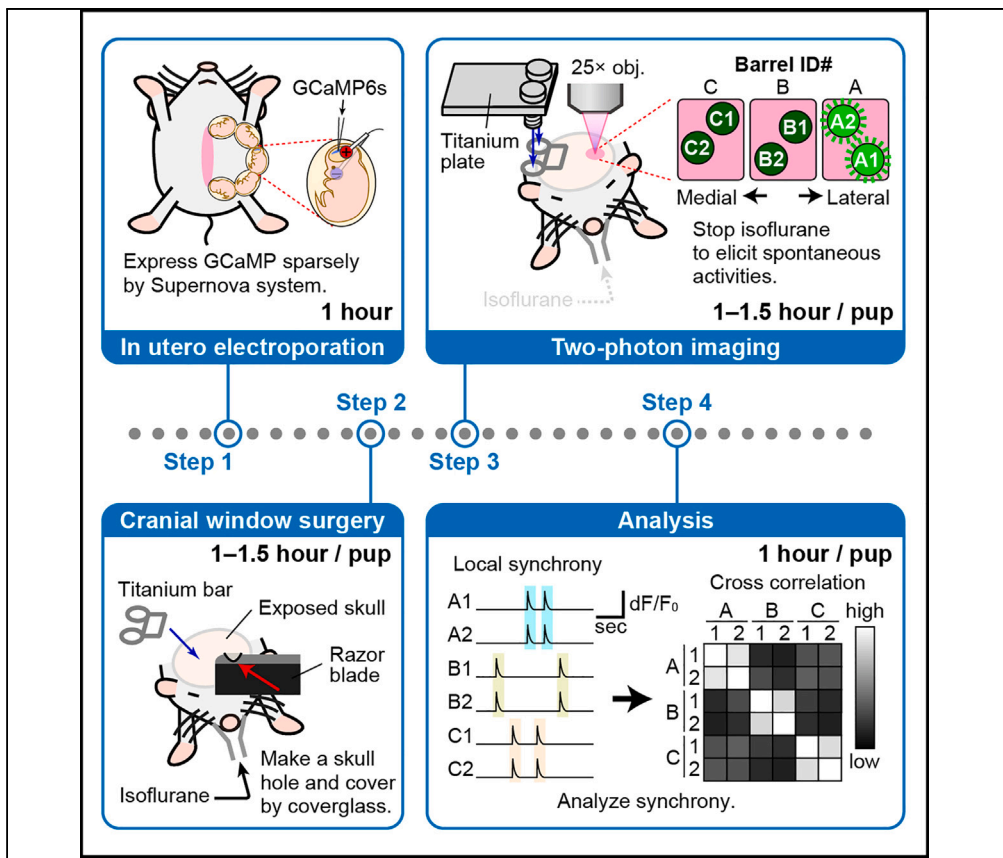


Protocol

In vivo two-photon calcium imaging of cortical neurons in neonatal mice



In vivo calcium imaging is essential to elucidate unique synchronous activities observed in the developing brain. Here, we present a protocol to image and analyze activity patterns in neonatal mouse neocortex in single-cell level. We describe steps for in utero electroporation, cranial window surgery, two-photon imaging, and activity correlation analysis. This protocol facilitates the understanding of neuronal activities and activity-dependent circuit formation during development.

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

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Highlights

Preparation of mice
by labeling sparse
cortical neurons using
GCaMP

Cranial window
surgery procedure for
in vivo 2-photon
imaging of neonatal
brain

Single-cell level *in
vivo* calcium imaging
in neonatal brain

Quantitative analysis
of activity correlation

Egashira et al., STAR Protocols
4, 102245
June 16, 2023 © 2023 The
Author(s).
[https://doi.org/10.1016/
j.xpro.2023.102245](https://doi.org/10.1016/j.xpro.2023.102245)



Protocol

In vivo two-photon calcium imaging of cortical neurons in neonatal miceTakamitsu Egashira,^{1,2,4} Nao Nakagawa-Tamagawa,^{3,4,5,*} Elvira Abzhanova,^{1,2,4} Yuzuki Kawae,¹ Ayami Kohara,¹ Ryoko Koitabashi,¹ Hiromi Mizuno,¹ and Hidenobu Mizuno^{1,2,5,6,*}¹Laboratory of Multi-Dimensional Imaging, International Research Center for Medical Sciences (IRCMS), Kumamoto University, Kumamoto 860-0811, Japan²Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-0811, Japan³Department of Physiology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8544, Japan⁴These authors contributed equally⁵Technical contact⁶Lead contact*Correspondence: naonaka@m.kufm.kagoshima-u.ac.jp (N.N.-T.), hmizuno@kumamoto-u.ac.jp (H.M.)
<https://doi.org/10.1016/j.xpro.2023.102245>

SUMMARY

In vivo calcium imaging is essential to elucidate unique synchronous activities observed in the developing brain. Here, we present a protocol to image and analyze activity patterns in neonatal mouse neocortex in a single-cell level. We describe steps for *in utero* electroporation, cranial window surgery, two-photon imaging, and activity correlation analysis. This protocol facilitates the understanding of neuronal activities and activity-dependent circuit formation during development.

For complete details on the use and execution of this protocol, please refer to Mizuno et al. (2014),¹ Mizuno et al. (2018a),² and Mizuno et al. (2018b).³

BEFORE YOU BEGIN

Institutional permissions (if applicable)

All experiments were conducted in accordance with the guidelines for animal experimentation at Kumamoto University and were approved by the animal experimentation committees. Readers who use this protocol will need to acquire permission from their relevant institutions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NaCl	Nacalai	31320-05
KCl	Nacalai	28514-75
Glucose	Nacalai	16806-25
HEPES	Gibco	15630-080
CaCl ₂ ·2H ₂ O	Nacalai	06731-05
MgSO ₄ ·7H ₂ O	Wako	131-00405
Trypan blue	Sigma	T8154
Sodium pentobarbital	Nacalai	26427-72
Isoflurane	Pfizer	N/A

(Continued on next page)



Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agarose	Sigma	A9793
Carprofen	Zoetis JP	Rimadyl
Saline	Otsuka	035175315
Phosphate buffered saline (PBS)	Nacalai	27575-31
4%-paraformaldehyde phosphate buffer solution (4% PFA)	Nacalai	09154-85
Experimental models: Organisms/strains		
Pregnant ICR female mouse (E14)	Nihon SLC	Slc:ICR
Delivered pups (P3–12, both sex)		Slc:ICR
Recombinant DNA		
Plasmid: TRE-FIpe-WPRE	Addgene	pK036
Plasmid: CAG-FRT-STOP-FRT-GCaMP6s		ask the corresponding author (HM)
Software and algorithms		
Las X	Leica Microsystems	https://www.leica-microsystems.com/
Fiji ImageJ	Schindelin et al. ⁴	https://fiji.sc/
Other		
250 mL vacuum filter/storage bottle system	Corning	431096
Precision calibrated micropipets	Drummond	2-000-050
Anesthesia unit	Univentor	410
Micropipette puller	Narishige	PC-100
Aspirator tube assembly	Drummond	2-040-000
Tweezers with platinum plate electrode, 5 mm diameter	BEX	CUY650P5
Electroporator	BEX	GEB14
Round cover glass, 3-mm-diameter	Matsunami	CS01078
Titanium bar	Endo Scientific Instrument	(Custom made)
Fluorescence stereo microscopy	Leica	M165FC
Tissue adhesive (Vetbond)	3M	1469SB
Titanium bar fixing plate	Customize	(Mizuno et al. ³)
Heat pad	Muromachi Kikai	FHC-HPS
Razor blade	Feather	FA-10
Gelfoam sterile sponge	Pfizer	No.12
Acrylic resin (UNIFAST II)	GC	N/A
Scopisol	Senju Pharmaceutical	N/A
Multiphoton microscope	Leica	SP8MP
Multiphoton laser	Spark Lasers	ALCOR 920-2W
Multiphoton laser	Coherent	Fidelity-2
25× objective lens (water immersion)	Leica	15506374
Stage for imaging living pup (two single-axis translation stage for XY positioning, two-axis goniometer, base plate, adjustable pillar for z positioning)	ThorLabs	LT1/M, GN2/M, BM2060/M, MLP01/M

MATERIALS AND EQUIPMENT

Plasmid solution

A plasmid solution of 10 μ L will be used for *in utero* electroporation in one pregnant mouse. See also the [key resources table](#).

Reagent	Final concentration	Amount
TRE-nCre (100 μ g/ μ L)	5 ng/ μ L	0.5 μ L
CAG-loxP-stop-loxP-GCaMP6s-ires-tTA-WPRE (10 μ g/ μ L)	1 μ g/ μ L	1 μ L
Trypan Blue (0.4%)	0.02%	0.5 μ L
MilliQ water	N/A	7 μ L
Total		10 μL

The solution can be stored at -20°C in a tightly sealed tube for at least several years.

Cortex buffer solution (Holtmaan et al.⁵)

A cortex buffer solution of 500 mL will be sufficient for cranial window surgery for up to 50–80 pups.

Reagent	Final concentration	Amount
NaCl	125 mM	3.65 g
KCl	5 mM	0.19 g
glucose	10 mM	0.90 g
HEPES	10 mM	1.19 g
CaCl ₂ ·2H ₂ O	2 mM	0.15 g
MgSO ₄ ·7H ₂ O	2 mM	0.25 g
ddH ₂ O	N/A	Up to 500 mL
1 M NaOH	N/A	Up to pH 7.4
Total	N/A	500 mL

After vacuum filter sterilization, the solution can be stored at 4°C for up to 3 months shielded from light.

STEP-BY-STEP METHOD DETAILS

In utero electroporation (optional)

⌚ Timing: 1 h/mouse

For *in vivo* calcium imaging, genetically-encoded calcium indicator (GECI)-expressing pups are necessary. This section describes the steps to sparsely introduce calcium indicator protein GCaMP6s to cortical layer 4 neurons using the Supernova system² by *in utero* electroporation.^{6–8} This step can be skipped if appropriate GECI-expressing transgenic mice are available.

1. Pull glass capillary using a puller (PC-100, Narishige) to make micropipettes for plasmid solution injection. Cut the tip to a diameter of ~50 μm using scissors.

Note: Beveled micropipette tips (obtained by scraping the tips) are more favorable.

2. Warm saline to 37°C in a 50 mL conical tube.
3. Attach an injection micropipette to an aspirator tube (2-040-000, Drummond) and fill the micropipette with the plasmid solution (1 μL per pup).
4. Anesthetize a pregnant mouse at embryonic day (E) 14 using a combination of sodium pentobarbital intraperitoneal injection (50 mg/kg body weight) and inhalation of isoflurane gas (0.8%–1.2% in air). [Troubleshooting 1](#).
5. Turn on a timer to record the time taken for the operation. Finish the surgery within 30 min to reduce the physical strain of pregnant mouse and obtain more viable pups.
6. Sterilize the incision surface with 70% ethanol. Make a midline laparotomy, place saline-moistened non-woven fabric (e.g., Kimwipe) around the incision, and expose one side of the uterus to the fabric.

Note: After exposing the uterus, dip warm saline constantly to prevent drying of the uterus. [Troubleshooting 2](#).

7. Using a micropipette, inject DNA solution into one side of the lateral ventricle of each embryo. [Troubleshooting 2](#).

Note: Change the micropipette when this becomes too blunt to penetrate the uterus wall.

Note: Avoid injecting the embryo closest to the vagina. If the embryo stops developing and dies, other embryos often die sequentially.

8. Pinch the embryo heads with a forceps-type electrode (CUI650P5, BEX) so that the target location is under the positive side of the electrode. Deliver square electric pulses (40 V, 50 ms) five times at 1 Hz using an electroporator (GEB14, BEX). [Troubleshooting 2](#).

Note: Check the current amplitude shown on the electroporator. For our conditions, the amplitude is 50–80 mA.

9. Expose the other side of the uterus, and reposition the electroporated side of the uterus. Repeat steps 7–8.
10. Place the exposed uterus back into the abdominal cavity. Fill the peritoneal cavity with warm saline and gently knead the stomach to relieve abdominal distension.
11. Suture the abdominal wall. Before closing completely, inject warm saline into the peritoneal cavity again to prevent adhesions between the internal organs.
12. Suture the abdominal skin.

Note: The skin can also be closed with metal clips.

13. Allow the mouse to recover on a heater (37°C). After recovery, return the mouse to the cage rack.

△ **CRITICAL:** To introduce genes in layer 5 or layer 2/3 neurons, in utero electroporation should be performed on E12–13 or E15, respectively. Electric pulse amplitude, duration, and numbers should also be altered.

Pup selection at postnatal days (P) 1–3 (optional)

⌚ **Timing:** 15 min

This section describes steps to select electroporated pups with appropriate gene expression.

14. Prepare a fluorescence stereo microscope.
15. Observe pup heads and check whether a GCaMP signal is seen in the electroporated side of the cerebral hemisphere.
16. Euthanize pups with no expression or inappropriate GCaMP expression.

Note: Shorten the time when pups are away from their mother as much as possible.

Note: Avoid selecting pups at the day of birth because the mother is nervous and would neglect the pups.

Note: If the number of pups with appropriate expression exceeds eight, euthanize some so the final number is eight or less; otherwise, too many offspring hinder their growth.

Skull exposure

⌚ **Timing:** 15 min/pup

This section describes the steps to expose the skull of pups (P3–12) and to fix the cut surface of the scalp with tissue adhesive (Vetbond). Though the protocol of skull exposure and the following cranial window surgery section has been described previously,³ we have included our modified protocol to provide additional advice.

17. Prepare a fluorescence stereo microscope.
18. Place saline into a 50 mL conical tube and maintain it at 20°C–25°C.

19. Fill a 1.5 mL tube with Vetbond.
20. Place the isoflurane-soaked fabric in an acrylic anesthetic container. Place a pup in the container and anesthetize.

△ CRITICAL: Pay attention to the pup's breathing during anesthesia at every step hereafter. A breathing arrest for a few seconds may stop the cerebral blood flow and cause critical brain damage even if the pup recovers normal breathing and movement later.

21. Place the pup in front of a tube cup where isoflurane gas (2.0%–2.5% in air) exits.

Note: To make the procedure easier, secure the tube cup to the stage with cellophane tape. Make a scalp incision of ~5 mm in diameter to expose the skull and view the location of GCaMP expression using the fluorescence stereo microscope (Methods video S1). Euthanize pups that have inappropriate expressions. [Troubleshooting 3](#).

22. Remove the scalp above the cerebral hemispheres with scissors to as wide as possible, especially on the side of making a cranial window.

Note: Avoid cutting the large blood vessels such as the branches of superficial temporal vein and supraorbital vein near the ears and eyes.

23. Wipe the skull with a saline-soaked cotton swab or use tweezers to remove connective tissue above the skull.
24. Wipe the skull with a dry cotton swab, and wait until the skull dries.
25. Apply Vetbond to fix the incised surface of the scalp to the skull.

Note: Avoid VetBond attaching to the location to make a cranial window.

26. After fixing the incised scalp surface for one round, wait for a few minutes, and then fix it for another round.

Note: Incomplete fixation can cause: (a) difficulty in holding the pup's head to open the skull hole with a razor blade (step 43), (b) scalp bleeding which makes the cranial window opaque, and (c) displacement of the brain region during imaging when the pup moves.

27. Return the pup to a plastic container on a 37°C heating pad to recover from anesthesia. Wait for at least 15 min to let Vetbond solidify.

▮▮ Pause point: The pup can be kept in a plastic container on a 37°C heating pad for ~1 h. Do not return the pup to the mouse cage, as the mother mouse often gnaws the Vetbond to bleed.

Preparation for cranial window surgery

⌚ **Timing:** 10 min

This section describes the steps to prepare for cranial window surgery ([Figure 1A](#)).

28. Add cortex buffer into a 50 mL conical tube and maintain it at 20°C–25°C.

Note: The solution should be kept at 20°C–25°C as warming the cortex facilitates bleeding during cranial window surgery (step 45).

29. Prepare agarose solution.

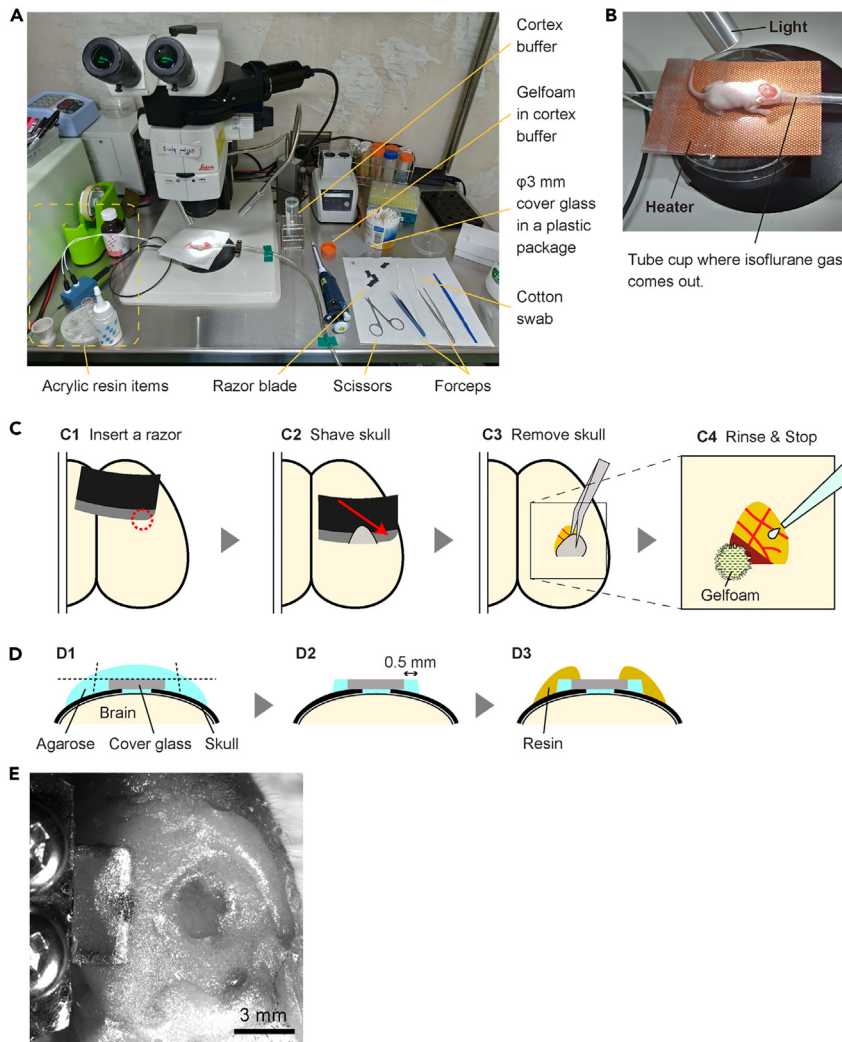


Figure 1. Set-up and procedures of cranial window surgery

(A) Operation desk set-up.

(B) A mouse pup under isoflurane anesthesia.

(C) Craniotomy. Curve the razor blade (reverse to the curvature of the skull) and insert the corner of the blade from the anterior side where a cranial window should be made (C1). Push the blade forward diagonally and slowly to shave off the skull and make a hole of appropriate size (C2). Pinch the cracked skull with tweezers and peel it off (C3). Rinse the brain surface with cortex buffer quickly using a micropipette until the bleeding stops completely. Put gelfoam to stop bleeding if needed (C4).

(D) After positioning a cover glass and adding agarose, remove any agarose on the glass and any agarose distant from the glass edge (about 0.5 mm) with tweezers (D1, D2). Agarose should be completely covered by resin (D3).

(E) An example image of a cranial window.

- a. Put 5 mL of cortex buffer and 0.05 g of agarose (Sigma, cat. no. A9793, gel point $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$) in a 50 mL conical tube without the lid. Microwave the solution to dissolve agarose.

Note: The solution should be almost boiled to dissolve agarose completely. Avoid overheating, to prevent the conical tube from bursting.

- b. Take ~ 1 mL of agarose solution in a 1.5 mL tube and maintain it at 42°C with a table-top incubator.

30. Select a titanium bar whose screw holes fit the screw holes of the titanium plate.

Note: Because titanium bars are thin and soft, some have screw holes that are unfit for the holes of the titanium plate.

31. Dilute analgesic (50 mg/mL carprofen, Rimadyl, Zoetis JP) 100× with saline to prepare analgesic solution.
32. Fold razor blades into four pieces.
33. Take some gelfoam (Pfizer No. 12), tear it into small pieces (~2 mm in diameter), and place them in a cortex buffer in a small container (e.g., a cap of a conical tube).

Cranial window surgery

⌚ Timing: 0.5–1 h/pup

This section describes the steps to make an imaging window.

34. Sterilize scissors, tweezers, and razor blades with 70% ethanol.
35. Turn on a timer to record the time taken for the surgery.
36. Anesthetize a pup with isoflurane in an acrylic anesthetic container.
37. Place the pup on a heating pad (35°C) under the stereo microscope. Place the tube cup where isoflurane gas (2.0%–2.5% in air) exits in front of the pup's nose (Figure 1B).
38. Observe GCaMP fluorescence using a fluorescence stereo microscope and mark the place to make a cranial window with a marker.
39. Apply one drop of cortex buffer to the skull of the hemisphere where a window should be made.

Note: Keep the skull wet while making a hole to prevent the dura from sticking to the razor blade and tearing.

40. Wipe away any blood on the skull, using a cotton swab wet with cortex buffer.

Note: Be careful not to make the skull too wet; otherwise, the Vetbond adhesion may be loosened.

41. Rotate the mouse so that the skull area where a cranial window should be made comes upward.
 - a. Curve the razor blade in reverse to the curvature of the skull, and insert the corner of the blade from the anterior side where the cranial window should be made (Figure 1C1).
 - b. Push the blade forward diagonally and slowly to shave off the skull and make a hole of an appropriate size (Figure 1C2).

Note: If there is strong resistance while inserting the blade, only the surface of the skull has been cut. The resistance decreases when the blade penetrates the skull. However, because the dura should remain intact, avoid inserting the blade too deep.

Note: Replace the blade when it is no longer sharp.

42. Pinch the cracked skull with tweezers and remove it (Figure 1C3). If a hole is successfully made, blood vessels can be seen.

Note: Bleeding tends to occur with the force of peeling off. Another choice is to rotate the razor blade 90° and cut the peeled bone away after a hole of the appropriate size is made.

43. If there is bleeding, quickly rinse the brain surface with cortex buffer using a micropipette. Repeat rinsing until the bleeding stops completely (Figure 1C4).

Note: If a blood clot is formed on the brain surface, take a piece of gelfoam with tweezers and gently wipe the surface to remove the clot. If the bleeding does not stop, place gelfoam in the bleeding position and let the blood soak into gelfoam (Figure 1C4). This will help to stop the bleeding, although the position covered by gelfoam tends to form a blood stain.

44. After the bleeding completely stops, apply one drop of cortex buffer on the cranial bone hole, and place a 3-mm-diameter round cover glass over the hole.

Note: If bleeding occurs at steps 43–45, wait a few minutes before moving on to the next step to improve the hemostasis and to reduce the risk of rebleeding.

45. After the buffer around the glass has dried, quickly apply warm agarose solution around the edge of the glass using a micropipette.

Note: During this step bleeding commonly occurs because vasodilation results from applying a warm agarose solution. If bleeding occurs, remove the glass and agarose, and restart from step 45. The glass can be reused after rinsing.

△ **CRITICAL:** Bleeding must be completely stopped. If bleeding occurs under the cover glass, imaging becomes impossible.

46. Remove the agarose on the glass approximately 0.5 mm distant from the glass edge with tweezers (Figure 1D1). Only cover the outer periphery of the glass with agarose (Figure 1D2).

Note: If air bubbles appear under the window, remove the glass and agarose, and retry from step 47.

47. Wait for the agarose to harden. Absorb any liquid on the skull with non-woven fabric and dry the skull entirely.

Note: Agarose gel shrinks as it hardens. If a space appears under the glass, add more agarose so that the agarose will finally cover the entire edge of the glass.

48. Mix acrylic resin liquid and powder in a rubber container and aspirate this with a micropipette.
49. Pour the resin on the skull away from the glass little by little and check the resin's hardness (resin hardens even after being aspirated by a micropipette). When the resin gets hard enough so that it does not flow laterally, fix the outer edge of the glass quickly.

Note: If the resin is placed while too fluid, it will flow under the glass and make imaging impossible.

Note: Cover the agarose completely with resin (Figure 1D3); otherwise, cerebral fluid will exit through the agarose and cause brain damage.

50. Fix the titanium bar with resin on the hemisphere contralateral to the window. Keep the bar angle parallel to the cover glass.

Note: If the titanium bar fixation is not tight enough, pup respiration makes imaging plane displacement during imaging.

51. Fix the entire skull surface with resin (Figure 1E).
52. After the surgery is finished, inject analgesics (carprofen 100× solutions, 0.1 μL/g weight; carprofen 5 μg/g weight) under the skin at the back of the neck.

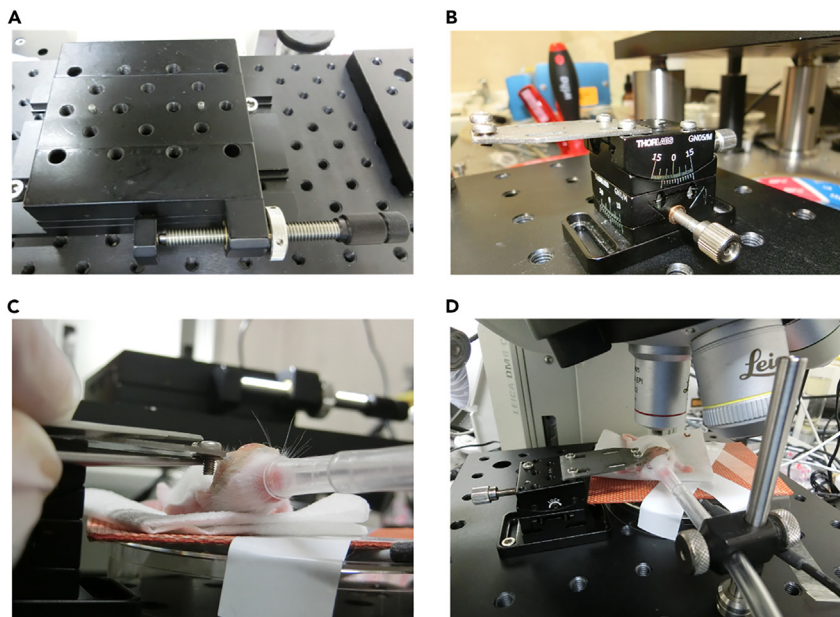


Figure 2. Two-photon imaging set-up

- (A) A stage plate with XY positioning.
 (B) Two-axis goniometer attached to the stage plate with a titanium plate on the top.
 (C) Fix the titanium bar that is attached to the pup's head to the titanium plate with screws. Place the tube cup for isoflurane gas on the pup's mouth.
 (D) Condition for observing the cranial window.

53. Return the pup to a plastic container on a 37°C heating pad to recover from anesthesia.
54. Record the time required for the surgery.
55. Wait for >60 min to solidify resin before imaging.

Note: Because isoflurane anesthesia suppresses cortical activity in neonatal brain,^{2,3,9,10} waiting before imaging is important to observe normal activity.

⏸ **Pause point:** The pup can be kept in a plastic container on a 37°C heating pad for a few hours. Do not return the pup to the mouse cage, as the mother mouse often gnaws the resin and exposes the brain.

Two-photon microscopy set-up

⌚ **Timing:** ~10 min

This section describes the steps required for two-photon imaging.

56. Turn on the microscope controller, confocal unit, PC, LasX, and Alcor Laser for GCaMP imaging.
57. Set a stage plate with XY positioning (Figure 2A). Attach a two-axis goniometer (ThorLabs GN2) with a titanium plate (Figure 2B) and a pillar with a tube cup where isoflurane gas exits to the stage plate.
58. Set a heating pad (35°C).
59. Set scan settings of LasX.
 - a. Format: 512 × 512
 - b. Speed: 400 Hz (possible to be raised up to 600 Hz).
 - c. Bidirectional: ON.

- d. Zoom Factor: 0.75 (with a 25× objective, the imaging area will be 590 × 590 μm).
- e. Averaging: none.

Preparation for two-photon imaging

⌚ Timing: 10 min/pup

This section describes the preparation for two-photon microscopy.

60. Turn on a timer to record the time taken for imaging.
61. Anesthetize a pup with isoflurane in an acrylic anesthetic container.
62. Place the pup on a heating pad. Fix the titanium bar on the pup's head to the titanium plate with screws (Figure 2C). Place the tube cup for isoflurane gas (2.0% in air) on the pup's mouth.
63. Adjust the window angle horizontally via the goniometer.
64. Wipe the window softly with an ethanol-soaked cotton swab to remove any dust.
65. Adjust the XY position of the stage to locate the window under the 5× objective lens (Figure 2D).
66. Turn on the backlight and ensure the window is visible through the eyepiece.

Note: The field of view through the eyepiece is usually rotated 180° from the actual orientation.

67. Put eye drops (Scopisol) on the cranial window and switch to the 25× water-immersion objective lens.

Note: Scopisol is recommended as this dehydrates more slowly than water does.

68. Adjust the z-position and make the window visible through the eyepiece. Confirm that blood flow is seen.

Note: The window angle can be again adjusted in this step. Blood vessels are good landmarks to ensure that the brain surface is horizontal.

Note: If blood flow is not seen in any or all the blood vessels, the pup may have received too much isoflurane in the previous steps.

69. Turn off the backlight.
70. Start Las X scanning using the one-photon mode. Increase the laser power for green fluorescence so that the autofluorescence of the glass or the brain surface is visible.
71. Lower the isoflurane concentration to 1.2%–1.5%.
72. Cover the microscope with a blackout curtain and turn off the room lights.

Note: Avoid intense light entering the microscope afterwards.

Two-photon imaging of spontaneous neuronal activity

⌚ Timing: 0.5–1 h/pup

This section describes steps for imaging spontaneous neuronal activity by two-photon microscopy.

73. Switch the imaging software to the two-photon mode.
74. Turn on the MP laser shutter for green fluorescence imaging. Select the imaging area and conditions. [Troubleshooting 4](#).
 - a. Adjust XYZ position to the field of interest where GCaMP positive cells are visible.
 - b. Increase the detector gain to 80%.
 - c. Increase the laser power. If the signal remains low, increase the detector gain to 100%.

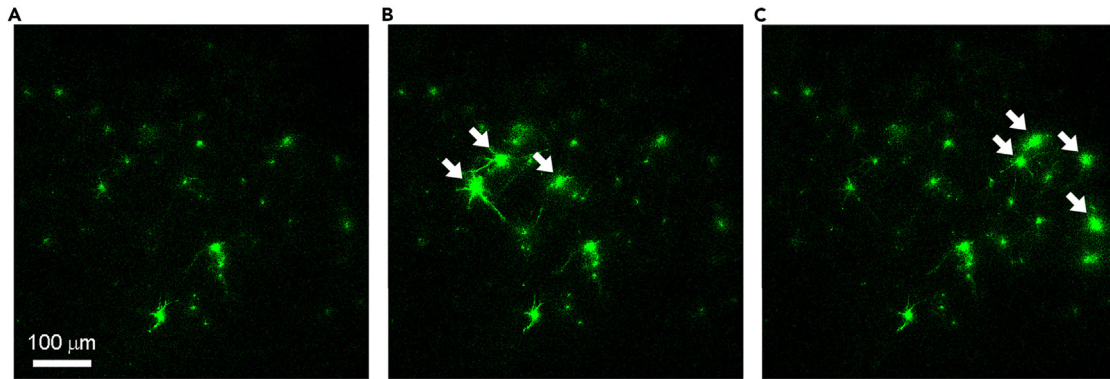


Figure 3. Two-photon imaging of spontaneous neuronal activity

Two-photon images obtained from a Supernova GCaMP6s-electroporated P5 pup.

(A) Baseline signals obtained during anesthesia.

(B and C) Representative GCaMP fluorescent changes (GCaMP-labeled cells with arrows).

Note: To avoid bleaching, increase the laser power gradually. The required laser power may change for GEC1-expressing transgenic animals.

75. Set time-lapse imaging parameters.
 - a. Set the scan rate to >1 Hz to observe the calcium transients in neonates.
 - b. Set the number of frames to scan >20 min.
76. Start time-lapse imaging and acquire GCaMP signals (Figure 3).
77. After 5 min of scanning of baseline activities, turn off the isoflurane.

Note: After ~5 min from the isoflurane offset, calcium transients will appear. After ~10 min from isoflurane offset, activity level becomes normal (see [limitations](#)).

78. When the pup becomes too active for imaging, quit imaging and anesthetize the pup by administering isoflurane (2% in air).
79. After the pup relaxes, repeat imaging from step 78. XYZ positions can be changed to scan another area as well.
80. When enough data has been obtained, end the session.
 - a. (optional) Euthanize the pup with an overdose of sodium pentobarbital. Fix the pup by transcardial perfusion of PBS, followed by 4% PFA. Remove the brain and post-fix in 4% PFA for 12~16 h to proceed with additional observation or immunohistochemistry.

Analysis

⌚ Timing: ~1 h/pup

This section describes steps to analyze spontaneous synchronous activity.

81. Open the imaging files with image analysis software such as Image with Fiji.⁴
82. Adjust XY drift between the time-lapse images using two-dimensional cross-correlation.¹¹ Remove images whose drift cannot be adjusted by this operation.
83. Select regions of interest (ROIs) for the analysis of GCaMP fluorescence changes. For the analysis of fluorescence in single cells, locate <20 μm-diameter circles on the center of GCaMP-labeled cells (Figure 4A).
84. Obtain dF/F wave, expressed as $dF/F = (F - F_0)/(F_0 - F_b)$, where F_0 is the baseline signal in the absence of calcium transients (step 79) and F_b is the background fluorescence (Figure 4B). [Troubleshooting 5](#).

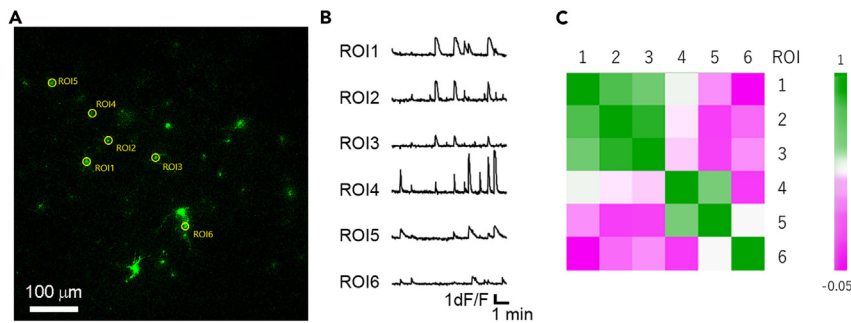


Figure 4. Analysis of calcium imaging data

(A) ROIs are centered on the cell bodies in Figure 3A.

(B) dF/F waves obtained from the ROIs.

(C) Representative correlation matrix obtained from the dF/F waves.

- a. Obtain F from the ROIs. To avoid the effects of anesthesia, use movies after 10 min after stopping isoflurane.
 - b. Calculate a threshold, the mean + 1 × standard deviation of the F wave.
 - c. Obtain F₀ by averaging the F wave below the threshold.
 - d. Obtain F_b by conducting a square box scanning (the pixel number must be approximately equal to the pixel numbers in the ROI) on the first image of the movie, and calculating the minimum value of the scan.
85. Using software such as Microsoft Excel, calculate Pearson's correlation coefficient by using dF/F obtained from each GCaMP-labeled cell, and generate a correlation matrix (Figure 4C).

Note: Correlation between inactive cells (unhealthy or dead cells) will be high. Therefore, only use active cells (we used cells that showed ≥ 5 calcium transients in 10 min).

EXPECTED OUTCOMES

Unique spontaneous neural activity patterns in the neonatal cortex have received widespread attention.^{2,9} However, the number of studies that imaged such neonatal activities in single-cell level has been limited, probably due to technical difficulties. This protocol will remove this bottleneck and encourage readers to study brain activities during development. This protocol will also facilitate the usage of advanced two-photon microscopy tools and applications, which have been revolutionized in the last 20 years.^{12–14}

A piezoelectric device and a resonant scanner, which are commercially available, enabled volume imaging of $>500 \times >500 \mu\text{m}$ plane for ten planes at ~ 2 Hz for adult studies.¹⁵ The neonatal cortex exhibits slower neuronal activities than the adult cortex, further allowing imaging of wider cortical depth with a scan rate sufficient for the synchrony analysis. These tools and advantages may enable the in-detail analysis of layer-specificity, inter-layer coordination, and intra-layer radial synchrony of spontaneous activities in neonates.

Though this protocol summarized the techniques to observe patchwork-like synchronous activity from the S1 barrel area, it can also be used to observe unique activities in other cortical areas, as the author observed retinal wave-derived activity in V1.² Additionally, some activities synchronize or propagate among multiple cortical areas. Thalamocortical inputs elicit inter-areal activities to modulate cortical maps at the early stages, which has mainly been studied with single-photon meso-scale imaging^{16–18} (see also Cardin et al.¹⁹). Combining with the burgeoning wide-field two-photon imaging technique,^{20,21} this protocol may enable single-cell level analysis of inter-areal activity in developing mice. The smaller cortical area of neonates further enables wide-field imaging.

This protocol is applicable for neonatal chronic activity imaging that enables the analysis of synchrony pattern changes for several days, as the author analyzed dendritic morphology changes.¹ The synchrony patterns are primarily shaped by spontaneous peripheral activity via thalamus,^{9,17,18} which are followed by sensory-evoked activities such as whisker movement-evoked activity in the S1 barrel area,²² sound-evoked activity in A1,²³ and light-evoked activity in V1.²⁴ Intracortical connections may also account for the synchrony pattern changes. Recurrent connections between excitatory neurons are mainly composed of chemical synapses in a mature stage, whereas gap junctions dominate in the neonatal stage and facilitate synchronous activities.^{15,25–27} The detailed analysis of synchrony pattern changes during development will help uncover the mystery of brain circuit formation.

LIMITATIONS

Spontaneous activity changes during the first two postnatal weeks,¹⁹ and therefore it is beneficial if the changes could be imaged for a longer period. However, the fixing of the exposed skull interferes with the increase in brain size, which potentially affects the development and activities of the neuronal circuit. The skull removal and the dura exposure during the cranial window surgery could induce an inflammatory response, affecting the imaging window's transparency. These factors limit the possible time window of chronic imaging.

Several postnatal manipulation techniques may be inapplicable with this protocol. For example, to label neurons that have specific projections using retrograde tracers, the neonate scalp is cut to inject tracer and then closed using tissue adhesive (e.g., Aron Alpha®). However, the adhesive attached to the skull interferes with its growth, making cranial window surgery difficult.

The smaller cell-cell distance in neonates increases the probability of signal contamination, which limits the accuracy of intercellular activity correlation. Sparse GCaMP expression is one solution to reduce contamination, as we used with the Supernova system, although this reduces data size. A number of tools and computational methods have been developed to reduce signal contamination in adult studies,^{28,29} which should also be tested in neonate studies.

Previous studies reported that isoflurane anesthesia affects the spontaneous neuronal activity in neonates.^{2,3,9,10} For calcium imaging, it is necessary to wait until the activity level becomes normal after stopping isoflurane anesthesia. In our experience, the frequency and pattern of calcium activity did not change beyond 10 min after cessation of isoflurane. These observations are consistent with cortical activity derived from retinal waves becoming observable 10 min after stopping isoflurane.⁹ However, the activity pattern we obtained might differ from that of a completely awake state, so careful consideration is required for its interpretation.

TROUBLESHOOTING

Problem 1

Pregnant mice die frequently.

Potential solution

Avoid isoflurane overdose, which is a frequent cause of pregnant mice dying during surgery. Observing the respiration and constantly adjusting the isoflurane level can avoid isoflurane overdose and choke.

Problem 2

Few pups are obtained because of miscarriages or pup rejection and cannibalism.

Potential solution

Avoid bleeding during the surgery. Inject plasmid solution to the lateral ventricle with a volume of ~0.5 μ L per embryo and by using as weak as possible pressure (0.1 μ L/s) to prevent hydrocephaly or fetal death.

Pinch the embryo head softly to avoid the amniotic sac rupturing. Dip saline constantly to prevent drying of the uterus. A shortened surgical time (<30 min) gives more viable pups.

Problem 3

GCaMP fluorescence is not seen through the skull.

Potential solution

Observe the appropriate cortical area (see [Methods video S1](#)). Spontaneous neuronal activities occur during low anesthesia; therefore, observe stimulus-dependent activities by deflecting the whiskers on the pup's snout.

Problem 4

GCaMP fluorescence change is not seen during imaging.

Potential solution

Avoid administering too much isoflurane and skull pushing, and reduce bleeding during the craniotomy.

Problem 5

Baseline of the dF/F wave fluctuates.

Potential solution

Avoid cutting the dura and fix the titanium bar to the skull tightly during the cranial window surgery.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Hidenobu Mizuno (hmizuno@kumamoto-u.ac.jp).

Materials availability

The plasmids and the titanium bar and plate information are available from the lead author upon request (see also Mizuno et al.³).

Data and code availability

The datasets in the current study have not been deposited in a public repository but are available from the corresponding author on request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2023.102245>.

ACKNOWLEDGMENTS

This work was supported by Japan Society for the Promotion of Science Grants-in-Aid for Transformative Research Areas (B) (22H05092, 22H05094) and for Scientific Research grants 20K06876, AMED under grant number 22wm0525015h0002, the Takeda Science Foundation, the Naito Foundation, the Kato Memorial Bioscience Foundation, and the Kowa Life Science Foundation (to Hid.M.) and Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research grants 19K06887 and 22K06446, the Kodama Memorial Fund for Medical Research, the Uehara Memorial Foundation, the Kato Memorial Bioscience Foundation, and the Takeda Science Foundation (to N.N.-T.).

AUTHOR CONTRIBUTIONS

T.E., N.N.-T., E.A., Y.K., A.K., R.K., Hiromi Mizuno, and Hidenobu Mizuno conducted the experiments and analyzed the data. T.E., N.N.-T., E.A., and Hidenobu Mizuno wrote the manuscript. All the authors checked and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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