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Protocol for calcium imaging and analysis of hippocampal CA1 activity evoked by non-spatial stimuli

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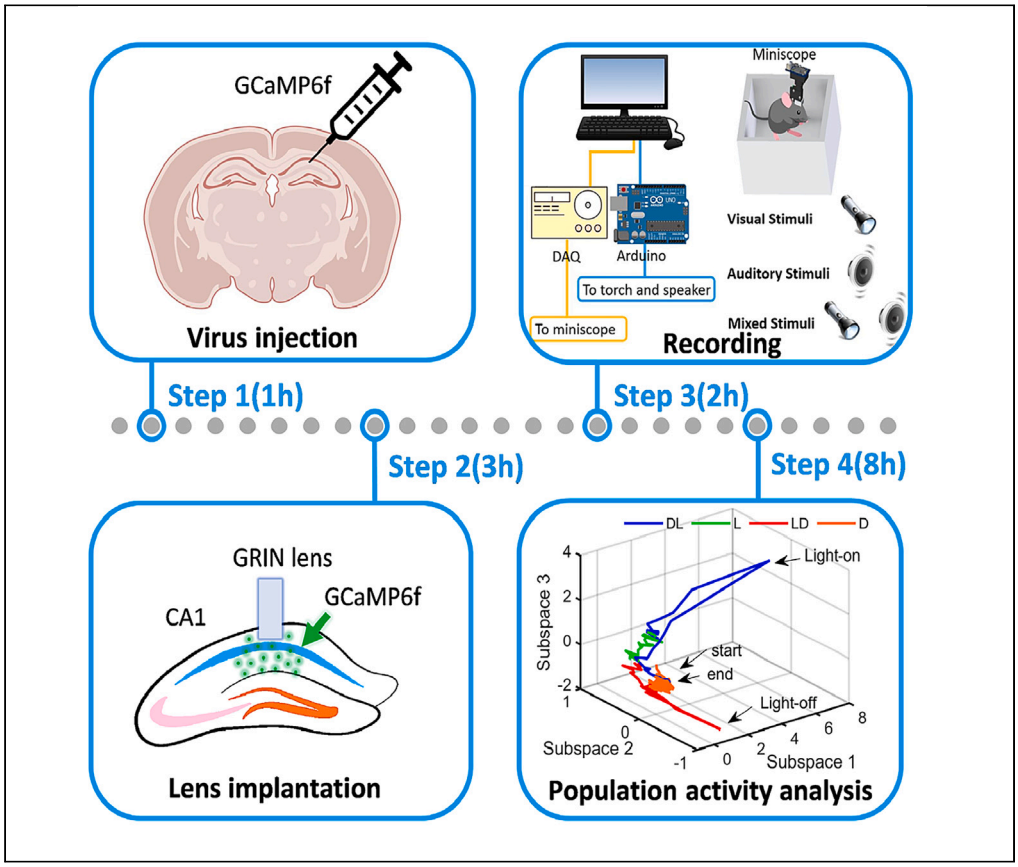

The hippocampus has a major role in processing spatial information but has been found to encode non-spatial information from multisensory modalities in recent studies. Here, we present a protocol for recording non-spatial stimuli (visual, auditory, and a combination) that evoked calcium activity of hippocampal CA1 neuronal ensembles in C57BL/6 mice using a miniaturized fluorescence microscope. We describe steps for experimental apparatus setup, surgical procedures, software development, and neuronal population activity analysis.

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

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Highlights

Instructions for measuring non-spatial stimuli that evoked hippocampal CA1 activity

Steps for calcium imaging in the mouse hippocampal CA1 region

Guidance on calcium imaging data analysis pipelines

Procedures for neuronal population activity analysis

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Protocol for calcium imaging and analysis of hippocampal CA1 activity evoked by non-spatial stimuli

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SUMMARY

The hippocampus has a major role in processing spatial information but has been found to encode non-spatial information from multisensory modalities in recent studies. Here, we present a protocol for recording non-spatial stimuli (visual, auditory, and a combination) that evoked calcium activity of hippocampal CA1 neuronal ensembles in C57BL/6 mice using a miniaturized fluorescence microscope. We describe steps for experimental apparatus setup, surgical procedures, software development, and neuronal population activity analysis.
For complete details on the use and execution of this protocol, please refer to Sun et al.[1]

BEFORE YOU BEGIN

In this protocol, we provide detailed instructions for using single-photon calcium imaging to record and analyze hippocampal neuronal population activity elicited by non-spatial stimuli in mice. Male C57BL/6 mice, aged 10–12 weeks and weighing 23–25 *g*, were obtained from the Australian Animal Resource Centre. Mice were group-housed, with 3–4 mice per cage, and maintained on a 12-h light/dark cycle with access to water and standard mouse chow *ad libitum* for at least one week before the start of the experiment.

Institutional permissions

All procedures were carried out under the Australian Animal Welfare Committee guidelines and approved by the Florey Animal Ethics Committee (No. 18-008UM).

Preparation for the virus injection and GRIN lens implantation surgery

Timing: 2–3 h

Here, we describe the steps for preparing tools for the surgery, including a virus injector, a lens holder, a brain tissue aspiration tool, and a virus loading tool, as well as preparing the virus.

1. Prepare the virus injector (Figure 1A).
   a. Use a microelectrode puller to pull a glass capillary tube (inner diameter: 0.9 mm, outer diameter: 1.5 mm), creating a sharp tip with an opening diameter in the range of 50 μm–100 μm.

Note: Researchers can use the following settings in the microelectrode puller to create a sharp tip - Heat:760, Pull:30, Velocity:120, Time:300, Pressure:250.

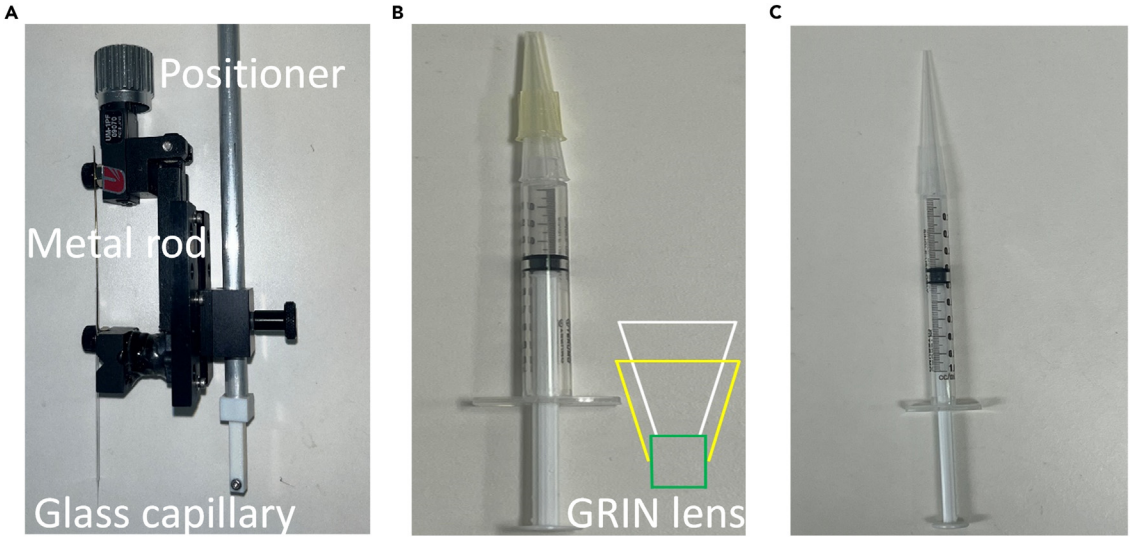

Figure 1. Miniscope surgery tools
(A) Virus injector.
(B) GRIN lens holder.
(C) Virus loading tool.

b. Insert a 0.8 mm diameter brass rod into the capillary from the flat end and ensure snug fit.

Note: If the diameter of the injector tip is less than 50 μm, use sharp tweezers to enlarge.

Note: Researchers may consider using transgenic mice expressing GCaMP6 in the hippocampus to avoid the virus injection step.[2,3]

2. Prepare the GRIN lens holder (Figure 1B).
   a. Source two 1 mL pipette tips. Use a sharp blade to trim the first one to a 1.5 mm diameter tip and the second one to a 2.1 mm diameter tip.
   b. Use fine sandpaper to smooth the cut for an even finish.
   c. Attach these two pipette tips to a 1 mL syringe and then connect them to a vacuum line using glue.

Note: The diameter of the GRIN lens used in this project is 1.8 mm. Adjust the pipette tip trimming accordingly if a different diameter GRIN lens is used.

3. Prepare the brain tissue aspiration tool.
   a. Attach a 27-gauge blunt needle to a 1 mL syringe.
   b. Remove the plunger and glue the syringe to a liquid trap, then connect the liquid trap to a vacuum line.
4. Prepare the virus loading tool (Figure 1C).
   a. Glue a 1 mL pipette tip to a 1 mL syringe.
   b. Cut the pipette tip to create an opening with a diameter of approximately 1.5 mm.
5. Load 5 μL of the virus (AAV1.Syn.GCaMP6f.WPRE.SV40)[4,5] into a 1.5 mL microcentrifuge tube and store it in a −80°C freezer.

Note: The concentration of the virus does not significantly impact its expression in the hippocampus. Diluting the virus in a saline solution (1 part virus to 3 parts saline) to achieve a titer value of 6.0 × 1012 GC/mL also works.

Pause point: The diluted virus can be stored for long term at −80°C.

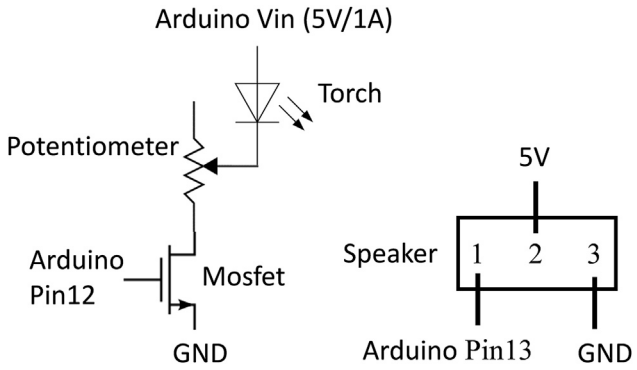

Figure 2. The torch and speaker circuit connection

The Arduino is powered by a 5V/1A adapter. The positive terminal of the torch is connected to the Vin pin of the Arduino board, and it is controlled to turn on and off using a potentiometer and a MOSFET, which is in turn controlled by pin 12 on the Arduino. The speaker is powered by a 5V pin of the Arduino board and is controlled by PWM through pin 13.

6. Prepare all necessary surgical tools and materials as specified in the key resources table for surgery.

Preparation for calcium signal recording experiment

Timing: 1–2 h

Here, we describe the steps for setting up experimental apparatus and recording equipment.

7. Download the recording software provided in this protocol. Links are available in the key resources table.

Note: The software is designed to synchronize the calcium recording with a speaker and an LED torch using an Arduino board or a Raspberry Pi. However, the software can be easily adapted to trigger other stimuli.

8. Connect the speaker and the torch to an Arduino board following the circuit shown in Figure 2.
9. Upload the code provided in this protocol to the Arduino.
10. Replace the achromatic lens inside the UCLA miniscope[6] V3 with a 7.5 mm focal length achromatic lens.

Note: The 0.23 pitch GRIN lens adapts well to the 7.5 mm focal length achromatic lens in our optical system, and the 0.25 pitch GRIN lens adapts well to the 15 mm focal length achromatic lens. The original optical system has a smaller magnification ratio and can provide a larger field of view.

Preparation for data analysis

Timing: 1–2 h

Here, we describe the steps for installing analysis software and packages required for data processing.

11. Download PyCharm and Anaconda. Use Anaconda to create a new environment for this project and install the TensorFlow package.[7]

12. Download MATLAB, CNMF-E, and the custom code provided in this protocol. Links are available in the key resources table.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
pAAV.Syn.GCaMP6f.WPRE.SV40	Addgene	100837
Chemicals, peptides, and recombinant proteins		
Isoflurane	VetOne	502017
Carprofen	Rimadyl	Small Animal Solution for Injection
Dexamethasone	Sigma	D4902
Hydrogen peroxide	Sigma	7722-84-1
Enrofloxacin	Baytril	Baytril 2.5%
Sodium chloride	Sigma	7647-14-5
Potassium chloride	Sigma	7447-40-7
Potassium dihydrogen phosphate	Sigma	13761-79-0
Sodium bicarbonate	Sigma	144-55-8
D-glucose	Sigma	50-99-7
Calcium chloride	Sigma	10043-52-4
Magnesium chloride	Sigma	7791-18-6
Antiseptic solution	Betadine	15055
Deposited data		
Demo data and video	https://doi.org/10.5281/zenodo.11118231	N/A
Experimental models: Organisms/strains		
Male C57BL/6 mice (male, 10–12 weeks, 23–25 *g*)	Australian Resources Centre	N/A
Software and algorithms		
MATLAB 2019a	MathWorks	N/A
CNMF-E	https://github.com/zhoupc/CNMF_E	N/A
NormCorre	https://github.com/flatironinstitute/NoRMCorre	N/A
Recording software	https://doi.org/10.5281/zenodo.11118231	N/A
Arduino code sketch	https://doi.org/10.5281/zenodo.11118231	N/A
Arduino IDE	Arduino	N/A
Python 3.7	Python Software Foundation	N/A
Analysis package	https://doi.org/10.5281/zenodo.11118231	N/A
Other		
GRIN lens	Edmund Optics	64-519
Miniscope	UCLA Miniscope	V3
Torch	Jaycar	ST3481
Speaker	Jaycar	XC3744
M1.2 stainless steel screw	Watch tools	N/A
Precision screwdriver	Craftright	Phillips #1
Stereotactic frame	World Precision Instruments	505388
Super glue	UHU	Ultra-fast
Dental cement powder	Paladur	Pink 100 *g*
Dental cement liquid	Paladur	80 mL
Microscope	KERN	OZL 961
Microelectrode puller	Sutter	P-1000
Glass capillary (ID: 0.9 mm, OD: 1.5 mm)	Narishige	G-1.5
Silicone gel	Barnes	SR-PINKY-T
Eye ointment	Terramycin	#8-763
Silicone oil	Sigma-Aldrich	378372
Miniscope baseplate	LabMaker	V3.2
Miniscope DAQ board	LabMaker	V3.2

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Blunt 27-gauge needles	Metcal	927050-TE
Brass rod (D: 0.8 mm)	Uxcell	a22060200ux0168
Drill burr (D: 2 mm)	Australian Jewellers Supplies	07-107-020
Drill burr (D: 0.9 mm)	Australian Jewellers Supplies	07-107-009
Drill burr (D: 0.4 mm)	Australian Jewellers Supplies	07-107-004
Silk sutures	LOOK	X776B
0-80 Hex key screwdriver	Base Lab Tools	HX-0.028
UV epoxy	Bondic	SK8024x2
Arduino board	Arduino	UNO
Linear positioner	Narishige	UM-1PF
Tweezers	Lindstrom	TL 4-SA SL
Drill set	Dremel	8220-2/45
MOSFET	DigiKey	IRLB8721PBF-ND
Potentiometer	Mouser	652-PDB181K420F103C
7.5 mm FL achromatic lens	Edmund	#45-407

STEP-BY-STEP METHOD DETAILS

GCaMP6f virus injection

Timing: 1 h

Here, we describe the steps for injecting virus into the hippocampal CA1 area.

Inject GCaMP6f AAV into the animal's brain to label hippocampal neurons.

1. Sterilize all surgical tools using a dry bead sterilizer.
2. Load the virus into the capillary tube.
   a. Take the virus microcentrifuge tube out of the −80°C freezer and allow it to thaw at 25°C.
   b. Connect the sharp tip of the capillary tube to the virus loading tool.
      i. Submerge the flat end of the capillary in the virus.
      ii. Gently pull the syringe plunger to draw the virus into the capillary.
   c. Load silicone oil into the capillary from the flat end using the same method mentioned above.

   Note: Minimize the air gap between the virus and the silicone oil; otherwise, it may impact the injection speed and stability.

   d. Insert the brass rod into the capillary and push the virus to the tip of the capillary.
   e. Use a fine marker pen to mark the virus level on the capillary.
3. Prepare the mouse for the surgery.
   a. Anesthetize the mouse in an induction chamber using an isoflurane induction concentration of 3% and an oxygen flow of 1 L/min.
   b. Secure the animal's head on a stereotaxic frame when it is fully anesthetized.
   c. Reduce the isoflurane to 2.5% and the oxygen to 0.25 L/min.

   Note: Verify the depth of anesthesia by performing a toe-pinch test and monitor the breathing rate during the surgery, ensuring it remains within the range of 68–80 breaths per minute.

   d. Shave the mouse's head and sterilize it using antiseptic solution (Betadine) and 80% ethanol. This should be done sequentially for at least 2–3 times.
   e. Apply eye ointment to ensure the protection of the eyes and prevent corneal dehydration.
4. Drill a craniotomy hole for the virus injection using the drill set.

Table 1. ACSF buffer

Reagent	Final concentration
Sodium chloride (NaCl)	127 mM
Potassium chloride (KCl)	1.2 mM
Calcium chloride ($CaCl_2$)	2.5 mM
Magnesium chloride ($MgCl_2$)	1.5 mM
Potassium dihydrogen phosphate (KH_2PO_4)	1.2 mM
D-glucose	10 mM
Sodium bicarbonate ($NaHCO_3$)	28 mM

[Note on storage conditions: Store at −4C for up to 2 weeks]

a. Make a single 8 mm incision along the midline of the scalp using a surgical blade.
b. Attach a syringe tip to the stereotaxic arm.
c. Use the syringe tip to align the bregma and lambda anatomical points at the same level.
d. Move the syringe tip to the virus injection coordinate (AP −2.1 mm, ML 2.0 mm), and mark the location on the scalp.
e. Drill a craniotomy hole at the marked location using a 0.4 mm drill burr.

⚠ CRITICAL: To prevent overheating during drilling, it is recommended to use slow drilling speeds and take breaks to allow the surface to cool down. Additionally, periodically bathing the skull in sterile saline and using a compressed air can facilitate cooling.

5. Inject the virus into the hippocampus.
   a. Secure the virus capillary to the stereotaxic arm and attach the rod to a single axis linear positioner.
   b. Lower the capillary tip perpendicularly into the craniotomy hole to a depth of 1.7 mm below the top of the skull.
   c. Inject the virus using the custom-made virus injector by advancing the rod in the capillary tube with the linear positioner.
   d. Lower the virus level by 1 mm, which equals 500 nL slowly, over a total injection period of approximately 10 min.
   e. Leave the injector in place for an extra 10 min to allow for viral diffusion.
6. Slowly withdraw the injector, and then suture the incision using silk sutures.

GRIN lens implantation

Timing: 2–3 h

Here, we describe the steps for implanting a GRIN lens into the brain to enable observation of hippocampal CA1 neurons.

After viral injection, allow one week for viral transduction. Then, implant a GRIN lens to touch the fimbria of the hippocampus CA1 region.

7. On the day of the surgery, prepare the artificial cerebrospinal fluid (ACSF) solution using the recipe listed below (Table 1). Bubble the solution with carbogen (5% CO_2, 95% O_2) for 1 h.
8. Weigh the animal, and then administer carprofen and dexamethasone subcutaneously at doses of 0.5 mg/kg and 0.06 mg/kg, respectively.
9. Sterilize all surgical tools and prepare the animal for the surgery using the method mentioned above.
10. Drill a round cranial window with a 2 mm diameter in the skull.
    a. Remove the scalp using scissors and apply hydrogen peroxide to clean the surface of the skull thoroughly.

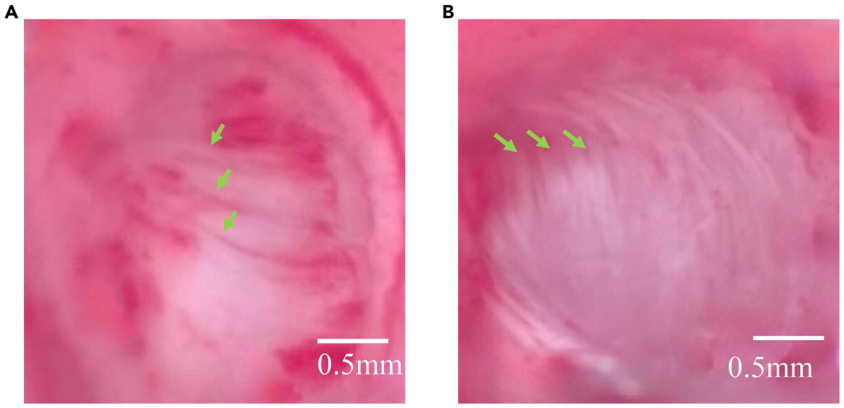

Figure 3. Cortex aspiration landmark
(A) Horizontal striations.
(B) Vertical striations. Arrows in figures indicate striations.

b. Detach neck muscles using tweezers.
c. Use alcohol to clean the skull.
d. Align the skull using bregma and lambda.
e. Drill holes using a 0.9 mm drill burr for anchor screws at the following coordinates: AP 1.8 mm, ML −2.5 mm; and AP −2.8 mm, ML −0.8 mm.
f. Locate a drill burr (2 mm in diameter) over CA1 at coordinates at AP: −2.1 mm, ML: 1.5 mm from the bregma.[8]
g. Drill through the skull to make a round cranial window until the brain tissue is identified under the microscope.

⚠ CRITICAL: Use slow drilling speeds during drilling and take breaks every five seconds to prevent overheating.

h. Use tweezers to remove any remaining bone fragments from the edge of the hole, ensuring that the grin lens can fit in.
i. Remove the dura using tweezers within the implantation site under the microscope.

⚠ CRITICAL: If there is any uncontrolled bleeding, apply sterile cotton swabs to the affected area, followed by gentle pressure to reduce bleeding.

j. Secure two anchor screws by twisting them in three turns each using the precision screwdriver (approximately 1.2 mm in distance).

Note: Ensure that the drill burr remains perpendicular to the skull and keep your hand steady while drilling; otherwise, the anchor screw may not securely fit into the holes.

11. Remove cortical tissue using the aspiration tool.
a. Load ACSF buffer into a 30G syringe needle and apply it to flush blood during aspiration.
b. Open the vacuum line (pressure: 0.08 Mpa) and use a 27G blunt needle of the aspiration tool to carefully aspirate cortical tissue.
c. Expose the horizontal hippocampal fimbria striations at a depth of approximately 1.2 mm (Figure 3A).
d. Carefully aspirate the horizontal striations to expose the vertical fimbria striations at a depth of approximately 1.35 mm (Figure 3B).
e. Allow the blood to dry for 2 min, then peel off the coagulated blood from the surface of the vertical striations.

Note: In case there is any uncontrolled bleeding, repeat steps (a) and (d).

12. Implant the GRIN lens, touching the hippocampal fimbria.
    a. Attach the GRIN lens holder to the stereotaxic arm.
    b. Use alcohol to sterilize the GRIN lens.
    c. Attach the GRIN lens to the holder and ensure it is aligned directly above the center of the cranial window.
    d. Lower the lens to the bottom of the craniotomy (−1.35 mm from the top of the skull).
    e. Absorb any residual liquid on the skull using a piece of tissue.

    ⚠ CRITICAL: The skull should be made dry.

    f. Apply super glue over the skull to secure the lens and anchor screws.
    g. Apply a thin layer of dental cement over the skull to further secure the lens and anchor screws.

    Note: Do not apply glue or dental cement to the top surface of the lens.

    h. Apply silicone gel over the lens for protection.
13. Administer daily injections of carprofen (0.5 mg/kg) and dexamethasone (0.06 mg/kg), and provide the animal enrofloxacin water (1:150 dilution, Baytril) for one week.

Baseplate mounting

Timing: 1–2 h

Here, we describe the steps for fixing a baseplate onto the animal's head, which serves to securely hold the miniscope in place for neuroimaging or recording purposes.

After four or five weeks following the implantation surgery, attach a miniscope baseplate to the animal head.

14. Set up the miniscope connection.
    a. Connect the miniscope to the DAQ board and the DAQ board to the laptop. Open the software and enable the miniscope camera.
    b. In the software, configure the settings.

    Note: Set the frame rate to 30 fps, the gain to the maximum (64), the exposure to the maximum (255), and the stimulating LED intensity to 4% (Figure 4). If the software crashes, consult Troubleshooting problem 1.

    c. Set the miniscope camera ID to "1" and connect to the camera.

    Note: The miniscope camera may be enumerated as "camera 0" or "camera 1", depending on the motherboard. Set the miniscope camera ID to "0" if camera "1" does not work.

    d. Attach the baseplate to the miniscope.
15. Anesthetize the animal and fix its head on the stereotaxic using the method mentioned above.
16. Fix the baseplate on the animal's head (Figure 5A).
    a. Use tweezers to remove the lens protection silicone gel.
    b. Place the miniscope with the baseplate attached on the GRIN lens.
    c. Adjust the miniscope focal length until the neurons come into focus (Figure 5B).

    Note: If there are no neurons in the field of view, consult Troubleshooting problem 2.

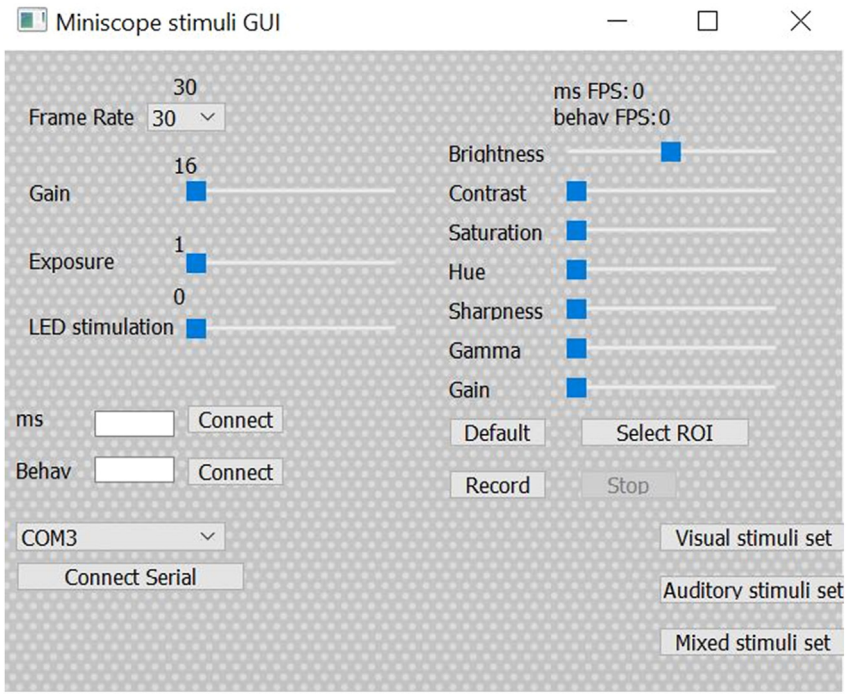

Figure 4. Miniscope controlling software setup
The miniscope camera ID (ms) should be set to "0" or "1", depending on the user's laptop motherboard.

d. Tighten the adjustment screw.

Note: You may have to decrease the miniscope sampling rate to 20 fps or increase the stimulation LED intensity to identify the neurons.

e. Apply UV glue on the skull and then place the miniscope on it.
f. Adjust the miniscope angle to find the best field of view.
g. Illuminate with UV light to harden the glue.

Note: The baseplate may cause the glue to squeeze onto the lens surface. Carefully clean any glue on the lens surface using alcohol before activating the UV light.

h. Apply dental cement around the baseplate to further secure it on the animal's head.
i. Detach the miniscope from the baseplate.
j. Secure the lens protection cap on the baseplate using the 0–80 Hex key screwdriver.

Animal habituation

Timing: 20 min per day for 3 days

Here, we describe the steps for habituating animals for the experiment.

Habituate the animal with the miniscope and the recording chamber.

17. Wrap the animal in a hand towel and use one hand to gently hold and release it several times until the animal is used to the activity.
18. Use the other hand to loosen the screw and remove the protective cap. Then, place the miniscope on the baseplate and fasten the screw.

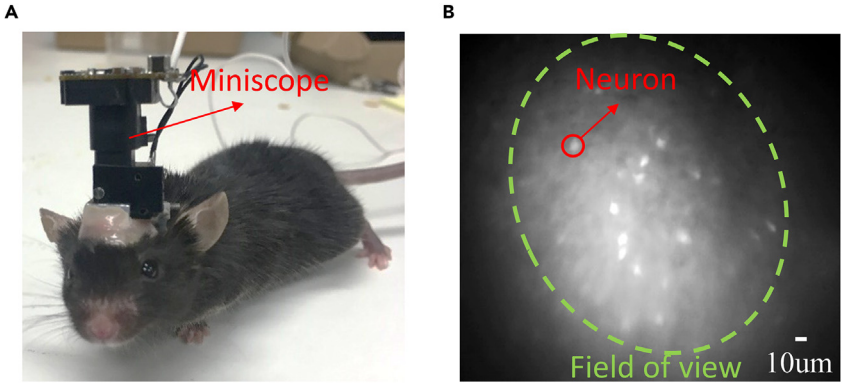

Figure 5. Hippocampal calcium signal
(A) A mouse with the miniscope.
(B) An example of the hippocampal neurons in the field of view.

19. Place the animal in the recording chamber and allow it to habituate for 20 min.
20. Repeat the above procedure until the animal no longer actively explores the chamber.

Calcium signal recording during non-spatial stimuli experiments

Timing: 1.5 h

Here, we describe the steps for using our customer designed software to record calcium signals in mice using the miniscope.

21. Set up the stimuli pattern on the Arduino board.
    a. Open the Serial Port settings from the Windows Device Manager. Connect the Arduino board and find its Serial COM port number.

Note: If the serial COM port does not appear, consult Troubleshooting problem 3.

    b. Open the Arduino IDE and load the sketch provided. Change the stimuli parameter as required.

Note: Previous work has found that neuronal populations exhibit widely distributed response latencies to stimuli, with a maximum around 2 s.[9] Consequently, we have selected a 2-s stimulus duration and employed a 3–3.5-s interval to minimize any significant influence of the previous epoch's stimulus on the current epoch's neuronal responses. Stimuli parameters are declared at the beginning of the template. In visual stimuli experiments, the light will be turned on for 2 s (light_on = 2) and turned off for 3–3.5 s (light_off = 3, extra_off = 0.5). This cycle will be repeated 600 times (iteration = 600). In auditory stimulus experiments, the speaker will be activated for 2 s (sound_on = 2) and deactivated for 3–3.5 s (sound_off = 3, extra_off = 0.5). The order of the sound frequencies is declared in the variable "sound_order". In mixed stimuli experiments, the animal experiences four different background environments: dark + silent, dark + sound, light + silent, and light + sound with a duration of 2 s (epoch_duration = 2). The order is declared in the variable "light_sound_order".

    c. Select the correct board "Arduino UNO" from the tool setting.
    d. Select the correct serial port number from the tool setting. Compile and upload the sketch to the Arduino board.

22. Place the animal in a quiet room and allow it to acclimate to the recording chamber for 30 min before starting the recording.
23. Set up the connection between the miniscope, Arduino board, and the computer.
    a. Mount the LED light source and the speaker above the recording chamber.
    b. Set up the miniscope connection and configure the settings in the software as mentioned earlier.
    c. Remove the protective cap and attach the miniscope to the baseplate.

    Note: Stimulation LED intensity may need to be optimized to achieve the best image, with a typical value being around 4%. If neurons do not fire, consult Troubleshooting problem 4.

    d. Depending on the type of experiment conducted, click on either "visual stimuli set", "auditory stimuli set", or "mixed stimuli set" in the software.

    Note: The original source code for the recording software, written in Python and utilizing PyQt5, is included in this protocol. It is self-explanatory, enabling users to customize the code as needed. Additionally, the software allows for the activation of a behavior camera to monitor the animal, which is synchronized with the miniscope camera.

    e. Turn off the room lights during the visual stimuli and mixed stimuli experiments. Note: Keep the room lights on during the auditory stimuli experiment.
    f. Select the correct Arduino serial COM port number, and then click "Connect Serial". g. Click "Record" to start the recording.
24. Click 'Stop' when the stimulus sequence is completed.

EXPECTED OUTCOMES

After successfully implementing this experimental protocol, researchers can monitor hippocampal CA1 neurons in mice. Ideally, a good field of view with hundreds of neurons will be observed, and the neurons should demonstrate clear firing patterns without photobleaching after 45 min. If this criterion is not met, the experiment can be delayed by one or two weeks or reduce the stimulation LED light intensity. For reference, a representative video demonstrating successful signals is provided in the protocol.

To detect non-obvious neuronal firing patterns of each neuron, an autoencoder is used to reduce the data dimensionality and extracted the most significant features. The autoencoder significantly improves discrimination, allowing activity in different temporal windows to be separated into distinct clusters. An example of the neuronal firing patterns in different temporal windows, extracted from visual stimuli experiments, is shown in Figure 7. Cortical neuronal population activity displays redundancy in several systems, wherein multiple neuronal populations may exhibit analogous patterns of activity despite processing different types of information or performing different tasks.[10] Thus, an autoencoder is used to reduce the dimensionality of the population activity. The results show that the majority of the variation in neuronal population activity (first three most significant features derived from the autoencoder) is represented by an intrinsic low-dimensional neural manifold, and encoding geometries follow relevant sensory-specific alterations. For example, in visual stimuli experiments, encoding geometries varies smoothly in time, but somewhat abruptly in auditory stimuli experiments. However, the population activity in mixed stimulus experiments shows certain overlap in neural manifolds. For more details regarding the experimental results, please refer to Sun et al.[1]

QUANTIFICATION AND STATISTICAL ANALYSIS

Extract neural calcium events and segment the data

Upon completion of the recording process, a motion correction algorithm will be applied to align the raw images. Subsequently, the extraction of neural calcium activity will be performed utilizing the constrained non-negative matrix factorization for endoscopic recordings algorithm (CNMF-E).[11]

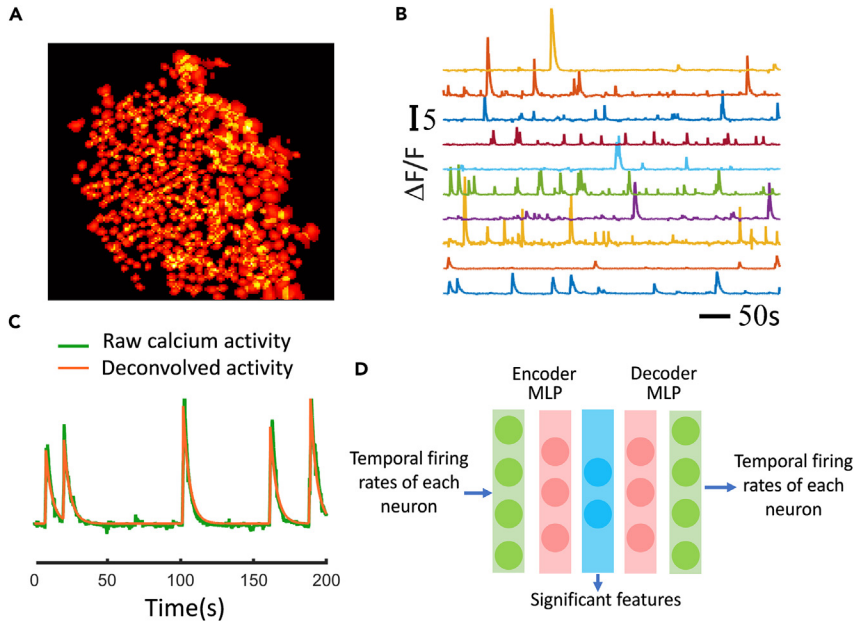

Figure 6. Calcium activity extracted using the CNMF-E algorithm and a diagram showing the autoencoder network

(A) Footprints of detected neurons.

(B) Raw calcium activity.

(C) Deconvolved calcium activity.

(D) The autoencoder is composed of an encoder and a decoder, both constructed as multiple layer perception networks. The inputs and outputs of the autoencoder are the temporal firing rates of each neuron. Significant features are then extracted from the last layer of the encoder. Figure reprinted and adapted with permission from Sun et al., 2024.

1. Open MATLAB, add the analysis package to the current working path, and execute the script. In brief, the script accomplishes the following:
   a. Align raw images using the NoRMCorre algorithm.[12]

   Note: To reduce computation time, we spatially downsampled the video by a factor of 3 and chose the rigid motion correction method (spatial_downsampling = 3, isnonrigid = true). We have tested different downsampling factors ranging from 1 to 3, and all of them have yielded comparable results. The choice between rigid or nonrigid motion correction methods does not significantly affect alignment results in our experiments.

   b. Extract neuronal footprints and calcium activity using the CNMF-E algorithm (Figures 6A–6C).

   Note: We find that setting the neuron diameter to 10 (gSiz = 10) and the minimum peak-to-noise ratio to 10 (min_pnr = 10) can yield good results with a high signal-to-noise ratio in our experiments. If an "out of memory" error pops up while running CNMF-E, consult Troubleshooting problem 5.

   c. Binarize the deconvolved calcium activity (ms.neuron.S) to obtain neuronal calcium events for further analysis.

   Note: The amplitude of the deconvolved calcium activity (ms.neuron.S) can be explained as the likelihood that a neuron fires at each time frame. To binarize this activity, a threshold can be set. There are several methods to determine the threshold, but we recommend using the mean amplitude minus two or three times the standard deviation.[13]

2. Segment the data into epochs according to the stimuli.

Note: Time stamps for the miniscope camera and stimuli are recorded in a text file (stamp.txt) and can be used to segment the data. The structure of epoch data in visual stimuli experiments is as follows: the light is turned on at 0.5 s and turned off at 2.5 s, remaining off for 3s. In auditory stimuli experiments, there are three types of epochs based on the sound frequency (4 kHz, 8 kHz, and 16 kHz). The sound is activated at time 0 s and deactivated at time 2 s, remaining off for 3 s. In mixed stimuli experiments, there are four types of epochs, each with a duration of 2 s (dark + silent, dark + sound, light + silent, and light + sound). The stimuli are triggered at time 0 s.

Stimuli sensitive neurons detection

"Mutual information" is a metric for quantifying the degree of interdependence between variables and can be used to identify stimuli sensitive neurons. However, its applicability to this study is limited. In contrast, Weighted Normalized Mutual Information (WNMI) incorporates the weights of deconvolved neural activities (ms.neuron.S) and integrates a normalization component. This adaptation is instrumental in detecting neurons that exhibit responses to diverse stimulus contexts. The metric of mutual information is characterized by a range extending from 0 to positive infinity, which can introduce varying levels of bias contingent on the dataset employed. The incorporation of normalization in WNMI constrains its value domain to [0, 1], and compensates for the bias towards multivalued features.[14] The calcium events underwent 500 shuffles, and the average WNMI was assessed for each permutation. To categorize a neuron as stimuli-sensitive, the average WNMI must exceed chance levels ($p < 0.05$) in comparison to the permutation results.

3. Use the script provided to detect stimuli sensitive neurons in each experiment.

Neuronal firing pattern extraction

In order to detect ''deep features'' from neuronal firing patterns, we use an autoencoder[15] to identify variations in these patterns across different contexts within a high-dimensional parameter space. This process effectively achieves dimensionality reduction for intricate data spaces.[16] The architecture of an autoencoder is bifurcated into two segments: the encoder and the decoder. The encoder's role is to compress the data dimensionality, whereas the decoder endeavors to reconstruct the initial input from this compressed form. Throughout the training phase, the encoder discerns the most significant components within the original dataset. Subsequently, this encoder is then utilized to extract "deep features" from the neuronal firing patterns, thereby facilitating a more nuanced understanding of these complex neural processes.

4. Create a new environment in Anaconda and install the Tensorflow package.
5. Build an autoencoder network to reduce the dimensionality of the neural calcium events epoch data (Figure 6D).

Note: The inputs of the model were the temporal firing rates of each neuron in each epoch. Both the encoder and decoder were built with three dense layers utilizing scaled exponential linear unit (SELU) activation functions. The model was trained using a binary cross-entropy loss function and an Adam optimizer. Additionally, a grid search methodology was implemented to optimally adjust various parameters, including the learning rate, the number of nodes in each layer, and the total number of training epochs. This approach was aimed at minimizing the error in the reconstructed data.

6. Apply principal component analysis to the outputs from the encoder for better visualization (Figure 7).

Neural manifolds analysis

A geometrical analysis of neural population activity has resulted in the identification of topologically defined state spaces known as "neural manifolds" characterized by reduced dimensionality.[10] There is a prevailing hypothesis that these manifolds are more adept at pinpointing functional structures

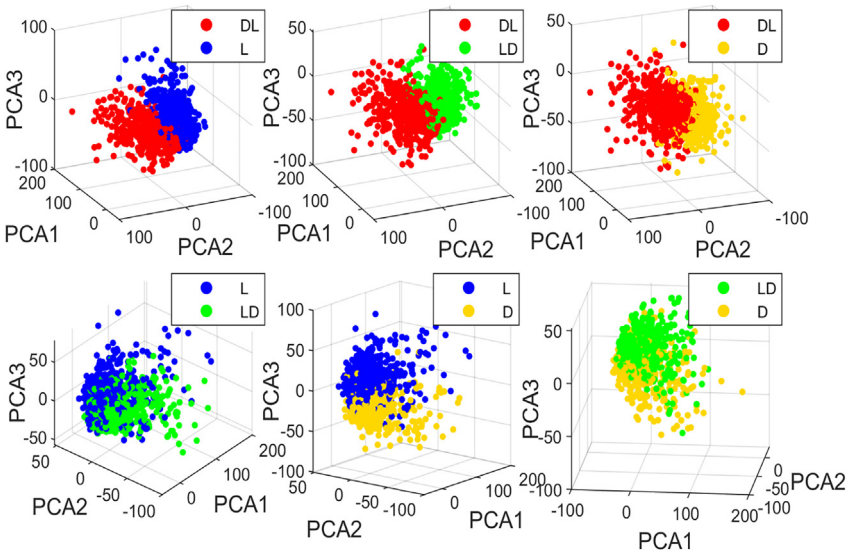

Figure 7. Neuronal firing patterns extracted in visual stimuli experiments

Neuronal firing patterns of each neuron in four temporal windows: dark-to-light transient (DL), light (L), light-to-dark transient (LD), and dark (D). Each dot represents the firing pattern of a specific neuron. Principal component analysis (PCA) is applied to the autoencoder results for better visualization. Figure reprinted and adapted with permission from Sun et al., 2024.

that are both dynamically and computationally pivotal.[17] To project the population activity from a high-dimensional neural state space onto lower-dimensional neural manifolds, an autoencoder is employed for dimensionality reduction. This approach allows for a more refined and manageable representation of the complex neural activity.

7. Build an autoencoder network to analyze neural manifolds activity (Figure 8).

> *Note:* The architecture of the autoencoder employed here mirrors that used in the extraction of neuronal firing patterns, including the adoption of the same optimization technique. However, the inputs for this model are the neural activity data from all neurons at each respective time point. The outputs generated by the encoder are then utilized for conducting manifold analysis. For visualization, the first three most significant features identified in this analysis are selected and used in plotting.

LIMITATIONS

Complete elimination of all spatial inputs to the animal is not feasible. Therefore, we confined the animal to a small recording chamber (20 cm × 20 cm) to minimize spatial influences as much as possible, allowing the animal to be passively exposed to stimuli. Remarkably, stimuli were introduced randomly throughout the experiment, a strategy that notably diminished the impact of spatial inputs. If neuronal responses genuinely represent feature-in-place responses, it is anticipated that discernible and consistent neuronal firing patterns would not be observed. Nevertheless, it is possible that some neurons encode both spatial and nonspatial information. Given our use of a very small recording chamber, relying solely on the animal's location within the chamber as an indicator of spatial inputs may not be a practical approach. The most reasonable method for detecting spatial inputs would have involved setting up an infrared eye-tracking system.[18] Unfortunately, this consideration was not part of the current experimental design.

TROUBLESHOOTING

Problem 1

Recording software crashes during recording.

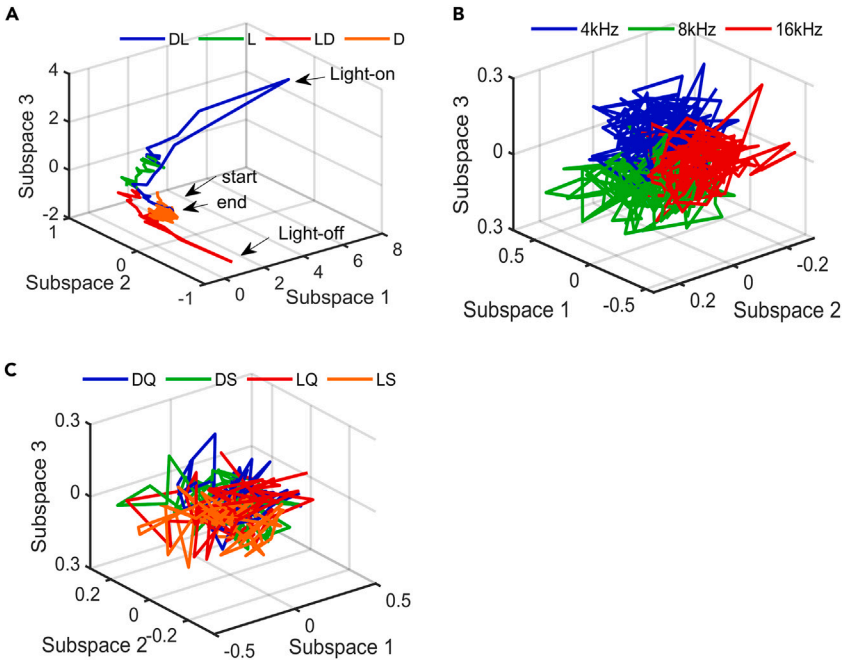

Figure 8. Hippocampal neural manifolds activity

Examples of neural manifold representations in (A) visual stimuli experiments, (B) auditory stimuli experiments, and (C) mixed stimuli experiments. The first three most significant features derived from the autoencoder are used for plotting. Figure reprinted and adapted with permission from Sun et al., 2024.

Potential solution

After clicking the "Record" button, the miniscope continues to capture images and loads them into a buffer. If the hard drive cannot efficiently handle the data in the buffer, it may become full, leading to software crashes. This issue sometimes may happen on laptops equipped with mechanical hard drives. To solve this problem, we recommend using a laptop equipped with a solid-state drive (M.2 interface; writing speed > 500 MB/s), which offers faster data transfer speeds and improved performance. The provided software stores the data in 'RAW' format, making the video file very large. If such precision is not required, modify the software code, such as replacing "codec = cv2.VideoWriter_fourcc('raw ')" with "codec = cv2.VideoWriter_fourcc('H264')".

Problem 2

No neurons in the field of view.

Potential solution

There are typically two possible reasons for this. If the background in the field of view is bright, it means the virus has been expressed, but the lens depth is not appropriate. On the other hand, if the background is dark, it indicates that the virus has not been expressed. Please wait for an additional two weeks to recheck the signal. If you still haven't observed any neurons, ensure that the virus injection coordinates are correct and verify if the virus is still viable.

Problem 3

In the recording software, the serial COM port does not appear when connecting the Arduino board.

Potential solution

This sometimes occurs in the Windows 8 or Windows 10 operating system. Install the serial COM port driver provided in this protocol.

Problem 4

Neurons do not fire when the animal is awake.

Potential solution

Several possible reasons could be causing this issue, such as inflammation or the virus concentration being too high. Please wait for an additional 2 weeks. If the signal still has not returned, it is very likely due to virus-related issues because a high concentration can be toxic to neurons. Double-check the virus injection depth and volume to ensure they are correct.

Problem 5

Encounter an "out of memory" error while running CNMF-E in MATLAB.

Potential solution

When analyzing very long recordings (e.g., 1 h recording, approximately 4 GB) using the CNMF-E package, MATLAB may encounter an "out of memory" error. We recommend increasing the virtual memory of your operating system to 200 GB and maximizing the MATLAB Java Heap Memory allocation.

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. Chris French (frenchc@unimelb.edu.au).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Dr. Dechuan Sun (dechuan.sun@unimelb.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The code and data described with this protocol is archived at Zenodo: https://doi.org/10.5281/zenodo.11118231.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.S., R.R.U., and C.F.; methodology, D.S.; writing – original draft, D.S. and M.A.; review and editing, R.R.U. and C.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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