracting the background using a spatial bandpass filter.

a. Use CaITracer as in step 50 to generate ROIs from a standard deviation projection of a spatially bandpassed copy of the raw movie.

Note: If not already done, the spatially bandpassed movie can be generated using IDPS. The projection can be generated by loading the movie into ImageJ, and using the Image>Stacks>Z-project function.

b. Generate traces as in step 51.
c. Load the offsets data from motion correction performed in step 48.
d. Use the code in the script `zmotion_modeling_analysis.m` to regress the traces against x and y motion, which are a proxy of z-motion.
e. Compare z-motion to activity.
i. Calculate and/or visualize z-motion changes in response to behavior using the same method for calculating activity changes in response to behavior.
ii. Calculate and/or visualize the same values at randomly chosen timepoints to judge the quality of the z-motion modeling. Z-motion traces should closely model the noise of traces generated as in step 50b.
iii. Visualize activity in response to behavior after subtracting the fitted z-motion from the activity traces.

⚠ CRITICAL: In cells where z-motion modeled noise well, activity responses to behavior should still be present. If instead they are eliminated by subtracting z-motion, any responses to this behavior in the real dataset may be due to residual z-motion.

56. Repeat steps 1–48 of this protocol using a virus encoding GFP instead of GCaMP.

Note: Because GFP is a static indicator, CNMF-E will not be able to identify cells in these movies. The effect of z-motion on activity in real movies can, however, be indirectly addressed by repeating the z-motion analysis as in step 55 on these GFP movies. Fluorescence should closely model x-y-motion, and subtraction of this motion should abolish any responses to behavior.

EXPECTED OUTCOMES

Traditional surgical and analytical methods for miniature microscopy and calcium imaging in rodents are not optimized for imaging the brainstem because of its location, motion during freely moving behavior, and correlated activity. This protocol adapts available methods to allow imaging in the mouse dorsal raphe nucleus. First, both viral injection and lens implantation are performed at an angle to avoid major sources of bleeding. The injection should allow for sufficient expression of GCaMP throughout the entire DRN, without leakage of the virus into the median raphe nucleus (MRN). Successful lens implantation results in a relatively stable and consistent field of view across multiple weeks, with approximately 20–60 cells detectable per session. After imaging, brains are harvested to determine lens placement. This protocol increases the integrity of the lens tract by prolonging fixation and describes the procedure for identifying the imaging plane from coronal sections of an angled lens tract.

Second, this protocol includes analytical methods to segment single cells from DRN calcium imaging movies. The motion correction algorithm deposited with this protocol reliably corrects approximately 20 pixels of motion that can be expected in movies from the brainstem. The cell segmentation protocol then adjusts for cell merging and splitting that occurs using common methods. Finally, this protocol describes methods for the validation of imaging datasets with high degrees of motion and correlation. Completion of this protocol in full yields robust single cell activity data from the mouse DRN during freely moving behavior.

LIMITATIONS

The primary limitation of this protocol compared to typical miniscope imaging protocols is the success rate of surgeries, i.e., the presence of 20+ cells in the field of view by 1–2 weeks after lens implantation. Our success rate is between 20% and 75% depending on the surgeon. In our experience, a surgeon's success rate for lens implantations in the DRN is approximately 25% lower than that for any other region for which the lens is implanted vertically.

During the processing of DRN imaging data, some movies cannot be motion corrected, or have residual motion artifacts in the activity traces. Additionally, the region has relatively low signal to noise ratio compared to other brain regions using GCaMP6m.

TROUBLESHOOTING

Problem 1

No cells in the field of view.

Potential solution

Sometimes cells are not visible during implantation (step 29) but are present several days later. In practice, we typically implant any animal with obvious fluorescence during lens implantation. If an animal does not have cells or fluorescence, the lens can be reused by retracting it and cleaning it gently with ethanol and lens paper.

If cells are never present, it is likely that targeting of the DRN was poor or the field of view is obscured by blood. If histological assessment of lens placement (step 45) reveals the lens to be in the correct location, ensure in future surgeries that the surgical field is free of bleeding before lens implantation. If instead the histological assessment reveals that the lens is systematically off target, adjust the implantation coordinates accordingly.

Problem 2

Loss of the field of view after lens implantation.

Potential solution

This is likely due to instability of the head cap or lens. For example, if the cement is not completely dry when the stereotax arm is retracted in step 31, the lens will shift as it dries. Allow the cement to dry completely. Stability can be further improved by ensuring the skull is dry, scoring the skull with the drill or a scalpel, or adding more screws.

Problem 3

Detached head cap.

Potential solution

If the mouse's head cap detaches at any stage of the experiment, the mouse should be euthanized. We recommend that immediately after discovering a detached head cap to perfuse the mouse for brain harvesting as described in steps 43–45. In some cases, it is still possible to identify the lens location.

Problem 4

Overcropping during motion correction.

Potential solution

Overcropping in step 48 is typically the result of a minority of frames with a large offset from the reference. A potential solution to this problem is to limit the offset values to a user-defined threshold. This can be done by un-commenting one section of the `mctophat` function. This section, headed `Account for extreme motion`, will alert you to the presence of frames with large shifts (`offset` > 25 pixels), and use the previous frame's offset value for that frame. As long as there aren't too many frames with large shifts, the resulting motion artifacts in segmented cells' activity traces should not affect further analysis. If there are many such frames, the movie may not be suitable for single-cell analysis. If such frames are restricted to a specific time period, you may consider trimming the movie or deleting the time period with high motion.

Alternatively, overcropping may result from assigning a reference frame that is not representative of the movie. To change the reference frame, edit the variable `template` in the section headed `Calculate x/y motion of each frame`. The new reference frame should replace the third input.

Problem 5

Poor motion correction.

Potential solution

Poor motion correction in step 48 often results from components of the video that move independently of one another. The most common cause of this is the edge of the lens but this could also result from debris on the lens. The top hat filter helps to eliminate these artifacts. If such artifacts are apparent in the `mcfiltered` video, you may still motion-correct the movie by using only a region of interest, with no artifacts, to calculate offsets. To do this, un-comment two sections: `restrict calculations to inner field to avoid conflict with lens edge` and `reset to full tophat movie`. The coordinates defining the region of interest can be adjusted in the first of these sections, in the cropping of `tophatstack`.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bradley R. Miller (bradley.miller@nyspi.columbia.edu).

Materials availability

This study did not generate unique reagents.

Data and code availability

The code described with this protocol is available on GitHub: github.com/Kang-Miller-Labs/DRN-Imaging-STAR-Protocols. The version of record is archived at Zenodo: https://doi.org/10.5281/zenodo.7497993.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.E.P., R.H., B.R.M.; methodology, G.E.P., K.C., B.R.M., C.O.L., P.Z.; software, G.E.P., C.O.L.; formal analysis, G.E.P.; investigation, G.E.P., K.C., B.R.M.; resources, C.O.L.; data curation, G.E.P.; writing – original draft, G.E.P.; writing – review and editing, G.E.P., K.C., B.R.M.; visualization, G.E.P.; supervision, R.H., B.R.M.; funding acquisition, G.E.P., R.H., B.R.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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