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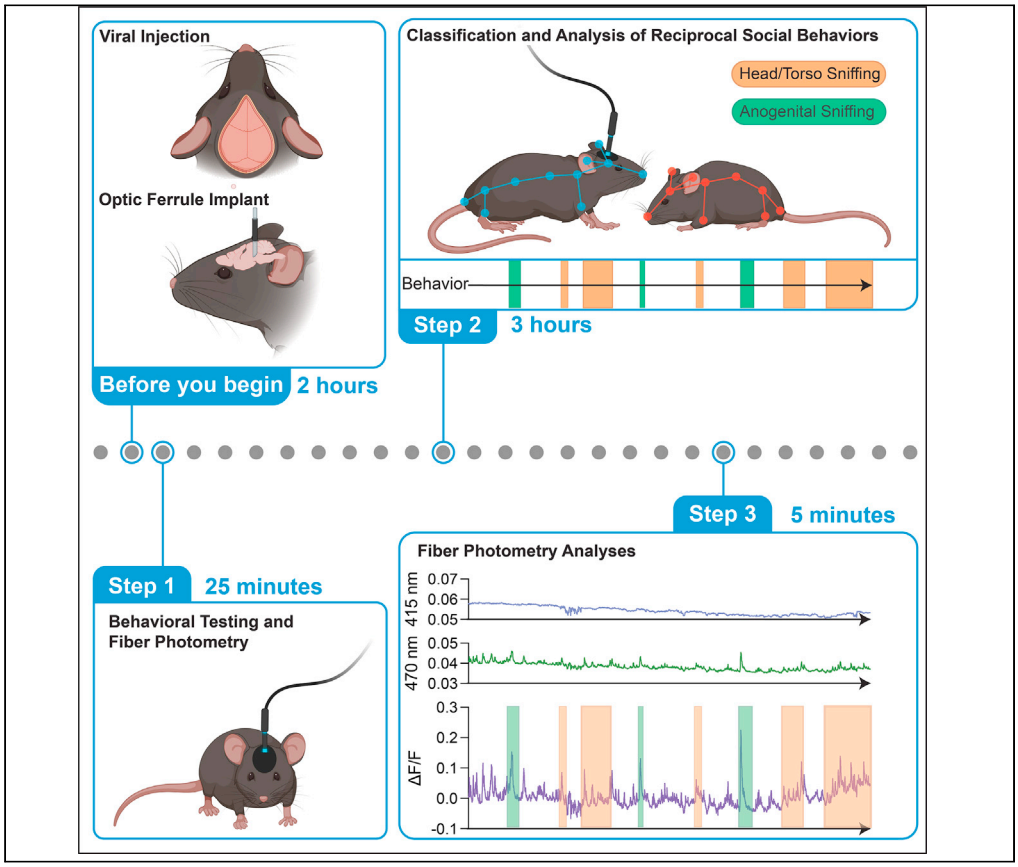
Protocol for the integration of fiber photometry and social behavior in rodent models



Fiber photometry offers insight into cell-type-specific activity underlying social interactions. We provide a protocol for the integration of fiber photometry recordings into the analysis of social behavior in rodent models. This includes considerations during surgery, notes on synchronizing fiber photometry with behavioral recordings, advice on using multi-animal behavioral tracking software, and scripts for the analysis of fiber photometry recordings.

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

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Highlights

Viral infusion and optic cannula implantation in the brain's region of interest

Acquisition of time-locked fiber photometry data and social interaction videos

Behavioral classification utilizing user-guided semi-automated analysis pipelines

Photometry and behavior alignment to study neuron activity during social behavior

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Protocol for the integration of fiber photometry and social behavior in rodent models

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SUMMARY

Fiber photometry offers insight into cell-type-specific activity underlying social interactions. We provide a protocol for the integration of fiber photometry recordings into the analysis of social behavior in rodent models. This includes considerations during surgery, notes on synchronizing fiber photometry with behavioral recordings, advice on using multi-animal behavioral tracking software, and scripts for the analysis of fiber photometry recordings.

For complete details on the use and execution of this protocol, please refer to Dawson et al. (2023).[1]

BEFORE YOU BEGIN

The protocol described below focuses on the integration of fiber photometry into social behavioral analyses. We used mice as the representative animal model, but each of the individual components of this pipeline has been applied to rat models. We used AAV9-CAG-flex-GcAMP6s.WRE.SV40 (Addgene viral prep # 100842-AAV9; 1.5 × 10^{13} vg/mL, 250 nL) in *Hcrt*$^{IRES\text{-}Cre}$ mice to record the activity of hypocretin/orexin neurons; however, these methods can also be applied using non-transgenic reporter mice and non-floxed viral constructs. To acquire fiber photometry recordings, we used a Doric Lenses fiber photometry system with an LED driver and console; however, we have also conducted comparable recordings and analyses using the FP3002 fiber photometry system from Neurophotometrics. We have no reason to believe that these approaches could not be modified to be compatible with any system which can synchronize to the behavior recording program via time-to-live pulse (TTL).

Institutional permissions

All experimental procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary. Readers will need to acquire permissions from their relevant institutions.

Stereotaxic surgery

Timing: 3 weeks prior to behavioral testing, duration: around 2 h

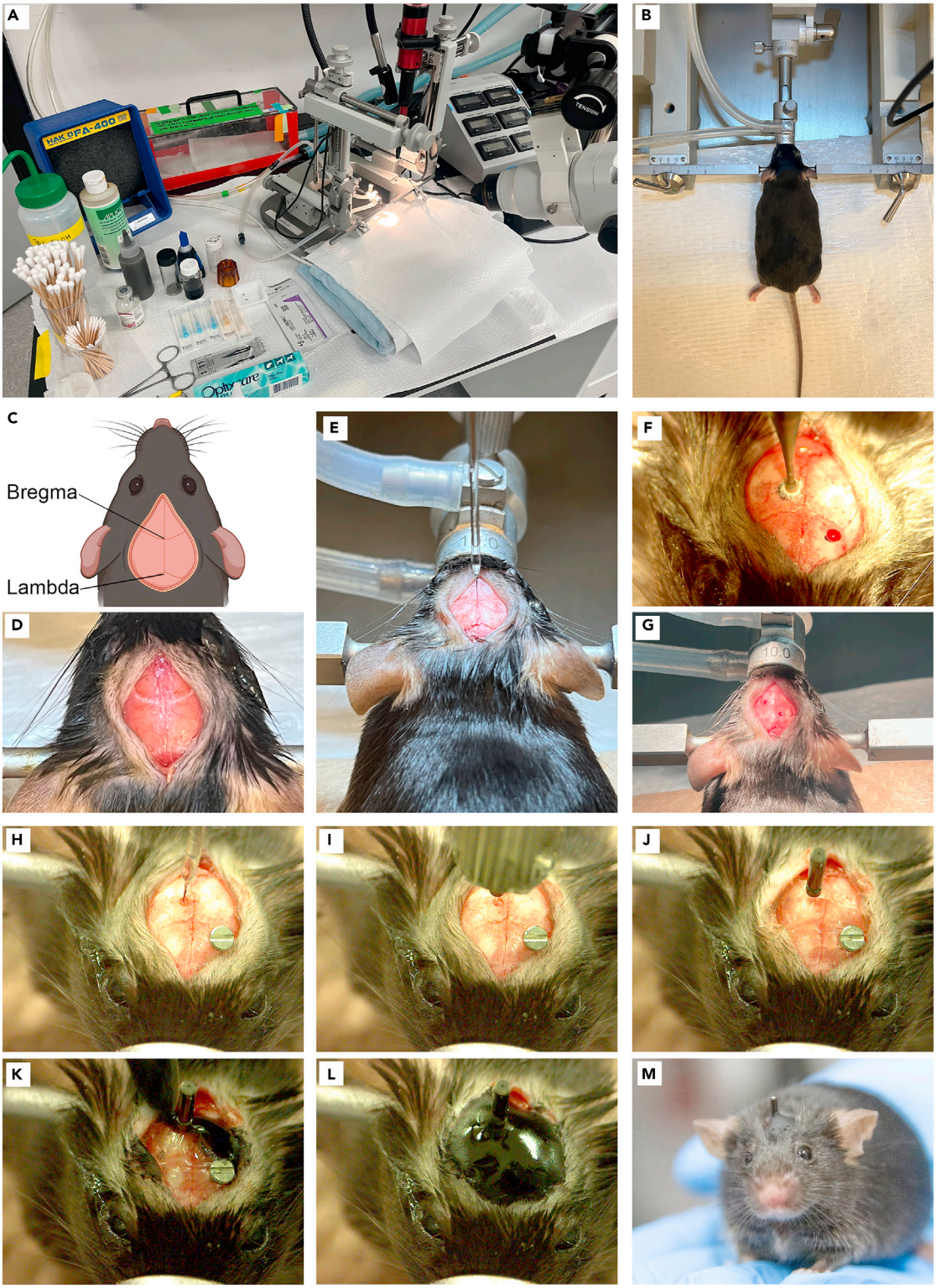

Figure 1. Stereotaxic surgery procedures for fiber photometry experiments

(A) Sterile and orderly surgical operating table with a stereotaxic frame.

(B) Mouse under anesthesia and securely placed in stereotaxic frame.

(C) Schematic illustrating bregma and lambda on the dorsal surface of a mouse skull.

Figure 1. *Continued*
(D) The dorsal surface of a mouse skull, cleaned to show bregma and lambda.
(E) Glass pipette with its tip set to bregma.
(F) The drilling of burr holes into the skull at the target coordinate. Note the additional burr hole on the contralateral side for a screw. See troubleshooting 1 for information on how the use of a support screw may help anchor a headcap to the skull.
(G) Surface of the skull is cleaned after drilling burr holes.
(H) Lower the glass pipette to the target and inject the virus. Note the optional screw for added headcap security, this was inserted prior to the injection of the virus.
(I) Fiber optic ferrule being held in place at the injection site.
(J) Super glue is used to attach the implanted fiber optic ferrule to the skull.
(K) A wide initial layer of dental cement is applied to the skull to form the base of a headcap.
(L) The dental cement headcap is gradually built-up to securely anchor the implanted fiber optic ferrule to the skull.
(M) Mouse with an implanted fiber optic ferrule, following recovery from surgical procedures.

These procedures outline the necessary steps for efficient and reliable stereotaxic viral infusion and optic fiber implantation. By the end of these procedures, experimenters should have mice which have been accurately infused with a fluorescent calcium indicator and implanted with a fiber optic ferrule. This ferrule should also be securely attached to the skull with a well-constructed dental cement headcap.

1. Cleave optic fiber(s) to appropriate length(s) for the desired target coordinate(s).

   Note: This step can be avoided by purchasing fibers which have been pre-cut to the appropriate length for the target coordinate(s). Alternatively, longer fibers can be cleaved by gently scoring the fiber at the desired length using either a carbide- or ruby-tipped scribe (e.g., Thorlabs S90C or S90R). Once scored, the excess length can be removed by flicking the fiber. During this process, it is important not to cut through the fiber with the scribe.

2. Prepare aliquots of viral constructs at a desired dilution.
3. Draw virus solution into the pulled glass micropipette inserted into a nanoinjector.
   a. For these experiments, we used a Nanoject III infusion system (Drummond Scientific).
4. Prepare a sterile and orderly operating table (Figure 1A).
5. Anesthetize the mouse initially at 5% isoflurane and maintain the surgical anesthetic plane with a constant flow of 1%–3% isoflurane.

   ⚠ CRITICAL: Closely monitor respiration rate and depth throughout all surgical procedures. Adjust isoflurane flow rate, if necessary, to maintain stable breathing under anesthesia.

6. Head-fix the mouse to the stereotaxic frame (Figure 1B).
7. Shave the scalp and sterilize the skin using alternating wipes with betadine and 70% isopropyl alcohol.
8. Administer lidocaine subcutaneously at the site where the incision will occur.
9. Open the skin with an incision running anterior to posterior along the center of the scalp to expose the skull surface.
   a. Clean the skull by lightly applying 3% hydrogen peroxide solution with a cotton-tipped applicator. Wash away any excess hydrogen peroxide solution with saline.
   b. Repeat step 9a until bregma and lambda are clearly visible (see Figure 1C for a graphical representation of these landmarks; Figure 1D for a picture of a mouse skull with these landmarks visible).
10. Using the fine point of the glass micropipette mounted to the stereotaxic frame, level the skull and calibrate the stereotaxic frame (Figure 1E).
    a. To ensure that the midline forms a straight line, assess the flatness of the skull in the stereotaxic. To do so, measure the medial-lateral coordinates of both bregma and lambda. Adjust the ear bars until these landmarks are aligned on the X axis (anterior-posterior).

b. To ensure that the ear bars and nose bar are at the correct vertical positions, assess the pitch of the skull. To do so, measure the dorsal-ventral coordinates of bregma and lambda. Adjust the vertical position of the nose bar until these landmarks are at the same depth.
c. To ensure that the skull does not tilt to the left or right, assess the roll of the skull. To do so, measure the dorsal-ventral coordinates of points 2 mm on either side of the midline between bregma and lambda. Adjust the tilt of the animal within the stereotaxic frame until these two points are aligned at the same depth.

⚠ CRITICAL: The accuracy of stereotaxic targeting depends on the proper leveling of the skull within the stereotaxic frame. The more care that is taken during this step, the more consistent and accurate the end targeting will be.

11. Prepare burr holes according to the experimental coordinates (Figure 1F).
12. Using sterile saline, clear the surface of the skull of any traces of blood, bone fragments, or debris from the drilling process (Figure 1G).
13. Lower the micropipette to the target depth and inject the virus solution (Figure 1H).
    a. For these experiments, we infused a total of 250 nL virus at a rate of 10 nL/s. Adjust the volume and rate according to the target brain region and the experimental animal species being studied.

Note: Generally, most photometry experiments involving mice use target volumes between 100 nL and 500 nL and inject at flow rates between 0.5 nL/s and 30 nL/s.

⚠ CRITICAL: After the injection, the micropipette should remain untouched at the target coordinates for 10 min to allow for virus diffusion. After this period, the needle should be slowly retracted from the tissue.

14. Load the optical fiber into a stereotaxic holder and calibrate its position to the stereotaxic setup by placing the tip of the fiber to bregma.
15. Implant the optic fiber 0.1 mm above the virus injection depth (Figure 1I).

⚠ CRITICAL: Ensure that the skull is clean following this implantation prior to constructing a headcap. Any blood on the skull at this point will interfere with the adhesion of the dental cement and compromise the integrity of the headcap.

16. Seal the implanted components and skull surface with dental cement.
    a. Apply superglue around the base of the optic fiber (Figure 1J).
    b. Gradually build a wide dental cement headcap to further anchor the optic fiber in place. The wider the footprint of this headcap, the more securely it will remain attached to the skull (Figure 1K,L).
    c. Wait for dental cement to have fully cured before proceeding.

⚠ CRITICAL: It is important that headcaps are built securely. See troubleshooting 1 for notes on additional precautions which can be taken to improve the adherence of headcaps to the skull.

17. Suture the wound.
18. Administer post-operative analgesic.
19. Turn off anesthesia and allow the mouse to recover on a heat pad in a clean cage for 30 min (Figure 1M).

⚠ CRITICAL: Administer appropriate post-operative support as approved by relevant institutions.

Note: It is highly recommended that users test the dynamic range of the fiber photometry signal, as well as its responsivity to external stimuli, during an initial signal test prior to starting behavioral procedures. See troubleshooting 2 for notes on what small signal dynamic range and poor responsivity to stimuli might indicate.

Pause point: Allow the research animal at least 2 weeks to recover from the surgical procedures before any further experiments.

Setting up software for fiber photometry and behavioral recordings

Timing: any time prior to behavioral testing

These procedures outline the necessary steps for setting up the software which will support the acquisition of photometry and behavioral recordings. Following these procedures, experimenters should be familiar with the software that supports the photometry system of their choosing.

20. Install appropriate drivers for operating the fiber photometry system and familiarize yourself with their operation manuals.
    a. If using a photometry system from Doric Lenses, these instructions and documents are available at the following link: https://www.doriclenses.com/downloads/ApplicationNotes/Fiber_Photometry_System_Getting_Started_Guide_v2.2.1.pdf.
    b. If using a photometry system from Neurophotometrics, these instructions and documents are available at the following link: https://static1.squarespace.com/static/60ff345fca665d50e1adc805/t/645405fc34c26c309dce5c6f/1683228157523/Manual-Hardware_20230504.pdf.
21. Install video acquisition software.
    a. Here, we used ANY-maze from https://www.any-maze.com. Alternatively, any video recording system with the capability to start recording upon receiving a TTL input or the capability to produce a TTL output trigger synchronized with the start of the recording could be used.

Setting up hardware for fiber photometry and behavioral recordings

Timing: 1+ h prior to behavioral testing

These procedures outline the necessary steps for setting up the hardware which will allow for the fiber photometry system to integrate with the system responsible for recording videos of the behavioral procedures. Following these procedures, experimenters should have means of TTL synchronization between video and fiber photometry recordings.

22. Time-to-live (TTL) synchronization.
    a. If using the Doric Lenses fiber photometry system, the console needs to be configured to send a TTL pulse which will trigger ANY-maze to start recording video overhead footage of the behavioral test. This ensures that video footage is time locked to the fiber photometry recording.
    b. If using the Neurophotometrics fiber photometry system, the most convenient way to integrate these signals is to send a TTL pulse from ANY-maze upon starting the video recording to the fiber photometry console.
    c. Either of these TTL synchronization steps requires an ANY-maze AMi-2 Digital interface (Cat# 60064).

Note: Individual data frames from both the behavioral recordings and the fiber photometry recordings will be aligned on a frame-by-frame basis during later photometry processing using nearest neighbor approximation, which will align frames based on their timestamps. Therefore, synchronization only requires the behavior and fiber photometry recordings to

be started together in a time-locked manner, such as the TTL synchronization used in this protocol.

23. Bleach optical patch cord with high intensity light (e.g., 10 mW) for at least 1 h prior to the start of behavioral testing.
24. After the optical patch cord has been bleached, calibrate the light power of each channel of the fiber photometry recording to deliver 30 μW light at the tip of the fiber using a photometer.
    a. For these experiments, a 470 nm LED was used to record the activity of the GCaMP signal.
    b. A second channel should be set to the isosbestic wavelength of 415 nm.
25. Select a recording frame rate which is appropriate for the fluorescent indicator being used.

Note: The selected frame rate should be faster than the rise time of the sensor to ensure that all real changes in the sensor are able to be captured in the fiber photometry signal. The GCaMP6s which was used in these experiments has a rise time of approximately 100–150 ms; therefore, with the frame rate of the fiber photometry system set to 120 fps we are able to fully capture the dynamics of this signal.[2]

⚠ CRITICAL: It is important to understand how the fiber photometry system handles recordings with multiple wavelengths. Rather than turning on each light source simultaneously, many systems, including those from Doric Lenses and Neurophotometrics, record from different channels sequentially and then interleave the results. In some systems, this may alter the effective framerate of the recording. Be mindful of how the system that you are using handles these scenarios and adjust the framerate accordingly. See troubleshooting 3 for a discussion on the importance of the framerate of the fiber photometry recording matching or exceeding that of the behavioral data within the context of these analyses.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mice: *Hcrt*$^{IRES\text{-}Cre}$ (females and males, 10–16 weeks old)	Dr. Gina Leinninger	N/A
Mice: *FlpO-Deleter*; *B6.129S4-Gt(ROSA)26Sor*$^{tm2(Flp*)Sor}$*/J* (females and males, 10–16 weeks old)	Jackson Laboratory	#012930
Mice: C57BL/6J (females and males, 10–16 weeks old)	Jackson Laboratory	#000664
Recombinant DNA		
AAV9-CAG-flex-GcAMP6s.WRE.SV40	Addgene	#100842-AAV9
Software and algorithms		
MATLAB	MathWorks	R2020a; https://matlab.mathworks.com/
ANY-maze	Stoelting Co.	https://www.any-maze.com
DeepLabCut	Lauer et al.[3]	http://www.mackenziemathislab.org/deeplabcut
SimBA	Nilsson et al.[4]	https://goldenneurolab.com/simba
Fiber photometry scripts	Dawson et al.[1]	GitHub: DOI: https://doi.org/10.5281/zenodo.8408555
Other		
USB video camera	Imaging Source	DMK 22AUC03
Doric Lenses basic fiber photometry system	Doric Lenses	FPS_S_GCaMP
Mono fiber-optic cannulae (400 μm core diameter)	Neurophotometrics	N/A
Carbide or ruby-tipped scribe	Thorlabs	S90C or S90R
Mono fiber-optic patch cord	Doric Lenses	MFP_200/220/900-0.37_2m_FCM-MF1.25
White ceramic split sleeve	Neurophotometrics	N/A
Mini cube filter set	Doric Lenses	iIFMC4-G3_IE(400-410)_E(460-490)_F(500-550)_S
Visible femtowatt photoreceiver module	Newport Co.	Model 2151
Photometer	Thorlabs	PM120D
ANY-maze AMi-2 digital interface	ANY-maze	60064

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nanoject III	Drummond Scientific	3-000-207
Stereotaxic head frame	Kopf Instruments	Model 940
Drill		
007 Drill bit	Meisinger	1-007-HP
Contemporary Ortho-Jet powder, dental cement powder	Lang Dental	N/A
Contemporary Ortho-Jet liquid, dental cement liquid	Lang Dental	N/A
Anesthesia system		
Surgical microscope		
Deposited data		
Custom MATLAB analysis for the integration of SimBA behavioral data with fiber photometry recordings	Dawson et al., 2023[1]	GitHub: DOI: https://doi.org/10.5281/zenodo.8408555

STEP-BY-STEP METHOD DETAILS

Fiber photometry recordings during a reciprocal social interaction test

Timing: 25 min per mouse

These procedures outline the necessary steps for the acquisition of fiber photometry data during a reciprocal social interaction test. By the end of these procedures, experimenters should have fiber photometry data which has been time-locked to video recordings of the behavioral tests.

1. Conduct all experiments in a dimly lit room. See Figure 2A for a schematic outlining of the behavioral procedures.
   a. These experiments were conducted under 50 lux lighting conditions.

   ⚠ CRITICAL: To minimize the potential impact of external lighting conditions on the photometry recordings, as well as on rodent anxiety during behavioral testing, tests should be conducted in a relatively dark setting.

2. Remove the appropriate experimental mouse from its home cage along with its familiar cage mate.
   a. For these experiments, mice were pair housed.
3. Clean the exposed end of the implanted fiber and then securely attach this fiber to the patch cord using a ceramic sleeve.
4. Return the experimental mouse to the home cage and start both the fiber photometry and behavioral recordings.
5. Allow the mouse 5 min (00:00–05:00) in the home cage on its own.

   Note: This recording serves as the first baseline recording.

6. After this first 5-min baseline recording, return the familiar cage mate to the cage and allow it to reciprocally interact with the experimental mouse for 5 min (05:01–10:00).

   Note: This period serves as the familiar interaction period.

7. After the familiar interaction period, remove the cage mate and leave the experimental mouse alone in the home cage for a second 5-min baseline recording period (10:01–15:00).
8. Introduce an unfamiliar, non-cage mate, stranger (intruder) mouse into the home cage with the experimental mouse for 5 min (15:01–20:00).

   Note: This period serves as the stranger interaction period.

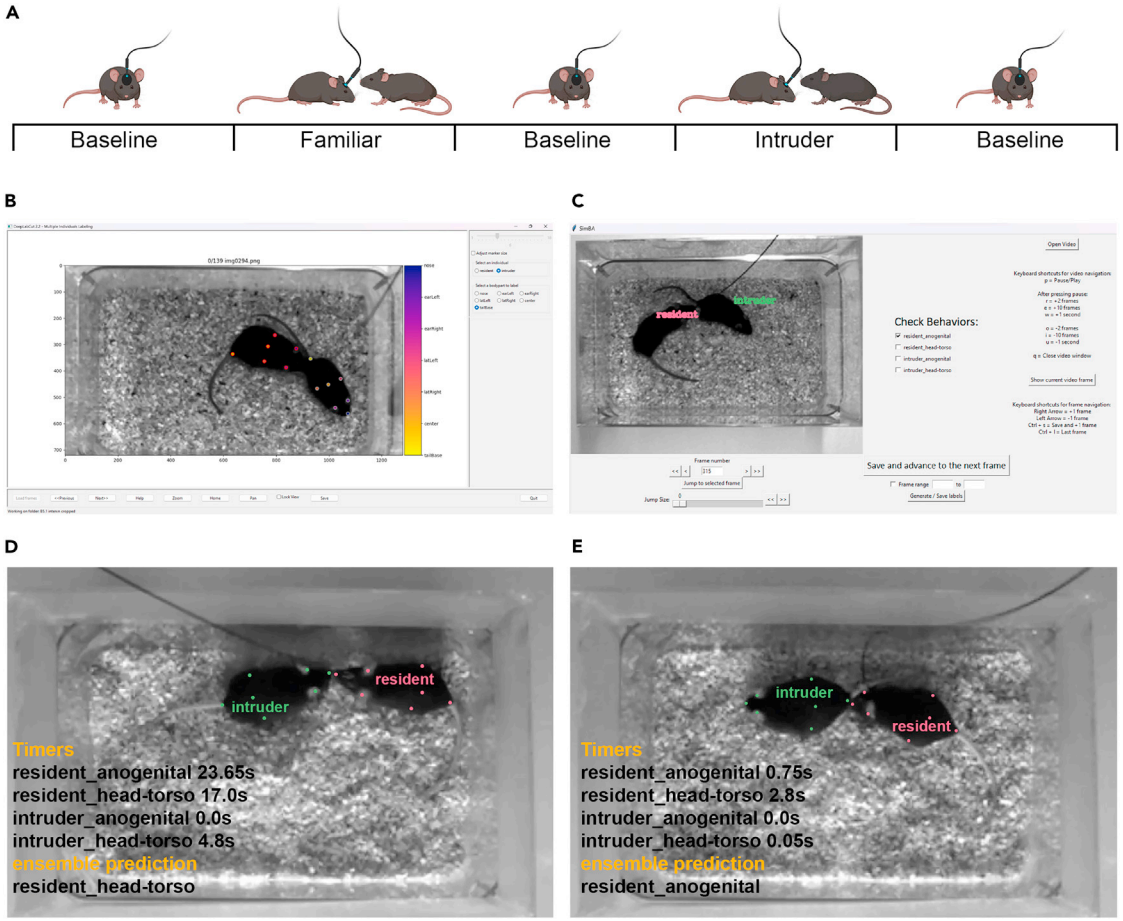

Figure 2. Classification and Analysis of Reciprocal Social Behaviors

(A) Schematic outlining the behavioral procedures.

(B) DeepLabCut graphical user interface during the annotation of training frames.

(C) SimBA graphical user interface during the validation of behaviors classified by the neural network.

(D) Example of the resident mouse engaging in head-torso sniffing behavior, as predicted by DeepLabCut and SimBA.

(E) Example of the resident mouse engaging in anogenital sniffing behavior, as predicted by DeepLabCut and SimBA.

Note: If the order of presentation of familiar and unfamiliar mice is counterbalanced during these procedures, this information should be incorporated during Process 02. Initialization – Subject Information in the MATLAB workflow during subsequent analysis of the data.

⚠ CRITICAL: Patch cord management is critical during interaction periods, as the patch cord can become tangled with increased locomotor activity. A tangled patch cord may compromise the quality of the photometry signal. Furthermore, if the patch cord is too slack in the cage, the stranger (intruder) mouse may be able to damage the cord. However, the use of rotary joints is not advisable during fiber photometry since they can introduce noise or a reduction in light transmission. One strategy that is particularly useful for effective patch cord management is draping the cord over a support located above the cage, which allows the cord to hang straight down towards the testing apparatus. As behavioral recordings across many tests, including these reciprocal social interaction tests, are gathered from an overhead camera, the support holding the camera in place can also serve as a suitable support for the patch cord. Manually feeding patch cord over this support towards the cage during the experiment while the animal is moving allows experimenters to ensure that cables do not become taut to the point of restricting behavior. Manually guiding

excessively slack patch cord back over the support away from the cage can help reduce the chances of fibers becoming tangled. Alternatively, the application of heat shrink tubing over the patch cord can help provide rigidity and reduce the likelihood of tangling.

9. Following the stranger interaction period, remove the stranger mouse and leave the experimental mouse alone in the home cage for one final 5-min baseline recording before the behavioral testing is complete and the photometry recording is turned off (20:01–25:00).
10. Detach the patch cord from the implanted fiber, being careful not to dislodge the headcap.

Behavioral classification – DeepLabCut and SimBA

Timing: 2–3 h, dependent on video quality

These procedures outline the necessary steps required for behavioral analysis during the reciprocal social interaction test. By the end of these procedures, experimenters should have data outlining the nature of these behavioral interactions presented as a time series.

The processes outlined in this section have been described in full in the original articles for DeepLabCut[3,5–7] and SimBA.[4] These markerless pose estimation and behavioral classification tools are versatile and can be tailored to a wide variety of experimental designs. The protocol for using these tools for unbiased, high-precision identification of head/torso and anogenital sniffing interactions has been outlined below.

Further information on how to use these tools can be found in the detailed guides linked below.

ANY-maze: https://www/any-maze.com/support/guides/

DeepLabCut: http://www.mackenziemathislab.org/deeplabcut.

SimBA: https://github.com/sgoldenlab/simba

⚠ CRITICAL: While these tools are very powerful in behavioral analyses, they each rely on high quality data input and careful training. Inconsistencies in data input quality and pose/behavior classification will introduce considerable variability in the accuracy of these results. It is recommended that users keep these input variables as consistent as possible. Furthermore, it is advised that users randomly sample their outputs at various stages and compare the accuracy of the pose estimation and behavioral classification to manual scoring. Spending extra time during these steps will help yield the most accurate behavioral classification possible.

11. Positional tracking and pose estimation with DeepLabCut.

Note: In this step, video files recorded in ANY-maze are exported and multi-animal tracking is conducted using a supervised-learning deep neural network. By the end of this step, users will have positional tracking data for each animal (experimental, familiar, and stranger) presented during the behavioral task (Figure 2B).

a. Export video files from ANY-maze as an MP4 format which can be analyzed using DeepLabCut.

Note: For the current experiment, the framerate of these videos was 20 frames per second.

b. Import videos into DeepLabCut, where a subset of videos are used to label body parts of the animals that the user intends to track.

Note: In the current protocol, these body parts are the nose, left and right ears, left and right latissimus dorsi, tail base, and center of the torso of each animal present in a user-selected subset of frames.

Note: This labelling will be used to train the neural network which will track mice in all videos. Therefore, it is important that the user-selected frames capture a wide range of the possible forms of interactions which can occur during these tests.

c. After this initial training, evaluate the results of the pose estimation.

Note: If these results are unsatisfactory, the user may choose to include more training frames and/or refine existing labels. These steps should be repeated until pose estimation is in line with manual scoring. In this case, manual scoring consists of the user manually labelling body parts and comparing with their position with estimations generated by DeepLabCut. Pose estimations are considered to be in line with manual scoring when the neural network consistently labels these body parts correctly.

d. Once satisfied with the quality of these results, process all videos using the trained neural network.

Note: To further ensure accurate tracking, the data from a subset of novel videos which were not included in the training dataset should be compared to manually scored results prior continuing to SimBA processing.

12. Predictive behavioral classification with SimBA.

Note: In this step, pose estimations and positional tracking data from DeepLabCut is classified into complex behaviors of interest using a supervised-learning deep neural network. By the end of this step, users will have data outlining the nature of each social interaction (in this case, head-torso and anogenital sniffing) performed during the behavioral test (Figures 2C–2E).

a. Import pose estimation data obtained from DeepLabCut.

Note: With these data, it is important to ensure that the framerate and the pixel size are properly calibrated. This can become an issue if the camera or behavioral apparatus is moved during the testing procedures. This further emphasizes the importance of minimizing variability in the experimental setup during data acquisition.

b. Manually annotate behaviors of interest in a subset of videos to build the behavioral classifier network.

Note: After annotating a subset of videos for the behaviors of interest, it is recommended that users evaluate the results of the classification. SimBA offers several tools to assist in this evaluation, including learning curves and means to visualize classification thresholds. If these results are unsatisfactory, the user may choose to include more training data and/or refine existing annotations. These processes should be repeated until behavioral classification is in line with manual scoring.

c. Once the experimenter is satisfied with the quality of the behavioral classification, all pose estimate data are analyzed using the trained neural network.

Note: To further ensure accurate classification, data from a subset of novel sessions which were not included in the training dataset should be compared to manually scored results.

Integrating behavioral classifications into fiber photometry analyses

Timing: 5 min per mouse

These procedures outline the necessary steps for integrating behavioral classifications obtained from DeepLabCut and SimBA with fiber photometry data. Following these procedures, experimenters should have information on neuronal population activity during specific social interaction types and fiber photometry and behavioral data which have been synchronized on a frame-by-frame basis for further analyses of their choosing.

During this step, the data from the behavioral classification is aligned with processed fiber photometry data to enable the analysis of fiber photometry data during specific behavioral epochs. This step requires MATLAB and the SarginFP_SimBA_v163.m file. This file, along with the steps outlined below, correspond with the data obtained using a Doric Lenses fiber photometry system. The organization of these inputs is different when using data from a Neurophotometrics fiber photometry system; however, the calculations are still similar. For users who would like to conduct these analyses using data collected from a Neurophotometrics fiber photometry system, a separate MATLAB file, SarginFP_SimBA_v163_NP.m, has been included in the supplementary materials of this manuscript.

Whether using the Doric Lenses or the Neurophotometrics fiber photometry analysis script, it is recommended that end users read through the documentation in the first section of the file. This section outlines required inputs, the conducted analyses, operational definitions, and any assumptions that the code makes during data analysis.

Note: It is recommended that users run through these analyses one process at a time using the ''Run Section'' command in MATLAB. Data can be saved and reloaded at any time using the save (lines 946–952) and load (lines 953–965) processes.

13. Complete process 01. Initialization – Group Information in the MATLAB workflow.

Note: In this process, the general parameters for the photometry analysis are defined. This section contains several editable variables which can be tailored to meet experimental needs. These variables are outlined below.

a. State the number of groups in the current experiment (line 171).

Note: This value is numeric. In the example below, there are two groups.

```
FP.info.groupnum = 2;
```

b. List the titles by which groups will be identified (line 173).

Note: Variables in this line are listed as character vectors, with each unique identifier written within quotation marks and the full list of variables written within square brackets and without commas separating entries. In the example below, the two groups are control and manipulation.

```
FP.info.groups = [''control'' ''manipulation''];
```

c. List the number of animals per group (line 175).

Note: This value is numeric. The number of animals should be presented in the same order as the identifiers were presented in the previous step, without commas separating entries, and with the full list of variables written within square brackets. Using the groups from step 13b, the example below shows 10 animals in the control condition and 11 animals in the manipulation condition.

```
FP.info.pergroup = [10 11];
```

d. Adjust the flag to indicate whether data is to be presented as a *Z* score or not (line 177).

Note: In this line, if the numeric variable is set to 0, the analyses will proceed without presenting the data as a *Z* score. If the numeric variable is set to 1, all analyses will be conducted on *Z* score ΔF/F data. In the below example, photometry traces will not be analyzed as *Z* scores.

```
FP.info.zscore = 0;
```

e. Adjust the flag to indicate whether data is to be presented as %ΔF/F or not (line 179).

Note: In this line, if the numeric variable is set to 0, the analyses will proceed without presenting the data as a percentage of the maximum ΔF/F for each given trace. If this variable is set to 1, analyses will proceed with subsequent analyses based on the ΔF/F trace as a percentage of the maximum ΔF/F values. In the below example, photometry traces will not be analyzed as %ΔF/F.

```
FP.info.percentdFF = 0;
```

Note: These functions for outputting data as *Z* score and %ΔF/F are not mutually exclusive. If both variables are set to 1, the data will be presented as a percentage of the maximum *Z* scored ΔF/F values. If both variables are set to 0, the data will be presented as ΔF/F without further processing.

f. Define the desired rolling window size for local peak detection (line 183).

Note: In this line, a rolling window is defined for subsequent local peak detection analyses. This numeric variable will be interpreted as seconds. In the below example, a rolling window of 30 s was used for local peak detection.

```
FP.info.windowtime = 30;
```

14. Complete process 02. Initialization – Subject Information in the MATLAB workflow.

Note: In this process, the user will follow the prompts in the command window and navigate to the appropriate fiber photometry and SimBA output files. This will initialize storage vectors for these data and perform all loading sequences. The user will also manually define whether the first interaction occurred with a familiar or an intruder mouse in response to a prompt in the MATLAB command window, which allows for counterbalancing of intruder/familiar presentation order. Several variables within this process will need to be modified depending on the

frame rate of the photometry recording and the duration of each test segment. These variables are described below.

a. Within the internal initialization subsection (lines 201–227), adjust the number of frames according to the times of each portion of the test and the frame rate of the fiber photometry recording.

Note: In the example below, the number of frames in the array was set to 180,705, to represent a framerate of 120 frames per second over the course of the full 25-min task and a buffer period at the end of the recording.

```
FP.inputs.doric.time.(FP.info.groups(ii))=zeros(180705,FP.info.pergroup(ii));
```

Note: This value represents the full length of the fiber photometry recording. If the length of this recording varies from mouse to mouse an error will occur. See troubleshooting 4 for potential workarounds when working with fiber photometry recordings with variable number of frames.

b. Adjust the number of frames to be allocated with the different segments of the behavioral task.

Note: In the below example, the segment of the test during which familiar interactions could occur was 5 min. Therefore, at a framerate of 120 frames per second, this period is a total of 36,000 frames, as shown in the example below.

```
FP.inputs.familiar.headtorso.(FP.info.groups(ii))=zeros(36000,FP.info.pergroup(ii));
```

c. Adjust the following lines to match the number of frames in the photometry array (from the above example, 180,705 frames): 239, 240, 265–267.
d. Adjust the following lines to match the number of frames in the behavioral array (from the above example, 36,000 frames): 260, 261, 269–272.
e. Adjust the column identifiers for their behaviors of interest, as needed.

Note: In the current study, the behaviors of interest were head-torso and anogenital sniffing, which were in columns 501 and 499 of the behavioral files respectively. The lines pertaining to these columns are as follows: 269–272.

15. Complete process 03. Photometry Processing – Behavior Independent in the MATLAB workflow. During this step the ΔF/F trace is generated from the raw photometry data.
    a. Define a baseline period (line 323).

Note: In this step, the user defines a baseline period which will be used for baseline adjustments to the ΔF/F trace. This variable is presented as a pair of numeric values, with the first representing the time at which the user-defined baseline period is to start and the second being the duration of this period. Both of these values have the unit of seconds. In the example below, the baseline period starts at 60 s and continues for 180 s until the 240th second of the recording.

```
FP.info.baseline = [60 180];
```

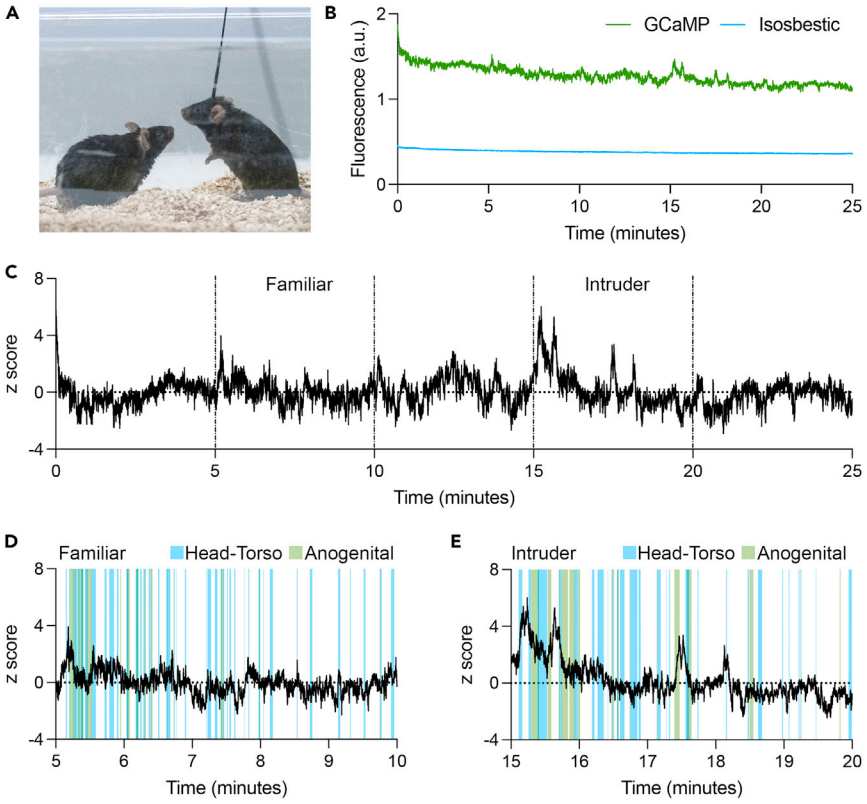

Figure 3. Fiber Photometry Analyses

(A) Representative image showing mice socially interacting during the acquisition of fiber photometry recordings.
(B) Raw fluorescence data from GCaMP (green) and isosbestic (blue) channels during the reciprocal social behavior task.
(C) Processed GCaMP fluorescence during the reciprocal social behavior task, presented as *Z* score ΔF/F.
(D) Processed GCaMP fluorescence, presented as *Z* score ΔF/F, from an experimental mouse during interaction with a familiar mouse. Bouts of head-torso sniffing are depicted in blue, while anogenital sniffing is depicted in green.
(E) Processed GCaMP fluorescence, presented as *Z* score ΔF/F, from an experimental mouse during interaction with an unfamiliar intruder mouse. Bouts of head-torso sniffing are depicted in blue, while anogenital sniffing is depicted in green.

⚠ CRITICAL: It is important to consider handling stress when defining a baseline period. When animals are initially placed into a testing environment, it is not uncommon for most fiber photometry signals to be elevated drastically above their normal baseline. For this reason, it is recommended that users select a baseline period that begins after this initial elevation in signal has decreased. This timing can be estimated by looking at the raw photometry data.

b. Prior to starting the batch processing portion, adjust the number of frames in lines 330 and 331, corresponding with the number of frames entered in line 239. This is the total number of frames in the fiber photometry trace during the full duration of the task itself. These lines create structure array elements to store the outputs of the fiber photometry analyses.

c. With these steps completed, the process can now be executed. The calculations have been outlined in full below.

During the first block of these analyses, data from the isosbestic channel is fit to a biexponential decay. This biexponential decay is then used to linearly scale the data from the channel of interest. Using the raw data and scaled data as references, a ΔF/F trace is calculated (See Figure 3B for raw fluorescence data from a GCaMP signal and its appropriate 415 nm isosbestic channel; Figure 3C shows these data presented as a *Z* score ΔF/F trace).

```
%load each vector
time=FP.inputs.doric.time.(FP.info.groups(ii))(:,iii);          %time column
sig=FP.inputs.doric.sig.(FP.info.groups(ii)(:,iii);             %signal of interest
iso=FP.inputs.doric.iso.(FP.info.groups(ii)(:,iii);             %isosbestic signal
temp_x=1:length(iso);                                           %vector counting rows
temp_x=temp_x';                                                 %transpose vector
isofit=fit(temp_x,iso,'exp2');                                  %fit isosbestic to a %biexponential decay
sigfit=robustfit(isofit(temp_x),sig);                           %linearly scale %biexponential decay %of the
                                                                isosbestic to %the raw signal of %interest
sigfit=isofit(temp_x)*sigfit(2)+sigfit(1);                      %apply linear fit
dFF=(sig-sigfit)./sigfit;                                       %calculate delta F/F
```

When examining a ΔF/F trace, it is not uncommon for variation in baseline intensity to be present. These can be indicative of differences in viral expression/cell density, or variability in the focal distance between the tip of the optic fiber and peak virus expression. To account for this, baseline adjustment is performed on all samples. In these steps, the minimum ΔF/F value during the behavioral task is compared to the mean ΔF/F value during a user-defined baseline period.[8] The ΔF/F trace during the behavioral task is then adjusted so that the minimum value recorded during the testing period corresponds with the mean value during the baseline period.

```
%baseline adjustment
[~,idx1]=min(abs(time-(FP.info.baseline(1))));          %identify the first frame %of the baseline period
[~,idx2]=min(abs(time-((FP.info.baseline(2)+FP.info.    %identify the last frame of %the baseline period
baseline(1)))));
baseline=dFF(idx1:idx2);                                %dF/F during the baseline %period
test=dFF(idx2:end);                                     %dF/F during the testing %period
meanbaseline=mean(baseline);                            %mean dF/F during the %baseline
mintest=min(test);                                      %minimum dF/F during the %test
adjust=diff([mintest,meanbaseline]);                    %difference between
                                                        the %mean dF/F during the %baseline and the
                                                        minimum %dF/F during the test
cordFF=dFF+abs(adjust);                                 %adjust dF/F based on %difference
```

If the user had previously defined further processing criteria, such as analyzing photometry data as a *Z* score ΔF/F or as a %ΔF/F, these are now applied.

```
%z-score

if FP.info.zscore(1) == 1

  cordFF=zscore(dFF);

  dFF=zscore(dFF);

else

  %proceed with non-z-scored data

end

%dF/F

if FP.info.percentdFF(1) == 1

  cordFF=((dFF-meanbaseline)./meanbaseline)*100;

else

  %proceed with non-%dF/F data

end
```

With fiber photometry data now preprocessed, initial behavior independent analyses are conducted to assess area under the curve, peak frequency, mean peak height, max peak height, max ΔF/F, and the time at which the max ΔF/F value occurs. These behavior and test-segment independent values are then stored to the FP.outputs.behaviourindependent element in the structure array.

Note: For peak analyses, a minimum peak height criterion is applied. In the block of code below, this is defined as 2 standard deviations above the median ΔF/F value within the user-defined rolling window.

```
%area under the curve calculation

auc=trapz(cordFF);

%peak detection and analysis

%Note: in this step the minimum peak height is defined as 2 standard deviations above the median dFF value within the rolling
window.

minpk=movmedian(dFF,FP.info.windowframes)+(2*movstd(dFF,FP.info.windowframes));

tracedif=ge(dFF,minpk);                                    %identify indices greater

                                          %than or equal to the

                                          %minimum peak threshold

pks=dFF(tracedif);                                  %identify peaks

%peak frequency

pkfreq=size(pks,1)/(FP.info.samplingrate(1)*size(time,1));

%mean peak height

meanpk=mean(cordFF(tracedif));

%index of maximum dF/F value

maxpkidx=find(dFF=max(dFF),1,'first');

%maximum dF/F value

maxpk=max(cordFF);

%time at which the maximum dF/F value occurs

maxpktime=time(maxpkidx);
```

16. Complete Process 04. Photometry Processing – Behavior Epoch Dependent. In this process, behavioral information will be integrated with the photometry data.
    a. Define the starting times and durations of each behavioral epoch in the initialization subsection.

Note: In the current manuscript, epoch refers to the entire testing period of interest. In the case of the social behavior task, the two epochs of interest are the 5-min block during which a familiar mouse is present and the 5-min block during which an intruder mouse is present. In the current design, regardless of counterbalancing, the first epoch occurs at the 5-min mark (300 s) while the second epoch occurs at the 15-min mark (900 s). Each of these epochs occurs for 300 s. Therefore, the initialization subsection would be as follows.

```
%initialization
FP.info.epoch.first = 300;                                    %start
FP.info.epoch.second = 900;                                   %start
FP.info.epoch.duration.familiar = 300;                        %duration
FP.info.epoch.duration.familiar = 300;                        %duration
```

b. Adjust the number of frames in lines 478–480 to match the number of frames in the photometry trace.

Note: These lines generate storage vectors for behavioral data at a framerate which coincides with the photometry data. In the below example, line 478 is set up for processing a photometry trace with a length of 180,705 frames. This value should match the user-entered value in line 239.

```
FP.outputs.behaviourdependent.behaviour.anogenital.familiar.(FP.info.groups(ii))
=zeros(180705,FP.info.pergroup(ii));
```

c. The script analyzes fiber photometry data by epoch and provides area under the curve, peak frequency, mean peak height, max peak height, max ΔF/F, and the time at which the max ΔF/F value occurs.
d. The script aligns behavioral and fiber photometry data using nearest neighbor approximation at the timestamps of individual data frames.

Note: Following epoch-level analyses, behavioral data is expanded using nearest neighbor approximation to align with the framerate of the fiber photometry recording. This approach allows for the assessment of fiber photometry analyses during specific behaviors within each epoch. For example, this approach allows users to assess area under the curve during head-torso sniffing interactions, which can then be compared between interactions with familiar and intruder mice. In the below example, behavioral data for head-torso and anogenital sniffing interactions is aligned to photometry data during the epoch with the familiar mouse. These vectors are then converted into logical arrays for the purpose of indexing into fiber photometry data. The logical vector is then used to index into the fiber photometry trace to extract only the ΔF/F values occurring during behaviors of interest. In the below example, the ΔF/F data occurring during head-torso sniffing interactions with a familiar mouse is isolated.

```
htf=fdFF(htfstretch);
```

⚠ CRITICAL: The nearest neighbor approach will not work properly if the framerate of the behavioral recording exceeds that of the fiber photometry recording. See troubleshooting 3 for notes on how to avoid this issue.

```
%familiar
famtime=(FP.info.epoch.familiar(1):(FP.info.epoch.familiar(2)...
/size(htf,1)):(FP.info.epoch.familiar(1)+FP.info.epoch.familiar(2)));
                    %dummy time series for %photometry during %familiar epoch
```

```
famtime=famtime(2:end)';          %trim leading zero

htfstretch=zeros(size(ftime,1),1);        %sink for head torso data

agfstretch=zeros(size(ftime,1),1);        %sink for anogenital data

for iv=1:size(ftime,1)             %loop through fp frames

  temp_time=ftime(iv);           %time frame from fp

  [~,ix]=min(abs(famtime-temp_time));     %find nearest time value %in behavioral dataset

  htfstretch(iv)=htf(ix,:);            %assign value from head-

                       %torso data at the index

                       %of nearest time value to

                       %the sink

  agfstretch(iv)=agf(ix,:);            %same process for

                       %anogenital data

end

htfstretch=(htfstretch==1);            %convert to a logical

agfstretch=(agfstretch==1);            %convert to a logical
```

e. The script applies fiber photometry analyses to these behavior and epoch specific fiber photometry traces to assess area under the curve, peak frequency, mean peak height, max peak height, max ΔF/F, and the time at which the max ΔF/F value occurs.

Note: These values are then stored to the FP.outputs.behaviourdependent.analyses element in the structure array (See Figures 3D and 3E for an example of social interaction data aligned to a fiber photometry recording).

17. Complete Process 05. Photometry Processing – Bout-by-Bout Information.

Note: In this process, photometry data will be analyzed on a bout-by-bout basis for each of the behavioral measures of interest. For example, this series of calculations will yield the area under the curve for the ΔF/F trace during each bout of head-torso interaction during the familiar epoch. There are no user-defined variables which will need to be edited in this process.

Note: All data from these analyses are stored under the FP.outputs.boutbybout element in the structure array. When interpreting these outputs under the FP.outputs.boutbybout.analyses element, each column represents a different mouse, with bout-by-bout information presented in chronological order down the column. For example, if the area under the curve during the first bout of head-torso sniffing for mouse 1 is 3, the cell at the intersection of the first column and the third row will be 3. NaN represents a placeholder value in these arrays. It is indicative of either a failure to meet a minimum criterion, such as a bout in which no points in the ΔF/F trace surpassed the minimum peak detection threshold, or that no further bouts have occurred. As a result, the bottom rows of each of these sheets will contain NaN values.

⚠ CRITICAL: Following the conclusion of all fiber photometry experiments, it is critical to assess the quality of viral expression and the placement of the fiber optic implant. Variability in each of these can influence the quality of the fiber photometry recording. Animals with poor viral expression, fiber optic implants which are more than the focal distance

from the center of the viral injection, or inconsistent regional targeting must be excluded from data analysis.

EXPECTED OUTCOMES

This protocol will allow assessment of neural signals (population calcium activity) underlying social behavior in freely interacting rodents. Example data using this approach is shown in Figure 2 of Dawson et al. 2023.[1]

LIMITATIONS

The success of fiber photometry recordings is highly dependent upon the expression of the fluorescent indicator and the precision of the surgical implant. Thus, the success rate and quality of these recordings can be highly variable if not piloted appropriately. To best overcome this limitation, it is recommended that viral titer and volume are reassessed each time that an experimental parameter (target region, model species, viral cocktail/preparation) is modified.

TROUBLESHOOTING

Problem 1

Dental cement headcaps are coming loose or falling off. As described in stereotaxic surgery, point 16.

Dental cement headcaps which are not securely fixed to the skull will cause the tip of the fiber optic ferrule to move during recordings, compromising the quality of the data collected. Furthermore, loose headcaps can rapidly detach and expose the skull, which would then call for the humane euthanasia of the animal. There are several methods to improve the adherence of your headcap to the skull.

Potential solution 1

Construct dental cement headcaps with wide bases. The larger the footprint of the headcap, the more likely it is to adhere securely to the skull. One method to help maximize the size of headcap footprint is by tucking the scalp under the ear bars. This approach requires a longer incision, but it will allow for the headcap to cover a much larger proportion of the dorsal surface of the skull.

Potential solution 2

Make the surface of the skull more abrasive to increase surface area. With a similar logic to the first potential solution, greater surface area improves the likelihood of the headcap adhering securely to the skull. By lightly etching a grid pattern into the skull using a blade, the skull can be made more abrasive.

Potential solution 3

The use of an anchor screw (Figure 1H). In some cases, it may be necessary to include a screw as an anchor which will help to keep the headcap securely attached to the skull. When using an anchor screw, a second hole is drilled elsewhere on the contralateral side of the skull to the target. A small screw is then inserted into this hole. It is critical that this screw is only inserted to a depth where it is flush with the inner surface of the skull. The screw should not touch the brain, as this will cause damage to this tissue. The risk of damaging the brain with an anchor screw can be further reduced by selecting a location where the skull is thick enough to permit drilling a hole that is deep enough to support a screw without going completely through the skull.

Problem 2

The signal from the fiber photometry recording has a very small dynamic range and/or seems non-responsive to external stimuli. As described in stereotaxic surgery, point 19.

While signal dynamics will vary depending on the fluorescent indicator being used, for most indicators the ideal recording will include a highly dynamic signal. However, there are several factors which may contribute to issues with a fiber photometry recording.

Potential solution 1

Ensure that the fiber optic patch cord is securely attached to the fiber optic ferrule. Especially during freely behaving tasks, movement can lead to a loosening of this coupling, which will disrupt the light path between the labeled cells and the photodetector.

Potential solution 2

Increase the waiting period post viral injection and/or adjust the viral titer. Low dynamic range may indicate low viral expression in some situations, which may be improved by longer wait times prior to recording. It is important to note that not all viral vectors display optimal expression as early as 2 weeks post-injection, with a notable example being the 6+ weeks that it can take for adequate viral expression to be detected at synaptic terminals.[9]

Problem 3

The framerate at which the behavioral analyses were conducted exceeds that of the fiber photometry recording. As described in setting up hardware for fiber photometry and behavioral recordings, point 25.

Given the nature of the nearest neighbor calculations during the alignment of fiber photometry and behavioral data, an error will occur if the framerate of the behavioral data exceeds that of the fiber photometry data.

Potential solution 1

The best way to avoid issues with this alignment is to have both the photometry recordings and video recordings set to the same frame rate. Due to technical limitations, this may not always be possible. In such cases, to avoid issues with alignment, the frame rate of the behavioral analyses should be less than that of the fiber photometry recording. This frame rate can be specified while exporting these videos from ANY-maze. It is also important to note that a low frame rate during the fiber photometry recordings will limit the temporal resolution of the behavioral analyses.

Problem 4

Photometry recordings for different mice within an experiment vary by length. As described in Integrating Behavioral Classifications in Fiber Photometry Analysis, point 14.

Due to data loading and management constraints, an error will occur when running these analyses on fiber photometry data with a variable number of frames across mice. This issue can be caused by inconsistent runtimes (e.g., the photometry recording not automatically ending at the same time as the behavioral recording) or dropped frames at the beginning or end of the recording as a consequence of de-interleaving data.

Potential solution 1

Trim frames from a buffer period at the end of the trial. Following the conclusion of each behavioral test, the fiber photometry recording is ended. However, in some recording setups, the end of the test will only alter a flag state in the fiber photometry data frame. In these cases, a buffer period may be included following the conclusion of the task which does not represent task-relevant neural activity. Because it is not data which is relevant to the task or will be included in the analysis, this buffer period can be deleted from the raw data file.

Potential solution 2

If there is no justifiable reason to trim recordings to be the same length, recordings can be analyzed individually by setting the numbers of groups and subjects per group to one. When analyzing traces in this manner, it is important to remember to assign unique identifiers to the save file generated in the section of the MATLAB workflow under the heading "Process X", in order to avoid overwriting previously analyzed data.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Derya Sargin (derya.sargin@ucalgary.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The protocol includes all datasets analyzed during this study. Sample data and code are available at: https://doi.org/10.5281/zenodo.8408555.

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AUTHOR CONTRIBUTIONS

D.J.T., M.D., J.R.E., and D.S. conceived the project and designed the analyses. M.T. and N.F.J. performed the surgeries. D.J.T. developed all analysis scripts unique to this protocol. D.J.T., M.D., J.R.E., and D.S. conducted the analyses. D.J.T., J.R.E., and D.S. wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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