Calcium imaging of the adult-born neurons in naturally sleeping mice

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**SUMMARY**

The adult-born granule cells in the dentate gyrus (abGCs) contributes to process memory and emotion both in physiological and pathological conditions including epilepsy, Alzheimer’s disease, and depression. To decipher the function of abGCs, calcium imaging under free moving condition enables to examine their event-related ensemble activities with genetically defined abGC population, although its temporal resolution is second to that of electrophysiological methods. Here, we present a complete protocol for the calcium imaging of abGCs in naturally sleeping mice using a miniaturized microscope. The preparatory surgery and the post-recording processing could be the major obstacles and this protocol provides solutions to them.

**HIGHLIGHT**

**GRAPHICAL ABSTRACT**

**BEFORE YOU BEGIN**

Timing: 3 – 4 months

1. Obtain at least two transgenic mice lines: one line expressing a fluorescent calcium indicator protein after exposure to Cre recombinase; the other line expressing a tamoxifen-inducible Cre recombinase in neural stem or progenitor cells.

Note: We have experience using Nestin-CreERT2 transgenic (nestin) mice from Kyoto University (Nestin-Cre ERT2 line 4, Imayoshi et al., 2008) and from Jackson laboratory (#016261), in which Cre recombinase is expressed in Nestin-positive cells in the adult brain by tamoxifen induction. Alternatively, the Ascl1-CreERT2 mice (Jackson #012882) could be examined to shorten the entire experimental period (e.g., Gregor-Alexander Pilz, Science, 2018, DOI:10.1126/science.aao5056) and control the target age of abGCs better, although we do not have experiences.

Note: In the best of our current knowledge, we recommend to use the GCaMP3 mice line Ai38 (RCL-GCaMP3) for expressing the fluorescent calcium indicator. Although next generation indicators are usually preferable, such as GCaMP6 and beyond, their mouse lines with Cre-inducible expression of the sensor showed very low/absent expression when expressed in abGCs in our experiments. For example, we crossed nestin mice with Ai96 (RCL-GCaMP6s), which resulted in no expression of GCaMP6s (unpublished results). We speculate two reasons: 1. expression of recent GCaMP sensor in neural progenitors interfere with Ca-signaling essential for immature abGC survival. 2. The adeno-associated virus vectors (AAVs) we used to deliver transgenes may affect the survival of abGCs.

Instead of using mice transgenic lines, induction of calcium indicators and/or Cre recombinase could be achieved by viral transduction. However, most AAVs are not suitable for neural stem cell research because of their low infection rate (Kotterman et al., 2015) and cytotoxicity (Johnston et al., 2020). Furthermore, lentivirus-mediated expression of Ca2+ sensors dramatically reduced the number of proliferating cells in the DG in our experimental conditions. Retrovirus-mediated expression works for two-photon microscopy in a head-fixed setup (Danielson et al., 2016), but their use in freely behaving microscopy is not described yet.

2. Cross and breed both transgenic lines, in accordance with corresponding guidelines, to generate animals in which the expression of the Ca2+ sensor is initiated by tamoxifen-inducible Cre recombinase in the adult neural stem/progenitor cells.

**KEY RESOURCES TABLE**

|  |  |  |
| --- | --- | --- |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Tamoxifen | Sigma | T5648-1G |
| Isoflurane inhalation solution | Pfizer |  |
| Xylocaine injection 1％ with epinephrine (lidocaine) | Aspen Japan |  |
| OCT mounting medium | Sakura Fine Tech. | 4583 |
| Vectashield | Vector | H-1000 |
| 4% Paraformaldehyde phosphate buffer solution | Nacalai Tesque | 09154-85 |
| Sucrose | Nacalai Tesque | 30404-45 |
| Corn oil | Sigma | C8267 |
| Flunixin meglumine | Fujita | FLUNIXIN INJ. 10% |
| Antibiotics (sulfadiazine/trimethoprim) | Kyoritsu Seiyaku | Tribrissen injection |
| Tarivid ophthalmic ointment 0.3％ (Ofloxacin) | Santen Pharmaceutical | 1319722M1056 |
| Experimental Models: Organisms/Strains | | |
| Mouse: Nestin-CreERT2 Line 4 | Kyoto University |  |
| Mouse: C57BL/6-Tg(Nes-cre/ERT2)KEisc/J | The Jackson Laboratory | 016261 |
| Mouse: Ai38(RCL-GCaMP3) | The Jackson Laboratory | 029043 |
| Software and Algorithms | | |
| nVista HD | Inscopix |  |
| Inscopix image decompressor | Inscopix |  |
| Electroencephalogram (EEG) and electromyogram (EMG) recording and analysis software | KISSEI COMTEC | Vistal Recorder and Sleep Sign |
| Mosaic | Inscopix |  |
| Matlab | Mathworks | 2016b |
| IgorPro 8 | WaveMetrics |  |
| Others | | |
| Gradient-index (GRIN) lens probe  1.0 mm diameter, ~4.0 mm length | Inscopix | 1050-004595 |
| Magnetic baseplate | Inscopix | 1050-004638 |
| nVista imaging system | Inscopix | nVista(2.0) |
| Baseplate cover | Inscopix | 1050-004639 |
| Fear conditioning setup | O’hara & CO |  |
| Microscope gripper | Inscopix | 1050-002199 |
| Dummy microscope | Inscopix | 1050-003762 |
| Isoflurane vaporizer | Shinano Seisakusyo | SN-487-OT |
| Stereotaxic frame | Narishige | SR-6M |
| Drill | Minitor | Minimo |
| Vacuum pump | Iwaki | VPUMP-140 |
| Surgical microscope | Leica | Leica GZ6 |
| Self-curing acrylic resin | Shofu Inc | PROVINICE |
| Electrodes for EEG recording | Yamazaki | ϕ1.0 × 2.0 |
| Electrodes for EMG recording | Cooner Wire | AS633 |
| Flat cable for EEG/EMG signal transfer | Hitachi Cable | 20528-ST LF |
| Super glue for temporally gluing the sockets | Toagosei | Alon Alpha A |
| EEG/EMG signal amplifier | KyotoBiotex inc | N/A |
| Analog-to-digital convertor for EEG/EMG signal | Contec | AD16-16U(PCIEV) |
| 4-pin header for EEG/EMG socket | Hirose | A3B-4PA-2DSA(71) |
| EEG/EMG Socket housing | Hirose | DF11-4DS-2C |
| EEG/EMG Socket | Hirose | DF11-30SC |
| Supebond dental cement | Sun Medical | Superbond C&B set |
| Metal frame | Narishige | CF-10 |
| Dental silicone | Shofu | DENTSILICONE-V |
| Head-fixing support | Narishige | MAG-1 |
| Perista perfusion pump | ATTO | AC2110 |
| Cryostat | Leica Microsystems | Leica CM 1850 |
| Confocal fluorescent microscope | Olympus | FV1200 |
| Glass electrodes | Harvard apparatus | GC150F-7.5 |
| Glass electrode puller | Narishige | PC-10 |

**STEP‐BY‐STEP METHOD DETAILS**

**Induction of Ca2+ sensor expression in abGCs**

Timing: 1 - 8 hours

GCaMP3 is expressed by the tamoxifen-inducible Cre recombinase in adult neural stem/progenitor cells.

3. Prepare a 20 mg/ml Tamoxifen solution.

a. Heat corn oil to 42℃ for 30 min.

b. Dissolve tamoxifen in corn oil at a concentration of 20 mg/ml in a ultra-sonic bath (any consumer product for glassware will do).

Critical: Tamoxifen is sensitive to light and must be made and stored away from it, in an amber or foil wrapped tube.

Note: It may take ~1 h to completely dissolve.

4. Inject tamoxifen to nestin:GCaMP3 mice

a. Select an appropriate number of the adult nestin:GCaMP3 mice.

Note: The definition of ‘adult’ is usually determined by sexual maturity, by which ~6 week old of age is usually accepted as adult in mice. Slightly older/younger mice can be used according to necessity. However, keep in mind that older mice show reduced levels of adult neurogenesis, decreasing the success rate of the imaging, while younger mice might be too small to endure the burden of the following surgical steps.

b. Administer 75 to 120 mg of tamoxifen/kg body weight into intraperitoneal cavity five times at 1- or 2-day intervals, with completion of the injection period within 10 days. Store the solution at 4°C until the next injection.

Optional: On the following day, heat the tamoxifen solution to 37°C confirming the re-dissolution by transparency, inject the same dose to the contralateral side of the first injection and store the solution again at 4°C. Repeat this step according to the experiment’s need.

Critical: Avoid using tamoxifen that has been stored at 4°C for more than a week. The efficiency of tamoxifen-induced recombination gets severely impaired when using old/light exposed tamoxifen.

Note: The duration of injection period will set the developmental variance of the labeled neurons. Meaning that, the longer the injection period, the higher the chances of recording neurons with different properties resulting from differences in their maturational stages.

**Maturation of labeled abGCs**

Timing: Dependent of experimental design

The abGCs mature along with the days passed from the tamoxifen injection.

5. Wait the determined period to allow for the maturation of the GCaMP3 labeled abGCs.

Note: The period of time to wait for the maturation of the abGCs will depend on the target age of the neurons at the time of the Ca2+ imaging. Bear in mind that one will need 1-2 weeks to prepare for the imaging. For example, if the target is 6 weeks old abGCs (for example), one should leave the tamoxifen-injected mice to rest for 4 weeks and then start the following steps. If the target abGCs is younger than 1 week old, consider implanting lens (next step) before the tamoxifen injection. So far, we have succeeded the Ca2+ imaging of abGCs as short as 3 days after the last tamoxifen injection.

Note: It can be useful to verify the GCaMP3 expression by histologically preparing a brain of a tamoxifen-injected mouse with confocal fluorescence microscope (Figure 1) before next step. This might avoid committing the rest of the animals to the next steps with failed recombination.

**GRIN lens implantation (Video 1)**

Timing: 2-3 hours per mouse

GRIN lens and EEG/EMG socket are surgically implanted into the brain of a tamoxifen-injected mouse

6. Anesthetize the animal with anesthetics of choice (we succeed in using isoflurane).

7. Open a cranial window large enough to permit lens and electrodes implantation

a. Trim hair from the surgical site

b. Secure the mouse’s head in the stereotaxic frame.

c. Cover eyes with white petrolatum cream to prevent from dryness .

d. Disinfect the skin from the surgical site

e. Cut a small incision in the skin using a spring scissor along the sagittal line of the skull.

f. Clean the exposed skull with cotton swabs.

g. Make exposed skull surface rough by creating fine scratches using drilling bits

h. Obtain flat skull position by bringing mediolateral skull (+/-3 mm from bregma) in same height and also bregma-lambda point in same height

i. Mark the desired stereotaxic coordinates for implantation of EEG, anchoring screw and GRIN lens on skull surface using stereotaxic manipulator and a surgical microscope

Note: The cranial window should be just large enough to accommodate the GRIN lens implant. We recommend using an Inscopix 1-mm diameter, 4-mm length lens to record abGCs in the dorsal dentate gyrus. In that case, a 1.2-mm2 cranial window centered on anterior-posterior (AP) -2 mm, medial-lateral (ML) +0.7 mm works well. The exact coordinate for each condition should be determined in each individual case.

j. Drill four holes in the skull, two for the implantation of the EEG electrodes, contralaterally to the side used to implant the GRIN lens and another two for the implantation of anchoring screw in the same side of GRIN lens (Figure 2a).

Note: Similar to the lens craniotomy, the electrode holes should be just large enough to accommodate the tip of the screws electrode attached to the wires. For the simultaneous recording of EEG and abGCs Ca2+ transients, the holes were drilled above the frontal and parietal cortices in both sides of skull, AP +1.5 mm and −3 mm, ML +/-1.5 mm and +/-1.7 mm respectively.

8. Implant anchoring screws, EEG electrodes and a GRIN lens

a. Insert the anchoring screws and EEG electrodes epidurally through the skull.

b. Aspirate the cortical tissue above the region of interest using glass pipettes connected to a vacuum pump.

Note: From this point onwards, some bleeding is normal during the procedure. Continuously irrigate the exposed brain tissue with sterile saline while aspiring the cortex in circular movements from the center to the periphery of the cranial window.

Critical: While aspirating the cortex, the brain tissue will have a homogeneous appearance at first. Stop aspiration when white matter tracts of corpus callosum are visible (Figure 2b), identifiable by its striations. Researchers need a surgical microscope to observe the differences in brain structure during the surgery. In our experience, damaging the CA1 region by aspiration completely destroys the structural organization of the DG.

c. After reaching the corpus callosum fibers, keep the exposed brain tissue irrigated with sterile saline while aspirating any blood present in the region to maintain visibility.

d. Attach a new GRIN lens to a stereotaxic manipulator and align it above the center of the craniotomy.

e. Carefully lower the lens in 0.1-mm dorso-ventral steps into the hippocampus. The final coordinate of the objective surface of the GRIN lens is 1.3 mm from the top of the skull.

Note: For the GRIN lens size used, we haven’t noticed any remarkable difference regarding different speed of lowering the GRIN lens. In our preparation, a rate of ~500 µm/min is perfectly acceptable.

Note: After reaching the final coordinate, the lens should compress the CA1 hippocampal area without penetrating into the tissue when researchers use the 1mm diameter lens (Figure 2c). This will introduce some degree of variance to the final position of the lens depending on the tissue swelling and relaxation that might decrease the success rate of the recordings. A thinner lens can penetrate the tissue and eliminate this variance. However, we do not recommend using it because the reduction in the area of the field of view makes it significantly harder to observe a population of abGCs that is sparse by default.

f. Fix the GRIN lens to the skull using a self-curing acrylic glue (Loctite) and connect it with all four screws using thin layer of Loctite. Let it solidify for approximately 10 min.

Optional: Put one drop of dental cement solution to quickly solidify the lens and screws in place. In this case, it is enough to wait for 5 min.

g. Release the lens from the stereotaxic manipulator

h. Cover the skull using a layer of the dental cement around the insertion point and wait until completely solid (Figure 2d).

i. Attach the electrode’s micro-socket to the skull using self-curing acrylic glue (Loctite) and put a drop of dental cement solution to solidify.

Critical: The EEG/EMG-socket should be fixated far enough from lens position to avoid the miniature microscope and the EEG/EMG-socket cables become too close to connect both systems to the head of the animal.

j. Insert two wires into the cervical portion of the trapezoid muscles for EMG recordings.

j. Apply carbon black powder mix dental cement to any exposed area of the skull, as well as to the area where the self-curing acrylic resin meets the GRIN lens, the EEG/EMG wires and cables, and let it solidify for approximately 10 min.

Critical: It is important to completely cover the EEG/EMG wires with the acrylic resin in order to prevent any damage to them (Figure 2f).

k. Apply super bond liquid to the outer border of dental cement (dental cement connects to surrounding skin) and let it solidify

9. Protect the GRIN lens from damage

Cover the lens and near surrounding area with a silicone gel (Figure 2g).

Critical: Make sure to apply enough silicone gel to cover lens and surrounding surface area sufficiently. The silicone prevents damages to the lens that would result from regular activity in the home cage.

10. Finish surgical procedure

a. Stop anesthesia.

b. Remove the mouse from the stereotaxic holder.

c. Administer postoperative 5% glucose solution and pain killer Ibuprofen in agreement with the animal care guidelines of the facility.

d. Place the operated animal in a clean cage and allow the mouse to recover.

Critical: After surgery, the animals should be housed individually. Otherwise, one mouse can remove the silicon cap from the other and expose the lens to structural damage. We recommend to place heat-pat partially covering the area of the cage bottom, which prevents hypothermia post-surgery.

Note: To prepare Ibuprofen (30 mg/kg) solution, 30 mg Ibuprofen powder is dissolved in 100µl of 100% EtOH and then 1000 µl sunflower oil is added. In the last step, ethanol was evaporated by centrifuge.

**Post-operative recovery**

Timing: At least 1 week

Note: We recommend keeping mice in sleeping chamber/room in post-surgery recovery period. The mouse gets habituated to sleeping environment in this way.

Allow the operated mice to rest for at least one week with minimal manipulation.

Note: Manipulation during this period may cause detachment of the lens and electrodes since the mouse might still present some degree of inflammation from the surgery, reducing the adhesion of the resin and dental cement to the skull.

**Microendoscope baseplate attachment (Video 2)**

Timing: 30 min per mouse

Magnetic baseplates for the miniature microscope imaging are attached to the operated mouse

11. Anesthetize the mouse with isoflurane.

12. Secure the mouse’s head in the stereotaxic frame

13. Prepare the miniature microscope

a. Attach the magnetic baseplate to the miniature microscope.

b. Secure the microendoscope to its gripper tool and attach it to a micromanipulator

c. Connect the recording hardware to the computer

d. Position the miniature microscope above the head of the mouse

e. Start the image acquisition software

14. Attach the magnetic baseplate

a. Remove the silicon protection from the skull of the animal

b. Adjust the position of the miniature microscope using the micromanipulator, centering it above the implanted lens.

c. Turn on the LED light of the miniature microscope and lower it until the top surface of the lens becomes visible through the camera.

d. Carefully continue lowering the microendoscope until the dentate gyrus becomes visible.

Note: The DG will be recognizable by its abundant vasculature. Due to the GCaMP3 expression in abGCs and our implantation method, this will be the first brain structure to be focusable.

e. From this point, lower the microscope ~50 µm to adjust the imaging focus to the granular/subgranular layer. The vasculature seen before should become out of focus by this point (figure 3a).

Optional: When the outlines of GCaMP3-expressing abGCs can be visualized, we recommend switching to the nVista software ∆F/F function to confirm whether there are dynamic changes in fluorescence corresponding of Ca2+ transients (figure 3b).

Note: Due to low signal/noise ratio of GCaMP3 and the small number of abGCs it is possible for the fluorescent signal from abGCs to be undetectable at this moment. Nonetheless, we can observe abGC’s activity with off-line image processing as long as the surgery is successful.

f. After choosing the optimal focal plane, permanently fix the baseplate using self-curing acrylic resin (figure 3c).

Critical: When fixing the baseplate, there are three points that should avoid getting resin directly onto them: the surface of the implanted lens, the screw of the magnetic baseplate and the microendoscope itself.

Note: Fixing the baseplate without any gaps will prevent dust and ambient illumination to interfere with the field of view during imaging.

g. Wait ~5 minutes for the resin to solidify.

15. Finish imaging preparation

a. Once the resin has solidified completely, release the miniature microscope from the gripper.

b. Detach it from the magnetic baseplate.

Note: The baseplate should remain in place attached to the skull cap.

c. Attach the baseplate cover in order to prevent dust from accumulating above the lens.

d. Release the mouse from the head-fixing support and return it to its home cage.

Note: From this point, mice are in principle ready to be recorded.

**Preparation for EEG/EMG recording and Ca2+ imaging during sleep**

Timing: ~6h per day, for at least 3 days, per mouse

16. Attach the dummy microscope and dummy EEG/EMG cables to the mouse.

a. Manipulate the awake mouse holding its head by both side of skullcap and attached to it.

Critical: While holding the animal by the implant avoid applying too much force. This may cause extra stress to the skullcap and mouse.

b. Remove the baseplate cover and attach the dummy microscope and dummy EEG/EMG cables to the mouse.

Note: The mouse might initially show difficulties moving around their environment with the microscope and EEG/EMG cables above their heads. To overcome this, we habituated them in an incremental manner for mice weight burden; we made mice habituated to the EEG/EMG cable first for 4 days before the baseplate implant, and then started to connect dummy miniscope for another 6 days together with the dummy EEG/EMG cable 48 hrs after the baseplate surgery. Since the goal is only to habituate the mouse to the recording cable in the sleeping chamber, it is not necessary to use the real recording system. The dummy tools are sufficient to simulate the physical and mechanical properties of the system.

**EEG/EMG recording and Ca2+ imaging during sleep: day 1**

Timing: 3-6 hours per mouse

Note: For illustrative purposes we will describe here one of the protocols for cued fear conditioning used in Kumar et al., 2020.

Record EEG/EMG and Ca2+ transients during sleep

22. Prepare the mouse for EEG/EMG recording and Ca2+ imaging.

a. Start the acquisition software and set the imaging parameters according to necessity.

Note: In order to eliminate possible differences in processing outcomes, it’s important to use the same imaging settings for all experimental animals.

Note: For long duration imaging as the ones in this protocol, we recommend setting a low acquisition rate of ~5 Hz in order to reduce the final size of the imaging file.

Note: Using an acquisition rate of ~5 Hz, we have seen that a LED intensity between 10 and 30% of its maximum power is sufficient to not oversaturate any detected pixel. We usually don’t observe any cell death resulting from phototoxicity but a small degree of photobleaching may be inevitable due to the long duration of the imaging.

Note: Since the signal/noise ratio of GCaMP3 is not as good as newer generation calcium sensors, we recommend leaving the gain at 1 in order to reduce the noise of the imaging.

b. Secure the microscope and EEG/EMG cables above the recording chamber.

Critical: To avoid damage to the cables, confirm that the cables do not hang next to the mouse. In addition, check that there is sufficient slack on the cables to avoid any movement-induced twisting and strain of them.

c. Remove the baseplate cover and attach the miniature microscope and EEG/EMG cables.

d. Start the EEG/EMG recording software.

23. Start the EEG/EMG recording and Ca2+ imaging simultaneously.

Critical: From time to time, monitor the state of the mouse and cables in video fitted in the sleeping chamber to ensure that there is no twisting of the cables and that the animal is behaving naturally.

24. Finish the recordings.

a. Stop both recordings.

b. Detach both microscope and EEG/EMG cables and reattached the dummy microscope and EEG cable

**EEG/EMG recording and Ca2+ imaging during sleep: day 2; conditioning**

Timing: 5-10 min per mouse.

Condition mice with tone/foot shock pairs

25. Prepare the mouse for fear conditioning

a. Remove the baseplate cover and attach the microscope (figure 4a)

b. Open recording softwares for Ca2+ imaging.

c. Arrange the cables above the conditioning chamber, taking the same precautions as with the previously described sleeping chamber.

Note: Use the dummy accessories instead of the real recoding equipment if it is not needed to record during conditioning.

26. Conditioning the mouse

a. Place the mouse inside the conditioning chamber (context A)

b. Image Ca2+ for 10 min in context A conditioning (figure 4b), as a pre-shock recording.

c. Stop the recording and detached the microscope and reattached the dummy (<1 min) to avoid a change in the field of view due to hitting the microscope against the wall during the shock.

d. Perform the shock conditioning in the same context with attached dummy microscope

e. Reattached the microscope (<1 min) and perform 5 min of Ca2+ imaging, as a post shock recording

f. Stop the Ca2+ recording and return mouse to sleeping chamber.

g. Connect with EEG cable and start the EEG/EMG recording and Ca2+ imaging simultaneously.

**EEG/EMG recording and Ca2+ imaging during sleep: day 2; post-learning**

Timing: 3-6 hours per mouse.

Record EEG/EMG and Ca2+ transients during sleep

27. Record EEG/EMG and Ca2+ transients during memory consolidation sleep

a. After conditioning, transfer the animal to the sleeping chamber.

Note: Avoid detaching the microscope and cables from the head of the mouse when transferring it to the sleeping chamber to keep the field of view unchanged. If necessary, disconnect the cables from the computer port and reconnect them after transferring the mouse.

b. Start the EEG/EMG and Ca2+ recordings simultaneously.

28. Finish the recordings as done in the previous day.

**Fear test: day 3**

Timing: 5-10 min per mouse

Confirm the formation of the fear memory.

29. Prepare the mouse for the conditioning chamber

Note: Take the same preparations as the ones described on the previous day when using the conditioning chamber. Similarly, if it is not necessary to record the Ca2+ transients at this step, use the dummy accessories instead of the real recoding equipment.

30. Verify the presence of the fear memory.

a. Start both recording and imaging softwares.

b. Place the mouse inside the conditioning chamber.

c. Start the ECoG/EMG recording and Ca2+ imaging simultaneously.

d. Leave the mouse inside the chamber for 5-10 minutes.

Critical: In order to confirm the formation of the fear memory, it is necessary to measure the immobility/freezing response exhibited by the mouse when it is placed inside the conditioning chamber.

31. Finish the recordings as done in the previous day.

**Anatomical confirmation of the recorded signals**

Timing: Dependent on the desired histological method

Verify the position of the implanted GRIN lens post-mortem.

Note: We recommend slicing the brain in the vibratome or cryostat and mount the slices in fluorescence preserving medium in order to store the labeled tissue in case it is needed for further analysis.

32. Preserve the brain of the mouse

a. After finishing the experiment, lethally anesthetize the mouse with injected anesthesia.

b. Using a peristaltic pump, transcardially perfuse the animal with a cold 4% PFA solution.

c. Remove the brain and leave it inside a tube with 4% PFA at 4°C for 24h.

Pause point: At this point, the brain can be stored at 4°C for several days. In this case, exchange the PFA solution for PBS and leave it protected from light. Expect a natural decay of the fluorescent signal when the tissue is left for long time in this manner. Also, long exposure to PFA may interfere with some immunohistochemical detection of antigens, some are critical for adult-neurogenesis research (e.g., double-cortin)(citation here: Moreno-Jimenez, Nat, Med, 2019, DOI. 10.1038/s41591-019-0375-9).

d. Replace the 4%PFA to 20-30% sucrose solution

33. Slice the brain of the mouse.

a. After the brain reaches the bottom of the tube, remove it and cryopreserve it in an OCT mounting medium block at -80°C.

Pause point: At this point, long term storage of the samples is possible by keeping the OCT blocks at -80°C.

b. Cut the brain using a cryostat with 50 μm thickness and collect the slides in plastic wells or dishes with PBS.

Pause point: Similar to the pause point after perfusion, the slices can be stored at 4°C for several days at this moment.

34. Confirm the position of the implanted lens and the fluorescent signal of GCaMP3

a. Mount the brain slices on microscope slides with using a fluorescent preserving medium.

b. Image the slides with a fluorescent microscope (figure 5).

Note: Usually, the fluorescence of GCaMP3 is bright enough to be detectable as it is in slice condition. If it is not possible to observe the fluorescent signal by any reasons, a simple antibody staining targeting the GFP molecule is sufficient to amplify the GCaMP3 signal.

**EXPECTED OUTCOMES**

Successful completion of this method allows for the detection of hippocampal abGCs Ca2+ transients in freely behaving mice. A recording session configured with the parameters here described will produce a raw recording file weighting ~1327 KB/frame.

Next, we will describe the methods to process raw video sessions into Ca2+ fluorescence time series. Here we use Mosaic software for preprocessing and motion correction and the MATLAB implementation of CNMF-E (Zhou et al., 2018) plus some custom script to optimize the extraction for abGCs.

**Extraction of calcium traces**

**Recommended system requirements:**

-128 GB or more RAM.

-AMD Ryzen processor for MATLAB 2020 or older. Most recent Intel Core i7 processor for older MATLAB versions.

-1TB SSD

**Decompression:**

1. Decompress the raw files using Inscopix image decompressor.

Note: We recommend down‑sampling the video by a factor of 4 during the decompression to save disc space and reduce loading times. Decompressing video files into ‘.hdf5’ format is recommended as this can substantially improve loading times in mosaic and MATLAB.

**Preprocessing** (Tutorial video 00:00-00:22).**:**

36. Preprocess the video file as indicated inMosaic standard workflow.

a. Import the video files into Mosaic software.

b. Preprocess file in mosaic (choose, fix defective pixels and fix isolated dropped frames. c. Spatial down-sampling is not necessary if already done during decompression).

**Motion correction** (Tutorial video 00:22-01:25).**:**

37. Perform motion correction as indicated in the Mosaic guideline.

a. Save data in .H5 format.

Note: To perform an optimal motion correction is ideal to choose as a reference point a region in the field of view where most of abGCs are located and if possible, with clear spatial marks (i.e. blood vessels). This is important because movements of the brain occur in three dimensions and might be non-rigid. Thus, perfect motion correction in the whole field of view is not possible in most cases. Instead, choose reference regions that minimize the motion in the region where neurons are located and consider cropping out portion of video that are not necessary. This will reduce the number of false positives and decrease processing time. Moreover, some regions in the field of view may move asynchronously. This usually happens by tissue or blood cloths that get stuck in between the lens and the focal plane, which create different planes of movement. Try to avoid those regions which contain desynchronized movement.

Note: detecting abGCs activity by the naked eye can be challenging. This is because abGCs are low in number (5-25 neurons per field of view) and have very sparse activity rate (~1 transient/min). Moreover, the GCaMP3 signal is weaker compared to most of the state‑of‑the‑art GCaMPs. To improve the identification of regions with active neurons, adjust the contrast between 40%-95% of the maximum observed signal. An estimation of neuron location can be obtained in the motion correction panel when you check “subtract spatial mean”, “invert image”, and “apply spatial mean”. Neurons are seen as black circular shapes in the field of view.

Critical: After running motion correction, be sure to check that no major signs of motion are visually detected. Usually, one run of the motion correction algorithm is not enough to solve all problems. Try using different combinations of reference region and motion correction type (i.e. translation, rotation, etc). We recommend to first use “Translation only” in the motion correction type. If there is still motion that could not be corrected, check whether this is due to rotations or expansions in the field of view and change the motion correction type accordingly.

Note: For comments on how to perform motion correction when tracking the same neurons through different recording session, see “tracking the same neurons”.

**Extraction of calcium traces** (Tutorial video 1:25-6:25)

38. Extract calcium traces in MATLAB using CNMF-E

a. Set up a folder with the “.h5” videos files to analyze.

b. Add the folder with the CNMF-E scripts provided in this paper to the MATLAB path.

c. Open the file “abGCs\_PV.m”.

d. Input the path of the folder with the video files in line 2.

e. Input the frame rate in line 46.

f. Run the code.

Note: For short video session run “demo\_large\_data\_1p.m” instead of “abGCs\_PV.m”.

39. Perform the following quality controls:

a. Run “neuron.viewNeurons([], neuron.C\_raw);” to visually inspect each neuron.

Note: Delete extracted components with temporal traces or spatial shapes that do not correspond to real neurons. If you must delete too many neurons, consider repeating the analysis with a higher peak-to-noise ratio (PNR) or local correlation (CORR) thresholds.

b. Run “neuron.show\_contours(0.6, [], neuron.PNR.\*neuron.Cn, 1)” to see the contours of the extracted components over the PNR image multiplied with the CORR image.

* Enlarged non-circular region usually reflects motion artifacts.
* If contours are not drawn over several neuron-like regions this suggests that the PNR or CORR threshold may have been set too high.
* If contours are drawn over several non-neuron-like regions this suggests that the PNR or CORR threshold may have been set too low.

c. Run “implay(cat(2,mat2gray(neuron.PNR\_all),mat2gray(neuron.Cn\_all)),5);” to see the PNR and the COOR image from consecutive temporal segments of 1000 frames. Check for signs of motion artifacts and to have a visual representation of the mean activity of neurons across different times.

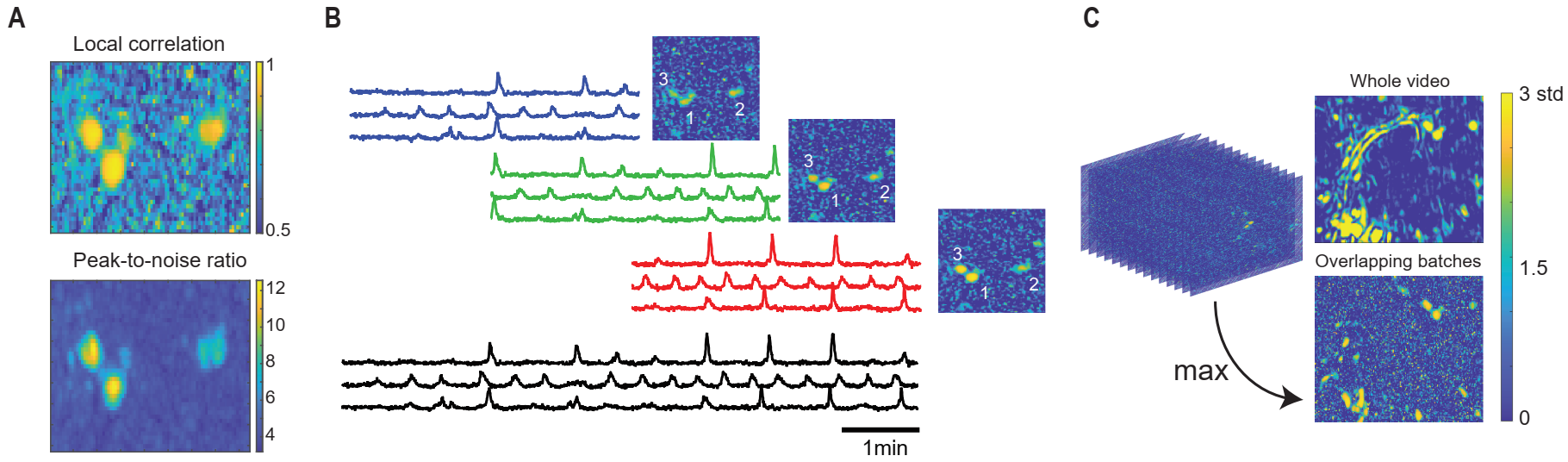
d. Run “stackedplot(neuron.C\_raw');” to see several calcium traces at the same time.

* Notice correlated activity across several neurons. Correlated ensemble activity is common in abGCs; however, perfectly overlapping rising dynamics with square‑like shapes may suggest motion artifacts. Check the video in the specific frames to corroborate whether this is real ensemble activity.

Pause point: For the extraction of calcium traces, we use CNMF-E (Zhou *et al.*, 2016) with some modifications. We recommend reading the original CNMF-E paper first to understand the main concepts behind the algorithm. We choose CNMF-E because is particularly good at extracting noisy signal and its code can be easily adapted for a wide range of experimental needs. We did some minor modifications to the original code that are necessary for long recordings (>10 min). Available in Mendeley Data (DOI: xxx, link: xxx). For short video session we recommend using the original CNMF-E

The parameters that mostly affect the CNMF-E results are the minimum local correlation (CORR) of a pixel and its neighbors (Figure 6A, left. Noted as min\_corr) and the minimum peak-to-noise ratio (PNR) of a pixel (Figure 6A right. Noted as min\_pnr). These parameters are thresholds used to obtain a first estimation of the spatial and temporal components that will be used to initialize the constrained non-negative matrix factorization (CNMF) algorithm. For sparse data, as occurring for abGCs, the total video length may affect the estimation of the CORR. This happens because, in 1p‑imaging, pixels associated with a neuron are highly correlated only when that neuron is active but not when it is not. Given that abGCs are most of the time inactive increasing the video length may be translated into a poor CORR, especially for files that are several hours long.

To solve this issue, we segmented the video in short overlapping batches of 1000 frames, which allowed us to estimate the spatial component of neurons from discrete temporal windows in which neurons are highly active. This is depicted in figure 6B where the CORR and the calcium traces of three close-by abGCs are shown for three consecutive batches. Note that abGCs display prominent calcium transients that are translated into a higher CORR particularly in the third batch. Because the spatial component of each neuron is shared across all batches, only this batch is necessary to initialize those neurons, even if other batches have CORR that are comparable to the background level. Having obtained a good initial estimation of the spatial components, the CNMF algorithm can extract the temporal traces from other batches, even if its CORR or PNR are below the defined threshold. An overlapping batch approach produce cleaner estimation of the CORR in contrast to the analysis of the whole video sequence (Figure 6C).



**Figure 6. Overlapping batch analysis:** (A) local correlation and peak-to-noise ratio image of three neurons in a recording file. (B) Calcium traces detected and CORR for three neurons in three consecutive batches. Black trace shows the final estimated signal for the whole recording. (C) Normalized CORR for a 3-hour long video obtained by analyzing the whole video sequence versus the maximum projection of the CORR images obtained by the overlapping batch implementation. Note that the CORR of the whole video sequence includes many highly correlated non-circular shapes that are unlikely to represent real abGCs. In practice, the whole video analysis introduces several false positive and noisier estimation of the calcium traces.

The multi-batch algorithm is implemented in the original CNMF-E paper; however, we found that in some cases artifacts may be introduced in the concatenation point between batches. Thus, we implemented a multi-batch algorithm using overlapping batches. We also included some utility functions to correct for variations in the baseline noise between batches and correction of some minor bugs producing empty matrices given the sparse activity of abGCs. We provide a code with optimized parameters that can extract calcium traces from abGCs in most of the cases.

**Cell tracking across sessions:**

40. To track the cells across different sessions we motion correct each session as described in (Ghandour *et al.*, 2019). In the mosaic software this is done as follows:

a. Motion correct each session independently.

b. Extract one frame from one session to use as a reference for others.

c. Motion correct each session relative to the extracted frame.

Note: sometimes using different reference frames, from different sessions, may provide better results. Consider the points mentioned in the motion correction section.

d. Concatenate the recording sessions. Be sure to corroborate that sessions are correctly aligned.

Pause point: Unfortunately tracking abGCs across several days using tracking algorithms such as (Sheintuch *et al.*, 2017) may not be possible given the reduced number of abGCs. However, we were able to track abGCs across different experimental sessions within one day by concatenating different recording sessions into one file. Spatial marks, such as blood vessels or neurons with persistent activity, can be used to align different experimental sessions.

One may be tempted to analyze sessions independently and then identify same neurons based on their footprints; however, this assumes that all neurons can be detected in each recording session. This is not always the case, especially considering abGCs sparse activity and the low signal-to-noise ratio of GCaMP3. This issue is illustrated in Figure 6B: note that in the first batch we can see three neurons that are active while displaying a CORR that is comparable to the background local correlation. If lower CORR and PNR threshold were used to detect these neurons many false-positive neurons would be included in the analysis, further hindering the tracking of neurons. Instead, we initialize these neurons given periods where they show high activity (e.g. the third batch in figure 6B). Following the CNMF algorithm is used to extract the calcium transient from other batches with a lower signal-to-noise ratio. This is particularly important when tracking cells, as some neurons may be more active in some recording sessions than others, and hence, be wrongly labeled as inactive if these sessions were analyzed independently.

**LIMITATIONS**

Although the development of miniature microscope imaging of the brain fueled the publication of hundreds of new discoveries regarding how the activity of individual neurons is able to generate specific behavior in freely moving animals, its limitations are well known.

The physical properties of the microscope itself is one of the limitations, since it only allows its use for certain behavioral tasks. For example, it is not possible to use it in any experimental setup that involves water, such as Morris Water Maze, because it is not waterproof. In addition, it is not possible to distinguish the activity of different types of labeled neurons by using other fluorescent markers since it only has one detection channel for now. A more serious limitation could be the resolution of z axis. Since Inscopix miniature microscope used in our experiment (Kumar et al., 2020) excites fluorescent molecule without focusing nor scanning the light. The microscope does not have pinhole to select the light path. Therefore, all the fluorescent molecule hundreds of micrometers below the microscope can be excited and detected at the same time. This could result in the larger number of neurons in the field of view compared to the imaging with 2 photon microscope. However, there is a possibility that the detected calcium trace stems from 2 neurons.

Other limitations of this method regard our specific neuronal target, the hippocampal abGCs. Most of our efforts to use more state-of-the-art calcium sensors were not successful, forcing us to go back a few generations and use GCaMP3 to tag these neurons. Compared to newer GCaMPs, GCaMP3 is not sensitive enough to reflect the activity of abGCs when they fire action potentials in a sparse manner.

The experimental preparation for dentate gyrus imaging in freely moving mice demonstrated here and in Kumar et al. (2020) results in a partial lesion of the ipsilateral CA1 area when implanting the GRIN lens. Less invasive techniques should be implemented in the future to prevent any possible circuit reorganization induced by surgery, as already pointed out in previous studies (Hainmueller and Bartos, 2018; Kirschen et al., 2017; Pilz et al., 2016).

Finally, the developing nature of the abGCs imposes a time constrain to all the experimental steps. Since their activity and behavioral significance changes with their maturation from immature GCs to mature ones (e.g., Kumar et al., 2020), it can become logistically challenging to use the same animals in multiple behavioral tasks and be certain that the recorded neurons have the same physiological proprieties during these multiple moments.

**TROUBLESHOOTING**

Problem

Low or absent of GCaMP3 expression

Potential solutions

Low expression of Ca2+ sensor at the histological level can be due to inefficient Cre induction/recombination by tamoxifen administration. Always prepare fresh tamoxifen solution, protecting them from light. Make sure that tamoxifen is completely dissolved before using it to avoid injecting a lower dose. In addition, when injecting, leave the syringe in place for a few seconds to avoid reflux of tamoxifen. It is possible to slightly increase the injected dose to see if it solves the issue.

When using different mice lines, confirm immunohistologically that Cre recombinase is being expressed and that there is no cell death induced by cytotoxicity of calcium sensors in abGCs.

Problem

Incorrect lens position

Potential solutions

This problem can occur due to inaccurate stereotaxic coordinates or to changes in the position of the lens during implantation. Take into account the direction of the lens when determining the optimal implanting coordinates and re-evaluate them as needed, always verifying the position of the lens histologically postmortem to fit to your specific surgical setting.

In our preparations, since we implant the lens into the hippocampal CA1 area without perforating it, resistance is generated, forcing the lens out of the brain. Only release the lens from the stereotaxic micromanipulator once the resin/dental cement used to fixate the lens to the skull is completely solidified. Otherwise, the lens might shift its position when released.

Problem

Detachment of the dental cement skullcap

Potential solutions

Detachment of the dental cement skullcap can occur when it is not well secured to the skull. Different adhesive materials present different recommended uses. Confirm the manufacture’s using instructions and change products accordingly. When applying several layers of different adhesives, pay attention that one layer is completely solidified before applying the next one, as mixing them might compromise their stability.

Infections and tissue inflammation can also decrease the adhesive strength of the resin/dental cement. Ensure adequate prophylactic practices during surgery and post-operative recovery to avoid this issue. In addition, do not try to immobilize the animals by their metal frame if they are not anesthetized, since the stress generated at the skullcap can also promote its detachment.

Problem

Excessive motion artifacts during data acquisition

Potential solution

Verify that the microscope is correctly attached to the magnetic baseplate and tightly secured in place by its lateral screw. Low frame rates may increase the amount of motion blur, which may reduce the performance of the motion correction algorithm,

Problem

Fluorescent signals do not show dynamic changes in intensity

Potential solutions

“False” fluorescent signals are usually derived from autofluorescent bodies and not from calcium sensor expressing cells. This autofluorescence can originate from damaged tissue or blood cells below the implanted lens. To prevent this from happening, ensure that the whole cortex above the selected stereotaxic coordinates is completely aspirated and that there is no residual bleeding before implanting the GRIN lens. Moreover, lowering the lens too quickly into the brain can also damage the tissue. Ensure that the lens is not being implanted to quickly and allow the tissue to settle every 150 μm step. If necessary, retract the lens ±25 μm before each decreasing step to further alleviate the pressure and prevent unnecessary damages.

Problem

Data processing is extremely slow/impossible

Potential solutions

Since the average sleep recording is at least 2-3h long, the final compressed file is about 200 GB. To be processed, the file will be decompressed and spatially down‑sampled (4x), resulting in a ~216 KB/frame file. However, because some MATLAB scripts require double‑precision, we recommend having at least 4 times more RAM than the down-sampled file size. Confirm that the computer has enough RAM size. When it is not possible to reduce the size of the data file by temporal and/or spatial binning, one strategy is to divide the complete recording into shorter ones by halting the recording in the middle. It is possible to perform the initial processing steps individually to each file and later concatenate them together before detecting active neurons using the batch implementation and disabling parallel computing (this can take several hours).

Problem

Low/Null number of detected cells from Ca2+ data

Potential solution

Not being able to detect cells from the Ca2+ recordings can be due to some issues already mentioned above. If there is no problem with the expression of the Ca2+ sensor and the coordinate of the lens implantation (both verified histologically postmortem), one final solution can be to change some of the parameters in the CNMF-E script. Modifying the minimum CORR and the minimum PNR values. In this case, the visual inspection of the cell shape and temporal dynamics of Ca2+ transients become more important, since the rate of false cell detection will increase with less restricted parameters.

Problem

MATLAB code crush in the middle of the analysis

Potential solution

Unfortunately, the code provided is not bug proof. In case of an error be sure to read the error message in the MATLAB command window:

Some common cause of errors may be:

-The recording has not active neurons: If no neurons are detected the code will crash. Visually confirm whether you can detect calcium activity. If active neurons can be visually detected in the raw file, consider using a lower PNR or CORR threshold.

- Out of memory problem: monitor your RAM usage when running the code. Consider upgrading your hardware or decrease recording duration as mentioned above.

**RESOURCE AVAILABILITY**

**Lead Contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Masanori Sakaguchi (sakaguchi.masa.fp@alumni.tsukuba.ac.jp).

**Materials Availability**

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

**Data and Code Availability**

Data underlying the results described in this manuscript, except raw data for RNA analysis, are available at: xxx

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**AUTHOR CONTRIBUTIONS**

Conceptualization, Y.S. M.K., and M.S.; Methodology, A.C.-R., Y.S. D.K., I.K., P.V., S.S., and M.S.; Investigation, A.C.-R., D.K., Y.S., I.K., S.S., and T.N.; Validation, A.C.-R, Y.S., D.K., P.V.; Formal Analysis, Y.S., A.C.-R., and P.V.; Data Curation, A. C.-R., Y.S. P.V., and M.S.; Writing – Original Draft, Y.S.; Writing – Review & Editing, Y.S., D.K., A.C.-R, P.V., M.S., and M.K.; Funding Acquisition, M.S., M.K., and Y.S.; Resources, M.K., M.S., T.S.; Supervision, M.K. and M.S.; Project Administration, Y.S., M.S., and M.K. All authors discussed and approved the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

**REFERENCES** (LATER)

List References alphabetically here; include the author list, year, article title, journal abbreviation, volume, and page range. Use the Harvard format for reference citations in the text (e.g. Smith et al., 2009).

**FIGURE LEGENDS** (LATER)

Figure 1: [Include a clear and concise figure legend for each figure.]

Table 1: [Include a clear and concise figure legend for each table.]

Video 1: [Include a clear and concise figure legend for each video. If possible, mention the relevant step.]