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In vivo 2-photon imaging of cortical neurons in neonatal mice

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Corresponding Author:	Hidenobu Mizuno International Research Center for Medical Sciences, Kumamoto University Kumamoto, Kumamoto JAPAN
Corresponding Author's Institution:	International Research Center for Medical Sciences, Kumamoto University
Corresponding Author E-Mail:	hmizuno@kumamoto-u.ac.jp
Order of Authors:	Hidenobu Mizuno
	Shingo Nakazawa
	Takuji Iwasato
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June 20th, 2018

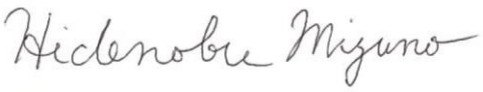
Phillip Steindel, Ph.D.

Review Editor, JoVE

Dear Dr. Steindel,

We were pleased by the positive responses of reviewers to our manuscript (JoVE58340). Acting upon the recommendation of the reviewers, we have incorporated new data **(Figures 2D-2F)**, into the revised version of our manuscript. The insightful comments and suggestions made by the reviewers have been helpful in improving the manuscript, and we have addressed their concerns as detailed below.

Yours sincerely,



Hidenobu Mizuno, Ph.D.
Associate Professor
International Research Center for Medical Sciences (IRCMS), Kumamoto University
2-2-1 Honjo, Chuo-ku, Kumamoto City 860-0811, JAPAN
Tel/Fax: +81-96-373-6740
e-mail: hmizuno@kumamoto-u.ac.jp

TITLE:

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

AUTHORS & AFFILIATIONS:

Hidenobu Mizuno[1,2,3], Shingo Nakazawa[2,3], Takuji Iwasato[2,3]

[1]International Research Center for Medical Science (IRCMS), Kumamoto University, Kumamoto, Japan

[2]Division of Neurogenetics, National Institute of Genetics, Mishima, Japan

[3]Department of Genetics, SOKENDAI (The Graduate University for Advanced Studies), Mishima, Japan

Corresponding Author:

Hidenobu Mizuno (hmizuno@kumamoto-u.ac.jp)

International Research Center for Medical Science (IRCMS), Kumamoto University,

2-2-1 Honjo, Chuo-ku, Kumamoto City 860-0811, Japan

Tel: +81-96-373-6740

E-mail Addresses of the Co-authors:

Shingo Nakazawa (snakazawa@nig.ac.jp)

Takuji Iwasato (tiwasato@nig.ac.jp)

KEYWORDS:

Newborn, two-photon, *in vivo* imaging, single-cell labeling, cerebral cortex, mouse

SHORT ABSTRACT:

We present an *in vivo* two-photon imaging protocol for imaging the cerebral cortex of neonatal mice. This method is suitable for analyzing the developmental dynamics of cortical neurons, the molecular mechanisms that control the neuronal dynamics, and the changes in neuronal dynamics in disease models.

LONG 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.

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[7-11].

1.1. Prepare timed-pregnant mice for IUE.

1.2. 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).

1.3. Perform regular IUE[7-11] to label cortical neurons. For the labeling of layer 4 neurons, use embryonic day-14 embryos.

1.4. Wait for pup delivery and growth.

2. Surgery

2.1. 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.

2.2. Sterilize the pup's skin covering the skull by wiping it with 70% ethanol.

2.3. Remove approximately 20 mm^2 of the skin covering the skull using scissors sterilized with 70% ethanol (**Figure 1A**).

2.4. Remove the fascia of the skull using sterilized forceps and a clean cotton swab (**Figure 1A**).

2.5. 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.

2.6. 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).

2.7. If necessary, apply more tissue adhesive and wait for it to dry and solidify.

3. Cranial Window Preparation

3.1. Anesthetize the pup using isoflurane (1.0% - 2.0%) and check the anesthesia level by a tail-pinch test.

3.2. 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.

3.3. 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.

3.4. 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 (**Figures 1D** and **1E**).

3.5. Secure the coverslip using dental cement (**Figure 1F**).

3.5.1. 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.

3.6. Attach a 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.

3.7. Cover the exposed skull with dental cement (**Figure 1H**).

3.8. Subcutaneously inject an analgesic (carprofen, 5 mg/kg).

3.9. 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).

4.1. 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.

4.2. Wipe the surface of the coverslip with 70% ethanol.

4.3. Anesthetize the pup using isoflurane (1.5% - 2.0%) and check the anesthesia level using a tail-pinch test.

4.4. Attach the pup to the titanium plate on the imaging stage using a titanium bar on the pup's head (**Figures 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).

4.5. Set the isoflurane concentration to 0.7% - 1.0%.

Note: A very high isoflurane concentration may cause accidental death of the pup during imaging.

4.6. Place the imaging stage under the objective lens (20X, NA 1.0) of the two-photon microscope (**Figures 2B** and **2C**).

4.7. 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.

4.8. Acquire z-stack images at 1.4-µm intervals. For layer 4 neuron imaging, set the z-width to 150 - 200 µm to image the entire dendritic morphology (**Figures 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

5.1. Detach the pup from the imaging stage.

5.2. Place the pup on a heater (37 °C) and allow it to recover (15 min).

5.3. 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

6.1. 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.

6.2. Locate the previously imaged neurons and acquire a z-stack image. The identification of neurons is easy owing to their sparse labeling with Supernova.

6.3. Repeat steps 5.1 - 6.2 until imaging is completed.

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 LEGENDS:

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.

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).

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[7-10]. 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] (also in Nakazawa *et al.*, submitted).

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 (Nakazawa *et al.*, submitted). 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.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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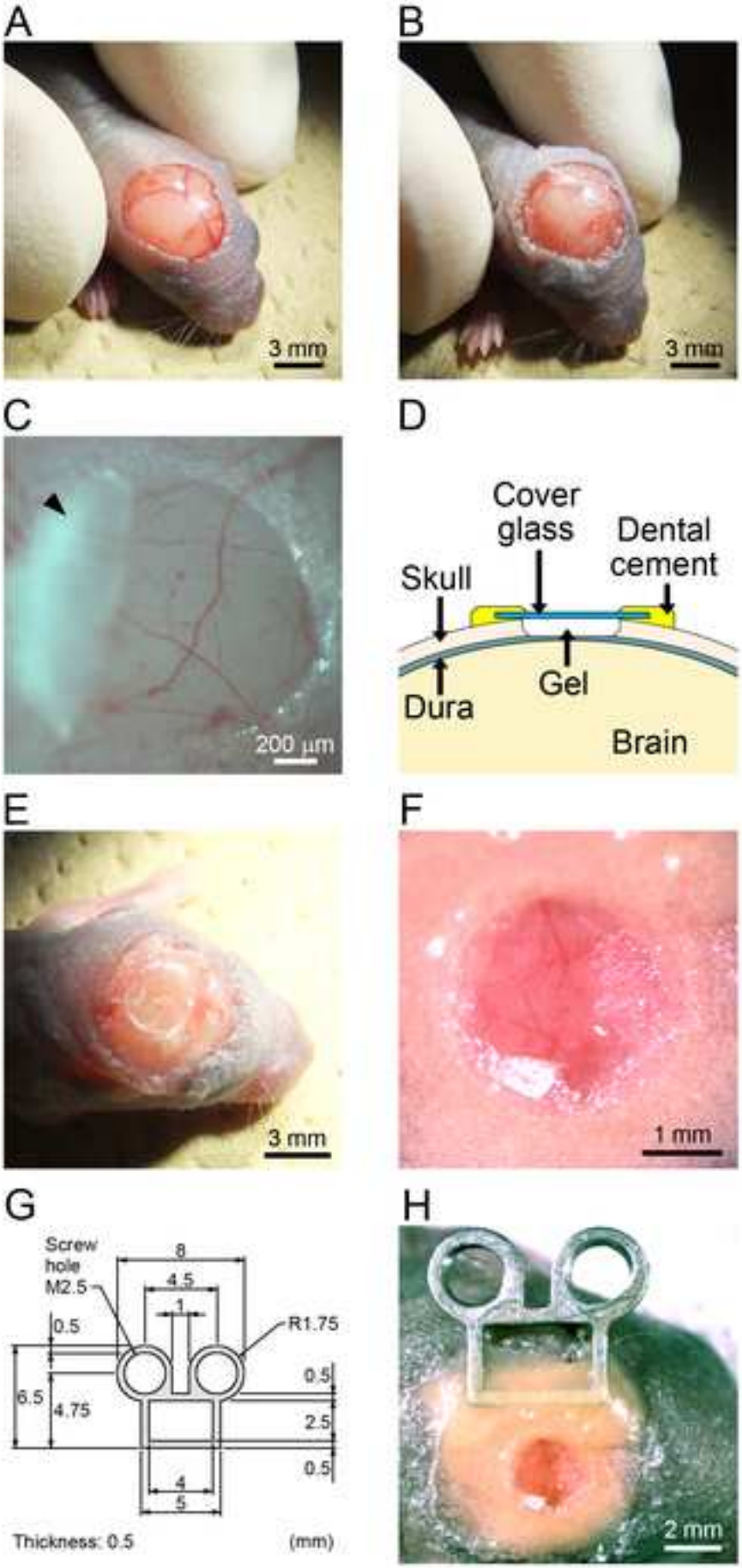

Figure 1

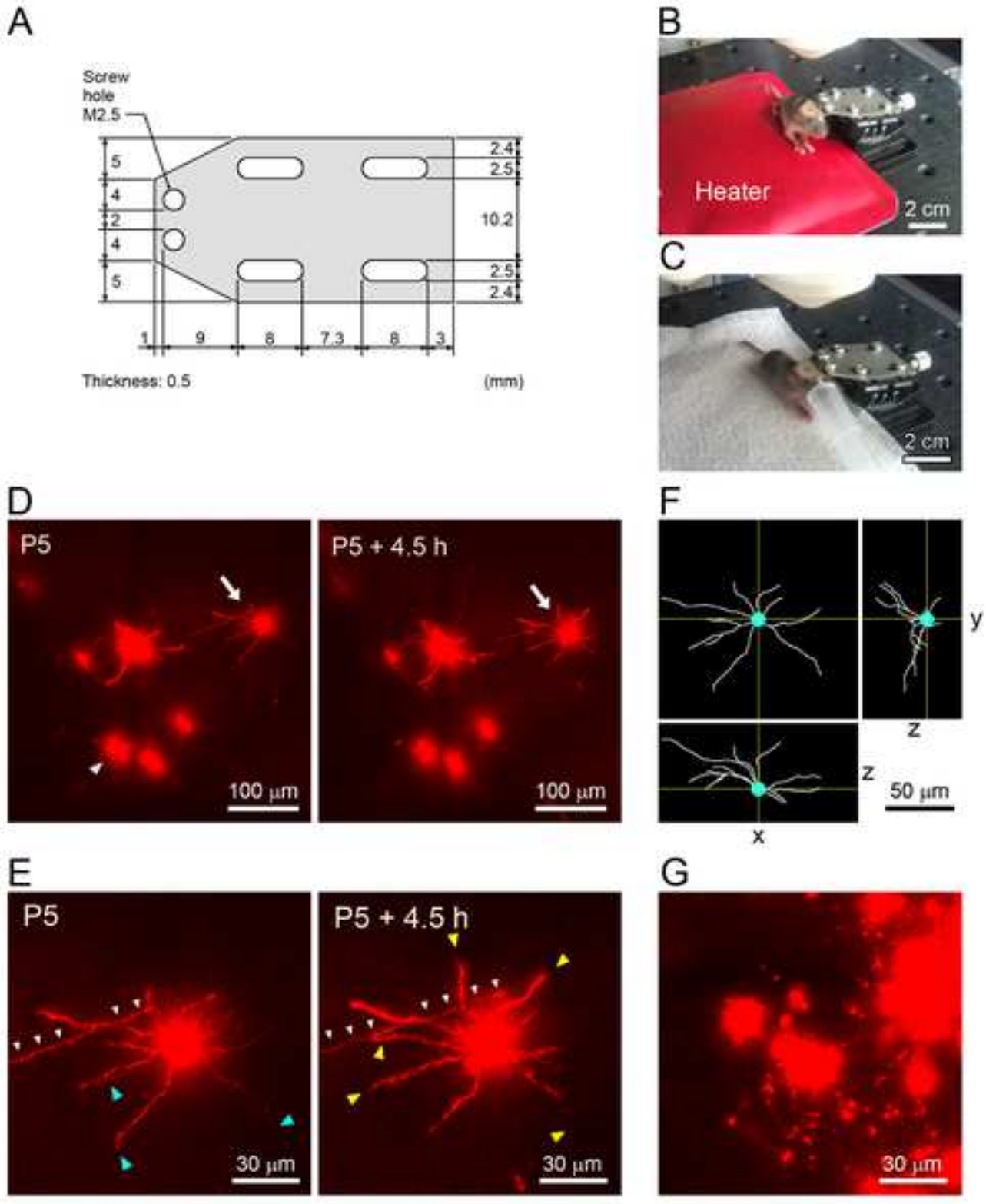

Figure 2

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
pK031. TRE-Cre	Authors	-
pK029. CAG-loxP-STOP-loxP-RFP-ires-tTA-WPRE	Authors	-
pK273. CAG-loxP-STOP-loxP-CyRFP-ires-tTA-WPRE	Authors	-
Isoflurane	Wako	099-06571
410 Anaesthesia Unit (isoflurane gas machine)	Univentor	8323101
Vetbond (tissue adhesive)	3M	084-1469SB
MμltiFlex Round (loading tip)	Sorenson	13810
Gelfoam (gelatin sponge)	Pfizer	09-0353-01
Agarose	Sigma	A9793
Round-shaped coverslip	Matsunami	-
Unifast 2 (dental cement)	GC	-
Titanium bar	Authors	-
Rimadyl (carprofen)	Zoetis	-
2-photon microscope	Zeiss	LSM7MP
Titanium-sapphire laser	Spertra-Physics	Mai-Tai eHPDS
Titanium plate	Authors	-
IMARIS, FilamentTracer, MeasurementPro	BITPLANE	
Goniometer stage	Thorlabs	GN2/M

Comments/Description
Available from RIKEN BRC and Addgene
Available from RIKEN BRC and Addgene
Available from authors
Low melting point
Custom made
Custom made (see Figure 1G)
Injectable
Custom made (see Figure 2A)

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CORRESPONDING AUTHOR:

Name:	Hidenobu Mizuno
Department:	International Research Center for Medical Sciences
Institution:	Kumamoto University
Article Title:	In vivo 2-photon imaging of cortical neurons in neonatal mice
Signature:	Hidenobu Mizuno
Date:	April 24th, 2018

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please define all abbreviations before use.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

4. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

5. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Univentor, Vetbond, 3M, Gelfoam (Pfizer), Sigma A9793, Matsunami, Zeiss, Spertra-Physics, MilliQ, etc.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

7. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

8. Please mention how proper anesthetization is confirmed.

9. Lines 74-81, 88-90: Please write the text in the imperative tense, include as "Note", or move it to Results or Discussion.

10. 1.3: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.

11. 6.1: Please mention how pup is anesthetized and how proper anesthetization is confirmed.

12. References: Please do not abbreviate journal titles.

13. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

We have revised all of the editorial comments accordingly.

For comment 10 (about IUE), as we previously discussed with a JoVE editor, Dr. Indrani Mukherjee, IUE protocol is out of focus of this paper. Instead we have added appropriate references.

Reviewers' comments:

Reviewer #1

Manuscript Summary:

This is a well written methods paper applying techniques in fluorescent imaging to interrogate neonatal cortical neurons in vivo. The authors utilize their experience and expertise developed in a previous publication involving in vivo imaging in neonatal mice in which they circumvent difficulties associated with the technique and address those caveats with the best available tools. We have made a few suggestions below that we think might improve the procedure or the clarity of the method.

We appreciate the reviewer's insightful comments, which were useful to improve our manuscript.

Major Concerns:

Line 98-101: Is a stereotaxic frame or similar head holding device necessary for the surgery? If so, please recommend an appropriate model. During anesthesia the pups must be kept warm by means of a heating pad to avoid anesthesia induced hypothermia, specially during long procedures like this one.

Usually we do not use a stereotaxic frame or head holding device because pups are quiet during surgery under anesthesia. The pups are kept warmed using a heating pad during surgery. We have added a sentence accordingly (lines 105-106).

Line 110 - 113: To shorten the procedure length, the use of Gelfoam (Pfizer) can help to stop bleeding to avoid the use of vetbond at this stage (30 min drying time). The skin wound could be closed later during the application of dental cement post window implantation to cover the exposed skull (line 138).

Thank you for your suggestion. The use of vetbond at this stage is necessary because the skin and the skull must be fixed to open the skull with razor blade.

Line 119: Gelfoam should be soaked in saline before contacting the exposed brain surface to avoid drying it. Gelfoam soaked in saline can still aid in stopping bleeding. It might be worth pointing out that the exposed brain surface must be kept moist with saline at all times.

Gelfoam is soaked in cortex buffer before contacting the brain surface in our protocol. We have modified the sentence accordingly (lines 127-128). As mentioned in our submitted manuscript, the brain surface is kept moist with cortex buffer (lines 130-131 in the revised manuscript).

Line 123: The draining of buffer and blood from the surface of the dura must be performed from the side of the craniotomy, being careful that dry gelfoam or other absorbent material does not get in contact with the dura as this might damage it.

We have corrected the sentence accordingly (lines 136-138).

Line 125: It would be relevant if the authors could specify the precise temperature at which the low melting point agarose can be applied on top of the brain.

The low melting point agarose is kept at 42°C and solidified at 37°C. It is difficult to measure the agarose temperature before application, because amount is quite small. However, we estimate that it is around 37°C, because it solidified in a moment.

Line 140: The surgery as described could easily take 2hs or more. Should the pups be fed milk to keep them hydrated and nourished before the (potentially also long) imaging session?

We give milk after the imaging session, because we think that pup should not have milk before long anesthesia (imaging session). We measured pup's body weight and confirmed no weight loss.

Line 150: Why is a higher % of isoflurane necessary for this step than for the surgery or the imaging session (line 155)?

That is because a higher % of isoflurane is necessary for induction of anesthesia. After induction, pup's anesthesia can be kept at a lower %.

Line 164: The authors should specify in more detail the recommended imaging parameters for assessing L4 neuron dendritic morphology: Optimal excitation wavelength for RFP, dychroic/filter sets, recommended PMT model and gain settings, FOV size and pixel resolution, optimal frame rate, 8 bit or 16 bit depth, etc...

We have added the details of imaging parameters (lines 190-193).

Line 184/line 260: Repetitive exposure to isoflurane can affect many neuronal processes:
https://link.springer.com/article/10.1007/s11064-016-2114-7
https://academic.oup.com/bja/article/115/5/752/230885
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0179588
This limitation and how it can affect conclusions made with this method must be mentioned and discussed.
Additionally and most importantly, after cranial window implantation surgery the brain develops inflammation, which can be evidenced by a GFAP staining to detect activated

microglia:

https://www.nature.com/articles/nprot.2009.89

In adult mice this can take some time, allowing for acute imaging immediately after cranial window implantation surgery. Nevertheless, in a chronic imaging paradigm as the one described in this method, brain inflammation would progressively increase throughout the imaging sessions. The authors should at least acknowledge this limitation of the chronic imaging paradigm they propose in this manuscript and elaborate on the possible impact it can have on the results obtained with their method.

We agree with the reviewer that repetitive exposure to isoflurane could affect neuronal process and the brain could develop inflammation after cranial window preparation. We consider that control experiments are essential for verifying the appropriateness of results obtained by *in vivo* imaging. In case of imaging of layer 4 neurons in the somatosensory cortex, we confirm an increase in total dendritic length and acquisition of biased dendrite morphology (elongation of the dendrites toward the barrel center where the thalamocortical axons make clusters). We have discussed about brain inflammation and effect of anesthesia accordingly (lines 303-312)

Line 190: Example images of a neuron with "disconnected dendrites" must be included to clarify what is meant by the term "disconnected dendrites". This also applies to neurons with "blurred dendritic trees", to help identify these cases.

We have added example images of neurons with disconnected dendrites (Figure 2G) and neurons with blurred dendritic trees (Figure 2D arrowhead).

Minor Concerns:

Figures need higher resolution.

Please check higher resolution images that can be downloaded from the links on the PDF files.

Reviewer #2

Manuscript Summary:

Mizuno et al. provide a useful protocol describing the fluorescent labeling of a sparse subset of neurons in neonatal mice using the Supernova system in combination with a cranial window implantation procedure for subsequent in vivo 2-photon microscopy. The Supernova system takes advantage of the leaky expression of TRE to drive the expression of the fluorescent protein/gene of interest in neurons which were transfected with the respective vectors by in utero electroporation. Since in vivo 2-photon imaging of neurons in neonatal mice is technically

very challenging, and thus rarely achieved, the presented protocol is of considerable interest for basic and applied research.

We appreciate the reviewer's kind comments.

Major Concerns:

None.

Minor Concerns:

-Is it possible to label also other cell types or pyramidal neurons in different cortical layers?

It is possible to label neurons in the hippocampus and different cortical layers. Please see Figure S2 of Luo, Mizuno et al., *Scientific Reports* 2016.

-Please specify the resolution of the acquired in vivo micrographs to analyze dendritic morphology.

We are using 512 x 512 pixels images (1.2 μm/pixel) to obtain clear dendritic morphology. We have added a sentence accordingly (lines 190-193).

-The limitations of the method should be discussed more thoroughly:

How long is the implanted window accessible to in vivo imaging (weeks or even months)?

So far we have reported 18 hour time-lapse imaging (Mizuno et al., Neuron 2014). Recently, we have succeeded in 72 hour time-lapse imaging by improving the protocol (Nakazawa et al., submitted). We have added sentences accordingly (lines 319-323).

Does growth of the skull limit the time period in which the implanted window remains open?

In 18 hour imaging, we did not notice limitation of the skull growth, and it appears that dendrites of imaged neurons grow normally (Figure 5E of Mizuno et al., Neuron 2014).

Are neonatal mice also amenable to the thinned-skull preparation method? In this case please specify the advantages/limitations of the open-skull preparation compared to the thinned-skull preparation method.

To the best of our knowledge, a neonatal brain imaging using the thinned-skull preparation method does not exist.

-There are some mistakes in the manuscript:

Legend of Fig. 2E: "Higher magnification image of the neuron in allow in (D)".

Line 251: "...SLICK have previously reported..."

Line 252: "... lower fluorescent intensity..."

Thank you very much.

Reviewer #3

Manuscript Summary:

The paper « In vivo 2-photon imaging of cortical neurons in neonatal mice » by Mizuno, Nakazawa and Iwasato is very important description of two-photon in vivo pups imaging. Multiphoton microscopy has been widely used for in vivo imaging in the adult mouse brain but only a small subset of labs are able to perform in vivo imaging in very young animal. The fine description of the protocol used by Mizuno et al. will allow the development of this approach. It will help the community in describing the developmental dynamics of cortical neurons or others brains areas.

However, to achieve this goal, the authors must provide additional explanations or develop multiple points:

We appreciate the reviewer's insightful comments.

Major Concerns:

The purpose of this protocol is to describe in vivo imaging during development. And there are no data showing the same neurons over a long period of time (more than 2 days). Unfortunately, the figure 2 only show a Z projection of one neuron at a single time. This type of data and morphology could be obtained by making brain slices and with a better quality. The advantages of the in vivo part is to look at the morphology across time. So the authors need to show data of the same neuron during developpement.

Moreover, it would be interesting to generalize this paper by presenting other possibilities than monitoring morphology over time. The authors already published a paper where they looked at calcium activity over time. Opening this protocol to biosensors genetically encoded would be a important to increase the visibility of the paper. I suggest that the authors add a GCaMP experiment in the figure 2.

We have added data for time-lapse morphology imaging as Figures 2D and 2E. About calcium imaging, we discussed with the JoVE editor before submission and the calcium imaging protocol is out of focus of this paper. Thank you for your suggestion.

Minor Concerns:

1. Preparation of pups for imaging

This part is described very quickly. I understand the space issues but some additional

clarification would be required. Like how the prepare the micropipette ? The size of this pipette ? How the target the cortex ? The voltage, shape of the pulse, frequency…
I'm also surprise that they didn't mention the type of anesthesia they use (isoflurane ? Or other) and if they use pain killer after the UIE and for how long.

We discussed with the editor that IUE protocol is also out of focus of this paper, because details of IUE protocol are previously described in other papers (e.g. Matsui et al., Jove 2011).

2. Surgery
Do they use pain killer at that step ? If yes they need to name it and give the dose.

We make a lot of efforts to reduce pup's pain in surgery. We anesthetize a pup with isoflurane during surgery and use carprofen as a pain killer. We have added sentences accordingly (line 155).

I did not find any mention on the maintenance of the animal's body temperature during the surgery. How they maintain the temperature of the pup ?

During surgery, we maintained the pup's body temperature with a heater (37 °C). We have added sentences accordingly (lines 105-106).

2.5 How they apply Vetbond and Gelfoam with a pipette tips ?

We are using loading tips (Sorenson) for Vetbond application. For Gelfoam, we cut it into small pieces (< 2 mm^3), and apply them using tweezers. We have added information (line 115 and lines 127-128, respectively).

Between 2.5 and 2.6 the pups is under anestesy during 30 minutes ? Place on a heat plate at 37°C ?

The pups are placed on a heat plate at 37°C between 2.5 and 2.6. We have added sentences accordingly (line 119).

3. Cranial window preparation
3.1 Can the authors give the osmolarity of their cortex solution ? This cortex solution is apply on the brain cold ? At room temperature ? Or 37°C ?

The osmolarity of the cortex buffer is approximately 300 mOsm/L, and it was applied at room temperature. We have added information accordingly (lines 128-130).

3.4 The authors discussed about a gel but which Gel ? Aggarose or the dental glue ?

It is about an agarose gel. We have changed a position of the sentence (3.3 lines 140-143).

The idea of Jove paper is to give the small tips the authors developped with their experience to help the community. And i think that these tips are missing all along this protocol. For exemple can they explain also how they remove the bubble (3.4) ? Explain how they applyied the dental cement and agarose ?

We sincerely appreciate your comments. To remove bubble, we poured excess of agarose gel between the coverslip and the agarose gel layer. We applied the dental cement and agarose gel by yellow tips before they become solidified. We have added sentences accordingly (lines 140-142 and lines 145-146). Also, we have added the details of the protocol throughout the manuscript.

3.5 is the positioning of the titanium bar a problem for the rest of the experiment? Should it be properly aligned ?

The titanium bar and the coverslip (the dura surface) should be aligned in parallel to easily get images. We have added the sentence accordingly (lines 150-151).

3.7 the mouse is kept under anesthesia for 1 hour?

No, the mouse is not kept under anesthesia (line 157).

4. Two-photon imaging. More details on the microscope is needed. Which emission filter they use, type of scanner, detector (non scanned?)…

We have added the detail of imaging parameters (lines 190-193).

4.1 why they didn't give us a unique wavelength ? Can they explain why the system is not tuned to optimal excitation wavelength.

We gave a unique wavelength in the revised manuscript (1,000 nm). We gave 940-1,000 nm in the submitted version, because some titanium-sapphire lasers cannot emit 1,000 nm wavelength.

They need also to give the power they use in mW/mm² (To give an idea of the amount of light needed for this deep imaging).

Beam Diameter $(1/e^2)^2$ of the Mai-Tai laser is 1.2 mm, and we use the laser at 500 mW (1,000 nm wavelength). So, we consider that approximately it is 450 mW/mm². We have added the information (lines 164-165).

4.3 type of screw they used ?

We are using M2.5 screw (2 mm in length). We have added the information in Figure 2A legend (line 244).

4.7 Can the authors precise the size of the image 512x512 and th field of view? Number of averaging, scan speed...

We have added precise scanning information (lines 190-193).

Can they discussed about bleaching issue of the sample.

We do not notice bleaching of the sample in our imaging condition.

They say « Use slow scanning and averaging to get clear images showing the neuronal morphology ». But the figure 2E (probably because no E are on the figure) shows a Z-projection of the stack. Could it be possible for showing the complete morphology to show a 3D reconstruction of the neuron ?

As suggested, we have added data showing 3D morphology of the neuron (Figure 2F).

With this technique can authors follow spines in vivo ?

We have seen very few spines in the first postnatal week. This is maybe because spine increases from the second postnatal week (Please see Figures 4G, 4H, 4N, 4O of Mizuno et al., Neuron 2014).

On my PDF file, figure 2 is very fuzzy and difficult to see detail of the setup.

Please check higher resolution images that can be downloaded from the links on the PDF files.

6. Re-imaging

Authors should specify how long can the animal be used? Because with the weight gain and the brain expansion, I guess the time window shouldn't be very big (more than a week?) ?

So far we have reported 18 hour time-lapse imaging (Mizuno et al., Neuron 2014), and now we are trying to extend an imaging period.

Missing part :

Authors never discussed data analysis. I suppose that they use specific programs for morphology reconstruction or quantification.

We are using the Imaris filament tracer (Bitplane) for data analysis such as dendrite tracing and measurement. We have added information (lines 216-217 and Table of Materials) and new data (Figure 2F).

Discussion

It would be interesting that authors discussed if with this technique they can monitor morphological changes more subtile like spines formation or dendrite/axon growth?

We previously reported dendrite growth in 18 hours (Mizuno et al., Neuron 2014). We have added new data showing morphological change of dendrites by our protocol (Figures 2D and 2E). Now we are trying to see spines and axon growth.

It's not clear which deepth they can reach and if this imaging can be generalize to other brain area like deep cortical layer or hippocampus.

We previously reported < 400 μm depth imaging (approx. 450 μm depth including the dura thickness). We feel that deep imaging in neonate is more difficult than that in adult, because the dura, which have high light scattering, cannot be removed in neonates. We have added discussion about depth issue (lines 313-318).

Reviewer #4

Manuscript Summary:

Nakazawa et al. described their protocols for sparse and bright labeling of cortical neurons in the developing brain and a surgical procedure for in vivo imaging from neonatal mice. The protocol is quite useful for various purposes to perform time-lapse imaging. This manuscript is basically worth publishing, but the authors should improve the manuscript before publication as written below.

We appreciate the reviewer's kind comments.

Major Concerns:

1) How long could the authors observe cortical neurons in healthy conditions in the time-lapse study? How often and in what interval could be images taken?

We previously reported 18 hour time-lapse imaging with 4.5 or 9 hour interval (Mizuno et al., 2014). By our protocol, layer 4 cortical neurons in the somatosensory cortex elongate their dendrites toward the barrel center where the thalamocortical axons make clusters, suggesting that neurons are healthy at least 18 hours.

2) How long could they keep pups with applying milk?

Pups can be kept at least for 18 hours without weight loss.

3) In the representative result, the authors could describe that the time lapse study showed morphological changes of dendrites, although they do not have to demonstrate more pictures.

As suggested, we have added new data for time-lapse pictures (Figures 2D and 2E).

4) It would be better to cite Glutzendler et al (2002) for 2-photon live imaging.

As recommended, we have cited Glutzendler et al (line 58).

Minor Concerns:

1) "E" is missing in Figure 2.

Thank you very much.