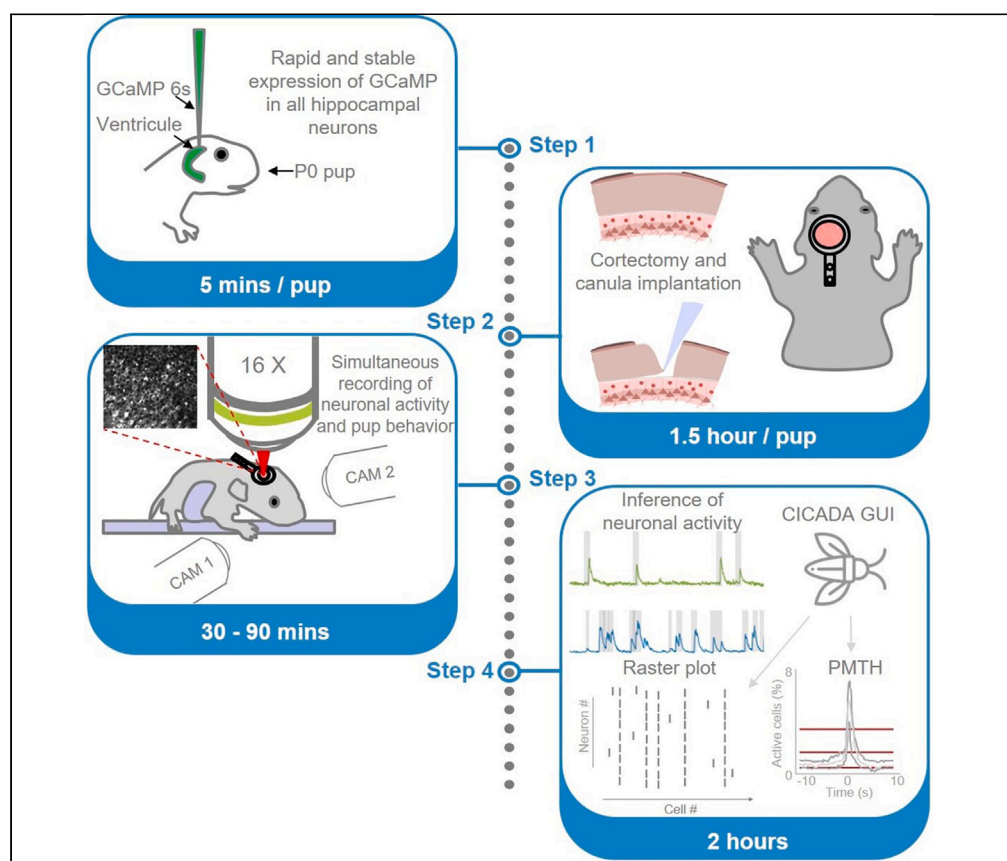


Protocol

Protocol to image and analyze hippocampal network dynamics in non-anesthetized mouse pups



Two-photon calcium imaging is a powerful technique that has revolutionized our understanding of how neural circuit dynamics supports different behaviors and cognitive processes. However, performing imaging during development remains challenging. Here, we provide a protocol to image CA1 neurons in mouse pups as well as a pipeline of analysis to analyze and share the data. We describe steps for intracerebroventricular injection, cranial window surgery, two-photon calcium imaging, and analysis of imaging data.

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

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Highlights
GCaMP expression
through
intracerebroventricu-
lar injection at birth

Cranial window
surgery with
cortectomy

Two-photon calcium
imaging of CA1
dynamics

DeepCINAC and
CICADA GUI to
analyze imaging data
formatted in NWB
files

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Protocol

Protocol to image and analyze hippocampal network dynamics in non-anesthetized mouse pups

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SUMMARY

Two-photon calcium imaging is a powerful technique that has revolutionized our understanding of how neural circuit dynamics supports different behaviors and cognitive processes. However, performing imaging during development remains challenging. Here, we provide a protocol to image CA1 neurons in mouse pups as well as a pipeline of analysis to analyze and share the data. We describe steps for intracerebroventricular injection, cranial window surgery, two-photon calcium imaging, and analysis of imaging data. For complete details on the use and execution of this protocol, please refer to Dard et al.¹ and Denis et al.²

BEFORE YOU BEGIN

It is worth noting that all of our experiments were conducted using GCaMP6s. This protocol can be adjusted to accommodate the most up-to-date version of GCaMP, both in terms of experimental procedures and analytical methods. This can involve fine-tuning Suite 2P parameters and training a new network with DeepCINAC.

Institutional permissions

All experiments were performed under the guidelines of the French National Ethics Committee for Sciences and Health report on 'Ethical Principles for Animal Experimentation' in agreement with the European Community Directive 86/609/EEC (Apafis #18–185 and #30–959).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
pAAV.Syn.GCaMP6s.WPRE.SV40 (AAV9)	Addgene	100843-AAV9
Chemicals, peptides, and recombinant proteins		
Trypan blue	Sigma-Aldrich	T8154
Isorane	Axience	03760087153613
Phosphate-buffered saline (PBS)	Thermo Fisher Scientific	12559069
Buprenorphine		
Povidone iodinated	Sigma-Aldrich	Y0000466
Experimental models: Organisms/strains		
Mouse: pregnant Swiss female	Janvier Labs	N/A

(Continued on next page)



Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
MATLAB	MathWorks	Version 2022a
MATLAB codes for motion correction	GitHub (this manuscript)	https://gitlab.com/cossartlab/motion-correction.git
Clampex	Molecular Devices	Version 10.7.0.3
Inspector Pro	LaVision BioTec	Version 7.3.6
Pylon viewer	Basler	Version 6.2.0.8205
Suite2p		https://github.com/MouseLand/suite2p
DeepCINAC	Denis et al. ²	https://gitlab.com/cossartlab/deepcinac.git https://doi.org/10.5281/zenodo.10041302
CICADA	Denis et al. ²	https://gitlab.com/cossartlab/cicada.git https://doi.org/10.5281/zenodo.10041434
Other		
3.5" Microinjection capillaries for Nanoject	Drummond	3-000-203-G/X
Pipettes puller	Narishige	PC-100
Homeothermic monitoring system	Harvard Apparatus	N/A
Tygon laboratory tubing R-3603	Sigma-Aldrich	T8913
10 mL syringe Terumo	Fisher Scientific	50-209-2975
1 mL Henke-Ject tuberculin	Henke Sass Wolf	4010-200V0
Stereotaxic frame for injection	Kopf	Model 940
Platform for surgery	Stoelting	51625
Compact anesthesia module	Minerve	N/A
Vacuum pump	World Precision Instruments	801963
Liquid Paladur	Kulzer	64707937
Powder Paladur, pink	Kulzer	64707945
Super-Bond C&B (green activator)	Sun Medical	
Cannula	MicroGroup	custom made
3 mm glass window	Warner Instruments	CS-3R-1
Norland Optical Adhesive 81	Norland Products	#36-428
Kwik-Sil and replacement tips	World Precision Instruments	600022
Liquid glue	Loctite	N/A
Cotton swabs	N/A	N/A
Parafilm Bemis American Can Company	Fisher Scientific	11747487
Fine-tip transfer pipette 2 mL Samco	Fisher Scientific	15397833
Headpost small round for P6 mice	Luigs & Neumann	200-200 500 2134
Headpost holder - dovetail type (headpost and adapter included included)	Luigs & Neumann	200-200 500 2132
Adapter plate type 1	Luigs & Neumann	200-200 500 2111
Eppendorf GELoader tips 0.5–20 µL	Thermo Fisher Scientific	10411193
BD Microfine insulin syringe, 30G, 0.3 mL	Becton Dickinson (BD)	324826A
26G needle Terumo	Fisher Scientific	15301557
Fine forceps	Fine Science Tools	11923-13
Spring scissors	Fine Science Tools	91501-09
Straight forceps Dumont	Fine Science Tools	11255-20
Angled fine forceps	Fine Science Tools	91110-10
Binocular	Olympus	SZ61
Custom-made 2-photon microscope	LaVision BioTec	TriM Scope II
Ti:sapphire excitation laser	Coherent	Chameleon Ultra II
GaAsP PMT	Hamamatsu	H7422-40
Bandpass filter	Semrock	510/25
16x water immersion objective	Nikon	NA 0.8
Digidata 1440A	Axon Instruments	N/A
Aluminium breadboard	Thorlabs	MB1015/M
Optical posts	Thorlabs	MS05 R/M
Screws	Thorlabs	MSRA90
Infrared cameras acA1920-155um	Basler	106879
TPL Vision SBAR infrared light source	Basler	SBAR-850-NL-DO
NWB formatted calcium imaging data	DANDI	RRID:SCR_015242

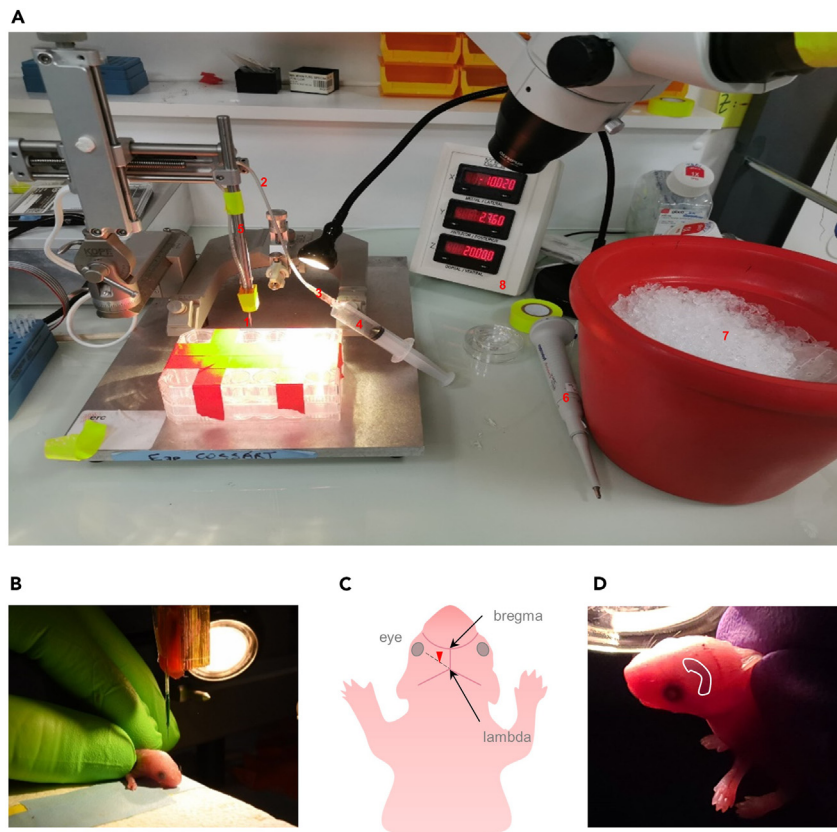


Figure 1. Intracerebroventricular injection

(A) Detailed picture of the injection set-up, it includes: the injection pipette (1), the silicon tube (2), the 18G needle (3), the 10 mL syringe (4), the stereotaxic arm (5), the P20 pipette (6), the container filled with ice and a Petri dish to put the pup in (7) and the digital display console (8).
 (B) P0 mouse pup being injected with a mix of viral solution and blue dye.
 (C) Injection site is located at $2/5$ on the imaginary line from the eye to lambda indicated by the red triangle.
 (D) Picture of previously injected pup. By transparency you should be able to see the shape of the ventricle.

STEP-BY-STEP METHOD DETAILS

Intracerebroventricular injection

⌚ Timing: 5 min per pup

In this section, we present the steps to induce the expression of the genetically encoded calcium indicator GCaMP6s³ in all hippocampal neurons using an already described intracerebroventricular injection protocol^{4,5} (Figure 1).

1. Make a glass pipette:
 - a. Take a 3.5" Drummond glass pipette (Figure 1A item 1) and pull using a Narishige puller (PC-100).
 - b. Cut the tip of the pipette to a diameter that ranges from 25 to 40 μ m.

Note: We only keep pipettes with a sharp opening and discard all the others (no need to polish the opening).

2. Prepare the injection system:
 - a. Connect the pipette to a silicon tube (Tygon R-3603) (Figure 1A item 2) that is connected to a 18G needle (Figure 1A item 3).
 - b. Connect the needle to a 10 mL syringe (Figure 1A item 4).
 - c. Clean the pipette with alcohol and rinse thoroughly with sterile water or sterile PBS. Let it dry.
3. Attach the system to a stereotaxic frame (Kopf Model 940) that will allow precise control of the injection depth (Figure 1A item 5).
4. Dilute the viral solution with 0.05% trypan blue (T8154 Sigma) to verify the injection site (see step 10, Figures 1B, 1C, and 1D).
5. Withdraw 2 μ L of this colored solution using a P20 pipette (Figure 1A item 6). Place this solution on parafilm and aspirate it.
6. Anesthetize a newborn (P0.5-P1) pup on ice for 3–5 min (Figure 1A item 7).
7. Gently hold the head of the animal between two fingers (Figure 1B). The injection site is approximately at two fifths of the imaginary line between lambda and the eye⁴ (Figure 1C and for more information see Figure 1 in Kim et al.⁵).
8. Place above the location site:
 - a. Place the pipette above the injection site.
 - b. Lower until it reaches the skin. Keep lowering the glass pipette and until a small deformation of the skull under this pressure is noticed.

Note: When piercing through the skull, its surface bounces back.

- c. At this step, zero the z-axis reference on the digital display console (Figure 1A item 8).
9. Descend to a depth of 400 μ m (150 μ m/s) and inject the viral solution (500 nL/s, this step should take roughly 10 s in total).

Note: You should see the ventricle filling up at this point (Figure 1D). Troubleshooting 1

10. Check the injection position by positioning the head of the pup against a source of light. Look through and you should see a “banana shape” labeling (Figure 1D).
11. Warm up the pup on a heating pad (set to 37°C) for 2 min after injecting before placing it back in the litter. Rinse the pipette with sterile PBS between each injection.

△ CRITICAL: To be effective, this injection needs to be performed a few hours after birth (typically works fine in pups less than 24 hours old). The longer you wait the more difficult the injection will be (mainly because of the skin transparency).

Preparation for cranial window surgery

⌚ **Timing:** 45 min

In this section, we will outline all the essential steps for preparing and executing a successful, high-quality cranial window surgery.^{1,6,7}

12. Preferably, choose the more robust animal in the litter (weight is a good indicator).

It’s important to wait a minimum of 5 days between the intracerebroventricular and the surgery to induce a strong and stable expression of GCAMP (see discussion).

13. Inject subcutaneously the analgesic (buprenorphine 0.05 mg/kg)⁸ at the back of the neck and keep the pup on a heating pad for 30 min. Troubleshooting 2.
14. Prepare your setup (Figure 2):
 - a. Ensure the entire setup is disinfected with alcohol.

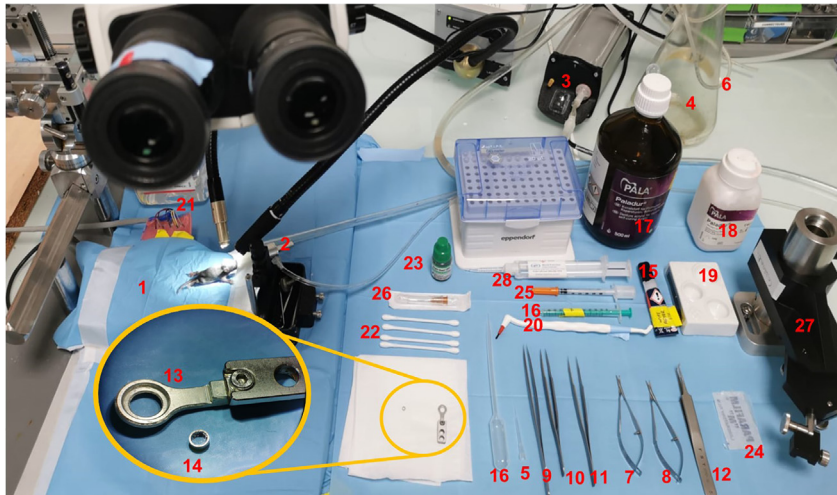


Figure 2. Cranial window implantation setup

Tools required for the cranial window surgery: heat pad (1), isoflurane mask (2), vacuum pump (3), sealed Erlenmeyer (4), thin GEloader pipette (5), flexible silicon tube (6), spring scissors (7) and (8), fine forceps (9), straight forceps Dumont (10) and (11), angled fine forceps (12), titanium headplate (13), cannula (14), stick of liquid glue (15), syringe (16), dental cement kit and brush (17, 18, 19, 20), Phosphate Buffered Saline (PBS) (21), cotton swabs (22), Super-Bond (23), Parafilm piece (24), 0.3 mL insulin syringe (25), 26G needle (26), headpost (27) and Kwik-Sil (28).

- b. Cover the heating pad (Figure 2 item 1) and the platform (Stoelting ref #51625) with a sterile diaper.
- c. Turn the heating pad on, with the heat stabilized at 37°. Fix the rectal probe with a tape, and place it underneath the pup. Fix the isoflurane mask (Minerve veterinary induction anesthesia machine, Figure 2 item 2) on the pad.
- d. Connect a vacuum pump (WPI ref. 801963; Figure 2 item 3) to a 500-mL sealed Erlenmeyer with a flexible silicon tube for aspiration (Figure 2 item 4). Fix a thin pipette (0.5–20 µL Eppendorf GEloader; Figure 2 item 5 and 6) at the end of the tube.
- e. Sterilize your scissors (Figure 2 items 7 and 8) and tweezers (Figure 2 items 9, 10, 11 and 12) in a hot bead sterilizer (Fine Science Tools sterilizer, at 240°C during 20 s).
- f. Rinse the headplate (Luigs and Neumann headpost small and round P6 + adapter plate type 1) and the cannula (custom made by MicroGroup, Gauge 11.5, welded. Outside Diameter: 0,1150 inches; Inside diameter: 0,0945 inches; Length: 0,060 inches) with ethanol to remove any impurities (Figure 2, yellow circle, items 13 and 14).

Note: A 3 mm glass window (Warner Instrument CS-3R-1) was previously glued (Norland Optical Adhesive 81) to one side of the cannula (Figure 2 item 14) and cured through UV light.

- g. On the side, set a sterile field and put the previously cleaned and sterilized tools (all listed in Figure 2), add a stick of liquid glue (Figure 2 item 15), a fine-tip transfer pipette (Figure 2 item 16), items for dental cement preparation (Figure 2 items 17, 18, 19 and 20), saline solution (Figure 2 item 21), cotton swabs (Figure 2 item 22), the Super-Bond (Sun Medical Super-Bond C&B, Figure 2 item 23), a parafilm piece (Figure 2 item 24), a needle (BD Microfine insulin syringe, 30 G, 0.3 mL, Figure 2 item 25), a 26G needle with its syringe (Figure 2 item 26), the headplate holder (Figure 2 item 27), and the Kwik-Sil syringe (Figure 2 item 28).

Cranial window surgery

© Timing: 1.5 h

In this section, we will review all the necessary steps involved during the cranial window surgery that are required to grant optical access to the hippocampus.

15. Induce the pup with isoflurane (3% in a 90% O₂ / 10% air mix at a rate of 0.4 L/min) in an anesthetic chamber. This step typically lasts 5 min.
16. Transfer the pup on the heating pad (37°C) and place its nose in the mask where the isoflurane exits (maintained between 0.5%–1.5% in a 90% O₂ / 10% air mix during the procedure. With this O₂ and air mixture concentration, we achieved a consistently stable state of anesthesia.). Troubleshooting 3.
17. Gently apply the povidone-iodine antiseptic (betadine) on the skin using a cotton swab.

Make a small incision at the midline between the eyes using curved scissors. The scalp should be taken off above the cerebral hemispheres until lambda has been reached.

18. Remove the connective tissues with a cotton swab soaked with saline solution. At the same time, use the cotton swab to gently push away the skin on the side.

Note: During this step, if the exposed area appears to be too small, extend it, especially on the side of the future cortectomy.

19. To dry out the surface of the skull, apply a tiny amount of Super-Bond onto the skull with a brush, spread and push the liquid on the sides. Wait for 20 s, and wipe the surface with cotton swabs soaked with saline solution.
20. To increase adherence, gently scratch the skull with a thin needle, in a grid pattern. Remove the impurities with a cotton swab soaked with saline solution and dry again.
21. Fix the headplate onto the skull with glue.

Note: The inner diameter of the headplate should follow the medial sinus and lambda.
Troubleshooting 4

22. Following up, apply a sufficient quantity of dental cement onto the skull surface. This will both anchor, and reinforce the overall strength of the headplate onto the skull.

Note: Make sure the cement only touches the skull, not the skin nor the hair. If so, the fixation will be unstable and movement artifacts will be observed during imaging.

23. Start the vacuum pump and cut the tip of the pipette with the curved scissors.
24. Using a small needle (BD Microfine insulin syringe, 30 G, 0.3 mL), pierce the skull following the edges of the headplate. Eventually the skull can be lifted up and removed with a pair of forceps.

Note: To prevent any damages to the cortex, the skull should be lifted up in one complete piece. If the skull is successfully removed, blood vessels should be visible and no skull pieces should exceed the edges.

25. With a hooked needle, gently scratch the surface of the pia mater. Continue to rinse and aspirate with saline. Gently pinch and remove the rest of the meninges using thin forceps.

△ CRITICAL: Make sure that no meninges are left on the edges; otherwise, they will prevent a correct flow of aspiration during the upcoming steps.

26. Remove the cortex and expose the fibers from the *corpus callosum* (Figure 3):
 - a. Start to remove several layers of the cortex on a small area with the tip (Figure 3A, B1 and B2).

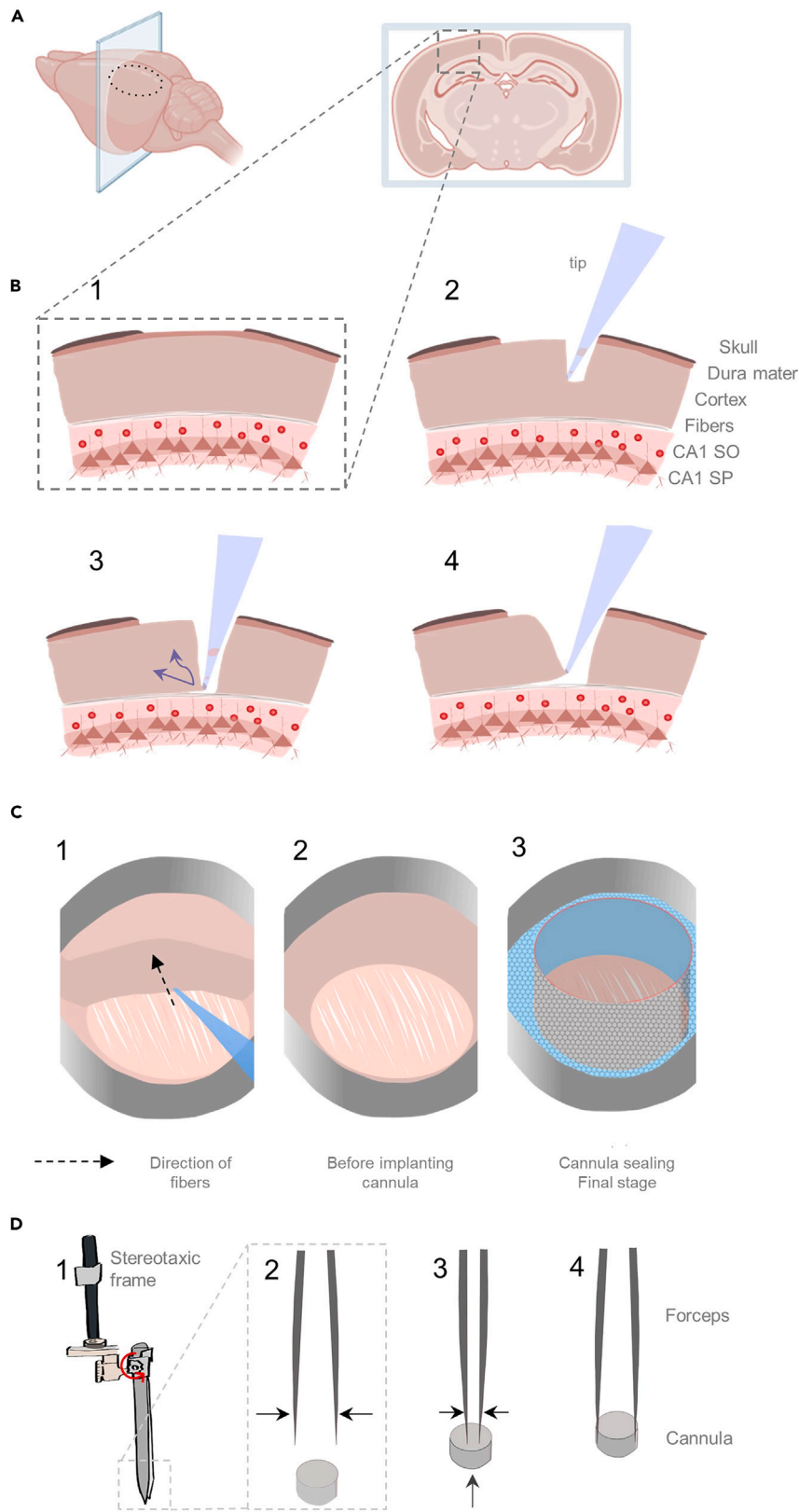


Figure 3. Schematic of the principal steps for cranial window surgery

(A) Coronal slice exposing the future location of the window indicated by the dotted circle.
 (B) Steps to follow until the fibers are reached: 1. Remove the skull 2. Remove the cortex starting from a small area 3. Dig deeper until the fibers are reached on the small area. Once there, expand the area by pushing the tip forward and up. 4. With the same movement, increasingly expose the fibers.
 (C) Top view of the cortex removal: 1. When fibers are reached, follow their directions while removing the cortex. 2. Before cannula implantation, the fibers should be visible, the surface flat and no bleedings left. 3. Implant the cannula and seal with dental cement if there is some space left between the cannula and the headplate.
 (D) Instructions for attaching the cannula to the forceps. 1. Secure the forceps firmly onto the stereotaxic frame, ensuring it is aligned vertically, and tighten the screw marked by the red arrow. 2. Grasp the end of the forceps and approach the cannula with the glass facing downward. 3. Maintain pressure and gently insert the ends of the forceps until they make contact with the glass at the base of the cannula. 4. Release the pressure on the forceps; the cannula will remain in position due to the tension exerted by the forceps against the cannula walls.

- b. Continuously adjust the focus on the aspirated area. Continue to remove the cortex until the fibers of the corpus callosum are reached (Figure 3B3 and B4).

Note: The fibers are distinguished by their stringy, elongated texture compared to the upper layers (Figure 3C). An adjustment of the light source onto the surface should bring them out.

- c. Once the fibers are well exposed, start vacuuming the remaining part of the cortex, by placing the aspiration tip on the lowest layer of cortex, right above the fibers.

Note: Aspirate the cortex in the same direction as the fibers in order to limit the damages to the fibers (Figure 3C1 and C2).

- d. To finalize this step, make sure that the field of view exposing the fibers is as flat as possible. Continue rinsing until the bleeding stops.

27. Next, attach the forceps to the stereotaxic frame, so that it's secured, and further pinch the clamp ends in order to insert the cannula (Figure 3D).
 - a. Using the pressure ends of the forceps to keep the cannula in place, move the cannula above the cortectomy (Figure 3D).
 - b. To ensure a perfect fit of the cannula for craniotomy, initiate an initial descent with the cannula, stopping just after it passes the skull level, and then return upward.

Note: It's important to ensure that the cannula is parallel to the surface of the brain.

Note: This step could trigger some bleeding during the final descent of the cannula.

28. Continuously rinse and aspirate the saline solution until the bleeding stops, and draw out the remaining saline.
29. Using a thin needle, lay a tiny droplet of transparent silicone (Kwik-Sil WPI) onto the surface of the brain.
 - a. Immediately descend the cannula and apply slight pressure so that the droplet of silicon spreads evenly and travels along the sides of the cannula, filling the area between the cannula walls and the brain (Figure 3C3).

△ **CRITICAL:** To prevent the formation of air bubbles in the silicone drop, the descent of the cannula and its implantation should not exceed 20 seconds.

△ **CRITICAL:** The imaged structure is localized deeply within the brain, therefore, the thickness of every element crossed by the laser should be optimized, more specifically, the thickness of the Kwik-Sil layer should be minimal and the top edge of the cannula must not be deeper than the lower bound of the headplate.

Note: The cannula should fit perfectly in the craniotomy, however, if there is some space left, fill it with Kwik-Sil.

30. Use liquid glue to seal the top edge of the cannula with the headplate.
31. Pinch the end of the forceps and lift it up using the stereotaxic frame.
32. Stop the anesthesia and remove the animal from the isoflurane mask.
33. Inject subcutaneously saline solution/physiological serum (same volume as buprenorphine) and leave the pup on a heating pad at 37°C.

Note: In neonatal mice, the anesthesia is likely to affect the brain activity;⁹ therefore, we leave the mouse to rest on a heating pad for at least one hour. After that, the imaging session can be started.

2 photon imaging in behaving pups

⌚ Timing: 0.5-2h

In this section, the central focus will be to outline all the essential steps required for the concurrent recording of neuronal activity and behavior (ranging from simple recordings of animal movements to any kind of behavioral tasks) in head fixed mouse pups.

34. Two-photon microscopy and behavior recordings setup (Figure 4A).
 - a. Turn on all necessary equipment to perform imaging experiments. This includes the laser, the fluorescent lamp, the data acquisition system, the heating lamp (adjusted to 37°C; Figure 4B item 1), and the xyz-stage controller.
 - b. Separately, on a small aluminum Breadboard (Thorlabs MB1015/M, Figure 4A item 1), fix two Thorlabs optical posts (Figure 4A items 2 and 3) using some screws and connect them with a third post (Thorlabs MS05 R/M and MSRA90; Figure 4A item 4).
 - i. Find a small c-shaped object (Figure 4A item 5) likely to hold the pup.
 - ii. Glue it to the metallic post and apply some medical silicon on the entire surface.

Note: This custom saddle can be adapted depending on the size of the pup (Figure 4A).

- c. Place the custom saddle on the main stage, right underneath the objective (Figure 4B item 2). On each side, fix an infrared camera (Basler camera acA 1920–155 µm; Figure 4B item 3 and 4) directed towards the saddle and finally the infrared light source (TPL VISION, SBAR-850-NL-DO).
35. Prepare the pup.
 - a. In order to avoid any leakage during the imaging session, contour thinly the outer edges of the headplate with some Kwik-Sil. Leave it to dry for 2 min.
 - b. Place the head-fixed mouse on the custom “saddle”; adjust the angles of the headpost, such that the cannula is perfectly orthogonal to the objective (Figure 4B).
 - c. Insert some water and verify that no air bubbles are present in the cannula. If you lose the meniscus during the acquisition, see Troubleshooting 5.
 - d. Find the surface of the hippocampus using the binocular and a fluorescent lamp. If you can’t find the surface of the brain, see Troubleshooting 6.
 - e. Start recording animal behavior as well as CA1 multi neuron dynamics (specific details about acquisition parameters/equipment use are given in the following section).
36. Two photon imaging of spontaneous CA1 dynamics in the hippocampus.
 - a. Use a single-beam multiphoton pulsed laser scanning system coupled to a microscope (TriM Scope II, LaVision BioTec).
 - b. Set the Ti:sapphire excitation laser (Chameleon Ultra II, Coherent) at 920 nm.

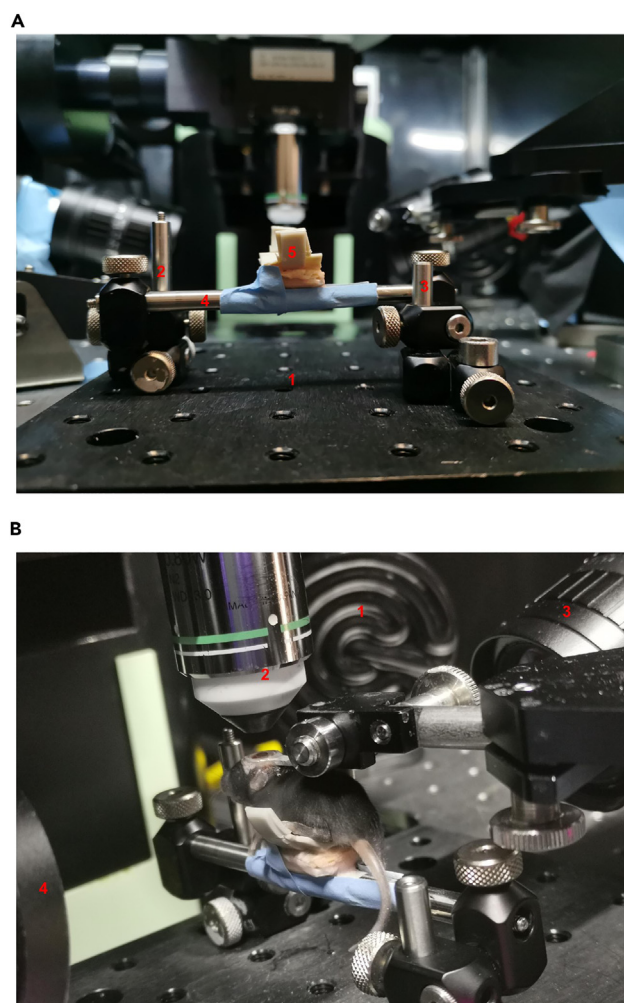


Figure 4. 2-photon calcium imaging set-up

(A) Custom-made saddle: The system relies on a small aluminum board (1), two optical posts (2) and (3) attached to the platform and connected by a third post (4). A C-shaped object (5) is fixated on (4) and acts as a saddle to support the animal during the recording.

(B) P8 animal under the objective: the heat lamp (1) is directed towards the saddle which is set right under the immersion objective x16 (2), infrared cameras (3) and (4) will closely record the animal behavior.

Note: GCaMP fluorescence is isolated using a bandpass filter (510/25). Images are acquired through a GaAsP PMT (H7422-40, Hamamatsu) using a 16X immersion objective (NIKON, NA 0.8 for a total duration of 33 min or 15000 frames).

- c. Record with Inspector software (LaVision BioTec), the fluorescence activity from a $400\ \mu\text{m} \times 400\ \mu\text{m}$ field of view at 9 Hz with a $1.85\ \mu\text{s}$ dwell time per pixel ($2\ \mu\text{m}/\text{pixel}$).

Note: Imaging fields of view are selected from the dorsal CA1 area and maximize the number of imaged neurons in the stratum pyramidale.

Note: For further details on the use of Inspector software, please refer to Figure 17 in this article.¹⁰

- d. To synchronize 2p images with the behavior, record the camera exposure time, and image triggered signals using a 1440A Digidata (Axon Instrument, 50 kHz sampling) and the AxoScope 10 software (Axon Instrument).

Analysis of two photon calcium imaging data

⌚ Timing: 2 h

This section describes the steps to preprocess and analyze the experimental data.

37. Motion correction and Segmentation (Figure 5, Step #1).

a. Motion correction.

To correct the possible motion artifacts occurring during the imaging session, we use custom MATLAB codes (adapted from Miri et al.¹¹) available at GitLab.¹²

Note: For this step, movies need to be in tiff. format.

Note: This step can also be performed using the Suite2p algorithm which uses .tiff movies as an input.

In brief, for each recording session, a subset of frames is selected as a reference and the shifts in the x and y axes are corrected with a rigid and / or non-rigid method.

b. Segmentation (cell detection and variation of fluorescence extraction).

i. Install Suite2p¹³ following the instructions.¹⁴

ii. Before running the algorithm, adapt the values to the current experimental values. The most important parameters to fine tune are the sampling rate 'fs' at 8, the Gaussian smoothing for registration 'smooth_sigma' to 1.15, 'tau' to 1.25, and the average diameter in pixels of ROIs in the recordings 'diameter' to 10.

iii. Run the algorithm. The output will be used in the next steps.

Note: Suite2p can perform a non-rigid motion correction simultaneously, if 'reg_tiff' and 'non_rigid' are set to 1.

38. Neuronal activity inference (Figure 5, Step #2).

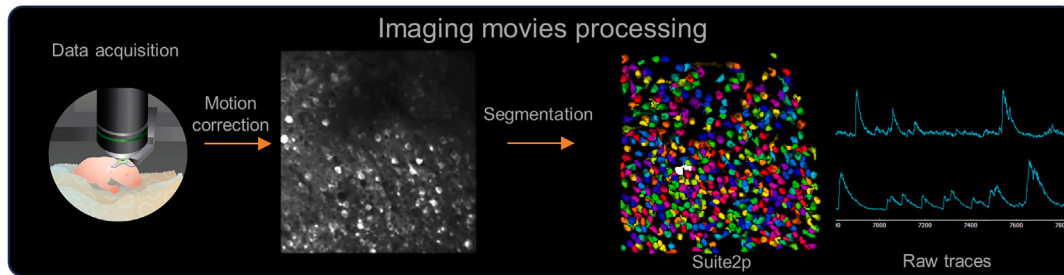
a. Neuronal activity extraction²

Note: Numerous algorithms have been developed to infer neuronal activity^{13,15–17} from calcium fluorescence signals. However, these algorithms rely on having several reference points established through simultaneous targeted patch clamp recordings and 2-photon calcium recordings^{3,18,19} of different cell types. Unfortunately, such data is unavailable when it comes to the developing hippocampus. As a result, we opted not to infer the spiking activity of every recorded neuron within our field of view. Instead, we assumed that neurons were active from the onset to the peak of any given calcium transient (depicted in Figure 5, blue and red stripes on calcium transient). Consequently, we developed DeepCINAC,² an innovative toolbox using a deep learning-based approach. This toolbox is designed to infer neuronal activity exclusively based on calcium imaging recordings (i.e., movies). The performance of this toolbox is impressive not only on developing hippocampus datasets, but also on recordings from other cortical areas, such as the barrel cortex.² It particularly excels in cases involving overlapping co-active cells, a common occurrence in densely packed layers like the CA1 pyramidal layer. Furthermore, DeepCINAC² has been trained to predict interneuron activity without any specific labeling to identify interneurons.

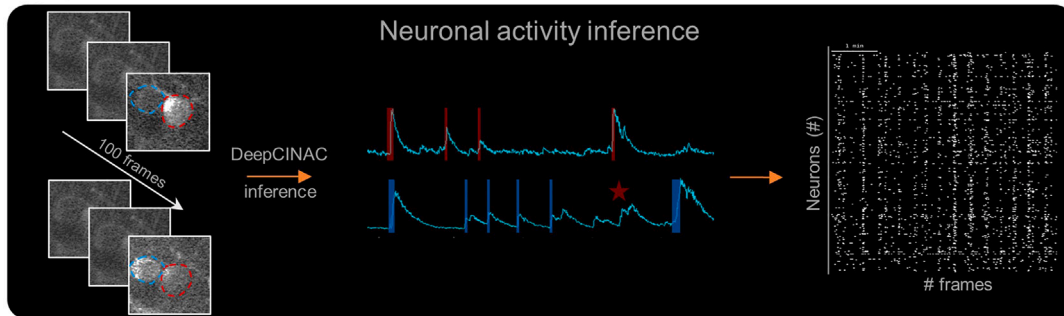
Since the algorithm is already published and available online, we will not detail how to infer activity with DeepCINAC; rather we invite you to follow the tutorial online on the GitLab page²⁰ in demos.

Processing steps for nwb creation

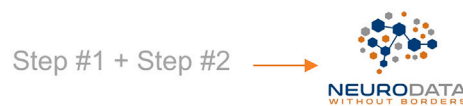
Step #1



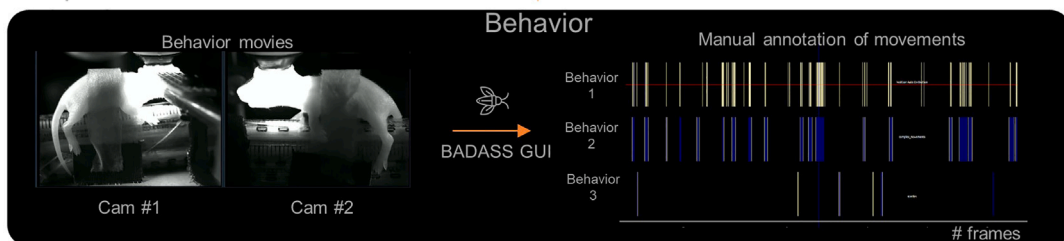
Step #2



Creation of a temporary NWB file



Step #3



Step #4

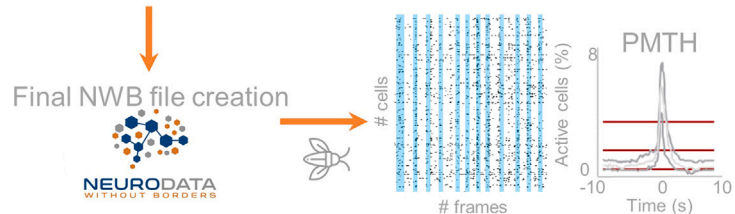


Figure 5. Analysis pipeline

Entire processing of calcium imaging data and behavioral movies to create the 'ready-to-analyze' NWB file. (step #1) Extraction of cells and their traces with Suite2p using the motion corrected movie. (step #2) Inference of neuronal activity using DeepCINAC takes as input the calcium imaging movie (left) and outputs a binary raster (right). The processing results and the data are stored in a temporary NWB file (step #1 + step #2). (step #3) Manual annotation of the behavior using the BADASS GUI, each line corresponds to one specific behavior. Blue and light-yellow stripes indicate the behavior epochs, i.e., periods during which the mouse is exhibiting a specific behavior. (step #4) Creation of the final NWB file. Peri Movement Time Histogram (PMTH), with the onset of the behavior at time 0 s and the visualization of the binary raster with behavior epochs in blue stripes. The cicada logo indicates the use of the Graphic User Interface of CICADA for analysis and manual annotation.

The code outputs a binary array with the activity of all cells as a function of time, with 1 if the cell is active at the current frame, 0 otherwise. (Figure 5 Step# 2).

Note: When used locally, the code needs to access the GPU to perform the prediction of activity, therefore, follow the instructions in the “inferring neuronal activity” section on the GitLab page.²⁰

The activity can also be predicted online on Google Colab.

△ **CRITICAL:** At the end of this step, it’s important to create a temporary Neurodata Without Borders^{21,22} (NWB) file (see below) to move to the next steps. The activity can also be predicted online on Google Colab.

39. Behavioral annotation.

CICADA and BADASS GUI.

Note: The goal of our experiment is to link neuronal activity (Steps #1 and #2) with pups’ behavior. To do so, we developed a graphical user interface (GUI) named CICADA (Calcium Imaging Complete Automated Data Analysis). This GUI accepts as inputs any NWB file to perform any kind of analysis. One example is BADASS (Behavioral Analysis of Data And Some Surprises) analysis that we use to manually annotate animal behavior during our imaging session (Figure 5 Step #3). To install and run it, refer to the instructions and the video on the GitLab repository.²³

Note: NWB formatting (not specific to this experiment): We decided to use NWB (Neurodata Without Border, Kavli Foundation) files as a data standard to share and store our data (including metadata). NWB files are similar to a container storing collections of data, including the raw data and the results of the preprocessing steps above. (Figure 5 Step #1+Step #2). These datasets are stored with the metadata describing each experimental condition or preprocessing parameters. Regardless of their content, for example two-photon imaging data or extracellular recordings data, NWB files are organized following a similar structure.^{21,22}

40. Final NWB file and analysis.

a. Final NWB file.

The final NWB gathers the content of the temporary version, namely the segmentation and the activity predictions (Figure 5 step #1, step #2) and the manual annotation performed in BADASS GUI (Figure 5 step #3) as well as the raw data. This final version is used as the input to CICADA GUI to perform analyses (see below).

b. Data analysis in CICADA.

To link pups’ behavior to neuronal activity, analyses such as Peri Movement Time Histogram (PMTH) or displaying the raster with the behavior epochs are performed on the final NWB. The outputs of such analyses are displayed on Figure 5 step #4.

Note: This entire step-by-step analysis pipeline is specific to our data which includes two photon calcium imaging and behavioral recordings. However, CICADA can be extended to other new avenues of analyses, of which can be implemented through CICADA following the template in.²³

EXPECTED OUTCOMES

This step-by-step protocol first allows an experimenter to achieve strong and stable expression of the calcium indicator GCaMP6s in CA1 neurons while avoiding cellular damages due to viral over-expression (Methods video S1). In a second step the protocol provides guidelines for a clean and stable (Methods videos S1 and S2) cranial window implant preserving healthy hippocampal activity

(see in¹ [Figure 1](#)—figure supplement 1). Even after optimization we expect a few imaging sessions to meet exclusion criteria. Consequently, before any further preprocessing steps; inspection of raw movies allows the experimenter to check for the absence of: a) z-motion artifact ([Methods video S3](#)), b) strong change in baseline fluorescence, c) absence of activity d) weird activity visible. We would recommend discarding the recording if one of these criteria was observed.

In the last step, NWB formatting facilitates both data sharing²⁴ and use of automated analysis pipelines such as CICADA²³ or other NWB compatible tools.²²

This protocol can be extended to the recordings of activity in other deep brain regions, across different mouse lines and other analyses.

LIMITATIONS

On the technical side, our protocol highlights two principal limitations: being quite invasive, as it requires a cortectomy, and the procedure cannot be performed before P5.

Even in young animals, the cortical thickness over the hippocampus is greater than 1 mm, which necessitates the use of invasive methods such as cortical aspiration. Although we have shown that early and mature hippocampal dynamics are not significantly altered by this surgery,^{1,6} not having to remove this part of the brain will simplify this surgery and improve our success rate. While 2P imaging has several advantages,²⁵ its main disadvantage is that it does not allow us to reach the hippocampus because of its limited penetration depth (limited to several hundreds of micrometers). To circumvent this technical limitation, red-shifted calcium indicators can be used for deeper imaging (Deep two-photon brain imaging with a red-shifted fluorometric Ca²⁺ indicator). However, compared to GCaMP, these indicators suffer from several limitations such as brightness and sensitivity.^{26,27} Using three-photon imaging and the latest generation of GCaMP indicators it may be possible to record CA1 dynamics without removing part of the cortex.²⁸

Another limitation of our protocols is that imaging sessions cannot be performed before P5, which corresponds to the time necessary to have a stable expression of GCaMP.⁵ One way around this limitation is to use either transgenic mice or in-utero electroporation. These techniques offer the possibility of imaging as early as P0. However, within transgenic lines, the expression of GCaMP is often restricted to a specific subpopulation of neurons and to promoters with a delayed expression, after the studied developmental period (after two weeks in the case of *thy1-GCaMP6* mice²⁹). On the other hand, in utero electroporation is quite a long and invasive technique compared to P0 injections. Moreover, it has been suggested that virus injection or plasmid electroporation at early stages of neuronal differentiation or migration is irreversibly toxic to neurons.³⁰

Regarding the analysis, a significant limitation of DeepCINAC is that it was exclusively trained using movies from developing pups expressing GCaMP6s. Despite demonstrating the algorithm's effectiveness in various brain regions and with different indicators,² it is not justifiable to assume its universal applicability across all brain regions and indicators without further training with appropriate datasets.

TROUBLESHOOTING

Problem 1

The viral solution is not going into the ventricle. Either the injection is superficial or the liquid is going into the third ventricle instead of the lateral.

Potential solution

If the blue pigmentation appears too posterior, the injection is too lateral: verify that when the pipette is touching the skin, the deformation is circular, if not, set the head of the pup straight and perpendicular to the pipette.

If the injection is too superficial, push the pipette further down and re-inject. Pay particular attention not to inject air. If the problem is not solved, sacrifice the animal.

If the pipette becomes obstructed, clear the tip using saline water and make another attempt. If the issue persists, replace the pipette.

Problem 2

Animal dies.

Potential solution

This is due to buprenorphine overdose. Adapt the dose.

Problem 3

The animal is not breathing normally.

Potential solution

Adjust the level of isoflurane according to the breathing of the pup. In case of gasping, reduce the level of anesthesia.

Problem 4

The head plate falls off.

Potential solution

Make sure that the skull is completely dry (i.e., no bleeding, no saline solution nor Super-Bond left) before fixing the headplate. If the scalp is too narrow to fix the headplate, expand it by pushing outwards with cotton swabs.

Problem 5

Leak in the "well".

Potential solution

If after several minutes you lose the meniscus between the objective and the surface of the glass, dry out everything, seal the leak, and fill with water. If the leakage is occurring because the animal is moving too much, or because you are imaging with an angle, you can use ultrasound gel instead of water.

Problem 6

Can't find the surface of the brain.

Potential solution

This is because the objective is touching the head of the animal which prevent the user to focus on the appropriate field of view. If the layer of cement is too thick, anesthetize the pup and shave it down. If the layer of Kwik-Sil below the glass is too thick (should not exceed 200 μm), try to thin the layer of cement to gain a bit of space. Otherwise, sacrifice the pup. Another indicator is the depth of the cannula: if the edges of the cannula must not be lower than the lowest bound of the headplate.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michel Picardo (michel.picardo@inserm.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the [key resources table](#).
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the [key resources table](#).
- Any additional information required to reanalyze the data 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.2023.102760>.

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AUTHOR CONTRIBUTIONS

Methodology and conceptualization, M.R.R., R.F.D., and M.A.P.; investigation, M.R.R. and R.F.D.; software and data curation, M.R.R., R.F.D., and J.D.; writing, M.R.R., R.F.D., M.A.P., and R.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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