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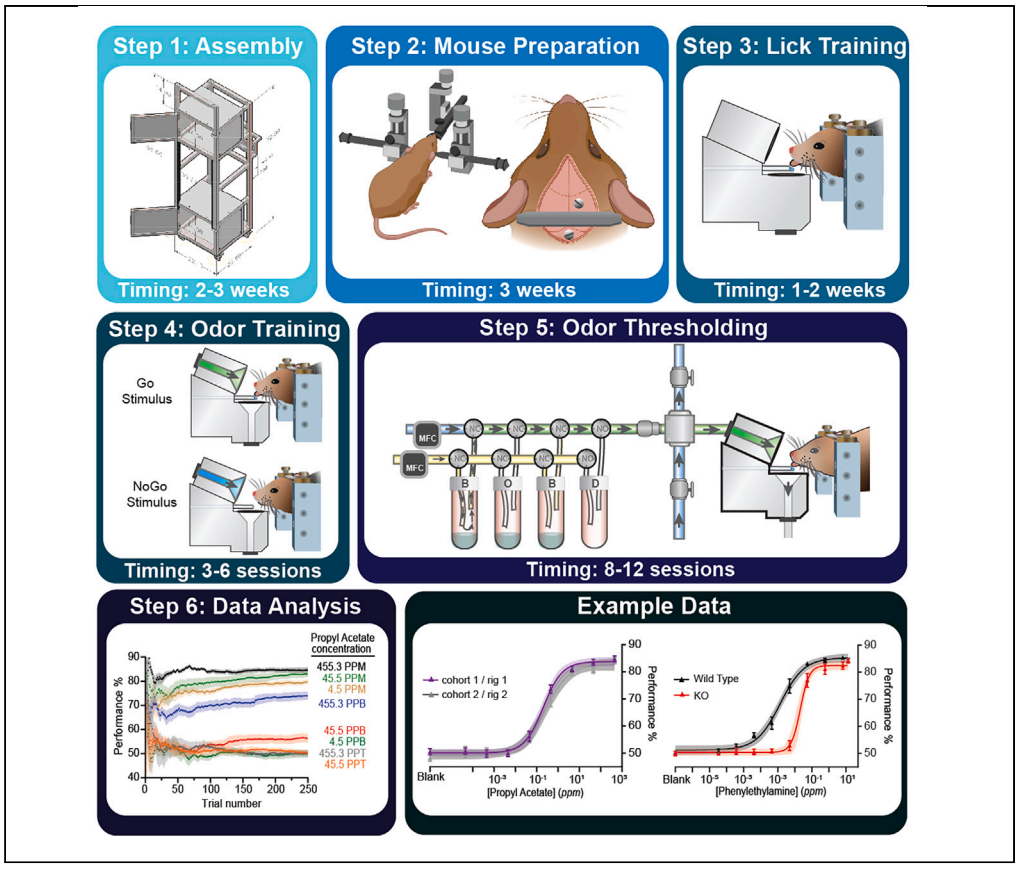
Protocol for quantifying the odor detection threshold of mice



Perceptual measures of odor threshold provide a mechanism to compare sensitivity across species and to gauge stimulus concentrations for functional experiments. Here, we present a protocol to precisely quantify the odor detection threshold of mice. We describe the construction of a head-fixed operant conditioning behavioral rig and provide details of the training and testing procedures. This approach can be used to compare the sensitivity of mice across odorants and to quantify detection differences associated with genetic mutations or pharmacological manipulations.

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

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Highlights

Construction of a behavioral chamber for measuring olfactory sensitivity in mice

Protocol for head fixation and water deprivation

Operant conditioning protocol using lick detection as an indicator of odor perception

Accurate quantification of odor detection thresholds in mice

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Protocol for quantifying the odor detection threshold of mice

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SUMMARY

Perceptual measures of odor threshold provide a mechanism to compare sensitivity across species and to gauge stimulus concentrations for functional experiments. Here, we present a protocol to precisely quantify the odor detection threshold of mice. We describe the construction of a head-fixed operant conditioning behavioral rig and provide details of the training and testing procedures. This approach can be used to compare the sensitivity of mice across odorants and to quantify detection differences associated with genetic mutations or pharmacological manipulations.

For complete details on the use and execution of this protocol, please refer to Johnson et al. (2023),[1] Jennings et al. (2022),[2] Williams and Dewan (2020),[3] and Dewan et al. (2018).[4]

BEFORE YOU BEGIN

This protocol consists of six main components and describes how to construct and utilize our head-fixed operant conditioning behavioral rigs to precisely measure the odor sensitivity of mice.[1–4] Important background information related to each step is provided below.

1. Construct the behavioral rig.

Note: The behavioral rig consists of the following components: 1) framed tower, 2) behavioral chambers, 3) olfactometer with the associated tubing, valves, and flow meters, 4) water delivery and lick detection system, and 5) software to trigger the olfactometer, actuate solenoid valves, control trial structure, and record licking behavior. To aid in the motility and compactness of our setup, we opted to mount two behavioral chambers within an 80/20 T-frame system on wheels. The 80/20 T-frame system is advantageous as it provides a mechanism to rack mount the computer, keyboard, and monitor, while also providing convenient attachment points for the olfactometer, flow meter panels, and water delivery system. Each behavioral chamber in the framed tower utilizes an independent computer and Arduino-based state machine but shares a single monitor using a KVM switch. However, we have also included the part number for a simplified version of our chamber without the associated wheeled frame (please see key resources table) that can be used in isolation.

Note: The inner portion of behavioral chamber is 19 in × 17 in × 14 in; however, researchers can use any size chamber, provided it is enclosed, sound dampened, and has sufficient air

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turn-over to remove any residual odor. The light level in our chamber ranges from 2–30 lux, depending on the status of the trial LED.

Note: Any liquid-dilution or flow-dilution olfactometer can be utilized, provided it can present consistent repeatable odor pulses. Our olfactometer is a customized version of the 220A Olfactometer from Aurora Scientific – originally designed by Dr. Dmitry Rinberg and licensed at HHMI Janelia farms (https://www.janelia.org/open-science/olfactometer). Our design allows for the olfactometer to be mounted on the back of the 80/20 framed tower in an easily removable manner.

Note: Each setup can be used to test 12–16 mice per day, as each session typically lasts 40–45 min. Our training protocols necessitate 7–13 sessions, while obtaining the full psychometric curve takes 7–10 sessions per odorant. Thus, to precisely measure the odor sensitivity of 12–16 mice, to three different odorants, using a single setup, should be expected to take 28–43 days.

2. Perform head-bar surgeries.

Note: Any institutionally approved surgical procedure for affixing a head-bar to the mouse skull may be utilized with our behavioral approach.

3. Complete lick training protocol.

Note: The goal of these training sessions is for the animal to acclimate head-fixation and learn to lick for a water reward.

Note: A 10 mL water syringe with a blunt tip needle is useful for helping stimulate licking behavior.

4. Complete odor training protocol.

Note: The goal of these odor training sessions is for the animal to learn to lick for a water reward in the absence of odor and to withhold from licking when they detect odor. This structure allows the collection of behavioral performance data below the animal's odor detection threshold using a standard Go/No-Go operant conditioning approach.

5. Perform odor thresholding experiment.

Note: This experiment measures an animal's olfactory sensitivity by determining their behavioral performance to a series of decreasing odorant concentrations.

Note: We only test mice on one odorant concentration per session. This approach eliminates any masking or adaptation effects resulting from the contamination of the olfactometer by higher concentrations of the target odor and results in a consistent estimation of behavioral performance at each concentration.

Note: One key difference between the thresholding experiment and odor training sessions is the inclusion of a "cheating check", which tests the animal's ability to distinguish between vials using non-odor cues on a per-session basis. The "checking check" refers to an additional solvent vial (i.e., Go stimulus) that is utilized by the program as a No-Go stimulus. This solvent No-Go vial should be indistinguishable from other solvent Go vials unless the animal is using non-odor cues to maximize their performance to receive the associated water reward. Thus, mice

are "cheating" at this task if they can reject (i.e., not lick) the solvent No-Go vial at a frequency higher than the percentage of misses (i.e., not licking during a blank Go vial).

Note: Because the cheating check is included in our example data, the maximum performance a mouse can attain using only odor cues is approximately 85% (in contrast to training sessions in which mice can achieve 100% behavioral performance). However, these cheating check trials can also be excluded from the analysis provided that the number of Go and No-Go trials are roughly equal.

6. Define behavioral threshold.

Note: We define threshold in the standard psychophysical manner as the concentration at which mice can discriminate the odor from the blank with half-maximal performance, typically represented by the inflection point of the psychometric curve.

Institutional permissions

All protocols and procedures were performed under the approval of the Institutional Animal Care and Use Committee at Florida State University, and in accordance with state and federal guidelines. Every effort was made to minimize the number of animals used and to ensure ethical treatment. Please note that all experiments conducted using animals require permission from the relevant institutions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Various chemical odorants (stimulus)	Sigma-Aldrich	N/A
70% Isopropanol (cleaning olfactometer)	Various	N/A
Software and algorithms		
Python Experimental Code	Dewan Lab GitHub	N/A
Arduino Experimental Code	Dewan Lab GitHub	N/A
Prism Analysis Software	GraphPad	N/A
Other		
Accessories: Flow Meter Panel	Dewan Lab GitHub	N/A
Accessories: Regulator and Pressure Gauge Panel	Dewan Lab GitHub	N/A
Accessories: Final Valve	NResearch	SH360T042
Accessories: 1/16″ Teflon Tubing (Odor Vial Tubing)	NResearch	TBGM109
Accessories: 1/8″ Teflon Tubing (Common Teflon Tubing)	NResearch	TBGM110
Accessories: Two-piece inert fitting set (TBGM109 Connectors)	NResearch	252P109-50
Accessories: 1/8″ Ferrule and Nuts (TBGM110 Connectors)	Idex	P-359X
Accessories: 40 mL Amber Odor Vials w/ Septum and Caps	VWR	66030–716
Accessories: Flow Meter 10L (Vacuum Line)	Cole-Palmer	EW-32003-14
Accessories: Flow Meter 2.5 L w/ knob (Air Line)	Cole-Palmer	EW-32003-10
Accessories: Flow Meter 2.5 L w/o knob (Exhaust Line)	Cole-Palmer	EW-32000-10
Accessories: Pressure Gauge (Air and Nitrogen Lines)	Cole-Parmer	EW-68008-03
Accessories: Female Adapter Push to Connect × FNPT (Regulator Pressure Gauge)	Grainger	1PEL6
Accessories: Male Adapter Push to Connect × FNPT (Flow Meters)	Grainger	36X026
Accessories: 1/4″ Push-to-Connect Y union	Grainger	1DDR5
Accessories: 1/4″ Push-to-Connect Equal union	Grainger	1PEV6
Accessories: 1/8″ × 1/4'' Push-to-Connect Unequal union	Grainger	1PEW2
Accessories: 1/8″ Push-to-Connect Equal union	Grainger	1PEV2
Accessories: Manual Needle Valve (Air and Exhaust Lines)	Grainger	4DGV3

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Accessories: Toggle Valves (Regulator and Pressure Gauge Panel)	Grainger	3ZVX3
Accessories: Rack-Mounted Regulator (Air, Vacuum, or Nitrogen Lines)	Grainger	4ZM06
Accessories: Blue Polyurethane Tube 1/4″ OD (Air Line)	MSC	48651442
Accessories: Red Polyurethane Tube 1/4″ OD (Exhaust Line)	MSC	48651681
Accessories: Black Polyurethane Tube 1/4″ OD (Nitrogen Line)	MSC	48651509
Accessories: Blue Polyurethane Tube 1/8'' (Air Line)	MSC	48655187
Accessories: Black Polyurethane Tube 1/8'' (Nitrogen Line)	MSC	74203712
Accessories: 1/8″ ID Tygon tubing (Vacuum Line)	Grainger	2LPR9
Accessories: Coalescing filter	Various	4ZL24
Accessories: 3-D Printed Organic Filter Holder	Dewan Lab GitHub	NA
Accessories: Super Clean Ultra-High-Capacity Hydrocarbon Filter (Air Line)	Restek	22030
Accessories: Filter Baseplate (Air Line)	Restek	22025
Accessories: Air Tank Regulator	Airgas	Y11215A590-AG
Accessories: Nitrogen Tank Regulator	Airgas	Y11215A580-AG
Accessories: Manual Needle Valve	Grainger	4DGW2
Accessories: Electronic Needle Valve	Clippard	EIVU-Z-ENS
Accessories: Stepper Motor Driver	Various	TB6600
Accessories: Zero Dead-Space fitting (Electronic Needle Valve)	Clippard	ZDVF-18
Behavioral Rig: Wheeled Tower	HPE Automation	DSD180713-RZ-1-E
Behavioral Rig: Standalone Behavioral Chamber	HPE Automation	DSD230619-RZ-1-A
Chamber: Sound-Proof Barrier (Foam Composite)	Soundproof Cow	Quiet Barrier Specialty Composite
Chamber: Acrylic Base	Dewan Lab GitHub	N/A
Chamber: Teflon Odor Port	Dewan Lab GitHub	N/A
Chamber: 3-D Printed Mouse Holder and Top	Dewan Lab GitHub	N/A
Chamber: 3-D Printed Mouse Holder Spacer	Dewan Lab GitHub	N/A
Chamber: 3-D Printed Odor Port Base	Dewan Lab GitHub	N/A
Chamber: 3-D Printed Exhaust Fan Duct	Dewan Lab GitHub	N/A
Chamber: 3-D Printed Final Valve Mount	Dewan Lab GitHub	N/A
Chamber: 3-D Printed Cleaning Bottle Holder	Dewan Lab GitHub	N/A
Chamber: 3-D Printed Electronic Needle Valve Mount	Dewan Lab GitHub	N/A
Chamber: Head-bar Clamp	Dewan Lab GitHub	N/A
Chamber: Bread Board (12″ × 12″)	Thorlabs	MB12
Chamber: 1/2'' Pedestal (Micromanipulator)	Thorlabs	RS05P
Chamber: 2″ Pedestal (Webcam)	Thorlabs	RS2P
Chamber: Pedestal Clamp for Micromanipulator	Thorlabs	CF125-P5
Chamber: Magnetic Base for Mouse Holder	Thorlabs	KB3X3
Chamber: 4″ Optical Post (LED)	Thorlabs	TR4
Chamber: Angle Bracket for Micromanipulator	Siskiyou	60050000E
Chamber: Micromanipulator	Siskiyou	62080000E
Chamber: Lick Tube	Various	304H22TW
Chamber: 4–40 Thumb Screw with Knob	Grainger	5RA21
Chamber: 4–40 Press Insert (Mouse Holder)	Grainger	1GUC6
Chamber: 1/4–20 Knurled Press Insert (Mouse Holder)	Grainger	4ZU39
Chamber: Webcam	Amazon	JUF4645
Chamber: Exhaust Fan	DigiKey	381-2335-ND
Computer Equipment: StarTech 2 Port USB 4K60Hz DisplayPort KVM Switch	CDW Government	SV211DPUA4K
Computer Equipment: 3-D Printed KVM Holder	Dewan Lab GitHub	NA
Computer Equipment: RackSolutions rack shelf 1U	CDW Government	115–4779
Computer Equipment: Middle Atlantic rack shelf 3U	CDW Government	RC-3
Computer Equipment: Middle Atlantic RM LCD PNLV LCD Panel rack mounting kit	CDW Government	RM-LCD-PNLV
Olfactometer: Commercially Available Olfactometer	Aurora Scientific	220A
Olfactometer: 3-D Printed Vial Holder	Dewan Lab GitHub	N/A
Olfactometer: Electronic Mounting Panel	Dewan Lab GitHub	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Olfactometer: 3-D Printed Manifold Mount	Dewan Lab GitHub	N/A
Olfactometer: Protective Acrylic Shield	Dewan Lab GitHub	N/A
Olfactometer: Mass Flow Controller (1 SLPM)	Alicat	MC-1SLPM-D/5M, 5IN
Olfactometer: Mass Flow Controller (100 SCCM)	Alicat	MC-100SCCM-D/5M, 5IN
Olfactometer: MFC to Manifold barb fitting	NResearch	FITM333
Olfactometer: Manifold with one normally open position	NResearch	225T082-1
Olfactometer: Manifold	NResearch	225T082
Olfactometer: Manifold Plug	NResearch	FITM128
Olfactometer: Pyrex cleaning bottle cap	Amazon	KSUS-GL45-3JM
Surgery: Head-bar	Dewan Lab GitHub	N/A
Surgery: Microscrews	Antrin Miniature Specialties	AMS120/1B ; 000-120 × 1/16 SL BIND MS SS
Water Delivery System: 60 mL syringe	VWR	15003–094
Water Delivery System: 3-D Printed Water Syringe Cap	Dewan Lab GitHub	N/A
Water Delivery System: Mounting Panel	Dewan Lab GitHub	N/A
Water Delivery System: 3-D Printed Water Dish Holder	Dewan Lab GitHub	N/A
Water Delivery System: Two-way Normally Closed Isolation Valve	NResearch	161T012
Water Delivery System: 1/32″ ID Tygon tubing	Grainger	22XH28

STEP-BY-STEP METHOD DETAILS

Constructing the behavioral rig

Timing: 1 week

1. Build the framed tower (optional).
   a. Construct the 80/20 T-frame system (Figure 1).
   b. Add rack-mounted monitor, computer, and keyboard.
2. Build the behavioral chamber.
   a. If applicable, construct the 80/20 isolated behavioral chamber kit.
   b. Drill a 3 in hole in the top panel for the fan.
   c. Drill a 2 in hole in one side panel for the odor, vacuum, and water tubing as well as the cords for the trial LED and webcam.
   d. Attach the fan and 3-D printed exhaust cover outside of the chamber.
   e. Line all inner chamber surfaces with sound-proofing material.
      i. Cut an "X" in the sound proofing material in line with the 2 in hole in the side panel to allow tubing and wires to pass through.
      ii. Cut a 3 in hole in the sound proofing material that lines up with the hole for the fan.
   f. Place the acrylic base on top of sound proofing material to serve as a stable base for the internal components of the chamber.
3. Assemble the components on a 12 in × 12 in breadboard (Figure 2).
   a. Assemble the odor port.
      i. 3-D print the odor port base with a high-density infill.
      ii. Attach the Teflon odor port to the 3-D printed odor port base with 2–56 screws.
      iii. Mount the odor port base on a 3-axis micromanipulator and connect it to the breadboard with 1/2 in pedestal and clamp.

Note: Once the odor exits the odor port, it is diluted by the ambient air. Thus, it is critical that the head position of the animal relative to the odor port is standardized using a micromanipulator.

      iv. Attach the vacuum line to the odor port base with a barbed connector and 1/8 in Tygon tubing.

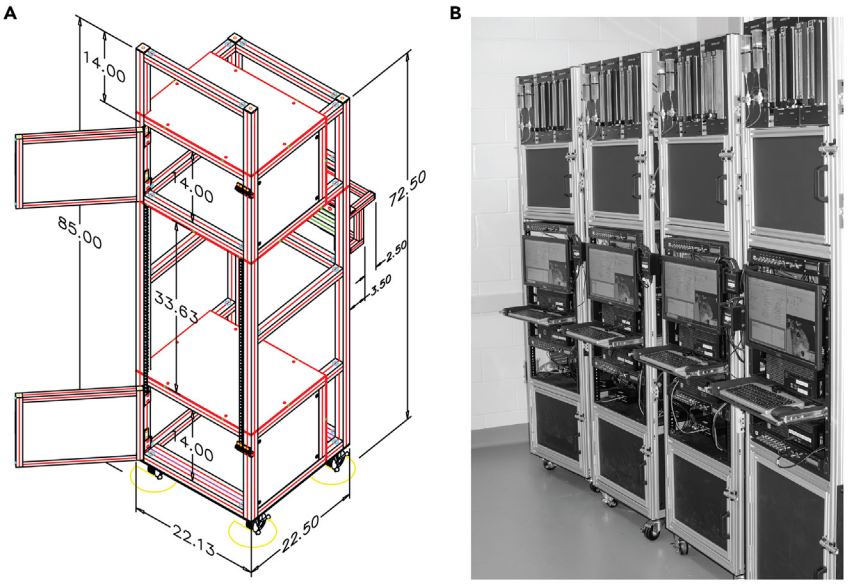

Figure 1. Building the behavioral rig towers

Each behavioral chamber is constructed using the 80/20 T-slot framing system.

(A) Blueprint of the wheeled tower version containing two behavioral chambers. Only one olfactometer frame is visible.

(B) Picture of four behavioral rigs within our laboratory space at Florida State University. One of the eight behavioral chambers is slightly larger to accommodate a freely moving odor investigation assay. The panels for the air and exhaust flow meters for both the top and bottom behavioral chambers are visible. The water delivery system for the top chamber is also visible. The water delivery system for the bottom chamber as well as the vacuum flow meters, olfactometers, final valves, and pressure gauges for the top and bottom chambers are not visible.

b. Assemble the mouse holder.
   i. 3-D print the mouse holder then attach the aluminum head-bar clamps with 4–40 knurled press inserts and 4–40 thumb screws.
   ii. Attach a male Molex pin to the outer clamp to attach to the lick-detector circuit.

Note: If the researcher uses the beam-break method for detecting licks, 3-D printed clamps can be used instead of metal clamps and the Molex pin.

   iii. Connect the mouse holder to the magnetic base with 1/4–20 knurled press inserts and 1/4–20 screws. Secure to the breadboard with 1/4–20 screws.

c. Set up the webcam and trial LED.
   i. Attach the webcam to a 2 in pedestal and connect to the breadboard. Position the webcam at the proper angle to visualize the mouse.
   ii. Position the trial LED anywhere in the chamber on a 4 in optical post and secure it to the breadboard.

d. Place the assembled breadboard on the acrylic base.

4. Obtain or construct the olfactometer.
   a. If necessary, 3-D print odor vial holders and manifold holders (see key resources table for other 3-D printed olfactometer accessories).
   b. If necessary, attach the protective acrylic shield on top of the olfactometer frame.
   c. Mount the olfactometer to the back of the 80/20 frame or position it near the behavioral chamber.

Note: In our setup, a nitrogen tank with regulator serves as the source for the carrier mass flow controller (MFC), while regulated compressed house air sent through coalescing and

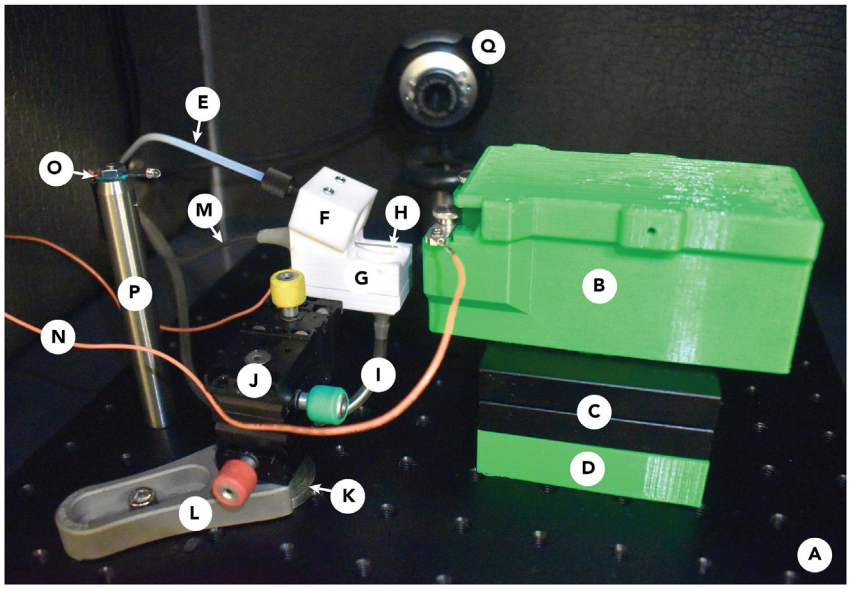

Figure 2. Inside the operant chamber

The mobile tower frames two behavioral chambers. A 12 in × 12 in breadboard (A) acts as an anchoring platform inside of the chamber. The mouse is head-fixed within the 3-D printed mouse holder (B) that is secured on a magnetic base (C) and a 3-D printed riser (D). Odorized or clean air is delivered via 1/8 in Teflon tubing (E) to the odor port (F). The odor port sits atop a 3-D printed odor port base (G), which accommodates a lick tube (H) and vacuum line (I). The combined odor port and base are mounted to a micromanipulator (J) to standardize the distance between the odor port and the nose of individual animals. The micromanipulator is mounted to the breadboard with a 1/2 in pedestal (K) and clamp (L). Water rewards are administered via 1/32 in Tygon tubing (M) to the lick tube (H) after a response has been made by the animal. Lick responses are monitored by an electronic lick circuit that connects to the animal's metal head-bar and the lick tube via two wires (N). A LED light (O) mounted on an optical post (P) indicates the trial initiation and duration. A vacuum line (I) is attached to the odor port base (G) and ensures the removal of prior stimuli before the next trial presentation. Any residual odor in the chamber is removed by an exhaust fan (not visible) placed in the top of the behavioral chamber. A webcam (Q) allows surveillance of an animal inside the chamber. Designs and files for the 3-D printed parts are available on GitHub: https://github.com/OlfactoryBehaviorLab/.

ultra-high-capacity hydrocarbon filters serves as the source for our dilutor MFC and clean air line (see below).

Note: Nitrogen gas is used to minimize odor oxidation across sessions.

5. Assemble the flow meters, valves, and tubing (Figure 3).
   a. Attach the final valve to the 3-D printed final valve mount and secure inside or outside of the behavioral chamber.

Note: When the final valve is actuated, the odorized air from the olfactometer is directed towards the animal while the clean air is directed towards the exhaust line. The resting state of the final valve sends odorized air from the olfactometer to the exhaust line, while the clean air is directed towards the animal. This setup allows the odor to reach a steady state concentration before it is presented to the animal– ensuring a repeatable square odor pulse with a minimal time delay.

⚠ CRITICAL: To shorten the time delay between actuating the final valve and the animal receiving the odor, it is necessary to minimize the distance between the final valve and odor port.

   b. Connect the olfactometer to the final valve with 1/8 in Teflon tubing, ferrules, and nuts.

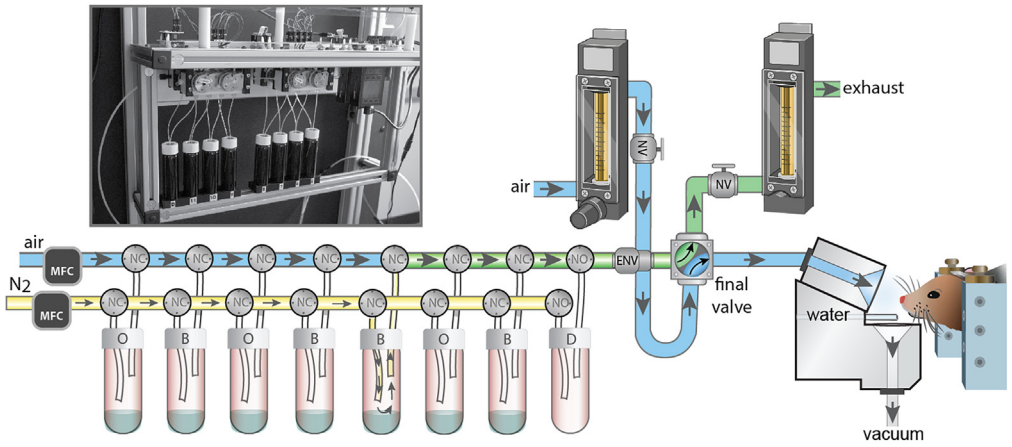

Figure 3. The odor delivery setup

Odor is delivered using an eight-channel olfactometer (inset) that switches between a pressure-balanced dummy (D) vial (via normally open valves, NO) and either odor (O) or blank (B) vials containing only the solvent (via normally closed valves, NC). Odorized air is directed to exhaust to allow the stimulus to reach equilibrium prior to stimulus delivery. Manual needle valves (NV) help equalize the pressure differences between the clean air line and olfactometer as well as the exhaust and odor port lines. An electronic needle valve (ENV) balances the pressure differences associated with different vials on a per trial basis. During stimulus application, the final valve re-directs pressure-balanced, odorized air from exhaust to the animal. At the conclusion of the trial, the final valve returns the pressure-balanced clean air to the animal.

i. If necessary, set up the electronic needle valve to minimize air pressure differences associated with individual odor vials.
ii. Attach the electronic needle valve to the 3-D printed electronic needle mount and secure to the framed tower between the olfactometer and final valve.
iii. Connect the electronic needle valve to the olfactometer and final valve with 1/8 in Teflon tubing, ferrules, and nuts.
iv. Control the electronic needle valve with the stepper motor driver.

c. Connect the 2.5 L clean air flow meter to the final valve with 1/8 in Teflon tubing, ferrules, and nuts.
   i. Mount the clean air flow meter onto a flow meter panel on the front of the framed tower to allow for easy visualization and adjustment.
   ii. Connect the input of the clean air flow meter to the regulated purified air line with 1/4 in polyurethane tubing and push-to-connect × FNPT fittings.

Note: An in-line manual needle valve can be added to equalize any resistance differences between the two final valve inputs (i.e., the olfactometer and clean air flow meter).

d. Connect the final valve to 2.5 L exhaust flow meter with 1/8 in Teflon tubing, ferrules, and nuts.
   i. Mount the exhaust flow meter onto a flow meter panel on the front of the framed tower to allow for easy visualization.
   ii. Direct the output of the exhaust flow meter to an area of negative pressure (e.g., a fume hood) using 1/4 in polyurethane tubing and push-to-connect × FNPT fittings.

Note: In-line manual needle valves can be added to equalize any resistance differences between the two final valve outputs (i.e., the odor port and exhaust flow meter).

⚠ CRITICAL: The exhaust flow meter is useful to diagnose leaks and pressure disparities in the system.

e. Attach the 10 L vacuum flow meter to the barbed connector on the odor port base with 1/8 in Tygon tubing, ferrules, and nuts.
   i. Mount the vacuum flow meter onto a flow meter panel on the back of the framed tower.
   ii. Connect the vacuum flow meter input to the regulated vacuum line with 1/4 in polyurethane tubing and push-to-connect × FNPT fittings.

6. Ensure the olfactometer can deliver a consistent and repeatable odor pulse.

Note: The kinetics and concentration of the odor pulse from the olfactometer can be tested using a photoionization detector (PID).

a. Determine the time delay between opening an odor vial and the concentration reaching a steady state at the final valve location.
   i. Position the inlet port of the PID at the final valve location and measure the time necessary for the odor to reach steady state.
   ii. Modify the training and testing scripts to include this time delay. This approach will ensure the animal will receive a consistent square pulse of odor.
b. Determine the time delay between final valve actuation and the odor reaching the animal.
   i. Position the inlet port of the PID 1 cm from the center of the odor port and measure the time course of the odor pulse relative to actuation of the final valve.

Note: To minimize this time delay, it may be necessary to move the final valve closer to the odor port.

c. Ensure that the headspace of an odor vial does not run out during a stimulus presentation and is sufficiently able to recharge during the inter-trial interval.
   i. Position the inlet port of the PID 1 cm from the center of the odor port and measure the amplitude and kinetics of the odor pulse over multiple trials.

7. Set up the gravity-fed water delivery system.
   a. Mount a 60 mL syringe with blunt tip needle and two-way normally closed isolation solenoid valve on the water panel.
      i. Connect the syringe needle to the solenoid valve with 1/32 in Tygon tubing and appropriate connectors.
   b. Mount water panel on the front of the framed tower.
   c. Connect the solenoid valve to the lick tube on the odor port base with 1/32 in Tygon tubing.
   d. Calibrate the water delivery system to deliver 1.2–2.0 μL (or 1.2–2.0 mg) per drop.
      i. Place a microcentrifuge tube under the lick tube and collect 100 droplets of water.
      ii. Each water reward will be equal to the difference in weight between the empty and water-filled tube divided by 100.
      iii. Adjust the volume of each water reward by varying the amount of time the two-way solenoid valve is open.
   e. Protect the opening of the syringe from dust or debris with a removable 3-D printed syringe cap.

8. Set up the software and valve drivers.

Note: Researchers should utilize the hardware and programming language that they are the most comfortable with and best suits their needs. Below, we include links to all the necessary documentation and information to reproduce our approach.

a. Download the python-based experimental software for use with an Arduino platform at (https://github.com/olfa-lab/Voyeur), produced in the Rinberg Lab.[5]
b. Download training and testing scripts at (https://github.com/OlfactoryBehaviorLab/).
c. Obtain the Arduino-based behavioral control system box licensed by HHMI Janelia Farms (https://www.janelia.org/open-science/behavioral-control-system-box).

Mouse surgeries and water deprivation

Timing: 17 days

9. Perform head-bar surgeries.
   a. Prepare for aseptic surgery.
      i. Anesthetize the animal to areflexia with isoflurane.
      ii. Administer an extended-release analgesic (e.g., Buprenex ER) and protect corneas with ophthalmic ointment.
      iii. Shave mouse head and clean the area to be incised with a Betadine scrub and 70% ethanol (3×).
   b. Perform head-bar surgical procedures.
      i. Perform a 'line' block of a local anesthetic (e.g., lidocaine) at the incision site.
      ii. Incise the scalp and retract to expose the skull. Gently remove the periosteum with a cotton swab and dry the skull.
      iii. Insert two or three microscrews without penetrating the skull. One microscrew is placed posterior to lambda, while the other two are rostral and lateral to the head-bar.

      Note: Ensure that there is enough space between screws to accommodate the width of the head-bar.

      iv. Place an aluminum head-bar (3 mm × 15 mm, <1 g) on the skull and close skin against the head-bar with a small amount of Vetbond or cyanoacrylate.
      v. Cement the head-bar in place using dental cement.
      vi. Affix a small label to the outside of the dental cement using cyanoacrylate to identify the animal.
      vii. Prior to discontinuing anesthesia, reapply ophthalmic ointment. Remove the animal from the stereotaxic head holder.
   c. Allow mice to recover post-surgery.
      i. Place mouse on a thermostatically controlled heating pad until they recover.
   d. Singularly house the animal with ad libitum access to food and water.
   e. Allow the mouse to recover for at least two days before starting water deprivation.
   f. Record the animal's baseline weight.
10. Begin water deprivation protocol.
   a. Visually inspect the hydration status and overall health of each animal daily.
   b. Pipette water into a petri dish that is housed inside of a 3-D printed water dish holder in the cage. Mice will have unrestricted access to this water.
      i. Mice with a weight in the range of 80%–100% of their baseline weight receive 1.0 mL of water.
      ii. Mice with a weight in the range of 75%–80% of their baseline weight receive 1.2 mL of water.
      iii. Mice with a weight in the range of 72%–75% of their baseline weight receive 1.3–1.5 mL of water.
      iv. Mice with a weight less than 70% of their baseline weight will 2.0 mL of water. If mice are unable to regain body weight to the desired 80% of their baseline, they are removed from the study.
   c. Continue water deprivation for at least 14 days before starting the lick training protocol.

Lick training

Timing: 4–7 sessions

11. Acclimate mice to head-fixation.

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Note: The initial acclimation to head-fixation can be stressful to the animal and should be performed with care. Thus, we prefer to gently guide the animal into being head-fixed using the guidelines below. If the animal exhibits excessive struggling or persistent vocalizations, it is removed from head-fixation.

a. Set the inter-trial interval to a max of 2 s and start the lick training script (https://github.com/OlfactoryBehaviorLab/).

Note: The short inter-trial interval is designed to increase the probability of receiving a water reward if the animal licks the lick tube.

b. Prime the water delivery system.

Note: It is critical that the animal is rewarded when it licks at the appropriate time. Thus, it is necessary to ensure that the water delivery system is working properly prior to the start of the session.

c. Place a few drops of water on the end of the lick tube to stimulate licking behavior.
d. Place the mouse in the mouse holder without securing the head-bar.
e. Gently guide the mouse to the lick tube by providing water with a 10 mL blunt tip syringe.
f. Once the animal is licking for water, secure the mouse by placing the head-bar in the grooves of the mouse holder.
g. Use the micromanipulator to adjust the lick tube position.
    i. Ensure that their tongue can reach the lick tube, and that their licks are registering in the program.
h. Close the behavioral chamber door and continue to monitor the animal via the webcam.
i. Allow the animal to receive water for 20–30 min, then return them to their home cage.
j. Record the amount of water they drank.
k. At the end of the day, give the mice their remaining water allotment in their home cage.

12. Train mice to lick for a water reward.

Note: Following successful acclimation, the animal will allow head-fixation without struggling and begin licking the lick tube for water rewards immediately upon entering the behavioral chamber.

a. Prime the water delivery system.
b. Secure mouse in the mouse holder.
c. Adjust lick tube position with the micromanipulator and start the lick training script (https://github.com/OlfactoryBehaviorLab/).
d. Close the behavioral chamber door and continue to monitor the animal via the webcam.
e. Allow the animal to lick for water for 30–50 min, then return them to their home cage.
f. Record the amount of water drank.
g. At the end of the day, give the mice their remaining water allotment in their home cage.

Note: Over successive sessions, the goal is to gradually acclimate the mice to longer time periods of head-fixation (~45 min) and to longer inter-trial interval (at least 8 s). The animal will learn that the small pressure spike associated with the final valve actuation and the trial LED signals the start of the next trial.

Odor training

Timing: 3–6 sessions

13. Condition mice to report the detection of a suprathreshold concentration of odor using a standard Go/No-Go paradigm.

Note: Correct Go responses during the 2 s stimulus window are rewarded with water (1.2–2 μL) and a short inter-trial interval (5–9 s). Correct No-Go responses receive only the short inter-trial interval, while incorrect responses are punished with a long inter-trial interval (15–19 s). Inter-trial intervals are randomized within these ranges to prevent mice from anticipating trial start times.

⚠ CRITICAL: Since over-motivation from increased thirst can mask true olfactory sensitivity,[6] the first ten trials of every session are Go trials and are not included in the analyses.

a. Prepare the olfactometer with vials containing a suprathreshold odor concentration and solvent only blanks.

Note: We typically utilize vapor-phase odor concentrations between 10–500 ppm for training.

b. Ensure that your selected odorant and solvent are guided by the solubility properties of the odorant.

Note: Utilize PubChem or another resource outlining the chemical properties of a given chemical prior to solvent selection. Whenever possible, we prefer mineral oil, as this solvent does not yield a detectable PID signal – allowing us to easily quantify odor pulses in our experimental setup.

c. Prime the water delivery system.
d. Secure mouse in the mouse holder and adjust the odor port and lick tube position with the micromanipulator.
e. Run the training session (https://github.com/OlfactoryBehaviorLab/).
   i. Terminate the session if the mouse misses three Go trials in a row or reaches 250 trials.
   ii. Record the amount of water they drank, then return the mouse to its home cage.
   iii. At the end of the day, give the mice their remaining water allotment in their home cage.
f. Calculate behavioral performance as the number of correct responses (hits + correct rejections) divided by the total number of trials (excluding the 10 initial Go trials).
g. Terminate odor training once the animal reaches two consecutive training sessions with $\geq$90% behavioral accuracy.

14. Calculate vapor-phase concentrations.

⚠ CRITICAL: The researcher cannot rely on liquid dilutions to extrapolate vapor-phase concentrations based on ideal gas behavior. For most chemicals, the relationship between liquid and vapor concentration deviates from these laws of proportionality due to interactions between the odorant and solvent.[7]

a. Estimate vapor-phase concentrations of diluted odorants using a PID by referring to our previously described method.[8]
   i. Measure the vapor-phase concentration of an odorant in your setup by positioning a calibrated PID in the location of the mouse.
   ii. Convert the resulting voltage to a vapor-phase concentration using your isobutylene calibration equation and the correction factor for the odorant. Please see Jennings et al., 2022[8] for more details.

Note: An archive of equilibrium equations for more than 100 odorant/solvent pairs (that can be utilized without performing any analytical measurements) is also available.[7,8]

15. Clean the olfactometer.
    For many odorants, our cleaning protocol involves flushing the manifolds and tubing with 70% isopropanol, then nanopure water, followed by air. However, additional solutions (e.g., acetone) may be necessary for more "sticky" odorants. Each solution is forced through all manifolds and tubing via a modified 500 mL glass bottle with a specialized cap to allow an inlet for compressed air and an outlet for the cleaning solution.

    Note: The olfactometer should be cleaned when the researcher changes odor identity or concentration.

    a. Prepare the olfactometer.
    b. Open the cleaning script (https://github.com/OlfactoryBehaviorLab/).
        i. Remove odor vials.
        ii. Disconnect the tubing from between the MFCs and manifolds.
        iii. Disconnect the olfactometer output tubing from electronic components.
    c. Direct tubing into a solution collection tub or waste container.

    Note: Liquid will be expelled from the olfactometer output tubing and the tubing from each vial when actuated.

    d. Load the 500 mL glass bottle with the cleaning solution and connect the output to both the carrier and dilutor manifolds.
        i. Flush ~300 mL of each cleaning solution through the manifolds over a period of approximately 60–75 s. The solenoids associated with each vial are actuated for approximately 5–6 s.
    e. Clean the vial septa, vial caps, and vial tubing.
        i. Fill a vial with cleaning solution and connect to the vial cap. Invert several times. Repeat for each vial.
    f. Allow clean air to flow through the manifolds and tubing for a minimum of 1 h.

    Note: The solenoids associated with each vial must be sporadically actuated to ensure that vial tubing is clean and dry. We typically allow the pressurized clean air to flow through the tubing and manifolds overnight.

Odor thresholding

Timing: 7–10 sessions

16. Perform the odor thresholding assay.
    This experiment measures an animal's olfactory sensitivity by determining their behavioral performance to a series of decreasing odor concentrations in the same Go/No-Go operant conditioning task described above.

    Note: The first session should utilize the training odor concentration.

    a. Prepare the olfactometer with the appropriate Go and No-Go stimuli. Utilize 3 Go vials, 3 No-Go vials, and one cheating check vial.

    Note: Vial locations are randomized daily.

b. Prime the water delivery system.
c. Secure mouse in the mouse holder and adjust the odor port and lick tube position via with the micromanipulator.
d. Run the experimental session (https://github.com/OlfactoryBehaviorLab/).
   i. Terminate the session if the mouse misses three Go trials in a row or reaches 250 trials.
   ii. Record the amount of water they drank, then return the mouse to its home cage.
   iii. At the end of the day, give the mice their remaining water allotment in their home cage.
e. Calculate behavioral performance.
   i. Behavioral performance is calculated as the number of correct responses (hits + correct rejections) divided by the total number of trials (excluding the 10 initial Go trials).
   ii. Researchers can also use signal detection theory (i.e., D-prime) to measure the mouse's ability to discriminate the signal from the noise at each odor concentration.

Behavioral threshold analysis

17. Fit behavioral performance data with the following four-parameter equation.

$$R = R_{min} + C^n * \frac{R_{max} - R_{min}}{\left[C^n + C_{1/2}^n\right]}$$

where R is behavioral accuracy, C is odor concentration, $C_{1/2}$ is the odor detection threshold, and n is the Hill coefficient.

EXPECTED OUTCOMES

The consistency of our approach is rooted in repeatedly measuring the animal's ability to perceive a stable odor pulse. Thus, it is critical that the odor concentration for each trial remain stable across the session. To test the consistency and timing of the odor pulse, a PID is placed in the same position as the animal. A single odor vial is actuated 250 times with a 15 s inter-trial interval and the resulting signal from the PID is recorded using the Arduino-based behavioral controller (Figure 4A). Mice typically learn to lick for a water reward rapidly within the first session. As stated above, we prefer to gently coax the animal into head-fixation, as we find that it reduces stress, promotes rapid learning, and increases the number of trials an animal is willing to perform. Upon completing lick training, the animals are trained to discriminate an odor stimulus from the solvent in a Go/No-Go paradigm. Mice begin to learn this task toward the end of the first session (Figure 4B). Specifically, behavioral performance (averaged with a sliding window of 100 trials) typically begins to rise around trial 200 and reaches the training criterion of $\geq$90% around 600–1200 trials or 2–6 sessions. For the odor thresholding assay, behavioral performance for a single concentration typically stabilizes after 150–175 trials (Figure 4C). The average behavioral performance of each animal at each concentration is fit with a Hill function to form a psychometric curve. Our behavioral method has resulted in estimates of olfactory sensitivity that are consistent within and between cohorts of mice, tested in different behavioral rigs, by different experimenters[1–4] (Figure 4D). In addition, this approach is sensitive enough to measure detection deficits associated with the genetic deletion of an entire class of olfactory receptors[4] (Figure 4E) or even a single olfactory receptor[4] (Figure 4F).

LIMITATIONS

The primary limitation of our approach is that it is time consuming. Specifically, our estimation of threshold typically takes 7–10 days (7–10 sessions) as compared to 1–4 days (1–8 sessions) using other methods.[9,10] Our longer time frame results from: 1) measuring only one odor concentration per day, 2) a solvent versus solvent session to verify that the animal cannot use non-odor cues to boost performance, and 3) measuring more than one sub-threshold concentration to produce a full psychometric curve. We favor the one concentration per day method because it prevents contamination of the common odor pathways by higher concentrations of the target odor. This

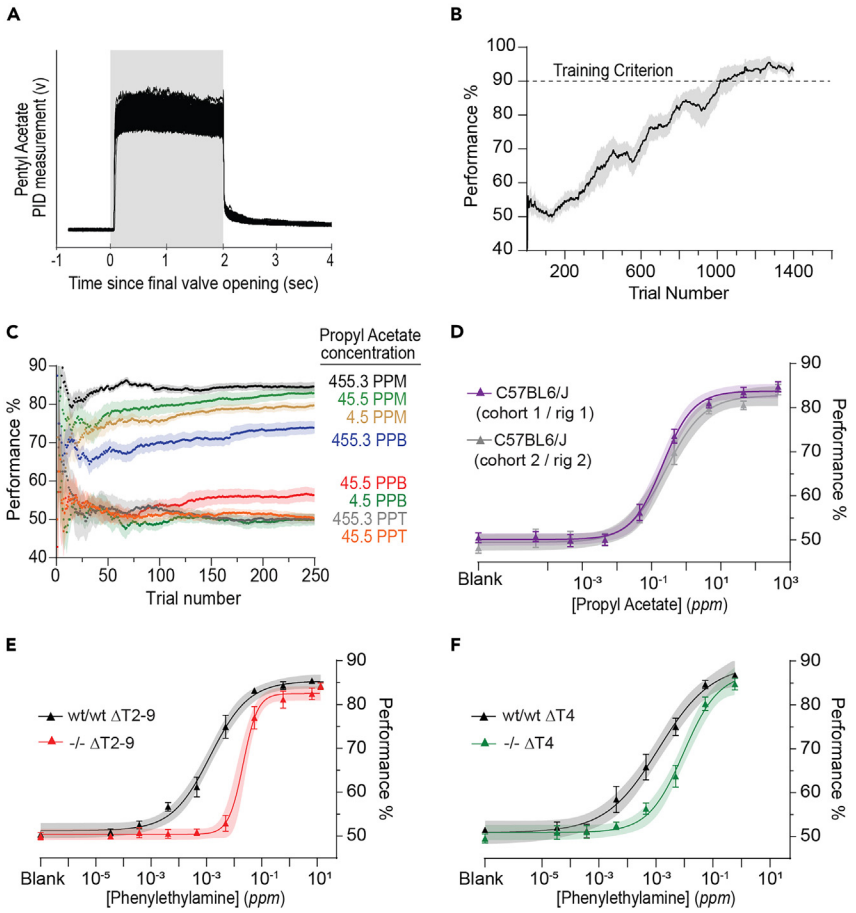

Figure 4. Example data

(A) Example odor pulse. Photoionization detector (PID) traces of 250 stimulus presentations of pentyl acetate. Shaded area signifies 2 s stimulus period. Data is from Jennings et al., 2022.[2]

(B) Example learning data. Running average (100-trial sliding window) of trial-by-trial behavioral performance of a single cohort of mice learning to discriminate between hexyl acetate (13 ppm) and a mineral oil blank. Data is from Johnson et al., 2023.[1]

(C) Average cumulative behavioral performance across 250 trials for all concentrations of propyl acetate. Initial Go trials are not included. Line signifies mean with shaded SE. Final behavioral performance for each concentration is plotted in the next panel (cohort 2/ rig 2). Data is from Jennings et al., 2022.[2]

(D) Our experimental approach does not differ across mouse cohorts, different behavioral setups or olfactometers, or even different experimenters. Data is from Jennings et al., 2022[2]; however, the consistency of our approach has been demonstrated multiple times.[1,3,4,6]

(E) Our experimental approach can detect behavioral sensitivity differences between wildtype littermates (wt/wt ΔT2-9; wt/wt ΔT4) and those lacking all of their olfactory trace amine-associated receptors (−/− ΔT2-9) or even mice lacking a single trace amine associate receptor (−/− ΔT4).

(F) Data is from Dewan et al., 2018.[4] Data (D–F) were fitted using a Hill function. Maximal behavioral performance for each odorant concentration is limited to ~85% (due to the inclusion of the cheating check). Plots show mean +/- SE with shaded 95% confidence interval.

is advantageous because the mouse's ability to detect near-threshold concentrations cannot be directly masked by residual contamination or indirectly masked by adaptation resulting from the presentation of higher concentrations of the target odor. Further, the test-retest reliability of our method is likely rooted in repeatedly measuring the animal's ability to perceive a stable odor pulse. Thus, we are also limited in the number sessions an animal will perform within a day before losing motivation for the water reward. Lastly, while this protocol focuses on olfactory sensitivity, we have also successfully used this setup to assess olfactory discrimination ability.[1]

TROUBLESHOOTING

Problem 1

Behavioral performance is artificially enhanced by a non-odor cue.

Potential solutions

Identify the non-odor cue that is artificially enhancing behavioral performance. The three most likely culprits are auditory, olfactory, or somatosensory cues.

- *Auditory*: A white noise machine can be used to mask distinct auditory cues resulting from the actuation of the solenoid valves associated with each vial.
- *Olfactory*: The olfactometer should always be properly cleaned to prevent unique odor blends that are associated with vial locations.
- *Somatosensory*: The flow through individual vials can result in minor pressure differences. For our setup, these pressure cues can be the source of artificially enhanced behavioral performance. To overcome this issue, an electronic needle valve can be programmed to match the pressure differences associated with each individual vial by adding a bi-directional change in resistance.

Problem 2

Animals are not reaching learning criterion.

Potential solutions

- *Clean the olfactometer.* Odor contamination could mask the detection of the training concentration. In addition to cleaning the olfactometer with the protocol described above, we recommend replacing the tubing, vial caps, and septa after every experiment.
- *Use a higher odor concentration.* It is important to start training the animal at a suprathreshold concentration.
- *Check the water delivery system.* Inconsistent water delivery can prevent the animals from associating the Go stimulus with the water reward, thereby limiting their ability to learn the task.
- *Check the vacuum line.* Insufficient suction can cause a buildup of odor inside of the behavioral chamber.

Problem 3

Mice tested at the end of the day have lower behavioral performance than those tested earlier in the day.

Potential solution

- *Use the PID to verify the odor concentration of each subsequent session.* It is critical to replace the odor vials prior to a significant drop in concentration.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Adam Dewan (dewan@psy.fsu.edu).

Materials availability

Materials are publicly available at (https://github.com/OlfactoryBehaviorLab/). Newly generated materials associated with this protocol will be uploaded to this link. Archived versions are available at (https://doi.org/10.5281/zenodo.8353698).

Data and code availability

Datasets and code are all available at (https://github.com/OlfactoryBehaviorLab/). Archived versions are available at (https://doi.org/10.5281/zenodo.8353698). For examples of data collected

using this protocol, please view Johnson et al. (2023),[1] Jennings et al. (2022),[2] Williams and Dewan (2020),[3] and Dewan et al. (2018).[4]

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AUTHOR CONTRIBUTIONS

E.W. and A.D. developed this protocol. E.W. collated the part list for the key resources table. C.E.J. and A.D. wrote the manuscript with input from E.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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