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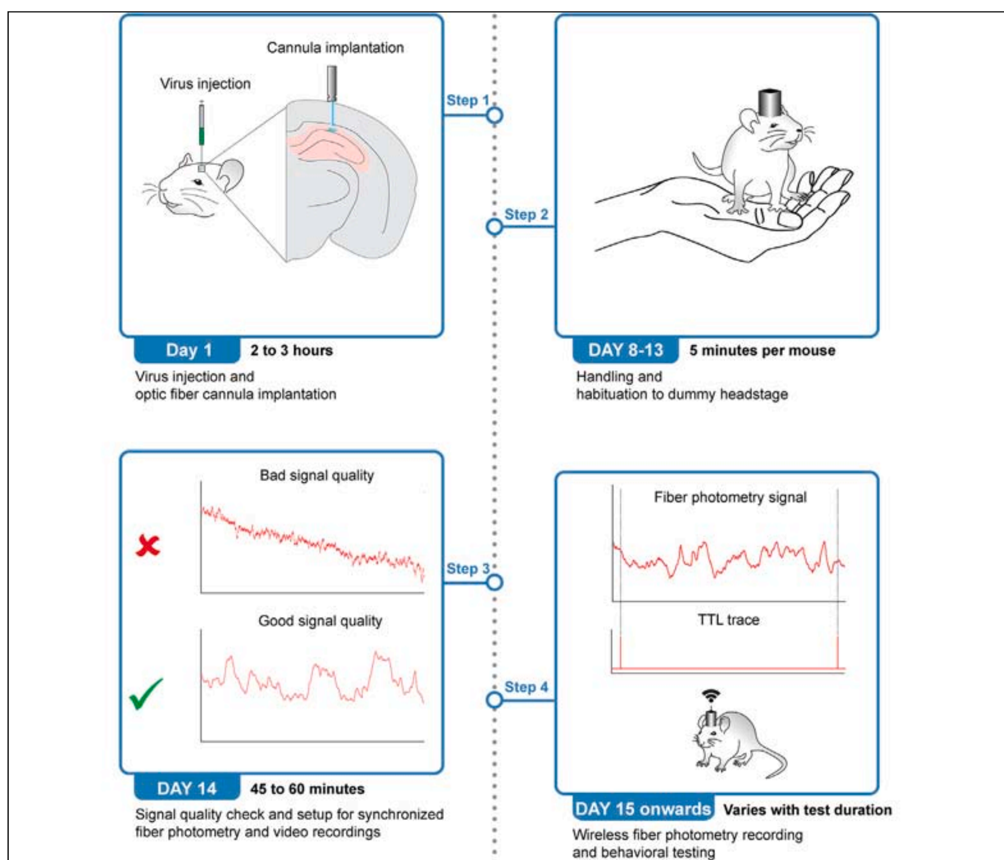
Protocol for synchronized wireless fiber photometry and video recordings in rodents during behavior



Fiber photometry technique allows investigation of *in vivo* neural activity during behavior allowing understanding of brain-behavior relationship. Here, we provide a protocol for synchronized wireless fiber photometry and video recordings in rodents during behavior. We explain the detailed steps for stereotaxic virus injection, optic fiber cannula implantation, setup for synchronized fiber photometry and behavioral recording, and analysis of photometry data. These protocol steps can be adapted for various animal models, photometry, and behavioral recording systems.

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

Suhel Tamboli, Dimitry Topolnik, Risna Radhakrishnan, Daphnée Veilleux-Lemieux, Lisa Topolnik

lisa.topolnik@bcm.ulaval.ca

Highlights

Step-by-step instructions for viral injections and optic fiber cannula implantation

Detailed setup and calibration guidelines for the wireless fiber photometry system

Configuration for synchronization of fiber photometry and behavioral video recordings

Comprehensive guidance on fiber photometry recordings, data extraction, and analysis

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Protocol for synchronized wireless fiber photometry and video recordings in rodents during behavior

Suhel Tamboli,[1,2,4] Dimitry Topolnik,[1,2] Risna Radhakrishnan,[1,2] Daphnée Veilleux-Lemieux,[3] and Lisa Topolnik[1,2,5,*]

[1]Neuroscience Axis, CRCHUQ-CHUL, Quebec City, PQ, Canada

[2]Department of Biochemistry, Microbiology, and Bioinformatics, Université Laval, Quebec City, PQ, Canada

[3]Direction des services vétérinaires, Université Laval, Quebec City, PQ, Canada

[4]Technical contact

[5]Lead contact

*Correspondence: lisa.topolnik@bcm.ulaval.ca
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SUMMARY

Fiber photometry technique allows investigation of *in vivo* neural activity during behavior allowing understanding of brain-behavior relationship. Here, we provide a protocol for synchronized wireless fiber photometry and video recordings in rodents during behavior. We explain the detailed steps for stereotaxic virus injection, optic fiber cannula implantation, setup for synchronized fiber photometry and behavioral recording, and analysis of photometry data. These protocol steps can be adapted for various animal models, photometry, and behavioral recording systems.

For complete details on the use and execution of this protocol, please refer to Tamboli et al.[1] and Amalyan et al.[2]

BEFORE YOU BEGIN

Fiber photometry technique involves acquiring fluorescent signals from a group of neurons in a particular brain region with the help of optic fibers. This technique is used to monitor the activity of specific neuronal populations in real-time, allowing an understanding of the dynamic interplay of neurons during a behavioral state or task in animals. Advancements in fiber photometry technique and the development of fluorescent markers accelerated the understanding of neuronal activity/ circuits underlying various animal behaviors.[3,4] Conventionally, wired setups of fiber photometry have been used in the field.[5,6] However, wired setups restrict naturalistic behavior and exploration, particularly in mice.[1] The recent development of wireless fiber photometry systems allows the acquisition of neuronal population signal while animals exhibit various behaviors/tasks without movement restriction.[1,2,7,8] To perform calcium imaging in freely behaving mice, we used the TeleFipho wireless fiber photometry system (Amuza Inc.). We believe that the methods mentioned in this article can be used/ adapted for other fiber photometry systems.

Our study was focused on understanding the *in vivo* activity of vasoactive intestinal polypeptide-expressing (VIP+) interneurons in the dorsal CA1 hippocampus. We synchronize photometry acquisition with behavioral recordings while mice perform various behavioral tasks. We used mouse as an animal model, but we believe this technique can also be implemented in other animals. To target VIP+ interneurons, we injected 200 nL of pGP-AAV-CAG-FLEX-jGCaMP7b-WPRE (Vigene Biosciences) in VIP-Cre mice. We also acquired signals from pyramidal neurons, somatostatin- and parvalbumin-expressing interneurons in CA1. Moreover, this system has been used to study the activity of motor cortex (M1) axons in C9orf72 female mice[2] as well as pyramidal neurons' and interneurons'

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populations in the dorsal CA1.[9] This methodology is applicable across various rodent models beyond transgenic mice strains and can employ diverse viral constructs carrying genetically encoded calcium indicators (GECIs). In addition to calcium imaging, this methodology is well-suited for studying neurochemical signals using the G-protein coupled receptor activation-based sensor (GRAB).[10–12]

Institutional permissions

All animal experiment protocols were approved by the Animal Protection Committee of Université Laval (CPAUL). The experiments were conducted in accordance with the guidelines of both CPAUL and the Canadian Council of Animal Care (CCAC). It is crucial for readers to acquire permission from their institute before conducting any experiments.

Virus injection

Timing: 2 weeks prior to the behavioral tests and fiber photometry recording, duration: 1–2 h

Note: The following instructions describe the procedure for the virus injection in the preparation of mice for fiber photometry experiments. These steps can be adapted to inject GECI or other viruses, depending on the specific requirements of the study.

1. Administer the mice with buprenorphine slow release (0.6 mg/mL) 45 min prior to anesthesia.

   Note: Buprenorphine slow release is administered 45 min before the start of the anesthesia and surgical procedures to ensure that the effect of analgesic is established.

2. Anesthetize the mice with isoflurane (induction: 3%–4%, maintenance: 1.5%–2%).
   a. Apply ophthalmic ointment to the eyes of the animal once it loses consciousness and reapply every 30 min in order to prevent corneal dryness.
   b. Administer Lactated ringer's solution (LRS; 5–10 mL/kg/h) to avoid dehydration.

   ⚠ CRITICAL: Provide a heat source (~37°C) to the animal throughout the entire duration of anesthesia to prevent hypothermia. Monitor the depth of anesthesia and adjust the level of isoflurane to maintain the breathing rate of 1 breath/second.

3. Administer lidocaine-bupivacaine (0.8 mg/mL) at the site of incision and under the ears.
4. Shave the incision site and clean it with Baxedin pre-op (contains 0.5% chlorhexidine and 70% isopropyl alcohol) before fixing the animal in the stereotaxic frame.
5. Make an incision over the midline of the scalp and clean the exposed skull using Baxedin pre-op.
6. Make sure the skull is straight by measuring the heights of bregma and lambda.
7. Locate the coordinates for CA1 (AP: −2.2 mm, ML: 2 mm, DV: −1.45 mm) and perform a craniotomy.
8. Clean the blood and bone debris from the skull by irrigating it with warmed LRS.
9. Fill a sterile glass pipette with the virus and inject 200 nL of the virus at the rate of 10 nL/s at the aforementioned coordinates using a nanoliter injector (Nanoliter 2000; World Precision Instruments).
10. Wait for 5 min for the virus to diffuse and slowly withdraw the pipette.

Optic fiber cannula implantation

Timing: After the virus injection procedure, duration: 45 min to 1 h

Note: The following steps describe the procedure of the implantation of an optic fiber cannula in mice. Experimenters can follow the stepwise procedure for cannula implantation and modify based on their requirements.

11. Drill an additional small hole on the contralateral side to accommodate the insertion of a screw, which serves to secure a headcap to the skull.

Note: It is advisable to insert the screw before the virus injection.

12. Insert the optic fiber cannula (400 μm core diameter) into the probe holder and connect it securely to the stereotaxic arm.
13. Position the fiber implant precisely above the region of interest using the stereotaxic arm.

Note: For the dorsal CA1 area, we used the following coordinates: AP-2.2 mm, ML-2 mm, and DV-1.35 mm.

⚠ CRITICAL: Maintain a controlled advancement rate of approximately 2 mm per minute, when inserting the optical fiber into the brain tissue, ensuring the fiber implant remains properly positioned.

14. Prepare a C&B Metabond Quick self-curing cement mixture.
    a. Dispense 4 drops of QuickBase into a designated well of the mixing dish.
    b. Add 2 scoops of L-Powder into the same well and dispense 1 drop of Gold Quick Catalyst.
    c. Stir the mixture for approximately 5 s to ensure proper incorporation of all components.
    d. Adjust the quantity of powder dispensed into the well, ranging from 1 to 2.5 scoops, based on the desired viscosity that is suitable for the seamless application across the cranium.

Note: This prepared mixture maintains its optimal workability for a duration of 2–3 min. The setting time is between 5 to 10 min.

15. Apply a thin and uniform layer of Metabond cement across the cranium and onto the lower section of the implant using a sterile pipette tip or toothpick.

Note: Ensure comprehensive coverage of the cranial surface with the base layer of Metabond.

16. Allow the Metabond mixture to dry thoroughly.
17. Apply successive even layers of Metabond cement to create a slight elevation over the cranium and surrounding the implant, allowing each layer to dry completely.

Note: Maintain a clear space of approximately 4–5 mm on the convex end of the cannula to ensure a smooth, unimpeded connection between the optic fiber implant and the TeleFipho transmitter headstage device.

Post-operative care

Timing: After the surgery procedure, duration: 30 min

18. Transfer the animal to the allocated warm recovery area.
19. Trim the animal's hind claws.
20. If more than 50 min have lapsed since the previous dose or if there was notable blood loss during the surgery, administer an additional warmed dose of LRS.
21. Administer Meloxicam (0.25 mg/mL) after the surgery and another dose within 24 h after the end of the surgery. Administer analgesics according to the protocol approved at your institution.

22. When the animal fully wakes up and breathes regularly, place it back in its cage.
23. Inspect the mouse daily for the next 5–7 days.

Note: If you observe any worrisome signs such as discharge, redness, or swelling, make a note on the postoperative follow-up and promptly inform the animal care staff (either the animal health technician or veterinarian).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
pGP-AAV-CAG-FLEX-jGCaMP7b-WPRE	Vigene Biosciences	Cat.# BS-12-CXBAAV9
AAV1-CamKII-GCaMP6f-WPRE	Penn Vector Core	N/A
AAV2-PHPeB-CaMKII-Cre-tdTomato	Canadian Neurophotonics Platform	RRID:SCR_016477
Experimental models: Organisms/strains		
Mice: VIP-IRES-Cre (homozygous for Vip<tm1(cre)Zjh>, 2–5 months old, males)	Jackson Laboratory	strain number: 010908
Mice: SST-IRES-Cre (homozygous for Sst<tm2.1(cre)Zjh>, 2–4 months old, males)	Jackson Laboratory	strain number: 013044
Mice: C57BL/6J (genotype: N/A, 2–4 months old, males)	Jackson Laboratory	strain number: 000664
Mice: B6.129P2-*Pvalb*$^{tm1(cre)Arbr}$/J (homozygous for Pvalb<tm1(cre)Arbr>, 2–4 months old, males)	Jackson Laboratory	strain number: 017320
Software and algorithms		
Igor Pro 8	WaveMetrics	https://www.waveme trics.com/products/ig orpro
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
Prism-GraphPad	GraphPad Software	https://www.graphpa d.com/features
ANY-maze	Stoelting Co.	https://www.anymaze.com/
TeleFipho software	Amuza Inc.	https://www.amuzainc.com/wireless-fiberphotometry/
Other		
Stereotaxic frame	RWD Life Science Co.	N/A
TeleFipho cannula	Amuza Inc.	N/A
Optic fiber cannula	RWD Life Science Co.	Cat. #R-FOC-BF400C39NA
Metabond cement (C&B)	Henry Schein Inc.	1861068
Gold Quick Catalyst	Patterson Dental Supply Inc.	S371
Lidocaine (2%)	Auro Pharma Inc.	DIN02462451
Bupivacaine hydrochloride injection USP	Aspen Pharmacare Canada Inc.	DIN01976141
Buprenorphine SR	Chiron Compounding Pharmacy Inc.	N/A
Lactated Ringer's solution	Baxter	JB2322
Meloxicam	Merck Animal Health	DIN 02452146
TeleFipho wireless fiber photometry system	Amuza Inc.	N/A
Digital USB 2.0 CMOS camera	Stoelting Co.	60516
Flaming/Brown micropipette puller	Sutter Instrument Co.	N/A
Nanoliter injector (Nanoliter 2000)	World Precision Instruments	N/A
ANY-maze BNC TTL I/O cable	Stoelting Co.	60058BNC
Ophthalmic ointment	Soothe	DIN 02125706
Isoflurane	Baxter	DIN 02188856
Baxedin pre-op	Omega Labs	DIN 01936441

STEP-BY-STEP METHOD DETAILS

Setup and calibration of fiber photometry system

Timing: Before beginning photometry acquisition with the TeleFipho setup, duration: 1–2 h

Note: TeleFipho transmitter headstage is equipped with adjustable switches to regulate offset and LED power levels. To identify the offset and LED power level settings suitable for achieving optimal signal, the device was calibrated. This subsection contains instructions for setting up and calibrating the fiber photometry system.

1. Install the software and required drivers before planning any testing or acquisition.
2. Connect the TeleFipho receiver to the computer and place it within the transmission range (2m) from the experimental/ testing setup.
3. Attach an optic cannula (TeleFipho cannula, core 400 mm, NA 0.39, cladding 425 mm, ferrule 2.5 mm) to the transmitter headstage.
   a. Fix the headstage above a fixed Leica sample at a distance of 500 μm.
   b. The headstage was attached to the stereotax with the help of a holder to fix it at a certain height from the fixed sample.

Note: The switches to adjust the offset and LED power can be rotated clockwise to increase the offset and power level. The switch can be rotated clockwise up to 270°. We divided this range of scale into 7 levels, with a scale interval level of 45°.

4. Switch off the room lights and ensure that no external light is incident on the headstage and the fixed sample setup.

Note: Calibration was conducted to find out the offset and LED power levels at which the photometry signal is not under or over-saturated.

5. Record the fiber photometry signal using the TeleFipho software at each of the seven LED power levels while keeping the offset level constant.
6. Repeat the same procedure for various offset levels while the LED power level is constant.
7. Extract the data from the software and plot the mean fluorescence values for all combinations of offset and LED power levels.[2]

We noted down the optimal offset and LED power level combinations i.e., the combinations where the fluorescence value was not close to either the low or high end of detection of the photosensor. This strategy was implemented to avoid choosing the setup where the photometry signal is under or oversaturated. The optimal range of fluorescence signal level recommended by Amuza is 30000–40000 arbitrary units.

Signal quality check and optimization of offset and LED power level

Timing: A few hours or a day before the start of the behavioral experiments, duration: 15 min

Note: The following procedure outlines the steps necessary for signal quality check and finding out the optimal settings for fiber photometry acquisition before real behavioral and imaging experiments. Before beginning behavioral experiments in animals with GECI virus injections and optic implants, it is essential to confirm the signal quality. The signal quality could be bad due to imprecise cannula implantation, inflammation near the implantation site, or inadequate expression of GECIs. These steps are conducted to find out the optimal offset

and power range settings for each animal. The optimal settings must be noted down and used for all imaging experiments.

8. Affix the transmitter headstage to the implanted cannula and place the animal in the homecage or a holding cage.
9. Make sure that the animal is within the 2m range of the TeleFipho receiver.
10. Adjust the offset levels – starting from the minimum to the maximum level.
11. Observe the signal quality and note down the fluorescence signal level from channel 1 in the TeleFipho software.

⚠ CRITICAL: It is important to standardize the procedure and details of the virus injection and cannula implantation prior to initiation of surgeries for fiber photometry recording. Check troubleshooting problem 1 for instructions on how to achieve good signal quality.

12. Adjust the LED power level if the optimal signal level cannot be achieved by adjusting the offset levels.

⚠ CRITICAL: In some cases, it is not possible to achieve the signal in the optimal range (30000–40000 arbitrary units) with the help of offset and LED power settings. See troubleshooting problem 2 to understand and pre-plan the modifications in the virus injections to avoid this issue.

13. Note down the settings for which the fluorescence level is in the optimal signal range.

⚠ CRITICAL: To avoid over-exposure of the tissue to the LED light and bleaching of the signal, it is recommended to avoid using the LED power at high levels.

Setup for synchronized wireless fiber photometry and behavioral recordings

⏲ Timing: Any time before the beginning of photometry acquisition during a behavioral test, duration: 20–30 min

Note: To understand the dynamics of neuronal population activity during a behavioral state or task, it is essential to synchronize the timing of fiber photometry signal with behavioral recording. To achieve this, the TeleFipho receiver and software used for behavioral recordings were synchronized.

Synchronization between photometry and video recording systems can be achieved by TTL (time to live) communication. We used the ANY-maze tracking system (Stoelting Co.) to record the mouse videos and send the TTL inputs to the TeleFipho receiver; however, experimenters can use any tracking software capable of sending or receiving TTL signals.

Preparation of ANY-maze protocol for TTL communication with the TeleFipho receiver

⏲ Timing: Any time before the experiments, duration: 10–15 min

ANY-maze and various other behavioral tracking software allow synchronization with fiber photometry recording software with the help of TTL communication. This subsection covers the procedure for creating a protocol for TTL communication of behavioral tracking software and fiber photometry software. Experimenters can also adapt these steps based on the requirements of the behavioral recording or fiber photometry software they work with.

14. Open a new file in the ANY-maze application. In the protocol section, choose the 'Video tracking mode with input/output' option.
15. In the 'Apparatus' window in the left column, click on 'I/O devices'. The input/output device can be added by following this path –Add item (top ribbon) > New I/O device.
16. Connect the USB to BNC cable (ANY-maze BNC TTL I/O cable, Stoelting Co.) to the 'IN' port of the TeleFipho receiver. The experimenter can rename the I/O device name in the I/O devices section if necessary.
17. The connected USB to BNC cable can be seen in the dropdown menu in the 'I/O devices' section. Click on this option.
18. Click on the 'Configure this device' button below to check or edit the configuration of the cable such as the assignment of input and output ports.

Note: If you need assistance to set up this device, use the link in the software in the 'I/O device' section.

19. Click on the 'On/off outputs' option in the 'INPUTS AND OUTPUTS' section in the left column. Then use the following steps to add a new output option –Add item (top ribbon) > New output item > New on/off output.
20. Click on the newly added output device in the left column under the 'On/off outputs' section.
21. Choose the appropriate cable name and port number in the 'Port to use' section, from the 'Port for the new apparatus' dropdown menu.

Note: The experimenter can achieve the TTL pulses to mark the beginning and end of the video either by sending a TTL signal at the beginning and end of the video or achieving a TTL step that starts at the initiation of the video recording and ends once the video recording has stopped. In ANY-maze software, the experimenter can specify the trial duration/ duration of the video recording in the 'stages' subsection under the 'Testing' section in the left column.

22. Use appropriate values of parameters like frequency, duty cycle, and duration of the output TTL pulse to achieve the TTL pulse/pulses that indicate the start and end of the trial/video recording.
23. Save the protocol file.

Hardware setup and validation of the synchronization

Timing: After preparation of protocol for TTL communication, duration: 10–15 min

Note: It is crucial to confirm the hardware setup between the fiber photometry system and the computer used for ANY-maze or other behavioral tracking software that allows TTL communication. Validation of synchronization before every experimental session minimizes the chance of misaligned data acquisition of fiber photometry and behavioral video recording. This subsection will help experimenters build the setup and ensure the synchronization between behavioral and fiber photometry recording software.

24. Connect the TeleFipho receiver to the computer with the power supply cable.
25. Connect the USB to BNC cable to the 'IN' port of the TeleFipho receiver.
26. Create a protocol in the ANY-maze software to send a TTL output at the time points when the video recording starts and ends. The duration of the TTL pulse can be defined in the ANY-maze software.
27. In the TeleFipho software, create a second channel (channel 2) that will display the TTL signal status.

Note: The second channel can be activated by following this path in the software – Setup > Channel Settings > choose 'Ch number' as 2.

28. To test the synchronization:
    a. Perform a photometry recording while the mouse is in the homecage or holding cage. This step can be conducted for a short duration (a few seconds or minutes).
    b. Extract the photometry data from the TeleFipho software as a 'TXT' type file. The exported file contains three columns of data with time, fluorescence values, and TTL values (from left to right).
    c. Transfer the data to an Excel file or any software of preference for data visualization.

Note: The data in column 3 will show the TTL value of '5.00001' at the start and end of the video recording. The TTL value 5.00001 marks the time points when the TTL signal was sent from ANY-maze software to the TeleFipho receiver. The experimenter can observe the trace of TTL values in 'channel 2' in the TeleFipho software while conducting the trial. If channel 2 shows a positive pulse (TTL pulse) when the video recording was started as well as at the end, the synchronization between the video recording software and photometry recording can be confirmed.

⚠ CRITICAL: If the time difference between the first and the second TTL pulse matches the duration of the video recording, the experimenter can conclude that the synchronization is correct.

Wireless fiber photometry recordings

Timing: After a 2-week recovery period post-surgery, duration: varies based on the behavioral paradigm

⚠ CRITICAL: Before the start of the fiber photometry recordings, mice should be habituated to the headstage attachment to the fiber cannula as well as carrying the headstage on their head for a few minutes. For this purpose, one week post-injection and fiber implantation, handle the mice and attach a dummy headstage on the implanted cannula. The dummy headstage weighs the same as the TeleFipho transmitter headstage and is about the same size. Affix the dummy headstage and place the mouse in their homecage for 5 min. It is important to perform all the handling procedures that will be conducted on the actual recording day to habituate the animals. Repeat this procedure once a day for 3 to 5 consecutive days before starting the real photometry recordings.

Note: After the handling/habituation of mice to the headstage, fiber photometry recordings can be started. The following steps should help experimenters perform fiber photometry recordings in rodents while they are subjected to a behavioral paradigm.

29. Bring the animals to the experimental room at least 30 min before the start of the photometry recording.
30. Check the connections to the TeleFipho receiver and TTL signal protocol in the ANY-maze or the software you are using for video recording.
31. Validate if the synchronization is working between the two software.
32. Place the behavioral arena within the 2m range of the TeleFipho receiver to allow uninterrupted communication between the headstage and the receiver.

⚠ CRITICAL: Make sure that there are no large metallic objects obstructing the space between the headstage and the receiver. See troubleshooting problem 3 for notes on precautions to avoid miscommunication errors in the signal.

33. Conduct all behavioral experiments in a room with uniform and subdued lighting. Ensure that there are no pronounced shadows within the arena.

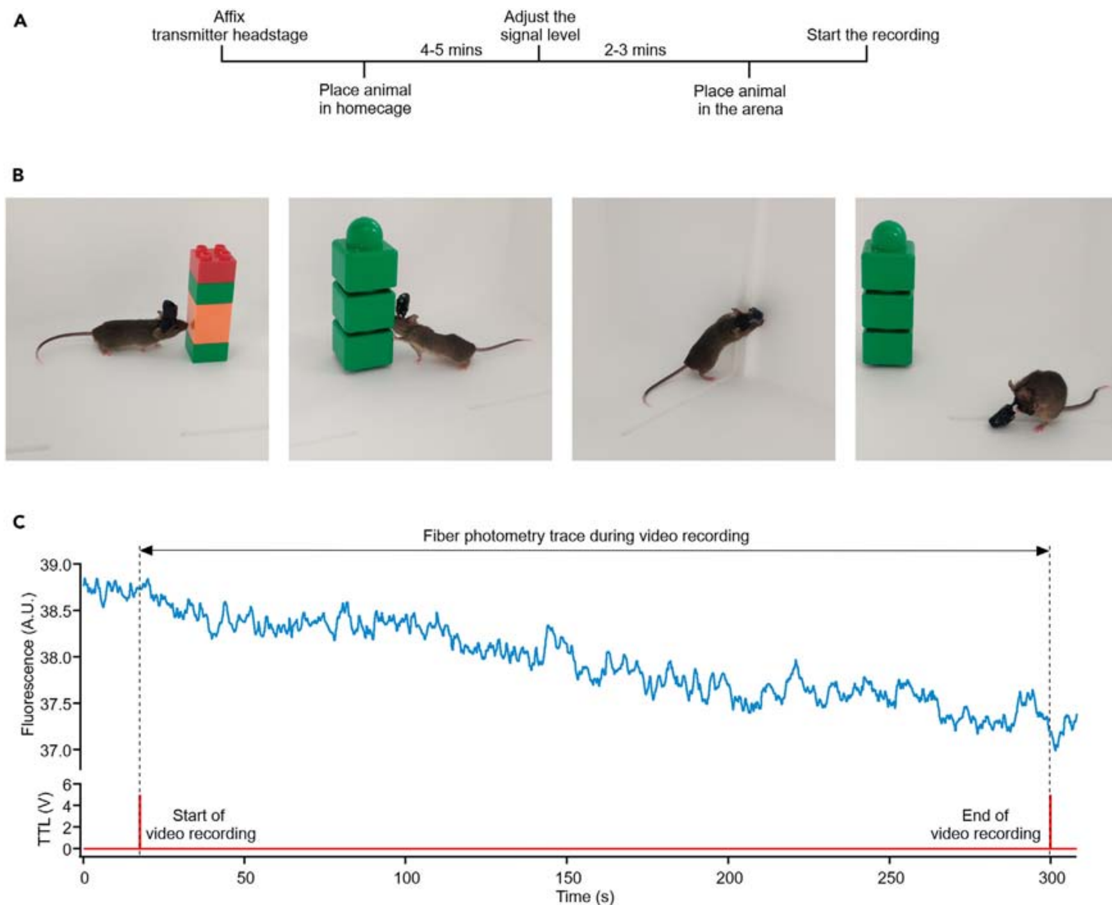

Figure 1. Acquisition of wireless fiber photometry signal and synchronization with video recording
(A) Schematic timeline of procedures on the day of fiber photometry recording.
(B) Images of a mouse with headstage transmitter approaching the object, exploring the object, rearing near the wall, and grooming (from left to right).
(C) Representative raw trace of fiber photometry recording (top) with the TTL trace showing pulses at the start and end of the video recording (bottom).

34. Attach and switch on the headstage on the implanted cannula following the same steps as performed during handling.

⚠ CRITICAL: It is crucial to fix the screw firmly when attaching the headstage to the cannula. A headstage that is loosely fitted can cause disruptions in the baseline of photometry recording, signal inconsistency, or potential removal of the headstage by the mouse during recording. Check troubleshooting problem 4 for suggestions on how to reduce the baseline changes and disturbances in the signal.

35. Place the mouse in the homecage or holding cage for 4–5 min. This allows the mouse to be more at ease (Figure 1A).
36. Observe the live photometry signal. Generally, the photometry signal shows relatively high photobleaching at the beginning. The wait period of a few minutes allows the photometry signal to stabilize. See troubleshooting problem 5 to reduce photobleaching in the photometry signal.
37. Observe the photometry signal and adjust the offset and LED power level using the settings obtained during 'signal check and optimization of offset and LED power level'.

Note: If optimal signal levels cannot be achieved using the previous settings, then adjust the parameters to obtain the signal in the optimal range and note down the new settings.

38. Start the fiber photometry recording after adjusting the offset and LED power level.
39. Place the mouse in the behavioral arena and start the video recording using the ANY-maze software.

Note: The start of the video recording can be automated in ANY-maze. If the experimenter chooses the option for the automatic start of the trial, the software will start the video recording as soon as it detects the mouse in the behavioral arena in the camera view.

40. Save the TeleFipho data file after the end of the recording.

Note: The small size and lightweight nature of the transmitter headstage allows us to acquire the fiber photometry signal while mice performed various spontaneous tasks. In Tamboli et al., 2024,[1] we examined the activity of the hippocampal CA1 VIP interneurons during various behavioral states like object exploration, rearing, and grooming (Figure 1B).

41. Check the presence of the TTL pulse in the TeleFipho software 'channel 2' at the start and the end of the video recording (Figure 1C).
42. Remove the animal from the arena and detach the headstage.
43. Place the plastic cap on the cannula to protect the cannula.

Calculation of *Z* score from the raw photometry signal

Timing: 5–10 min per mouse

Note: The following steps can be employed to convert the raw fiber photometry trace into a statistical measure that experimenters can use to compare and understand the neuronal activity at certain time points or behavioral states. In this procedure, the data from wireless fiber photometry is matched with the video recordings and the artifacts. The physiologically irrelevant changes in the activity baseline should be removed to reveal the real signal prior to its conversion to Z-score.

The code is written in MATLAB (MathWorks) where time series, fluorescence values (arbitrary units), and TTL values were used as input. In this case, these data series can be extracted from the TeleFipho software; however, the code process data from any recording software provided that the input data series follow the same format.

44. Open the pre-recorded fiber photometry file (h5 file) using the TeleFipho software, extract the data in 'TXT' format, and transfer the data to an Excel file.

Note: It is more convenient to paste the data of several trials of the same mouse or data of different mice in separate sheets in the same Excel file.

45. Import the data to MATLAB and align the fiber photometry data with the video recordings.
    a. Mention the number of sheets/trials to be processed from the Excel file (line 1).
    b. Adjust the code in line 2 based on the trial duration/ duration of the video recording from the ANY-maze software.
    c. Import the time, fluorescence, and TTL values from the Excel file (lines 3–5).

Note: To align the fiber photometry recording with the video recording, the recording before and after the TTL pulses needs to be neglected (see preparation of ANY-maze protocol for TTL communication with the TeleFipho receiver and hardware setup and validation of the synchronization). The lines below identify the first and the last TTL pulses. Further, it eliminates the fiber photometry data from before and after the video recording to achieve the alignment.

```
>Pulse_points=find(TTL==5.00001);
>first_TTL_pulse= Pulse_points(1);
>i=1:(first_TTL_pulse-1);
>TTL(i)=[];
>time(i)=[];
>F_value(i)=[];
>Pulse_points_new=find(TTL==5.00001);
>last_TTL_pulse= Pulse_points_new(length(Pulse_points_new));
>i=(last_TTL_pulse+1):(length(TTL));
>TTL(i)=[];
>time(i)=[];
>F_value(i)=[];
```

d. Adjust the length of the fiber photometry recording if it does not match with the video recording and export the corrected data to the Excel file (lines 32–40).

46. Elimination of artifacts from the photometry signal.
    a. Differentiate the fiber photometry signal (lines 47–48).

Note: The fiber photometry signal trace was differentiated to find out the instances where the rise or drop in signal is not physiological.

b. Define a threshold (lines 50–51) and a time window to detect artifacts.

Note: The threshold was set as 20 times the standard deviation away from the mean of the differentiated trace i.e., values above mean + 20*(std. dev (differentiated trace)) and values below mean - 20*(std. dev (differentiated trace)).

```
>upper_bound = DIF_average + (20*(Std_dev));
>lower_bound = DIF_average - (20*(Std_dev));
```

Note: The artifacts occur largely due to momentary miscommunication between the head-stage and the receiver. In these cases, the duration of the artifacts is less than 400 ms. A window was defined of 40 data points (approximately 400 ms) to detect the positive and negative artifacts in the data (line 42). Movement artifacts (if any) were shorter than the 400 ms time window. This value was set based on the observation of raw data from several mice and manual counting of the duration of the evident artifacts.

c. Detect and remove the data points that cross the threshold.

Note: With the help of the window, all the data points that do not lie within the threshold were removed (lines 53–203). The differentiated trace is plotted with the start and end points of the positive (values 20 times std. dev. above the mean) and negative (values 20 times std. dev. below the mean) artifacts highlighted for visualization and validation of the efficiency of the artifact removal (line 205).

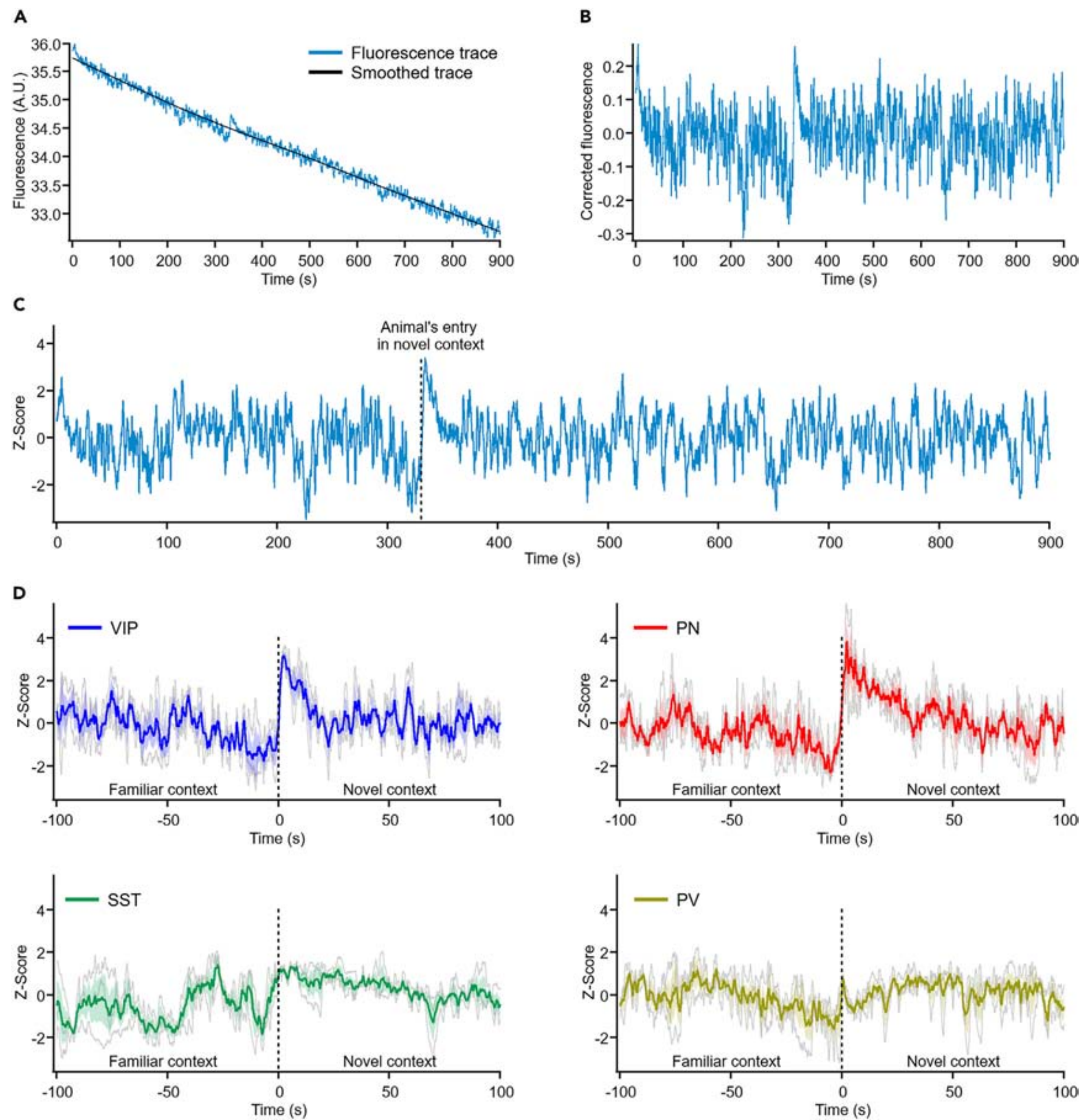

Figure 2. Fiber photometry signal during free exploration and analysis of the photometry signal

(A) Representative raw fiber photometry trace (blue) and the loess-smoothed trace (black).

(B) Corrected fiber photometry data after elimination of the effect of photobleaching from the raw fiber photometry data.

(C) Fiber photometry data presented as Z-score. The dotted line indicates the time at which the mouse entered a novel context.

(D) Photometry signals of vasoactive intestinal polypeptide-expressing (VIP), pyramidal neurons (PN), somatostatin- (SST), and parvalbumin-expressing (PV) presented as Z-score. The dotted line shows the animal's entry in a novel context. Data from individual mouse are shown in gray. Solid lines and shaded regions indicate mean and SEM, respectively.

47. De-trending the photometry signal when photobleaching is evident.
    a. Smooth the signal using Loess function[13] (Figure 2A).

Note: During fiber photometry, photobleaching can occur due to high light intensity and long light exposure along with parameters like fluorophore properties, photon-induced chemical reaction, etc. To remove the effect of the photobleaching from the data before data analysis and interpretation, the raw photometry trace (after the removal of artifacts) was smoothed using the Loess function of order one. After smoothing, the data was plotted to check the

precision of the fit using the Loess function (lines 208–217). Experimenters could also use one- or two-degree exponential or polynomial functions to choose the best fit for the trend in the signal due to bleaching.[13,14]

```
>%Loess smoothing of the 'F' trace
>F_smooth = smooth(time,F,0.5,'loess');
>figure(2)
>plot(time,F);
>hold on
>xlim([time(1),time(end)])
>xlabel('Time (s)')
>ylabel('F')
>plot (time,F_smooth,'m');
>exportgraphics(gcf, sprintf('Animal_%d_F & F-Smooth.png', q));
>close(gcf)
```

b. Eliminate the effect of photobleaching from the signal by subtracting the smoothed trace from the original trace (Figure 2B).
c. Calculate *Z* score (Figure 2C).

Note: The Z-score of the entire trace can be calculated using the mean and standard deviation of the corrected trace (i.e., the trace obtained by subtraction of the smoothed trace from the original trace) (lines 231–233).

```
>Mean_of_Fcorr = mean(F_corrected);
>S_deviation = std(F_corrected);
>% Formula for Z-score
>Z_score = (((F_corrected) - Mean_of_Fcorr )/S_deviation);
```

The Z-score values can be exported to the Excel file (lines 243–249) and used for further analysis and comparison of activity during certain time periods or behavioral states.

EXPECTED OUTCOMES

The protocol will guide the reader to synchronize the fiber photometry with the behavioral video recording and analyze the raw data. The representative signal traces obtained with the abovementioned protocol can be found in Figures 2C and 2D, also Figure 4 of Tamboli et al., 2024,[1] and Figure 4 of Amalyan et al., 2022.[2]

LIMITATIONS

The fiber photometry signal quality is dependent on precise cannula implantation and the optimal quantity of virus injection. Imprecise cannula location or injection volume leads to unsuccessful recordings and the mice cannot be used for experiments. This limitation can only be overcome by practice and standardization of virus injection and cannula implantation procedures, before the start of photometry recording of a new neuronal subtype. Further, the technique allows the

understanding of neuronal population activity but lacks information on single neuron activity dynamics and the diversity of the activity in the given neuronal population. One technical limitation of the TeleFipho fiber photometry system is that it can only perform recordings for approximately 2 h due to its limited battery size (when using 10% of the maximum LED power), unlike wired systems that can acquire data for several hours. While the wireless nature of the device reduces the chances of motion artifacts in the photometry data, the system does not acquire the isosbestic signal using 405 nm light. Additionally, the demanding surgical procedure and weight of the transmitter headstage (3 g) make it impractical to conduct photometry recordings in juvenile mice without affecting their natural behavior.

TROUBLESHOOTING

Problem 1

The fiber photometry signal is noisy, and the calcium transient amplitude range is small (related to - signal quality check and optimization of offset and LED power level, step 11).

The fiber photometry signal quality could depend on various factors. The signal quality may vary depending on the type of GECI used for photometry acquisition, the injection coordinates, the location of the fiber cannula, and the quality of the brain tissue near the imaging site. Here are a few solutions to reduce the chances of getting a noisy signal.

Potential solutions

- Weak expression of GECI can potentially lead to a small range of amplitude and noisy signal. Before beginning the fiber photometry recordings, standardize the coordinates and the volume of the virus injection.
- If using a low volume of virus, increase the wait period between the surgery and imaging sessions to allow more time for the expression of the GECIs.
- Imprecise implantation of the optic fiber cannula can deteriorate the signal quality. While setting up the coordinates of the cannula implantation, identify and note down the combinations of virus and cannula implantation coordinates that do not lead to noisy signals.
- During the implantation of the fiber cannula, lower the cannula gradually in the brain tissue, taking 4–5 min to ensure minimal tissue damage. If the cannula is lowered rapidly, there is a chance of damaging or pushing the brain which could result in inflammation or deformation of the tissue near the imaging site leading to noisy signal quality.

Problem 2

The optimal signal quality cannot be achieved with any offset or LED settings in some mice (related to - signal quality check and optimization of offset and LED power level, step 12).

In some mice, it is not possible to obtain fiber photometry signal in the optimal range (30000–40000 arbitrary units), when the type of neurons being imaged is present abundantly in the brain region of interest, for example, the pyramidal cells in the CA1 hippocampus. The signal level is oversaturated irrespective of the settings of the offset and LED power. In this situation, these mice cannot be used for imaging purposes.

Potential solution

- Identify the neuronal cell type in which the GECI expression could be very high and standardize the virus injection volume, so the expression is sparse near the imaging site.

Problem 3

Photometry signal jumps to oversaturated or drops down to below-detection level for a brief period, generally, about 200 ms (related to – wireless fiber photometry recordings, pt 32).

These sudden and step-like jumps/ drops in fiber photometry signals are caused by a momentary loss of communication between the TeleFipho receiver and headstage. The loss of communication could possibly lead to misalignment in the photometry recording and behavioral recording synchronization.

Potential solutions

- Ensure that the distance between the TeleFipho receiver and the headstage is less than 2 m. The chances of miscommunication increase as the distance increases.
- Avoid placing many objects, especially metallic objects between the headstage and the receiver to avoid miscommunication.
- The status of the headstage battery level also affects communication strength. Charge the headstage sufficiently before beginning any prolonged photometry recording.
- The jumps or drops in signal baseline due to loss of communication are easily noticeable and can be removed from the data with the help of the code. The code removes these events without disturbing the time data points for the rest of the data. After removing these events, the photometry data can be used to understand the neuronal population activity during various behavioral states.

Problem 4

The signal baseline may abruptly change during the recording (related to - wireless fiber photometry recordings, pt 34).

Often appearing as a sudden jump or drop that, in some cases, returns to the normal baseline after some time. The primary cause for these baseline shifts is a disturbance in the coupling between the headstage and the cannula. This can occur if the animal strikes the headstage against the walls of the behavioral arena during active exploration or if the headstage is not properly secured.

Potential solutions

- Before starting the imaging session, firmly secure the headstage onto the fiber cannula using the screw and double-check its fixation manually. The latest generation of headstage is equipped with screws on both sides, providing a better grip on the cannula and reducing the chances of baseline shifts due to micro-movements of the headstage.
- Ensure that the walls of the behavioral apparatus are smooth and, if possible, cover them with a material that is not too rigid. Reducing the impact force can help minimize the likelihood of baseline shifts during recording.

Problem 5

Strong photobleaching in the photometry signal (related to – wireless fiber photometry recordings, pt 36).

Potential solutions

- The effect of photobleaching seemed stronger in the few minutes after attaching the transmitter headstage to the implanted cannula. To lessen the effect of the photobleaching on the photometry recordings, place the animal in the homecage after attaching the headstage and wait for 4–5 min. The waiting period in the homecage allows the photometry signal to stabilize and helps mice to acclimatize to the headstage weight. Further, a study by Nakamura and colleagues suggests that the optical fiber should be pre-bleached by exposure of 20 μW light power for 15 min before the photometry recordings to reduce photobleaching during the recordings.[15]

- The effect of photobleaching on the fiber photometry signal can be variable from animal to animal. If the decline in signal due to photobleaching does not diminish after a few minutes, note down the details of the animal and examine the cannula location, and the protein expression level in the brain sections post-hoc. In case of imprecise cannula location or abnormal expression level, remove the animal from the analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lisa Topolnik (lisa.topolnik@bcm.ulaval.ca).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Suhel Tamboli (suhel.tamboli.1@ulaval.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The protocol includes all datasets analyzed during this study. The original code has been deposited at Zenodo (https://zenodo.org/records/10819265).

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AUTHOR CONTRIBUTIONS

Surgeries, S.T. and D.T.; data analysis, S.T. and R.R.; development of scripts for analysis, S.T.; writing, S.T., R.R., and D.T. with inputs from D.V.-L and L.T.; graphical abstract, D.T.; funding acquisition and supervision, L.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103407.

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