**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://link.springer.com/article/10.1007/s11064-016-2114-7)[*https://academic.oup.com/bja/article/115/5/752/230885*](https://academic.oup.com/bja/article/115/5/752/230885)[*http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0179588*](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*](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 m/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 mm3), 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/e²)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 m depth imaging (approx. 450 m 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.