Video Article

In Vivo Two-photon Imaging of Cortical Neurons in Neonatal Mice

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Abstract

Two-photon imaging is a powerful tool for the *in vivo* analysis of neuronal circuits in the mammalian brain. However, a limited number of *in vivo* imaging methods exist for examining the brain tissue of live newborn mammals. Herein we summarize a protocol for imaging individual cortical neurons in living neonatal mice. This protocol includes the following two methodologies: (1) the Supernova system for sparse and bright labeling of cortical neurons in the developing brain, and (2) a surgical procedure for the fragile neonatal skull. This protocol allows the observation of temporal changes of individual cortical neurites during neonatal stages with a high signal-to-noise ratio. Labeled cell-specific gene silencing and knockout can also be achieved by combining the Supernova with RNA interference and CRISPR/Cas9 gene editing systems. This protocol can, thus, be used for analyzing the developmental dynamics of cortical neurons, molecular mechanisms that control the neuronal dynamics, and changes in neuronal dynamics in disease models.

Video Link

The video component of this article can be found at https://www.jove.com/video/58340/

Introduction

The precise wiring of neuronal circuits in the cerebral cortex is essential for higher brain functions including perception, cognition, and learning and memory. Cortical circuits are dynamically refined during postnatal development. Studies have investigated the process of cortical circuit formation using histological and *in vitro* culture analyses. However, the dynamics of circuit formation in living mammals has remained mostly unexplored.

Two-photon microscopy has been widely used for the *in vivo* analyses of neuronal circuits in the adult mouse brain[1,2]. However, owing to technical challenges, only a limited number of studies have addressed neuronal circuit formation in newborn mice. For example, Carrillo *et al.* performed the time-lapse imaging of climbing fibers in the cerebellum in the second postnatal week[3]. Portera-Cailliau *et al.* reported the imaging of axons in cortical layer 1 in the first postnatal week[4]. In the present study, we summarize a protocol for the observation of layer 4 cortical neurons and their dendrites in newborn mice. Results obtained by applying this protocol, which includes two methodologies, are reported in our recent publication[5]. First, we use the Supernova vector system[5,6] for labeling individual neurons in the neonatal brain. In the Supernova system, the fluorescent proteins used for neuronal labeling are exchangeable and labeled cell-specific gene knockdown and editing/knockout analyses are also possible. Second, we describe a surgical procedure for cranial window preparation in fragile neonatal mice. Together, these methodologies allow the *in vivo* observation of individual neurons in neonatal brains.

Protocol

Experiments should be performed in accordance with the animal welfare guidelines prescribed by the experimenter's institution.

1. Preparation of Pups for Imaging

NOTE: Pups with sparsely labeled cortical neurons can be obtained by *in utero* electroporation (IUE) of Supernova vectors[5,6]. The Supernova system consists of the following two vectors: TRE-Cre and CAG-loxP-STOP-loxP-Gene X-ires-tTA-WPRE. In this system, sparse labeling relies on TRE leakage. In a sparse population of transfected neurons, TRE drives the weak expression of Cre and tTA. Subsequently, only in these cells, the expression of gene X is facilitated by a positive feedback of the tTA-TRE cycles. The achieved sparse and bright labeling allows the visualization of morphological details of individual neurons *in vivo*. Details of the IUE procedure are not described in this protocol since they have been described elsewhere[7,8,9,10,11].

1. Prepare timed-pregnant mice for IUE.

2. Prepare a DNA solution for IUE. For sparse labeling with RFP, use a solution containing pK031:TRE-Cre (5 ng/μL) and pK029:CAG-loxP-STOP-loxP-RFP-ires-tTA-WPRE (1 μg/μL) or a solution containing pK031:TRE-Cre (5 ng/μL) and pK273:CAG-loxP-STOP-loxP-CyRFP-ires-tTA-WPRE (1 μg/μL).
   NOTE: Various proteins can be expressed in the labeled neurons using different combinations of vectors. Also, various genes can be knocked-down or knocked-out specifically in labeled cells[5,6] (*e.g.*, a series of vectors for the Supernova system are available from RIKEN BioResource Research Center and from Addgene).
3. Perform regular IUE[7,8,9,10,11] to label cortical neurons. For the labeling of layer 4 neurons, use embryonic day-14 embryos.
4. Wait for pup delivery and growth.

2. Surgery

1. Anesthetize the postnatal day-5 (P5) pup using isoflurane gas (1.0%). Perform a tail-pinch test to check the level of anesthesia. If the pup responds to the pinch, increase the isoflurane concentration (up to 2.0%) or wait until the response disappears. Maintain the pup's body temperature during surgery using a heating pad.
2. Subcutaneously inject an analgesic (carprofen, 5 mg/kg).
3. Sterilize the pup's skin covering the skull by wiping it with 70% ethanol three times.
4. Remove approximately 20 mm^2 of the skin covering the skull using scissors sterilized with 70% ethanol (**Figure 1A**).
5. Remove the fascia of the skull using sterilized forceps and a clean, sterile cotton swab (**Figure 1A**).
6. Apply tissue adhesive using loading tips to the incised skin surface to stop the bleeding. Do not apply tissue adhesive to the imaging area (**Figure 1B**) because this makes the opening of the skull more difficult.
7. Place the pup on a heating pad (37 °C) and allow it to recover from anesthesia. Wait until the tissue adhesive has dried and solidified (approximately 30 min).
8. If necessary, apply more tissue adhesive and wait for it to dry and solidify.

3. Cranial Window Preparation

1. Anesthetize the pup using isoflurane (1.0% - 2.0%) and check the anesthesia level by a tail-pinch test.
2. Carefully open the skull with a sterilized razor blade leaving the dura intact (1 mm in diameter) (**Figure 1C**). Use a gelatin sponge (cut into small pieces, approximately < 2 mm^3, using sterilized scissors, and apply them using tweezers) soaked in cortex buffer[12] (125 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L glucose, 10 mmol/L HEPES, 2 mmol/L $CaCl_2$, and 2 mmol/L $MgSO_4$; pH 7.4; 300 mOsm/L; room temperature) to stop the bleeding. When opening the skull, apply a cortex buffer to keep the brain surface moist.
3. Remove any buffer and blood from the dural surface using a gelatin sponge. Apply a thin layer of 1.0% low-melting-point agarose (dissolved in cortex buffer) using yellow tips. Using a heat block machine, maintain the temperature of the agarose solution at 42°C until application.
   NOTE: The draining of buffer and blood must be performed from the side of the craniotomy while taking care that the dry gelatin sponge does not come in contact with the dura. Failure to do so may damage the dura.
4. Apply a round glass coverslip (No. 1, 3 mm in diameter) onto the agarose gel layer. Remove all bubbles between the coverslip and the agarose gel layer by pouring an excess of agarose gel between them. Remove the excess gel protruding from under the coverslip using tweezers (**Figure 1D** and **1E**).
5. Secure the coverslip using dental cement (**Figure 1F**).
6. Mix the cement powder and the cement liquid. Apply the mixture using yellow tips before it becomes solidified. Do not apply dental cement onto the dura, because this may damage the brain.
7. Attach a sterile titanium bar (custom made, approximately 30 mg, see **Figure 1G**) on the cranial bone using dental cement. Align the titanium bar and the coverslip (on the surface of the dura) in parallel to easily capture images.
8. Cover the exposed skull with dental cement (**Figure 1H**).
9. Recover the pup from anesthesia. Keep it on a heater (37 °C) until the dental cement has solidified (1 h).

4. Two-photon Imaging

NOTE: The *in vivo* images in **Figure 2** were acquired using a two-photon microscope with a titanium-sapphire laser (beam diameter $[1/e^2]^2$: 1.2 mm).

1. Set the two-photon laser wavelength. For RFP excitation, use 1,000 nm (450 mW/mm^2 at 400 μm of depth).
   NOTE: The laser power should be reduced as the z-position moves up.
2. Wipe the surface of the coverslip with 70% ethanol.
3. Anesthetize the pup using isoflurane (1.5% - 2.0%) and check the anesthesia level using a tail-pinch test.
4. Attach the pup to the titanium plate on the imaging stage using a titanium bar on the pup's head (**Figure 2A** and **2B**). Adjust the head such that the coverslip is parallel to the objective lens using the goniometer stage (**Figure 2B**). Maintain body temperature of the pup using a heating pad (37 °C).
5. Set the isoflurane concentration to 0.7% - 1.0%.
   NOTE: A very high isoflurane concentration may cause accidental death of the pup during imaging.
6. Place the imaging stage under the objective lens (20X, NA 1.0) of the two-photon microscope (**Figure 2B** and **2C**).
7. Apply one drop of water onto the coverslip. Use epi-fluorescence to locate the fluorescent protein-labeled neurons in the area where the dura has been exposed.
8. Acquire z-stack images at 1.4-μm intervals. For layer 4 neuron imaging, set the z-width to 150 - 300 μm to image the entire dendritic morphology (**Figure 2D** and **2E**). Use slow scanning and averaging to get clear images showing the neuronal morphology (it usually takes > 20 min to acquire the entire dendritic morphology).

NOTE: The following parameters are recommended for imaging. Excitation wavelength: 1,000 nm, scanner: galvanometer type, dichroic mirror: 690 nm, emission filter: 575 - 620 nm bandpass, detector: GaAsP type, gain setting > 100, image size > 512 x 512 µm, field of view > 600 x 600 µm, pixel resolution < 1.2 µm.

5. Recovery and Nursing

1. Detach the pup from the imaging stage.
2. Place the pup on a heater (37 °C) and allow it to recover (15 min).
3. Feed the pup warm milk using a micropipette at 2-h intervals and gently stimulate the stomach to allow excretion. Confirm that the pup is drinking milk by measuring its body weight.

6. Re-imaging

1. Anesthetize the pup with isoflurane (1.5% - 2.0%), and check the anesthesia level using a tail-pinch test. Attach it to the imaging stage.
2. Locate the previously imaged neurons and acquire a z-stack image. The identification of neurons is easy owing to their sparse labeling with Supernova.
3. Repeat steps 5.1 - 6.2 until imaging is completed. NOTE: The pup can be imaged up to 18 h without losing weight.

Representative Results

Figures 2D - **2F** show representative results of two-photon time-lapse imaging of layer 4 cortical neurons using the present protocol. For the purpose of analysis, select neurons with clear dendritic morphology throughout the imaging periods. We analyzed the dendritic morphology of imaged neurons using morphological analysis software. Representative dendritic morphology reconstruction is shown in **Figure 2F**. Neurons showing disconnected dendrites (**Figure 2G**) should be excluded from analyses, because disconnected dendrites indicate cell death induced by damage during surgery or imaging. In addition, neurons with blurred dendritic tips should be excluded (*e.g.*, the neuron with the arrowhead in **Figure 2D**).

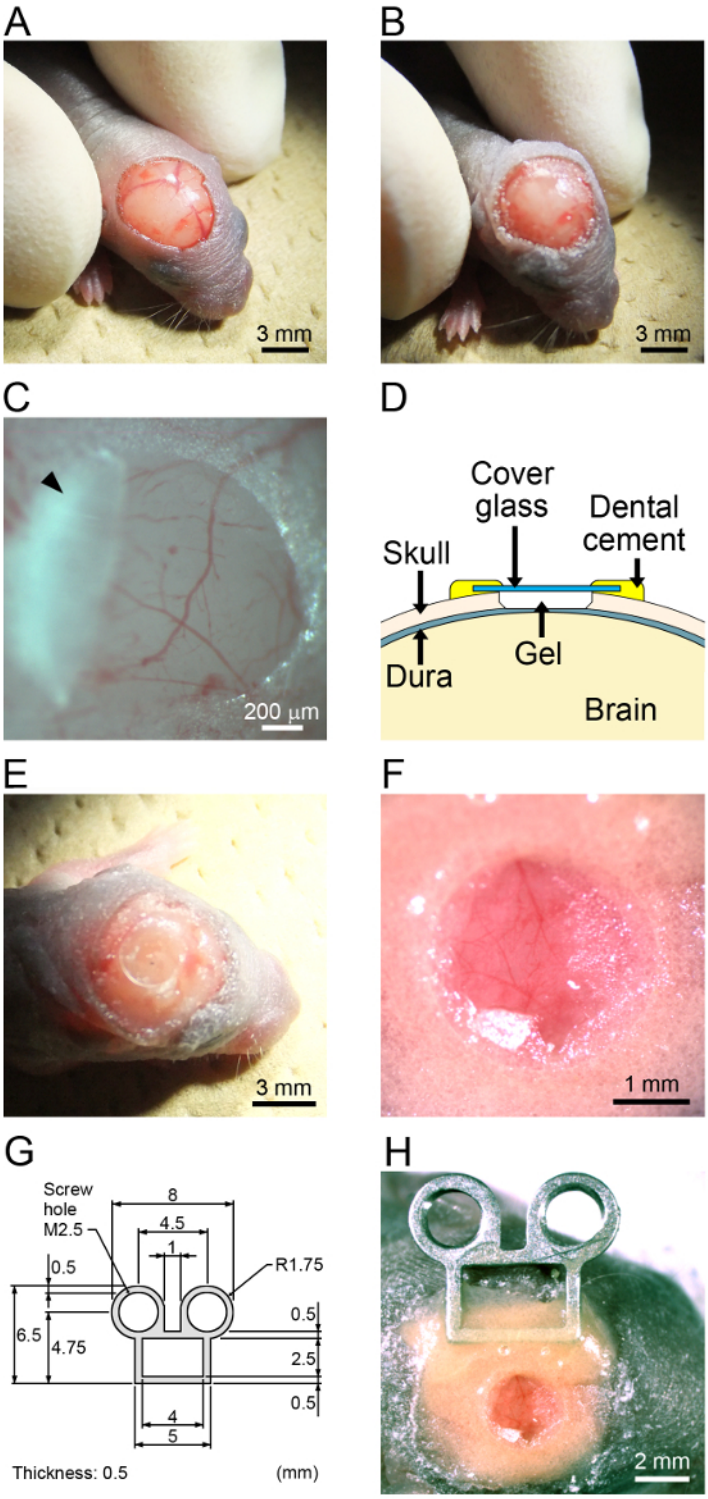

Figure 1: Surgery, cranial window preparation, and attachment of the titanium bar. (**A**) This panel shows the removal of the skin covering the skull. (**B**) This panel shows the fixation of the gap between the skin and the skull. Be careful not to apply the bond to the imaging area. (**C**) This is an image of the exposed dura. A razor blade was inserted between the skull and the dura, and the skull was flapped open to the left of the exposed area (arrowhead). The bone can then be easily removed using forceps. (**D**) This is a schematic design showing a vertical view of the cranial window. The gap between the cover glass and the dura is filled with a thin layer of agarose gel. The coverslip is fixed to the skull using dental cement. (**E**) A round-shaped coverslip is placed on the agarose gel layer. (**F**) This is an image of the secured coverslip. (**G**) This panel shows the design of the titanium bar. The titanium bar contains two screw holes for attachment to the imaging stage (see **Figure 2**) and

one flat rectangular part that is attached to the pup's head. (**H**) The rectangular part of the titanium bar is attached to the pup's skull using dental cement. Please click here to view a larger version of this figure.

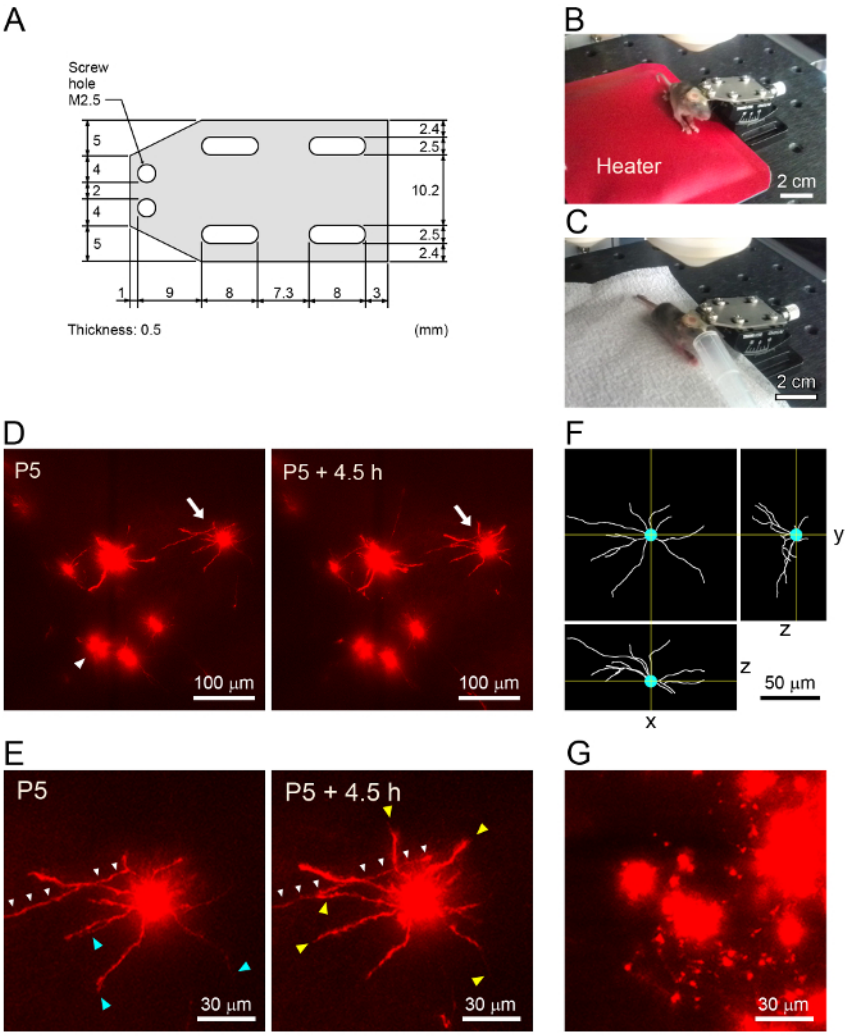

Figure 2: *In vivo* two-photon imaging of cortical neurons in neonatal mice. (**A**) This panel shows the design of the titanium plate. The titanium plate has two screw holes for attaching the titanium bar and four screw holes for attaching the goniometer stage, which is placed on the imaging stage. 2.5 mm (in diameter) x 2 mm (in length) screws are used for fixation. (**B**) This is a representative image of the pup attached to the imaging stage. The pup's body temperature is maintained using a heater. (**C**) The pup is anesthetized using isoflurane during the *in vivo* imaging procedure. (**D**) This panel shows a representative Z-stack time-lapse image of layer 4 cortical neurons of a P5 pup. The arrowhead indicates the neuron with blurred dendrites, which should be removed from the analysis. (**E**) This panel shows higher magnification time-lapse images of the neuron with arrows in panel **D**. Blue arrowheads: dendritic tips that are retracted in 4.5 h, yellow arrowheads: dendritic tips that are elongated in 4.5 h, small white arrowheads: axon of a neighboring cell. (**F**) This is a 3-D model of the dendritic trace of neuron in the left figure of panel **E**. Blue circles indicate the position of the cell body. (**G**) This panel shows representative neurons with disconnected dendrites, which are not included in the analysis. The sample data in panels **D** - **G** contain NR1 (an essential subunit of NMDA-type glutamate receptor)-knocked-out neurons (because of limited data available from the authors). Please click here to view a larger version of this figure.

Discussion

Critical Steps in the Protocol and Troubleshooting:

The most critical step of the protocol is the removal of the skull (Protocol step 3.2). Upon insertion, the razor blade often adheres to the dura, causing dural bleeding and damage to the brain. This can be avoided by adding a drop of cortex buffer on the skull and removing the skull in cortex buffer.

Bleeding from the dura and the skin after cranial window preparation leads to occlusion of the window. To avoid this, the tissue adhesive and dental cement used should be allowed to completely dry before proceeding to the next step. Multiple pups with a cranial window should be

prepared since it is difficult to completely avoid bleeding. In general, if the cranial window remains clear and stable for 2 - 3 h post-surgery, the pup can be used for time-lapse imaging.

Significance of the Method with Respect to Existing/Alternative Methods:

Here, we have described a method for *in vivo* two-photon imaging of the neonatal cortex. This protocol has several merits compared with previously reported methods. These are listed as follows.

1) Cortical neurons can be sparsely and brightly labeled by transfecting Supernova vectors using IUE. IUE has been widely used for the labeling of cortical neurons during developing stages. However, a simple IUE is unsuitable for the imaging of individual neurons since neurons may be labeled too densely[7,8,9,10]. Moreover, using the Supernova system, various types of fluorescent proteins can be used for labeling sparse populations of neurons. For example, using Supernova-mediated sparse labeling of a genetically encoded calcium indicator GCaMP[13,14], we have recently performed a functional analysis of individual neurons in the developing cortex layer 4 (P3 to P13)[15].

2) The method described in this study is suitable for elucidating the molecular mechanisms underlying neuronal circuit development. The Supernova system allows sparsely labeled cell-specific knockout of any gene. Thus, the dynamics of a neuron containing a specific gene disruption can be observed. For this purpose, genetics-based systems such as MADM[16] and SLICK[17] have been previously reported. However, because the breeding of mouse lines is essential for these systems, they require much time and cost than the protocol described here.

3) This study utilizes a razor blade for skull removal. This minimizes bleeding from the dura and allows the opening of a wide area of the skull (< 2 mm in diameter). Using this procedure, it was possible to observe the spontaneous activity of layer 4 neurons in the entire large barrel area within the somatosensory cortex[15].

Limitations of the method:

A disadvantage of *in vivo* imaging of the mouse neonatal brain, compared with the imaging of transparent animals such as zebrafish larvae and *Xenopus* tadpoles, is the lower spatial and temporal resolution. Slow scanning and averaging should be performed for yielding clear images of the neuronal morphology because more light scattering occurs in the mouse brain. Light scattering may possibly be reduced using a longer wavelength laser for fluorescent protein excitation and proteins with longer fluorescence emission wavelengths.

Another limitation of the present protocol could be that the surgery for cranial window implantation may affect the formation of a normal cortical circuit due to brain inflammation[12]. However, there is a high likelihood that *in vivo* imaging is more physiological compared with *in vitro* imaging such as time-lapse imaging of the brain slice preparation, which should also give rise to severe inflammation by ischemia and slicing. It has also been reported that repetitive exposure to isoflurane may affect several neuronal processes[18]. Control experiments should be performed for verifying the appropriateness of results obtained by *in vivo* imaging. In the case of our imaging of layer 4 neurons in the somatosensory cortex, we confirmed a normal increase in total dendritic length and acquisition of orientation bias of dendritic projections of spiny stellate neurons[5,20].

Recent studies reported > 1-mm-depth imaging in an adult mouse brain wherein the dura was removed during surgery[19]. On the other hand, we have been able to report up to 400-μm-depth imaging in neonates[5]. Since the dura cannot be removed in neonates and this, in turn, leads to a high light scattering, we consider that deep imaging in neonates is more challenging than in adults. Future improvement in fluorescent probes, lasers, and detectors should allow deep imaging in neonatal brains.

Thus far, we have reported 18-hour time-lapse imaging using the present protocol[5]. Recently, we have succeeded in 72-hour time-lapse imaging by improving the protocol[20]. We will continue to refine the protocol presented here (*e.g.*, longer-term, higher time, and/or spatial resolution imaging) for revealing dynamic mechanisms of neuronal circuit development.

Disclosures

The authors have nothing to disclose.

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