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Multiphoton intravital calcium imaging

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Abstract

Multiphoton intravital calcium imaging is a powerful technique that enables high resolution longitudinal monitoring of cellular and subcellular activity hundreds of microns deep in the living organism. This unit addresses the application of 2-photon microscopy to imaging of genetically encoded calcium indicators (GECIs) in the mouse brain. The protocols in this unit enable real-time intravital imaging of intracellular calcium concentration simultaneously in hundreds of neurons, or at the resolution of single synapses, as mice respond to sensory stimuli or perform behavioral tasks. Protocols are presented for implantation of a cranial imaging window to provide optical access to the brain and for 2-photon image acquisition. Protocols for implantation of both open skull and thinned skull windows for single or multi-session imaging are described.

Keywords

multiphoton imaging; intravital imaging; genetically-encoded calcium indicator; cranial window; brain; *in vivo* imaging; 2-photon microscopy

INTRODUCTION

In excitable cells, membrane depolarization results in calcium influx through one or more types of voltage-gated calcium channels. This may also trigger calcium release from intracellular stores, which further amplifies the depolarization-induced rise in intracellular calcium concentration. Calcium is also a ubiquitous signaling molecule in all cells, impacting virtually all aspects of cellular function. Hence, calcium imaging can provide a real-time readout of activity at both the cellular and subcellular level in both excitable and non-excitable cells. Intravital imaging enables these cellular and subcellular calcium signals to be measured over time *in situ* in the intact organism. The advent of multiphoton (typically 2-photon) excitation microscopy has made intravital calcium imaging at far greater depths possible.

This unit will focus on multiphoton intravital calcium imaging using genetically-encoded calcium indicators (GECIs) in the mouse brain. GECIs are fluorescent protein-based calcium sensors that can be expressed in specific cell types in a spatially and temporally controlled manner. Intravital image acquisition and analysis in other tissues and organs is similar to the protocols provided for brain imaging, although a different surgical preparation will be required. The protocols described here are also readily adaptable to intravital calcium

imaging in the brains of larger mammals such as rats and monkeys, although it is important to note that the dura mater is thicker and so must be opened to permit imaging of the underlying brain. The protocols presented here assume the use of transgenic or knock-in mouse lines that already express a GECI in the desired cells types. However, these open skull cranial window protocols described are readily adaptable to incorporate a virus injection to deliver the GECI, either several weeks prior to implantation of an acute window, or at the time of chronic window implantation. These protocols also describe the use of anesthetized mice for intravital calcium imaging; procedures for multiphoton calcium imaging in awake mice (Dombeck, Khabbaz, Collman, Adelman, & Tank, 2007) are becoming widespread but are beyond the scope of this unit.

The unit first presents protocols for cranial window implantation to provide optical access to the brain. The choice of cranial window type will depend on the cell type(s) to be imaged as well as the experimental goals. Protocols for acute window implantation for single-session imaging, as well as chronic window implantation for multi-session imaging over days to months, are provided. Furthermore, both open skull and thinned skull preparations are described. The unit then provides a protocol for 2-photon image acquisition and points to consider during image analysis.

BASIC PROTOCOL 1. CHRONIC OPEN SKULL CRANIAL WINDOW IMPLANTATION

Chronic cranial windows enable repeated imaging of the same cells or subcellular structures over time periods of days, weeks or even months. When combined with expression of a genetically encoded calcium indicator, this provides a longitudinal readout of cellular activity. The ability to repeatedly image the same animals enables within-subject baseline data to be collected prior to any experimental manipulation. Furthermore, the statistical power provided by repeated measures from individual subjects will reduce the number of animals required for a study.

The protocol involves replacing a small area of the skull with a glass coverslip to provide optical access to the underlying brain. Chronic cranial window implantation must be performed under aseptic conditions. The scalp is opened, a small craniotomy is performed, and a glass coverslip is attached to the skull using superglue, followed by dental cement. The scalp is then sutured over the cranial window, and re-opened two weeks later, at which point multiphoton imaging can begin. Acute open skull cranial window implantation is described in Alternate Protocol 1 and 2.

Materials

Mice expressing a genetically-encoded calcium indicator, typically obtained from commercial vendors e.g. Jackson labs, MMRRC

Sterile synthetic absorbable sutures (e.g. 5-0 Vicryl, Ethicon). Avoid using silk sutures as they cause greater tissue inflammation, which can delay wound healing and result in deposition of serous exudate that adheres the scalp to the underlying skull.

Isoflurane

Oxygen cylinder and 2-stage regulator plus flow meter

Dexamethasone (use at 2 mg/kg)

Ketoprofen (use at 5 mg/kg) or meloxicam (use at 5 mg/kg)

Enrofloxacin (use at 5 mg/kg)

Sterile eye lubricant e.g. Puralube, Altalube

Sterile lactated Ringer's solution

Sterile saline (for making up drug solutions)

70% ethanol

Povidone-iodine (e.g. Betadine) or chlorhexidine solution

Cotton swab sticks

Disposable biopsy punches of the desired diameter (Miltex)

#1 coverslips of the desired diameter, or appropriately sized coverslip to be cut down to fit craniotomy (e.g. 3mm diameter circular coverslips, Harvard Apparatus)

Cold sterile cortex buffer (see Reagents and Solutions)

Vetbond

Superglue (e.g. Loctite Ultra Gel Control Superglue)

Dental cement (e.g. Jet repair, Lang Dental)

Sterile Gelfoam

Sterile absorbent surgical spears

Sterile drape, autoclaved foil

Sterile syringes and needles (e.g. Insulin syringes)

Sterile surgical gloves, mask and hair cover, lab coat (follow the protocols outlined by your Institutional Animal Care and Use Committee or equivalent)

Stereotaxic frame and appropriate ear bars (non-rupture "cuff" style ear bars are recommended for mice to avoid damage to the inner ear and prevent inward compression of the skull, which can impede the airway)

Feedback-controlled heat pad

Isoflurane vaporizer and scavenger system (active scavenging via building vacuum or dedicated scavenger unit is recommended to minimize personnel exposure to isoflurane)

Surgical microscope equipped with cold lamp or LED light source to illuminate surgical field without heating

High-speed hand-held microdrill with 0.45mm drill bit (e.g. Foredom K.1070) or high-speed dental drill

Hot bead sterilizer (to sterilize tool tips between animals in the same cohort)

Autoclave-sterilized tools (#3, #5 and #55 forceps; small surgical scissors; scalpel handle to fit #10 blades; ridged forceps or needle holders for suturing; 2 surgical clamps e.g. 1.5" Johns Hopkins bulldog clamp; 0.45mm drill bits [Stoelting])

Clean recovery cage placed half-on a second heat pad

Head bars that enable attachment to an imaging frame under the multiphoton microscope (design appropriate to brain region to be imaged)

1. Before beginning, ensure that you have institutional and national approval for all procedures to be conducted on animals.
2. Prepare drug solutions and autoclave tools, drapes and foil.
3. Administer subcutaneous dexamethasone approximately 30 minutes prior to beginning surgery

This reduces inflammation and potential brain edema
4. Set up the surgical area: clean with chlorhexidine and lay out a sterile drape. Open sterile items into the sterile area i.e. autoclaved tools, sutures, etc. Place autoclaved foil over microscope focus and zoom controls, stereotax controls, vaporizer controls etc to avoid touching any non-sterile surface once surgery has begun.
5. Prepare the mouse in a separate area: anesthetize with 4% isoflurane in 1 l/min O₂. Administer ketoprofen or meloxicam by subcutaneous or intraperitoneal injection.

If approved by the Institutional Animal Care and Use Committee or equivalent, ketamine/xylazine anesthesia can be used in place of isoflurane
6. Use clippers or small non-sterile scissors to remove fur on the dorsal surface of the head.

If using clippers, take care not to remove the whiskers. Depilatory cream can be used but application time should be minimized to avoid causing chemical damage to or over-drying of the skin, which make suturing difficult and can cause inflammation
7. Apply sterile eye lubricant to the eyes using cotton swabs to prevent drying
8. Sterilize the scalp using three repeats of betadine scrub followed by 70% ethanol. Using a clean cotton swab, start at the center of the area and work outwards in a circular motion.
9. Place the mouse on a feedback-controlled heat pad on the stereotax, and maintain anesthesia using 1.5–2% isoflurane in 1 l/min O₂.

- 10.** Secure the head in the stereotax using the tooth bar and ear bars.

This is typically achieved by holding the head with one hand and manipulating the ear bars, which should be at the same height, with the other. “Cuff” style ear bars are not inserted into the ear, but instead are used to hold the zygomatic arch. If right-handed, position the left ear bar first, then the right. Check that the head is flat between the ear bars with a ruler, and that the head cannot move side-to-side but the nose can still be raised. Then insert the incisors into the tooth bar, and position the nose cone or clamp to secure the nose. Adjust the nose bar position so that the head is level. Alternatively, the incisors can first be positioned in the tooth bar to stabilize the head for ear bar placement, in which case the tooth bar height should then be adjusted after ear bar placement to level the head.

- 11.** Ensure that the head is held firmly but the airway is not impeded.
- 12.** Confirm the absence of a pedal reflex, indicating that a surgical plane of anesthesia has been achieved
- 13.** Ensure that all required items are available in the sterile area, and put surgical gloves on.

From this point until step 29 is complete, do not touch anything outside the sterile area.

- 14.** Make an incision along the center of the scalp using a scalpel or small sharp scissors. The length of the incision will depend on the location of the cranial window, but ensure that the incision is long enough to provide unrestricted access to the craniotomy site.
- 15.** Secure the scalp either side of the craniotomy location with clamps, ensuring that the skin is pulled away from the area of skull to be drilled.
- 16.** Remove the periosteum from the surface of the skull.

This is a layer of connective tissue and can be removed by gentling peeling off from the dry skull with fine forceps, or by gentle scraping of the skull surface with a sterile cotton swab. Any residual periosteum can be removed from the craniotomy site by very gently drilling that area of the skull. In young mice, which have thin flexible skulls, take great care not to exert downward pressure on the brain during this step.

- 17.** Check that the head is level using an arm holding a syringe with needle mounted to the stereotax. Move the needle until it is positioned at the intersection of lambda with the midline and lower to the surface of the skull. Record the dorsal-ventral co-ordinate. Raise the needle, move to the intersection of bregma with the midline, and lower to the surface of the skull. Record the dorsal-ventral and check that the two dorsal-ventral co-ordinates are the same. If not, raise the needle and adjust the height of the tooth bar to level the head and re-check co-ordinates.

- 18.** Determine and mark the position of the center of the craniotomy.

This is typically achieved using stereotaxic co-ordinates, although there are exceptions e.g. for cranial windows over the olfactory bulb. If stereotaxic co-ordinates are used, first record the dorsal-ventral, medial-lateral and anterior-posterior co-ordinates at the intersection of bregma and the midline (similar to step 9), then move the needle to the required location and mark the position on the skull. Inspect the surrounding area of skull that will be removed: try to avoid major bone sutures and underlying blood vessels, although this may not always be possible.

- 19.** Place a biopsy punch of the same diameter as the desired cranial window on the skull surface, centered on the mark made in step 10. Rotate the biopsy punch in either direction to produce a shallow groove in the skull.

Note that the skull thickness may vary across the diameter of the window e.g. it is typically thicker on the lateral than the medial side.

- 20.** Deepen the groove using the handheld drill. Drill carefully and only for short bursts in any given area to avoid heating the underlying brain tissue. Pause to apply cold sterile cortex buffer to cool the brain, reduce any bleeding and enable visualization of the underlying vasculature as the skull in the groove is gradually thinned.

The amount of bleeding can vary from mouse to mouse, and also depends on the age and strain of the mouse and the location of the craniotomy. The skull consists of a layer of highly vascularized trabecular (spongy) bone sandwiched between two compact bone layers; hence, bleeding typically subsides once the inner compact bone layer is reached.

- 21.** Once the bone in the groove is thin and transparent i.e. the underlying blood vessels can clearly be seen under cortex buffer, test the thickness by gently tapping on the central bone island with #5 forceps. If the groove is sufficiently thin, it will flex slightly when the central island is tapped. Continue very carefully drilling any thicker regions within the groove.
- 22.** Check that a coverslip of the desired size will fit into the groove that you have drilled. If it is slightly too small, you can enlarge it slightly by drilling around the outer walls of the groove.
- 23.** Place cortex buffer in the groove and wait 10 minutes.

This will soften the skull slightly.

- 24.** Use a new 26G needle to make a very small hole in the thinned bone groove, close to the central island. Insert one tip of the #5 forceps into the hole, taking care not to pierce the underlying dura, and gently lift the central bone island upwards.

Ideally, this should be removed in one piece, but if it breaks, the remaining skull within the craniotomy can be removed with #5 forceps, taking care not to damage the dura.

25. Immediately apply cold cortex buffer to the dural surface and either wick away using sterile absorbent swabs or remove using a vacuum aspirator.

Take great care not to touch the surface of the brain with anything other than Gelfoam, and if using a vacuum line, ensure that the suction tip is outside the craniotomy and suction is not exerted directly on the brain.

26. Continue reapplying cold buffer until all bleeding has stopped: this prevents blood from clotting on the surface of the dura. Gelfoam that has been pre-soaked in sterile cortex buffer can also be applied to the surface of the brain to aid coagulation.

Some minor bleeding is to be expected, as blood vessels in the dura can be continuous with the inner compact bone layer. The amount of bleeding will depend on the craniotomy location, age and strain of the mouse, and level of surgical expertise.

27. Inspect the craniotomy for any subdural bleeding, which will appear as a diffuse dark red area resembling a bruise. If present, exclude the mouse from the study as the brain has been damaged.
28. Remove a coverslip of the desired size from storage in 70% ethanol and dry with lens paper. Handle the coverslip with a dedicated pair of #5 forceps to keep it clean and hold only close to the edge.
29. Once all bleeding has stopped, remove the buffer and apply a small drop of fresh buffer to the craniotomy.

There should be enough liquid present to fill the window once the coverslip is placed, but no so much that the coverslip will float away from the desired location.

30. Place the coverslip carefully into the craniotomy. Ideally, you should not need to move it, but if misplaced, lift and re-lower the coverslip rather than trying to slide it sideways, as this may cause the dura to bleed.

The coverslip should fit snugly into the craniotomy; do not place the coverslip on top of the skull, as this will enable the skull to regrow under the window. Remove any excess buffer using absorbent surgical spears.

31. Apply a small amount of superglue close to one edge of the coverslip and use a 26G needle to draw the glue around the edges of the coverslip.

Depending on the location of the craniotomy and whether any edema has occurred, you may need to gently hold the coverslip in place using #5 forceps. Exert the minimum amount of downward pressure required to keep the coverslip in place. Ensure that no glue runs onto the surface

of the brain. If this occurs, discard the mouse. Ensure that you use a gel formula superglue to minimize the risk of glue running under the window, and do not use Vetbond as it is insufficiently viscous.

32. Allow the superglue to dry completely.

33. Suture the scalp using a continuous subcuticular stitch.

Suturing the scalp over the window provides a protected environment that appears to promote the long-term clarity of chronic cranial windows (Li et al., 2017).

34. Administer ~200 µl bolus of warmed lactated Ringer's solution and enrofloxacin subcutaneously

A single dose of enrofloxacin helps to prevent infection, while lactated Ringer's improves hydration.

35. Remove the mouse from the stereotax and place in a clean recovery cage placed partially on a heat pad.

36. Monitor the mouse until it has regained ambulatory behavior, then return to the home cage, and wait at least 14 days.

Removal of the skull results in an inflammatory response that includes microglial and astrocytic activation. Previous studies have shown that microglia and astrocyte density and morphology return to normal within 30 days of chronic open skull cranial window implantation over the visual or somatosensory cortex (Holtmaat et al., 2009). It may be necessary to determine empirically the optimum time to wait before beginning 2-photon imaging through a chronic cranial window in order to avoid confounds due to inflammation. Note that the scalp can still be re-opened at 14 days, even if imaging sessions will not commence immediately.

37. Anesthetize the mouse with isoflurane, remove the fur on the scalp and clean with three alternating swabs of povidone-iodine and 70% ethanol.

38. Place the mouse on a heat pad and secure in the stereotaxic frame

39. Make an incision along the center of the scalp using a scalpel or small sharp scissors (the sutures will already have been absorbed). Again, the length of the incision will depend on the location of the cranial window but must also allow sufficient space for attachment of the head bar, which must be far enough away from the cranial window to provide access for the microscope objective, for subsequent imaging.

40. Remove a strip of skin along either side of the incision and glue the skin margins to the skull using Vetbond.

41. Remove the periosteum from the skull using cotton swabs following by gently drilling the entire exposed surface of the skull.

This both removes any residual periosteum and roughens the skull to promote adherence of glue and dental cement.

42. Attach the head bar to the appropriate location on the skull using super glue. Hold the head bar in place for ~2 minutes, until it has stably adhered. Apply additional superglue around the head bar to provide secure attachment to the skull, and also spread a thin layer of superglue over the entire exposed surface of the skull, up to the edges of the skin and the cranial window.
43. Once the glue has dried, add a layer of dental cement over the entire exposed skull and around the head bar. If desired, you can also use dental cement to construct a shallow well around the cranial window to hold water for subsequent imaging.

However, take care to allow sufficient space for the objective to achieve the required working distance (typically ~2 mm for high numerical aperture water immersion objectives). The dental cement well is optional, as surface tension is typically sufficient to retain the immersion water column between the coverslip and the objective.

44. Allow the dental cement to dry, and clean the cranial window.

Loose debris can be removed using a cotton swab dipped in 70% ethanol. Any superglue on the surface of the glass can be removed by carefully scraping with the side of a 26G needle (take great care not to remove the glue holding the coverslip to the skull, or to exert so much force that you crack the coverslip).
45. The mouse is now ready for multiphoton intravital imaging (see basic protocol 3).

ALTERNATE PROTOCOL 1. ACUTE OPEN SKULL CRANIAL WINDOW IMPLANTATION

For some experiments, data acquisition at only a single time point is required, or data can be obtained during a single time-lapse imaging session a few hours in duration. In this case, a cranial window can be implanted acutely, immediately prior to collection of images. This approach also enables implantation of a cranial window smaller than the smallest commercially available circular coverslips, since skull regrowth under the window does not need to be considered.

The scalp is removed, a craniotomy is performed, and a glass coverslip is implanted. A head bar is then attached to enable positioning of the mouse in a custom frame under the microscope. As the mouse will not recover from anesthesia, acute cranial window implantation does not need to be performed under aseptic conditions unless the nature of the experiment specifically necessitates it.

Materials

Mice expressing a genetically-encoded calcium indicator, typically obtained from commercial vendors e.g. Jackson labs, MMRRC

Isoflurane

Oxygen cylinder and 2-stage regulator plus flow meter

Dexamethasone (use at 2 mg/kg)

Ketoprofen (use at 5 mg/kg) or meloxicam (use at 5 mg/kg)

Sterile eye lubricant e.g. Puralube, Altalube

Sterile lactated Ringer's solution

Sterile saline (for making up drug solutions)

70% ethanol

Cotton swab sticks

Disposable biopsy punches of the desired diameter (Miltex) [optional]

#1 coverslips of the desired diameter, or appropriately sized coverslip to be cut down to fit craniotomy (e.g. 3mm diameter circular coverslips, Harvard Apparatus)

Cold cortex buffer (see Reagents and Solutions)

Superglue (e.g. Loctite Ultra Gel Control Superglue)

Dental cement (e.g. Jet repair, Lang Dental)

Gelfoam

Sterile syringes and needles (e.g. Insulin syringes)

Stereotaxic frame and appropriate ear bars (non-rupture “cuff” style ear bars are recommended for mice to avoid damage to the inner ear and prevent inward compression of the skull, which can impede the airway)

Feedback-controlled heat pad

Isoflurane vaporizer and scavenger system (active scavenging via building vacuum or dedicated scavenger unit is recommended to minimize personnel exposure to isoflurane)

Surgical microscope equipped with cold lamp or LED light source to illuminate surgical field without heating

High-speed hand-held microdrill with 0.45mm drill bit (e.g. Foredom K.1070) or high-speed dental drill

Tools (#3, #5 and #55 forceps; small surgical scissors; scalpel handle to fit #10 blades; 2 surgical clamps e.g. 1.5” Johns Hopkins bulldog clamp; 0.45mm drill bits [Stoelting])

#5 forceps and small scissors dedicated for cutting the glass coverslip (optional)

Head bars that enable attachment to an imaging frame under the multiphoton microscope (design appropriate to brain region to be imaged)

1. Before beginning, ensure that you have institutional and national approval for all procedures to be conducted on animals.
2. Prepare drug solutions
3. Administer subcutaneous dexamethasone approximately 30 minutes prior to beginning surgery

This reduces inflammation and potential brain edema

4. Anesthetize the mouse with 4% isoflurane in 1 l/min O₂
5. Remove the fur using clippers, small scissors or depilatory cream
Take care not to remove the whiskers if using clippers, and apply depilatory cream for only 45–60 seconds to prevent chemical damage and/or excessive drying of the skin.
6. Administer subcutaneous ketoprofen (5 mg/kg) or meloxicam (5 mg/kg)
7. Apply sterile eye lubricant to the eyes using cotton swabs to prevent drying
8. Secure the head in the stereotax using the tooth bar and ear bars.

This is typically achieved by holding the head with one hand and manipulating the ear bars, which should be at the same height, with the other. “Cuff” style ear bars are not inserted into the ear, but instead are used to hold the zygomatic arch. If right-handed, position the left ear bar first, then the right. Check that the head is flat between the ear bars with a ruler, and that the head cannot move side-to-side but the nose can still be raised. Then insert the incisors into the tooth bar, and position the nose cone or clamp to secure the nose. Adjust the nose bar position so that the head is level. Alternatively, the incisors can first be positioned in the tooth bar to stabilize the head for ear bar placement, in which case the tooth bar height should then be adjusted after ear bar placement to level the head.

9. Ensure that the head is held firmly but the airway is not impeded.
10. Confirm the absence of a pedal reflex, indicating that a surgical plane of anesthesia has been achieved
11. Make a midline incision of the scalp, typically from behind the ears to the eyes. Remove a strip of skin on either side of this incision to provide access to the skull.

Ensure that the craniotomy site and the location where the head bar will be attached are accessible.

12. Use clamps to retract the skin adjacent to the craniotomy site

13. Remove the periosteum using cotton swabs. The skull surface can be drilled gently to remove any residual periosteum.
14. Check that the head is level using an arm holding a syringe with needle mounted to the stereotax. Move the needle until it is positioned at the intersection of lambda with the midline and lower to the surface of the skull. Record the dorsal-ventral co-ordinate. Raise the needle, move to the intersection of bregma with the midline, and lower to the surface of the skull. Record the dorsal-ventral and check that the two dorsal-ventral co-ordinates are the same. If not, raise the needle and adjust the height of the tooth bar to level the head and re-check co-ordinates.
15. Locate and mark the center of the craniotomy using stereotaxic co-ordinates or skull landmarks.

If stereotaxic co-ordinates are used, first record the dorsal-ventral, medial-lateral and anterior-posterior co-ordinates at the intersection of bregma and the midline (similar to step 14), then move the needle to the required location and mark the position on the skull.
16. If a craniotomy the size of a commercially available coverslip will be made, use a biopsy punch of the same size to mark the margins of the craniotomy: center the biopsy punch on the mark, and rotate the biopsy punch so that the teeth mark a groove in the skull. If a non-circular or non-standard sized craniotomy will be made, use the drill to make a shallow groove of the appropriate dimensions in the outer compact bone layer of the skull.
17. Next, use the drill to deepen the groove. Drill slowly and pause frequently to apply cold cortex buffer.

This keeps the underlying brain cool, helps to constrict any bleeding vessels, and enables the skull thickness to be assessed based on visibility of the brain vasculature through the skull in the groove. The central trabecular (spongy) bone layer of the skull is highly vascularized and may bleed when you are drilling through this layer. Bleeding is typically minor and resolves once that specific area of the skull has been removed.
18. Test the thickness of the bone in the groove by gently tapping the central bone island. If the bone in the groove is sufficiently thin to enable removal, it will flex slightly when the central island is tapped. Continue to carefully drill any remaining thicker areas of bone in the groove.
19. Place buffer in the groove and leave for 10 minutes
20. Meanwhile, prepare the coverslip. If a circular craniotomy of 3mm or larger diameter was performed, remove a round coverslip of the desired size from storage in 70% ethanol and dry with lens paper. If a smaller or irregularly sized craniotomy was made, cut the coverslip to a size slightly larger than the craniotomy and clean with 70% ethanol.

It is preferable to start with a small circular coverslip to provide a curved edge, although a window can be cut from a larger square coverslip. Ensure that the coverslip is not so large that it will contact the skin margins, as this will cause motion artifacts during imaging.

21. Under the buffer, very carefully make a small hole in the groove using a 26G needle, close to the central bone island. Insert one tip of a pair of #5 forceps carefully into the hole and gently lift the central bone island.

Ideally the central bone island can be removed in one piece, but if the skull breaks, remaining skull can be removed carefully using #5 or #55 forceps.

22. Immediately apply cold cortex buffer to the dural surface and either wick away using sterile absorbent swabs or remove using a vacuum aspirator.

Take great care not to touch the surface of the brain with anything other than Gelfoam, and if using a vacuum line, ensure that the suction tip is outside the craniotomy and suction is not exerted directly on the brain.

23. Continue reapplying cold buffer until all bleeding has stopped: this prevents blood from clotting on the surface of the dura. Gelfoam that has been pre-soaked in sterile cortex buffer can also be applied to the surface of the brain to aid coagulation.

Some minor bleeding is to be expected, as blood vessels in the dura can be continuous with the inner compact bone layer. The amount of bleeding will depend on the craniotomy location, age and strain of the mouse, and level of surgical expertise.

24. Inspect the craniotomy for any subdural bleeding, which will appear as a diffuse dark red area resembling a bruise. If present, exclude the mouse from the study as the brain has been damaged.

25. Once all bleeding has stopped, remove the buffer and apply a small drop of fresh buffer to the craniotomy.

There should be enough liquid present to fill the window once the coverslip is placed, but no so much that the coverslip will float away from the desired location.

26. Place the coverslip carefully over the craniotomy. Ideally, you should not need to move it, but if misplaced, lift and re-lower the coverslip rather than trying to slide it sideways, as this may cause the dura to bleed.

The coverslip should fit into the craniotomy if the size matches that of a commercially available coverslip. If the coverslip was cut to fit the craniotomy, it should sit on the skull at the edges of the craniotomy; this will minimize the chance of glue running on to the surface of the brain.

27. Apply a small amount of superglue close to one edge of the coverslip and use a 26G needle to draw the glue around the edges of the coverslip.

Depending on the location of the craniotomy and whether any edema has occurred, you may need to gently hold the coverslip in place using #5 forceps. Exert the minimum amount of downward pressure required to keep the coverslip in place. Ensure that no glue runs onto the surface of the brain. If this occurs, discard the mouse. Do not use Vetbond as it is insufficiently viscous.

28. Allow the superglue to dry completely.
29. Attach the head bar to the appropriate location on the skull using super glue. Hold the head bar in place for ~2 minutes, until it has stably adhered. Apply additional superglue around the head bar to provide secure attachment to the skull, and also spread a thin layer of superglue over the entire exposed surface of the skull, up to the edges of the skin and the cranial window.

Ensure that there is sufficient clearance between the head bar and the cranial window such that movement of the objective lens will not be impeded by the head bar.

30. Once the glue has dried, add a layer of dental cement over the entire exposed skull and around the head bar. If desired, you can also use dental cement to construct a shallow well around the cranial window to hold water for subsequent imaging.

Take care to allow sufficient space for the objective to achieve the required working distance (typically ~2 mm for high numerical aperture water immersion objectives). The dental cement well is optional, as surface tension is typically sufficient to retain the immersion water column between the coverslip and the objective.

31. Allow the dental cement to dry and ensure that there is no glue on the surface of the coverslip; if there is, use the side of a 26G needle to gently scrape the glue off the coverslip. Do not exert any downward pressure on the window, as this may cause bleeding or loosen the attachment of the coverslip to the skull.
32. Administer ~200 μ l bolus of warmed lactated Ringer's solution
33. The mouse is now ready for multiphoton intravital imaging (see basic protocol 3).

ALTERNATE PROTOCOL 2. ALTERNATIVE CRANIOTOMY PROTOCOL FOR ACUTE OPEN SKULL CRANIAL WINDOWS

This protocol describes an alternative strategy for skull removal. Rather than a standard craniotomy, wherein a groove is drilled and the central piece of skull is removed, this method involves careful thinning of the skull, which is then removed. This protocol is well suited to small cranial windows (e.g. 1mm diameter) and is particularly advantageous in young mice (less than postnatal day 28), which have thin, flexible skulls. The flexibility of the skull can make it difficult to drill a groove without causing damage to the underlying

brain. This protocol is not recommended for larger craniotomies, as it is difficult to thin the skull uniformly over a large area without causing subdural bleeding.

Materials

Mice expressing a genetically-encoded calcium indicator, typically obtained from commercial vendors e.g. Jackson labs, MMRRC

Isoflurane

Oxygen cylinder and 2-stage regulator plus flow meter

Dexamethasone (use at 2 mg/kg)

Ketoprofen (use at 5 mg/kg) or meloxicam (use at 5 mg/kg)

Sterile eye lubricant e.g. Puralube, Altalube

Sterile lactated Ringer's solution

Sterile saline (for making up drug solutions)

70% ethanol

Cotton swab sticks

Appropriately sized coverslip to be cut down to fit craniotomy (e.g. 3mm diameter circular coverslips, Harvard Apparatus)

Cold cortex buffer (see Reagents and Solutions)

Superglue (e.g. Loctite Ultra Gel Control Superglue)

Dental cement (e.g. Jet repair, Lang Dental)

Gelfoam

Absorbent surgical sponges

Sterile syringes and needles (e.g. Insulin syringes)

Dental microblade (e.g. Surgistar 6961)

Stereotaxic frame and appropriate ear bars (non-rupture "cuff" style ear bars are recommended for mice to avoid damage to the inner ear and prevent inward compression of the skull, which can impede the airway)

Feedback-controlled heat pad

Isoflurane vaporizer and scavenger system (active scavenging via building vacuum or dedicated scavenger unit is recommended to minimize personnel exposure to isoflurane)

Surgical microscope equipped with cold lamp or LED light source to illuminate surgical field without heating

High-speed hand-held microdrill with 0.45mm drill bit (e.g. Freedom K.1070) or high-speed dental drill

Tools (#3, #5 and #55 forceps; small surgical scissors; scalpel handle to fit #10 blades; 2 surgical clamps e.g. 1.5" Johns Hopkins bulldog clamp; 0.45mm drill bits [Stoelting])

#5 forceps and small scissors dedicated for cutting the glass coverslip (optional)

Head bars that enable attachment to an imaging frame under the multiphoton microscope (design appropriate to brain region to be imaged)

- 1 Before beginning, ensure that you have institutional and national approval for all procedures to be conducted on animals.
- 2 Prepare drug solutions.
- 3 Administer subcutaneous dexamethasone approximately 30 minutes prior to beginning surgery.

This reduces inflammation and potential brain edema.
- 4 Anesthetize the mouse with 4% isoflurane in 1 l/min O₂
- 5 Remove the fur using clippers, small scissors or depilatory cream.

Take care not to remove the whiskers if using clippers, and apply depilatory cream for only 45–60 seconds to prevent chemical damage and/or excessive drying of the skin.
- 6 Administer subcutaneous ketoprofen (5 mg/kg) or meloxicam (5 mg/kg).
- 7 Apply sterile eye lubricant to the eyes using cotton swabs to prevent drying.
- 8 Secure the head in the stereotax using the tooth bar and ear bars.

This is typically achieved by holding the head with one hand and manipulating the ear bars, which should be at the same height, with the other. "Cuff" style ear bars are not inserted into the ear, but instead are used to hold the zygomatic arch. If right-handed, position the left ear bar first, then the right. Check that the head is flat between the ear bars with a ruler, and that the head cannot move side-to-side but the nose can still be raised. Then insert the incisors into the tooth bar, and position the nose cone or clamp to secure the nose. Adjust the nose bar position so that the head is level. Alternatively, the incisors can first be positioned in the tooth bar to stabilize the head for ear bar placement, in which case the tooth bar height should then be adjusted after ear bar placement to level the head.
- 9 Ensure that the head is held firmly but the airway is not impeded.
- 10 Confirm the absence of a pedal reflex, indicating that a surgical plane of anesthesia has been achieved
- 11 Make a midline incision of the scalp, typically from behind the ears to the eyes. Remove a strip of skin either side of this incision to provide access to the skull.

Ensure that the craniotomy site and the location where the head bar will be attached are accessible.

- 7 Use clamps to retract the skin adjacent to the craniotomy site.
- 8 Remove the periosteum using cotton swabs. The skull surface can be drilled gently to remove any residual periosteum.
- 9 Check that the head is level using an arm holding a syringe with needle mounted to the stereotax. Move the needle until it is positioned at the intersection of lambda with the midline and lower to the surface of the skull. Record the dorsal-ventral co-ordinate. Raise the needle, move to the intersection of bregma with the midline, and lower to the surface of the skull. Record the dorsal-ventral and check that the two dorsal-ventral co-ordinates are the same. If not, raise the needle and adjust the height of the tooth bar to level the head and re-check co-ordinates.
- 10 Define the edges of the area of skull to be thinned.

If stereotaxic co-ordinates are used, first record the dorsal-ventral, medial-lateral and anterior-posterior co-ordinates at the intersection of bregma and the midline (similar to step 9), then move the needle to the required location and mark the position on the skull.
- 11 Begin gently thinning the skull with the drill. Keep the drill moving continuously and do not drill any one area of the skull for more than a few seconds. Pause frequently and apply cold cortex buffer to minimize heating of the underlying brain.
- 12 Continue carefully thinning the skull.

As the skull becomes thinner, the brain vasculature will become visible when buffer is applied, and the skull will flex when it is tapped gently.
- 13 The final stage of skull thinning is best performed by hand using a dental microblade.

Hold the microblade at approximately 45° to the skull and exert only lateral pressure using the microblade; do not press downwards.
- 14 Apply cortex buffer to the thinned skull and wait 10 minutes.
- 15 Meanwhile, cut a coverslip to the desired size using a dedicated pair of small scissors and handling only with a dedicated pair of #5 forceps.

The coverslip should be slightly larger than the area of skull that will be removed.
- 16 Using a new 26G needle, make a small hole or slit in one edge of the thinned area.
- 17 Carefully insert one tip of a pair of #55 forceps into the hole. Gently slide the tip of the forceps further under the thinned skull, exerting gentle upward pressure as

you slide the forceps to prevent the tip from damaging the dura. Lift up a small flap of the skull.

- 18** Repeat to remove remaining thinned areas of skull. Do not remove any skull that has not been thinned.

- 19** Rinse the surface of the dura by applying cold cortex buffer and either wicking away with an absorbent spear or carefully using a vacuum aspirator. Only ever apply the aspirator from the side of the craniotomy.

Typically, only minor bleeding occurs; if necessary, Gelfoam can be applied to promote clotting.

- 20** Once all bleeding has stopped, apply fresh buffer to the surface of the dura.

There should be a sufficient volume of buffer to fill the craniotomy once the coverslip has been placed, but not so much that the coverslip will float away.

- 21** Ensure that the coverslip has been cut to the correct size for the exposed area of brain, and if necessary trim excess glass or cut a new coverslip.

- 22** Carefully place the coverslip over the craniotomy, ensuring that the glass extends beyond the exposed brain on all sides. If the coverslip needs to be repositioned, lift it and place it in the correct position; do not slide it sideways as this will drag on the dura and may cause bleeding.

- 23** Dry any excess buffer from around the coverslip; ensure that the buffer still extends to the edges of the coverslip so that no glue can run under it. However, the surrounding skull should be dry to allow the glue to adhere.

- 24** Apply a small drop of superglue to the skull close to the craniotomy and use the tip of a 26G needle to carefully draw the glue around the edge of the coverslip.

Typically, because of the small size of the craniotomy, the glass will sit flat on the skull at the edges of the craniotomy, but if necessary, the coverslip can be gently held in place using clear #5 forceps. Exert minimal downward pressure to avoid compressing the underlying brain. Ensure that the coverslip is not glued to the skin, as this will introduce motion artifacts.

- 25** Allow the glue to set. Meanwhile, glue the head bar to the skull, remove the clamps, and spread a thin layer of glue over the entire surface of the skull.

This provides additional stability, especially in young mice, in which the skull is more flexible. Also ensure that the scalp margins are glued to the skull.

- 26** In young mice, it is beneficial to apply a thin layer of dental cement over the skull to provide additional mechanical stability. Either superglue or cement can also be used to build a shallow well around the coverslip.

Take care that the well is sufficiently large in diameter to accommodate the microscope objective.

- 27 Ensure that there is no glue on the surface of the coverslip; if there is, use the side of a 26G needle to gently scrape the glue off the coverslip. Do not exert any downward pressure on the window, as this may cause bleeding or loosen the attachment of the coverslip to the skull.
- 28 Administer ~200 μ l bolus of warmed lactated Ringer's solution
- 29 The mouse is now ready for multiphoton intravital imaging (see basic protocol 3).

BASIC PROTOCOL 2. THINNED SKULL CHRONIC CRANIAL WINDOW

For multiphoton intravital calcium imaging of certain cell types, such as microglia, in which function is altered by removal of the skull, it is necessary to use a thinned skull cranial window. This method involves thinning the bone over the region to be imaged down to just 20 – 30 μ m, sufficiently transparent to enable high resolution imaging with minimal aberration being introduced by the remaining skull.

It is possible to repeatedly image through a thinned skull window, but the skull must be re-thinned before each imaging session. It is therefore important to ensure that the skull is re-thinned to the same thickness as during the initial imaging sessions to avoid alterations in image clarity. The need for re-thinning may also limit the total number of imaging sessions that can be performed; however, they can be spaced at any time interval.

A thinned skull window can be used for either chronic imaging (this protocol) or for acute i.e. single session imaging (see ALTERNATE PROTOCOL 3).

Materials

Mice expressing a genetically-encoded calcium indicator, typically obtained from commercial vendors e.g. Jackson labs, MMRRRC

Sterile synthetic absorbable sutures (e.g. 5-0 Vicryl, Ethicon). Avoid using silk sutures as they cause greater tissue inflammation, which can delay wound healing and result in deposition of serous exudate that adheres the scalp to the underlying skull.

Isoflurane

Oxygen cylinder and 2-stage regulator plus flow meter

Dexamethasone (use at 2 mg/kg)

Ketoprofen (use at 5 mg/kg) or meloxicam (use at 5 mg/kg)

Enrofloxacin (use at 5 mg/kg)

Sterile eye lubricant e.g. Puralube, Altalube

Sterile lactated Ringer's solution

Sterile saline (for making up drug solutions)

70% ethanol

Povidone-iodine (e.g. Betadine) or chlorhexidine solution

Cotton swab sticks

Disposable biopsy punches of the desired diameter (Miltex)

#1 coverslips of the desired diameter, or appropriately sized coverslip to be cut down to fit craniotomy (e.g. 3mm diameter circular coverslips, Harvard Apparatus)

Cold sterile cortex buffer (see Reagents and Solutions)

Vetbond

Superglue (e.g. Loctite Ultra Gel Control Superglue)

Dental cement (e.g. Jet repair, Lang Dental)

Sterile Gelfoam

Sterile drape, autoclaved foil

Sterile syringes and needles (e.g. Insulin syringes)

Dental microblade (e.g. Surgistar 6961)

Sterile surgical gloves, mask and hair cover, lab coat (follow the protocols outlined by your Institutional Animal Care and Use Committee or equivalent)

Stereotaxic frame and appropriate ear bars (non-rupture “cuff” style ear bars are recommended for mice to avoid damage to the inner ear and prevent inward compression of the skull, which can impede the airway)

Feedback-controlled heat pad

Isoflurane vaporizer and scavenger system (active scavenging via building vacuum or dedicated scavenger unit is recommended to minimize personnel exposure to isoflurane)

Surgical microscope equipped with cold lamp or LED light source to illuminate surgical field without heating

High-speed hand-held microdrill with 0.45mm drill bit (e.g. Freedom K.1070) or high-speed dental drill

Hot bead sterilizer (to sterilize tool tips between animals in the same cohort)

Autoclave-sterilized tools (#3, #5 and #55 forceps; small surgical scissors; scalpel handle to fit #10 blades; ridged forceps or needle holders for suturing; 2 surgical clamps e.g. 1.5” Johns Hopkins bulldog clamp; 0.45mm drill bits [Stoelting])

Clean recovery cage placed half-on a second heat pad

Head bars that enable attachment to an imaging frame under the multiphoton microscope (design appropriate to brain region to be imaged)

- 1 Before beginning, ensure that you have institutional and national approval for all procedures to be conducted on animals.
- 2 Prepare drug solutions and autoclave tools, drapes and foil.
- 3 Administer subcutaneous dexamethasone approximately 30 minutes prior to beginning surgery.

This reduces inflammation and potential brain edema.
- 4 Set up the surgical area: clean with chlorhexidine and lay out a sterile drape. Open sterile items into the sterile area i.e. autoclaved tools, sutures, etc. Place autoclaved foil over microscope focus and zoom controls, stereotax controls, vaporizer controls etc to avoid touching any non-sterile surface once surgery has begun.
- 5 Prepare the mouse in a separate area: anesthetize with 4% isoflurane in 1 l/min O₂. Administer ketoprofen or meloxicam by subcutaneous or intraperitoneal injection.

Ketamine/xylazine anesthesia can be used in place of isoflurane if preferred.
- 6 Use clippers or small non-sterile scissors to remove fur on the dorsal surface of the head.

If using clippers, take care not to remove the whiskers. Depilatory cream can be used but application time should be minimized (45–60 seconds) to avoid chemical damage to or over-drying of the skin, which make suturing difficult and can cause inflammation.
- 7 Apply sterile eye lubricant to the eyes using cotton swabs to prevent drying.
- 8 Sterilize the scalp using three repeats of betadine scrub followed by 70% ethanol. Using a clean cotton swab, start at the center of the area and work outwards in a circular motion.
- 9 Place the mouse on a feedback-controlled heat pad on the stereotax, and maintain anesthesia using 1.5–2% isoflurane in 1 l/min O₂.
- 10 Secure the head in the stereotax using the tooth bar and ear bars.

This is typically achieved by holding the head with one hand and manipulating the ear bars, which should be at the same height, with the other. “Cuff” style ear bars are not inserted into the ear, but instead are used to hold the zygomatic arch. If right-handed, position the left ear bar first, then the right. Check that the head is flat between the ear bars with a ruler, and that the head cannot move side-to-side but the nose can still be raised. Then insert the incisors into the tooth bar, and position the nose cone or clamp to secure the nose. Adjust the nose bar position so that the head is level. Alternatively, the incisors can first be positioned in the tooth bar to stabilize the head for ear bar placement, in

which case the tooth bar height should then be adjusted after ear bar placement to level the head.

- 11 Ensure that the head is held firmly but the airway is not impeded.
- 12 Confirm the absence of a pedal reflex, indicating that a surgical plane of anesthesia has been achieved
- 13 Ensure that all required items are available in the sterile area, and put surgical gloves on.

From this point until step 29 is complete, do not touch anything outside the sterile area.

- 14 Make an incision along the center of the scalp using a scalpel or small sharp scissors. The length of the incision will depend on the location of the cranial window, but ensure that the incision is long enough to provide unrestricted access to the craniotomy site.

- 15 Secure the scalp either side of the craniotomy location with clamps, ensuring that the skin is pulled away from the area of skull to be drilled.

- 16 Remove the periosteum from the surface of the skull.

This is a layer of connective tissue and can be removed by gentle scraping of the skull surface with a sterile cotton swab. Any residual periosteum can be removed from the craniotomy site by very gently drilling that area of the skull.

- 13 Check that the head is level using an arm holding a syringe with needle mounted to the stereotax. Move the needle until it is positioned at the intersection of lambda with the midline and lower to the surface of the skull. Record the dorsal-ventral co-ordinate. Raise the needle, move to the intersection of bregma with the midline, and lower to the surface of the skull. Record the dorsal-ventral and check that the two dorsal-ventral co-ordinates are the same. If not, raise the needle and adjust the height of the tooth bar to level the head and re-check co-ordinates.

- 14 Define the edges of the area of skull to be thinned

If stereotaxic co-ordinates are used, first record the dorsal-ventral, medial-lateral and anterior-posterior co-ordinates at the intersection of bregma and the midline (similar to step 13), then move the needle to the required location and mark the position on the skull.

- 15 Begin gently thinning the skull with the drill. Keep the drill moving continuously and do not drill any one area of the skull for more than a few seconds. Pause frequently and apply cold cortex buffer to minimize heating of the underlying brain.
- 16 Continue carefully thinning the skull.

As the skull becomes thinner, the brain vasculature will become visible when buffer is applied, and the skull will flex when it is tapped gently.

- 17** The final stage of skull thinning is best performed by hand using a dental microblade.

Hold the microblade at approximately 45° to the skull and exert only lateral pressure using the microblade; do not press downwards.
- 18** Apply cortex buffer and Gelfoam to the thinned skull.

This ensures that the thinned skull is not exposed to super glue in the next step
- 19** Attach the head bar to the appropriate location on the skull using super glue. Hold the head bar in place for ~2 minutes, until it has stably adhered.

The head bar will need to be removed at the end of the imaging session so that the scalp can be sutured.
- 20** Once the glue is completely dry, remove the Gelfoam, but ensure that the thinned skull remains covered with buffer
- 21** The final skull thickness should be 20–30 µm throughout the region to be imaged. When beginning to use this preparation, it is helpful to check the skull thickness at this step by securing the mouse under the multiphoton microscope. Skull autofluorescence at an excitation wavelength of ~900nm can be used to estimate the skull thickness by measuring the distance in z between the upper and lower surface of the skull. If necessary, the mouse can be moved back to the stereotax to thin the skull further with the microblade.
- 22** The mouse is now ready for 2-photon imaging (see BASIC PROTOCOL 3).
- 23** Once imaging is complete, carefully remove the head bar and suture the scalp
- 24** Suture the scalp using a continuous subcuticular stitch.
- 25** Administer ~200 µl bolus of warmed lactated Ringer's solution
- 26** Remove the mouse from the stereotax and place in a clean recovery cage placed partially on a heat pad.
- 27** Monitor the mouse until it has regained ambulatory behavior, then return to the home cage, and wait until the next imaging time point.
- 28** On the next imaging day, repeat steps 1–12.
- 29** Locate the area of skull that was previously thinned and begin carefully re-thinning the same area using the drill and/or dental microblade
- 30** As before, pause frequently to apply cold sterile cortex buffer and to inspect the surface vasculature.
- 31** Proceed until the skull is sufficiently thin for imaging.

The degree of skull regrowth will depend on the length of time between imaging sessions, the age of the mouse and the location of the thinned skull region.

- 32** Place cold sterile cortex buffer and Gelfoam over the thinned skull and glue the head bar on.

The head bar should be in as similar a location and orientation as possible to the previous imaging session.

- 33** Allow the glue to dry completely, then remove the Gelfoam.
- 34** The mouse is now ready for 2-photon imaging (see BASIC PROTOCOL 3).
- 35** Repeat steps 22–33 as required to obtain the planned number of imaging time points.

Note that repeatedly re-thinning the skull without damaging the underlying brain is a highly skilled task, and most labs confine the number of imaging time points with this preparation to two to five.

ALTERNATE PROTOCOL 3. ACUTE THINNED SKULL CRANIAL WINDOW

Materials

Mice expressing a genetically-encoded calcium indicator, typically obtained from commercial vendors e.g. Jackson labs, MMRRC

Isoflurane

Oxygen cylinder and 2-stage regulator plus flow meter

Ketoprofen (use at 5 mg/kg) or meloxicam (use at 5 mg/kg)

Sterile eye lubricant e.g. Puralube, Altalube

Sterile lactated Ringer's solution

Sterile saline (for making up drug solutions)

70% ethanol

Cotton swab sticks

Cold cortex buffer (see Reagents and Solutions)

Superglue (e.g. Loctite Ultra Gel Control Superglue)

Dental cement (e.g. Jet repair, Lang Dental)

Gelfoam

Sterile syringes and needles (e.g. Insulin syringes)

Dental microblade (e.g. Surgistar 6961)

Stereotaxic frame and appropriate ear bars (non-rupture “cuff” style ear bars are recommended for mice to avoid damage to the inner ear and prevent inward compression of the skull, which can impede the airway)

Feedback-controlled heat pad

Isoflurane vaporizer and scavenger system (active scavenging via building vacuum or dedicated scavenger unit is recommended to minimize personnel exposure to isoflurane)

Surgical microscope equipped with cold lamp or LED light source to illuminate surgical field without heating

High-speed hand-held microdrill with 0.45mm drill bit (e.g. Foredom K.1070) or high-speed dental drill

Tools (#3, #5 and #55 forceps; small surgical scissors; scalpel handle to fit #10 blades; 2 surgical clamps e.g. 1.5” Johns Hopkins bulldog clamp; 0.45mm drill bits [Stoelting])

Head bars that enable attachment to an imaging frame under the multiphoton microscope (design appropriate to brain region to be imaged)

1. Before beginning, ensure that you have institutional and national approval for all procedures to be conducted on animals.
2. Prepare drug solutions.
3. Anesthetize the mouse with 4% isoflurane in 1 l/min O₂.
4. Remove the fur using clippers, small scissors or depilatory cream.

Take care not to remove the whiskers if using clippers, and apply depilatory cream for only 45–60 seconds to prevent chemical damage and/or excessive drying of the skin.
5. Administer subcutaneous ketoprofen (5 mg/kg) or meloxicam (5 mg/kg).
6. Apply sterile eye lubricant to the eyes using cotton swabs to prevent drying.
7. Secure the head in the stereotax using the tooth bar and ear bars.

This is typically achieved by holding the head with one hand and manipulating the ear bars, which should be at the same height, with the other. “Cuff” style ear bars are not inserted into the ear, but instead are used to hold the zygomatic arch. If right-handed, position the left ear bar first, then the right. Check that the head is flat between the ear bars with a ruler, and that the head cannot move side-to-side but the nose can still be raised. Then insert the incisors into the tooth bar, and position the nose cone or clamp to secure the nose. Adjust the nose bar position so that the head is level. Alternatively, the incisors can first be positioned in the tooth bar to stabilize the head for ear bar placement, in which case the tooth bar height should then be adjusted after ear bar placement to level the head.

8. Ensure that the head is held firmly but the airway is not impeded.
9. Confirm the absence of a pedal reflex, indicating that a surgical plane of anesthesia has been achieved
10. Make a midline incision of the scalp, typically from behind the ears to the eyes. Remove a strip of skin either side of this incision to provide access to the skull.

Ensure that the craniotomy site and the location where the head bar will be attached are accessible.
11. Use clamps to retract the skin adjacent to the craniotomy site.
12. Remove the periosteum using cotton swabs. The skull surface can be drilled gently to remove any residual periosteum.
13. Check that the head is level using an arm holding a syringe with needle mounted to the stereotax. Move the needle until it is positioned at the intersection of lambda with the midline and lower to the surface of the skull. Record the dorsal-ventral co-ordinate. Raise the needle, move to the intersection of bregma with the midline, and lower to the surface of the skull. Record the dorsal-ventral and check that the two dorsal-ventral co-ordinates are the same. If not, raise the needle and adjust the height of the tooth bar to level the head and re-check co-ordinates.
14. Define the edges of the area of skull to be thinned.

If stereotaxic co-ordinates are used, first record the dorsal-ventral, medial-lateral and anterior-posterior co-ordinates at the intersection of bregma and the midline (similar to step 13), then move the needle to the required location and mark the position on the skull.
15. Begin gently thinning the skull with the drill. Keep the drill moving continuously and do not drill any one area of the skull for more than a few seconds. Pause frequently and apply cold cortex buffer to minimize heating of the underlying brain.
16. Continue carefully thinning the skull.

As the skull becomes thinner, the brain vasculature will become visible when buffer is applied, and the skull will flex when it is tapped gently.
17. The final stage of skull thinning is best performed by hand using a dental microblade.

Hold the microblade at approximately 45° to the skull and exert only lateral pressure using the microblade; do not press downwards.
18. Apply cortex buffer and Gelfoam to the thinned skull.
19. Meanwhile, glue the head bar to the skull, remove the clamps, and glue the skin margins to the skull.
20. Administer ~200 µl bolus of warmed lactated Ringer's solution.

21. Once the glue is completely dry, remove the Gelfoam, but ensure that the thinned skull remains covered with buffer.
22. The final skull thickness should be 20–30 μm throughout the region to be imaged. When beginning to use this preparation, it is helpful to check the skull thickness at this step by securing the mouse under the multiphoton microscope. Skull autofluorescence at an excitation wavelength of $\sim 900\text{nm}$ can be used to estimate the skull thickness by measuring the distance in z between the upper and lower surface of the skull. If necessary, the mouse can be moved back to the stereotax to thin the skull further with the microblade.
23. The mouse is now ready for multiphoton intravital imaging (see basic protocol 3).

BASIC PROTOCOL 3. 2-PHOTON INTRAVITAL CALCIUM IMAGING THROUGH A CRANIAL WINDOW

Materials

GECI-expressing mouse implanted with acute or chronic cranial window and head bar

Cotton swabs

70% ethanol

Materials required to attach head bar to imaging frame e.g. screws

2-photon laser-scanning microscope equipped with tunable femtosecond pulsed infrared laser and high numerical aperture (NA) water immersion objective (popular and reasonably-priced choices are the Nikon 16x 0.8 NA for population calcium imaging or the Olympus 20x 1.0 NA)

Suitable image acquisition software: this may be provided by the microscope vendor, or there are open source options e.g. ScanImage (Pologruto, Sabatini, & Svoboda, 2003) or SciScan (Ward, Murray, & Wilms, 2017)

Equipment for stimulus delivery (optional; this will depend on the nature of the experiment)

Frame to which head bar can be attached to position mouse under the microscope

Surgical microscope equipped with camera

Feedback-controlled heat pads on stereotax, imaging frame and under recovery cage

Anesthesia and scavenging equipment on stereotax and microscope

Recovery cage partially on a heat pad

1. Anesthetize the mouse and either place mouse in stereotax or attach head bar to imaging frame (if it can be removed from the microscope and placed under the surgical microscope).

2. Under the surgical microscope, clean the window carefully with 70% ethanol using fine cotton swabs.
3. Capture an image of the cranial window showing the surface vasculature.

This is essential for experiments requiring repeated imaging at multiple time points, to enable the imaged region to be relocated, and optional for acute (single session) experiments.
4. Transfer the mouse to the microscope and position so that the cranial window is centered under the microscope objective.
5. Carefully place a drop of water (or buffer if using a thinned skull window) over the window and lower the objective into the liquid.

Ensure that there is sufficient liquid under the objective to maintain a 'column' between the cranial window and the end of the objective. Also ensure that liquid is not dripping off the side of the head; if it is, raise the objective, dry the skull and skin completely, and reapply water or buffer. Once the liquid has found a path off the head, it will continue to follow that path and it is unlikely you will be able to collect data successfully.
6. Use visible light illumination to focus on the surface of the brain. Either use white light illumination to focus on the surface vasculature, or fluorescence illumination to locate the cells or structure of interest.

Note that some of the newer GECIs have low baseline fluorescence, so unless a non-calcium sensitive fluorescent protein is also expressed in the same or nearby cells, locating the surface vasculature under white light illumination is likely to be the best approach.
7. Locate a region of interest to be imaged; for chronic windows, ensure that you will be able to relocate this region based on the pattern of surface vasculature. Do not rely solely on the co-ordinate system of the microscope.
8. Set up the required image acquisition parameters: zoom, pixel size, pixel dwell time, z-stack parameters (size, number of optical sections, step size between optical sections) and time series parameters, and stimulus triggering. The details of these parameters will depend on the microscope and GECI being used, and the experimental goals.

There will be a tradeoff between acquisition speed and resolution, depending on the experimental goals.
9. Continue collecting data as required. Throughout the experiment, carefully monitor anesthesia, and check the water column under the objective at frequent intervals and top up as required.
10. Once data acquisition is complete for an experimental design comprising multiple imaging sessions, administer a ~200 μ l bolus of subcutaneous warmed lactated Ringer's solution and recover the mouse in a clean cage placed partially

on a heat pad. Monitor the mouse until ambulatory behavior has recovered, then return to the home cage.

11. For an acute experiment, or the final time point of a chronic experiment, the mouse should be euthanized according to institutional and national guidelines. If histological analysis of tissue is required, the mouse can undergo transcardial perfusion with appropriate fixatives at this point.
12. For subsequent imaging sessions, repeat steps 1–6. In step 4, take particular care to secure the mouse at the same angle as used in the previous imaging session.

Rotational shifts will prevent the same population of cells or structures from being visible in any given optical session

13. Relocate the previously imaged region and align the field of view to the same x, y, and z positions. For the first region of interest, beginning with the settings used in the previous session, carefully adjust the excitation power to obtain images with baseline fluorescence emission intensity as similar to the previous session as possible.

Note that increasing the laser power above ~50 mW at the back focal plane of the objective may cause photodamage, depending on the scan speed used. Note also that the amplitude of spontaneous or stimulus-evoked calcium transients may have changed between imaging sessions as a result of experimental manipulations being performed, e.g. behavioral training, altered sensory experience, so use of baseline GECI fluorescence, or fluorescence from a non-calcium responsive fluorescent protein should be used to set excitation power.

14. Repeat for the other regions of interest, then repeat steps 9–12 as required to collect calcium imaging data at all time points necessary.

REAGENTS AND SOLUTIONS

MAKING STERILE CORTX BUFFER

This is a protocol for making sterile HEPES-buffered artificial cerebrospinal fluid (ACSF). Cold sterile cortex buffer is applied to the surface of the brain during cranial window surgery to cool the brain between drilling sessions, clear and prevent any bleeding, and promote visualization of the underlying vasculature. The buffer contains (in mM): 125 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 CaCl₂, 2 MgSO₄.

Materials

Sodium chloride
Potassium chloride
Glucose
HEPES
1M CaCl₂ solution

1M MgSO₄ solution
Deionized water
Autoclavable glass bottle
Autoclave tape
Sterile 15ml conical tubes
Autoclave
pH meter
Magnetic stirrer

Solution preparation

1. Dissolve 7.21g NaCl, 0.37g KCl, 1.80g glucose and 2.38g HEPES in 900ml deionized water
2. Add 2ml of 1M CaCl₂ solution, and 2ml 1M MgSO₄ solution.
3. Check that pH is 7.4 and adjust with a small volume of HCl or NaOH if required (pH should be close to 7.4; if not, check the pH of the deionized water and if not close to pH7, find an alternative source)
4. Make solution up to 1000 ml and transfer to autoclavable glass bottle
5. Autoclave solution for at least 20 minutes to sterilize.
6. Allow to cool, make 10ml aliquots in sterile 15ml tubes and store at –20 °C
7. To use, thaw an aliquot completely and keep on ice

COMMENTARY

Background Information

Most biologically relevant applications of multiphoton microscopy in fact refer to 2-photon fluorescence microscopy. For fluorescence to occur, a photon of sufficient energy to raise an electron from its ground state to an excited state must be absorbed. The energy of a photon is inversely proportional to its wavelength. Two-photon excitation occurs when a fluorophore absorbs two photons of half the energy (double the wavelength) required for single photon excitation near-simultaneously i.e. within ~1 attosecond (10^{-18} second). A high photon flux is therefore required to provide a significant probability that 2-photon excitation will occur. This is achieved by using mode-locked i.e. ultra-fast pulsed laser sources at a high repetition rate; by concentrating all the photons into these discrete pulses, the peak power is much higher than the average power of the laser. Hence, the high peak power allows 2-photon excitation to occur, while the low average power minimizes photodamage.

Two-photon excitation increases the depth at which high resolution fluorescence imaging can be performed in two main ways. First, the longer wavelength light used for excitation undergoes less scattering within the tissue. Second, because absorption depends on the square of the excitation power, photon density is sufficiently high for 2-photon absorption

only in the focal plane. As well as minimizing out-of-plane photobleaching and phototoxicity, this means that optical sectioning is achieved without the use of a pinhole in front of the detector(s). Hence, scattering of emitted photons, which increases with tissue depth, is less detrimental than in confocal microscopy because many scattered photons can still reach the detector. 3-photon excitation is analogous but involves the near-simultaneous absorption of 3 photons of approximately three times the wavelength required for single photon excitation. Although some specialized applications have been published, the requirement for 10-fold greater peak power than for 2-photon excitation means that 3-photon excitation microscopy has not been widely used for intravital imaging.

Early *in vivo* 2-photon calcium imaging studies in neurons were performed by loading organic calcium indicator dyes into individual neurons via intracellular recording pipettes (Chapack, Mertz, Beaurepaire, Moreaux, & Delaney, 2001; Helmchen, Svoboda, Denk, & Tank, 1999; Svoboda, Denk, Kleinfeld, & Tank, 1997; Svoboda, Helmchen, Denk, & Tank, 1999). For example, the first such study used 2-photon imaging of iontophoretically-loaded calcium green-1 responses in dendrites of individual pyramidal cells in rat whisker barrel cortex to image spontaneous and sensory-evoked activity (Svoboda et al., 1997).

The next advance was the ability to bulk load populations of neurons with organic calcium indicators. Taking advantage of the anatomical organization of the mouse olfactory system, olfactory sensory neurons in the nose were bulk loaded with calcium green dextran, which was trafficked anterogradely, enabling 2-photon imaging of odor-evoked calcium responses in olfactory sensory neuron axons in the olfactory bulb (Wachowiak & Cohen, 2001). A more broadly applicable multi-cell bolus loading approach was developed shortly thereafter (Stosiek, Garaschuk, Holthoff, & Konnerth, 2003). This involved pressure ejection of membrane-permeant acetoxymethyl (AM) esters of dyes such as Fura-2 or Fluo-4 into the brain, and enabled simultaneous imaging of calcium signals in tens to hundreds of neurons for several hours (Stosiek et al., 2003).

The advent of genetically-encoded calcium indicators (GECIs) has enabled calcium imaging to be performed in a cell type-specific and temporally controlled manner; expression to be directed to specific subcellular compartments; and longitudinal experimental designs comprising multiple imaging sessions spread over days to months to be employed. The first GECIs were Förster resonance energy transfer (FRET)-based calcium sensors developed in the lab of Roger Tsien (Miyawaki et al., 1997) and dubbed ‘cameleons’ because fluorescence emission shifted from the blue/cyan wavelength range to the green/yellow wavelength range in the presence of calcium. They consisted of a tandem fusion of a blue or cyan fluorescent protein (B/CFP), calmodulin (CaM), the CaM-binding peptide M13 (derived from myosin light-chain kinase), and green or yellow fluorescent protein (G/YFP). Calcium binding to CaM caused a conformational change that enables it to wrap around M13, increasing FRET between B/CFP and G/YFP such that excitation at the short wavelengths that elicit only B/CFP fluorescence in the absence of calcium, also elicit G/YFP fluorescence.

Over the ensuing 20 years, numerous labs have worked both to generate novel GECIs and to improve the sensitivity, kinetics and signal to noise ratios of existing GECIs by structure-

guided mutagenesis. GECIs fall into two broad classes: single fluorophore sensors, and (FRET)-based sensors. The family of GECIs most widely used for intravital multiphoton imaging is the GCaMP indicators. They consist of circularly permuted GFP (cpGFP) fused to CaM and the M13 peptide. In the absence of calcium, the chromophore is protonated and fluorescence emission is low. Calcium binding to CaM enables it to wrap around M13, eliminating solvent access to the chromophore, which is rapidly deprotonated resulting in bright fluorescence. The current published generation, GCaMP6 (available in fast (f), medium (m) and slow (s) variants which differ in their kinetics), is the first to surpass the kinetics and sensitivity of the best organic calcium dyes (Chen et al., 2013), with GCaMP6f enabling detection of single action potentials *in vivo*. GECIs with more red-shifted excitation and emission spectra and sensitivity that approaches that of GCaMP6 have also recently been developed (Dana et al., 2016; Inoue et al., 2015). There are several advantages to red-emitting GECIs, including greater tissue penetration with the same excitation power due to reduced scattering and tissue absorption of longer excitation and emission wavelengths; lack of spectral overlap with channelrhodopsins, permitting all-optical stimulation and recording; and the ability to perform simultaneous multi-color calcium imaging in different neuronal types or subcellular structures.

An alternative approach to monitor the activity of excitable cells is to use a genetically encoded voltage indicator (GEVI) to provide a direct readout of electrical signals. GEVIs in principle offer two main advantages over GECIs: millisecond temporal precision that should enable trains of action potentials to be followed, and the detection of hyperpolarization. The first GEVI, a fusion protein of a modified GFP with a potassium channel voltage-sensing domain, was described in 1997 (Siegel & Isacoff, 1997). Since then, protein engineering has resulted in a palette of GEVIs with improved kinetics and signal to noise ratios (Gong, 2015; Lin & Schnitzer, 2016). There are two broad classes. The first consists of fluorescent proteins fused to voltage-sensing domains of ion channels or voltage-sensitive phosphatases. A voltage readout is provided by changes in the conformation of the voltage-sensing domain, which either directly alters the environment of the fluorescent protein chromophore to affect fluorescence emission, or alters FRET between a pair of attached fluorescent proteins. The second, more recently developed, class of GEVIs are fusions of the microbial proton pump Archaelhodopsin 3 and fluorescent proteins (Kralj, Douglass, Hochbaum, MacLaurin, & Cohen, 2011). However, although development is ongoing, current GEVIs in both classes still lack the sensitivity required for intravital imaging in the mouse brain.

The invention of the 2-photon fluorescence microscope was published in 1990 (Denk, Strickler, & Webb, 1990): this was the first application of the experimentally demonstrated principle of 2-photon excitation (Kaiser & Garrett, 1961) to laser scanning microscopy. The first commercial system became available in 1996, and numerous 2-photon microscope systems, from self-built designs to turnkey commercial systems, are now in labs worldwide. Femtosecond-pulsed titanium sapphire sources that are continuously tunable from ~680 – 1040 nm (Spectra-Physics Mai Tai; Coherent Chameleon; ThorLabs Tiberius) have become the workhorse laser for biological 2-photon microscopy. Near-infrared lasers with a broader tuning range (680 – 1300 nm) and increased peak power at ~900nm, which is of greater utility for excitation of fluorescent proteins, GECIs and optogenetic actuators, are also becoming increasingly popular (Spectra-Physics Insight DeepSee and X3; Coherent

Discovery). 2-photon microscopes are also continuously being updated, with many modern systems now benefiting from fast acquisition due to resonant-galvanometer scanners and increased sensitivity due to the incorporation of GaASP detectors.

There are several advantages to using 2-photon microscopy for intravital calcium imaging; chief among these is the increased tissue depth at which imaging can be performed while minimizing photodamage. By comparison, greater scattering of visible excitation wavelengths combined with loss of emitted photons due to the requirement for a pinhole in order to achieve optical sectioning mean that confocal microscopy is not a viable approach at depths above 100 microns. Photobleaching due to out-of-plane excitation further limits the utility of confocal microscopy for intravital imaging. However, it should be noted that 2-photon microscopy does not provide any resolution increase relative to confocal microscopy.

Light sheet or selective plane illumination microscopy (SPIM) has also been used for intravital calcium imaging, and offers higher axial (z) resolution than 2-photon imaging. SPIM employs an illumination light sheet perpendicular to the imaging axis, resulting in an optical section the thickness of the light sheet and by confining fluorescence to the illuminated plane, minimizes photodamage in a manner analogous to 2-photon imaging. However, the perpendicular geometry of the illumination source precludes its use for intravital imaging through a cranial window. Widefield single-photon intravital calcium imaging lacks the spatial resolution to image subcellular structures and the depth penetration of 2-photon excitation. However, cellular resolution widefield calcium imaging can be performed through a cranial window, or if low spatial resolution imaging over a large field of view (mesoscopic imaging) is the goal, even through the intact skull (Silasi, Xiao, Vanni, Chen, & Murphy, 2016; Steinmetz et al., 2017). Remarkably, implantable 2-photon (Helmchen, Fee, Tank, & Denk, 2001) and widefield (Cai et al., 2016; Flusberg et al., 2008) microscopes have also been developed, which enable the detection of cellular resolution calcium signals in freely moving mice.

Critical Parameters

- The success of intravital imaging is critically dependent on both the GECI expression level and the density of GECI-expressing cells being appropriate to the goals of the experiment (see Troubleshooting). For example, population calcium imaging requires dense expression, whereas longitudinal tracking of calcium responses at individual axonal boutons requires much sparser expression.
- The clarity of the cranial window is also critical. For acute cranial windows, it is vital that blood does not clot on the surface of the dura, and that there is no additional dural bleeding once the coverslip has been implanted. Clotted blood on the dural surface will prevent imaging under that specific area, while additional dural bleeding will result in a gradual reduction in the clarity of the window and hence the signal to noise ratio of the acquired images.
- For chronic cranial windows, and particularly for experiments in which there is a long interval between imaging time points, the location of the selected regions to be imaged within the window is important. The skull is likely to regrow

gradually over time, so imaged regions close to the edges of the window, or close to any residual skull, may become obscured at subsequent time points.

- Be aware of the capabilities and limitations of the laser and microscope that you will be using when designing an experiment. For example, titanium-sapphire lasers may not provide sufficient power at the longer wavelengths required for excitation of red GECIs, and a resonant scanner may be required to achieve sufficient temporal resolution. Consult published 2-photon absorption spectra, which are available for many of the widely used fluorescent proteins (Drobizhev, Makarov, Tillo, Hughes, & Rebane, 2011; Drobizhev, Tillo, Makarov, Hughes, & Rebane, 2009), from which most GECIs are derived.
- The laser power delivered to the sample should be carefully calibrated to provide sufficient power for 2-photon excitation, without causing photobleaching or phototoxicity. Typically the laser power delivered through the objective should be 10 – 50mW to enable data collection at multiple time points without inducing photodamage.
- For chronic imaging, the ability to reposition the mouse at exactly the same angle as in the previous imaging session is essential to enable the same cells and/or structures to be repeatedly imaged over time. Careful consideration should be given in advance to the design of the head bar, imaging frame and microscope in order to permit this.

Troubleshooting

See Table 1.

Statistical Analyses

The details of statistical analysis will depend on the exact nature of the experimental design. Some general points to consider are:

1. Whether parametric statistics are appropriate.
2. That a repeated measures design is likely required for data collected from the same cells or structures at multiple time points.
3. Whether n should be considered the number of cells/synapses/regions of interest, or the number of animals; and how potential clustering of cells from the same animal can be controlled for, for example using multiple linear regression.
4. The threshold used to determine whether a calcium response is considered significant. This must be set carefully to minimize both false positive and false negative classification of responses.

Anticipated Results

The protocols described in this unit enable collection of intravital calcium imaging data from populations of hundreds of cells (typically neurons) simultaneously, or from tiny subcellular compartments such as dendritic spines and axonal boutons. Detailed protocols for a range of cranial window types are provided to permit experimental designs spanning from a single

imaging session of a few hours, to chronic longitudinal imaging of the same regions of interest for days, weeks or even months. The number of cells or structures from which calcium responses can be collected will depend upon the cell type of interest, the proportion of that cell type that express the GECI, and the clarity of the cranial window.

A detailed protocol for analysis of intravital calcium imaging data is not provided, as the analytical approach and design will be strongly dependent on the type of data that has been collected. One important decision is the software to be used for image analysis: many of the microscope manufacturers have analysis modules that are either included with the acquisition software, or can be purchased separately. Commercial image analysis platforms, such as Imaris (RRID:SCR_007370 <http://www.bitplane.com/Imaris/Imaris>) or MetaMorph (RRID:SCR_002368 <http://www.moleculardevices.com/Products/Software/Meta-Imaging-Series/MetaMorph.html>) provide a second option. Alternatively, many labs prefer to write their own image analysis code in ImageJ (RRID:SCR_003070 <https://imagej.nih.gov/ij/>) or Matlab (RRID:SCR_001622 <http://www.mathworks.com/products/matlab/>). Another important decision is what data need to be extracted from the acquired images. Typically, this will consist of a fluorescence time course for each cell, region or structure of interest, which is then used to calculate the $\Delta F/F$, i.e. the change in fluorescence relative to the baseline, and kinetic parameters of the responses.

Figure 1 provides an example of 2-photon intravital calcium imaging data acquired through an acute open skull window over the mouse olfactory bulb. The responses of neurons (Fig. 1Bi) and dendrites (Fig. 1Bii) are also quantified as the $\Delta F/F$. Figure 2 provides an example of data acquired through a chronic cranial window, implanted over the olfactory bulb 2–3 weeks prior to 2-photon image acquisition. This figure illustrates both within-session data collected in response to different olfactory stimuli, and longitudinal data collected at different time points.

Time Considerations

Cranial window implantation—Each of the surgical procedures for cranial window implantation will take 1–3 hours, depending on the thickness of the skull and the amount of bleeding from the trabecular bone and/or dura that is encountered.

Image acquisition—The length of the imaging session will depend on the experimental design. For repeated longitudinal imaging designs using chronic cranial windows, each imaging session will typically be 45 min – 1.5h. For single acute imaging sessions, the duration will depend upon the experimental design. The total time for which anesthesia can be stably maintained, which will depend on the age of the mouse and the anesthetic agent, and may vary with genotype, needs to be accounted for when designing the imaging experiment.

Image analysis—The time required for image analysis will typically be significantly longer than that needed for image acquisition, and for each mouse, can range from hours to days. The amount of time required will depend on the image analysis software used; the degree to which the analysis is automated; the number of time points and the number of cells and/or regions of interest from which data have been collected. Significant time may also be

required initially to establish image analysis procedures and/or write the necessary image analysis code.

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Significance Statement

This unit describes protocols for monitoring of intracellular calcium concentration in the brains of living mice. Two-photon microscopy is an imaging technique that can provide detailed 3-dimensional images of either large numbers of cells or tiny subcellular compartments over time, without damaging the brain. Changes in the brightness of fluorescent calcium indicators provide a readout of the amount of calcium present over time. This can provide important information about neuronal activity and signaling pathways within cells, for example in response to sensory stimuli or during learning. The protocols in this unit describe how to create an imaging window by replacing a small piece of the skull with glass, and how to acquire 2-photon calcium data.

Figure 1. 2-photon intravital calcium imaging of tufted cells expressing GCaMP6f under control of the CCK promoter in the mouse olfactory bulb

(A) Two trials showing peak responses of GCaMP6f-expressing neurons and their dendrites to a three second duration odor stimulus. Overlaid letters and numbers in Trial 2 correspond to traces shown in B. Cell A responded to the odor while cells B-G did not. (Bi) F/F for numbered tufted cells shown in lower left panel of A. (Bii) F/F for tufted cell dendrites in regions of interest (ROIs) marked in lower right panel of A. ROIs 1–5 are odor-responsive; ROIs 6–7 are not. Significant responses had a peak amplitude of at least three times the standard deviation of the baseline during the one second period prior to odor delivery. Modified with permission from (Cheetham, Grier, & Belluscio, 2015) under a CC BY license.

Figure 2. 2-photon intravital calcium imaging of olfactory bulb neurons transduced with AAV2/1-CAG-GCaMP6s

Data were collected from an adult C57BL6/J mouse (RRID:IMSR_JAX:000664) 2–3 weeks after chronic open skull cranial window implantation. Central panel shows odor evoked responses of multiple neurons. Left panel shows responses of the same neurons to a different odor, within the same imaging session. Right panel shows the same field of view imaged on a different day. Stimulus-evoked responses have changed following manipulation of sensory experience.

Table 1

Troubleshooting

Problem encountered	Causes and potential solutions
GECI expression is too sparse	Only a subpopulation of the cell type of interest are driven to express the GECI by the selected promoter. Consider alternative promoters or expression strategies.
GECI expression is too dim or weak	This suggests a low expression level. Try using an alternative strategy e.g. tetO driver lines typically drive stronger expression than Cre drivers; or consider using a viral expression strategy.
GECI-expressing cells are too dense	This may arise simply due to the anatomy of the region being imaged. Consider using an inducible expression strategy such as a tamoxifen-inducible Cre line, or a viral strategy in which the titer can be titrated to achieve the desired labeling density.
GECI expression is too strong, resulting in toxicity to expressing cells or other side effects e.g. seizures	Use a different expression strategy. Note that mice expressing both Emx1-Cre (RRID:IMSR_JAX: 005628) and Ai93 (RRID:IMSR_JAX: 024104) transgenes exhibit widespread large amplitude electrical activity and occasionally undergo seizures under baseline conditions (Steinmetz et al., 2017)
Excessive bleeding from the skull	Apply Gelfoam soaked in cortex buffer and wait. If the bleeding area is on the side of the bone, apply lateral pressure with a cotton swab. Do not exert downward pressure as this will damage the brain.
Bruised appearance of the brain when skull is removed or under thinned skull window	Indicates subdural bleeding and likely damage to underlying brain. Exclude the mouse. In future preparations, drill more slowly and apply cold cortex buffer frequently.
Glue or dental cement runs onto surface of dura	Exclude the mouse. In future preparations ensure that sufficient buffer is present under the coverslip and if coverslip does not lie flat on skull, hold gently with fine forceps as glue is applied.
The chronic cranial window is milky when the scalp is re-opened	The window is infected. Administer systemic antibiotics and monitor the window for an additional 1–2 weeks. If clarity does not improve, exclude the mouse.
Bone has regrown under the cranial window	If bone has completely regrown, discard the mouse, or remove the coverslip and regrown skull to obtain a final imaging time point. If bone regrowth is partial and the experiment requires only days to a week of imaging, it may still be possible to obtain data: choose regions of interest as far from the encroaching skull as possible.
Gradual increase in the laser power required to obtain a comparable signal across experimental days when imaging through a chronic cranial window	This may indicate thickening of the dura or gradual encroachment of a thin sheet of bone under the window. Monitor the excitation power carefully and do not increase to a level that will induce photodamage. Also monitor the signal to noise ratio and consider a minimum cutoff value beyond which further data will not be collected.
Loose or broken coverslip in chronically implanted mice	Mouse may have knocked window on cage roof, food hopper, etc. or there may have been insufficient attachment with glue and/or dental cement. Exclude the mouse.
Previously bright structures gradually become dimmer during the imaging session	The water column between the coverslip and objective is being lost; reapply water. There is dural bleeding, releasing red blood cells under the window; check for this under white light illumination. The experiment may have to be stopped.
Fluorescence signal is suddenly lost	The water column has been abruptly lost. Reapply water.
Large amplitude motion artifacts appear in the image	Mouse is waking up from anesthesia; check pedal reflex and increase concentration of inhalant anesthetic or administer top-up dose of injectable anesthetic or sedative. Mouse is gasping due to an excessive concentration of inhalant anesthetic; check for absence of pedal reflex and reduce percentage anesthetic. This agonal breathing usually indicates imminent death. Head bar has come loose from the skull; stop imaging and attempt to reattach the head bar. Beware that the orientation of the window will change unless the head bar is reattached in exactly the same position.
Very bright spots appear in the image and rapidly increase in area	Indicates tissue photodamage; reduce laser power. Check that mouse has not died; blood circulation continuously cools the tissue, preventing photodamage.