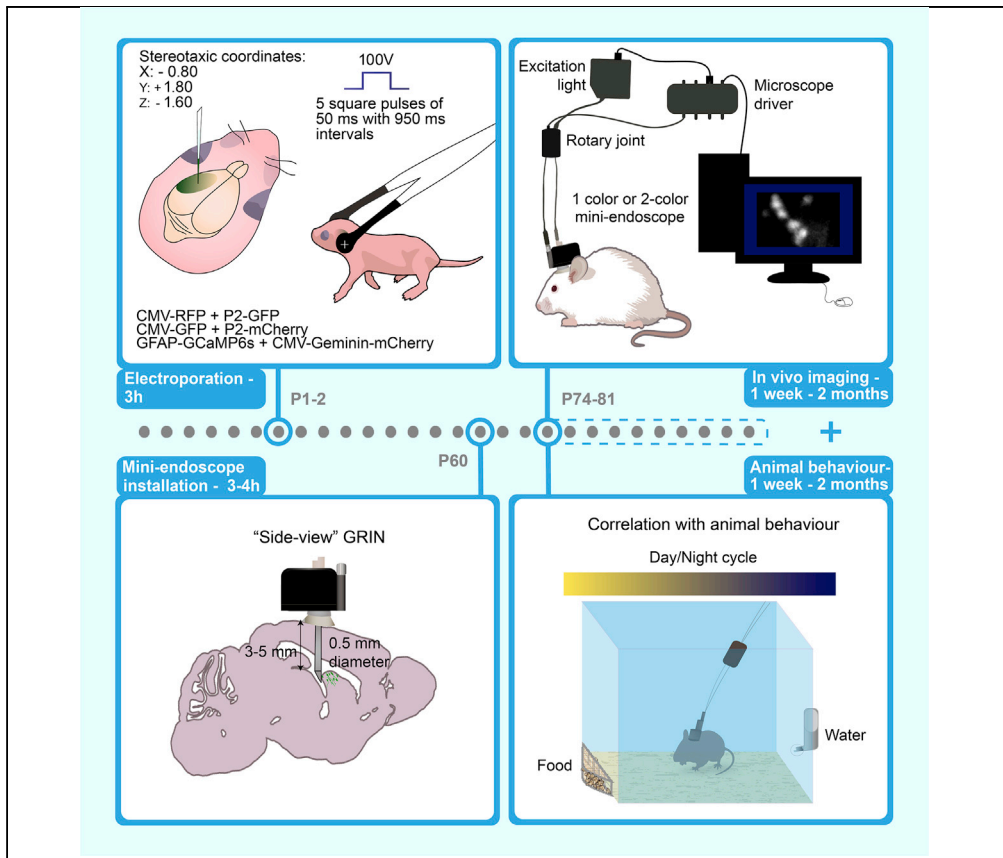


Protocol

Live imaging of adult neural stem cells in freely behaving mice using mini-endoscopes



During adulthood, the activation of adult neural stem cells (NSCs) has been mostly studied *ex vivo* in post-mortem tissues or *in vivo* in anesthetized animals. This protocol presents an approach that allows for the long-term and minimally invasive investigation of adult NSC activation and physiology in freely behaving animals. By combining specific NSC labeling and mini-endoscopic microscopy, live imaging of NSC division and Ca^{2+} activity can be performed continuously for 2–3 days and even up to several months.

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Highlights

A protocol for mini-endoscopic imaging of adult neural stem cells (NSCs)

Live imaging of NSCs activation in freely behaving mice

In vivo mini-endoscopic imaging and analysis of Ca^{2+} dynamics in NSCs

A protocol for labeling adult NSCs based on coincident activity of two promoters

Malvaut et al., STAR Protocols
2, 100596
June 18, 2021 © 2021 The Author(s).
<https://doi.org/10.1016/j.xpro.2021.100596>



Protocol

Live imaging of adult neural stem cells in freely behaving mice using mini-endoscopes

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<https://doi.org/10.1016/j.xpro.2021.100596>

SUMMARY

During adulthood, the activation of adult neural stem cells (NSCs) has been mostly studied *ex vivo* in post-mortem tissues or *in vivo* in anesthetized animals. This protocol presents an approach that allows for the long-term and minimally invasive investigation of adult NSC activation and physiology in freely behaving animals. By combining specific NSC labeling and mini-endoscopic microscopy, live imaging of NSC division and Ca²⁺ activity can be performed continuously for 2–3 days and even up to several months.

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

BEFORE YOU BEGIN

Plasmid preparation for postnatal electroporation

⌚ Timing: 10 min

Plasmids used for *in vivo* imaging of NSCs (Gengatharan et al., 2021):

- One-color imaging of NSC activation: CMV-GFP plasmid.
- Two-color imaging of NSC activation: CMV-RFP and prominin (P2)-GFP (or CMV-GFP and P2-mCherry) plasmid combination, where prominin promoter-driven fluorophore expression makes it possible to narrow down the identity of imaged cells while ubiquitous CMV expression makes it possible to follow daughter cells and avoid losing fluorescent signal because of a lack of promoter activity.
- Ca²⁺ imaging in active NSCs (aNSCs) and NSCs/astrocytes: GFAP-GCaMP6s is used to record Ca²⁺ transients and CMV-geminin-mCherry is used to identify dividing cells and distinguish between aNSCs and quiescent NSCs/astrocytes.

1. Preparation of DNA, Solutions, and Glass Pipettes

- a. Prepare high purity (OD 260/280 ≥ 2.00) and high concentration (> 4 µg/µL) endotoxin-free plasmid DNA. Consider higher concentrations if using a combination of plasmids.
- b. Dilute plasmid DNA to a final concentration of 1–4 µg/µL with endotoxin-free Tris-EDTA buffer. Inject approximately 1 µL per animal.

Note: For the electroporation of calcium indicators such as GCaMP6, we recommend using a plasmid concentration ≤ 1 µg/µL since at higher concentrations these indicators buffer intracellular Ca²⁺ in the cells where they are expressed and may therefore affect NSC's physiology.



- c. Supplement the DNA solution with 1% Fast Green to visualize the site of injection and the filling of the ventricle with the plasmid solution.
- d. Prepare pulled glass capillaries (inner diameter (ID) = 0.530 mm \pm 25 μ m, outer diameter (OD) = 1.14 mm) for the DNA injections.

Mini-endoscope preparation

⌚ Timing: 10–15 min

2. For mini-endoscopic imaging, the imaging cannula composed of gradient index (GRIN) lens has to be implanted in the brain first. On the day the GRIN lens is implanted, the appropriate GRIN lens has to be selected, which depends on the experimental paradigm. GRIN lenses are available in different diameters and lengths, and the selection of the appropriate lens mostly depends on the size of the desired region of interest (ROI) that will be imaged and the depth of the brain region that is to be targeted. In our study we used 0.5-mm diameter lenses to cause the least damage to overlying brain structures.

In addition, two models of GRIN lenses are available:

- Flat-tip GRIN lenses that make it possible to perform imaging from the top.
- Side-view GRIN lenses where the GRIN lens is coupled to a 45° prism that makes it possible to image from the side. These side-view lenses cause less damage when inserted into the brain.

Note: Prism-coupled GRIN lenses make it possible to insert the cannula directly into the lateral ventricle and to detect fluorescently labeled cells during implantation, which is much more challenging when installing flat-tip GRIN lenses. As NSCs are a rare population of cells and are distributed throughout the dorso-ventral and medio-lateral axis of the subventricular zone (SVZ), visualizing labeled cells during installation is important for deciding when to stop lowering the GRIN lens into the tissue. Another important advantage of prism-coupled, side-view GRIN lenses is that imaging is performed in the intact SVZ given that the GRIN lens is installed in the lateral ventricle.

Note: We used mini-endoscopes from Doric Lenses, and all the steps in this protocol are based on these specific products. If mini-endoscopes from other suppliers are used, some adjustments of the installation steps will be required.

Note: In this protocol, we describe the implantation of a specific model of GRIN lens cannula, the snap-in cannula.

3. Based on the depth of implantation, select the appropriate protrusion ring to screw onto the GRIN lens cannula. This ring, also called the spacer, is used to adjust the distance between cannula and skull and to securely fix the cannula to the skull by increasing the surface of fixation using cement (more details are given in the [step-by-step method details section](#)).
 - a. Screw the selected protrusion ring onto the GRIN lens cannula, with the widest part facing the animal's skull.
 - b. Seal the protrusion ring to the cannula by putting a small drop of liquid crazy glue at a site where the camera will not be located during imaging.
4. Before implantation, prepare the camera that will be used for the installation of the GRIN lens cannula and assessing fluorescence changes in the tissue during the procedure. Remove the optic fiber from the camera and fix the fluorescence microscope holder to the camera. Connect the HDMI cable to the CMOS input on the microscope driver device.

△ **CRITICAL:** It is important to note that, in the case of two-color imaging with a dual-color mini-endoscope, the camera contains two CMOS (Complementary Metal Oxide Semiconductor) detectors that are placed at different distances from the GRIN lens. This is essential because of the resulting chromatic aberration produced (the longer the GRIN lens the bigger the aberration). To prevent such chromatic aberrations, the CMOS are not located at the same position. For more details, please see (Malvaut et al., 2020).

△ **CRITICAL:** Selecting the appropriate thickness of the protrusion ring is critical as this will also make it possible to fill any potential space between the animal's skull and the GRIN lens cannula. If the space is too large, it would be more challenging to fill it with glue and cement during implantation and would also result in greater instability during imaging.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|----------------------------|---|
| Chemicals, peptides, and recombinant proteins | | |
| Tris-EDTA buffer (for the preparation and dilution of plasmids) | QIAGEN | Cat# 12362 |
| Mineral oil | Sigma | Cat# M5904 CAS: 8042-47-5 |
| Fast Green FCF | Sigma | Cat# 44715 |
| Electrode Gel Spectra 360 | Parker | https://www.parkerlabs.com/spectra-360.asp |
| 3M Vetbond glue | Vetbond | Cat# 1469SB |
| Anhydrous Ethyl Alcohol | Commercial Alcohols | Cat# P016EAAN |
| Sodium chloride (NaCl) | Fisher Scientific | Cat# S271-3; CAS: 7647-14-5 |
| Paraformaldehyde | MilliporeSigma | Cat# P6148; CAS: 30525-89-4 |
| C&B Metabond Quick Base | Parkell | Cat# S398 |
| Amalgambond/Metabond Catalyst 7 mL Bt | Parkell | Cat# S371 |
| C&B Metabond Powder Clear 3 g Bt | Parkell | Cat# S399 |
| Gel Clear Surface Insensitive Glue | Loctite | Loctite 454 |
| Ethyl Ultra Speed Adhesive (Quick Bond) | Aron Alpha | Aron Alpha 200 |
| Critical commercial assays | | |
| EndoFree Plasmid Maxi Kit | QIAGEN | Cat# 12362 |
| Experimental models: organisms/strains | | |
| Mouse: CD1 | Charles River | Strain code: 022 |
| Recombinant DNA | | |
| GFAP-GCaMP6s | (Gengatharan et al., 2021) | Modified from Plasmid # 40753, Addgene |
| CMV-GFP | Clontech | Plasmid # U55763 |
| P2-mCherry | (Gengatharan et al., 2021) | Modified from Plasmid # 64324, Addgene |
| CMV-geminin-mCherry | (Gengatharan et al., 2021) | Sequence modified from (Sakaue-Sawano et al., 2008) |
| P2-GFP | (Gengatharan et al., 2021) | Modified from Plasmid # U55763, Clontech |
| CMV-RFP | Addgene | Plasmid #54764 |
| Software and algorithms | | |
| Doric Neuroscience Studio | Doric Lenses | http://doriclenses.com/life-sciences/software/955-doric-neuroscience-studio.html |
| ImageJ | NIH | https://imagej.net/Welcome |
| MATLAB 2016a | MathWorks | N/A |
| MATLAB scripts for Ca ²⁺ imaging analysis | This paper | https://github.com/SagLab-CERVO/Calcium_analysis_in_NSC |

(Continued on next page)

| <i>Continued</i> | | |
|---|-----------------------------|---|
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| <i>Other</i> | | |
| Flaming/Brown Micropipette Puller | Sutter Instrument | Cat# P-97 |
| Glass Capillaries | World Precision Instruments | Cat# 504949 |
| Electroporator ECM 830 | Harvard Apparatus | https://www.harvardapparatus.com/media/harvard/pdf/W3_9_359.pdf |
| Footswitch | Harvard Apparatus | Model: 1250FS |
| Platinum Tweezerrode, 5 mm 45-0489 | Harvard Apparatus | https://www.btxonline.com/tweezerrodes-electrodes.html |
| Nanoliter Injector 2010 | World Precision Instruments | Cat# NANOLITER2010 |
| Microdrill | RWD Life Science | Cat# 78001 |
| Snap-in Imaging Cannula, Model L-V Prism orientation 1 Depth range: 2.9 mm–5.9 mm | Doric Lenses | Cat# SICL_V_500_80_P_1 |
| Snap-in Imaging Cannula, Model L-V Depth range: 2.87 mm–5.96 mm | Doric Lenses | Cat# SICL_V_500_80 |
| Basic Fluorescence Microscopy System - Deep Brain | Doric Lenses | Cat#BFMS-L_UFGJ_1000_900_458_D |
| 2-color Fluorescence Microscopy System - Deep Brain | Doric Lenses | Cat# 2CMS-LD_UFGJ_1000_900_458/561 |
| Fluorescence Microscope Holder | Doric Lenses | Cat# FMH |
| Dummy Snap-in Microscope Body-L | Doric Lenses | Cat# DSMB-L |
| Metabond Ceramic Mixing Dish | Parkell | Cat# S387 |
| Disposable Brushes | Centrix Incorporated | Cat# 370101 |
| Absorbable Hemostatic Gelatin Sponge | (Spongostan Dental) Ethicon | Cat# MS0005 |

STEP-BY-STEP METHOD DETAILS

Postnatal electroporation

⌚ Timing: 1–3 h

1. Animal Preparation, Glass Pipette Loading, and Plasmid Injection
 - a. Prepare a mix of ice and water for animal anesthesia by hypothermia.
 - b. Install a glass pipette filled with mineral oil on an injector arm. Cut the tip of the pipette, and lower the metal plunger of the injector to expel some oil from the pipette.
 - c. Lower the tip of the pipette into a 1- μ L drop of DNA solution, and pull the metal plunger upward to draw the solution into the pipette.
 - d. Set the voltage of the electroporation generator for 5 square pulses, 50 ms/pulse at 100 volts, with 950 ms intervals.
 - e. Place one postnatal day 0–1 pup (P0–P1) on ice for anesthesia.
 - f. After approximately 5 min, determine the state of anesthesia using the foot pinch response. If no movement occurs, proceed with an intraventricular injection of plasmids.
 - g. Place the pup in the stereotaxic apparatus, making sure that the head is straight (Figure 1A).
 - h. Sterilize the head with ethanol and chlorhexidine using cotton swabs.
 - i. Open the skin with the scissors by approximately 2 mm along the sagittal line of the skull starting from the lambda.
 - j. Lower the pipette to the level of the lambda and zero all coordinates. Lift the pipette and move it along the anterior-posterior (AP) axis in an anterior direction by 1.8 mm.
 - k. Readjust the medio-lateral (ML) coordinate by zeroing on the sagittal suture and move 0.8 mm laterally (hemisphere of choice).
 - l. Touch the skull and zero dorso-ventral (DV) coordinates.
 - m. Lower the pipette slowly into the brain by 1.6 mm.

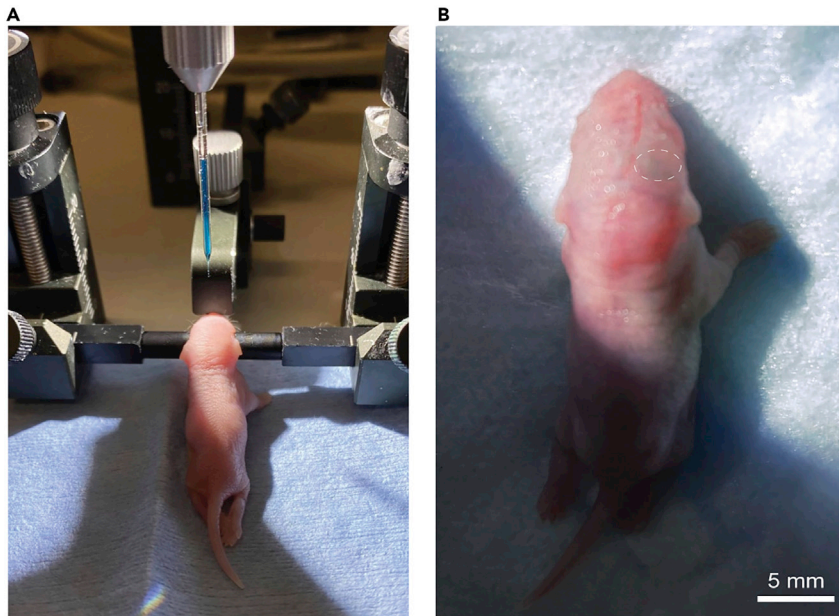


Figure 1. Electroporation procedure

(A) Anesthetized animal placed on stereotaxic setup. Note that the head is properly fixed and straight.
(B) Photograph illustrating the pup following electroporation. The site of injection is visualized with Fast Green dye.

- n. Inject the DNA solution.
- o. Place electrodes coated with conductive gel on the surface of the bones with the positive side on the lateral side of the hemisphere where the DNA was injected.
- p. Initiate current transfer by pressing the pulse footswitch pedal.

△ **CRITICAL:** Current transfer should be initiated as fast as possible after DNA injection.

Note: For better area covering, sweep the electrodes from the dorsal to the lateral side at $\sim 25^\circ$ angle intervals. For more details and how to target different NSC pools see also (Fernandez et al., 2011). For detailed video protocols of the electroporation procedure see publications by Feliciano et al. and Sonogo et al. (Feliciano et al., 2013; Sonogo et al., 2013).

- q. Close the wound with forceps and apply Vetbond surgical glue.

△ **CRITICAL:** If Fast Green was used, the lateral ventricle should be visible (Figure 1B). If the dye is not visualized or present in the 3rd ventricle the SVZ cells will not be labeled.

- r. Place the electroporated pup on a heating pad for recovery.
- s. Reunite the pup with the mother.

Electroporation efficiency checking

⌚ Timing: 24 h

Following electroporation and before implanting the GRIN lens cannula, we recommend checking electroporation efficiency by sacrificing 1–2 pups from the electroporated litter. To study adult NSCs, we use P60–P90 mice as only slowly dividing cells such as NSCs or non-dividing cells such as niche astrocytes retain the label at this age. A comparison of the number of cells in the SVZ at post-electroporation days 10 and 60 is presented in Figure 2A. The adult NSCs co-expressing GFP and mCherry proteins driven by two different promoters are shown in Figure 2B.

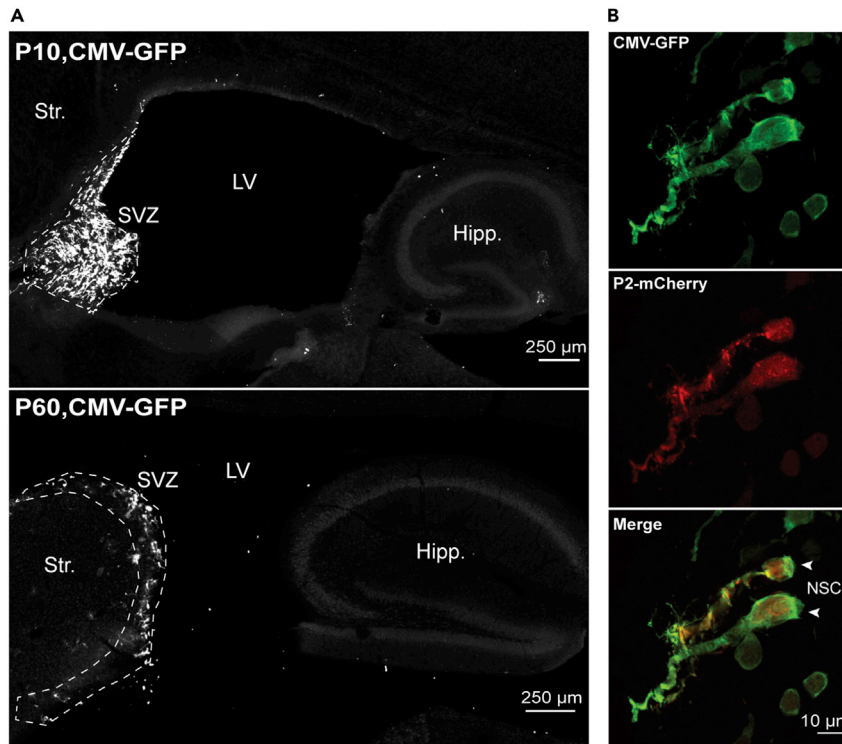


Figure 2. NSCs labeling in the adult SVZ

(A) Sagittal sections of P10 and P60 mouse brains that had been electroporated at P0-P1 with CMV-GFP plasmid. Note reduction in the number of electroporated cells at P60 because of plasmid dilution. LV, hipp. and str. indicate, respectively, lateral ventricle, hippocampus and striatum.

(B) Images of adult NSCs co-labeled with CMV-GFP and P2-mCherry plasmids.

2. Perfuse the animals to collect their brains.
 - a. Deeply anesthetize the animals using sodium pentobarbital (12 mg/mL; 0.1 mL per 10 g of body weight).
 - b. Perfuse the animals intracardially with 0.9% NaCl followed by 4% paraformaldehyde (PFA).
 - c. Collect the brains and post-fix them from 12 to 16 h at 4°C in 4% PFA.
3. Cut sagittal sections of the brains using a vibratome.
4. Check for fluorescently labeled NSCs in the SVZ.

Note: Verifying the amount and location of fluorescently labeled NSCs in the SVZ is useful for adjusting the implantation coordinates along the dorso-ventral and/or media-lateral coordinates, depending on the location of electroporated cells in the SVZ (see also [troubleshooting 2](#)).

Mini-endoscope installation and fixation

© Timing: 2–4 h

The following section describes the installation of the mini-endoscope on 2- to 3-month-old animals electroporated at P0-P1.

5. Perform isoflurane anesthesia (2%–3% isoflurane, oxygen flow at 1) by placing the animal in an induction chamber.

6. When the animal is anesthetized, shave the hair on the head and inject a local anesthetic and a non-inflammatory drug subcutaneously.
7. Place the animal on the stereotaxic setup under isoflurane anesthesia (1.5%–2% isoflurane, oxygen flow at 1) and remove any remaining hair on the head using depilatory cream. Disinfect the surgical site.
8. Cut the skin on top of the head from between the ears to the anterior region of the eyes. On each side of the initial cut, also cut out a small piece of skin in the region that will be surrounding the cannula once it is put in place. Use four small clamps to pull aside the skin in front and in back of the cut to expose as much skull as possible (Figure 3A).
9. Prepare a glass pipette (the same type as that used for performing the electroporation) and install it on an injector arm. Cut the tip of the pipette to get an approximately 50–75- μm diameter tip.
10. After zeroing the coordinates at the bregma, move to the targeted coordinates for implantation. Drill a hole at this coordinate using a medium tip burr (Figure 3B).

Note: The diameter of the drilled hole should be about two times larger than the diameter of the GRIN lens to allow for adjusting the coordinates during implantation and to prevent the GRIN lens from touching the sides of the hole. Avoid making the hole too large as this may result in too much pressure being put on the brain tissue when the GRIN lens is inserted.

11. Lower the glass pipette progressively into the brain tissue to prevent bleeding. The path created by the pipette will be used as a “track” in the brain to facilitate the insertion of the GRIN lens.

Note: Lower the pipette into the hole multiple times without going as deep as the targeted coordinates. For instance, if your targeted z depth is 3 mm, do not lower the pipette any further than 2.5–2.6 mm into the brain.

12. Clean the surface of the brain well using sterile saline solution and presoaked hemostatic sponges.

△ CRITICAL: It is important that there is no bleeding on the surface of the brain before starting the insertion of the GRIN lens since it can alter imaging quality.

13. Remove the injector from the stereotaxic frame and install the fluorescence microscope holder to which the camera is attached.
14. Carefully fix the GRIN lens cannula with the protrusion ring onto the microscope.

Note: To equilibrate the weight of the microscope on the head of the mouse, it is important to place the microscope in such a way that the back of the camera is positioned backwards on the animal's head. The camera may also be slightly oriented toward the center if GRIN lens cannula is implanted in a lateral region.

15. Turn on the microscope driver and launch the imaging software (Doric Neuroscience Studio).
16. Set microscope exposure time, gain, and LED efficiency to be able to visualize background fluorescence.
17. Start live imaging with the imaging software and make sure that the excitation light correctly exits the GRIN lens.

Note: It is possible to use GRIN lenses several times (see implant removal steps). If a GRIN lens that has already been used is to be installed, it is important to make sure that the light spot coming out of the GRIN lens is circular (this can be done using a small piece of white paper) and that there is no major background on the image. If there is major background, carefully clean the tip and sides of the GRIN with water and soap and then with 70% ethanol using a piece of Kimtech tissue.

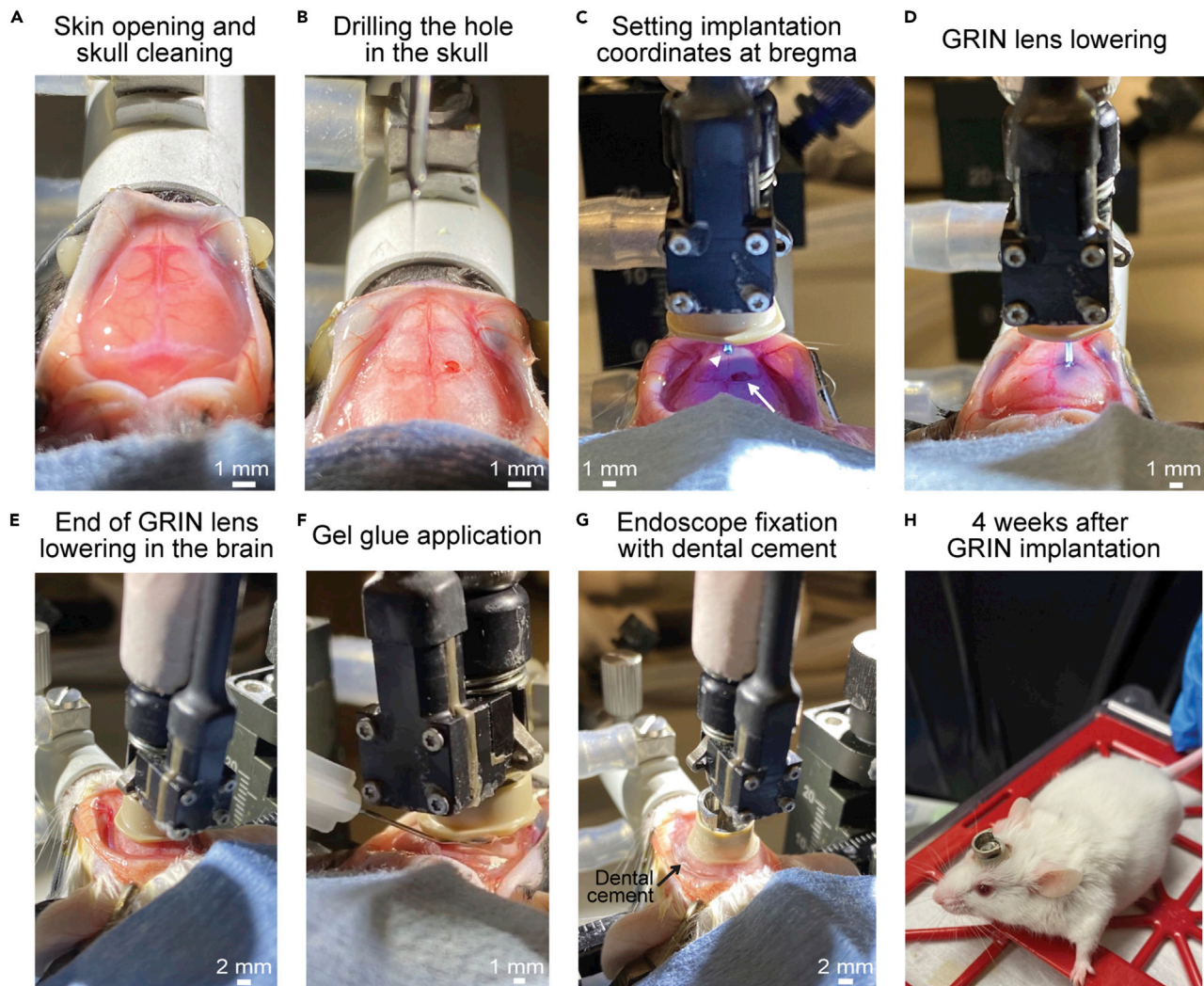


Figure 3. Mini-endoscope installation and fixation on the skull

(A) Photograph illustrating opening of animal's skin and skull cleaning prior to cannula implantation.

(B) Photograph illustrating drilling of a hole in the skull above the targeted region.

(C) Photograph illustrating the lowering of a prism-coupled GRIN lens on top of the bregma. The white arrowhead indicates the prism-tip of the GRIN and the white arrow indicates the hole that was drilled above the implantation coordinates in the right hemisphere of the animal.

(D) Positioning of the GRIN lens at the targeted XY coordinates, above the hole previously drilled in the skull. The GRIN is lowered along the z axis to approach it to the surface of the brain.

(E) Photograph illustrating the end of the descent of the GRIN lens into the brain. Note the small space between the protrusion ring and the skull of the animal.

(F) Application of gel glue with a needle to fill the space between the skull and the protrusion ring.

(G) Photograph illustrating the sealing of the cannula to the animal's head using dental cement and after snapping off the imaging camera. Note that dental cement was applied all around the GRIN lens implant.

(H) Photograph showing a mouse with the implant, 4 weeks post-implantation.

18. Lower the GRIN lens to the bregma and zero coordinates (Figure 3C).

Note: The excitation light makes it easier to perform this step as the diameter of the blue circle decreases as the GRIN lens is lowered close to the surface of the skull. Center the excitation light spot on the bregma and set the XY coordinates to 0. If a 45 μ m prism-coupled GRIN lens is used, center the bregma at the midline of the prism.

19. Position the GRIN lens above the hole previously drilled in the skull at the targeted XY coordinates and lower along the z axis to approach it to the surface of the brain (Figure 3D). Set the z coordinates to 0.
20. Draw an ROI on the acquired image using the imaging software.

Note: Drawing an ROI makes it possible to assess changes in fluorescence intensity on the graph of fluorescence intensity in the Doric Neuroscience Studio software during the implantation of the GRIN lens.

21. Implant the GRIN lens in the brain
 - a. Lower the GRIN lens into the brain using a relatively fast first push of approximately 100–200 μm to break into the tissue. Wait approximately 3–5 min for the brain to stabilize.

Note: You should be able to clearly visualize the moment at which the GRIN lens enters the brain on the resulting fluorescence graph by changes in background fluorescence that then stabilizes as the tissue surrounding the implant stabilizes.

- b. Continue lowering the GRIN lens along the z axis in 50–100- μm increments. Wait approximately 30 s to 1 min between each increment (Figure 3E).

△ CRITICAL: Track any significant change in background fluorescence levels when lowering the GRIN lens into the brain. If the fluorescence intensity decreases while lowering the GRIN lens, this indicates that the brain tissue is being pushed too much, which results in tissue squeezing. If this happens, we recommend decreasing the speed of insertion or increasing the pause between each increment. Alternatively, completely remove the GRIN lens and begin lowering it into the brain again. If regular small increases in fluorescence intensity occur at every increment, this may either indicate that the surface of GRIN is getting dirty or that some bleeding is occurring. In this case, we recommend removing the GRIN lens from the brain, cleaning it with 70% ethanol, and lowering it into the brain again.

- c. As the GRIN lens gets closer to the targeted z depth, slow down the insertion and check for any change/increase in background fluorescence intensity on the fluorescence graph. An increase may be a sign that the region containing NSCs has been reached. Since NSCs are a rare population of cells and are sparsely labeled, it is possible that only a small increase in background fluorescence will be observed or that some cell “shadows” may appear in the field of view without causing any increase in overall background fluorescence. When using a prism-coupled GRIN lens, fluorescently labeled cells can already be seen in the field of view during installation, which is a great advantage when working with a rare population of cells such as NSCs.

△ CRITICAL: Regularly check the remaining distance between the skull and the protrusion ring. When the ring starts to touch the skull, stop lowering the implant. On the other hand, if the target z depth is reached and the distance between the spacer and the skull is still too large (1 mm approximately), this will make it more difficult to fill the gap with glue and dental cement, and may lead to instability during long-term imaging. If the distance is too large, we recommend removing the GRIN lens from the brain, changing the protrusion ring for a thicker ring, and restarting the implantation using the previously described steps (steps 15 to 21).

Optional: If after lowering the GRIN lens no significant change in background fluorescence is visible, the GRIN lens may be removed from the brain. It can be cleaned and re-installed at slightly adjusted XY coordinates. This operation, however, should not be repeated more than 2–3 times.

22. Fix the implanted GRIN lens cannula on the animal's skull (see also [troubleshooting 3](#) and [4](#)) ([Figures 3E–3G](#), [Methods video S1](#)).
- Fill the space between the skull and the protrusion ring using a needle and gel glue, being careful not to touch the camera and the place where the baseplate is attached to the camera. Concentrate on filling the parts where there is the most space, especially if GRIN lens cannula is installed at lateral coordinates ([Figure 3F](#)).
 - While the glue is drying, prepare the Metabond dental cement that will be used for the first layer. We recommend mixing 2 spoons of cement powder with 4 drops of solvent and 1 drop of catalyst, according to the manufacturer instructions.
 - Apply the first layer of cement with a disposable brush, being careful not to put dental cement on the camera or where the baseplate is attached to the camera ([Figure 3G](#)).
 - Prepare cement for a second layer by mixing two spoons of cement powder with 2–3 drops of solvent and 1 drop of catalyst, and clean the brush with ethanol if you wish to reuse it.
 - When the second layer of cement is dry, carefully remove the camera from the GRIN lens cannula ([Figure 3G](#)).
 - Apply a third layer of cement, mixing half as much of every ingredient in order to fill any remaining empty spaces between the protrusion ring and the skull.

Notes:

- We recommend that the cement used for the first layer be more liquid than the cement used for the second and third layers.
 - To make sure that no drift in the implant position occurs when sealing with the glue and cement, we recommend taking snapshots of the imaged field of view while the glue and cement are being applied.
23. When the cement is completely dry, remove the four clamps holding the skin, place the skin back on top of the skull covered with dental cement. Since small pieces of skin on each side of the initial cut were removed, the skin should now surround the GRIN lens cannula baseplate.

△ CRITICAL: Before putting the skin back in place, check for any cement on the skin and ensure that the edges of the cement are rounded and adhere well to the skull. Sharp edges, especially near the eyes, may be harmful for the animal and cause excessive scratching. If such sharp edges are present, cut them with a small pair of scissors.

24. Sew the skin back together in front of and behind the implant.

△ CRITICAL: Caution should be taken when sewing the skin back close to the animal's eyes. Too much tension in the skin may cause some discomfort to the animal and cause it to scratch and possibly hurt itself.

Note: We recommend cutting the nails of the implanted animals once a week to prevent any scratching-related injuries.

▣ Pause point: Post-surgery, mini-endoscopic imaging can usually start 10–14 days after the insertion of the side-view GRIN lens and 3–4 weeks after the implantation of the flat GRIN lens ([Figure 3H](#)).

In vivo imaging of adult NSC activation and physiology

⌚ Timing: 3–5 h (Ca²⁺ imaging) to 2–3 days (NSC division imaging)

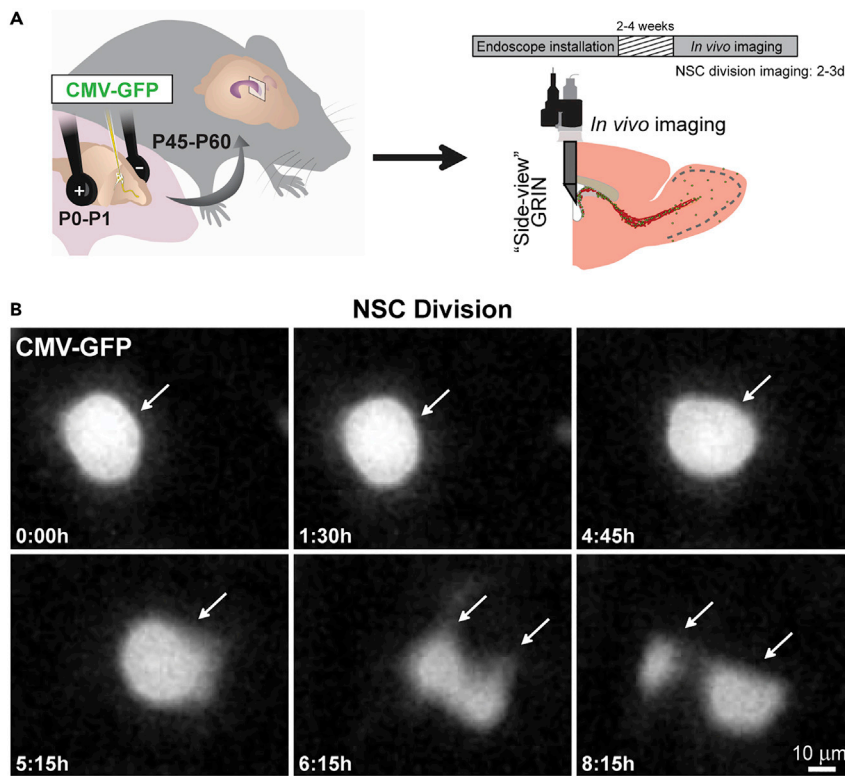


Figure 4. Imaging NSC activation *in vivo* in freely behaving animals

(A) Schematic of the electroporation of a CMV-GFP plasmid in the lateral ventricle of P0-P1 pups that were then implanted with a side-view GRIN lens at P45-P60. Mini-endoscopic imaging started 2-4 weeks post-installation. (B) Snapshot images illustrating the division of an NSC (white arrow) labeled with the CMV-GFP plasmid. In this particular example, cell division started at 6:15 h, which corresponded to the first time-frame preceding the reduction in fluorescence intensity. The cell division ended at 8:15 h when two daughter cells were clearly distinguishable. For other examples of snapshot images and videos of NSC division see (Gengatharan et al., 2021).

Following the recovery of the animal and the stabilization of the tissue, *in vivo* imaging of adult NSCs can be performed. We recommend habituating the mice to mini-endoscopic imaging by progressively increasing the time of consecutive imaging sessions from 1–2 h to 24–48 h (Figure 4A).

25. Carefully clean the lens of the camera as well as the top and sides of the implanted cannula with 70% ethanol or isopropyl alcohol using a cotton swab.

Note: It is important, especially when cleaning the microscope, to use a swab that is not too wet to prevent any liquid from penetrating below the lens.

26. Dry the camera lens and the top of the cannula well using compressed air to remove residual dust or humidity.
27. Snap the mini-endoscope camera into the cannula baseplate using forceps to separate the lateral brackets.

Note: To reduce as much as possible the pressure applied on animal's head and skull due to consecutive attachments of the microscope body with the cannula during the 2–3-month period, we recommend positioning the animal with the top of the head facing down while attaching the microscope on its baseplate.

28. Transfer the animal to the imaging chamber.

29. Connect the wires (camera cable and optic fiber) to the mini-endoscope driver.
30. Launch Doric Studio imaging software.
31. Set the acquisition parameters (exposure time, gain, and LED efficiency) for one or both channels, depending on the microscope used (i.e., one- or two-color mini-microscope).
32. Launch live imaging to check for fluorescent labeling in the field of view and adapt the acquisition parameters, if needed (see also troubleshooting 3 and 5).
33. If the labeling is satisfactory, launch acquisition.

Notes:

- For one-color imaging of GFP or GCaMP6, we used 458/35 and 525/40 excitation and emission filters, respectively. For two-color mini-endoscopic imaging, we used LED and YAG illumination sources with 430–477 nm and 558–564 nm excitation filters for GCaMP6s (or GFP) and mCherry imaging, respectively, and 520/35 and 615/45 emission filters.
- To image NSC division, we acquired images at a frame rate of 5–10 images (which were averaged offline to obtain a single image) every 15 min continuously for 2–3 days. An example of NSC division is shown in [Figure 4B](#).
- To perform Ca²⁺ imaging of adult NSCs, 5–10 images, which were averaged offline to obtain a single image, were acquired every 30 s for 2–3 h.

Optional: During adult NSC imaging, animal activity in the arena can also be monitored. In addition, some naturalistic behaviors such as food and water intakes can be evaluated by the addition of photobeams at the food and water ports of the cage. To do so, we used custom-made, 30 cm × 24 cm cages with water and food ports equipped with photobeams as shown in our previous publication ([Gengatharan et al., 2021](#)) or a slightly modified version where food port is placed in middle right part of the cage ([Methods video S2](#)).

34. At the end of the acquisition period, stop the imaging software, unplug the cables from the driver, and remove the animal from the arena.
35. Disconnect the camera from the GRIN lens cannula and put the animal back in its housing cage.

Note: Like when the microscope body is fixed to the animal's head, we recommend holding the mouse in the hand upside-down (the top of the head facing down). Lateral brackets on the sides of the microscope body can be rapidly detached from the baseplate using forceps, which quickly unplug the microscope body from the baseplate. This minimizes the impact of microscope manipulation on the animal's head.

GRIN lens cannula removal

⌚ **Timing:** 30min – 1h

The following section describes the steps to remove the GRIN lens and to clean it at the end of an experiment (see also [Methods video S3](#)).

36. Perform isoflurane anesthesia (2%–3% isoflurane, oxygen flow at 1) by placing the animal in an induction chamber.
37. When anesthetized, place the animal on the stereotaxic setup under isoflurane anesthesia (1.5%–2% isoflurane, oxygen flow at 1).
38. Fix the dummy version of the microscope on the fluorescence microscope holder and install it on the stereotaxic frame.
39. Snap the microscope to the GRIN lens baseplate by lowering it down the z-axis arm.
40. Carefully drill the dental cement surrounding the baseplate using a medium tip burr.

Note: This step will produce a large amount of dental cement dust. We recommend that this dust be regularly removed using compressed air to maintain good visibility around the implant.

41. Continue drilling around the implant until no residual resistance is experienced when the fluorescence microscope holder is pulled up using the z-axis arm and the baseplate is no longer attached to the animal's skull.

△ **CRITICAL:** Proper and complete drilling is required to remove the GRIN lens from the animal's head to avoid damaging the brain, especially if fluorescent labelling or correct GRIN lens location needs to be evaluated.

42. To perform post-mortem analysis of implanted brain tissues once the implant is removed, stop the isoflurane anesthesia and perfuse the animal to collect the brains as per steps 2 to 4 above.
43. To reuse a GRIN lens for another experiment, carefully clean it as follows:
 - a. Use a small blade to remove the spot of glue used during the GRIN lens cannula preparation steps to adhere the protrusion ring to the implant baseplate
 - b. Unscrew the protrusion ring from the implant baseplate.

Note: The protrusion ring can occasionally be difficult to unscrew, especially after being fixed in place on the animal's skull for several months. Soaking the implant, when it is still attached to the ring, in a mixture of water and soap for approximately 12h may facilitate this step by dissolving residues that have built up between the parts.

- c. Soak the implant for approximately 12 h in a mixture of distilled water and soap.
- d. Carefully and thoroughly clean the implant using a cotton swab or a piece of Kimtech paper and 70% ethanol.

Note: The quality of the implant cleaning is of particular importance if you plan to use the GRIN lens for another experiment. The last two steps for cleaning the GRIN lens can be performed several times if some deposits or background are visible on the image before the lens is implanted in the brain.

EXPECTED OUTCOMES

Successful completion of this step-by-step protocol makes it possible to assess NSC function and activation in freely behaving mice. When coupled with behavioral monitoring, NSC division and their Ca^{2+} activity may also be investigated during several behavioral states. Representative video examples of NSC activation *in vivo* in freely behaving mice and of Ca^{2+} activity in NSCs can be found in a previous publication ([Gengatharan et al., 2021](#)).

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of adult NSC activation and division

Images obtained at the 15-min acquisition time-points were averaged to obtain a single image per time-point. The resulting time-lapse movie was then corrected for XY drift using the StackReg ImageJ plugin, as warranted. NSC division was then manually determined and was defined as a complete mitosis of the mother cell. In fact, mitosis of a high fluorescence intensity mother cell resulted in two daughter cells exhibiting reduced fluorescence intensity due the dilution of the fluorophore in the two daughter cells. For examples of videos of *in vivo* NSC division, please see ([Gengatharan et al., 2021](#)). The start of cell division is defined as the first time-frame preceding a reduction in fluorescence intensity and the end of cell division when two clearly discernable daughter cells are observed ([Figure 4B](#)). Note that this analysis focuses on cell division, i.e., the M-phase, and does not measure the full cell cycle length, including the typically long G1 phase.

Analysis of Ca²⁺ activity in adult NSCs

Ca²⁺ events are analyzed using a custom-written script in MATLAB (MathWorks Inc., USA), which is available through a GitHub link provided in this protocol as well as in our previous publication ([Gengatharan et al., 2021](#)).

1. Perform motion correction using the StackReg ImageJ plugin, as warranted.
2. Add the folder with the MATLAB scripts provided in this article to the MATLAB path.
3. Launch the Main_part.m file and open your movie in .tif format.
4. Draw an ROI around the soma of the cell to extract the GCaMP6s fluorescence signal.
5. Draw an ROI near a cell body to define a background.
6. Proceed with peak detection by applying a multiple threshold algorithm ([Danielson et al., 2017](#); [Gengatharan et al., 2021](#)) to the Ca²⁺ time series of the defined ROI.

▮▮ Pause point: The mean standard deviation (SD) of the Ca²⁺ trace is calculated first, and all peaks with an amplitude greater than 2 times the SD are measured. These peaks are then removed from the trace, making it possible to detect smaller peaks by recalculating the mean SD and selecting events greater than 2 times the new SD. This procedure is repeated once more, and the third threshold is applied. Multiple thresholding allows all Ca²⁺ events to be detected regardless of their amplitudes.

7. Detected peaks in the interactive window may be added or removed by pressing the “add” or “delete” button after pressing on the detected peak.

Note: Detected peaks are represented by circles and are color coded based on the threshold during which they were selected.

8. After proofreading the automatic peak detection, approve the result by pressing the “save” button, and type the acquisition frequency of imaging to calculate the frequency of events.

Note: Ca²⁺ activity is calculated as the relative change in the percentage of $dF/F = (F-F_0)/F_0$, where F is the GCaMP6 intensity in the ROI and F₀ is the baseline signal. The baseline signal is defined as a mean of the trace after removing all detected peaks.

9. Save the result, which is an Excel file with the mean amplitudes and frequencies and graphs of the time series in .pdf format.

LIMITATIONS

While this protocol makes it possible to track the same NSC for up to several months (three months being the longest period that we imaged), the ability to monitor the activity of the same cells during such an extended period of time may be compromised because of several challenges. Postnatal electroporation itself gives rise to sparse labeling in the adult SVZ. Over time and with consecutive cycles of division, dilution of the plasmid may substantially decrease the fluorescence intensity of NSCs in the field of view. In addition, the daughter cells may differentiate to a neuronal lineage and migrate away, leading to the disappearance of daughter cells from the field on view.

The ubiquitous CMV promoter should be used in order to reliably detect fluorescent cells after cell division. However, this promoter leaves the identity of the daughter cells unknown. If cell-type specific promoters are used (e.g., GFAP or P2) to define NSCs ([Beckervordersandforth et al., 2010](#); [Codega et al., 2014](#)), the division event can be missed because of the lack of GFAP and/or P2 promoter activity in one or both daughter cells, because of differentiating asymmetric or symmetric NSC division, respectively. This issue can be partially solved by using the two-color mini-endoscope, which

allows the coincident expression of CMV-RFP and P2-GFP (or GFAP-GFP) in order to detect different modes of NSCs division.

Lastly, the imaging technique used in this protocol has a non-negligible drawback that is its inability, once the GRIN lens is installed, to adjust the focus during an acquisition in order to better focus on NSCs in the field of view, track other NSCs in different z locations, or track NSC division in the z axis. Recent advances in the field of electronically tunable miniature endoscopy (Aharoni and Hoogland, 2019; Malvaut et al., 2020) that make it possible to adjust the focal plan and also track cells in z axis may help resolve this issue.

The advent of wireless miniature endoscopy may also make it possible to use this technique in a wider range of behavioral contexts such as social interactions and more complex social behaviors and may also reduce the impact of cables on animal behavior.

TROUBLESHOOTING

Problem 1

Plasmid injection is not visualized (step 1).

Potential solution

Make sure that the stereotaxic coordinates are properly adjusted. It is also important to correctly position the head of the animal. If the head is not straight, the plasmid can go into the third ventricle. In this case, the dye will be visualized under the lambda.

Problem 2

The injection site is correct and is clearly visualized but no or very faint NSC labeling is visible during electroporation efficiency checking (steps 2–4).

Potential solution

1. Make sure that a current is passing between the electrodes. If not, change the electroporation gel and make sure that the electrode surfaces are completely covered. Another possible source of this problem is that the electrodes are barely touching the bone of that animal and that no pressure is applied. If this is the case, try pressing a bit more firmly. The time between injecting the plasmid and applying the current is also crucial. Apply the current very quickly after injecting the plasmid.

2. Since the plasmid solution is often quite viscous, mix it well before checking the concentration and before preparing the injection solution to make sure that your final concentration is correct. The recommended concentration for Ca²⁺ indicators is 1 µg/µL and is 3–4 µg/µL for the other plasmids mentioned above.

Problem 3

No labeling is visible during imaging (steps 28–32).

Potential solution

As mentioned in the [step-by-step method details section](#), it is quite often possible to start *in vivo* imaging 10–14 days after the insertion of the side-view GRIN lens and 2–4 weeks after the implantation of the flat GRIN lens. However, there is some variability and an extended recovery time may occasionally be required to allow for clear visualization of the fluorescent labeling.

If after an additional 2–3 weeks of recovery fluorescent labeling is still not visible, we recommend sacrificing the animal in order to check the SVZ for labeling and the placement of the GRIN lens. This step may also help adapt the implantation coordinates for future animals.

Problem 4

The implant detaches from the skull when the camera is attached (step 27).

Potential solution

The detachment of the implant during the attachment of the camera may reflect instability caused when the GRIN lens cannula is installed. This instability may be associated with one of the steps used to secure the implant on the skull.

The free space between the protrusion ring and the surface of the skull is filled with gel glue. However, if excess glue is used because of a large space between the protrusion ring and the skull this may increase implant instability that can, in some cases, result in the detachment of the implant during the fixation/removal of the camera. This instability may also indicate that the glue was not sufficiently dry before the cement was applied.

We recommend that the cement be applied in a homogenous way in order to secure the implant tightly to the animal's head. It is important to make sure that cement is applied to the entire space around the implant. As with the glue, it is important that each layer of cement is completely dry before applying the next layer. Lastly, the cement used to secure the implant to the animal's head is crucial for the long-term stability of the cannula. Some cements become friable over time, which in turn can considerably reduce the stability of the implant over time.

Problem 5

The field of view moves significantly during imaging (step 32).

Potential solution

Movement of the field of view during imaging may reflect several potential issues. First, make sure that the camera is correctly fixed to the cannula by detaching the microscope and reattaching it to the implant. A clue that the movement of the field of view may be linked to the fixation of the camera is a change in fluorescence background when the animal moves around the arena.

If the problem persists after making sure that the camera is correctly fixed to the GRIN lens cannula, an additional recovery period may occasionally help improve implant stabilization and reduce motion artifacts.

If it is not too important, image movement in XY may be corrected using a motion correction plugin such as StackReg ImageJ plugin. However, z axis movement is much more challenging to correct, and if substantial movement persists after several imaging sessions, we recommend stopping 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, Dr. Armen Saghatelian (armen.saghatelian@fmed.ulaval.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The MatLab code for the analysis of Ca^{2+} activity in NSCs is available at https://github.com/SagLab-CERVO/Calcium_analysis_in_NSC.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.100596>.

ACKNOWLEDGMENTS

This work was supported by a Canadian Institute of Health Research (CIHR) grant to A.S. S.M. is supported by a MITACs post-doctoral fellowship.

AUTHOR CONTRIBUTIONS

S.M., A.M., and A.S. wrote the manuscript. A.G. established the electroporation approach for NSC labeling in the laboratory. A.S. developed the *in vivo* mini-endoscopic imaging approach for NSCs, which was further refined by S.M. A.G. and S.M. performed *in vivo* mini-endoscopic imaging of NSCs.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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