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TITLE:

Precise Brain Mapping to Perform Repetitive In Vivo Imaging of Neuro-Immune Dynamics in Mice

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SUMMARY:

This protocol describes a chronic cranial window implantation technique that can be used for longitudinal imaging of neuro-glio-vascular structures, interactions, and function in both healthy and diseased conditions. It serves as a complementary alternative to the transcranial imaging approach that, while often preferred, possesses some critical limitations.

ABSTRACT:

The central nervous system (CNS) is regulated by a complex interplay of neuronal, glial, stromal, and vascular cells that facilitate its proper function. Although studying these cells in isolation in vitro or together ex vivo provides useful physiological information; salient features of neural cell physiology will be missed in such contexts. Therefore, there is a need for studying neural cells in their native in vivo environment. The protocol detailed here describes repetitive in vivo two-photon imaging of neural cells in the rodent cortex as a tool to visualize and study specific cells over extended periods of time from hours to months. We describe in detail the use of the grossly stable brain vasculature as a coarse map or fluorescently labeled dendrites as a fine map of select brain regions of interest. Using these maps as a visual key, we show how neural cells can be precisely relocated for subsequent repetitive in vivo imaging. Using examples of in vivo imaging of fluorescently-labeled microglia, neurons, and $NG2^+$ cells, this protocol demonstrates the ability of this technique to allow repetitive visualization of cellular dynamics in the same brain location over extended time periods, that can further aid in understanding the structural and functional responses of these cells in normal physiology or following pathological insults. Where necessary, this approach can be coupled to functional imaging of neural cells, e.g., with calcium imaging. This approach is especially a powerful technique to visualize the physical interaction between different cell types of the CNS in vivo when genetic mouse models or specific dyes with distinct

fluorescent tags to label the cells of interest are available.

INTRODUCTION:

The central nervous system (CNS) is governed by a complex interplay of interactions between various resident cell types including neurons, glia and vessel-associated cells. Traditionally, neural cells were studied in isolated, co-cultured[1-5] (in vitro) or excised brain tissue (ex vivo)[6-10] contexts. However, there is need to further understand neural cell behavior and interactions in the native environment of the intact brain in vivo. In this protocol, we describe a method to map in vivo regions of interest and precisely re-image those regions in future imaging sessions to track the complex interactions between the various CNS cell types over extended periods of time.

The development of in vivo imaging approaches has provided significant gains for the proper understanding of neural function [11-15]. Specifically, these approaches provide several advantages over traditional in vitro and ex vivo approaches. First, in vivo imaging systems have physiologically relevant cell and tissue components such as the vasculature with the full repertoire of cellular interactions to provide a complete understanding of neural network physiology. Second, recent findings suggest that when removed from their native environment, certain neural cells (such as microglia) lose important features of their identity and thus physiology[16,17] which can be preserved in the in vivo setting. Third, in vivo imaging systems provide the opportunity for stable longitudinal investigations of weeks to months to study CNS cellular interactions. Finally, given the growing evidence for contributions from the peripheral immune system[18,19] and the microbiome[20,21] in CNS physiology, in vivo systems provide a platform to interrogate such contributions and effects on CNS cells. Thus, approaches that employ longitudinal in vivo imaging to study neuro-immune physiology and interactions in healthy, injured, and diseased states are a great complementary addition to traditional approaches.

In this protocol, we describe a reliable approach to image different cell types in the brain including microglia, neurons and $NG2^+$ cells as examples. Two approaches to visualize neural cells in vivo have been developed: the thinned skull approach and the open skull with a cranial window approach. Although thinned skull approaches are in use and are preferred because they overcome some of the disadvantages of the open skull approach such as glial cell activation, higher-than-physiological spine dynamics and the use of anti-inflammatory agents[22-25], thinned skull approaches also show a few critical drawbacks. First, the thinning procedure is a very delicate procedure that many researchers find difficult to perfect especially when re-thinning is necessary. This is the case because it is often difficult for experimenters to ascertain that they have thinned the skull to a ~20 µm depth. Second, for adequate comparisons between mice, thinning would need to be identical and a variety of thinning success between imaging sessions or mice could complicate visualization of neural structures. Third, when employed for longitudinal imaging, animals with thinned skulls can only be used for a limited number of sessions when re-thinning of the skull is employed. Forth, since some of the bone tissue still remains, clarity in depth of imaging could be compromised from the thinned skull approach allowing for great visualization of more superficial but not as much with deeper regions. In the light of this, deeper brain structures such as the hippocampus, cannot be successfully imaged with the thinned skull approach. These considerations raise the need for alternative and

complementary approaches that could overcome these concerns.

Alternative to the thinned skull approach, the open skull window implantation approach uses a procedure in which the skull is replaced with an optically clear glass coverslip. This allows for a near-unlimited number of imaging sessions. Moreover, given the replacement of the skull with the glass coverslip, this method allows for a clear viewing window of fluorescently tagged brain cells for extensive periods of times from hours to months and, therefore, can be employed to study cell activity and interactions that are relevant for physiology, aging and pathology.

Overall, we detail steps that can be followed to do implant chronic cranial windows through a stereotaxic craniotomy that enables in vivo imaging of brain regions of interest. We also describe how the grossly stable brain vasculature or the fluorescently labeled dendrites could be used to generate a coarse or a fine map, respectively of the brain regions of interest. This approach can then be used for repeated imaging over several sessions. The importance of this technique, therefore, lies in its ability to image the long-term changes or stasis in brain elements including the arrangement, morphology, and interactions of the different cellular types.

PROTOCOL:

All steps are in accordance with the guidelines set and approved by the Institutional Animal Care and Use Committee of the University of Virginia.

1. Mouse preparation for cranial window implantation

NOTE: Various transgenic mouse lines with florescent tags are suitable for imaging.

1.1 Use CX3CR1$^{GFP/+}$ mice[26] to visualize microglia in vivo. Typically, juvenile to young adult 4 to 10-week-old mice that weigh 17-25 g are used.

NOTE: Although, this approach is even apt for pre-weaned mice, the need to return the mice to their cage with their mothers for feeding, may complicate recovery if the mother does not take adequate care of pups post-surgery. Therefore, the use of mice post-weaning is recommended.

1.2 Anesthetize the mouse using isoflurane (5% flow in oxygen for induction for 1 min) in an anesthetic chamber. Check that the mouse doesn't show any movement or twitching responses to toe and/or tail pinches. Take the mouse out of the chamber and in open air thoroughly shave the hair on the head between the ears from about the eye level to the top of the neck region using a hair trimmer.

NOTE: The concentration of isoflurane used would depend on the size of the induction chamber. Therefore, for smaller chambers, 3-4% isoflurane can be used to effectively induce anesthesia while larger chambers will require up to 5%.

1.3 Move the mouse to the stereotactic surgery station nose cone for anesthesia (1.5-2% for maintenance for the surgery), stabilize its head using ear bars, and maintain the mouse on a

heating pad to keep the body temperature warm.

1.4 Lubricate both eyes with eye ointment. Inject 100 µL of 0.25% bupivicaine (to provide local analgesia to the mouse that will last 8-12 h) and 100 µL of 4 mg/mL dexamethasone (to reduce the inflammation that may result from the surgery procedure) subcutaneously at the incision site. Allow the mouse to sit for at least 5 min before moving to the next step.

1.5 Clean the shaved head with three alternating swabs of betadine and 70% alcohol. Make a midline scalp incision using surgical blade or scissors extending from the back of the skull region between the ears to the frontal area between the eyes. The remaining skin is cut to expose the skull.

1.6 Clean the connective tissue located between the scalp and the underlying skull with 3% hydrogen peroxide (H_2O_2) and localize the brain area to be imaged with stereotactic coordinates.

NOTE: There is often some bleeding (step 1.5) from the incision on the skull surface. This bleeding usually resolves by itself within 3-5 min. Cleaning with the peroxide helps. Prior bupivacaine treatment (step 1.4) is also noted to limit the amount of bleeding during this time.

2. Mouse cranial window implantation surgery

2.1 Drill a circular opening ~4 mm into the skull using a dental drill bit (0.7 mm tip diameter) and carefully remove this portion of the skull using pointed forceps. For imaging the somatosensory cortex of 6-8-week-old mice, locate the center of the craniotomy at -2.5 posterior and ± 2.0 lateral to bregma. During drilling, regularly moisten the skull with sterile saline and cotton swabs to cool the brain, clean off bone debris and soften the skull bone for eventual removal.

NOTE: The coordinates for the craniotomy would vary depending on the region of interest and the age of the mice.

2.2 After the skull is removed, carefully place a small coverglass (size #0 at 0.1 ± 0.02 mm thickness) moistened with saline in the craniotomy. Dry off excess saline using a sterile wipe.

2.3 Using a pointed applicator (such as a pipette tip or the pointed end of a broken wooden cotton swab stick), apply cyanoacrylate glue around the window and allow it to attach to the brain and skull. Apply the primer glue to the rest of the skull and cure it with a curing light for 20-40 s. Prepare a well around the window with the final glue and cure with a curing light for 20-40 s.

2.4 Glue a small head plate on to the skull on the contralateral hemisphere of the craniotomy first with the primer glue as a primer and then with the final glue. Cure both with the curing light for 20-40 s each.

NOTE: Sutures are not needed if the skull is totally covered with the glue during this procedure.

3. Post-surgery care

3.1 Allow the mouse to wake up in the absence of anesthesia (recovery done on a heating pad shortens the recovery time) and return it to its home cage once fully awake. Inject one subcutaneous dose of buprenorphine SR (0.5 mg/kg) as post-operative analgesia that is sufficient for 72 h.

3.2 To facilitate a healthy recovery from the surgery, provide the mouse an extra soft food, which can be in the form of regular solid chow in water to soften the chow or food in the form of a gel.

NOTE: A one-time provision of the soft food immediately after the surgery is sufficient.

3.3 Monitor the mouse daily for health and proper recovery for the first 72 h of the surgery procedure. Afterwards, perform imaging from as early as 2 weeks from the window implantation surgery.

NOTE: If done well, mice recover well showing normal ambulatory behaviors, sufficient cage exploration, good hydration, stable weight gain and extensive interactions with other mice in the cage and other items in the cage. Mice showing lethargy, dehydration and greater than 10% weight loss following the surgery are euthanized and removed from the study.

4. Two-photon brain mapping for initial imaging

4.1 Anesthetize the mouse (Isoflurane, 5 % induction and 1.5 % maintenance). Stabilize the head using screws to mount the headplate on the two-photon microscope stage, being maintained on a heating plate at 35 ˚C. Inject intraperitoneally 100 µL of blood vessel dye such as Rhodamine B (2 mg/mL).

NOTE: Imaging could also be done in awake mice without anesthesia. However, recent studies indicate that anesthesia affects microglial surveillance dynamics[27-29] and that head fixation for two photon imaging in awake mice increases stress even during chronic imaging for at least 25 days (see Juczewski et al., 2020)[30].

4.2 Clean the surface of the cranial window gently using a cotton swab dabbed in 70% ethanol. Put a few drops of water or saline on the cranial window and lower the objective lens into the solution since the objective is an immersion lens.

4.3 Hand-draw a coarse map to denote the major blood vessel landmarks in a lab notebook while looking through the eyepiece by epiflorescence. Use this drawing to identify the specific regions during two photon imaging. Alternatively, take pictures of the blood vessels either through a camera fitted to the microscope or through a hand-held camera or phone.

NOTE: These hand-drawn images and pictures are to facilitate revisiting the same broad regions under the microscope before two photon imaging. These are not precise image mapping.

4.4 Under two photon imaging, collect images of florescent cells and vessels as needed. Take careful notes with appropriate coordinates to ensure that that the precise regions can be revisited for subsequent imaging. Collect several fields of view in this initial imaging session e.g. acquire z-stack images every 1-25 µm through a volume of tissue.

NOTE: While collecting images by two photon, the blood vessel landmarks are used for coarse mapping. If fine mapping is needed, YFP-labeled dendrites from Thy1-YFP[31] mice are used.

4.4.1. Use these recommended parameters for imaging: a wavelength of 880-900 nm is optimal; for GFP and/or dsRed / Rhodamine excitation, a 565 nm dichroic mirror with 525/50 nm (green channel) and 620/60 nm (red channel) emission filters are used; for GFP and YFP separation, a 509 nm dichroic mirror with 500/15 and 537/26 nm emission filters are used; the power at the brain is maintained at 25 mW or below; image resolution is 1024 x 1024 pixels, the field of view taken with a 25X 0.9 NA objective at a 1.5X zoom factor is 295.24 x 295.24 µm.

4.5 At the end of the imaging, take the mouse off the stage, allow it to wake up from anesthesia and return to its home cage until a future imaging session.

5. Two-photon imaging and re-imaging

5.1 For future subsequent imaging sessions, which could be anywhere from a few hours to months after the initial imaging session, anesthetize the mouse (Isoflurane, 5% induction and 1.5% maintenance), mount on the two-photon microscope, maintain on a heating plate and re-inject 100 µl a blood vessel dye such as Rhodamine B (2 mg/mL).

5.2 Using the notes from the previous session, identify the same areas and carefully re-image them.

5.3 Repeat this for as long as the imaging window is clear or as essential for the extent of the study.

REPRESENTATIVE RESULTS:

To visualize microglial dynamics in vivo, double transgenic CX3CR1$^{GFP/+}$:Thy1^{YFP} mice were used. The Thy1-YFP H line is used as opposed to the Thy1-GFP M line to avoid florescence overlap of microglia (GFP) and neurons (YFP). Alternative approaches could use a reporter line in which microglia are labeled with e.g., tdTomato and then the Thy1-GFP M line can be used. A drawback of the H line is that YFP labels a lot of neurons and the label increases with increasing age (personal observation). The M line exhibits sparse labeling of neurons. Between 2 – 4 weeks of the window implantation surgery, microglial dynamics can be followed by repeated imaging. Large blood vessels are used to localize specific regions and then the YFP-labeled dendrites are

used for fine mapping of brain regions. With this approach, specific dendrites can be used as stable landmarks for the fine mapping of brain regions (**Figure 1**, arrows in **Figure 1b**). While dendrites are stable, some microglia move daily (**Figure 1b**).

In addition, this approach is sufficient for weekly longitudinal imaging in the long-term. Thus, single transgenic CX3CR1$^{GFP/+}$ mice were used to follow microglia coupled with intraperitoneal injections of Rhodamine B to label the vasculature during each imaging session for up to 8 weeks (**Figure 2**). Alternatively, as discussed above, Thy1 mice could be used for longitudinal fine mapping. When weekly imaging is performed, the vasculature is noted to be stably fixed, but microglia can be seen to be dynamic as shown in three specific regions of interest (ROI, dashed circles) in **Figure 2**. In the top ROI, microglia begin to enter the ROI by the 4th week of imaging and continue through the 8th week of imaging. In the middle ROI with a bifurcated vessel, a microglial cell emerges around the lower vessel in the 3rd week, is lost on the 6th week and another microglia emerges on the upper blood vessel in the 7th week and is maintained into the 8th week. Finally, in the bottom ROI, a microglial cell in maintained through the 6th week and lost in the 7th and 8th weeks of imaging. These results indicate dynamic changes in the microglial positional network over weeks to months.

This approach can also be used to investigate cellular dynamics following acute injury or during pathological disease progression. Single transgenic CX3CR1$^{GFP/+}$ mice were used to follow microglia coupled with intraperitoneal injections of Rhodamine B to label the vasculature before (data not shown) and following severe seizures induced by kainic acid (**Figure 3**). Following seizures, the vascular bed structure is maintained without overt perturbations (**Figure 3a**). However, the microglial cellular network and positional landscape is transiently changed (some cells are "gained" and others are "lost" in the field of view) with greater changes within 24-48 h of seizures that is restored to normal by 72 h (**Figure 3b**) as we previously reported[32].

Finally, this approach can also be used to investigate cell-cell interactions or compare dynamics between neural cell types. Double transgenic CX3CR1$^{GFP/+}$:NG2$^{dsRed/+}$ mice were used to track microglia and NG2^{+} cells in vivo. Without labelling the vasculature, microglia and NG2 cells can be identified (**Figure 4a**). NG2 is a proteoglycan that labels both vessel-associated pericytes and oligodendrocyte precursor cells (OPCs)[33,34]. Pericytes typically have elongated processes that follow along the vascular wall (presumptively identified with arrowheads in **Figure 4a**) and OPCs typically show larger cell bodies that reside in the brain parenchyma away from the vasculature (presumptively identified with arrows in **Figure 4a**). To adequately distinguish pericytes and OPCs, the vasculature is labeled with Rhodamine B. The brighter florescence of NG2^{+}-vessel associated cells (pericytes, arrowheads) can be distinguished from the fainter florescence of luminal Rhodamine despite similar excitation by two photon imaging (**Figure 4b,c**). Daily imaging shows that pericytes are stably positioned, while OPCs (asterisks in **Figure 4b**) and microglia (circles in **Figure 4b**) are dynamic consistent with previous reports[32,35,36].

FIGURE AND TABLE LEGENDS:

Figure 1: Daily imaging of microglia using fine mapping with neuronal dendrites in double transgenic CX3CR1$^{GFP/+}$:Thy1^{YFP} mice. (**a**) Representative two-photon image of microglia (green)

and dendrites (red) from a double transgenic CX3CR1$^{GFP/+}$:Thy1^{YFP} mouse. (**b-c**), Daily images of boxed region in (**a**) showing repeatedly imaged dendrites (arrows in b) and dendrites with microglia (**c**). While dendritic structures were positionally stable, some microglia were noted to translocate from their original position in subsequent days. Such cells were identified with a number (1, 2 or 3). On the previous day, their position was noted with a white asterisk and on a subsequent day, their position was noted with a yellow asterisk.

Figure 2: Long-term weekly imaging of microglia in CX3CR1$^{GFP/+}$ mice for several months. (**a-h**), Representative two-photon images of microglia (green) from a CX3CR1$^{GFP/+}$ mouse during repeated imaging using acutely labeled vasculature (red, Rhodamine, 2 mg/mL, i.p.) as a coarse landmark to track the microglial network for up to 8 weeks. The vasculature was structurally stable through the imaging period. Three small regions with the vasculature (dashed circles) were highlighted to indicate the movement of microglial somata into (top two dashed circles) or out of (bottom circle) those regions.

Figure 3: Long-term daily imaging of microglia in CX3CR1$^{GFP/+}$ mice following seizures. (**a-b**), Representative two-photon images during daily imaging of the same field of view of a specific brain region with microglia (green) and acute labeling of the vasculature (red, Rhodamine, 2 mg/mL, i.p.). Imaging begins after induction of severe seizures using a chemoconvulsive agent, kainic acid. The vascular structure was maintained as individual vascular segments (arrows) can be identified through time (**a**). However, the microglial network dynamics was increased during the first two days following the seizures and returns to normal levels by the third day (**b**).

Figure 4: Daily imaging of microglia and NG2^{+} cells in CX3CR1$^{GFP/+}$:NG2$^{dsRed/+}$ mice. (**a**) A representative two-photon image of microglia (green) and NG2^{+} cells (red) in vivo. The unlabeled vasculature fails to distinguish NG2 cells (pericytes, presumptively identified with arrowheads) and NG2 cells not associated with the vasculature (oligodendrocyte precursor cells or OPCs, presumptively identified with arrows). (**b**) Representative two-photon images of microglia (green) and NG2^{+} cells (red) in consecutive days of imaging with the vasculature labeled with Rhodamine. Pericytes (arrowheads) are stationary while OPCs are dynamic (white to yellow asterisks). Microglia are also dynamic (circles: dashed circles represent a position without microglia and filled circle represents a position with a corresponding microglia).

DISCUSSION:

The advent of in vivo two-photon imaging has opened opportunities to explore the plethora of cellular interactions and dynamics that occur in the healthy brain. Initial studies focused on using the open skull craniotomy approach to image neuronal dendrites by both acute and chronic imaging[37,38]. This can also be used to elucidate neuroimmune interactions in the brain. This protocol describes a method for the reliable imaging of fluorescently tagged cells (especially microglia, the resident immune cell of the brain) for extended periods of time in the short or long term. The use of dye-labeled vascular and / or fluorescently tagged dendrites is detailed for coarse or fine mapping of the brain regions of interest to allow repeated, reliable imaging of cells. Although the Thy1^{YFP} line is suggested for use for fine brain mapping, alternative approaches could use other techniques or mouse lines for labeling select neuronal populations such as in

utero electroporation[39,40], early postnatal AAV injections[41] or the use of TRAP mice[42]. Furthermore, although cortical imaging was the focus of this discussion, this approach can be adapted to visualize deep brain structures in the long-term as well[43].

For this protocol, the surgery procedure on each mouse can be completed in 30-60 min from the initiation of anesthesia until the recovery from anesthesia. During the surgery, the skull is carefully removed and replaced with a sterile coverglass which is implanted for long-term imaging after at least two weeks. Mortality is extremely rare (less than 5%) in wildtype mice, though mice with clotting problems, such as P2Y12R knockout mice, show higher mortality and surgery failure. In such mice, bleeding may persist for longer periods of time and mice may die within the first 48 h of the craniotomy presumably due to complications from internal bleeding. Mice with implanted windows from this protocol have not been noticed to show any signs of infection and the protocol can be reliably used to generate clear windows for long-term imaging in 50-80% of mice.

Alternative to the current chronic window implantation approach, the thin skull approach exists to visualize brain cells in the intact brain repeatedly. Several studies have highlighted the value and even priority of choice of the thin skull approach over the window implantation approach[22-25]. The promise of that approach should not be ignored as, when done properly, it counteracts several salient limitations or disadvantages of the current approach including the lack of activation of glial cells, the low turnover of spines which is more physiological, and the lack of need for the use of anti-inflammatory agents which could also affect brain physiology. In selecting an approach for specific research questions, these serious limitations should be considered before choosing the approach detailed in this protocol.

However, the appeal of this approach is four-fold. First, the cranial window implantation approach is attractive because of the ease of mastery of this procedure relative to the thin skull procedure. Appropriate skull thinning cannot always be mastered by experimenters and if not done well can result in glial activation limiting its appeal. Second, this cranial window implantation approach gives powerful depth clarity of brain structures as the brain is imaged through an optically clear window. The window available for imaging is also usually much larger than that used in the thin skull approach allowing access to a larger volume of tissue for analysis. Third, like depth clarity, this approach allows for a uniform clarity through the window since the glass coverslip is uniformly thin and clear. This facilitates comparisons between sessions and between animals. Special expertise is required for the thin skull technique to ensure even clarity across the window during repeated sessions and between animals. Finally, this approach offers much flexibility in the *frequency* of imaging from hours, to days to weeks to months and even years. For the thin skull approach, a maximum of five repeats has been suggested[24].

Future applications of this approach are many. First, applications could involve elucidating novel neuro-glio-vascular interactions in the brain in both normal physiology and pathology. Second, although resident cells are discussed in this procedure, the approach can be used to study the dynamics and interactions of infiltrating immune cells as occurs e.g., during acute injury, chronic brain infection, and/or neurodegenerative conditions as long as mice with the respective

fluorescently tagged cells are available. Finally, this approach has been discussed mainly in the context of structural studies of brain cells. However, with the advent of functional imaging e.g. using calcium[44-46] or voltage imaging techniques[47,48], this approach can be used for functional imaging over time in health and disease.

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DISCLOSURES:

The authors have nothing to disclose

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Fig 1

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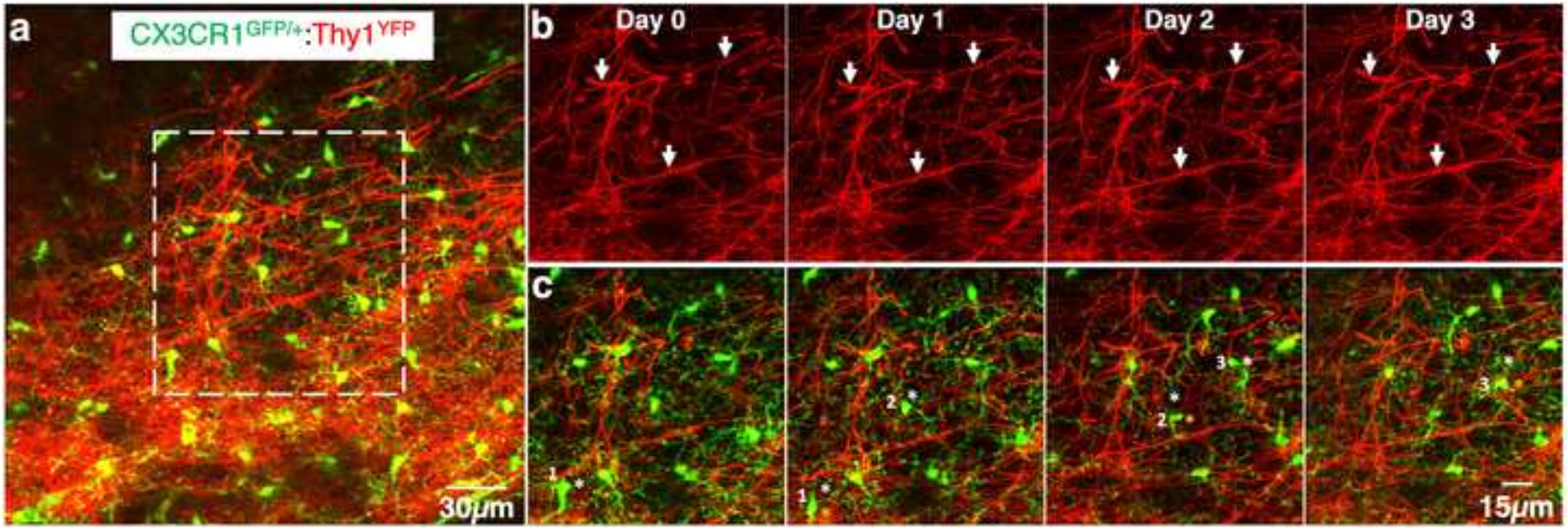

Fig 2

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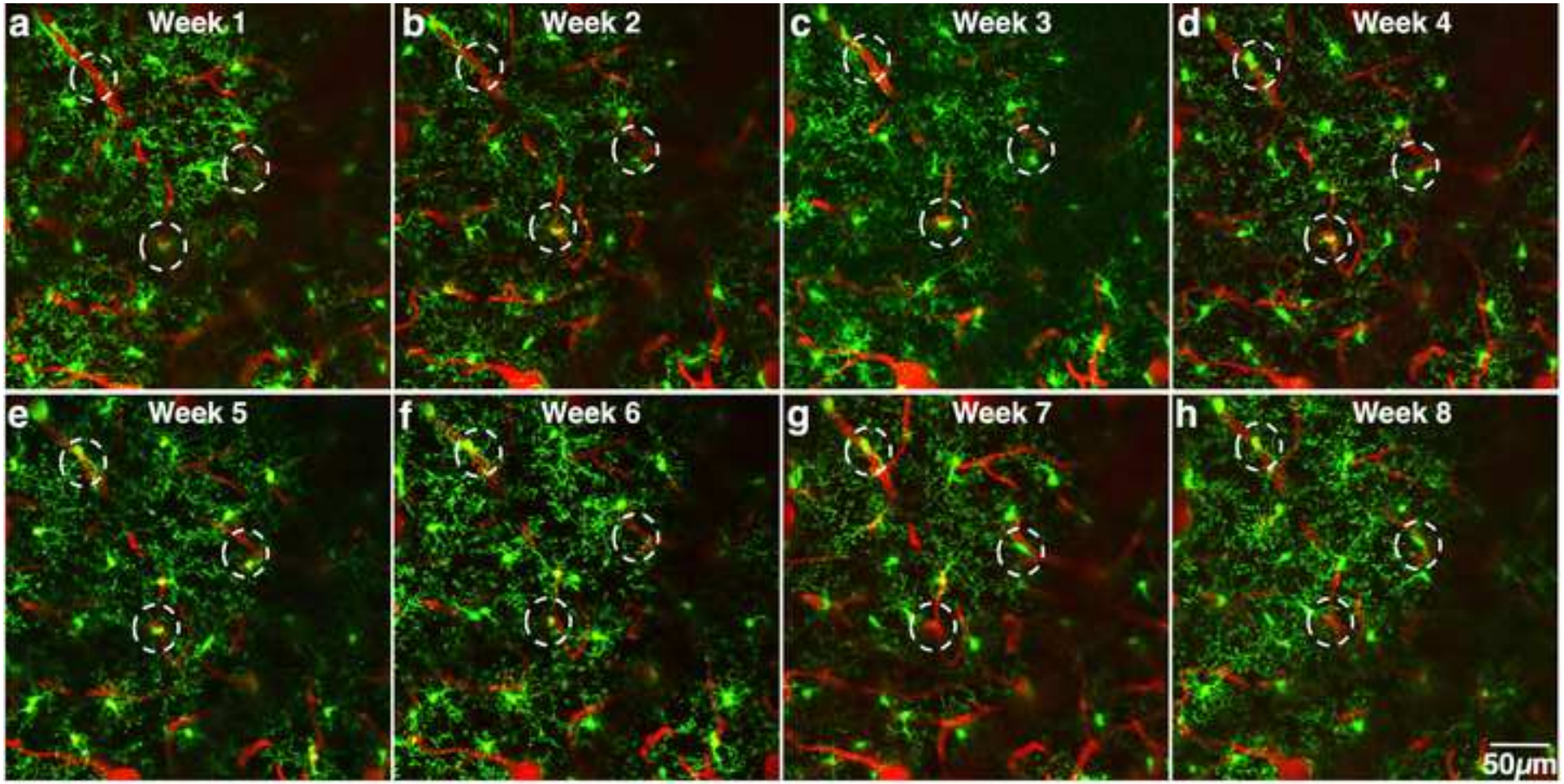

Fig 3

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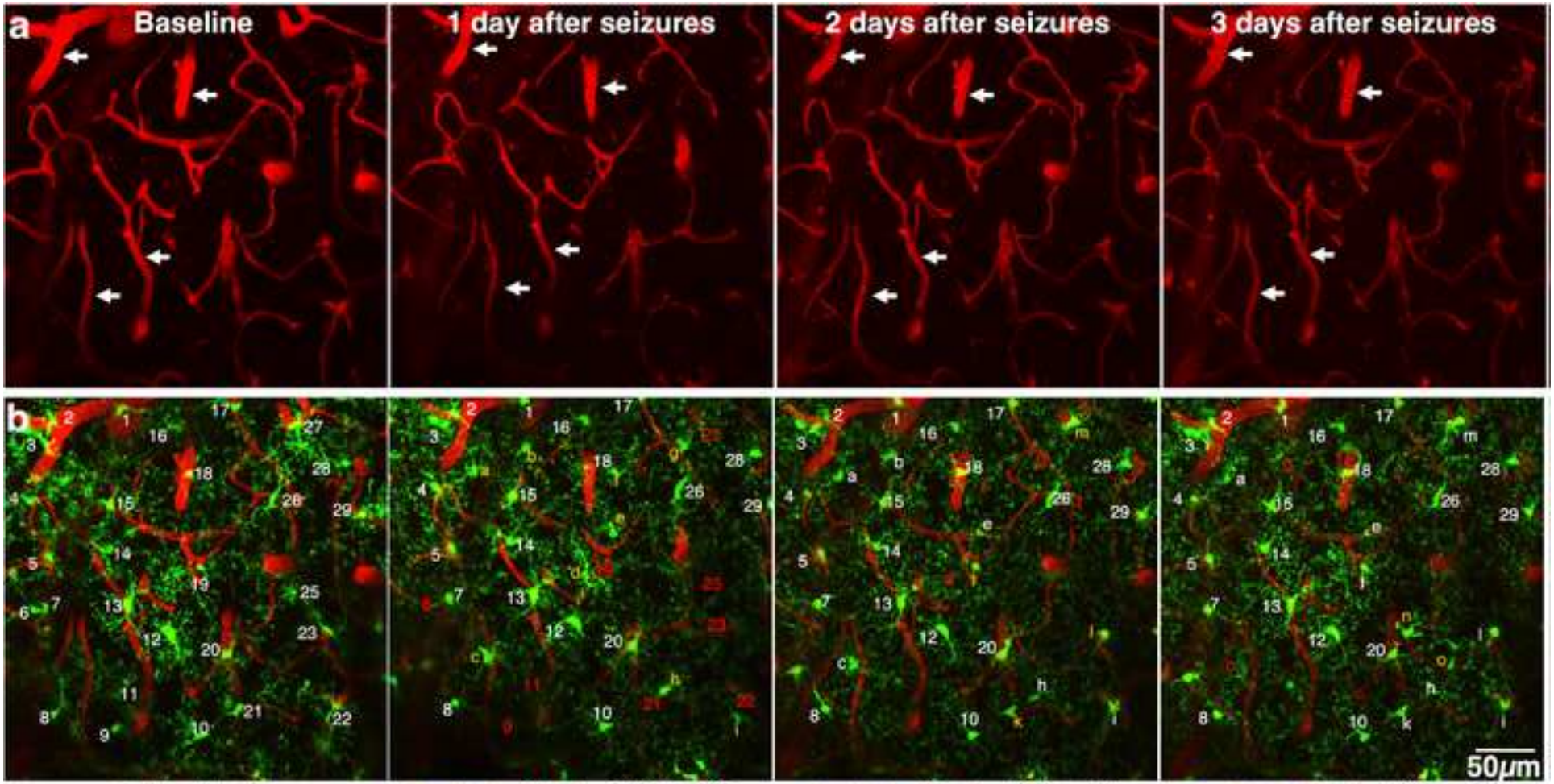
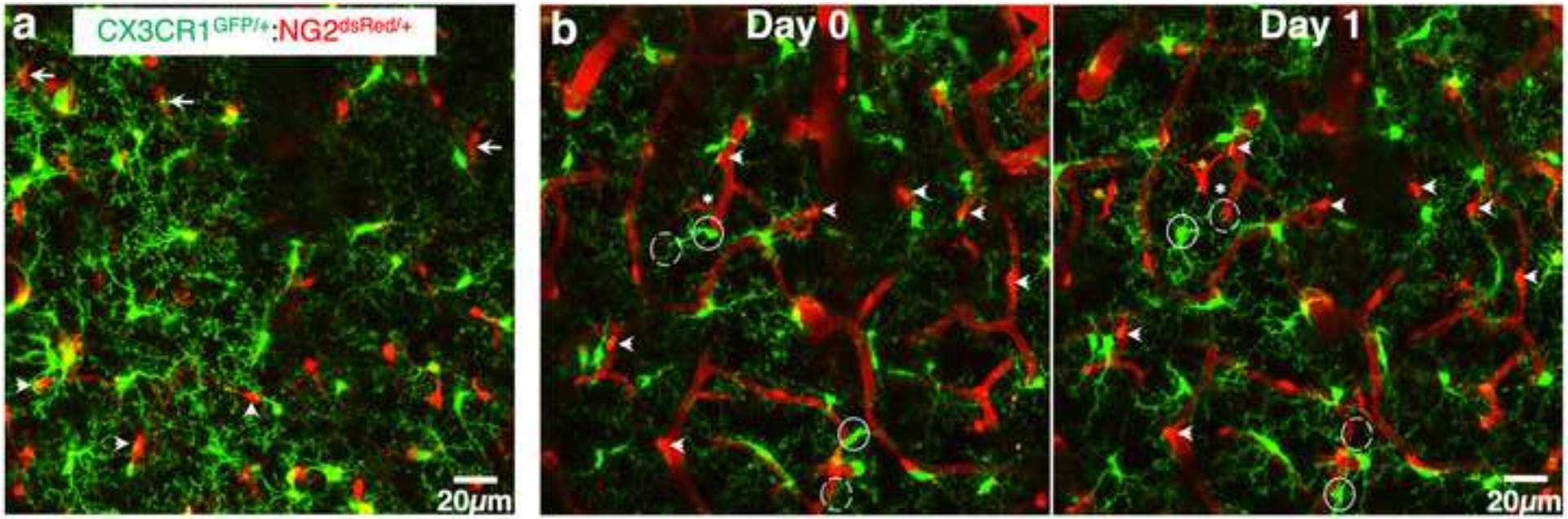


Fig 4

Click here to access/download;Figure;Fig 4.tif



Name of Material/Equipment	Company
Coverglass (3mm)	Warner Instruments
Cyanoacrylate glue (Krazy Glue)	Amazon
Demi Ultra LED Curing Light System	Dental Health Products, Inc
Dental Drill	Osada: www.osadausa.edu
Drill Bit	Fine Science Tools
Eye Ointment	Henry Schien
iBond Total Etch (Primer glue)	Chase Dental Supply (Heraeus Kulzer)
Rhodamine B	Millipore Sigma
Tetris Evoflow glue (Final glue)	Top Dent (Ivoclar Vivadent)
Wahl Brav Mini+	Amazon

Catalog Number	Comments/Description
64-0726	
https://www.amazon.com/Krazy-Glue-Original-Purpose-Instant/dp/B07GSF31WZ/ref=sr_1_2?keywords=krazy+glue&qid=1583856837&s=pet-supplies&sr=8-2	
910860-1	
EXL-M40 #19008-07 1338333	
66040094 81-88-9 (R6626)	
#595956 https://www.amazon.com/Wahl-Professional-Animal-BravMini-41590-0438/dp/B00IN24ILE/ref=asc_df_B00IN24ILE/?tag=hyprod-20&linkCode=df0&hvadid=167141013968&hvpos=&hvnetw=g&hvrand=12368793083893626704&hvpone=&hvptwo=&hvqmt=&hvdev=c&hvdvcmdl=&hvlocint=&hvlocphy=9008337&hvtargid=pla-332197544154&psc=1	

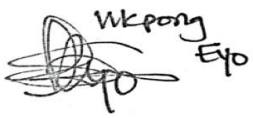
Dear Dr. Vineeta Bajaj,

Thank you again for all your additional inputs for our manuscript, "**Precise Brain Mapping To Perform Repetitive In Vivo Imaging of Neuro-Immune Dynamics in Mice**". Please see below a point-by-point response to all your comments in the manuscript.

Again, as previously followed, we have included all the original comments in black, whereas the changes made by us to address those concerns are given in blue. In the accompanying text, the changes made to the original submission are also indicated in blue text. We hope that all the changes made by us adequately address the editorial concerns.

We thank you for all your thoughtful concerns and hope that the manuscript now fulfils all the criteria for publication in JoVE.

Yours Sincerely,



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1. Title: Precise brain mapping to perform repetitive in vivo imaging of neuro- immune dynamics? Since the precise brain mapping is the crux of the protocol. Please proofread the manuscript well.
   Thank you for the suggestion. We have accepted this title and edited the manuscript accordingly.

2. Intro needs its opening as well with respect to CNS, in vivo imaging, NMJ and brain mapping. Please reformat the Introduction to include all of the following with citations in paragraph style:
   a) A clear statement of the overall goal of this method.
      This is now included as the last sentence of the first paragraph: "In this protocol, we describe a method to map *in vivo* regions of interest and precisely re-image those regions in future imaging sessions to track the complex interactions between the various CNS cell types over extended periods of time". We have also included and additional final paragraph in the Introduction to express this: "Overall, we detail steps that can be followed to do implant chronic cranial windows through a stereotaxic craniotomy that enables in vivo imaging of brain regions of interest. We also describe how the grossly stable brain vasculature or the fluorescently labeled dendrites could be used to generate a coarse or a fine map, respectively of the brain regions of interest. This approach can then be used for repeated imaging over several sessions. The importance of this technique therefore lies in its ability to image the long-term changes or stasis in brain elements including the arrangement, morphology, and interactions of the different cellular types".

   b) The rationale behind the development and/or use of this technique.
      This is stated in the third sentence of the first paragraph: "there is need to further understand neural cell behavior and interactions in the native environment of the intact brain *in vivo*".

   c) The advantages over alternative techniques with applicable references to previous studies.
      Most of the third and all of the forth paragraphs argue for the advantages given the stated 4 limitations of the thin skull approach.

   d) A description of the context of the technique in the wider body of literature.
      We have included many more citations in this revised version that are relevant to the points discussed.

   e) Information to help readers to determine whether the method is appropriate for their application.
      Again, the third and fourth paragraphs provide sufficient information for this purpose.

3. 5%of isoflurane isn't it too high? Anesthesia steps cannot be filmed, so highlights removed.
   This is what we use. Our anesthesia chamber can fit up to 5 mice easily so for us we use this much to effectively saturate the chamber. But perhaps others who use smaller chambers would use a lower percent. We have now included a note as follows in Step 1.2 of the protocol:

   NOTE: The concentration of isoflurane used would depend on the size of the induction chamber. Therefore, for smaller chambers, 3-4% isoflurane can be used to effectively induce anesthesia while larger chambers will require up to 5%.

4. How big is the midline scalp incision?
   We are not quite sure what is required by this question. We noted that our cuts are made from the back of the skull to about the midpoint between the eyes. The length of this would differ depending on the age of the mouse and thus the size of the mouse's head.

5. Please include this as an in text citation and include the bioRxiv paper as reference.
   We are not sure how to include a bioRxiv pre-print as an intext citation with EndNote but we have manually included it here and in the references.

6. Step 4.3: This won't be accurate.
   Thank you for this comment. From this, we realize that we conflated two aspects in our note for 4.3. 4.3 is about taking *rough* images by epiflorescence while 4.4 is about taking *precise* images by two photon. The step described in 4.3 helps the experimenter to get the microscope in the rough area of the 3mm window while that described in 4.3 helps the experimenter to get to the specific local area of interest. Therefore, we have added a note to 4.3 as follows:

   "These hand-drawn images and pictures are to facilitate revisiting the same broad regions under the microscope before two photon imaging. They are not sufficient precise image mapping"

   and moved the previous note to 4.4 which is more relevant to the 2P imaging:

   "While collecting images by two photon, the blood vessel landmarks are used for coarse mapping. If fine mapping is needed, YFP-labeled dendrites from Thy1-YFP[13] mice are used"

   We also added a sentence in 4.4 to clarify that image collection here is by 2P as follows:

   "Under two photon imaging, collect images of florescent cells and vessels as needed."

7. Please include steps to show how two photon imaging was performed.
   We appreciate the recognition of this omission. We have now included a new step in 4.2 that details the process of setting up the objective lens for epifluorescence:

   "Clean the surface of the cranial window gently using a cotton swab dabbed in 70% ethanol. Put a few drops of water or saline on the cranial window and lower the objective lens into the solution since the objective is an immersion lens"

   Then we added these parameters in a note for Step 4.4:

   "These are the recommended parameters for imaging: a wavelength of 880-900nm is optimal; for GFP and/or dsRed / Rhodamine excitation, a 565nm dichroic mirror with 525/50nm (green channel) and 620/60nm (red channel) emission filters are used; for GFP and YFP separation, a 509nm dichroic mirror with 500/15 and 537/26 nm emission filters are used; the power at the brain is maintained at 25mW or below; image resolution is 1024 x 1024 pixels, the field of view taken with a 25X 0.9 NA objective at a 1.5X zoom factor is 295.24 x 295.24 µm"