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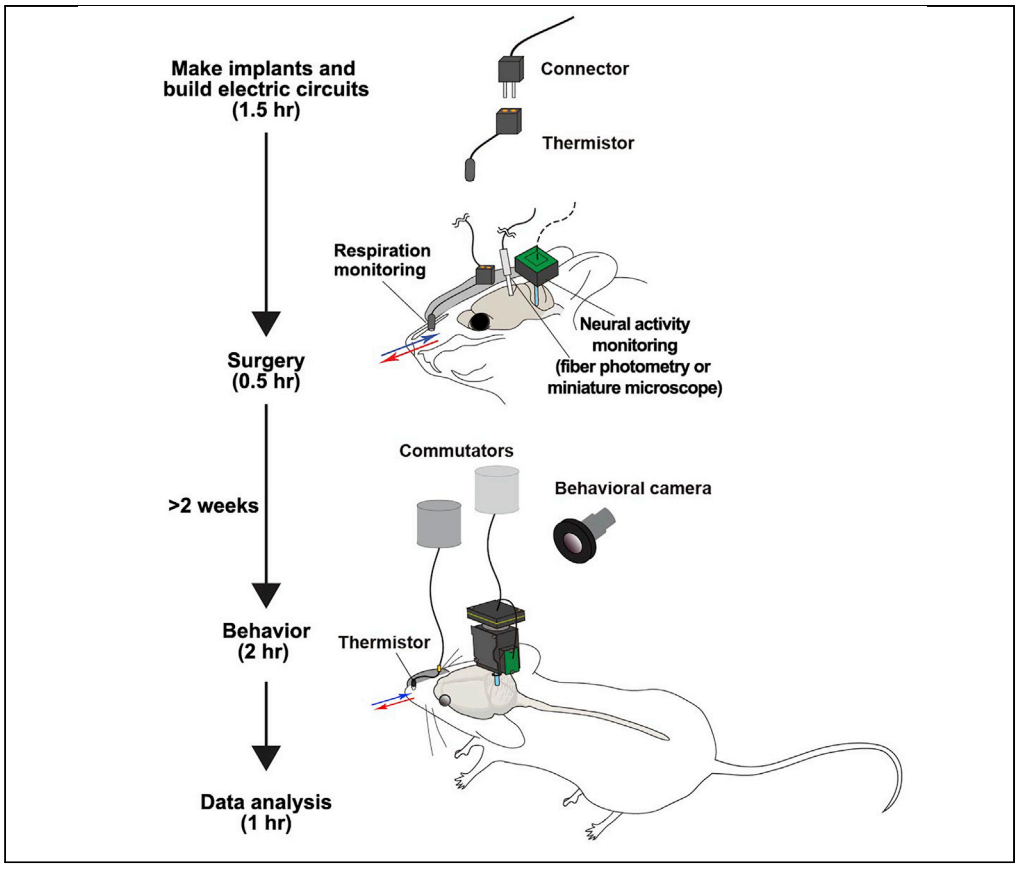
Protocol

Simultaneous recording of breathing and neural activity in awake behaving mice



This protocol provides a pipeline for simultaneous recording of breathing and neural activities in awake, behaving mice. Breathing is recorded with thermistor probes implanted in the nasal cavity, which can be easily integrated with neural activity monitoring approaches such as fiber photometry. Here, we detail the procedures of the thermistor probe assembly, surgery, recording system setup, and data analysis. This protocol can be applied to investigate respiratory physiology and breathing changes during natural behaviors.

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

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Highlights

Protocols for constructing a simple, lightweight breathing sensor

Instructions for sensor implantation surgery and behavior setup

Tips on improving the signal quality and stability

Flexibility to integrate with multiple recording and manipulation platforms

Liu & Han, STAR Protocols 3, 101412
June 17, 2022 © 2022 The Authors.
https://doi.org/10.1016/j.xpro.2022.101412

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Simultaneous recording of breathing and neural activity in awake behaving mice

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https://doi.org/10.1016/j.xpro.2022.101412

SUMMARY

This protocol provides a pipeline for simultaneous recording of breathing and neural activities in awake, behaving mice. Breathing is recorded with thermistor probes implanted in the nasal cavity, which can be easily integrated with neural activity monitoring approaches such as fiber photometry. Here, we detail the procedures of the thermistor probe assembly, surgery, recording system setup, and data analysis. This protocol can be applied to investigate respiratory physiology and breathing changes during natural behaviors.

For complete details on the use and execution of this protocol, please refer to Liu et al. (2022).

BEFORE YOU BEGIN

Breathing is an essential physiological process that also plays important roles in various behaviors such as exploration, social interaction, and learning. However, standard approaches for breathing recording, such as tracheostomy and whole-body plethysmography, often involve restraining or anesthetizing the animal, thus making it hard to access the brain and record or manipulate neural activities.

Here, we introduce an approach to integrate breathing recording with neural activity monitoring in awake, unrestrained, behaving mice. Breathing is recorded with a thermistor-based breathing sensor implanted into the nasal cavity (McAfee et al., 2016). The thermistor sensor detects temperature differences between inhaled and exhaled air and converts this difference into voltage signals. On the other hand, neural activity can be recorded with fiber photometry or a miniature microscope.

This protocol provides step-by-step instructions to construct thermistor-based breathing sensors, implant sensors, and record the respiratory activity simultaneously with calcium signals in awake behaving mice. We have applied this protocol to investigate the relationship between breathing and calcium signals recorded with fiber photometry or a miniature microscope for home-cage animals. Additionally, we have also used this protocol to examine breathing and neural activities during emotional, social, and motor behaviors.

Institutional permissions

All protocols for animal experiments were approved by the IACUC of the Salk Institute for Biological Studies according to NIH guidelines for animal experimentation. We would also like to remind the readers that they will need to acquire permissions from the relevant institutions before carrying out the procedures outlined in this protocol.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
AAV1-syn-FLEX-jGCaMP7s-WPRE	Dana et al. (2019)	Addgene viral prep # 104491-AAV1
Deposited data		
Custom MATLAB scripts for data analysis	This paper	Zenodo: https://doi.org/10.5281/zenodo.6466532
Experimental models: Organisms/strains		
Mouse: *Oprm1*-Cre:GFP +/-, 8–52 weeks, males and females	Laboratory of Dr. Richard Palmiter	N/A
Software and algorithms		
MATLAB	MathWorks	R2019a
Python	Anaconda Distribution	Version 3.7
LabChart	ADInstruments	Version 8
Other		
PowerLab data acquisition system	ADInstruments	Cat#PL3508
400 μm 0.37 NA fiber-optic cannula	Laboratory of Dr. Sung Han	N/A
ProView™ integrated lens 0.6 mm × 7.3 mm	Inscopix	Cat#1050-004413
Negative Temperature Coefficient (NTC) thermistor	TE Connectivity	Cat#GAG22K7MCD419
Hemostat	Fine Science Tools	Cat#13014-14
Wire cutter	Amazon	ASIN#B000X4X23U
Lighter	Amazon	ASIN#B086R3LFBW
Light-curing epoxy	Ivoclar	Cat#595953US
Dental curing light	Azdent	Cat#1070800105
Heat shrink tubing	Amazon	ASIN#B09JK6RYBD
Interconnector	Mill-Max	Cat#851-93-050-10-001000
Voltage divider	Phidgets	Cat#1121_0
Silicone wire	Amazon	ASIN#B07DHQK4JY or B01K4RPD00
Commutator (for breathing sensor only)	Adafruit	Cat#SRC012-6
Commutator (for breathing sensor and fiber optics)	Doric	Cat#HRJ-OE_12_FC_HARWIN
5 V power outlet	Amazon	ASIN#B01D4JJ41O
BNC male connector	Amazon	ASIN#B01IYI2GEM
Drill burr	Fine Science Tools	Cat#19007-05
Loctite gel superglue	Amazon	ASIN#B01EZTPXEO
Ortho-Jet liquid	Lang Dental	Cat#B1303
Metabond	Parkell	Cat#S380
Raspberry Pi	Raspberry Pi Foundation	Version 4 model B
Lubricant eye ointment	Amazon	ASIN#B004RHPZRK
Cotton tipped applicators	Amazon	ASIN#B003IT74DG
Nair hair removal body cream	Amazon	ASIN#B001E6OAM8
White spray paint	Amazon	ASIN#B002BWOS7G

STEP-BY-STEP METHOD DETAILS

Constructing the thermistor-based respiration sensor

Timing: 20 min

This section describes the construction of respiration sensor that will be implanted into the nasal cavity.

1. Prepare the thermistor, the pins, and the socket.
   a. Cut the thermistor head to 3-cm long.
   b. Dissemble the original connector stripe by pushing out the pins with a hemostat.
   c. Cut the black socket stripe using a wire cutter into small pieces that contain only two holes (Figure 1A).

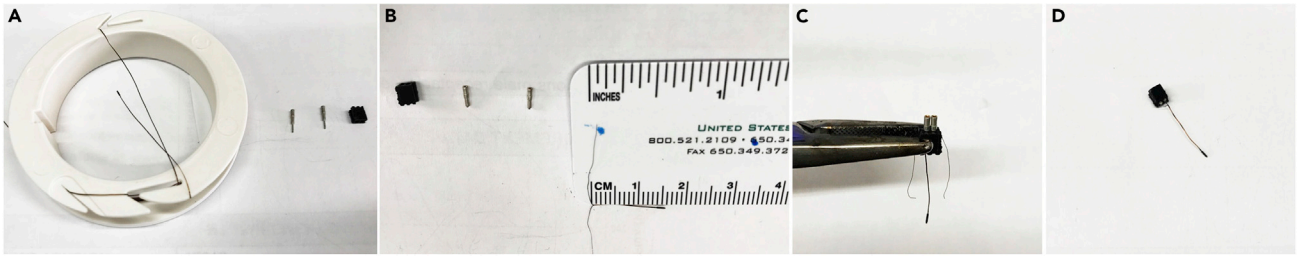

Figure 1. Constructing the breathing sensor

(A) The starting material: thermistor, pins, and socket.

(B) Parts for assembling the breathing sensor: thermistor with wires separated, pins with bottom part cut, and socket.

(C) Positioning the parts before pushing the pins into the socket.

(D) An assembled thermistor sensor.

d. Cut off the bottom of the pins.
e. Cut off the thermistor.
f. Use the flame to burn the wires so that the ends separate into two strands.
g. Separate the thermistor wires so that the total length of the thermistor head and intact wire is around ~1.5 cm (Figure 1B).

Note: Adjust the length according to the implantation coordinates.

2. Build the thermistor.
   a. Insert two wires of thermistor into the socket.
   b. Insert the pins (with the bottom cut) into the socket (Figure 1C).
   c. Gently push the pins down with a hemostat to fit perfectly within the socket. [trouble-shooting 1].
   d. The metal pin should now have a tight connection with the exposed wire of the thermistor without the need to solder (Figure 1D).
   e. Apply some epoxy, such as LED-curable epoxy, to the bottom of the sensor to protect the bare wires.

Note: We recommend the direct connection rather than soldering because the wires are very thin. The integrity of the sensor can be tested by plugging the sensor into the patch cord and exhaling towards the sensor (step 14) before applying the epoxy.

Making the patch cord that connects to the thermistor

Timing: 20 min

This section describes the construction of patch cord that connects the thermistor and electric circuit.

3. Prepare two pins (with the bottom intact) and a socket. Insert the pins into the socket to make a connector (Figure 2A).
4. Prepare a patch cord, separate its two ends, strip off the coating, insert two metal ends into each socket, and then solder the wire with the socket to electronically couple the wire and socket (Figure 2B).
5. Use heat shrink tubing to secure the connector and patch cord (Figure 2C).
6. Connect the patch cord with the thermistor sensor (Figure 2D).
7. Using the same design, build a holder for a thermistor sensor that can be used during the stereotaxic implant.

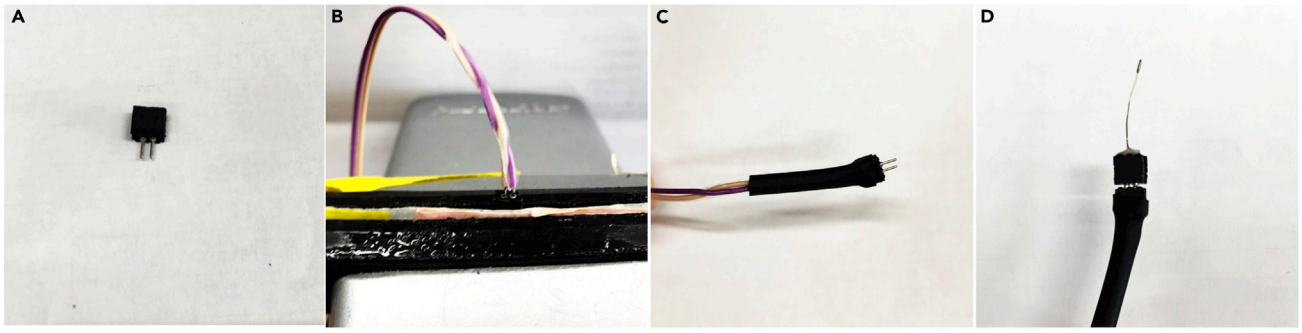

Figure 2. Constructing the patch cord that connects to the breathing sensor
(A) An assembled connector.
(B) Soldering the patch cord to the connector (the black part inside the clamp).
(C) Using heat shrink tubing to protect the connection.
(D) Attaching the thermistor sensor to the patch cord.

Building the electric circuit

Timing: 30 min

This section describes the construction of the electric circuitry that transmits thermistor signals into the data acquisition system.

8. In the setting below, a voltage divider is used to adjust the voltage output of the thermistor sensor to amplify the breathing signals. The voltage divider is composed of three different wires (Figure 3A) (Phidgets Inc., 2022a).
   a. "Power" (red) and "Ground" (black) wires are connected to the power outlet.
   b. "Data" (white) and "Ground" wires are connected to the Data Acquisition (DAQ) system for receiving signals from the thermistor.
   c. The "Ground" wire needs to be duplicated so that it can connect with both the "Power" and the "Data" wires.
9. The goal is to assemble the following system:
   a. One end of the voltage divider (Figure 3B, two green screws on top of the voltage divider) should be connected to the patch cord with the thermistor sensor attached.
   b. The other end of the voltage divider should be connected to the power outlet (the yellow/red/black strands wrapped with the red tape in Figure 3B) and the DAQ system (the white/yellow strands wrapped with the green tape in Figure 3B).
10. To assemble the system:
    a. Connect the "Data" (white) wire to the BNC male connector.
    b. Connect the "Power" (red) wire to the 5 V power outlet.
    c. Extend the "Ground" (black) wire with silicone wire to duplicate it. Connect one duplication to the BNC male connector and the other one to the power outlet.

Optional: Add a commutator between the voltage divider and the thermistor patch cord to help release the torque of the thermistor patch cord due to the animal's movement.

Test the thermistor

Timing: 10 min

This section describes how to examine the integrity of the thermistor sensor before proceeding to implantation.

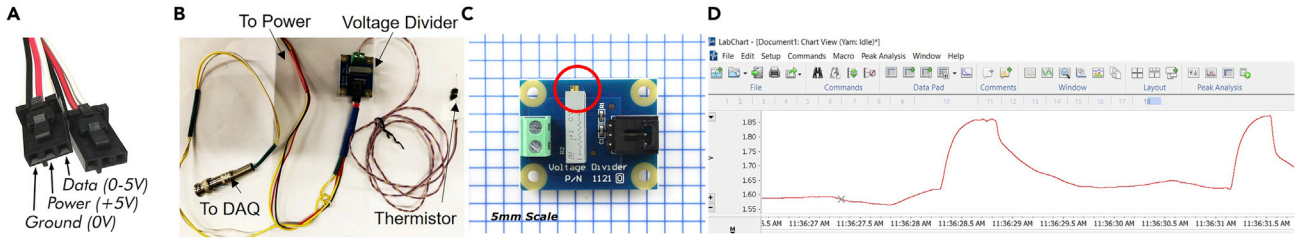

Figure 3. Assembling the recording system and testing the breathing sensor

(A) Wire connections of the voltage divider (Phidgets Inc., 2022a).

(B) An assembled electric circuit for connecting the thermistor to the DAQ.

(C) The knob of the Variable Resistor (screw inside the red circle) for adjusting baseline voltage (Phidgets Inc., 2022b).

(D) Example trace shown in LabChart software when the experimenter exhales towards the sensor.

11. Connect the data BNC cable to the DAQ system, such as the PowerLab.

Note: PowerLab is one of the common DAQ systems in the physiology labs, and the built-in peak detection function of its associated LabChart software is also more intuitive, flexible, and reliable than the customized code. We are also aware that alternative options exist for using the open-source platform (such as LabView and MATLAB) to acquire and process breathing signals.

12. Open the LabChart software and create a new document to view the BNC input.
13. Adjust the baseline voltage by turning the knob of the Variable Resistor (small screw on the side of the gray box) (Figure 3C, red circle) (Phidgets Inc., 2022b) on the voltage divider. We found the signal-to-noise ratio is the best when the baseline voltage is 1.5–1.7 V.
14. Test the thermistor sensor by exhaling towards the sensor. You are expected to see an immediate change in voltage during exhalation (Figure 3D).

Pause point: The procedure can be paused between any two steps in the above sections.

Implanting the thermistor into the nasal cavity

Timing: 30 min

This section describes the procedure of sensor implantation with stereotaxic surgery in mice.

15. The surgery procedures are revised based on a previous report (McAfee et al., 2016). The goal is to implant the thermistor probe into the nasal cavity securely. The sensor head should be fully inserted into the airway to detect temperature changes between the inhaled and exhaled air.
16. Anesthetize the animal and place the animal in the stereotaxic frame. We used isoflurane anesthesia for induction (flow rate: 4–5 L/min) and maintenance (flow rate: 1–1.5 L/min); however, other types of anesthesia can also be used.

Note: The head should be well fixed because the nose cone cannot be securely attached to the nose during the sensor implantation.

17. Move the nose cone close to the animal without blocking access to the skull.

Note: This placement will still result in the leakage of isoflurane, so an exhausting system for isoflurane is strongly suggested.

18. Apply lubricant eye ointment.
19. Use cotton tips to apply Nair Hair Removal Body Cream onto the fur above the surgical area.

20. Wait for 30–60 s, then use new cotton tips to remove the cream and clean the surface with water and 70% ethanol.

Note: Hair removal cream is preferred over shaving because the hair on the anterior side is short. Only certain types of hair removal cream will work for this purpose (for example, Nair for sensitive skin will not work).

⚠ CRITICAL: Be careful not to touch the eyes when removing the cream. Re-apply lubricant eye ointment if necessary.

21. Make an incision along the midline, from the most anterior part of the head to Bregma.
22. Remove the skins on both sides to create space for implantation.
23. Use the stereotaxic frame to locate the site of implantation. An example coordinate is Anterior-Posterior (AP) 3.5 anterior from the nasal fissure (McAfee et al., 2016) and Medial-Lateral (ML) 0.4 from the midline. Since the nasal cavity are two parallel canals on both sides of the midline, anywhere between AP 3–6.5 and ML 0.3–0.5 would also work.
24. Drill a hole big enough to allow the sensor to be inserted. Push the skull down with forceps during drilling to reduce the movement of the skull. You should be able to see the brain tissue rather than the membrane. There should be little to no bleeding. [troubleshooting 2].
25. Attach the thermistor with a custom-built holder. Gently lower the thermistor into the drilled hole with a ~45-degree angle (tip pointing anterior) so that most of the sensor body goes inside the cavity, which secures the implant. [troubleshooting 3].
26. Fix the sensor head onto the skull with a small amount of gel-like superglue and fix the rest of the sensor body with Metabond. Other adhesives of choice may work as well. [troubleshooting 4].
27. Inject the genetically encoded calcium indicator and implant the optic fiber (for fiber photometry) or GRIN lens (for single-cell calcium activity monitoring with miniature microscopes).
28. Wait for 3–5 days for the breathing recording experiment. Stable recording can be achieved for up to 10 months. [troubleshooting 5].
29. Wait for at least 2 weeks for the calcium signal recording experiment.

Note: The optic fiber or lens implant can be performed during the same surgery as the thermistor implant, or one procedure can be done weeks after the other. The thermistor is typically implanted at last since its signal may deteriorate over time if the implantation is not secure. If multiple implants are performed during different surgeries, enough skull surface should be left for later implants.

Simultaneous recording of breathing rate and neural activities

Timing: 30 min–2 h

This section describes the procedure for setting up the recording system and performing breathing/calcium signal recording.

30. Design the setup for simultaneous recording of breathing and calcium activity together with video recording of behavior. Use LabChart to record breathing signals and fiber photometry to record calcium activities.
31. For breathing recording, set the sampling rate at 100 Hz, low-pass filter at 10 Hz, and smooth with a 100-ms moving window.

Note: Adjust the parameters above to improve signal-to-noise ratio of the respiratory waveform.

32. In different channels, calculate real-time breathing rate and relative amplitude by detecting respiratory peaks.

Note: Adjust the parameters to ensure that breathing peaks can be detected across different breathing rates.

33. Use Raspberry Pi to control the recording camera and send two simultaneous digital inputs to the LabChart and the fiber photometry system for synchronization.

Note: LabChart has a "trigger mode" to allow breathing recording to be triggered by an external digital input.

34. Habituate the animals at least three times before behavioral experiments. Practice a technique where the animal's skull is firmly held with the thumb and index fingers, and the body is gently held in the palm. Use the other hand to attach the patch cords for breathing and calcium recording quickly.

Note: The researcher may use gas anesthesia during the attachment of the sensors to reduce pain/stress to the animal. We decided not to use anesthesia to avoid any long-lasting effects on neural activity and locomotion.

35. Perform the behavioral experiments.

Note: When using video tracking software, the patch cords can be sprayed with white paint to facilitate automatic detection of the animal.

Note: A similar setup can be used to examine breathing changes during neural activity manipulations.

Analysis of breathing rate and calcium signals

Timing: 1 h

This section describes the procedure for data analysis.

36. Export the breathing and calcium data to the format of your choice (.mat, .txt, etc.).
37. Align the onset of both sets of data and resample if necessary.
38. Calculate breathing rate by counting the number of peaks per minute (which can be automatically calculated with LabChart).
39. Estimate breathing amplitude by the plethysmograph amplitude, i.e., the height of the plethysmograph waveform (which can be automatically calculated with LabChart).
40. Plot different breathing parameters with calcium signals and examine their correlation during specific behavioral episodes.

Note: The MATLAB code for data analysis can be found in the key resources table.

EXPECTED OUTCOMES

This protocol will allow one to record the breathing parameters (breathing rate, plethysmograph amplitude) and neural signals (population calcium activity) in awake behaving animals. Example data from a home-cage recording are shown in Figure 1 of Liu et al. (2022).

LIMITATIONS

The success of the thermistor recording is highly dependent on the surgical implant. Thus, the success rate and implantation quality are highly variable but can be improved with practice.

The breathing sensor can output an accurate breathing rate but not amplitude. The temperature detected by the thermistor sensor can be determined by multiple factors, such as the gas exchange volume, gas exchange rate, and ambient temperature. Therefore, the output voltage trace amplitude only reflects the relative plethysmograph amplitude but not the actual tidal volume.

The throughput of the sensor implant-based method is relatively low. Other approaches to measuring breathing have been suggested (Grimaud and Murthy, 2018), each with its strengths and limitations.

TROUBLESHOOTING

Problem 1

The separated wires at the end of the sensor break when the pins are being pushed into the socket (step 2c).

Potential solution

Push the pins down perpendicularly. Also, test the sensor before applying the epoxy. In step 1e, keep the wires as long as possible so that if the wire breaks, you can still cut off the leftover part to make a new sensor.

Problem 2

Suboptimal drilling of the hole: too shallow, too deep, too big, or too small will lead to suboptimal insertion of the breathing sensor (step 24).

Potential solution

Use the smallest drill burr and fix it to the stereotaxic frame with an angle when drilling. Do not handhold the drill. Instead, gently drill multiple times by pushing down the burr against the surface of the skull.

Problem 3

Suboptimal insertion of the breathing sensor: too shallow will make the breathing sensor unable to detect the temperature fluctuations in the airway; too deep will cause bleeding and scar tissue formation around the sensor, leading to a deterioration of signals (step 25).

Potential solution

Insert the breathing sensor gently into the hole without pushing down the epithelium.
Record the length of sensor insertion and compare it across successful or unsuccessful experiments.

Slight bleeding from the hole will not negatively affect the results. However, if there is excessive bleeding, it might be safer to drill on the other side or use another animal.

Problem 4

Suboptimal fixation of the thermistor sensor: this will cause sensor detachment during the behavioral recording or deteriorate quickly after each attachment-detachment (step 26).

Potential solution

Expose as much skull surface as possible.

Apply a small amount of Ortho-Jet liquid to help cure the superglue. However, do not contact the sensor head directly with acrylic solutions since they can damage the thermistor.

Create a strong attachment of the sensor to the skull. Make scores on the surface, implant a screw or two to facilitate fixation, and use dental cement to create a strong bond.

If the hole is too big, it will be more challenging to achieve a stable, clean implant.

Problem 5

The thermistor signal goes bad suddenly during the experiment or slowly over time (step 28).

Potential solution

Secure the implant during the surgery (see problem 3).

Use two commutators for breathing and calcium activity monitoring during both habituation and actual behavior experiments. Since the two commutators and patch cords are made of different materials, they have different levels of friction which could cause the entangling of the two cables. Therefore, we also provide an option of using one commutator for both breathing sensor and fiber optics (see key resources table).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sung Han (sunghan@salk.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- Original code for data analyses has been deposited at Zenodo (https://doi.org/10.5281/zenodo.6466532).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

S.H. is supported by 1R01MH116203 from NIMH and Brain Research Foundation Fay/Frank Seed Grant. S.L. is supported by the Salk Women & Science Special Award, the Mary K. Chapman Foundation, and the Jesse & Caryl Philips Foundation.

AUTHOR CONTRIBUTIONS

Methodology, S.H.; writing, S.L.; funding acquisition, S.H. and S.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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