Two-Photon Microscopy Functional Assays for Serial Imaging of Brain Microvessels and the Neurovascular Unit Through Disease Progression

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Microvascular impairments are a major issue in many diseases of the brain. In fact, they are often considered the earliest pathological phenomena in neurodegenerative diseases like Alzheimer's Disease or the locus of pathology as in stroke. Still, little is known about the mechanistic and cellular level events that contribute to these impairments. Given the importance of the neurovascular unit (NVU) in maintaining functional brain tissue, alterations to NVU cell types are important to study in the context of disease progression. With the use of two-photon microscopy, microvessels and cells can be imaged and evaluated throughout disease progression. Herein we aim to provide a comprehensive protocol for setting up and using two-photon microscopy for serial imaging of neurovascular unit cell types (i.e., pericytes, astrocytes, and microglia). We also describe interpreting results from cell and vessel changes based on disease or functional tests through multiple timepoints. © 2025 The Author(s). Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Mouse set up for serial, live 2-photon imaging
Basic Protocol 2: Field of view acquisitions over multiple timepoints
Basic Protocol 3: Line scan acquisitions for acquiring red blood cell metrics
Basic Protocol 4: Quantitative measurements of vessel and cell changes
Basic Protocol 5: Documenting changes in astrocyte morphology following induction of transient focal photothrombotic stroke using Rose Bengal

Keywords: blood–brain barrier • microvascular • neurovascular unit • serial brain imaging • two-photon microscopy

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INTRODUCTION

The brain microvasculature forms the blood–brain barrier (BBB), a physical boundary that isolates the neural tissue from the systemic blood compartment and protects the brain from harmful substances in the bloodstream (e.g., pathogens and xenobiotics). Endothelial cells, pericytes, microglia, astrocytes, and neurons collocate as a functional unit referred to as the neurovascular unit (NVU), which maintains the structural integrity of the BBB, as well as regulates cerebral blood flow (CBF) (El-Ghazawi et al., 2024). In diseases like Alzheimer's Disease, increased vascular permeability and lowered CBF are thought to be the earliest pathological phenomena, preceding clinical disease onset by years to decades (Hays et al., 2016). There are conflicting data regarding the cellular mechanisms underlying these vascular impairments; hence, it is imperative to observe these cells over the course of disease progression or through functional assays so that therapeutic targets can be properly identified.

Two-photon microscopy allows for live imaging of animal models with lineage traced cells of the NVU. Current methods do not provide a guide for performing this imaging in the same brain area over an extended period. They also do not provide the various ways of evaluating these different cells of the NVU and their relation to vessel impairment. Two-photon microscopy leverages the principle of two-photon excitation, where a fluorescent molecule at the focal plane of the objective absorbs two photons of infrared light simultaneously (within a femtosecond), instead of one single photon. The infrared light penetrates tissue more deeply than the standard excitation light used in fluorescence microscopy. Due to its low energy, infrared light is additionally less damaging, and therefore especially useful for in vivo imaging (see Two-Photon & Multiphoton Microscopy, Principle & Solutions, at *https://ibidi.com/content/217-two-photon-microscopy)*. This microscopy method has many applications: it has been used for determining mural cell organization on vascular structures of mouse brains (Grant et al., 2019), understanding astrocyte plasticity via cell ablation or following focal photothrombotic stroke (Mills et al., 2022), determining the role of microglia in seizure (Gibbs-Shelton et al., 2023), as well as several other applications.

This imaging technique generates insights into live cell behaviors that are not possible with ex vivo tissue staining methods. It also provides an authentic context for the cell behaviors to occur in a fully living system, which is not possible with in vitro culture systems. One disadvantage of this technique is that the depth into the brain at which images can be acquired is typically limited to $<$500 μm (Oheim et al., 2001) without making additional modifications to commercially available systems (Stamenkovic et al., 2024).

Basic Protocol 1 describes the mouse preparation that is done prior to image acquisition. We detail the cranial window surgery, how to label the vasculature via retro-orbital injections, and how to set the mouse up on the microscope stage for imaging. Basic Protocol 2 details the z-stack acquisition of the same field of view over multiple timepoints. This acquisition allows for diameter and cell behavior analyses over time. In Basic Protocol 3, the acquisition of line scans from the same vessels over multiple timepoints is described. We describe this to obtain blood flow information by way of measuring red blood cell (RBC) metrics. Basic Protocol 4 describes how to analyze the acquired images from Basic Protocols 2 and 3. This describes quantifying diameter changes, RBC velocity and flux, changes in cellular behavior, and changes in vessel volume across time. Finally, in Basic Protocol 5 we describe how to induce a transient focal photothrombotic stroke and how to observe the cellular response to this induced stroke. We expect that the information presented here will allow for researchers to perform reproducible longitudinal 2-photon imaging experiments in vivo for neurovascular studies in a multitude of disease contexts.

STRATEGIC PLANNING

Facilities

It is imperative before beginning experimentation to identify the Biohazard Safety Level (BSL) status of your planned intervention or observational study. As an example, longitudinal imaging with a transgenic mouse reporter line might require different BSL status facilities than procedures involving viral injections or human-derived cell lines. The Institutional Animal Care and Use Committee (IACUC), at a minimum, will require you to define terminal timepoints for all experiments as well as benchmarks for determining if/when an animal needs to be sacrificed before the end of an experiment. This typically involves weight loss from some percentage of baseline, abnormal behavior (e.g., persistent hovering in a corner for a prolonged period away from cage mates despite attempts to obtain attention), some injury to peripheral organs (i.e., eyes, skin, ears, etc.), obvious internal organ malformations, and loss of head cap following surgery. Furthermore, IACUC may require you to transfer the mice to a separate facility after performing surgery. After obtaining required approval from governing institutional ethical bodies, such as IACUC and IBC, confirm that rooms possess the equipment needed to support your experiments. See the Materials section in Basic Protocol 1 or Shih et al. (2012) for a list of the necessary equipment to support mouse surgery. If using anesthesia for imaging, ensure that your imaging room can store gas canisters safely and has up-to-date vaporizers. Further ensure that light leakage in the room is minimized as much as possible and that all laser safety requirements are adhered to and signage displayed. Last, it is recommended to have your laser plugged into a backup generator in case your building loses power. Lasers typically require a programmed shut down procedure and sudden loss of power can/will result in serious damage to your equipment.

Animal welfare and biohazards

All animal procedures were approved by the University of Virginia Institutional Animal Care and Use Committee (IACUC Protocol #4237) and conformed to NIH guidelines for the care and use of laboratory animals. Mice were housed in standard cages with a 12:12 light-dark cycle and provided ad libitum access to food and water. To ensure animal welfare during longitudinal imaging, animals were monitored daily for signs of distress, including reduced grooming, abnormal posture, and altered activity. Analgesics were provided pre- and post-operatively as needed. During surgery and imaging, body temperature was maintained with a heating pad and eyes were protected from drying by administering ophthalmic lubricant. All biohazardous materials were handled in accordance with institutional biosafety guidelines, and appropriate personal protective equipment (PPE) and waste disposal procedures were followed throughout.

Mouse preparation/cranial window surgery

It is important to determine if the study design involves the use of a mouse model that utilizes inducible Cre and if any disease/treatment inductions will be needed. If this is the case, we recommend starting with the induction by administering soft tamoxifen feed or intraperitoneal injections of tamoxifen, as specified by the vendor. We typically use a soft tamoxifen feed for 2 weeks (e.g., Teklad) followed by a change to regular chow for a 2-week clearance period. Cranial window surgery can follow at this stage with at least 2 weeks of recovery for the inflammatory response from the surgery to resolve. Any disease/treatment inductions should be planned around the imaging timeline. A general outline is provided in Figure 1.

Acquisition hardware

Prior to beginning imaging experiments, one needs to know the excitation and emission detection capabilities of their system. This includes knowing what barrier filters,

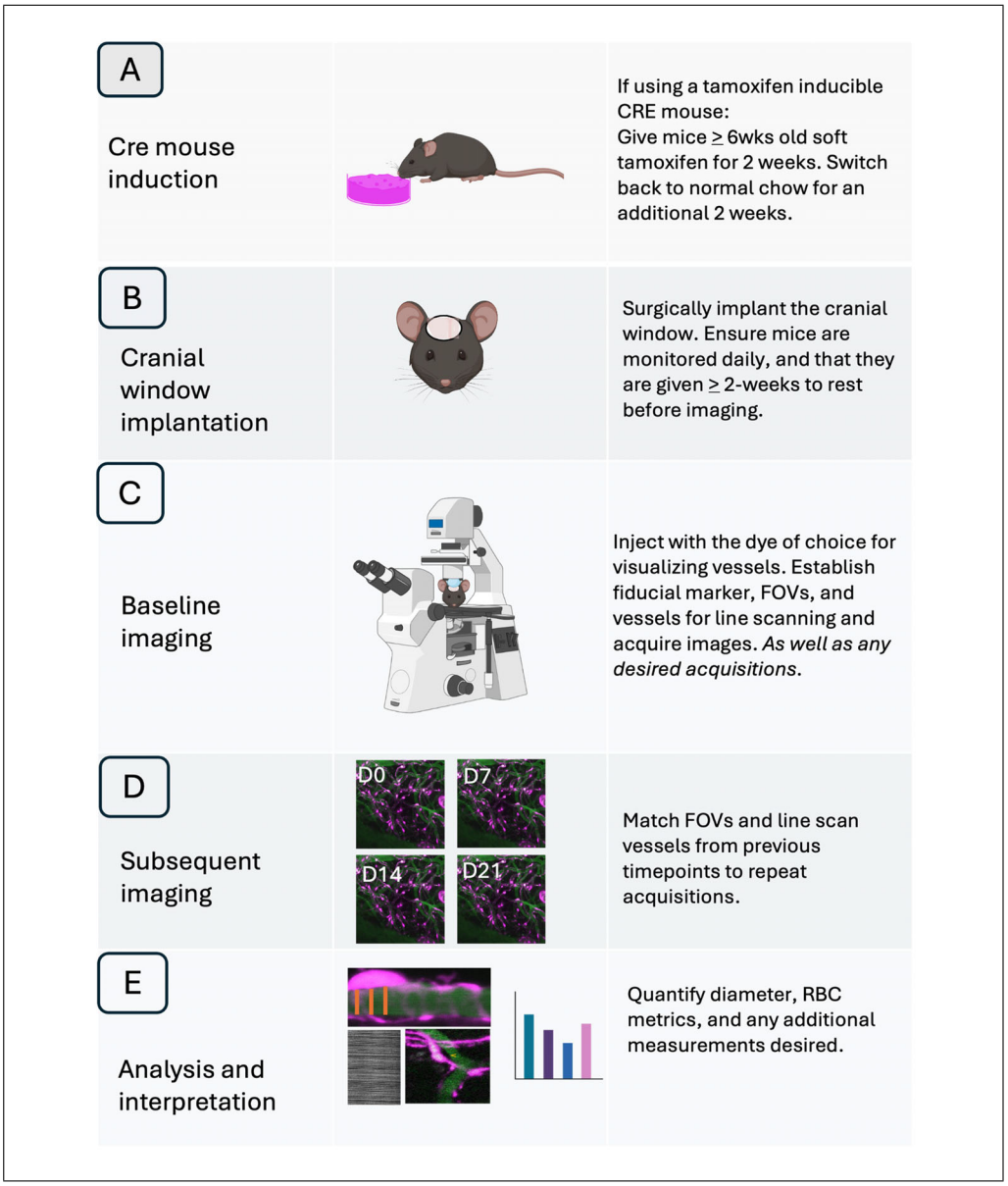

Figure 1 Overview of experimental procedure. Steps (**A**-**B**) in total require at least 6 weeks. Subsequent steps are variable in the amount of time required.

detectors, and dichroic mirrors are installed in their system. This information can be obtained from a company representative. With this knowledge, one can then determine what fluorophores are best suited for their hardware. Websites such as *https://searchlight.idex-hs.com/* are very helpful. Understanding the wavelength range of your system is further necessary. If you need to image deeper in the brain, for example, then longer wavelengths will be best for your experiment. Finally, one needs to understand the quality of their objective. As mentioned in Basic Protocol 1, the immersion medium (or lack thereof) will determine the thickness of cover glass used in cranial window implantation so that one does not run into refractive index mismatch.

Physiological parameters to control for effects of anesthesia

This depends on the experimental intervention. We have experience mostly with whole cell ablations and tamoxifen injection paradigms under isoflurane anesthesia. The best method to control for anesthesia is to minimally place the mouse under isoflurane such that their hind limbs move gently. Gradually and slowly increase this until the animal

stops moving but breath rate remains high. Count breath rate at this point. We find that for tamoxifen injections or observational studies using longitudinal imaging, matching breath rate is obtainable. However, matching breath rate following whole cell microglial ablation using PLX3397 in older wild-type and 5×FAD mice is not feasible. Hence, we bring our mice just to the point where they stop moving, count breath rate, and note our isoflurane vaporizer setting. We then follow this same procedure at subsequent timepoints, aiming to match breath rate and vaporizer settings as close as possible.

Note-taking considerations for optimal imaging across multiple timepoints

If a user does not like note-taking within their imaging software graphical user interface (GUI), we highly recommend the use of a tablet (e.g., iPad) along with an electronic notebook or a note-taking application (e.g., Notability). This will allow for pictures to be captured with the tablet or a phone during imaging. Having these pictures with your notes will allow for easier mapping between fiducial markers and fields of view, and easier identification of individual vessels to acquire line scans from. Using a split-view function on the tablet will allow for a side by side of your previous timepoint with your current, allowing for faster and more confident identification of the same regions and vessels. Throughout the protocol, we identify where notes should be taken, and at the end we show how these notes could be applied to a baseline image in the Olympus FV31S-SW software (this is optional and not necessary if you do not have access to the software).

MOUSE SET UP FOR SERIAL LIVE 2-PHOTON IMAGING

BASIC PROTOCOL 1

In this protocol, we demonstrate how to prepare a mouse for imaging on a 2-photon microscope. We describe the surgical implantation of a cranial window, the retro-orbital dye injection for visualizing blood flow within vessels, and the setting up of the mouse on the microscope for a successful imaging session. If conducted properly, this protocol allows users to have cranial window–implanted mice set up on their 2-photon microscope ready for imaging of their cortical vessels and associated NVU cells.

Materials

Genetically engineered mouse (Jackson Laboratory, Cyagen, etc.)
70% ethanol
Ice-cold 1× phosphate-buffered saline (PBS) (Current Protocols, 2006) or artificial cerebrospinal fluid (aCSF) (Tocris, cat. no. 3525)
Systemic and local analgesic, IACUC-approved, e.g., buprenorphine SR (Fidelis Animal Health, Ethiqa XR RL-LA000799) and bupivacaine (Hospira, NDC 0409-1159-18, PAA150062)
Eye lubricant (e.g., Optixcare eye lubricating gel, Amazon, cat. no. OPX-4242)
Nair hair remover lotion (Amazon, cat. no. LL1322)
Betadine (Medline, REF: MDS093945)
Veterinary tissue adhesive glue (Oasis, cat. no. TA5)
Canned air
Vessel dye (e.g., 70 kDa MW FITC-dextran, Thermo Fisher, cat. no. D1822; Rose Bengal, Millipore Sigma, cat. no. 330000; Alexa 633 hydrazide, Thermo Fisher, cat. no. A30634)
C&B Metabond adhesive luting cement system (Parkell, cat. no. S380)
Isoflurane-oxygen tank setup
Proparacaine hydrochloride ophthalmic solution, USP 0.5% (e.g., Bausch & Lomb, Bernell, cat. no. AK2D15DS)
Isopropyl alcohol

No. 1 cover glass, 3-mm diameter, CS-3R (Warner Instruments, cat. no. 64-0720)
Surgifoam (Ethicon, cat. no. 1972)

Heat pad, ideally with feedback regulation
Isofluorane vaporizer
Dissecting scope
Small cotton-tipped applicators (Fisherbrand, cat. no. 22363167)
27G to 30G needle and syringe (e.g., EasyTouch syringes at 30-50 cc)
Fine scissors, ToughCut (Fine Science Tools, cat. no. 14058-11)
Iris forceps (Fine Science Tools, cat. no. 11066-07)
Dumont #5AC forceps (Fine Science Tools, cat. no. 11258-20)
Scalpel handle #4 (Fine Science Tools, cat. no. 10004-13)
#10 scalpel blades (World Precision Instruments, cat. no. 500239)
Electric powered hand drill (air-powered drill with foot pedal control is also suitable)
Carbide inverted cone burr, size 33-1/2 (Roboz Surgical Instrument, cat. no. RS-6282C-33-1/2)
Transfer pipets (Fisherbrand, cat. no. 13-711-7M)
Dumont #55 fine forceps (Fine Science Tools, cat. no. 11295-51)
Bonn micro probe (Fine Science Tools, cat. no. 10031-13)
Stereotax with stereotactic arm (Kopf) and toothpicks
BVI Weck-Cel eye surgical spear (Beaver-Visitec, cat. no. 0008680)
Straight halsted-mosquito hemostat (Fine Science Tools, cat. no. 13008-12)
Plate for head fixation or a Kopf 3D stereotax (we purchase from a local machine shop; contact us for details)
2-photon microscope and acquisition software (e.g., LEICA DM6000 CFS microscope with LEICA LASX software, or Olympus dual beam FVMPE-RS microscope with FV31S-SW software)
Timer

Cranial window implantation

1. Weigh the mouse prior to surgery. Record all IACUC mandated information including record of surgery and analgesics administered in surgery log, surgery record cage card, drug log, and charcoal canister weight (if applicable). Only perform surgical procedures approved by your IACUC.
2. Pre-soak glass coverslips in 70% ethanol and surgifoam in ice-cold 1× PBS or aCSF. Place recovery cage on separate heating pad, with half the cage on and the other half off.

   Note that the thickness of your glass coverslips should match the refractive index of the immersion medium used for image acquisition.

3. Induce a surgical plane of anesthesia. This is confirmed by a lack of pain response to toe pinch. We initially utilize the max setting of our isoflurane vaporizer but then quickly bring down to the minimal requirement for no response to toe pinch.
4. Once the animal is secured in the stereotactic frame and centered in the field of view through the dissecting scope, immediately apply eye lube using small cotton-tipped applicators.
5. Using a 27G to 30G needle and syringe, inject local analgesic (Bupivicaine for our surgeries) at the site of surgery prior to incision.
6. Apply Nair to the scalp.
   a. Allow enough time for the Nair to remove hair from the scalp but do not wait too long as it can be a skin irritant.
   b. One should be able to remove the hair with back-and-forth motions as opposed to downward forceful motions.

c. Alternatively, one could shave the scalp with an electrical razor, but we get the cleanest site of surgery using Nair.

7. Remove all Nair and clean the incision site with three alternating rounds of betadine followed by 70% ethanol.

8. After ensuring no pain response to an aggressive toe pinch (using minimal anesthesia for this), use fine scissors and Iris forceps to remove scalp. Make an initial incision at the plane just behind the ears and cut to between the eyes. Expose the entire dorsal surface of the skull.

9. Pick up a pre-soaked glass coverslip using Dumont #5AC forceps.

10. Use scalpel handle #4 and #10 scalpel blades to remove the periosteum from the surface of the skull. Score the skull with the scalpel blade. Try to minimize downward pressure on the skull as you do both steps.

11. Use veterinary tissue adhesive superglue with applicators to glue down the skin to the edges of the skull.

12. Place glass coverslip along the location you want to implant your window. We typically place the 10/11 o'clock position ($\frac{5\pi}{6}$ *to* $\frac{2\pi}{3}$) at Bregma, or just beside such that the far-left portion of the window (9 o'clock position or π) is parallel to and directly beside the sagittal suture.

13. Begin to drill using an electric powered hand drill with a carbide inverted cone burr, size 33-1/2, along the dimensions visualized through the dissecting scope.

    a. Only pass once over a coordinate along the 360° arc to minimize heat generated beneath the skull.
    b. After this first round of drilling, apply ice-cold 1× PBS or aCSF with a transfer pipette to allow for cooling. The goal is to generate an accurate sketch of the skull region to remove.
    c. Adjust dimensions by placing window coverslip in drilled region.
    d. Repeat alternating drilling and cooling until ready to remove the skull flap.
    e. Dry the skull after each cooling by either using a cotton tip applicator or using canned air.

14. Once the skull flap is ready to remove as evidenced by the appearance of spongy bone, apply ice-cold 1× PBS or aCSF. It is imperative to keep the brain moist once the skull flap is removed. Use Dumont #55 fine tip forceps to remove the skull flap. It should require no effort to place the fine tips underneath the skull flap. Keep the forceps parallel to the brain surface to minimize the chance of injury to brain tissue.

15. If not performing a durotomy, skip to step 17.

    a. To remove the dura, first once again bath apply ice-cold 1× PBS or aCSF to the brain surface.
    b. Use the Bonn micro probe to create an initial tear in the dura.
    c. Keep the point of the hook facing upward to the ceiling, choose a location away from a surface vessel, and tuck the probe underneath your index fingernail.
    d. Gently move the probe until a portion of dural skin catches.
    e. You can then decide to continue to use the probe for complete dural removal or move back to the Dumont #55 fine tip forceps.
    f. Ensure that the brain remains moist.

    Note that sometime the dura will fuse to large pial vessels. We do not remove dura here as it will lead to bleeding.

16. Reapply ice-cold 1× PBS or aCSF to the surface of the brain. Use pre-soaked surgifoam to stop any bleeding. Apply dyes to brain surface if experiment calls for it.
17. Place glass coverslip on the exposed brain. Place stereotactic arm with attached toothpick in place. Use the arm to gently push down the window over the exposed portion of brain. This step should look a lot like mounting a slide at the end of immunohistochemistry, with the ice-cold 1× PBS or aCSF flowing underneath the glass coverslip.
18. Once coverslip is in place, use the Weck-Cel held by your hemostat to dry the skull. Glue down the edges of the window to the skull. We recommend initially placing glue away from the window and then using the fine tip applicator to push over towards the edge of the window.
19. Apply dental cement.

For our C&B metabond dental cement, we use one scoop of powder, 3 drops of base, sharpie ink, and finally one drop of catalyst. Mix and add once the cement starts to cure (as evidenced by beading on the edge of a toothpick or applicator provided with the kit). If using a head plate for imaging, this is the step to mount that in. We always place left of midline between the eyes, ensuring our objective will be able to image the entire window without obstruction from the headplate.

20. Inject buprenorphine SR (as approved in our IACUC protocol).
21. Remove mouse from stereotax and place in pre-warmed recovery cage on the side that is on the heating pad. Place moist food in the cage as well. We allow our mice to recover for 2 weeks before imaging.
22. Clean tools. Best practices for this include autoclaving before the next procedure (Shih et al., 2012).

Retro-orbital injection

23. Anesthetize the mouse using isoflurane mixed with oxygen (≤2% isoflurane).
24. Administer a drop of ophthalmic solution on the eye of the mouse and leave it for 2 to 3 min.
25. Blink the eye of the mouse.
26. Load the syringe with an appropriate volume of the desired dye.

    For td-Tomato reporter mice we use a 70 kDa FITC-Dextran at 12.5 mg/ml in sterile saline and we inject 80 µl retro-orbitally. Injection volume should not exceed 150 µl in adult mice (Yardeni et al., 2011).

27. Allow the dye to circulate for at least 5 min before moving to the next step.

Mouse set up on the microscope stage

28. Anesthetize the mouse and place the mouse on the head plate holder platform and tighten the screw so that the head is fixed. Alternatively, one can use a Kopf 3D stereotax and rotate until the plane of the window is parallel to the plane of the objective.
29. Place the mouthpiece over the mouse's nose and ensure anesthesia is being delivered through the mouthpiece.

    We typically keep the isoflurane level at 1% to 1.5% at this stage to minimize vasodilatory effects of isoflurane and the level chosen is kept consistent throughout the imaging time course.

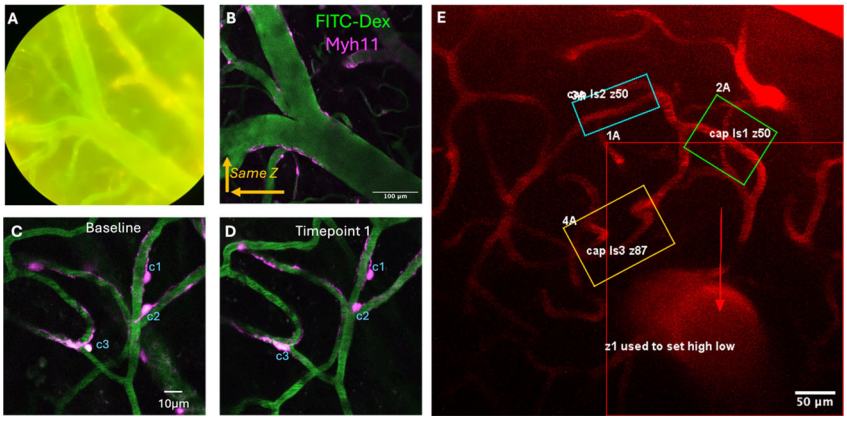

Figure 2 Establishing a marker to identify the same FOV over time. (**A**) A fiducial marker identified through the eyepiece of the microscope. (**B**) The same view using the 2-photon laser in the LEICA software. Orange arrows indicate the location of the FOV in relation to the marker, and orange writing indicates the z-plane of the FOV in relation to the marker. (**C**) The FOV at a single z-plane on the baseline timepoint, where blue writing indicates labels for cells. (**D**) The same FOV identified at a later timepoint with the same cells labeled. (**E**) A maximum intensity projection showing a field of view at baseline acquired using the Olympus 2-Photon system where notes and ROIs have been added to the image. These include the location of line scans (ls) as well as where in the image laser power was determined to optimize high–low settings. This same location was used at subsequent timepoint imaging.

30. Place a drop of eye lubricant on each eye.
31. Place the mouse on a heat pad.

    We typically set this to be ~35°C.
32. Clean the window of any potential debris with a cotton swab sprayed with isopropyl alcohol. Alternatively, use lens cleaning paper and fine-tip forceps.
33. Align the window with the fluorescent IL-Shutter.

    A blue light should appear from the objective.
34. Add a drop of water to immerse the lens and set the stage as low as possible without losing the water drop connecting the lens and window

    A Vaseline well can be formed around the window if the water drop is not connecting the objective and the window.
35. Adjust the Z-plane until vessels come into focus and move in the X–Y directions to identify a fiducial marker (Fig. 2A).

    This marker should match between weeks. Keep an image on your electronic notebook for reference or draw a rough sketch to identify.
36. Note the location of the fluorescent light within the window.

    We typically use a tablet device and draw a circle to represent the window and color in a spot of where the light is shining on the window.

    Use this spot to identify where to shine the fluorescent light each week so that it is the same spot.
37. Set a timer for 1 min and count the mouse's breath rate.

    This is to keep the breath rate consistent between weeks.

BASIC PROTOCOL 2

FIELD OF VIEW ACQUISITIONS OVER MULTIPLE TIMEPOINTS

In this protocol, we demonstrate how to image the same field of view (FOV) at multiple timepoints. We describe the suggested settings and the technique for aligning FOVs to obtain the same z-stack at different timepoints. If conducted properly, this protocol allows users to have multiple timepoints of the same FOV imaged in a mouse brain.

Materials

Cranial window−implanted mouse (see Basic Protocol 1)
Intraluminal dyes (e.g., dextran or rhodamine, see Basic Protocol 1)
2-photon microscope and acquisition software (e.g., LEICA DM6000 CFS microscope with LEICA LASX software, or Olympus dual beam FVMPE-RS microscope with FV31S-SW software)

1. Turn off all lights and cover the stage from any possible external light.
2. Set the format and speed to the desired values.
3. Set the desired excitation wavelength, laser intensity, and gain.

   The wavelength is dependent on the channel(s) your subject has. We typically set the electro-optic modulator intensity to 1, the laser percentage is initially set to 2%, and gain is initially set to 100.

4. Turn on the appropriate laser detector(s).

   This is dependent upon the configuration of the barrier filters in your system. Know what those are, their wavelength detection range, and how they are configured to detectors prior to image acquisition.

5. Take an image of the marker with all desired channels turned on (Fig. 2B).
6. Move to the desired FOV and note where it is in relation to the marker (Fig. 2B).

   If using an electronic notebook, ensure that images are taken along the marker and added to your notebook as a guide for moving to the FOV.

7. Align the vessels to match the FOV from previous weeks (Fig. 2C-D).

   If it is the first timepoint of imaging, then make note of how it is aligned. We use the ruler tool to align based on xy-coordinates of certain cells and vessels. Make sure to note the z-position of the FOV and the z-position of the shallowest viewable vessel in the window, as this can be used to approximate cortical depth.

   As an alternative to the notebook, you can apply annotations in your baseline image with detailed notes if your GUI allows (Fig. 2E).

8. Use the z-compensation setting to set the start of the z-stack (shallow) at the lowest laser percentage that vessels and cells can be seen at and to set the end of the z-stack (deep).

   The reason for this setting is to be able to set a higher laser power deeper in the FOV where needed. Be careful of not setting the laser power too high at any point so that it does not significantly damage the tissue. We have found that 8% is a good upper limit to set.

9. Set the number of steps desired and start the acquisition.

BASIC PROTOCOL 3

LINE SCAN ACQUISITIONS FOR RED BLOOD CELL METRICS

In this protocol, we demonstrate how to acquire line scans while imaging at a given timepoint. We demonstrate how to align and set up the vessel for a line-scan, as well as acquiring it. If conducted properly, the user will have red blood cell (RBC) data between timepoints that can be used for insights on blood flow.

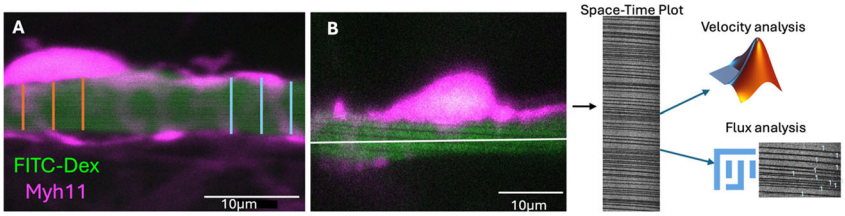

Figure 3 Diameter and RBC measurements for quantitative analysis. (**A**) The locations where 3 measurements of vessel diameter were taken at the cell soma (orange lines) and away from the cell soma (cyan lines). (**B**) The alignment for a line-scan acquisition (white line) and its subsequent conversion to a space–time plot for further analysis. Black streaks on the space–time plot indicate RBCs, and this plot can be used with MATLAB for velocity analysis and with FIJI for flux analysis.

Materials

Cranial window–implanted mouse (see Basic Protocol 1)
Intraluminal dyes (e.g., dextran or rhodamine)
2-photon microscope and acquisition software (e.g., LEICA DM6000 CFS microscope with LEICA LASX software, or Olympus dual beam FVMPE-RS microscope with FV31S-SW software)

1. Once the z-stack is acquired, go back into "Live Mode" and adjust the z back to the starting point of the z-stack.
2. Identify a vessel of interest as you go through the z-stack manually and note the depth it is located at.

   This is a good place to snap an image of the field to include in your electronic notebook. Circle the vessel of interest for ease of identification in subsequent timepoints.

3. Align the vessel horizontally using the rotation slider and zoom in until the length of the vessel fills the screen (e.g., orientation in Fig. 3A-B).

   Snap a picture here also and note the zoom factor and laser percentage used. This will make acquisition and comparison of the same vessel at subsequent timepoints easier.

4. Turn off "Live Mode" and switch the "Acquisition Mode" from xyz to xt.
5. Set the desired duration of your line scan and press start.

QUANTITATIVE MEASUREMENTS OF VESSEL AND CELL CHANGES

BASIC PROTOCOL 4

In this protocol, we describe how to analyze the acquired images. We identify methods for quantifying diameter changes, RBC flux and velocity, changes in cellular behavior, and changes in vessel volume across time. If conducted properly, users will have quantified measurements from their analysis software of choice to use for statistical analysis and interpretation. We also highlight how to do specific measurements in NIS-Elements.

Materials

Software:
FIJI (*https://imagej.net/software/fiji/downloads*)
FIJI Vasometrics (*https://github.com/mcdowellkonnor/Vasometrics*)
MATLAB (*https://www.mathworks.com/help/install/ug/install-products-with-internet-connection.html*)
RBC MATLAB script (*https://sourceforge.net/projects/lspivsupplement/files/*)
NIS-Elements, optional (https://microscope.healthcare.nikon.com/products/software/nis-elements)

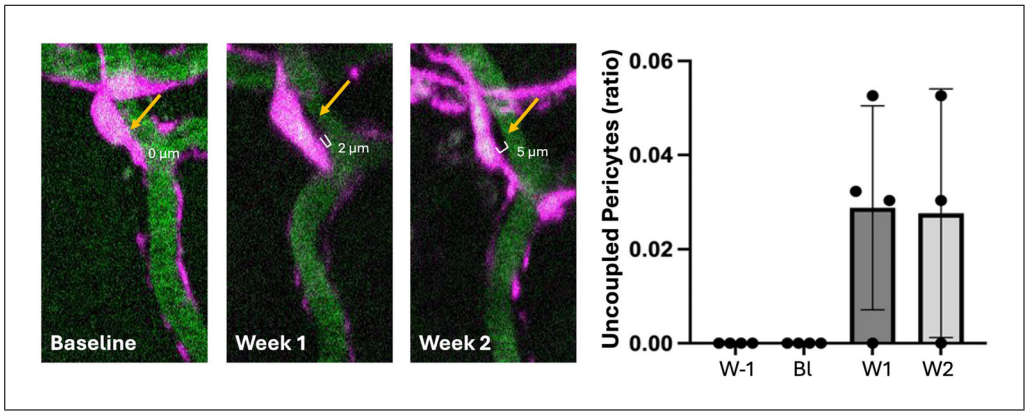

Figure 4 Example of cell behavior alteration over time in response to a hyperglycemic insult. The same perivascular cell (Myh11+, fuchsia) is seen detaching from its associated capillary (visualized by FITC-Dextran perfusion) over the course of 2 weeks, as indicated by the orange arrows. White brackets indicate the increasing gap between the pericyte and capillary over time. The ratio of uncoupled to total pericytes was quantified for 1 mouse at 1 week prior to baseline, baseline, 1-week-post-hyperglycemic insult, and 2-weeks-post-hyperglycemic insult.

Quantitative measurements

1. Use FIJI to create max projections of sub-stacks based on cortical layer.
2. Label the cells in the baseline timepoint with a unique identifier and match the labels for the subsequent timepoints.
3. To take diameter measurements, use FIJI to draw a straight line at the point of contact with the cell soma and away from the cell soma (Fig. 3A) and take those same measurements for the subsequent timepoints.

   Make sure that you associate the diameter measurement with the cell label made in step 2. Diameter can also be obtained using the Vasometrics macro in FIJI (McDowell et al., 2021).

4. To obtain RBC velocity, convert the generated space time plot to a 16-bit TIFF file in FIJI and load that into the published MATLAB script (Fig. 3B) (Kim et al., 2012).
5. To obtain RBC flux, use the 16-bit TIFF in FIJI with the cell-counter macro to count the dark streaks (Fig. 3B).

   We typically acquire for 1 min, so the same 1-s portion is cropped for all images and used to estimate flux.

6. Behavioral changes are measured in FIJI, depending on the criteria. For example, we assess pericyte uncoupling from capillaries, which is defined as a 1-µm distance between pericyte and capillary (Fig. 4).
7. Volumetric changes in individual capillaries, arterioles, and venules can be analyzed using the rotating rectangle feature in NIS-Elements (Nikon) or an equivalent feature in other software packages that isolates a region of interest without changing the underlying metadata. If one does not have a scope matching their analysis software of choice, they must first convert from the native file format of their microscope to TIFF.
   a. File conversion (prior to beginning this, record step size of each image and the pixel to micron ratio value).
      i. Open file in FIJI, duplicate image, and then split channels by navigating to image -> color -> split channels. Save individual channels in a way that you will be able to find and pair them again in NIS-Elements or your analysis software of choice.

ii. Launch NIS-Elements or your analysis software of choice.
iii. Open individual channels for an image that you saved as TIFF files in substep "i."
iv. Input correct z-step size for individual channels.
v. Rename all individuals channels and select desired pseudocolor. Recombine into one composite image. In NIS-Elements, the opened TIFF channels will be labeled as Mono. Right click on this Mono tab in the bottom left of the image. Select channel properties. Rename the channel appropriately, select the correct imaging modality, and choose desired pseudocolor for the channel. Repeat for all channels. To combine channels into one image, click the bottom left and drag to the center of another channel.
vi. Set the image calibration. One can do this in NIS-Elements by navigating to calibration -> recalibrate document. Input the known micron length of 1 pixel.
vii. Save this combined channel composite image as a ".nd2" file or native file format for your analysis software of choice. This workflow is presented in Supplementary Figure 1 (see Supporting Information).

b. Individual vessel volumetric analysis.
i. Duplicate the image. In NIS-Elements, you can do this by selecting "All" and dragging away from the image into the main GUI. Close the original image.
ii. Find desired vessel to image and the optical section range corresponding to its location within the image. In NIS-Elements, hold the shift key as you click and drag from the starting optical section to the ending optical section. Click "All" and drag out of image into main GUI space. A duplicated image with this *z*-series will appear. Do this for all relevant timepoints of the same vessel, ensuring to start as close as possible in the same location for both timepoints.
iii. Draw a square region of interest (ROI) marker over vessel to measure. Copy and paste this ROI onto the images from later timepoints.
iv. Use the rotating rectangle or equivalent feature to crop out the vessel to be measured from the main image. In NIS-Elements, do this by navigating to Image -> Rotate -> Rotate Rectangle. Draw the rectangle over the applied ROI in step iii and then rotate as needed to select the desired vessel. The ROI applied to all images in step iii will ensure the rotating rectangle will be applied at the same size for all images. Right click when done.
v. Click the "Show Volume View" icon.
vi. At this point the image can go through standard background subtraction, filtering, and threshold application for volumetric analysis, which is available in common analysis software packages and FIJI. In NIS-Elements, background subtraction can be found by navigating to image -> background -> rolling ball correction. Filters can be found under image on the toolbar. Volumetric thresholding can be performed by navigating to Binary -> define 3D threshold. Use the same values for vessels compared to themself across time. In FIJI, background subtraction can be found by navigating to process -> subtract background. Filter options can be found under process -> filters. Thresholding can be found under image -> adjust -> threshold.

One crucial point to raise is that volumetric analysis can only be successful if you maximize your histogram the same way for each timepoint imaged. In our baseline image notes, we record where in the field of view we optimized the histogram when imaging in high low mode. Alternatively, if you wanted to measure intensity data, as with dye extravasation for blood–brain barrier studies, you would use the exact same image parameters for each timepoint. Note that we rarely must change the laser power values even for volumetric measurements at different timepoints.

DOCUMENTING CHANGES IN ASTROCYTE MORPHOLOGY FOLLOWING INDUCTION OF TRANSIENT FOCAL PHOTOTHROMBOTIC STROKE USING ROSE BENGAL

In this protocol, we demonstrate how to induce a transient focal photothrombotic stroke using Rose Bengal. If conducted properly, the user will have the ability to observe the cellular response to transient focal photothrombotic stroke of virtually any cell type. Here, we present a protocol for astrocytes, but one can apply to this protocol to any fluorescently tagged cell type in vivo. Additionally, one could apply this protocol in a CreERT2 fl/fl system to observe how cellular responses change following cell-specific genetic ablation.

Materials

See Basic Protocol 1

1. It is best to know which penetrating arterioles you want to induce a transient focal photothrombotic stroke in prior to injecting Rose Bengal. Acquire this information prior to starting using the Basic Protocol 1, steps 16 to 37, and Basic Protocol 2, steps 1 to 5. Alexa 633 hydrazide can be used to label arterioles. If documenting changes in cell morphology following focal photothrombotic stroke, a baseline image prior to stroke induction will be needed (see Mills et al., 2022, Fig. 7A-C).

   Typically, we select an arteriole based on either positive labeling with Alexa 633 hydrazide (Shen et al., 2012), direction of blood flow, or minimal capillary branching relative to venules.

2. Retro-orbitally (RO) inject a very small amount of Rose Bengal dye at 20 mg/ml in sterile 1× PBS or artificial cerebrospinal fluid. In our hands, all mice <20 g receive 25 µl. Mice >20 g receive a 50 µl injection.

3. Upon RO injecting Rose Bengal dye and getting the animal ready for imaging, keep the laser power as low as possible. Only use what is necessary to accurately and quickly find the vessel to induce dye nucleation in. If you have laser power high enough, the dye will nucleate in multiple places, potentially killing the mouse.

   In our hands, all dye nucleation is induced in a power range of 50 to 90 mW, with power usage determined based on average intensity of the Rose Bengal dye at the beginning of the experiment.

4. Place a square ROI over the penetrating arteriole to induce dye nucleation in, matching the dimensions of the ROI to the dimensions of the vessel.

   We typically place ROIs 90 µm from the surface of the brain.

5. If only part of the vessel is occluded at the end of 2 min, image the vessel in laser scanning mode until dye nucleation is complete. Fig. 5 shows successful dye nucleation in a penetrating arteriole.

6. Upon successful dye nucleation in the entire vessel, acquire another z-stack to confirm volumetric presence of a clot (see Mills et al., 2022, Fig. 7C-F).

7. One can then characterize changes in astrocyte morphology at timepoints following clot formation, and in many instances, reperfusion following clot formation (see Mills et al., 2022, Fig. 7G-J).

   You could compare to astrocytes in the same mouse at a maximally distal arteriole where you perform the same protocol, only use 70 kDa-TRITC Dextran as a control.

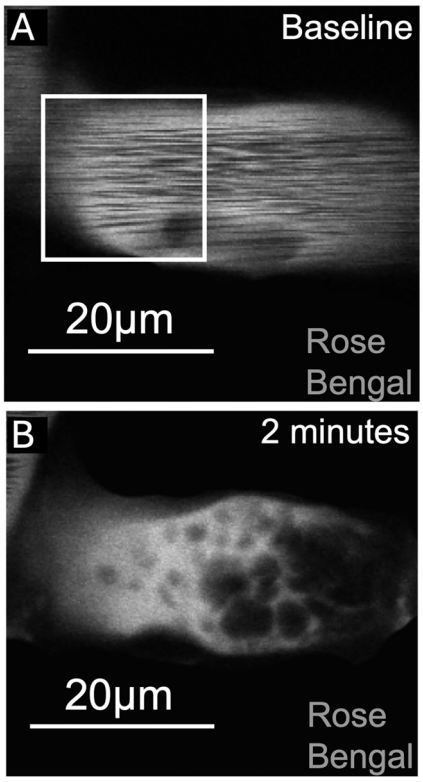

Figure 5 Rose Bengal dye nucleation following 2 min of scanning at 50-90 mW. (**A**) Maximum intensity projection showing a penetrating arteriole at baseline. The square box represents the ROI used to induce dye nucleation. (**B**) That same penetrating arteriole 2 min later. Note the bright dye, the location of scanning and downstream clot formation.

COMMENTARY

Critical Parameters

RO injection

Without a proper RO injection, visualization of the vessels will not be possible. This will affect the visualization and the ability to perform proper line scan acquisitions. When injecting, follow Yardeni et al. (2011). If the dye leaks, or there is excessive bleeding, redo the injection on the contralateral eye.

Effects of isoflurane

Isoflurane can act as a vasodilator (Sullender et al., 2022); thus, it is imperative to control for this and minimize this effect. Keeping the concentration <2% and ensuring that the same percentage is used for an approximately similar amount of time between timepoints is the best way to address this. Additionally, keeping track of the mouse's breath rate helps with controlling for this as well. Alternatively, one could perform all these acquisitions in awake mice.

Troubleshooting

For a list of problems associated with the imaging and image analysis, and their solutions, refer to Table 1.

Statistical Analysis

For measurements of the same capillary/cell type at multiple timepoints for one mouse, a repeated measures ANOVA is recommended. To compare imaging timepoints with the baseline timepoint, we recommend using a Dunnett's post-hoc test. For measurements of the same capillary/cell type at multiple timepoints for multiple mice, a linear mixed effects model is recommended.

Understanding Results

Through this technique there are several measurements that can be obtained. Essentially, anything about cell and vessel changes over the course of days to months (Fig. 2C-D). Here, we present a few measurements that we have conducted for our purposes: changes in astrocyte morphology following induced photothrombotic stroke (Fig. 5), vessel diameter

Table 1 Potential Problems and Solutions for Imaging and Image Analysis

Problem	Possible cause	Solution
Blurry or unclear field of imaging	Unclear window; dirty objective lens	Begin with cleaning the objective using lens paper. If this does not fix it, the window itself may be unclear which would make the mouse unusable. This could be because you did not keep the brain moist during surgery, having ice-cold 1× PBS or aCSF ready to flood the brain prior to removing the skull flap!
Reporter illuminated but vessels are not	Poor RO injection	Redo the RO injection in the contralateral eye
Inconsistent thresholding between images	Inconsistent image acquisition parameters between timepoints	Image in high–low mode to optimize the histogram the same across timepoints

at and away from the point of contact with pericytes (Fig. 3A), RBC flux and velocity (Fig. 3B), changes in cell behavior (Fig. 4), and volumetric vessel measurements (Supplementary Fig. 1; see Supporting Information). The diameter measurements can be taken for the same cell/vessel over the time course if they are not subject to pruning. This can help indicate the role of NVU cells in regulating capillary diameter in a progressing disease context. Average diameter measurements per FOV can also be acquired, to give a more general idea of how/if vessel diameters change in disease progression. RBC metrics and cell behavior changes can be interpreted in a similar manner, from a group of the same vessels and cells or from a larger group to obtain averages at each timepoint.

Time Considerations

If using an inducible Cre-mouse, the induction in total takes ∼4 weeks (2 weeks of tamoxifen and 2 weeks of normal chow). After performing the cranial window implant, the mice need at least 2 weeks of recovery time to account for inflammation. Imaging time per mouse is highly variable and can take anywhere from 30 min to 3 hr, depending on the number of FOVs and measurements being taken during imaging. For a mouse with 2 to 3 FOVs and 9-line scan acquisitions, imaging takes 1 to 2 hr. When imaging >2 hr in one session, consider injecting lactated ringer's solution (Vet One, cat. no. 501033) intraperitoneally at 3 ml/kg.

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Author Contributions

Kareem El-Ghazawi: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; visualization; writing—original draft; writing—review and editing. **William Mills III**: Data curation; formal analysis; investigation; methodology; project administration; validation; visualization; writing—original draft; writing—review and editing. **Shayn Peirce-Cottler**: Funding acquisition; supervision; writing—review and editing. **Ukpong Eyo**: Funding acquisition; supervision; writing—review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data, tools, and material (or their source) that support the protocol are available from the corresponding author upon reasonable request.

Supporting Information

cpz170167-sup-0001-FigureS1.docx
Supplementary Figure 1.

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