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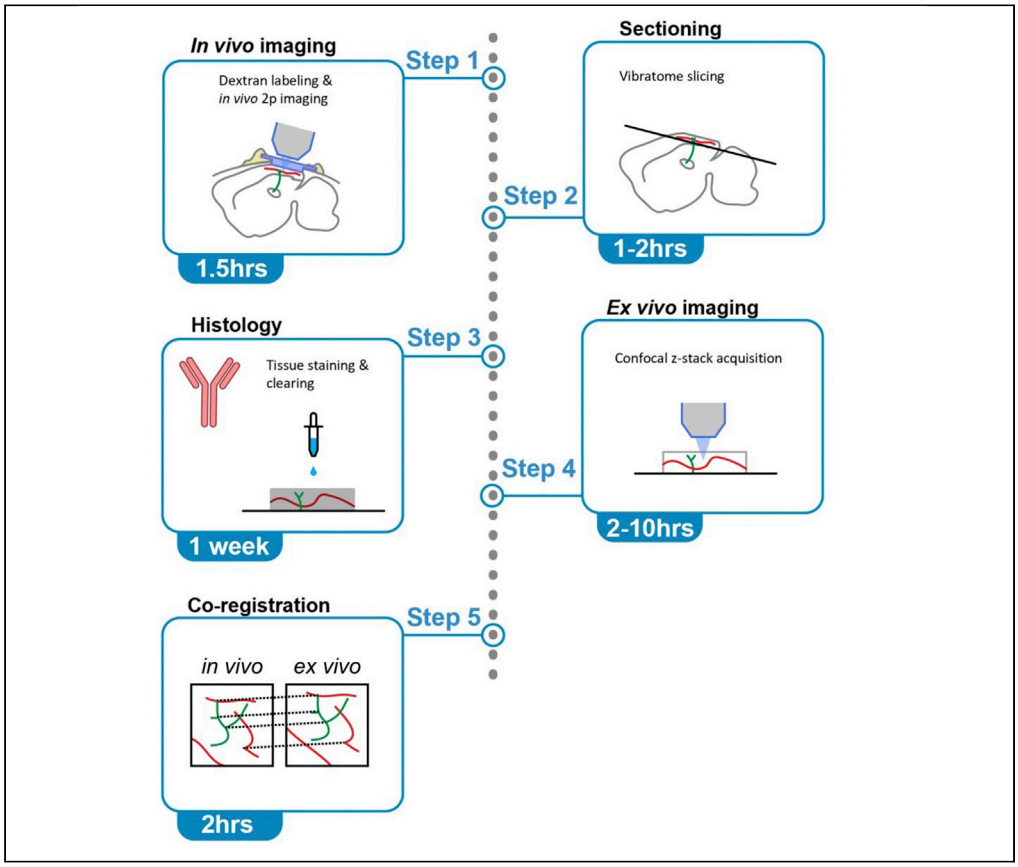
Protocol

Co-registration of mouse cortical structures between *in vivo* and *ex vivo* images using tangential sectioning



Co-registration of neuronal structures between *in vivo* and *ex vivo* imaging is necessary to study structure-function correspondence in the mammalian brain. Here we describe a protocol based on tangential sectioning of the mouse brain. This protocol aligns *in vivo* two-photon calcium imaging volumes with *ex vivo* confocal imaging volumes and registers the same cortical structures in both volumes. This approach allows detailed analysis of the corresponding function and structure of these entities.

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

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Highlights

Tangential sectioning facilitates co-registration between imaging modalities

In vivo two-photon calcium imaging followed by *ex vivo* confocal imaging of mouse brain

Blood vessels are labeled as fiducial landmarks for *in vivo* and *ex vivo* image alignment

In vivo to *ex vivo* co-registration enables studying function-structure correspondence

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Co-registration of mouse cortical structures between *in vivo* and *ex vivo* images using tangential sectioning

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SUMMARY

Co-registration of neuronal structures between *in vivo* and *ex vivo* imaging is necessary to study structure-function correspondence in the mammalian brain. Here we describe a protocol based on tangential sectioning of the mouse brain. This protocol aligns *in vivo* two-photon calcium imaging volumes with *ex vivo* confocal imaging volumes and registers the same cortical structures in both volumes. This approach allows detailed analysis of the corresponding function and structure of these entities.

For complete details on the use and execution of this protocol, please refer to Zhuang et al. (2021).

BEFORE YOU BEGIN

This protocol is designed to co-register the same cortical structures between *in vivo* functional imaging and *ex vivo* structural imaging, allowing the investigation of function-structure correspondence. The high-level workflow follows six parts:

Part 1. Label the neuronal structure with calcium indicator (GCaMP, Figure 1A).

Part 2. Use two-photon *in vivo* calcium imaging to functionally characterize the labeled structures while recording fiducial landmarks (vasculature patterns) for co-registration (Figure 1B).

Part 3. Extract tissue using tangential sectioning (Figure 1C).

Part 4. Stain the structures of interest (e.g., those labeled with GCaMP) and fiducial landmarks and clear the tissue (Figure 1D).

Part 5. Use high-resolution *ex vivo* imaging to record the labeled structures and the fiducial landmarks (Figure 1E).

Part 6. Use the fiducial landmarks to align the *in vivo* and *ex vivo* image volumes and co-register GCaMP labeled entities between these two volumes (Figures 1F and 1G).

This protocol will focus on parts 2–6 by assuming the users are familiar with the surgical procedures of viral/transgenic labeling and cranial window implantation. All animal experiments should be approved by the Institutional Animal Care and Use Committee (IACUC) before implementation.

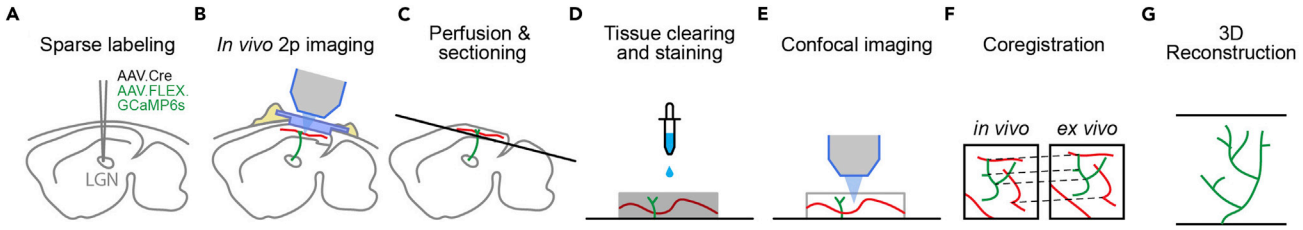

Figure 1. Sketches indicating this protocol's workflow

(A) The neuronal structures of interest (dLGN axons) are sparsely labeled by calcium indicator via viral injection.

(B) two-photon *in vivo* calcium imaging to functionally characterize the labeled structures. Fiducial landmarks (blood vessels, red) are also labeled during imaging sessions.

(C) Tangential sectioning after perfusion and brain fixation.

(D) GCaMP signals are enhanced by immunohistochemistry, blood vessels are labeled by lectin, and the section is cleared .

(E) Regions of interest are imaged by a confocal microscope.

(F) Using structure landmarks (blood vessels), the *in vivo* and *ex vivo* imaging volumes are aligned, and the same GCaMP labeled entities are registered in both volumes.

(G) The morphology of functionally identified neuronal structures in *in vivo* experiments can be reconstructed from the *ex vivo* confocal imaging volume. This figure is modified from Zhuang et al. (2021) with permission.

Institutional permissions

All animal procedures performed in this document were approved by the Institutional Animal Care and Use Committee (IACUC) at the Allen Institute for Brain Science.

Preparation one: Labeling with calcium indicator (GCaMP)

Timing: 1–2 h

Delivery of GCaMP can be achieved by using transgenic and/or viral tools. The sparsity of the labeling depends on the structure of interest and level of detail in the structural reconstruction. In general, more detailed reconstruction (e.g., axon morphology reconstruction) will require higher sparsity.

1. To sparsely label the thalamocortical neurons projecting from dorsal lateral geniculate nucleus (dLGN) to primary visual cortex in mouse (Zhuang et al., 2021):
   a. Deliver a 1:1 mixture of AAV9-hSyn-Cre (1:40000 dilution, Addgene: 105553-AAV9, titer: 3.3 × 1013 vg/mL) and AAV1-Syn-FLEX-GCaMP6s (Addgene: 100845-AAV1, titer: 2.5 × 1013 vg/mL) to mouse dLGN via stereotaxic injection (100 nL per mouse).

   Alternatives: Other viral (e.g., Lin et al., 2018) or genetic tools (e.g., Veldman et al., 2020) are available to achieve sparse labeling.

   Note: The methods for viral delivery of GCaMP to the neurons in mouse brains are standard and widely used in neuroscience research (Harris, JA et al., 2012; Yardeni et al., 2011), so they are not elaborated here.

Preparation two: Cranial window

Timing: 2–3 h

For *in vivo* two-photon imaging, a cranial window is implanted for optical access to the cortical areas of interest; and a headpost is implanted to stabilize the skull during imaging sessions.

2. Following the published protocols, create a 5 mm diameter circular craniotomy over the area of interest. In this case, over visual cortex.

3. Implant a circular glass window (diameter 5 mm) and a headpost over posterior cortex to image calcium activity in visual cortex (Goldey et al., 2014; Groblewski et al., 2020).
4. After surgery, let the animal recover for a minimum of 5 days and then handle and habituate it to the imaging rig for two additional weeks before *in vivo* two-photon imaging (de Vries et al., 2020).

Note: The sectioning, histology, *ex vivo* imaging procedures and the placement of the cranial window described in this protocol are applicable to other cortical regions.

Note: Preparations one and two can be done during a single surgery under anesthesia, with the cranial window implantation immediately following the viral injection.

⚠ CRITICAL: It is important that the chronic cranial window flattens the cortical surface of interest. This facilitates the mounting and tangential sectioning methods described in this protocol. In our case, the 5 mm glass cranial window was fully in contact with cortical surface, resulting a flat cortical surface after perfusion (See more discussion in Problem #3 of troubleshooting).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken polyclonal anti-GFP antibody	Abcam	ab13970; RRID: AB_300798 See materials and equipment for dilution
Alexa Fluor 488 Donkey Anti-Chicken IgY (IgG) (H+L)	Jackson ImmunoResearch	703-545-155; RRID: AB_2340375 See materials and equipment for dilution
Bacterial and virus strains		
AAV1-Syn-FLEX-GCaMP6s	Chen et al. (2013)	Addgene: 100845-AAV1
AAV9-hSyn-Cre	James M. Wilson Lab	Addgene: 105553-AAV9
Experimental models: Organisms/strains		
Mouse: C57BL/6J Wildtype or transgenic P28 or older of either sex	The Jackson Laboratory	Strain#000664
Chemicals, peptides, and recombinant proteins		
Isoflurane	Patterson Veterinary	Cat# 07-878-1636
Sodium Azide	VWR	Cat# BDH7465-2
Paraformaldehyde powder	Sigma-Aldrich	Cat# 441244
10× PBS	Ambion	Cat# 9625
5 M NaOH solution	VWR	Cat# BDH3225-1
1 N HCl solution	Sigma-Aldrich	Cas 7647-01-0
Lectin, DyLight® 649	Vector Laboratories	Cat# DL1178
Dextran Texas Red	Thermo Fisher Scientific	Cat# D3328
0.9% Sodium Chloride Inj., USP	Hospira	Cat# 00409488810
Urea	Sigma-Aldrich	Cat# U5378
Triton X-100	Sigma-Aldrich	Cat# X100
Quadrol® (N,N,N′,N′-Tetrakis(2-Hydroxypropyl)ethylenediamine)	Sigma-Aldrich	Cat# 122262
Normal Donkey Serum	Sigma-Aldrich	Cat# D9663
10× PBS (phosphate buffered saline)	Invitrogen	Cat# AM9624
Sodium Dodecyl Sulfate	Sigma-Aldrich	Cat# L6026
Agarose	Sigma-Aldrich	Cat# A0169
Clear Nail Polish	Electron Microscopy Sciences	Cat# 72180
Recombinant DNA		
AAV pCAG-mRuby3-WPRE	This study	Addgene: 107744

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Small animal stereotaxic instrument with digital display console	KOPF	Cat# 940
Spacers	SunJin Labs	iSpacer, #IS101 and #IS001
#1 coverglass	VWR	Cat# 16004-332
Glass slide	Electron Microscopy Sciences	Cat# 71867-01
Two-photon microscope	SUTTER INSTRUMENT	MOM
Confocal Microscope	Olympus	FV3000
Vibratome	Leica Biosystems	Leica VT1000 S
Personna Double-Edge Blade	EMS	Cat# 72000
Metal cylinder	Thorlabs	SM1S10M
Vortex-Genie 2	Scientific Industries	SI-0236
Peristaltic Pump	Harvard Apparatus	MA1-55-7766
Anesthesia System	VetEquip	Cat# 901806
Software and algorithms		
Fiji software	Schindelin et al. (2012)	https://fiji.sc/Fiji
TeraStitcher	GitHub	https://abria.github.io/TeraStitcher/ Bria and Iannello (2012)

MATERIALS AND EQUIPMENT

Dextran solution (for labeling blood vessels *in vivo*)		
Reagent	Final concentration	Amount
Texas Red - Dextran, 3 kD MW conjugate, lysine fixable	2.5%	10 mg
0.9% Sodium Chloride	n/a	400 μL
Total	**n/a**	**400 μL**

Note: Vortex to mix. Aliquot and store at −20°C for up to three months.

4% paraformaldehyde (PFA) (for perfusion and fixation)		
Reagent	Final concentration	Amount
Paraformaldehyde powder	4%	8.4 g
5 M NaOH	n/a	1 mL
10× PBS	n/a	20 mL
Milli-Q water	n/a	Add to 200 mL
Total	**n/a**	**200 mL**

Heat stir at 65°C to allow paraformaldehyde to dissolve. While stirring add HCl dropwise to adjust pH to 7.4. After fully dissolved and pH adjusted, let the solution to cool to ~20°C–27°C, then vacuum filter the solution by using a Corning filter system with a 0.45 μm filter. Stored at 4°C for up to one week.

Note: Paraformaldehyde is toxic. The whole procedure should be performed in a fume hood with PPE (gloves, lab coat, and eye protection).

NDSTU (for immunostaining)		
Reagent	Final concentration	Amount
Urea	4 M	48.0 g
Donkey Serum	5%	10 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Triton X-100	0.2%	400 μL
1× PBS	n/a	add until final volume is 200 mL
Total	**n/a**	**200 mL**

Note: Mix with stir bar until Triton X-100 and Urea are fully dissolved. Store at 4°C for up to one month.

CUBIC-1 clearing solution (for tissue clearing and mounting)		
Reagent	Final concentration	Amount
Urea	25 wt %	50 g
Triton X-100	15 wt %	30 g
Quadrol	25 wt %	50 g
Milli-Q water	n/a	70 g
Total	**n/a**	**200 g**

Note: Quadrol is extremely viscous. We found it easiest to pour it directly into a container on a scale and use that container as the final container for the CUBIC-1 solution. Degas the reagent with a vacuum desiccator (~0.1 MPa, ~30 min). CUBIC-1 can be stored at 20°C–25°C for up to one month. (Susaki et al., 2015).

PBST (rinse solution for immunostaining)		
Reagent	Final concentration	Amount
1× PBS	n/a	1996 mL
Triton X-100	0.2%	4 mL
Total	**n/a**	**2000 mL**

Note: Mix with a stir bar until Triton is fully dissolved. Store at 20°C–25°C for up to one month.

Primary antibody working solution (for immunostaining)		
Reagent	Final concentration	Amount
NDSTU	n/a	2 mL
Chicken polyclonal anti-GFP antibody (Abcam ab13970)	1: 500	0.4 μL
Total	**n/a**	**2 mL**

Note: Prepare solution immediately before use.

Secondary antibody stock solution (for immunostaining)		
Reagent	Final concentration	Amount
Alexa Fluor 488 Donkey Anti-Chicken (Jackson Immuno 703-545-155)	1.25 mg/mL	0.5 mg
Milli-Q water	n/a	400 μL
Total	**n/a**	**400 μL**

Note: Product is stable for at least 6 weeks at 2°C–8°C as an undiluted liquid.

Secondary antibody working solution (for immunostaining)		
Reagent	Final concentration	Amount
NDSTU	n/a	1992 µL
Secondary antibody stock solution	1: 500	4 µL
DyLight 649 (Vector Laboratories, DL1178)	2.5 µg/mL	4 µL
Total	**n/a**	**2 mL**

Note: Prepare solution immediately before use.

Agarose (for mounting fixed brain before sectioning)		
Reagent	Final concentration	Amount
Agarose	4%	2.0 g
1× PBS	n/a	50 mL
Total	**n/a**	**50 mL**

Note: Heat briefly in microwave until agarose is fully dissolved. Let cool to approximately 40°C before embedding brain. Prepare solution immediately before use.

PBS + 0.01% sodium azide (for long-term storage of fixed brain)		
Reagent	Final concentration	Amount
Sodium azide 5% solution	0.01%	4.0 mL
1× PBS	n/a	1996 mL
Total	**n/a**	**2000 mL**

⚠ CRITICAL: Care should be taken when handling sodium azide. It is toxic and carcinogenic. Do not breathe dust, do not use metal utensils. Use appropriate PPE. Dispose of stock Sodium azide waste in a segregated waste stream. Store at 20°C–25°C for three months or longer.

8% SDS solution (for antigen retrieval before immunostaining)		
Reagent	Final concentration	Amount
Sodium Dodecyl Sulfate	8%	4.0 g
1× PBS	n/a	50 mL
Total	**n/a**	**50 mL**

Note: Heat in 37°C water bath and stir until SDS is fully dissolved. Store at 20°C–25°C for up to one month. If stored at less than 25°C, the SDS will precipitate out of solution.

STEP-BY-STEP METHOD DETAILS

In vivo imaging

Timing: 1–2 h

This section describes the procedure of *in vivo* two-photon calcium imaging. At least two separate image sets will be saved: an image time series containing calcium activities for *in vivo* function characterization and a static 3D imaging volume for *in vivo* to *in vitro* co-registration.

1. After the animal is fully recovered from surgery and habituated to the imaging setup, capture a widefield image showing the surface vasculature pattern of the entire cranial window (Figure 3A).

   Note: Since this vasculature pattern is relatively stable overtime, this "zoomed-out" widefield image can be captured on a different day from the two-photon imaging session.

2. Before a two-photon imaging session, thaw and inject 30 μL of diluted dextran solution (materials and equipment) subcutaneously under skin on the back without anesthesia, using a syringe with 31G needle. Blood vessel labeling will appear within 5 min and last for at least two hours.
3. Perform functional two-photon calcium imaging of the cortical structures of interest. This should be specific to each research group.

In our case, for imaging thalamocortical axons:

   a. Zoom and resolution: 180 μm × 180 μm field of view, 0.35 μm × 0.35 μm per pixel (for imaging cell soma, a larger field of view and lower resolution can be used).
   b. Excitation: 920 nm.
   c. Emission filter for GCaMP: 470–588 nm.
   d. Emission filter for dextran-Texas Red: 600–722 nm.
   e. Objective: Nikon 16× 0.8NA.

   ⚠ CRITICAL: After functional imaging, without moving the objective XY position, record a two-photon z-stack with both GCaMP and dextran channels. The 3D vasculature pattern and structures of interest should be visible in this stack.

   a. Zoom: same as functional image.
   b. Starting depth: ~10 μm above highest point of pia surface.
   c. Ending depth: 500 μm below pia surface or 100 μm below the structure of interest.
   d. Z-Step depth: 4 μm.
   e. Average 100 frames per step.

   Optional: Without moving the objective XY position, record a surface two-photon z-stack in both channels with the largest field of view.

   a. Zoom: allowing the largest field of view (716 μm × 716 μm with 1.4 μm resolution on our setup).
   b. Starting depth: ~10 μm above highest point of pia surface.
   c. Ending depth: 100 μm below pia surface.
   d. Z-Step depth: 10 μm.
   e. Average 100 frames per step.
   f. Save the mean projection of the stack as two-photon surface vasculature pattern.

   Optional: Without moving the objective XY position, record a local widefield surface vasculature stack through the same objective (if this has larger field of view than the two-photon field of view).

   a. Starting depth: ~10 μm above highest point of pia surface.
   b. Ending depth: where the surface vasculature pattern is defocused.
   c. Z-Step depth: 10 μm.
   d. 1 image per step.

e. Save the mean projection of the stack as local wide-field surface vasculature pattern.

Note: Steps 1–6 can be repeated in multiple sessions/days to either image the same structures repeatedly (by aligning two-photon field of view) or image different structures at different locations. For each two-photon field of view, a corresponding two-photon z-stack (step 4), two-photon surface z-stack (step 5), and a local widefield surface stack (step 6) should be recorded.

Note: The two-photon surface vasculature pattern and the local wide-field surface vasculature pattern recorded in steps 5 and 6, respectively, can aid the alignment with *ex vivo* volume when the field of view of functional imaging is small and does not contain enough structural information for alignment.

Perfusion and tissue fixation

Timing: 1 h

After two-photon imaging was concluded, a standard transcardial perfusion was performed to fix brain tissue. Since this procedure is standard and has been documented (Gage et al., 2012; Wu et al., 2021), it is only described briefly here.

4. Under isoflurane (initial: 5%, maintain: 3%) anesthesia, expose thoracic cavity.
5. Insert the blunt perfusion needle (which is attached to a peristaltic pump, Harvard Apparatus, MA1-55-7766) into the left ventricle directing the tip of the needle toward the aorta.
6. Perfuse 10 mL saline (0.9% Sodium Chloride) using the peristaltic pump through the circulatory system at a flow rate of 9 mL/min or until the liver is clear.
7. Perfuse 50 mL freshly prepared 4% PFA (materials and equipment) at a flow rate of 9 mL/min.
8. Extract the brain and immerse it in 4% PFA at 20°C–25°C for 3–6 h and then at 4°C for 12–24 h.
9. After fixation, immerse the brain in 1 × PBS with 0.01% sodium azide (materials and equipment) at 4°C in the dark for long term storage (up to one year). This can be a pause point.

Histology: Sectioning

Timing: 1–2 h

A vibratome (Leica VT1000 S) was used to section the brain tangential to the area that was flattened under the cranial window. It is crucial to preserve the top 350 μm–400 μm of cortex including the pial surface in one intact section (due to the shrinkage effect from PFA and the compression by the glass cranial window, we found, this depth range incorporates almost the entire cortical depth within the 5 mm cranial window over visual cortex, Zhuang et al., 2021, please see Problem #5 in troubleshooting for more discussion). Typically, during vibratome sectioning, the blade is automatically lowered at a set interval and slices through an agarose block until it begins to section the embedded tissue. This method would result in an unknown thickness for the first crucial section. To avoid this, the blade must be aligned as closely as possible to the surface of cortex before the first section is cut. The following steps describe this process. The resulting section of cortex will be preserved in its flattened state to aid the alignment with *in vivo* images.

10. Take the fixed brain out of PBS + 0.01% sodium azide (materials and equipment) and gently press the flattened area of cortex facing down against a clean glass slide.

⚠ CRITICAL: The flattened area must be fully in contact with the glass.

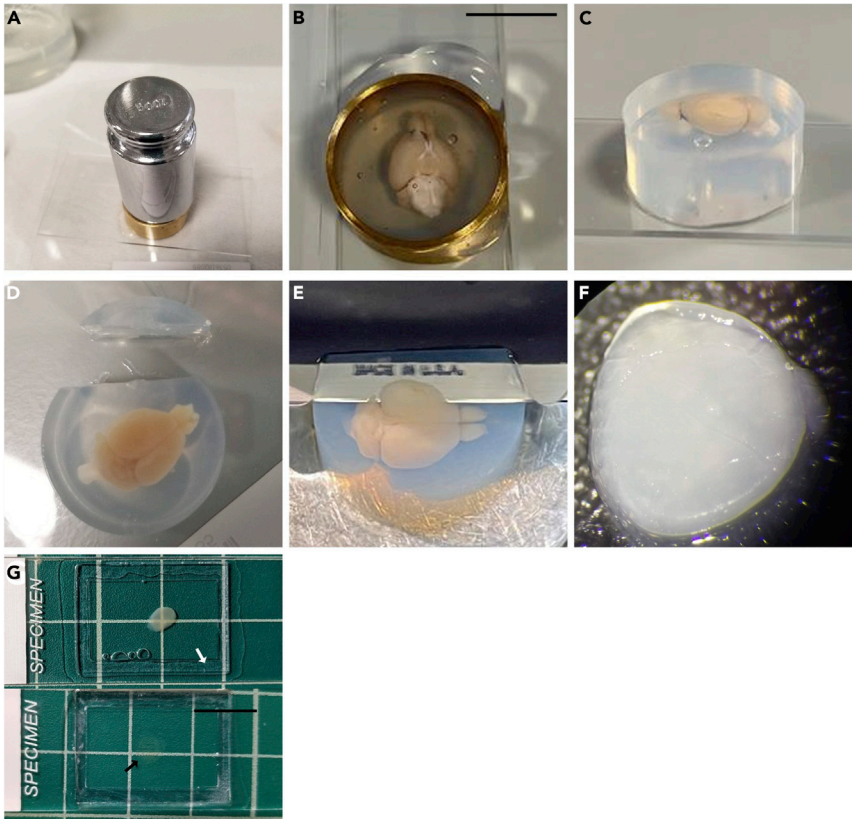

Figure 2. Photos taken to demonstrate the tissue processing steps

(A) The brain is placed inside a metal ring on a glass slide (with the flat part of cortex facing down), and the ring is then filled with agarose. An additional glass slide is placed on top, followed by a weight to hold it in place. This helps ensure that the top and bottom of the solidified agar block will be flat and parallel.

(B and C) (B) The agarose is solidified, and the block is ready to be removed. It is gently pushed out of the ring and onto a glass slide, as shown in image (C).

(D) Agarose on one side is trimmed away, and the block is then glued to the vibratome stage with the trimmed side facing the blade.

(E) A section with thickness no less than 350 μm is cut.

(F) The flat tangential thick section is ready for staining.

(G) A mounted uncleared section (top) and a mounted cleared section (bottom). White arrow: spacer for making a well to accommodate tissue thickness. Black arrow: cleared section. Scale bar: 0.5 inch.

11. Place a metal ring around the brain and rest it on top of the glass slide to create a mold. Carefully pour warmed liquid agarose (materials and equipment) in the mold to embed the brain. The agarose should be filled to the top of the mold to prevent unevenness and bubbles.

⚠ CRITICAL: Special care should be taken to ensure the cortical surface is flat against the glass slide. It is possible the brain may move out of position if the agarose is not poured carefully.

⚠ CRITICAL: When embedding the brain, ensure that the agarose has cooled to approximately 40°C to avoid causing tissue damage.

12. Before the agarose hardens, place another glass slide on top of the metal ring so both ends of agarose block will have a level and flat surface. Place a weight (100 g or similar) on top of this slide to make sure it stays in contact with the metal ring and the agarose. (Figure 2A).

⚠ CRITICAL: The top and bottom of agarose block must be as flat and parallel as possible so the sections can be cut evenly on the vibratome.

13. Wait about ~10–15 min for the agarose to cool to 20°C–25°C (or sufficiently hardened) and remove the agarose cylinder with the embedded brain from the mold. (Figures 2B and 2C).
14. Trim off one round edge of the resulting agarose cylinder with a razor blade on the side where the cutting would begin, so that the vibratome blade approaches parallel to the trimmed edge. This will make it easier to see that the vibratome blade is aligned with the top surface of the block in later steps. (Figure 2D).
15. Position the agarose cylinder so the flat cortex side is facing up and the trimmed side is facing the blade. Affix the agarose to the vibratome stage with a thin layer of super glue. Fill the chamber with PBS just below the surface of the agarose cylinder. This makes it possible to see the blade and top of the agarose cylinder clearly for alignment.
16. Set the blade advancing speed at 0.21 mm/s and the blade vibration frequency at 90 Hz. Set cutting depth to a small increment such as 50 μm and, with the blade above the agarose block, slowly lower the blade step by step. When the blade is barely cutting agarose across the full width of the cylinder, STOP the blade BEFORE it reaches the brain tissue. This will be the zero position where sectioning will begin.

⚠ CRITICAL: If the blade makes contact along only part of the width of the cylinder, reverse the blade, lower, and repeat until the full width of the cylinder contacts the blade. If the blade is not cutting across the full width of the agarose at the zero position, it means the zero position may be too high and the first brain section may be thinner than desired.

⚠ CRITICAL: The purpose of this step is to carefully align the blade to the top surface of the agarose block (hence the cortical surface). The agarose block should only be barely touched, and the brain tissue should NOT be touched by the blade in this step.

17. From the zero position, with the blade fully retracted, lower the blade to the desired depth, e.g., 350 μm. Fill the chamber with more PBS so the agarose is completely submerged and cut the first brain section. We find that a relatively thick slice of tissue (~350–400 μm) will ensure a complete first section that encompasses the entire surface under the cranial window (Figure 2E).

Optional: Check the thickness of the section by placing it on a glass slide and imaging it on a microscope with z measurement. The thickness can be measured as the depth difference in the focal planes between the surface of the section and the surface of the glass slide.

Histology: Staining and clearing

Timing: 1 week

In this step, the endogenous fluorescence will be enhanced using standard immunohistochemistry. During the staining procedure, lectin conjugated dye will also be used to label blood vessels in a different wavelength. After staining, the tissue will be cleared before mounting to facilitate *ex vivo* imaging.

Antigen retrieval (optional)

18. Place tangential sections into 8% SDS in PBS (materials and equipment) at 37°C on a shaker for two days, then rinse in PBS 5 times for 5 min each. This step will increase antibody binding by exposing more epitopes, but will cause some tissue degradation. In our preparation, it had only a small effect on the results. Skip this step if the tissue to be stained is delicate.

Immunohistochemistry and blood vessel labeling

19. Rinse: If the optional antigen retrieval step was not performed, rinse sections on a shaker in PBS 5 times for 5 min each.
20. Blocking: Place tissue in blocking buffer NDSTU (materials and equipment) on a shaker at 20°C–25°C for 1 h.
21. Primary antibody incubation: Incubate tissue for 2 days on a shaker at 20°C–25°C in primary antibody working solution (materials and equipment).
22. Rinse: On a shaker, rinse the tissue at least 5 times for 5 min each in PBST (materials and equipment).
23. Secondary antibody incubation: Incubate tissue for 2 days on a shaker at 20°C–25°C in secondary antibody working solution (materials and equipment).

In this step, the secondary antibody will greatly enhance the endogenous fluorescence of GCaMP in the 488 nm channel and the Lectin 649 will label blood vessels in the 649 nm channel.

24. Rinse: On a shaker, rinse 5 times for 5 min in PBST (materials and equipment), then 5 times for 5 min in PBS.

Note: We found that adding 4 M urea to our staining buffer (NDSTU, materials and equipment) helped with penetration of antibody labeling of the thick (>350 μm) sections.

Note: We found the labeling quality of lectin to be temperature sensitive, with 20°C–25°C providing satisfactory staining.

Clearing

25. Place the tissue into CUBIC-1 (materials and equipment) solution for ~30 min at 20°C–25°C. Use this solution for the following mounting step. Do not rinse the tissue in between clearing and mounting.

Note: We found the CUBIC clearing crucial for the image quality of confocal imaging. Specifically, it greatly reduces the scattering in deep tissue (>100 μm below the section surface). Without CUBIC clearing, the image quality at the bottom half of the section will not be good enough for extracting spatial information even with increased laser power.

Mounting

26. Create a chamber for the section by stacking spacers (SunJin Labs, iSpacer, thickness: 150 μm #IS101 or 200 μm #IS001) on a glass slide (Electron Microscopy Sciences, 71867-01) up to an appropriate height, e.g., 350 μm or 400 μm.
27. Mount the section and fill the chamber with 100 μL CUBIC-1 solution (materials and equipment) solution.
28. Apply a cover glass (VWR, 16004-332) on top of the chamber (start from one side and slowly lower it toward the other side, avoid creating any bubbles).
29. Seal the edges with clear nail polish (Electron Microscopy Sciences, 72180). The cleared and mounted section and its uncleared comparison are shown in Figure 2G.

Note: The pia surface should be facing up. We recommended mounting the tissue with consistent orientation (e.g., lateral side facing left and medial side facing right). This will aid the procedures listed in "Alignment and co-registration".

Note: CUBIC-1 will crystallize overtime. We recommend performing *ex vivo* imaging within a week.

Ex vivo confocal imaging

Timing: Hours (depending on the size of image volume)

We used an Olympus FV3000 laser scanning confocal microscope, but the section can be imaged by any generic laser scanning confocal microscope with appropriate filter sets and resolution.

30. Take a low-resolution, single z-plane overview of the sample using a 4× objective.

⚠ CRITICAL: surface blood vessels should be visible in the red channel and can be matched to *in vivo* widefield surface images.

a. XY resolution: 800 × 800 pixels.
b. Objective: Lens UPLSAPO 4×, air objective.
c. Pinhole: maximum.
d. Sampling speed: 2.0 μs/pixel.
e. Optical filter cube:
GCaMP: excitation 488 nm, emission: 500–540 nm.
Lectin labeled blood vessels: excitation 640 nm, emission: 650–750 nm.

31. Compare the vasculature patterns in the 4× overview to the *in vivo* widefield and two-photon surface vasculature patterns (Figures 3A and 3B), and locate the regions of interest for high-resolution volume imaging.
    a. resolution: specific to the structure of interest (in our study, we used XY resolution: 0.414 μm/pixel, depth step: 0.5 μm for dLGN axons).
    b. Objective: UPLSAPO 30XS, oil immersion, 30.0×, NA 1.05 (This is for studying axonal morphology, user should select the appropriate objective depending on the structure of interest).
    c. Pinhole: 1.00.
    d. Sampling speed 2.0 μs/pixel.
    e. Optical filter cube:
    GCaMP: excitation 488 nm, emission: 500–540 nm.
    Lectin labeled blood vessels: excitation 640 nm, emission: 650–750 nm.
32. Post process (align and stitch) the tiled confocal depth stacks using TeraStitcher (https://abria.github.io/TeraStitcher/ Bria and Iannello, 2012), or other software compatible with the data format.

Alignment and co-registration

Timing: 1–2 h

33. Transform all surface images and image volumes into standard orientation (e.g., top-down view, top: anterior, bottom: posterior, left: lateral, right: medial).
34. Compare the *in vivo* widefield surface image and the *ex vivo* confocal surface overview and locate the matching major blood vessels (Figures 3A–3D).

Note: only a subset of blood vessels will be labeled in the confocal image (Figures 3A and 3B), usually due to damage to the dura/pia during perfusion and tissue handling. However, we found in almost all cases, the labeled surface blood vessels were sufficient for locating the region of interest at coarse scale.

35. Compare the *in vivo* widefield surface image and the *in vivo* two-photon surface view and locate the two-photon field of view in the widefield surface image (Figure 3E).

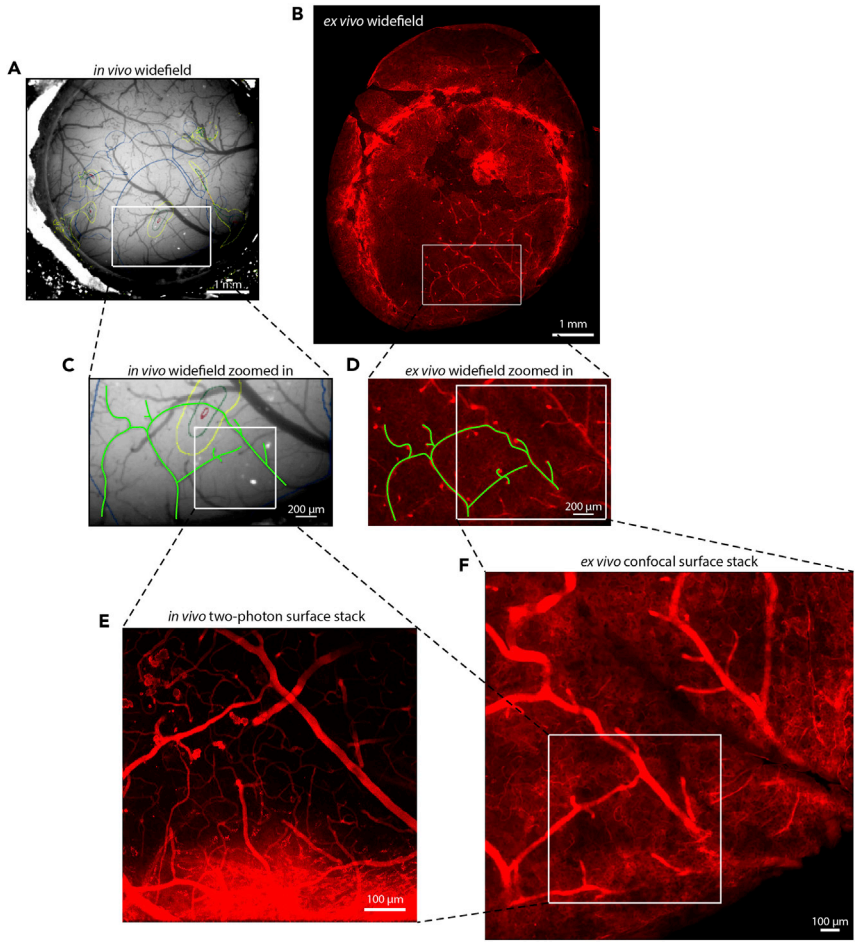

Figure 3. Alignment between *in vivo* and *ex vivo* images to locate the field of view in both image volumes

(A) *in vivo* widefield image of a 5 mm cranial window over a mouse's posterior cortical surface. Colored contour marks the visual area and retinotopic eccentricity.

(B) *ex vivo* widefield image of the surface of the tangential section from the same mouse. Vasculature patterns were revealed by lectin labeling of blood vessels.

(C) Zoomed-in view of the region marked in (A).

(D) Zoomed-in view of the region marked in (B). Green outlines in (C and D), matched surface blood vessels between *in vivo* and *ex vivo* surface images.

(E) Surface view of a two-photon volume that can be located in (C) (white box).

(F) Surface view of a confocal volume that can be located in (D) (white box). Importantly, the region of two-photon field of view can be located in (F) (white box).

36. Through the matching widefield surface image and confocal surface image (done in step 38), locate the *in vivo* two-photon field of view in the *ex vivo* confocal surface image (Figure 3F).
37. With matching surfaces of *in vivo* two-photon volume and *ex vivo* confocal volume side by side, slowly move down through the cortical depth in both volumes (color composite with green indicating GCaMP and red indicating blood vessels), keeping track of matching descending blood vessels until the target depth is reached (Figure 4B).
38. At the matching location between *in vivo* and *ex vivo* volumes, search for corresponding neuronal structures labeled by GCaMP in the green channel (Figure 4B). We found that if matching blood vessels can be identified and followed from surface to the targeted region, given a certain level of labeling sparsity, the corresponding brain structures can be easily identified by their unique shape or distribution pattern with naked eye (Figure 4).

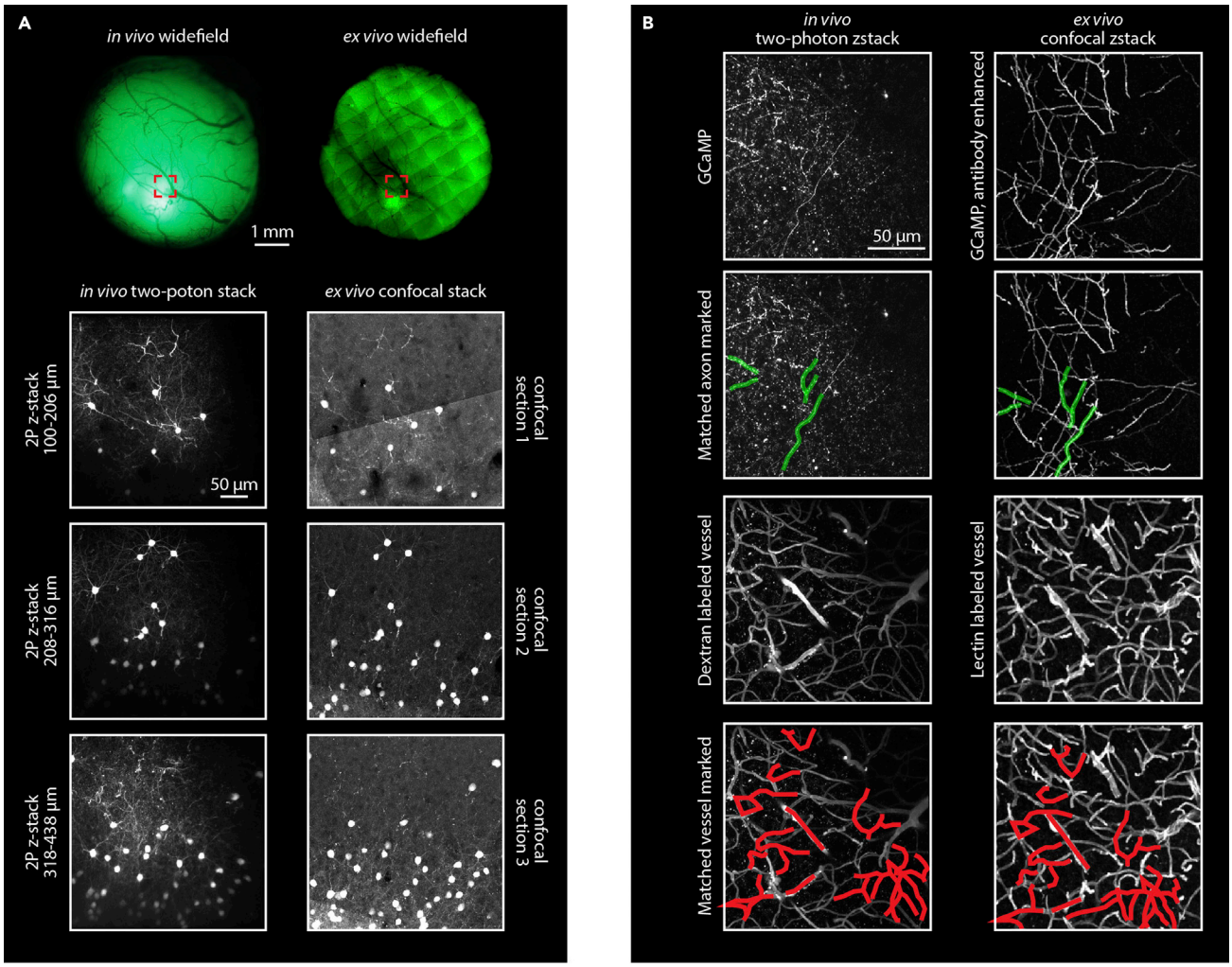

Figure 4. Co-registration of GFP/GCaMP labeled structures between *in vivo* and *ex vivo* imaging volumes

(A) Co-registration of cell bodies between *in vivo* and *ex vivo* volumes. V1 cortical cells were labeled by injecting SiR-Cre (Ciabatti et al., 2017) in Snap25-LSL-F2A-GFP mice. Labeled cortical cells in an *in vivo* cortical stack (left column) can be matched to the confocal stacks imaged from three continuous tangential sections (right column).

(B) Co-registration of GCaMP labeled dLGN axons between *in vivo* and *ex vivo* volumes. All panels show the same field of view. The images in the second row and the fourth row are the same as images in the first row and the third row, respectively, but with the relevant structures marked by colored outlines. For *in vivo* two-photon images (left column), the dLGN axons were sparsely labeled with GCaMP and blood vessels were labeled by dextran Texas-Red. For *ex vivo* confocal images (right column), the GCaMP signal was enhanced by antibody labeling, and blood vessels were labeled by lectin. Labeled blood vessels were used as fiducial landmarks for cross-modality registration (bottom row, red lines). After registration, the same GCaMP positive axons were identified in both the *in vivo* two-photon images and ex vivo confocal images (second row, green lines). (B) is modified from Zhuang et al. (2021) with permission.

Note: In some cases, the steps in stacks (especially in confocal volume) are too thin to provide enough spatial information, we found it is helpful to generate and compare stepped z projections (e.g., maximum or average z projections of small volumes every 50 µm in depth) which can be achieved in FIJI software (Image → Stacks → Tools → Grouped Z Project).

39. Once the corresponding entity (e.g., soma, spine, or bouton) is found in both volumes, the functional characteristics of the entity can be extracted from *in vivo* calcium activity recorded by two-photon imaging and the morphology can be reconstructed from the confocal volume (reconstruction procedure not covered in this protocol, but see Zhuang et al., 2021).

EXPECTED OUTCOMES

GCaMP labeled structures and blood vessels should be clearly visible in green and red channels, respectively, in both *in vivo* two-photon volume and *ex vivo* confocal volume.

The corresponding major blood vessels should be easily identified between the two imaging volumes (Figure 3).

Within the field of view of interest, the corresponding descending vessels can be identified and followed through the cortical depth and the field of view of *in vivo* imaging sessions can be located in the *ex vivo* confocal imaging volume.

Within the located field of view, GCaMP labeled structures (e.g., cell soma or subcellular structures) in *in vivo* images can be identified in the *ex vivo* confocal imaging volume (Figure 4).

LIMITATIONS

Antibody penetration and the working distance of the confocal microscope both limit the section thickness. In our setup, the maximum tissue thickness is 400 μm, which covers almost the entire cortical depth due to the compression from the cranial window and tissue shrinkage in PFA. It is possible that the tissue compression can be less if a smaller cranial window is used. In this case, 400 μm will likely not cover the entire cortical depth. See more discussion in Problem #5 in troubleshooting.

This technique is limited to cortical structures only, as traditional *in vivo* two-photon imaging cannot reach deep structures.

The flat tangential section only covers the region under cranial window. For co-registration and alignment on whole cortical tangential sections at mesoscale, please follow Zhuang et al. (2018).

TROUBLESHOOTING

Problem 1

The first tangential section of the brain is uneven, resulting in lost area of interest (steps: 10–17).

Potential solution

During embedding, the flat windowed surface of the brain must be pressed as flush as possible against the bottom of the mold. If there is an uneven layer of agarose above the flattened area, you will get an uneven section, and the area of interest may be lost.

Occasionally there may be areas of the brain that bulge around the perimeter of the windowed region. This will also result in an uneven section. To resolve this issue, carefully use a surgical blade to shave off any areas causing unevenness around the perimeters of the windowed region.

After the agarose block has solidified, inspect the surface of the agarose block to ensure that the flat cortex is flush with the surface. If the flattened area of cortex is not flush with the flat surface of the cylinder, remove the brain from the agarose and re-embed.

Problem 2

Lectin stain is dim (steps: 19–24).

Potential solution

We found the labeling quality of Lectin to be temperature sensitive, with 20°C–25°C providing the most effective staining.

Problem 3

Brain cannot sit stably with flattened surface facing down when mounted for tangential sectioning (step: 10).

Potential solution

This is due to that the flattened surface does not cover the center of mass when placing it against and on top of a glass slide. In our case (5 mm flattened surface over visual cortex), this never happened. However, this problem may occur when the cranial window is smaller and is at a more off-centered location. We recommend using some mechanical aid to keep the brain in place (e.g., using a smaller ring as a constraining mold in Figure 2B) or trim the brain into a smaller tissue block so that the center of mass is covered by the flattened surface. However, we have not tested these solutions.

Problem 4

Cannot find corresponding vasculature patterns between *in vivo* and *ex vivo* imaging volumes (steps: 33–39).

Potential solution

First, make sure the *in vivo* and *ex vivo* images are from the same animal.

Second, make sure to transform all the images (widefield vasculature, two-photon stack, confocal overview, confocal stack, etc.) to the same orientation (no flip or rotational differences).

Third, locate regions of interest at lowest zoom first (i.e., between widefield vasculature, biggest zoom two-photon surface stack and confocal overview), then zoom in.

Forth, start from the surface and follow the descending blood vessels in both *in vivo* and *ex vivo* stacks. If the single z-plane is too thin and cannot provide enough spatial information (due to a tight point spread function), we found it is helpful to generate and compare stepped z projections (max or average z projections of small volumes every 50 μm in depth) which can be achieved in FIJI software (Image → Stacks → Tools → Grouped Z Project).

Problem 5

The section does not cover entire cortical depth (steps: 10–17).

Potential solution

In our case, a tangential section with a thickness of ~400 μm can cover almost the entire cortical depth (Zhuang et al., 2021), likely due to the tissue shrinkage in PFA and the compression from cranial window. If a smaller cranial window is used or a cranial window is placed at a cortical location with less curvature, the compression from the cranial window will be less, and a 400 μm section will likely not cover the entire cortical depth. In this case, a thicker section can be cut. In this protocol, the section thickness is not limited by the sectioning procedure but by the antibody penetration and the working distance of the confocal objective. In our setup, we used an oil immersion 30× objective with a working distance of less than 450 μm (limited by the detachment of immersion medium). With a thicker section, we recommend increasing immunostaining duration and using an objective with a longer working distance (ideally without immersion medium). However, we have not tested sections thicker than 450 μm.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jun Zhuang (junz@alleninstitute.org).

Materials availability

The DNA plasmids generated in this study (AAV pCAG-mRuby3-WPRE) can be acquired from Addgene (Catalog#: 107744).

Data and code availability

The data that support the findings of this study are available from the lead contact, Jun Zhuang (junz@alleninstitute.org), upon reasonable request. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.Z.; Investigation, J.Z. and N.D.O.; Formal Analysis, J.Z. and N.D.O.; Methodology, J.Z., N.D.O., and E.T.; Writing – Original Draft, J.Z., N.D.O., and E.T.; Writing – Review & Editing, J.Z., N.D.O., and E.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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