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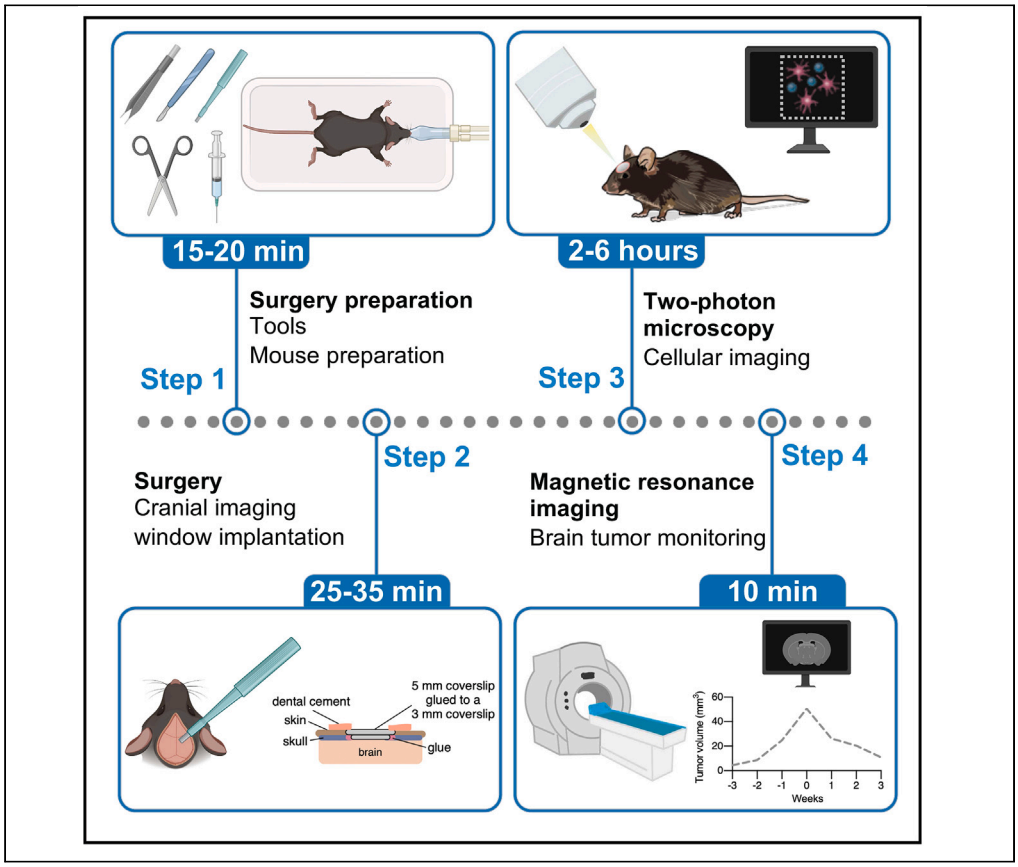
Cranial imaging window implantation technique for longitudinal multimodal imaging of the brain environment in live mice



Intravital two-photon microscopy of the mouse brain requires visual access without affecting normal cognitive functions, which is crucial for longitudinal imaging studies that may last several months. In this protocol we describe the surgical implantation of a metal-free cranial imaging window, which can be used to perform two-photon microscopy and magnetic resonance imaging in the same animal. This multimodal imaging platform enables the investigation of dynamic processes in the central nervous system at a cellular and macroscopic level.

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

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Highlights

Cranial imaging window (CIW) for multimodal imaging by MRI and two-photon microscopy

Craniotomy made using a biopsy punch allows for precise placement of the CIW

MRI and two-photon microscopy data can be collected from the same individual mice

This allows the correlation of macroscopic and microscopic changes in biology and disease

Croci et al., STAR Protocols 4, 102197
June 16, 2023
https://doi.org/10.1016/j.xpro.2023.102197

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Cranial imaging window implantation technique for longitudinal multimodal imaging of the brain environment in live mice

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https://doi.org/10.1016/j.xpro.2023.102197

SUMMARY

Intravital two-photon microscopy of the mouse brain requires visual access without affecting normal cognitive functions, which is crucial for longitudinal imaging studies that may last several months. In this protocol, we describe the surgical implantation of a metal-free cranial imaging window, which can be used to perform two-photon microscopy and magnetic resonance imaging in the same animal. This multimodal imaging platform enables the investigation of dynamic processes in the central nervous system at a cellular and macroscopic level.

For complete details on the use and execution of this protocol in the context of brain cancer, please refer to Zomer et al.[1]

BEFORE YOU BEGIN

Two-photon intravital microscopy (2P-IVM) imaging allows the *in vivo* assessment of complex cellular processes under normal conditions, such as stem cell behavior[2] and the immune system,[3] or under pathological conditions including cancer[4] and infection.[3,5] In contrast to static analyses, 2P-IVM enables the tracking of cellular dynamics in a longitudinal manner in the same individual animal.[1,6] Central to this approach is the development of imaging windows that can be surgically implanted in mice to allow visual access to internal organs or tissues over weeks, while minimally affecting the animal welfare. Here we describe a surgical procedure for the implantation of a cranial imaging window (CIW) into the skull, thereby permitting 2P-IVM of the meninges and the underlying brain parenchyma. The detailed protocol presented herein has been previously used to assess the dynamic changes of the tumor microenvironment (TME) in genetically-engineered mouse models (GEMM) of gliomagenesis.[1] Importantly, our CIW does not contain any metallic components, thereby allowing magnetic resonance imaging (MRI) of the brain. MRI is fundamental in the clinical management of brain pathologies such as multiple sclerosis,[7] traumatic brain injury[8] and cancer,[9] and has been shown to be a potential tool to investigate the TME non-invasively in murine models of brain malignancies.[10] Combining 2P-IVM and MRI in the same animal allows one to correlate (sub) cellular dynamics measured by 2P-IVM with disease stage measured by MRI. In the long-term, this approach may promote the clinical translation of cellular MRI approaches and help to explain the cellular basis of MRI changes in patients.

In the protocol presented herein, we describe the CIW preparation, followed by explanation of the surgical procedure to implant the CIW into the skull. Optionally, high-grade gliomas can be induced by taking advantage of the replication-competent ALV-splice acceptor (RCAS) retroviral delivery strategy. This method allows the introduction of particular genetic alterations in a cell type-specific manner in cells that express tva, the receptor for the RCAS virus.[11] We describe the production of the head-bar, based on earlier designs,[12,13] enabling the mouse's head to be secured during 2P-IVM experiments, and to place the CIW perpendicular to the microscope's objective. For this purpose, we provide schematic drawings of the head-bar and head-bar holder which can be used for 3D printing; the STL files for production of this head-bar and head-bar holder are provided in the supplemental information. The device presented herein allows immobilization of the mouse's head during 2P-IVM experiments in almost every angle and position of interest. However, other immobilization devices can also facilitate exact re-positioning of the same mouse over multiple 2P-IVM sessions, albeit with less flexibility, and may be compatible with inverted two-photon microscope systems. Such setups include a stainless steel[6] or plastic ring[14] that can be fixed in a imaging box containing a CIW insert.

Institutional permissions

All mice were bred within the University of Lausanne animal facilities, and all animal studies were approved by the Institutional Animal Care and Use Committees of the University of Lausanne and Canton Vaud, Switzerland (License numbers: VD3269 and VD3636). Standard autoclaved lab diet and water were provided *ad libitum*. Mice were housed in an individually ventilated cage (IVC) housing system under a 12-h light/ dark schedule at 22°C, in the presence of 1–4 cage mates (2–5 mice per cage in total). We used standard IVC cages (GM500 from Tecniplast) with a regular wire lid. In our studies, we never found a mouse stuck with its head bar in the wire lid. To prevent this from occurring, we recommend to use a wire lid in which the distance between individual wires is larger than the width of the head bar (6.5 mm). Mice did not touch or try to remove each other's CIWs and/or head bars, so there was no requirement to isolate individual animals. Only exceptionally, male mice were housed individually, when they were the last experimental animal in a cage. We used standard cage enrichment, including paper or plastic tunnels and houses. The mice adapted quickly (< 3 days) to their head bar/ CIW, and were perfectly able to navigate through their cage enrichment materials.

Cranial imaging window preparation

Timing: 1 h 30 min

This section describes the steps necessary to prepare the cranial imaging windows (CIWs) prior to the surgery. It is recommended to prepare more CIWs than necessary, since they can break. The glue that is used to prepare the CIWs, Norland optical adhesive 61, is designed for bonding lenses, prisms and mirrors for military, aerospace and commercial optics, and its characteristics achieve long term performance under dynamic environments. We did not test the shelf-life of glued CIWs, but based on the physical properties of cured Norland optical adhesive 61, we expect that unused CIWs can be stored for an unlimited amount of time.

1. Aliquot one drop of Norland optical adhesive 61 in a 1.5 mL Eppendorf tube.
2. Place the 5-mm diameter coverslips in a 60 mm Petri dish.

⚠ CRITICAL: The coverslips are fragile. Use surgical forceps to gently handle them. Use blunt forceps to minimize the risk of scratching the coverslips.

Note: The coverslips do not need to be sterile, and the gluing does not need to be performed in a laminar flow hood. Sterilization will be achieved through exposure to UV light for 1 h in step 5.

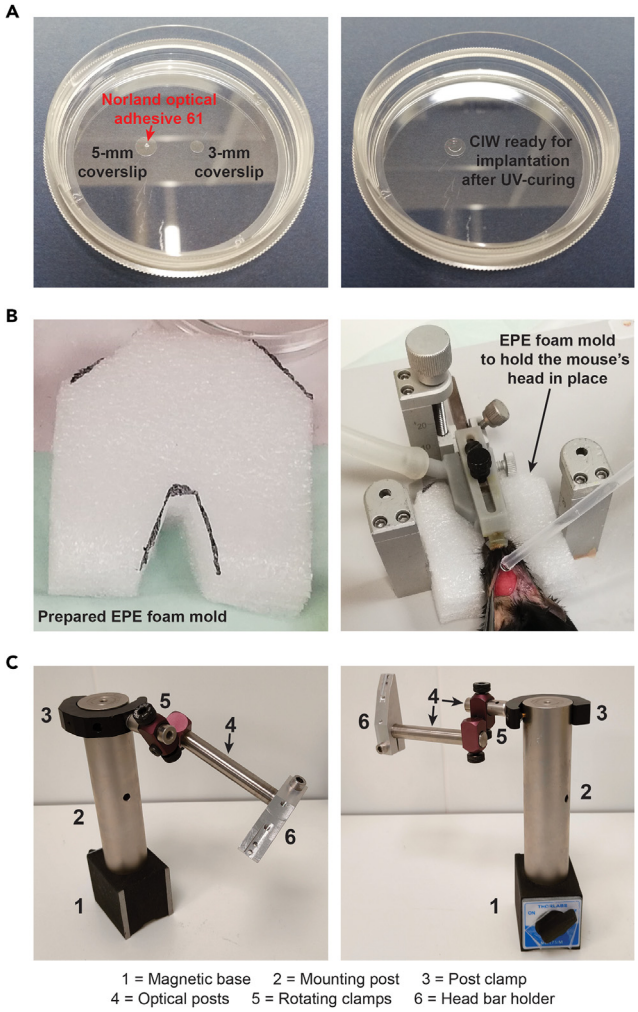

Figure 1. Before you begin: Prepare CIWs, EPE foam molds, and the head-bar holder

(A) Prepare CIWs by gluing a 3-mm coverslip to a 5-mm coverslip using Norland optical adhesive 61.

(B) Prepare EPE foam mold (left) to fix the mouse's head during the surgery (right).

(C) Build the head-bar holder to fix the mouse with its head bar during subsequent 2P-IVM experiments. Components 1–5 can be purchased at Thorlabs (see key resources table), and component 6 can be 3D-printed (see supplemental information for STL files of the head-bar holder and the head bar).

3. With the aid of a 10 µL pipette, put a tiny drop of Norland optical adhesive 61 in the center of the 5-mm diameter coverslip, simply by touching the coverslip with the pipette tip that was dipped in Norland optical adhesive 61 (Figure 1A).
4. Place one 3-mm diameter coverslip on top of the drop of adhesive on each 5-mm diameter coverslip.

Note: If the Norland optical adhesive 61 does not spread homogenously between the two coverslips automatically, gently apply some pressure with blunt forceps.

Note: When gluing the CIWs, one may want to vary the position of the 3-mm coverslip relative to the 5-mm coverslip with some coverslips slightly off-center. During the surgery, the amount of space that is available for the fixation of the coverslip to the skull may be limited, especially on the side of the eye, and in those cases an "off-centered" CIW, with a smaller surface for fixation on one side, can be used.

⚠ CRITICAL: Do not exceed the recommended amount of Norland optical adhesive 61 on the coverslip. If a large amount leaks out from the edge of the 3-mm diameter coverslip, it is preferable to discard the CIW, as it might not subsequently fit the surgical aperture in the skull. Conversely, make sure to not use insufficient amounts of glue, as this may lead to air between the coverslips.

5. Place the coverslips in a tissue-culture hood under the UV lamp emitting UV-C radiation at 254 nm for 1 h to cure the Norland optical adhesive 61. Alternatively, another UV lamp or source can be used; in this case the adhesive curing time may be different and needs to be tested empirically.

Note: Store the prepared CIWs in a petri dish until use. On the day of the surgery, fill the petri dish with 70% ethanol as an additional sterilization step. 70% ethanol is not reported as a solvent of Norland optical adhesive 61, but to ensure a prolonged proper adhesion of the CIW, we advise to not keep the coverslips in 70% ethanol longer than 1 day, and to not re-use or store CIWs that have been exposed to 70% ethanol.

Expanded polyethylene (EPE) foam mold

Timing: 15 min

Ear bars that are usually used in a stereotaxic frame during mouse brain surgeries limit the freedom of movement during the CIW implantation. Therefore, we recommend to remove the ear bars and associated supports from the stereotaxic frame. Instead, we make use of home-made EPE foam molds (Figure 1B) to hold the mouse's head in place during the surgery described herein. When the mold is made for the first time, it is best to fit and adjust the EPE foam sheet in the stereotaxic frame until there is a perfect fit. This mold can then be used as a standard for new molds.

6. We advise to remove the ear bars and screws of the ear bar holders from the stereotaxic device, to increase the freedom of movement during the CIW implantation.
7. Bring a piece of ~2 cm-thick EPE foam to the stereotaxic device, and cut it in such a way that it fits between the ear bar holders.
8. At the location of the mouse snout clamp and tooth bar, remove one or more layers of EPE foam to allow the snout clamp and tooth bar to "sink in" the EPE foam when lowering them.
9. Completely remove the EPE foam at the predicted location of the mouse's head.
10. Test the mold by fixing an anesthetized mouse inside it.

Note: The mouse's head must be held in place when slightly turning its tail and the mouse must be laying in a vertical and straight position on the stereotaxic frame. Adjust the foam where necessary.

Note: The perfect-sized mold should hold the head of the mouse in place, while retaining freedom of movement for the surgery.

⚠ CRITICAL: Carefully check that the EPE foam mold is not restricting the mouse's breathing. If this is the case, remove a bit more foam to release the pressure on the mouse's respiratory system.

Optional: DF-1 cell preparation

Timing: 30 min

DF-1 cells allow the delivery of the RCAS virus to induce glioma formation in Nestin-Tva-positive transgenic mice. For further details on this glioma model, see.[11] Alternatively, other cell lines can

be used depending on the required brain tumor model, or another brain disease can be induced (other than cancer). In the case of studying brain cancer, we recommend to induce tumor formation simultaneously with the surgical implantation of the CIW. Advanced brain tumors may adhere to the skull; therefore, removing a piece of the skull to implant a CIW in a later stage of tumorigenesis may impact the tumor and its environment, and may lead to bleeding.

Note: PDGF-B GFP DF-1 cells are freshly prepared before starting the surgery, as described below. DF-1 cells were cultured under standard conditions in a 75 cm^2 tissue-culture flask.

11. Prepare DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S), and a smaller aliquot of DMEM with 1% FBS (without P/S). Prewarm both media at 37°C in a water bath.
12. Remove the culture medium and rinse the tissue-culture flask with 1× PBS.
13. Incubate the cells with 2 mL of 0.05% Trypsin-EDTA (1×) for 2–4 min at 37°C.
14. Once the cells detach from the bottom of the tissue culture flask, resuspend them in 10 mL DMEM + 10% FBS + P/S, and transfer the cells to a 15 mL Falcon tube.
15. Count the cell density, either manually or using an automated cell counter.
16. In the meantime, spin the cells for 5 min at 300 × *g*, 4°C.
17. Remove the supernatant and resuspend at a concentration of 100,000 cells/µL in DMEM + 1% FBS in a 1.5 mL Eppendorf tube. The presence of a small percentage of FBS improves cell viability.
18. Place the cells on ice.

⚠ CRITICAL: We recommend not keeping the cells on ice for more than 3 hours. If more time is required for the surgeries, we suggest preparing multiple batches of fresh cells throughout the day.

Build head-bar holder

Timing: 15 min

The head-bar holder is used to fix the mouse during 2P-IVM experiments.

19. 3D print the head-bars and head-bar holder (see supplemental information for STL files).

Note: We recommended to use a strong, biocompatible and autoclavable plastic for the head-bars, for example polyether ether ketone (PEEK).[15–18] For the head-bar holder, a metal such as stainless steel or aluminum can be used.

20. Build the head-bar holder using the Thorlabs components (see key resources table) and according to the instructions in Figure 1C.

Note: We recommend producing multiple head-bars to be able to perform surgeries on several mice at the same time. After the experiment, the head-bar can be removed, cleaned with acetone to remove the dental cement and subsequently autoclaved and re-used.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dental cement powder	Paladur, Kaladent	2260240 - 100 g
Dental cement liquid	Paladur, Kaladent	2260210 - 80 mL

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Norland optical adhesive 61	AMP Technica	431508
Loctite 401 super glue (or any other cyanoacrylate)	Henkel	123011
0.5% bupivacaine	Sintetica SA	-
2% lidocaine	Streuli Pharma	-
Temgesic (Buprenorphine)	Indivior	6664853
Phosphoric acid solution	Chem Cruz	Sc-301545
Dafalgan (paracetamol) powder, sweet-tasting formulation	UPSA	-
Other		
Peek head bars	This paper, home-made	supplemental information
Head bar holder	This paper, home-made	supplemental information
Magnetic base 175# pull with M6 mounting hole	Thorlabs	MB175/M
Mounting Post, M6 Taps, L = 150 mm	Thorlabs	P150/M - Ø1.5"
Slip-On Post Clamp, Metric	Thorlabs	C1498/M - Ø1.5"
Optical Post, SS, M4 Setscrew, M6 Tap, L = 40 mm	Thorlabs	TR40/M-P5 - Ø12.7 mm
Rotating Clamp for Ø1/2" Posts, 360° Continuously Adjustable, 5 mm Hex	Thorlabs	SWC/M-P5
3 mm diameter coverslips, thickness "#0" (= 0.085 mm)	Multichannel Systems	640726
5 mm diameter coverslips, thickness "#0" (= 0.085 mm)	Multichannel Systems	640731
Stereotactic frame with integrated heating pad	Stoelting	53800/53850
Bed Mat	Hartmann	161066
3 mm biopsy punches	Integra Miltex	33-32
Lacryvisc (eye ointment)	Alcon Switzerland SA	530
Sterile alcohol prep pads	Fisherbrand	22-363-750
Betadine solution swab pads	Purdue Products L.P.	-
Scalpels	Swann Morton	0501
Sliding lock forceps	Fine Science Tools	11062-10
Disposable wooden cotton tipped swabs	Jiangsu Suyun Medical Materials	-
Gelfoam	Pfizer	09-0891-04-079
Disposable sterile plastic Pasteur's pipettes	VWR	05072021
Ringer's solution	Clearline	993850
HydroGel	ClearH2O	70-01-5022
0.9% NaCl	Braun	3535800
Micro-drill	Roboz Surgical	RS6300
Multi-photon microscope	Leica	TCS SP8 DIVE

MATERIALS AND EQUIPMENT

Culture medium for DF-1 cells

Reagent	Final concentration	Volume
DMEM	N/A	500 mL
FBS	10%	50 mL
Penicillin–streptomycin	1%	5 mL
Total	N/A	555 mL

Storage at 4°C for up to 1 month.

Phosphoric acid

Reagent	Final concentration	Volume
50% phosphoric acid solution	35%	70 mL
Sterile Milli-Q water	N/A	30 mL
Total	N/A	100 mL

Storage in a glass bottle, at 20°C–25°C in a laboratory safety cabinet. Do not use the diluted phosphoric acid solution past the expiration date of the 50% phosphoric acid solution.

Systemic buprenorphine analgesia		
Reagent	Final concentration	Volume
0.3 mg/mL buprenorphine	0.02 mg/mL (0.05–0.1 mg/kg in mice)	10 μL
Sterile water or 0.9% NaCl	N/A	140 μL
Total	N/A	150 μL
Prepare on the day of surgery and keep at 20°C–25°C until use. Do not store and re-use remaining diluted buprenorphine.		

Local lidocaine/bupivacaine analgesia		
Reagent	Final concentration	Volume
2% lidocaine	4.4 mg/mL (5.5–11 mg/kg)	2 mL
0.5% bupivacaine	2.8 mg/mL (3.5–7 mg/kg)	5 mL
Sterile water	N/A	2 mL
Total	N/A	9 mL
Storage at 4°C, do not use past the earliest expiration date of either lidocaine or bupivacaine.		

Oral paracetamol analgesia		
Reagent	Final concentration	Amount
Paracetamol powder, sweet-tasting formulation	2 g/L (200–400 mg/kg/day per mouse assuming a water intake of 4 mL/day)	500 mg
Drinking water	N/A	250 mL
Total	N/A	250 mL
Protect drinking bottle from light. Prepare on the day of surgery, and use immediately for up to 1 week.		

STEP-BY-STEP METHOD DETAILS

PART 1: Mouse preparation

Timing: 15–20 min

This section will explain how to anesthetize and prepare the mouse before initiating the surgery.

Note: Prepare all autoclaved surgical tools and other materials required during the surgery before starting, and place them next to the stereotactic apparatus (Figures 2A and 2B). The Petri dish containing the CIWs should be filled with 70% ethanol at the start of the surgery for sterilization purposes. The same surgical tools can be used for multiple CIW surgeries, but make sure to extensively clean them with water and subsequently rinse them with 70% ethanol and/or sterilize in a glass bead sterilizer before each individual surgery.

1. Prepare the shaver, one ethanol pad and one betadine pad, ~10 sterile disposable cotton swabs, a scalpel, one 0.5 mL syringe containing 50 μL of the mixture of 4.4 mg/mL lidocaine and 2.8 mg/mL bupivacaine, and one 1 mL syringe containing 100 μL of 0.02 mg/mL buprenorphine.
2. Anesthetize the mouse with isoflurane inhalation anesthesia (3.5% isoflurane/O_2 for induction, and 2% isoflurane/O_2 for maintenance).

⚠ CRITICAL: To assess that the mouse is fully anesthetized, pinch the toes with surgical tweezers. If fully asleep, the mouse will not react.

3. Place the mouse on a heating pad covered by a liquid-absorbable bed mat and ensure continuous isoflurane inhalation anesthesia through a nose mask.

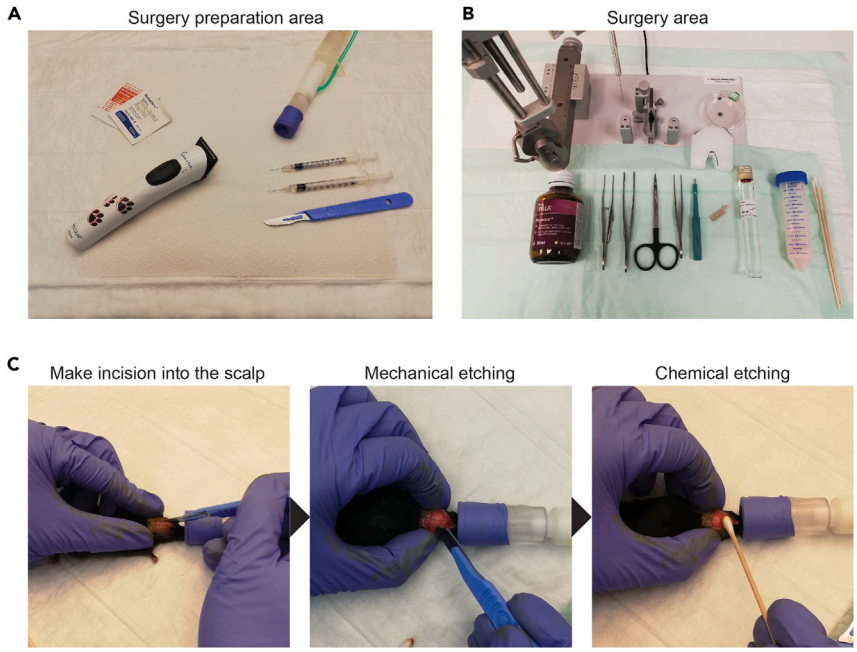

Figure 2. Preparation for the CIW implantation surgery

(A) Prepare the surgery preparation area by placing the following items on a heating pad covered by an absorbent bed mat: a shaver, ethanol and betadine pads, a scalpel, an insulin syringe containing lidocaine/bupivacaine analgesia, an insulin syringe containing buprenorphine analgesia, and the isoflurane anesthesia mask.

(B) Prepare the CIW surgery area by placing the following items on an absorbent bed mat next to the stereotaxic frame: CIWs in 70% ethanol, EPE foam mold, dental cement liquid and powder, surgery tools including forceps and scissors, a 3-mm biopsy punch, an autoclaved head bar, Ringer's solution and sterile cotton swabs.

(C) Prepare the mouse for the CIW implantation surgery by making an incision into the shaved and clean scalp (left image), and by mechanically (middle image) and chemically (right image) etching the skull.

⚠ CRITICAL: Heat loss is rapid in anesthetized mice. Providing a safe supplemental heat source and preventing contact with cold surfaces is critical for maintaining normal body temperature during anesthesia.

4. Shave the fur on the top of the scalp.
5. Apply eye ointment.
6. Clean the shaved scalp with the ethanol and betadine pads sequentially, and repeat this procedure 3 times.
7. Apply eye ointment again.
8. Inject 50 μL of the mixture of 4.4 mg/mL lidocaine and 2.8 mg/mL bupivacaine subcutaneously under the scalp; a small bulge appears.
9. Inject 100 μL of 0.02 mg/mL buprenorphine subcutaneously in the upper back of the mouse.
10. After 2 min, allowing the lidocaine/bupivacaine mixture to take effect, use the scalpel to make an incision into the skin overlaying the scalp (Figure 2C). Cut longitudinally between the eyes as far as the back of the skull.

⚠ CRITICAL: Keep the scalpel blade flat and cut very gently in order to avoid damaging blood vessels in the skull underneath. If bleeding occurs, place a piece of Gelfoam on the damaged site while gently applying pressure and wait for the bleeding to stop.

11. Dry the skull and remove the periosteum with a cotton swab.

12. Mechanically etch the skull with the scalpel by making a pattern of crosses on the bone (Figure 2C).

⚠ CRITICAL: While etching the skull mechanically, avoid cutting or touching the major blood vessels as this can result in substantial bleeding.

13. Chemically etch the skull by wiping it once with a cotton swab moistened in 35% phosphoric acid (Figure 2C).

Note: Etching the skull chemically and mechanically improves the long-term adherence of the CIW and dental cement applied at the end of the surgical procedure. To assess the proper etching of the skull, clean the skull with a cotton swab again. An etched bone should provide an uneven and coarse surface.

⚠ CRITICAL: 35% phosphoric acid is not expected to be an inhalation hazard unless heated or misted, but it can cause skin corrosion and eye irritation upon contact, so we advise wearing adequate personal protective equipment.

PART 2: CIW implantation

Timing: 25–35 min

The following section explains the surgical procedure to implant the CIW in the mouse's skull.

⚠ CRITICAL: Heat loss is rapid in anesthetized mice. Use a safe supplemental heat source, for example a stereotaxic apparatus with an integrated warming base, to maintain normal body temperature during anesthesia.

14. Move the mouse on the stereotactic apparatus and restrain the head in the EPE foam mold. Apply new eye ointment if necessary.
15. Use a disposable Pasteur pipette to moisten and clean the exposed skull with Ringer's solution (Figure 3A).

⚠ CRITICAL: Perform this step regularly between each of the following steps to ensure the proper hydration of the tissues and brain throughout the procedure.

16. Use surgical scissors to remove part of the scalp (Figure 3B).

⚠ CRITICAL: Remove approximately 4 mm of skin frontally and caudally, and 2 mm of skin laterally on each side. This step allows the investigator to create physical space to perform the next surgery steps. Be careful not to remove too much skin close to the eyes, as this may later result in discomfort for the animal.

17. Use forceps with a sliding lock to grasp the skin on the side of the craniotomy to have better access to the surgical site (Figure 3C).
18. Use a sterile cotton swab to remove the periosteum and to dry the area of the craniotomy, which will be located in the skull overlaying the right frontal cortex.
19. Use a 3-mm biopsy punch to make a craniotomy.
    a. Place the biopsy punch on the skull, and slowly turn it in both directions to etch the bone (Figure 3D).
    b. Continue until the round bone flap becomes loose and almost detaches from the skull so that it can be carefully removed with a forceps.

Figure 3. CIW implantation surgery

(A) Place the mouse on the stereotaxic frame, and rinse the skull extensively with Ringer's solution to remove excess 35% phosphoric acid.

(B) Remove a part of the scalp, while leaving sufficient skin around the eyes.

(C) Place sliding lock forceps on the side of the planned craniotomy to gain better access to the surgical area.

(D) Perform craniotomy using the 3-mm biopsy punch and carefully remove the bone flap.

(E) Optional: induce glioblastoma formation by injecting RCAS virus-producing DF-1 cells into Tva transgenic mice using a Hamilton syringe.

(F) Place the CIW (left image) and put Loctite super glue on the edge of the 5-mm coverslip while gently pressing the CIW with a blunt forceps.

(G) Glue head bar next to the implanted CIW using Loctite super glue.

(H) Apply dental cement to the exposed skull and wait until it solidifies (several minutes) before removing the mouse from the surgery table.

⚠ CRITICAL: We recommend to perform the craniotomy using a biopsy punch instead of using a micro-drill. In our hands, it is more precise, allowing the 3-mm coverslip to completely seal the craniotomy. This minimizes the risk of Loctite super glue running into the brain tissue when gluing the CIW, and infections under the CIW. Morever, when choosing the correct position of the craniotomy, the risk of major bleedings is minimal. Make sure that there is ample space between the craniotomy and the major blood vessels in the skull, including the sagittal suture and bregma. We recommend to take the necessary time to slowly turn

the biopsy punch while applying minimal pressure. Rough movements can lead to the breaking of the skull and eventually damage the brain. Regularly check if the bone flap starts to detach by gently applying some pressure on the craniotomy with a forceps. If the bone is detaching, the bone flap starts to move when applying a minimal amount of pressure. This method can also be used to evaluate on which side the bone flap is still attached to the skull. If this is the case, slightly tilt the biopsy punch when making the turning movements to the side where the bone still needs to be cut. Doing these checks regularly during the punching procedure prevents cutting too deeply thereby damaging the meningeal layers and/or brain tissue.

Note: The bone flap can be stored in 1× PBS to later confirm the absence of the dura mater on the piece of removed bone.

20. Extensively rinse the site of the craniotomy with Ringer's solution.

⚠ CRITICAL: The exposed brain tissue should be covered by Ringer's solution throughout the surgery to prevent any permanent damage to the brain tissue at the site of the craniotomy.

21. Remove one CIW from the ethanol and allow it to dry while proceeding with steps 22–25.

⚠ CRITICAL: Use blunt forceps to handle the CIWs to minimize the risk of scratches and damage.

Optional (to induce high-grade gliomas):

22. Resuspend the DF-1 cells by flicking the Eppendorf tube and prepare 2 μL in the Hamilton syringe.
23. Attach the Hamilton syringe to the stereotactic apparatus.
    a. Use the stereotactic apparatus to precisely position the syringe and to inject the cells into the right frontal cortex (2 mm frontal, 1.5 mm lateral from bregma, 2 mm deep) (Figure 3E).
    b. Inject the cells slowly over a time frame of 10 s, and then leave the needle in place for an additional 10 s before slowly removing it.

Note: Depending on the cells to be injected, an injecting pump may be needed to control the injection rate. In our experimental setting herein, the injected cells (DF1) are not tumorigenic per se, and it is therefore not essential to prevent leakage by lowering the injection speed. However, we do advise to lower the injection rate to 1 μL/minute when cancer cells are injected, which will prevent the formation of extraparenchymal tumors.

24. Prepare some Loctite super glue in a Petri dish and break a cotton swab in the middle in order to create a small spatula.
25. Carefully dry the skull surrounding the craniotomy while leaving some Ringer's solution on top of the brain tissue.

Note: Drying the skull will support a proper adherence of the Loctite super glue (step 27 below), and will prevent the glue running over the skull and into the skin which complicates the proper closure of the wound.

26. Verify that the CIW is free of any residual ethanol, and use surgical tweezers to gently place the CIW on the craniotomy hole (Figure 3F).

⚠ CRITICAL: The CIW must be placed with the 3-mm diameter coverslip facing the brain. The 3-mm diameter coverslip will perfectly fit the size of the 3-mm craniotomy, allowing the craniotomy hole to be fully sealed and thereby minimizing the chance of bone regrowth. The upper 5-mm diameter coverslip allows gluing of the CIW to the skull (Figure 3F). When the CIW is correctly placed, one can feel that it is firmly inserted inside the craniotomy hole and it will not move.

27. While gently keeping the CIW in place using blunt tweezers with one hand, use the wooden end of the broken cotton swab in the other hand as a spatula to apply the Loctite super glue at the edges of the 5-mm diameter coverslip (Figure 3F). Try to avoid any running over of the glue across the middle of the CIW.
28. Wait 1–2 min with the tweezers in place to ensure drying of the glue.
29. Take one head bar and immerge the smaller side into the Loctite super glue, then place it on the skull in the lower back corner (diagonal to the CIW) and allow it to dry while keeping it in place with surgical tweezers (Figure 3G).
30. Prepare the dental cement by mixing an equal volume of dental cement powder and solution in a Petri dish.

Note: The dental cement is required to close and seal the surgical site, while keeping the skin and head bar in place.

⚠ CRITICAL: We recommend to prepare a rather fluid dental cement; the recommended mixing ratio is 1 g powder : 0.6 mL liquid. Wait until it starts to solidify, instead of exceeding the initial powder content. Start to apply the dental cement only once it becomes viscous and slowly drops from the surgical spatula.

31. Apply the dental cement slowly by dropping it in the empty spaces between skin and skull, starting from the skin of the neck, proximal to the head bar. Then, cover the smaller side of the head bar and seal the space around the CIW (Figure 3H).

⚠ CRITICAL: Pay attention not to cover the CIW itself with dental cement. A firmly viscous dental cement will help in performing this last step. If needed, the spatula can be used to make refinements to the dental cement shape in the first minutes after making the cement, and the cement may still be pushed away from the central CIW area. Also, small refinements to the dental cement can still be made using a micro-drill prior to the first imaging session, see step 40. We do not advise to adjust the shape of the dental cement in this step to prevent the detachment of the CIW (the Loctite super glue and/or dental cement may not have set completely). Moreover, if there was a bleeding during the surgery that was successfully stopped (see troubleshooting problem 3), drilling the dental cement may reinduce the bleeding.

32. Remove the mouse from the stereotaxic apparatus and turn off the isoflurane anesthesia.
33. Weigh the mouse to have a baseline measurement for subsequent weight monitoring.
34. Place the mouse on a heating pad.
35. Administer 300 μL of a 0.9% NaCl solution by intraperitoneal injection.
36. Monitor the mouse until it becomes conscious and then move it back to the housing cage.
37. Add paracetamol to the mouse's drinking water for the first 7 days after surgery (500 mg paracetamol per 250 mL water).

Note: Diet gel, placed in the bottom of the cage, provides easy access to water, calories and minerals, and may help speeding up the post-surgery recovery. We recommend to closely monitor the mouse at least daily over the weeks following surgery, and to administer 0.9%

NaCl daily (300 μL by intraperitoneal injection) until the mouse's weight is equal to, or greater than, its starting weight.

PART 3: Multimodal imaging

CIW-bearing mice can be used for multimodal imaging – in this protocol we describe 2P-IVM and MRI, but it is possible to extend the application to even more (functional) imaging modalities, such as molecular bioluminescence imaging[19] or positron emission tomography.[20] We advise to wait at least 3 weeks before performing the first imaging experiments described below, to allow any wound healing response triggered by the CIW surgery to fully resolve.[21]

Note: We advise to use monitoring equipment (for example from Harvard Apparatus) to check the mouse's vital signs such as heart and respiration rate, and body temperature during 2P-IVM and MRI. This allows the investigator to assess the depth of anesthesia and adjustment of isoflurane levels if necessary, ensuring proper recovery of the animal after each imaging experiment.

Two-photon microscope imaging

Timing: 2–6 h

In this section we explain a standard two-photon microscopy protocol that allows acquisition of an overview of the area below the CIW, and the recording of high-resolution time lapses of specific regions of interest. The exact imaging settings depend on the scientific question and on the specifications of the microscope.

⚠ CRITICAL: Switch on the two-photon microscope before anesthetizing the mouse. Also switch on the climate chamber surrounding the microscope or any other heating system to keep the mouse warm during the imaging and in order for the optic elements of the microscope to reach a stable temperature before starting the imaging.

38. Anesthetize the mouse with isoflurane inhalation anesthesia (3.5% isoflurane/O_2 mixture) and place it on a heating pad with a nose mask to ensure continuous anesthesia (use 2% isoflurane/O_2 mixture for maintenance).
39. Apply eye ointment.
40. Use a micro-drill to reshape the dental cement around the CIW (Figure 4A).

⚠ CRITICAL: Excessive amounts of dental cement may limit the range of movement of the microscope's objective and preclude imaging the full area underneath the CIW. While reshaping the dental cement, it is important to create or maintain the conical shape (Figure 4A) which allows it to hold the immersion liquid for the microscope objective on top of the CIW for the duration of the 2P-IVM experiment.

41. Screw the head bar to the head-bar holder and place the mouse on the stage of the two-photon microscope with its nose into the anesthesia mask (Figure 4B). Adjust isoflurane levels as necessary – the optimal level is typically around 1%.
42. Adjust the head-bar holder to ensure that the CIW is positioned perpendicular to the objective, allowing optimal imaging time.
43. Depending on the type of objective, add immersion liquid in the dental cement pocket above the CIW to immerse the objective (Figure 4B). The mouse is now ready to be imaged with the settings required for the specific study.

Note: The total imaging duration and the time interval greatly depend on the specific scientific research question. If one is mainly interested in the overall changes in the brain

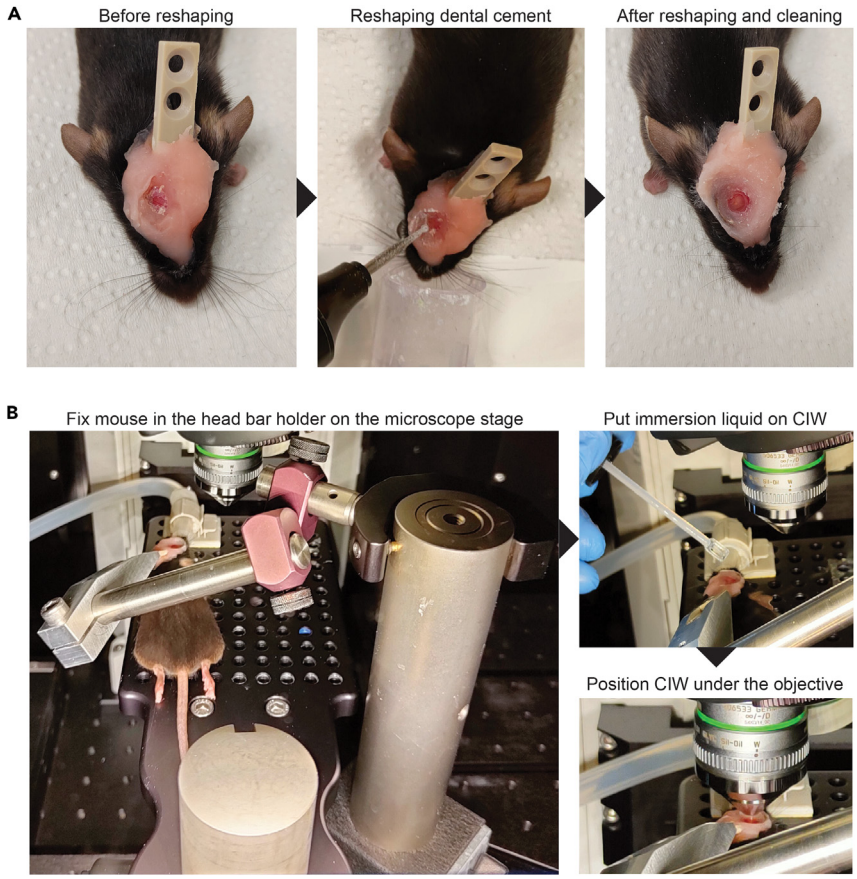

Figure 4. Preparing the mouse for 2P-IVM

(A) Before the first 2P-IVM session it is often necessary to reshape the dental cement to obtain an optimal field-of-view and to remove super glue and/or dental cement residues from the CIW. The dental cement is reshaped using a micro-drill, aiming for a conical shape to keep immersion liquid in place during 2P-IVM experiments. Subsequently, the CIW is cleaned with acetone until it is free of glue residues.

(B) The mouse is fixed in the head-bar holder and is constantly kept under isoflurane anesthesia (left image). Dependent on the microscope objective to be used, immersion liquid is added on top of the CIW. Completely fill the conical shape of the dental cement so that there is sufficient immersion liquid for the duration of the experiment (top right image). Lastly, position the CIW under and perpendicular to the objective (lower right image).

environment without tracking individual cells, it may be sufficient to collect a tile-scan overview image of the area underneath the CIW at defined time-points. The expected timing is 30 min–1 h, depending on the objective used, the field-of-view imaged and the imaging settings. For example, with a 16× objective (HC FLUOTAR L N.A. 0.6 FWD 2.5 mm), it takes approximately 30 minutes to image 16 individual tiles (covering the area under the CIW) at 512 × 512 pixels, 400 Hz, with a total Z-depth of 300 μm and a Z-step size of 10 μm.

If one is interested in tracking individual cells, the total time duration depends on the cell type of interest. Tumor cells generally migrate more slowly than immune cells, in the range of 2–4 μm/h[22,23] and therefore we would advise a total imaging time of ∼5 h with a time interval of ∼20 min. For immune cells, migrating in the range of 100–200 μm/h[1] a total imaging duration of 20–30 min with a minimal time interval (usually around 1 min when scanning at 1024 × 1024 pixels, 400 Hz, a total Z-depth of 300 μm and a Z-step size of 5 μm) would be sufficient to detect migrating cells. In case the time interval needs to be shortened further, for example when studying extremely fast-moving cells or other rapid dynamic behaviors, different considerations will have to be made.

Limitations in scanning speed can be overcome by using a resonance scanner with a scan frequency up to 12,000 Hz; however, this is associated with extremely short pixel dwell times and may therefore, depending on the fluorescence intensity and background signal, provide an insufficient signal-to-noise ratio. Another option would be to decrease image size, for example from 1024 × 1024 pixels to 512 × 512 pixels, at the cost of resolution. Lastly, one can decide to decrease the Z-depth or to increase the Z-step size, but this may result in cells moving outside the Z-stack or Z-planes.

In the situation where the exact same brain environment from a previous imaging session needs to be relocated, it is advised to first make a (low-resolution) overview tile-scan of the tissue underneath the CIW. Visual landmarks, such as patterns in the vasculature or extracellular matrix, can be used to navigate to the region-of-interest.

44. After the 2P-IVM session, remove the mouse from the microscope and inject ~0.5 mL of 0.9% NaCl solution intraperitoneally to rehydrate the mouse. Return the mouse to its cage, place the cage on a heating pad and visually inspect the mouse until it has fully recovered from anesthesia.

Note: For in-depth analysis of 2P-IVM data, we advise to use Imaris for image processing, cell segmentation, and extracting single cell measurements, followed by further processing and analyses-of-interest in R.

Magnetic resonance imaging

Timing: 5–10 min

In this section we describe a standard MRI protocol to monitor tumor growth or regression. However, MRI can also be used to investigate other brain pathologies, including multiple sclerosis[7] and traumatic brain injury.[8]

⚠ CRITICAL: Switch on the MRI machine before anesthetizing the mouse. Also switch on the water bath pumping warm water to the MRI-compatible heating pad inserted in the mouse cradle. For our experiments, we are using a BioSpec 3T preclinical machine (Bruker).

45. Anesthetize the mouse with isoflurane inhalation anesthesia (3.5% isoflurane/O_2 mixture) and apply eye ointment once the mouse is anesthetized.
46. Transfer the mouse in its natural position (belly positioned towards the ground) to the continuously heated MR cradle and fix the mouse in the tooth bar. Adjust isoflurane levels to 1.5–2%.
47. Make sure the mouse's head is positioned straight and is not tilted before positioning the MRI surface coil.
48. Insert the cradle into the MR bore, placing the animal's head at the center of the magnet.
49. Run a 3-slice localizer to assess the position of the mouse's head, and initial MR preparation scans suitable for your experimental design.

Note: This step may include adjustments to tune and match the frequency of the coil, to the radiofrequency power, and to compensate for MR field inhomogeneities.

50. Start the desired protocol on the MRI machine. To visualize high-grade gliomas, we suggest to use a 2D turbo rapid acquisition relaxation enhancement (Turbo-RARE) T2-weighted acquisition with the following pulse sequence parameters: TR = 3000 ms, TE = 75 ms, NA = 6, number of slices 10, slice thickness (ST) = 0.7 mm, FOV = 20 × 20 mm^2, pixel size 0.156 × 0.156 mm^2, (ETL = 12, T_{acq} = 3 min) with images being acquired in axial planes.

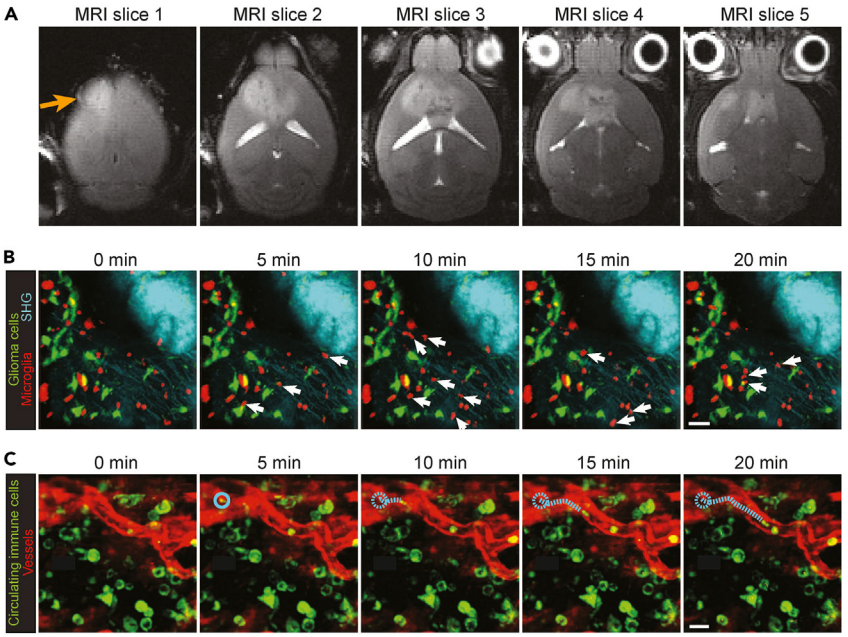

Figure 5. Typical multimodal MRI – 2P-IVM results

(A) Representative MRI images of a mouse with a CIW. The different images show different slices of the same mouse brain (slice thickness = 0.7 mm), where MRI slice 1 shows the top of the brain and MRI slices 2–5 going deeper in a stepwise manner. The orange arrow in MRI slice 1 indicates the border of the CIW with a high-grade glioma directly underneath (bright signal that extends deep into the brain, also visible in MRI slices 2–5).

(B) 2P-IVM images of the CX3CR1 lineage-tracing mouse model showing GFP^+ high-grade glioma cells in green, $tdTomato^+$ microglia in red, and second harmonic generation (SHG) signal in cyan (for details on the tumor and lineage tracing mouse model, see[1]). The white arrows indicate migrating microglia in the tumor microenvironment. Scale bar: 50 μm.

(C) 2P-IVM images of the FLT3 lineage tracing mouse model showing GFP^+ circulating immune cells in green and $tdTomato^+$ vessels in red (for details on the tumor and lineage tracing mouse model, see[1]). The cyan circle outline and track indicate an immune cell displaying rolling behavior inside a vessel within the tumor. Scale bar: 20 μm.

51. After the MRI session, remove the mouse from the cradle and return it to its cage. Visually inspect the mouse until it has fully recovered from anesthesia. This happens usually within 1 min when the total duration of the MRI scan is short (5–10 min if monitoring brain tumor growth).

Note: We suggest to use the free MIPAV (Medical Image Processing, Analysis, and Visualization) application (National Institutes of Health, USA) for quantification of tumor volumes.

EXPECTED OUTCOMES

The mice should fully recover from anesthesia briefly (< 15 min) after the surgery. After a small initial decrease in their body weight, the animals start gaining weight 3–4 days post-CIW implantation.[1] The metal-free setup allows investigators to safely image tumor growth in the brain by magnetic resonance imaging (Figure 5A). Once the tumor is detected, usually between 4-5 weeks post-injection, mice can be imaged weekly during the time of tumor progression and in response to therapy.[1] The main imaging readout is dependent on the choice of the cell-lineage tracing model. We have used the protocol described herein to monitor the dynamics of GFP-labeled cancer cells, brain-resident microglia and recruited immune cells (Figures 5B and 5C). Other cells and/or structures of interest can be visualized by implanting the CIW in other genetically-engineered cell-lineage tracing mouse models. In addition, diseases and pathologies other than cancer can be investigated using the method described herein, for example multiple sclerosis or brain infections.

LIMITATIONS

Multimodal imaging modalities

This protocol describes multimodal imaging using 2P-IVM and MRI. We have used this specific combination of imaging instruments to integrate dynamics at a cellular level (measured by 2P-IVM) with tumor volume, to determine the stage of cancer progression and/or regression (measured by MRI).[1] However, depending on the specific research question, other imaging modalities such as molecular bioluminescence imaging[19] or positron emission tomography[20] may be more suitable. The final choice of instruments may also depend on resource availability.

Imaging area

The 2P-IVM in this protocol is confined to a restricted area; 3 mm in diameter with a typical imaging depth of 300–500 μm. As a consequence, only the upper cortical layers of the brain or the most superficial part of a brain tumor can be imaged. For specific applications the dura mater may be removed for increased imaging depth (adding approximately 40 μm), however, we advise to leave the dura mater in place to preserve the composition of the natural brain environment. With recent studies demonstrating the presence of cervical lymph node-draining lymphatics in the dura mater,[24,25] this may represent an opportunity to further investigate cellular interactions at the brain cortex-meninges interface, especially upon brain infection or tumor formation.

Experimental timeframe

The advantage of the protocol we present herein is that the craniotomy is fully sealed with the 3-mm diameter coverslip, thereby minimizing the chance of bone regrowth and craniotomy closure. However, in rare cases it may be that the bone starts to grow underneath the craniotomy / CIW, thereby limiting the field-of-view and total experimental time in case the experiment needs to be terminated. Another limitation relates to the frequency and duration of individual imaging sessions. For our experiments, mice on a C57BL/6 background were imaged once per week by MRI (5–10 min) and once per week by 2P-IVM (~2 h), and this could be repeated for at least up 4 months, which was the scientific endpoint of our studies. However, we did notice that repeatedly increasing either the frequency of imaging sessions or the length of individual imaging sessions can affect the well-being of the mice. We therefore advise to carefully plan your experiments, and adjust the experimental design to the specific research question. For example, if a long 2P-IVM imaging session of ~6 h is needed and the desired total duration of the experiment is 4 months, it may be necessary to lower the 2P-IVM frequency to once every 2 weeks. These adjustments may also depend on the mouse strain and/or sex of the animal.

TROUBLESHOOTING

Problem 1

The skull cracks or breaks while performing the craniotomy (step 19). If the skull breaks during the CIW implantation surgery, it usually results in an incomplete craniotomy and complicates the rest of the surgery. The risk of bleeding and damage to the brain tissue significantly increases when trying to remove the rest of the skull to make the craniotomy complete.

Potential solution

In our experience, the main reason for the skull breaking during the craniotomy is excessive chemical etching with 35% phosphoric acid (step 13), making the bone less hard. We advise to only wipe the skull once with 35% phosphoric acid, and then clean the skull with Ringer's solution. Another suggestion is to perform the craniotomy slowly, and without putting too much pressure on the biopsy punch (step 19).

Problem 2

Difficulties taking out the craniotomy (step 19).

Potential solution

First verify that all sides of the craniotomy are detached from the rest of the skull by gently pressing on the craniotomy with forceps; when the craniotomy is detached it slightly moves. If the craniotomy is complete, but it is difficult to take it out with forceps, we advise to slightly bend the tip of an insulin syringe to create a "hook". This can be used to then lift the craniotomy while grabbing it with forceps held in the other hand.

Problem 3

Bleeding during the surgery (the procedures with the highest risk on bleeding include steps 13 and 19).

Potential solution

If bleeding occurs, put a piece of Gelfoam on the damaged site while gently applying pressure and wait for the bleeding to stop (usually 1–2 min). Sometimes it is necessary to repeat this procedure several times.

Problem 4

The implanted CIW is not completely transparent, interfering with 2P-IVM experiments (step 43).

Potential solution

Try to avoid the accumulation of Loctite super glue and/or dental cement on top of the coverslip during the CIW implantation (steps 27 and 31). Any potential Loctite super glue and/or dental cement traces present on the CIW can be removed with a cotton swab moistened with acetone. It may be needed to repeat this procedure several times to completely clean the CIW. Make sure to clean the CIW carefully and not to exert too much pressure. This may result in breaking the CIW or in pressure-induced brain damage.

Problem 5

Movement artefacts during 2P-IVM (step 43).

Potential solution

Visually inspect the mouse and isoflurane levels: either the isoflurane levels are too low and the mouse is awake, or the levels are too high, resulting in irregular breathing and gasping. In either of these scenarios it is necessary to adjust the isoflurane levels; the ideal breathing frequency is ~55–65 breaths per minute. Please note that it usually takes around 10 min until a mouse has fully responded to a change in isoflurane levels. It is critical to not leave the mouse under too deep anesthesia for too long, as it may be difficult to re-establish a regular breathing pattern, and it may hamper a quick recovery for the animal after the experiment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Johanna A. Joyce (johanna.joyce@unil.ch).

Materials availability

This study did not generate new unique reagents.

Data and code availability

For additional information on the downstream analyses of 2P-IVM experiments we refer to our original research paper.[1] Imaging data from this publication have been deposited at Mendeley data: https://data.mendeley.com/datasets/yt99rmbcdt/1. Corresponding two-photon microscopy images and time-lapses will be shared by the lead contact upon request. All original code that was used for downstream analysis of exported Imaris data in R has been deposited at Mendeley

data: https://data.mendeley.com/datasets/yt99rmbcdt/1. Any additional information required to set up the protocol reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102197.

ACKNOWLEDGMENTS

We thank members of J.A.J.'s lab for insightful discussion. We are grateful to Dr. Leon van Gurp for developing part of the data analysis pipeline, as published previously.[1] We would also like to thank Rui Santalla Méndez for providing pictures for the figures, Prof. Anthony Holtmaat and Prof. Alan Carleton for providing the original drawings of the head bars and head bar holder, and Maj-Britt Buchholz for providing STL files for 3D printing. Several of the cartoons in the graphical abstract were created using BioRender.com. We are grateful to the In Vivo Imaging Facility at the University of Lausanne for assistance and advice, in particular Dr. Alexandre Bénéchet. We thank Dr. E. Holland for generously providing the RCAS vectors and Nestin-Tva mice. Research in J.A.J.'s lab for this project was provided in part by the Charlie Teo Foundation, Swiss Cancer Research Foundation (KFS-3390-08-2016; KFS-5280-02-2021), the Ludwig Institute for Cancer Research, and the University of Lausanne. A.Z. was supported in part by a fellowship from the Human Frontier Science Program (LT000240/2017-L) and a Veni fellowship from the Netherlands Organization for Scientific Research (09150161910076).

AUTHOR CONTRIBUTIONS

D.C., A.Z., and J.A.J. wrote the protocol; D.C., A.Z., and J.K. performed the experiments; and J.A.J. supervised the research. All authors edited or commented on the manuscript.

DECLARATION OF INTERESTS

D.C. has received consulting fees from Seed Biosciences SA; J.K. currently works at Lunaphore Technologies SA, Switzerland; J.A.J. has received honoraria for speaking at a research symposium organized by Bristol Meyers Squibb (last 3 years) and currently serves on the scientific advisory board of Pionyr Immunotherapeutics.

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