We present a protocol to prepare mouse cranial window implantation for in vivo two-photon wide-field calcium imaging. This protocol uses G-CaMP9a transgenic mice, which express a genetically encoded calcium indicator with high signal-to-noise ratio. We describe in utero electroporation, followed by headplate fixation and cranial window implantation. This protocol can be used for measuring neural activity and is suitable for long-term imaging in large populations. Moreover, this protocol does not require preparation of Flp-expressing transgenic mice.

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

In utero electroporation and cranial window implantation for in vivo wide-field two-photon calcium imaging using G-CaMP9a transgenic mice

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https://doi.org/10.1016/j.xpro.2022.101421

SUMMARY

We present a protocol to prepare mouse cranial window implantation for in vivo two-photon wide-field calcium imaging. This protocol uses G-CaMP9a transgenic mice, which express a genetically encoded calcium indicator with high signal-to-noise ratio. We describe in utero electroporation, followed by headplate fixation and cranial window implantation. This protocol can be used for measuring neural activity and is suitable for long-term imaging in large populations. Moreover, this protocol does not require preparation of Flp-expressing transgenic mice. For complete details on the use and execution of this protocol, please refer to Sakamoto et al. (2022).

BEFORE YOU BEGIN

The protocol below describes the materials and methods for in utero electroporation and cranial window implantation for in vivo two-photon calcium imaging using G-CaMP9a knockin mouse (Sakamoto et al., 2022). This protocol can allow to monitor the neural activity of the barrel cortex from several hundreds of neurons simultaneously with single-cell resolution.

Institutional permissions

All recombinant DNA and animal experiments in this study were performed in accordance with regulations and guidelines for the care and use of experimental animals at the University of Tokyo and approved by the institutional review committees of the University of Tokyo Graduate School of Medicine.

Preparation of G-CaMP9a transgenic mice for in utero electroporation

© Timing: 4 months

1. Obtain G-CaMP9a transgenic mice [C57BL/6N-Gt (ROSA)26Sor<tm1(CAG-G-CaMP9a, R-CaMP2) Hbto>] - RIKEN BRC (Cat#010815)] in which fast and high signal-to-noise ratio
genetically encoded calcium indicator G-CaM9a can be expressed in a Flp dependent manner (Sadowski 1995; Chowdhury et al., 2019).

2. Genotyping of the G-CaMP9a transgenic mouse.
   a. Isolate genomic DNA from tail clippings after weaning.
      i. Cut 0.1–0.3 cm mouse tail and transfer into a 1.5 mL microcentrifuge tube.
      ii. Add 100 μL tail lysis buffer (see materials and equipment) containing proteinase K (1.0 μL of 20 mg/mL Proteinase K for 100 μL of Tail Lysis Buffer) and vortex briefly.
      iii. Incubate at 55°C in a hybridization oven with rocking overnight (16–20 h).
      iv. Vortex briefly to mix.
      v. Incubate at 85°C for 45 min to inactivate Proteinase K.
      vi. Spin down the tube briefly.
      vii. Use the supernatant for PCR.

   b. Perform PCR for genotyping.
      i. Prepare the PCR Reaction mixture as shown in Table 1.
      ii. PCR parameters to run the samples for genotyping are shown in Table 2.
   c. PCR products are separated on a 1.5% agarose gel, stained with ethidium bromide dye, and photographed (Figure 1).

   Note: Genotyping of the G-CaMP9a transgenic mice was determined by PCR using the following oligonucleotides: Rosa26 forward primer (5'-AGTCGCTCTGAGTTGTTATC-3'), Rosa26 reverse primer (5'-GGATCTCAAGCAGGAGAGTA-3'), pCAG reverse primer (5'-GATGGGGAGAGTGAAGCAGAACGT-3'). Rosa26 forward primer and Rosa26 reverse primer correspond to the wild-type Rosa26 locus, and pCAG reverse primer corresponds to the transgene. Rosa26 forward primer and Rosa26 reverse primer amplified a 500-bp fragment specific to the wild-type allele. Rosa26 forward primer and pCAG reverse primer amplified a 684-bp fragment specific to the knock-in allele.

3. The heterozygous transgenic G-CaMP9a mice were crossed with the heterozygous transgenic G-CaMP9a mice to get the homozygous strain.

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### Table 1. PCR reaction mix

| Reagent                              | Final concentration | Amount |
|--------------------------------------|---------------------|--------|
| DNA template                         | n/a                 | 0.5 μL |
| MightyAmp DNA polymerase             | 0.125 U/20 μL       | 0.1 μL |
| 10 μM Rosa26 forward primer          | 0.3 μM              | 0.6 μL |
| 10 μM Rosa26 reverse primer          | 0.3 μM              | 0.6 μL |
| 10 μM pCAG reverse primer            | 0.3 μM              | 0.6 μL |
| 2X MightyAmp Buffer Ver.2            | n/a                 | 10 μL  |
| ddH₂O                                | n/a                 | 7.6 μL |

### Table 2. PCR cycling conditions

| Steps       | Temperature | Time  | Cycles |
|-------------|-------------|-------|--------|
| Initial Denaturation | 98°C       | 3 min | 1      |
| Denaturation | 98°C       | 10 s  | 32 cycles |
| Annealing   | 58°C       | 15 s  |        |
| Extension   | 68°C       | 1 min |        |
| Final extension | 68°C     | 2 min | 1      |
| Hold        | 4°C        | Forever |    |
4. Cross homozygous G-CaMP9a male mice with wild-type female mice. The day when a vaginal plug is found is counted as embryonic day 0.5 (E 0.5).

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| Proteinase K | Wako | Cat# 161-28701 |
| Tail lysis buffer | Viagen Biotech | Cat# 102-T |
| MightyAmp DNA Polymerase | Takara | Cat# R071A |
| Agarose | NIPPON GENE | Cat# 318-01195 |
| Ethidium bromide dye | Nacalai Tesque | Cat# 14631-94 |
| Medetomidine Hydrochloride | ZENOAQ | N/A |
| Midazolam | Maruishi Pharmaceutical | N/A |
| Butorphanol tartrate | Meiji Seika Pharma | N/A |
| Atipamezole Hydrochloride | ZENOAQ | N/A |
| Carprofen | Zoetis | N/A |
| Dexamethasone sodium phosphate | Wako | Cat# 046-30813 |
| White petrolatum ointment | Maruishi Pharmaceutical | N/A |
| Ethanol | Nacalai Tesque | Cat# 14710-54 |
| Lidocaine jelly | Aspen | N/A |
| Isoflurane | Wako | Cat# 099-06571 |
| Saline | Otsuka | N/A |
| Fast Green | Wako | Cat# 061-00031 |
| **Critical commercial assays** | | |
| NucleoBond Xtra Midi EF | MACHEREY-NAGEL | Cat# 740420 |
| **Experimental models: Organisms/strains** | | |
| Mouse: C57BL/6N (> P60, females) | Japan SLC | N/A |
| Mouse: G-CaMP9a (C57BL/6N-Gt (ROSA)26Sor<tm1(CAG-G-CaMP9a,-R-CaMP2)Hbto>) (> P60, heterozygous, both females and males) | Sakamoto et al. (2022) | RIKEN BRC: RBRC11575 |
| **Oligonucleotides** | | |
| Rosa26 forward primer 5'-AGTGCCTCTGAGTTGTATTAC-3' | Thermo Fisher Scientific | Sakamoto et al. (2022) |
| Rosa26 reverse primer 5'-GGATCTCAACGAGGAGTA-3' | Thermo Fisher Scientific | Sakamoto et al. (2022) |
| pCAG reverse primer 5'-GATGGGAGAGTGAAGCACGT-3' | Thermo Fisher Scientific | Sakamoto et al. (2022) |
| **Recombinant DNA** | | |
| pCAG-Fipo | This paper | RIKEN BRC: RDB19603 |
| pCAG-mCherry | This paper | RIKEN BRC: RDB19604 |

(Continued on next page)
Continued

### REAGENT or RESOURCE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Software and algorithms |        |            |
| ImageJ | Schneider et al. (2012) | https://imagej.nih.gov/ij/ |
| Other |        |            |
| Fine scissors | FST | Cat# 14060-10 |
| Micro-adson forceps | FST | Cat# 11018-12 |
| Fine forceps | FST | Cat# 11295-10 |
| Ring forceps | Natsume Seisakusho | Cat# A-26 |
| Microneedle holder | Natsume Seisakusho | Cat# MC-40 |
| Electroporator | BTX | ECM 830 |
| Electrode | Nepa Gene | CUY650P5 |
| Glass capillary | Harvard | Cat# 30-0066 |
| Suction tube | Drummond | Cat# 2-040-000 |
| Syringe Filter | AS ONE | Cat# 1-3199-01 |
| Luer fitting | Bio-Rad | Cat# 731-8224 |
| Puller | Sutter Instrument | P-97 |
| Heating pad | Minimal LAND | RH-207 |
| Surgical drape | ASKUL | Cat# IRL-SS1 |
| Surgical tape | Hakujuij | N/A |
| Sterilized gauze | ASKUL | Cat# 021-774000 |
| Razor | Feather | Cat# FA-10 |
| Water bath | AS ONE | IMB-100 |
| Transfer pipette | Samco Scientific | Cat# 202 |
| Suture | Natsume Seisakusho | Cat# RK-RSU-NGVXSX10 |
| Surgical needle (3/8 Circle) | Natsume Seisakusho | N/A |
| Isoflurane anesthesia system | Muromachi | MK-AT210D |
| LED light for mCherry fluorescence | Relyon Handy Green Pro Plus |
| Mouse adaptor | WPI | Cat# 502063 |
| Hair-removal cream | Veet | N/A |
| Small cotton tips | Sanritsu | Cat# 4935089230108 |
| Dental acrylic (kit) | SUN MEDICAL | Cat# 204610555 |
| Superglue | Toagosei | Cat# 30424 |
| Surgical drill handpiece | NSK | Volvere i7 |
| Diamond bar | Minitor | Cat# AD1401 |
| Titanium headplate | This paper | N/A |
| Vacuum | Nitto Kohki | HK-435A |
| Gelatin sponge | LTL Pharma | N/A |
| Coverglass (#0 thickness, 3 mm diameter) | Warner Instruments | Cat# 64-0726 |
| Toothpick | AS ONE | Cat# 1-5980-01 |
| Surgical superglue (Aron Alpha A) | Sankyo | N/A |
| 27G blunt needle | Nipro | Cat# 02-165 |
| Silicone sealant | WPI | KWIK-CAST |
| Two-photon microscope | Olympus | FVMPER-S5 |

### MATERIALS AND EQUIPMENT

#### Anesthetic mixture

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Medetomidine Hydrochloride | 0.075 mg/mL | 1.875 mL |
| Midazolam | 0.40 mg/mL | 2.0 mL |
| Butorphanol tartrate | 0.50 mg/mL | 2.5 mL |
| Saline | n/a | 18.625 mL |
| Total | n/a | 25 mL |
Note: The anesthetic mixture can be stored for 1 month at room temperature (22°C–26°C).

### STEP-BY-STEP METHOD DETAILS

#### In utero electroporation

**Timing:** 30 min per pregnant mouse

This protocol describes the critical steps of *in utero* electroporation to introduce Fli recombinase in layer 2/3 pyramidal neurons in the right hemisphere of the barrel cortex.

1. Prepare glass capillaries (I.D.: 1.17 mm, O.D.: 1.50 mm) using a puller (P-97, Sutter). The detail of the parameters is shown in Table 3. After pulling, break the tip of the capillaries with fine forceps so that the tip diameter is approximately 50 μm.

   **Note:** Pulled glass capillaries are irradiated with U.V. for 15 min before use.

2. Add 1/20 volume of 1.0% dye (Fast Green) to the plasmid solution used for injection and fill the glass capillary.

   **CRITICAL:** We used the mixture of pCAG-Flpo and pCAG-mCherry plasmids, in which Flpo and mCherry are expressed under the control of the CAG promoter (Miyazaki et al., 1989; Seita et al., 2016). The plasmid solution (see materials and equipment section) should be purified using an endotoxin-free kit. The G-CaMP9a fluorescence induced by Flp recombination is very dim. Therefore, to identify the electroporated region, we recommend mixing a plasmid encoding red fluorescent protein (e.g., mCherry).

   **Note:** The plasmid solution is made each before use.

3. Attach the glass capillary to the suction tube and suck up 10 μL plasmid solution *(Figure 2)*.

   **CRITICAL:** The plasmid solution should be loaded very slowly to prevent air bubbles. Injection of bubbles will likely result in the embryo’s death.

   **Note:** For safety, a syringe filter with a 0.22 μm pore size is installed between the mouthpiece and the tube via a luer fitting.

   **Note:** Use a ruler to mark every 1 mm on the outside of the capillary. One millimeter corresponds to 1 μL.

### Table 3. Parameter of puller

| Parameter   | Value  |
|-------------|--------|
| Heat Ramp   | 15     |
| Pull        | 80     |
| Velocity    | 50     |
| Time        | 50     |
4. Anesthetize a pregnant mouse (E15.5) with an intraperitoneal injection of an anesthetic mixture (see materials and equipment). An anesthetic mixture is administered at 100 μL per 10 g of body weight (BW).  

Alternatives: Some alternative anesthetic methods are available, including ketamine/xylazine and isoflurane.

5. Wait 10 min and make sure that the mouse has been anesthetized by pedal reflex and breathing deeply and steadily.

6. Administer carprofen (dissolved in saline; 5.0 mg/kg-BW, subcutaneously) and dexamethasone (dissolved in saline; 2.0 mg/Kg-BW, intraperitoneally) to relieve pain and inflammation, respectively.

7. Place the mouse on its back on the operating table and fix the limbs with surgical tape (Figure 3A).

Note: We use an operating table shown in Figure 4. To better position and fix the animal, when the pregnant mouse is placed on the table with a heating pad, styrofoam platforms at both ends will be the same height as the limbs. We lay a surgical drape on the table during the surgery. The surgical tape connects the two sides of the styrofoam platforms. Make sure the mouse limbs are secured.

△ CRITICAL: Warm the mouse on a heating pad at 37°C during the surgery.

8. Apply white petrolatum ointment to the eyes for physical protection and prevent dehydration using small cotton tips.
9. Sterilize the abdomen with 70% ethanol.
10. Remove hairs from the abdomen with a razor (Figure 3B).
11. Pinch the skin through the slit with micro-adson forceps and make a ~30-mm-long midline incision through the skin and then the abdominal wall with fine scissors (Figures 3C and 3D).

⚠️ CRITICAL: Avoid hurting the nipples of the dam. It will result in poor feeding of the pups.

⚠️ CRITICAL: The surgery should be finished within 30 min completely (from incision to the closure of the skin). Prolonged procedures cause embryo death and abortion.

12. Cover the abdomen with sterilized gauze with a ~30 mm slit in its center.
13. Wet the gauze with warm saline (40°C) by transfer pipette.

Memo: The saline is transferred to a sterile beaker and warmed in a water bath.

14. Attach a ring forceps to the uterus at a gap between embryos and carefully pull the uterus out of the abdominal cavity (Figure 3E).

⚠️ CRITICAL: Immediately after uterus exposure and before electroporation, the embryos should be wet with warm saline to avoid excessive cooling and to favor current flow during electroporation.
15. Inject with 1 µL plasmid solution into the lateral ventricles of the embryos by exhalation (Methods video S1).

**Note:** The capillary is inserted vertically into the lateral ventricles. It is approximately 1 mm depth from the uterine wall.

⚠️ **CRITICAL:** When plasmids with dye are appropriately placed in the lateral ventricle, a blue crescent-like line can be visualized on the injection site (Figure 3F).

16. Hold the plasmid-injected head of the embryo with forceps-type electrodes and deliver five electric pulses with an electroporator (Figure 3G). The detail of the parameters is shown in Table 4.

⚠️ **CRITICAL:** The position and polarity of the electrodes are very critical. Make sure the anode is pointing at the targeted cortical area. In addition, make sure that the electrodes (especially the platinum tip) are clean. Before delivering electric pulses to embryos, make sure that air bubbles appear on the electrodes’ surface when electric pulses are delivered into saline.

⚠️ **CRITICAL:** Pulse intensity and duration are key parameters. When increasing these parameters, the efficiency of the electroporation will increase. However, if these are too high, neurons expressing G-CaMP9a will be unhealthy or dead. In some cases, it can lead to abortion. Therefore, we recommend a voltage in 25–35 V.

**Alternatives:** We use a 5 mm platinum disk electrode. Another 3 mm platinum disk electrode (CUY650P3, Nepa Gene) is also available. In that case, the electroporated region will be smaller.

17. Return the uterus carefully into the abdominal cavity.
18. Fill the abdominal cavity with warm saline by a transfer pipette.

19. Suture the abdominal cavity with microneedle holder and suture with surgical needle (Figure 3H).
20. Suture the skin with microneedle holder and suture with surgical needle (Figure 3I).
21. Administer atipamezole hydrochloride (0.75 mg/kg-BW, intraperitoneally) to reverse medetomidine-induced anesthesia.
22. Remove the surgical tape and transfer the mouse to a clean recovery cage.
23. Warm the mouse on a heating pad at 37°C until the mouse recovers from the anesthetic.
24. Transfer the mouse to the original cage.
Note: Within 48 h of birth, the electroporated site is confirmed by detecting red fluorescence expression (filler plasmid) over the skin under green light LED illumination (Figure 5). The expression can be seen in the entire somatosensory cortex and sometimes in the visual cortex. Because of the low copy number of G-CaMP9a, it is not possible to see the G-CaMP9a fluorescence on the skin by blue light LED illumination. After the screening, we remove pups that do not have red fluorescence at that time.

Headplate fixation

© Timing: 40 min per mouse

This part describes the critical steps of headplate attachment for in vivo two-photon calcium imaging. This surgery is performed after one month of age. The headplate is attached to the barrel cortex of the right hemisphere.

25. Transfer the mouse into an anesthesia induction chamber and induce deep anesthesia with 3% isoflurane and 1.0 L/min airflow.
26. Transfer the mouse from the chamber to the mouse adaptor and anesthetize with 1.5%–2.0% isoflurane and 1.0 L/min airflow.

Optional: Injecting anesthetic mixture is also available.

27. Administer carprofen (dissolved in saline; 5.0 mg/kg-BW, subcutaneously) and dexamethasone (dissolved in saline; 2.0 mg/Kg-BW, intraperitoneally) for the relief of pain and inflammation, respectively.
28. Apply white petrolatum ointment to the eyes for physical protection and prevent dehydration using small cotton tips.
29. Trim the hair on the scalp with a pair of fine scissors.
30. Apply hair-removal cream over the trimmed scalp and wait 5 min. Then, clean it off using small cotton tips.

△ CRITICAL: Make sure that hair-removal cream does not leave any residue on the eyes.

31. Apply 70% ethanol over the trimmed scalp for sterilization and wash off residual hair and hair-removal cream using small cotton tips.
32. Secure the head in a mouse adaptor by engaging ear bars (Figure 6A).
33. Apply lidocaine jelly (2%, 0.3 g) over the trimmed scalp for local anesthesia using small cotton tips. Wait 5 min for local anesthesia.
34. Incise the scalp.
   a. Open the skin above the skull (~30 mm) with a fine scissor (Figure 6B).
   b. Cut the scalp skin (~30 mm) on both sides of the incision (Figure 6C).

Note: Only skin without hair should be removed. Otherwise, cutting hair will irritate the wound.

Table 4. Parameter of the electric pulse

| Parameter               | Value |
|-------------------------|-------|
| Voltage (V)             | 30 V  |
| Duration (ms)           | 50 ms |
| Interval (ms)           | 1000 ms|
| The number of pulses    | 5     |

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c. Push remaining skin to the side with small cotton tips (Figure 6D).
d. Scratch the exposed skull with fine forceps (Figures 6E–6G).

Note: Superglue and dental acrylic bond better to the rough surface.

35. Apply superglue to the titanium headplate (Figure 6H).
36. Attach a custom-made titanium headplate to the skull with superglue (Figure 6I).

△ CRITICAL: Keep pushing the headplate from the top for a while to get firmly attached to the headplate cranium.

37. Wait at least 10 min to dry the superglue completely.
38. Glue the headplate with dental acrylic to the skull by filling the space under the headplate and exposing the skull. (Figures 6K and 6L).

△ CRITICAL: Cover all the exposed bone because the open wound might get infected. Since the ear bars block space below the headplate, the mouse must be detached from the adaptor. Then, continue filling dental acrylic under the headplate with the mouse in your hand (Figure 6L).

△ CRITICAL: To dry dental acrylic completely, wait 10 min.

Cranial window implantation

⏱ Timing: 120 min per mouse

This part describes the critical steps of cranial window implantation.

39. Place the coverglass (#0 thickness, 3 mm diameter) over the skull and drill around it to mark where the cranial window will be made (Figure 6M).

Note: The skull where the cranial window will be made is removed in step 41. If the skull is removed beyond 3 mm in diameter, the exposed brain will not fit within the coverglass. Otherwise, surgical superglue (see step 44) and dental acrylic (see step 46) penetrate the brain.

40. Drill the skull inside of the circle made in step 39. Continue to thin until there is a small crack or the skull can be easily pushed with gentle pressure with fine forceps (Figure 6N).

△ CRITICAL: Rash punctures cause long-term inflammation leading to high autofluorescence of the brain, cell death, and consequently poor quality of neuronal imaging. It is best to use
Figure 6. Significant steps of headplate fixation and cranial window implantation

(A) Remove hair over the scalp.
(B) Incise the scalp.
(C) Cut the scalp skin on both sides of the incision.
(D) Push remaining skin to the side with small cotton tips.
(E and F) Before scratching the skull with fine forceps. Panel F is a higher magnification of the skull in panel E.
(G) After scratching the skull with fine forceps.
(H) Apply superglue on a titanium headplate.
(I and J) Attach the headplate with superglue. Panel J is a higher magnification of the skull in panel I.
(K) Glue the headplate with dental acrylic on the headplate.
(L) Glue the headplate with dental acrylic to the skull by filling the space under the headplate and exposing the skull.
(M) Mark the area where the cranial window will be made.
(N) Drill the skull of the inside of the circle made in panel M.
(O) Peel away the thinned skull with fine forceps.
(P) Clean the dura.
the drill speed at 3,000–5,000 rpm. Also, prolonged drilling in the same position will cause overheating and damage the brain tissue and blood vessels. Such damage may cause bruising or bleeding under the bone in the thinned part of the skull. The skull should be cooled and cleaned with saline and gently sprayed periodically with compressed air.

41. Peel away the thinned skull gently with fine forceps when some cracks are visible (Figure 6O).

△ CRITICAL: Do not try to force the bones to strip off. If you force it to come off, it will damage blood vessels and cause bleeding. If it is still hard, drill further to remove a thin layer of bone. If bleeding occurs, use a gelatin sponge moisturized with saline to stop the bleeding immediately.

△ CRITICAL: It is reported that two-photon imaging in the deep brain region (at a depth of 600–800 μm) is possible in the presence of dura (Augustinaite and Kuhn, 2020). Therefore, we think the dura should be kept intact throughout the surgery. Attempted dura removal can lead to bleeding.

42. Clean the dura with a gelatin sponge moisturized with saline by a transfer pipette (Figure 6P).

△ CRITICAL: Make sure no bleeding when adding a drop of saline to the dura.

43. Place the coverglass on the dura pressing on its center with a toothpick, and make sure there is no bleeding (Figure 6Q).

Note: Clean the coverslip with 70% ethanol and sterilize them with U.V. light before use.

△ CRITICAL: Do not proceed if bleeding starts. Remove the coverglass and clean the dura with a gelatin sponge moisturized with saline.

44. While pressing on the center of the coverglass with a toothpick, apply surgical superglue (Aron alpha A) to the edge of the coverglass using a blunt needle (Figure 6R).

△ CRITICAL: Pressing the coverslip prevents the spread of the surgical superglue under the coverglass. It is also important to reduce motion artifacts during imaging.

45. Wait 10 min to dry the surgical superglue completely.

△ CRITICAL: Make sure that the surgical superglue completely seals the glass periphery. If not, apply an additional seal to it.

46. Apply dental acrylic to hide the exposed cranium except for the coverglass (Figures 6S and 6T).

△ CRITICAL: To dry dental acrylic completely, wait 10 min.

47. Apply silicone sealant to protect the exposed region from light.
48. Remove the mouse from the mouse adaptor.
49. Transfer the anesthetized mouse to a clean recovery cage.
50. Warm the mouse on a heating pad at 37°C until the mouse recovers from the anesthetic.

51. After waking up from anesthesia, transfer the mouse to the original cage.

EXPECTED OUTCOMES

After the electroporation, pups are born and develop normally. The expression can be seen in the entire somatosensory cortex and sometimes in the visual cortex. The electroporated site is confirmed within 48 h of birth by detecting red fluorescence expression (filler plasmid) over the skin under green light LED illumination. Because of the low copy number of G-CaMP9a, it is not possible to see the G-CaMP9a fluorescence on the skin by blue light illumination.

Furthermore, after the cranial window implantation, in vivo two-photon calcium imaging can observe spontaneous and sensory-evoked calcium transients in subsets of neurons with single-cell resolution (Methods video S2). Some transgenic animals expressing genetically encoded calcium indicators show significant abnormalities in their brain activity (Daigle et al., 2018; Steinmetz et al., 2017). In our transgenic animal, however, we did not observe any abnormalities in the cell morphology and brain activity (Sakamoto et al., 2022). In addition, G-CaMP9a fluorescence was stable under continuous in vivo imaging for 30 min. Thus, making a high-quality cranial window can make it possible to monitor neuronal activity continuously on several consecutive days. Furthermore, our transgenic mice might be suitable for wide-field two-photon microscopy with cellular resolution (Ota et al., 2021; Sofroniew et al., 2016; Stirman et al., 2016).

LIMITATIONS

To introduce gene expression from transgenic mice by site-specific recombination system, it is necessary to prepare other transgenic mice expressing Flp recombinase. Utilizing in utero electroporation, in vivo two-photon imaging of pyramidal neurons in the cerebral cortex can be performed without Flp drivers. Stable homogeneous G-CaMP9a expression without apparent adverse effects over five months is suitable for long-term imaging and accurate deconvolution of calcium imaging data (Sakamoto et al., 2022). However, targeting other brain regions or inhibitory neurons is more challenging by in utero electroporation, and results can be more inconsistent. Some alternatives have been developed with modified electrodes to significantly increase plasmid targeting (dal Maschio et al., 2012).

TROUBLESHOOTING

Problem 1
Poor efficiency of electroporation (step 16).

Potential solution
Typically, when electroporation is successful, the expression of G-CaMP9a can be observed by two-photon microscopy. The expression can be seen in the entire somatosensory cortex and sometimes in the visual cortex. However, if the efficiency of electroporation is low, the number of neurons expressing G-CaMP9a will be small. The reasons for such inefficiency may be as follows.

- **Injection site:** Beginners often inject plasmids into the subcortical parenchyma by mistaking the injection angle and depth. In this case, electroporated neurons are usually found in subcortical areas like the striatum. When plasmids with dye are adequately placed in the lateral ventricle, a blue crescent-like line can be observed on the injection site (Figure 3F). This line is an indicator that the injection was successful.

- **Electrodes:** The position and polarity of the electrodes are very critical. Make sure the anode is pointing at the targeted cortical area. Also, make sure that the orientation of the anode and cathode are correct. In addition, make sure that the electrodes (especially the platinum tip) are clean. We recommend cleaning the tip with a toothbrush after the experiment. Moreover, before
delivering electric pulses to embryos, make sure that air bubbles appear on the electrode’s surface when electric pulses are delivered into saline. If you plan to target regions other than the barrel cortex like the prefrontal cortex and cerebellum, a protocol with triple electrodes is helpful (dal Maschio et al., 2012).

- **Parameters of electrical pulse:** Pulse intensity and duration are key parameters. When increasing the pulse intensity and frequency, the efficiency of the electroporation will increase. However, neurons will be unhealthy or dead if they are too strong. In some cases, these strong parameters will lead to abortion.

**Problem 2**
Poor survival after in utero electroporation (step 24).

**Potential solution**
If the survival rate of embryos is low, the possible reasons are as follows.

- **Surgery time:** The surgery should be finished within 30 min (from incision to the closure of the skin). Prolonged procedures cause embryo death and abortion.
- **Maternal care:** Maternal stress around/following delivery causes neglecting litter. C57BL/6 background is especially prone to stress and rejecting litter. We strongly recommend using a foster mother from an ICR background.
- **Electroporation parameter** (related to Problem 1): High pulse intensity and frequency cause damage to both mother and her embryos.
- **DNA quality:** Low-quality plasmid is toxic for embryos. Plasmids for injection should be purified by an endotoxin-free plasmid kit. Also, purified plasmids should be handled on a clean bench to avoid pathogenic contamination. Also, repeated freeze and thaw would degrade the quality of the DNA. Therefore, we recommend making aliquots and keeping them in the freezer.
- **Breeding environment:** Make sure that environmental conditions such as temperature, humidity, and dirt in the air are optimal in the animal facility. These abnormalities cause substantial stress to the animals.

**Problem 3**
Headplate comes off (step 36).

**Potential solution**
Fixing the headplate with dental acrylic bonds is essential (step 38). However, the process of attaching the headplate with superglue is also very critical (step 36). It is important to keep pushing the headplate from the top for a while to get firmly attached to the headplate cranium. Wait for at least 10 min to make sure that the superglue is completely dry before proceeding to the next step. In addition, scratching an exposed skull with fine forceps will strengthen the bond since the dental acrylic bonds better to the rough surface.

**Problem 4**
Bleeding and damage during the surgery (step 40).

**Potential solution**
Bleeding and brain damage will occur if the drill bit breaks through the skull while drilling. The skull is thick and takes time to thin but be careful not to increase the speed of the drill too much.

**Problem 5**
No G-CaMP9a fluorescence under the two-photon microscope (expected outcomes).
Potential solution
Genotyping error might cause. Make sure that genotyping of the G-CaMP9a transgenic mouse is correct. Either wild-type allele or transgenic allele hands should be detected.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Haruhiko Bito (hbito@m.u-tokyo.ac.jp).

Materials availability
Plasmids used in this study have been deposited to the RIKEN BRC (catalog number: RDB19603 and RDB19604).

Data and code availability
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101421.

ACKNOWLEDGMENTS
This work was supported in part by grants from Precursory Research for Embryonic Science and Technology (PRESTO)-JST (JPMJPR1906 to M.S.), Grant-in-Aid for Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS) (JP20dm020706 to M.S., JP19dm020709 to H.B.), Strategic Research Program for Brain Sciences (JP16dm0107149 and JP21wm0525004 to M.S., JP16dm0107132 to H.F.), Interstellar Initiative (JP18jm0610009 to M.S.), Brain Information Dynamics (BID) (JP17H06312 to H.B.), JSPS KAKENHI (JP21K19429, JP20H04122, JP19H04898, JP18K19493, JP17H05941, and JP16H06278 to M.S.; JP17K13270 to H.F.; and JP19H01007 to H.B.), Takeda Science Foundation (to M.S., H.F., and H.B.), Uehara Memorial Foundation (to M.S.), Kowa Life Science Foundation (to M.S.), Tokyo Biochemical Research Foundation (to M.S.), Research Foundation for Opto-Science and Technology (to M.S.), Lotte Foundation (to M.S.), The Konica Minolta Science and Technology Foundation (to M.S.), Brain Science Foundation (to M.S.), Nakatani Foundation (to M.S. and H.B.), Astellas Foundation for Research on Metabolic Disorders (to H.F.), and Hitachi Global Foundation (to H.B.).

AUTHOR CONTRIBUTIONS
Conceptualization, M.S. and H.B.; Methodology, M.S., K.O., Y.K., and M.O.; Acquisition of data, M.S., Y.K., and M.O.; Writing—original draft, M.S. and H.B.; Writing—review & editing, M.S. and H.B.; Supervision, H.F. and H.B.

DECLARATION OF INTERESTS
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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