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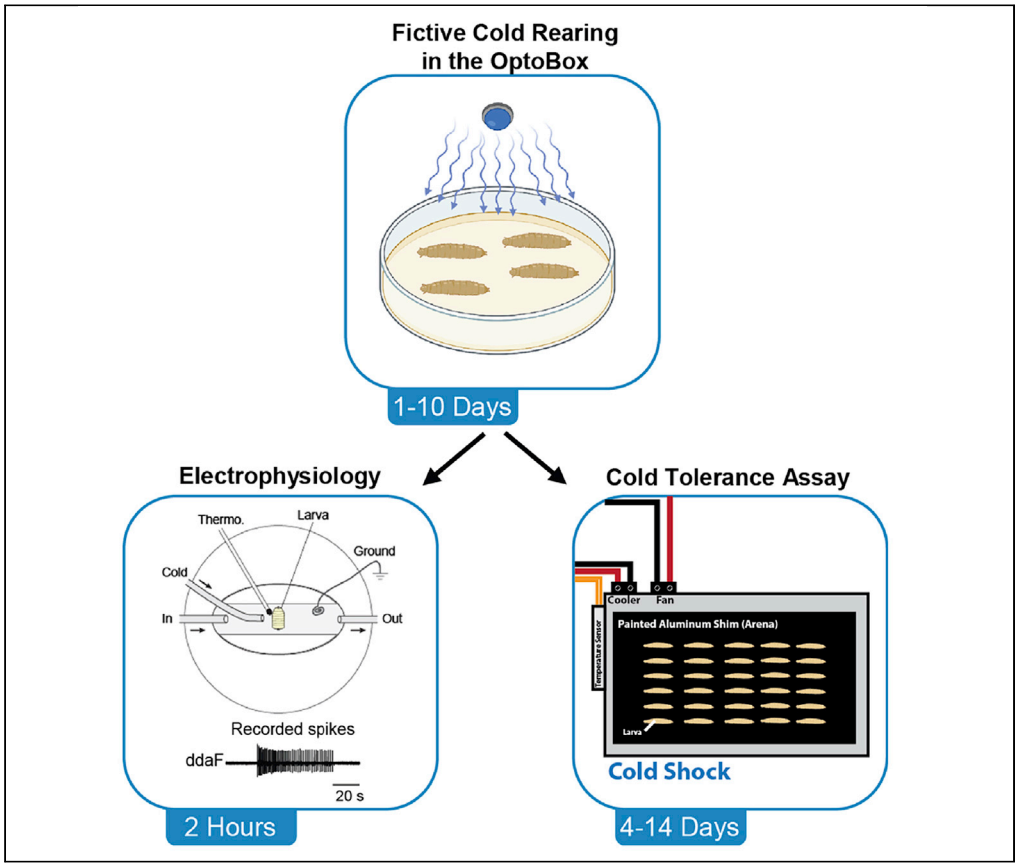
Protocols for measuring cold-evoked neural activity and cold tolerance in *Drosophila* larvae following fictive cold acclimation



Here, we outline protocols to study cold acclimation in *Drosophila* from a neurobiological perspective, starting with fictive cold acclimation using a custom-built optogenetics-housing apparatus we call the OptoBox. We also provide detailed steps for single-unit electrophysiological recordings from larval cold nociceptors and a high-throughput cold-tolerance assay. These protocols expand the toolkit for the study of insect cold acclimation and nociception.

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

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Highlights

Design for the OptoBox, a rig for developmental optogenetic activation of *Drosophila*

Protocol for fictive cold acclimation via the OptoBox

Protocol for electrophysiological recordings from larval cold-sensing neurons

Protocol for a cold-tolerance assay adapted from a cold-evoked behavior assay

Himmel et al., STAR Protocols 3, 101510
September 16, 2022
https://doi.org/10.1016/j.xpro.2022.101510

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Protocols for measuring cold-evoked neural activity and cold tolerance in *Drosophila* larvae following fictive cold acclimation

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https://doi.org/10.1016/j.xpro.2022.101510

SUMMARY

Here, we outline protocols to study cold acclimation in *Drosophila* from a neurobiological perspective, starting with fictive cold acclimation using a custom-built optogenetics-housing apparatus we call the OptoBox. We also provide detailed steps for single-unit electrophysiological recordings from larval cold nociceptors and a high-throughput cold-tolerance assay. These protocols expand the toolkit for the study of insect cold acclimation and nociception.

For complete details on the use and execution of this protocol, please refer to Himmel et al. (2021).

BEFORE YOU BEGIN

The ability of insects to acclimate to changes in temperature is critical to their survival, distribution, and evolution. In our recent work, we have shown that noxious cold-sensing neurons (cold nociceptors) participate in the ability of *Drosophila melanogaster* larvae to acclimate to cold (Himmel et al., 2021). Previous work outlines methods for the study of insect cold tolerance (e.g., (Andersen et al., 2015)); here, we give a detailed outline of methods for rearing larvae under fictive cold via optogenetics, a method we have shown can cause cold acclimation in the absence of cold temperatures (Himmel et al., 2021). We also provide a step-by-step instruction for single-unit electrophysiological recordings from larval cold nociceptors and describe a novel method for assessing cold tolerance in larvae, making use of a relatively inexpensive rig modified from a previously described cold-plate behavior assay (Himmel et al., 2020; Patel and Cox, 2017; Turner et al., 2016).

Before you begin, you will need to construct the OptoBox and prepare materials for the electrophysiological experiments.

Construction of the OptoBox

Timing: 5–7 days

The OptoBox was designed to serve as a high-throughput, portable enclosure which enables custom control of developmental optogenetic activation in *Drosophila* larval neuro-behavioral experiments. In the experimental paper linked to this protocol, larvae expressing channelrhodopsins in cold-sensing neurons were reared under regular optogenetic activation, thereby delivering what we call a fictive-cold stimulus. This method is not, strictly speaking, an alternative to rearing animals at cold temperatures; rather, this allows for the selective study of cold acclimation resulting from

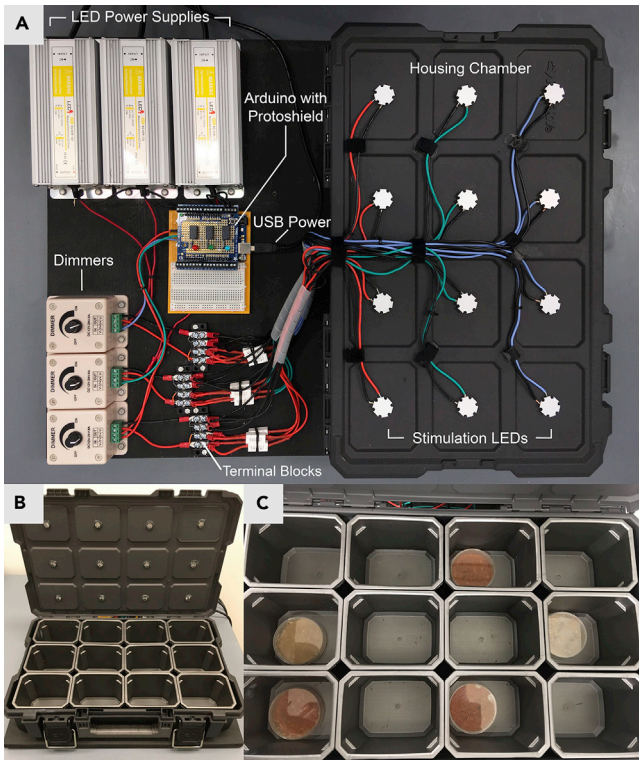

Figure 1. The OptoBox

(A) Overhead view of entire device, including all components and wiring with lid closed.

(B) Optobox open, with the 12 lenses visible on the lid.

(C) Examples of dishes housing larvae inside OptoBox chambers. Darker orange dishes are those containing ATR.

chronic cold-sensing neural activation, thereby excluding any other effects resulting from chronic exposure to actual cold. In this context, this method should allow for the continued dissection of the neural component of insect cold acclimation.

The OptoBox device is constructed from readily available components from online vendors or local hardware stores (see key resources table). Minimal assembly skills are necessary beyond basic soldering and drilling. Here, we made use of a 12-chamber Husky brand small-parts organizer purchased from a hardware store; however, any chamber of sufficient size can be used for this purpose.

Arduino control software was written to control three 5-volt outputs which activated and deactivated the associated relays (code available at the Cox Lab GitHub site: https://github.com/CoxLabGSU). This mechanism allows for constant, immediate, availability of current from the power supplies to the LEDs, similar to a light switch. Users can assign millisecond values to the duration and spacing of pulses. In its current configuration, stimulation occurs until a user turns off the Arduino by disconnecting the USB cable from a computer monitor (or another USB power source). Alternatively, one could alter the control script to cease stimulation at a given number of cycles or at a specific time of day.

1. Prepare the housing chamber (Figure 1).
   a. Select an appropriate housing chamber (box); ideally, this chamber will have several independent compartments large enough to house standard 60 × 15 mm Petri dishes, and will have a lid which can be opened/closed.
      i. The box should be made light impermeable; if it contains any transparent parts, a spray paint can be used to cover those parts.

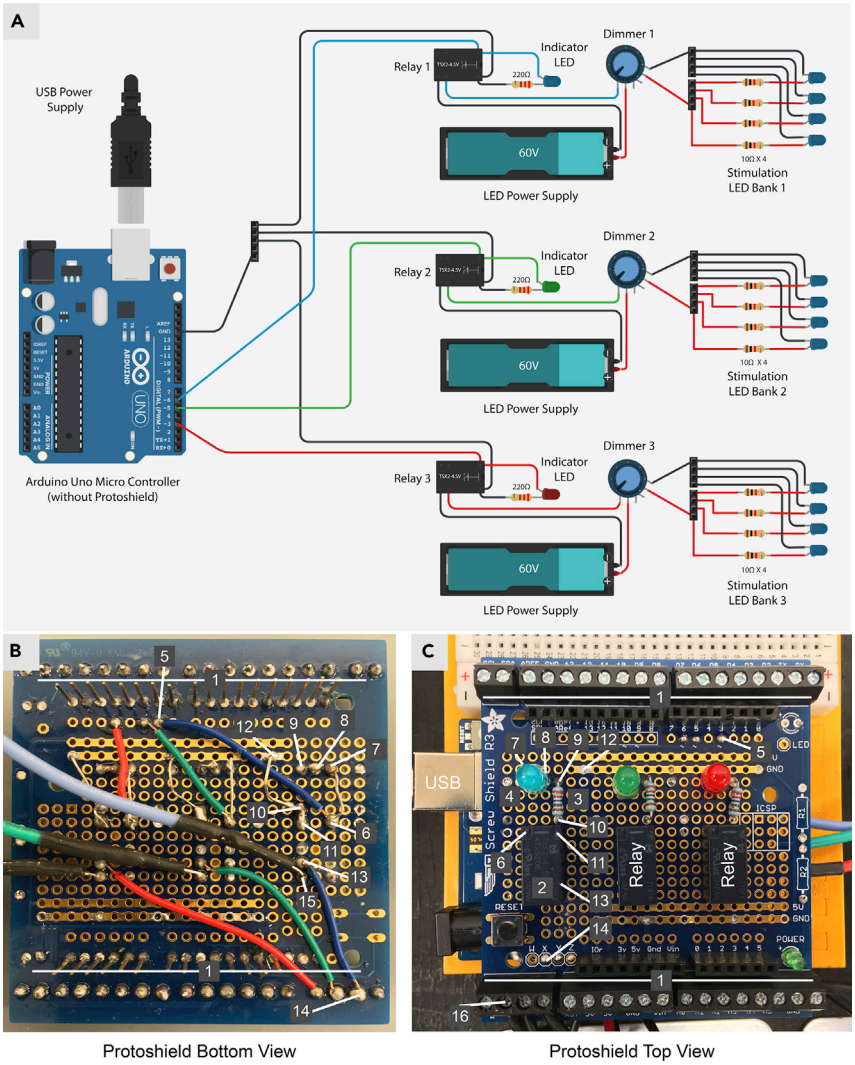

Figure 2. Wiring diagram for the OptoBox

(A) Wiring diagram.

(B and C) Images of Arduino Protoshield circuit wiring when viewed from bottom (B) and top (C) of shield. Optional use of this shield enables wiring consolidation while maintaining modular flexibility for alternate experimental configurations.

b. Drill holes in the lid of each of the chambers using a spade bit, making sure the hole is centered above each underlying compartment.

2. Attach the LEDs.
   a. Epoxy lenses to the underside of each of the holes and allow to dry overnight.
   b. Superglue a single LED to each of the lenses from the outside of the enclosure, with positive and negative wires soldered to the appropriate terminals on the LEDs.
3. Wire the OptoBox (Figure 2).
   a. Solder 10 ohm resistors inline on the anode (positive) side exiting the LED.
   b. Terminate these wires with spade connectors attached to terminal blocks, splitting the 12 individual LEDs into sets of 4.
   c. Using short lengths of 20 g wire (orange and gray wires in Figure 1A), connect adjacent terminal block lanes to create these sets.
   d. Connect each set to a separate dimmer controller and relay (Dimmer Out V+ and V-).

4. Set up Proto-Screw Shield to receive connections from the dimmers and relays and connect to the relevant Arduino microcomputer inputs and outputs. While the Optobox control circuit (Figure 2) can be assembled with standard jumper wires and an adjacent prototyping breadboard, we opted instead to use an Arduino Proto-Screwshield. This allows for increased modularity for future circuit upgrades, more reliable connections, and consolidation of wiring. All shield connections are made via soldering of components from the underside of the shield unless otherwise noted. Components and connections are numerically annotated in Figure 2B to aid in the assembly process.
   a. Attach and solder the following components.
      i. Shield risers (1).
      ii. All contact points of the 3 relays (2).
      iii. All contact points of the 3 resistors (3).
      iv. All 3 indicator LEDs (4).
   b. Once all components are attached, connect them by soldering the 20 g wire included in the jumper wire kit. The following connections are for one of the 3 stimulation LED banks. The same general circuit is mirrored for the other two banks with the appropriate digital out (DO5,DO6) and power supply connection (Protoshield Y,Z) changed.
      i. Arduino Digital Out D3 (5) to Relay pin 1 (6).
      ii. Anode (+) side of indicator LED (7) to Relay pin 1 (6).
      iii. Cathode (-) side of indicator LED (8) to resistor side 1 (9).
      iv. Resistor side 2 (10) to Relay pin 12 (11).
      v. Resistor side 2 (10) to Ground (12).
      vi. Relay pin 9 (13) to Protoshield X output (14).
      vii. Solder a short portion (2 cm) of 20 g wire to the larger 18 g wire and cover joint with heat-shrink tubing. Connect 18 g end to Relay pin 8 (15) and the 20 g end to Dimmer In V-.
5. Connect the negative wire (black wire) from a 60 V power supply to Protoshield screw terminal X (16).
6. Connect the positive wire (red wire) from the same 60 V power supply to the corresponding In-V+ terminal of a dimmer.
7. Repeat steps 4b–6 for the remaining two LED bank circuits.
8. Secure Protoshield into Arduino by making sure all header pins are straight in sockets and then gently pressing down.
9. Secure the housing chamber, Arduino, power supplies, terminal blocks and dimmers to a piece of $^{3}/_{4}$" thick plywood using screws. This will enable the entire device to be moved efficiently and safely by a single person to other experimental areas, as needed.

Construction of the chamber for electrophysiological experiments

Timing: 2 days

To perform electrophysiological recordings, an experimental chamber, which also serves as a dissection dish, must be prepared by pouring a 10:1 mixture of Sylgard resin and catalyst into a 35 mm Petri dish.

10. First, pour the resin into dish at a depth of approximately 2 mm to create the bottom layer.
11. After the bottom layer is solidified, place an oval-shaped mold (e.g., the bottom of a small disposable lighter, such as a BIC mini lighter or similar kind) in the center of the dish, and pour more resin to form the second layer.
12. Pour a second batch of resin, again 2 mm thick, in a second dish.
13. From the second batch cut two small pieces, long and thin with a rounded edge on one side, with a razor blade. These will serve as flow guides in the final experimental chamber (dish).

Making micro-pins for use in larval dissection

Timing: 1 h

Micro-pins are required for larval dissection prior to electrophysiology. These can be made using the protocol below:

14. Prepare a 35 mm Petri dish lined with Sylgard resin on the bottom, as above, and add 1.5 mL of 1 M NaOH solution.
15. Place an insect pin (1″ length) on the edge of the dish.
16. Connect an output from an autotransformer to the insect pin. Connect the other output to the end of forceps with alligator cords. Set the output to 1–2 V.
17. Place a 0.2-mm tungsten wire, cut approximately 1 cm long, in the NaOH solution, electropolishing it to make the entire wire thinner and the tip sharper.
18. After polishing, the micro-pins should be washed with distilled water and stored by sticking them onto a piece of Sylgard resin.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
All-*trans*-Retinal (ATR)	Sigma-Aldrich	R2500
NaCl	Sigma-Aldrich	S9888
KCl	Sigma-Aldrich	P3911
$CaCl_2 \cdot 2H_2O$	Sigma-Aldrich	C3881
$MgCl_2 \cdot 6H_2O$	Sigma-Aldrich	M2393
$NaHCO_3$	Sigma-Aldrich	S6014
Trehalose	Fisher Scientific	BP268725
Sucrose	Sigma-Aldrich	S8501
HEPES	Sigma-Aldrich	H3375
Experimental models: Organisms/strains		
Drosophila melanogaster GAL4-19-12 (various ages depending on experimental design, mixed sex)	Bloomington *Drosophila* Stock Center	36369
Drosophila melanogaster GAL4-nompC (various ages depending on experimental design, mixed sex)	Bloomington *Drosophila* Stock Center	36361
Drosophila melanogaster UAS-ChETA::YFP (various ages depending on experimental design, mixed sex)	Bloomington *Drosophila* Stock Center	36495
Drosophila melanogaster UAS-mCD8::GFP (various ages depending on experimental design, mixed sex)	Bloomington *Drosophila* Stock Center	5130
Software and algorithms		
Spike2	Cambridge Electronic Design	https://ced.co.uk/products/spkovin
OptoBox Software	This report	https://github.com/CoxLabGSU/OptoBox
Other		
10-Compartment Interlocking Small Parts Organizer	Husky	35587
$^3/_4$-inch Plywood Board	The Home Depot	N/A
12 oz. Flat Black General Purpose Spray Paint	Rust-Oleum	334020
PWM Rotary Dimming Controller	Genssi	N/A
Cree XP-E2 blue LEDs with 80° lens	Rapid LED	XPEBBL-L1-0000-00301
Kwikweld Epoxy	The Home Depot	N/A
Arduino Uno Starter Kit	Arduino	K000007
Adafruit Proto-Screwshield Kit	Mouser/Adafruit	196
Dimmers	LEDwholesalers	X0028C24E9
TXS2-4.5 V Relay	Digi-Key	255-1896-ND
Power Supplies	LEDwholesalers Power Supplies	3204
10-ohm ceramic resistors	Bettal	1A30033
Eaton barrier terminal blocks	Mouser Electronics	TB100-04
Heat shrink wrap kit	Eventronic	asd-123
18 AWG Wire	Striveday	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Spade terminals	Utilitech	0423953
Jumper wire kit	WayinTop	WYTP13
Soldering iron kit	Anbes	FBA_D-950
20 V MAX XR brushless drill/driver	DeWalt	DCD991P2
6PC spade bit set	Bosch	DSB5006
1/8-inch Black and Gold split point drill bit	DEWALT	DW1108
Computer Repair Screwdriver Set	UnaMela	UMT0014
#8 × 3/4-inch external hex flange hex-head self-drilling screws	Teks	21312
#8 × 3/4-inch self-tapping slotted pan head screws	Bolt Depot	13866
60 × 15 mm Petri dish	Kord-Valmark	2901
35 mm Petri dish	Falcon	351008
Cold/Peltier plate	TE Technology	CP-031
Cold plate temperature controller	TE Technology	TC-48-20
Colt plate power supply	TE Technology	PS-12-8.4A
Aluminum shim for arena (cut 7.5 × 11.5 mm)	Global Equipment	WBB512969
Microscope	Leica	MZ16FA
Patch-clamp amplifier	Molecular Devices	Multiclamp 700A
Electrode holder	Molecular Devices	1-HL-U
A/D converter	Cambridge Electronic Design	Micro1401
Inline solution cooler	Warner Instruments	SC-20
Temperature controller	Warner Instruments	CL-100
Temperature probe	Physitemp	BAT-12
Micromanipulators	Siskiyou	MX1600
Data acquisition interface	Cambridge Electronic Design	Micro1401-4
Fine forceps (Dumont #5)	Fine Science Tools	11251-30
Dissection scissors (Vannas spring scissors)	Fine Science Tools	15000-08
Syringes	BD Syringe	09646
0.45 micron syringe filter	AQ Syringe Filters	58045-CA04-C
Polyethylene tubing	Fisher Scientific	BD427435
Sylgard 184 silicone elastomer kit	Dow Corning	DC4019862
Insect pins	Fine Science Tools	26000-70
Tungsten wire (0.002″)	A-M Systems	795500
Variable autotransformer	Staco Energy Products Co.	Type 3PN1010
Alligator cords	VWR	470122-138
Flaming/Brown type micropipette puller	Sutter Instrument	P-97
Borosilicate capillary glass	Sutter Instrument	B100-75-10

MATERIALS AND EQUIPMENT

HL-3 Saline

Reagent	Final concentration	Amount
NaCl	70 mM	4.09 g
KCl	5 mM	0.37 g
CaCl2	1.5 mM	0.17 g
MgCl2	20 mM	4.07 g
NaHCO3	10 mM	0.84 g
trehalose	5 mM	1.89 g
sucrose	115 mM	39.4 g
HEPES	5 mM	1.19 g
ddH_2O (final volume)	n/a	1 L
Total	**n/a**	**1 L**

HL-3 can be stored at 4°C for up to one month (be certain to discard stock if sucrose lumps or mold form).

STEP-BY-STEP METHOD DETAILS

Fictive cold rearing in the OptoBox

Timing: 1–10 days (depending on desired life stage and ambient temperature)

Note: The flies you use will depend upon your experimental design, as this setup can be adapted for chronic optogenetic activation of any number or type of neurons. In our study (Himmel et al., 2021), we made use of flies expressing *UAS-ChETA::YFP* under the control of $GAL4^{19\text{-}12}$, which targets Class III (CIII) neurons. CIII neurons are a segmentally-repeated class of peripheral noxious cold-sensing neurons (cold nociceptors). Here, we compared animals of the same genotype ($GAL4\text{-}^{19\text{-}12}$ > *UAS-ChETA::YFP)*, where the control group did not receive all-*trans*-retinol, a necessary cofactor for ChETA function. The goal of this experiment was to determine whether chronic activation of CIII nociceptors, in the absence of cold, had any effect on the ability of animals to survive subsequent cold shocks. This protocol is inspired by a similar approach employed by Kaneko et al. (2017) for studying (de)sensitization of mechanical/high temperature nociceptors and can be feasibly adapted to study any number of systems.

1. Prepare housing for *Drosophila*.
   a. Prepare *Drosophila* food (according to your laboratory/experimental needs); while the food is still liquid (approximately 45°C–50°C), use a pipette to transfer 2.5 mL of food to a 60 × 15 mm Petri dish.
      i. The food must be spread thin to be sure animals receive full doses of optogenetic light during their stay in the OptoBox (troubleshooting 1).
      ii. For experimental animals, before pouring food use a micropipette to add all-*trans*-retinol (ATR). Bring the final concentration of ATR in the food to 1.5 mM.

Note: This protocol assumes two groups of the same genotype, where the presence of ATR (and thus active ChETA) is the independent variable. It may be desirable to also control for the presence of ATR, and thus to also use a wild-type or genetic-background control strain which is fed ATR.

2. Once the food has cooled and solidified, introduce ~15 adult *Drosophila* to the Petri dish. Anesthetize adult flies with carbon dioxide, then place the adults on the lid of the plastic dish, closing the dish with the base/food on top. Once adults are mobile, flip the dish and keep it food-side down.
3. House the dish in a dark, temperature-controlled area.
4. After allowing for egg-lay (no longer than 24 h), remove adults from the dish (by aspiration or by simply releasing them in a controlled area). Leaving adults in the dish may lead to too much food being eaten before larvae mature.
5. Monitor the plates until your life-stage of interest is present. Closely monitor the quality of the food over time (troubleshooting 2).

Note: Alternatively to steps 2–5, eggs, larvae, or adults can be transferred to the dish, and subsequently to the OptoBox, at the desired life stage.

6. Transfer each of the plates, in the dark, to an OptoBox compartment. Close the OptoBox compartment and start the Arduino microcontroller.
7. After a length of time determined by your experimental design, remove each Petri dish and continue at either step 8 (larval dissection and single unit electrophysiological recordings) or step 13 (cold-tolerance assay by cold plate).

Note: Following fictive cold acclimation, one might also perform behavioral analyses by either the cold-plate (Himmel et al., 2020; Patel and Cox, 2017) or cold-probe assays (Turner et al., 2017), as previously described.

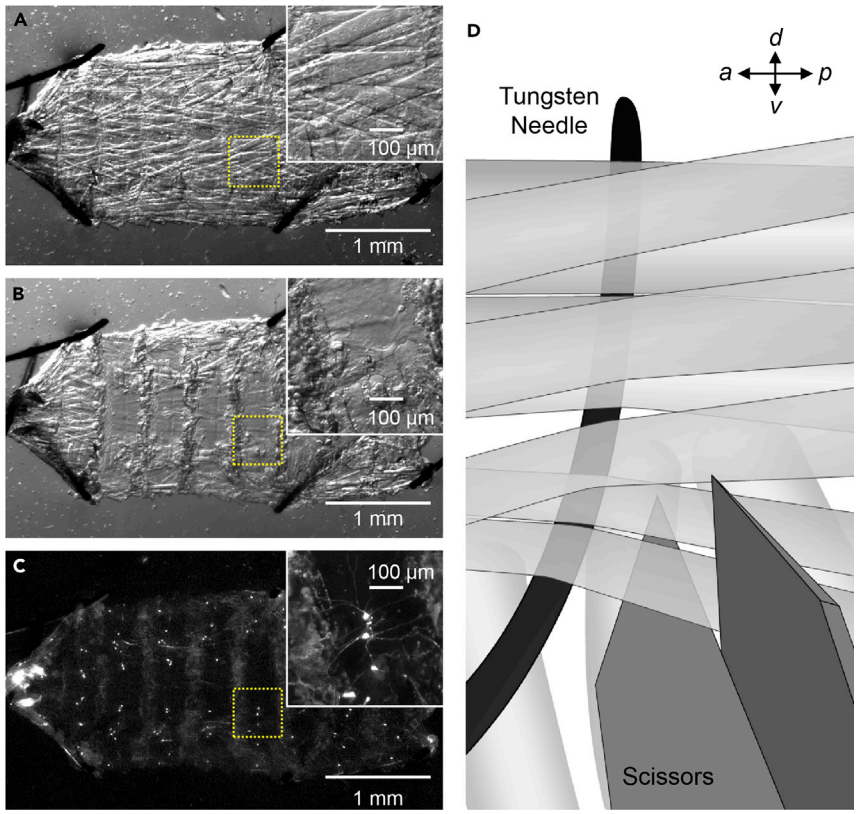

Figure 3. Making a fillet preparation

(A) The body wall of a 3rd instar larva cut longitudinally, spread out, and pinned with micro-pins onto the floor of experimental chamber.

(B) Cutting the longitudinal muscles exposes the lumen of the body wall.

(C) GFP-labeled CIII neurons visible under fluorescent stereomicroscope.

(D) A schematic drawing of how to transect the longitudinal muscles with a tungsten needle and a microscissors.

Larval dissection and single unit electrophysiological recordings

Timing: 2 h (to recording)

Using the protocol below, one can record cold-evoked electrophysiological activity in larval CIII neurons (Figures 3 and 4). Ideally, one will be able to visualize neurons of interest using fluorescence microscopy; in the linked study, CIII neurons were visualized by *GAL4*$^{19\text{-}12}$*>UAS-mCD8::GFP*.

8. Prepare HL-3 saline for use throughout. Prepare 4 L of solution.
9. Select and fillet the larva:
   a. Arrange larva ventral-side up on the bottom of the experimental chamber (dish) by pinning the anterior and posterior ends of the body with 1–2 mm tungsten micropins (previously made).
   b. Using dissection scissors, cut open the ventral body wall of the final posterior segment horizontally, then cut longitudinally in a straight line toward the anterior.
   c. Fill the experimental chamber with approximately 0.5 mL of HL-3 saline (Figure 3A).
   d. Spread the body wall and pin it onto the Sylgard floor of the experimental chamber.
   e. Loosen the internal organs (brain, intestine, salivary glands, tracheae, and fat tissues) by gently pipetting, then remove them with micro-scissors and forceps.
   f. Using a tungsten needle and dissection scissors, remove the dorsal longitudinal muscles to expose the dorsal cluster of sensory neurons. Insert a micro pin (glued to the top of

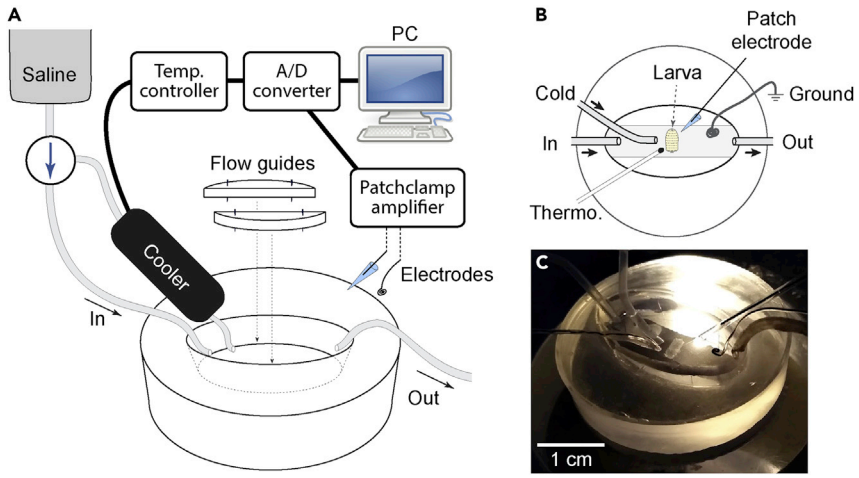

Figure 4. Electrophysiology prep

(A) The dish is constantly superfused with gravity-dripped physiological saline. The tube carrying the saline splits into two paths via a three-way stopcock (circled arrow), one (In) leading directly to the experimental chamber and the other via the cooler. The saline solution is aspirated and drained from the opposite edge by an outlet tube (Out) connected to a vacuum. After pinning the specimen at the center of the chamber, two flow guides made of Sylgard resin are installed to ensure that the fluid will pass straight through. The temperature of the cooler is directly controlled by the temperature controller, which is operated manually by operator or by an command waveform produced by the computer (PC) via an A/D converter.

(B) For the experiment, the fillet preparation is pinned in the center of the oval chamber. The inlet (In) and outlet (Out) tubes are placed at both ends of the chamber, and the tube that comes out of the cooler (Cold) is placed closer to the specimen. The grounding Ag-AgCl wire (Ground) is placed in the corner of the chamber and the thermometer probe (Thermo.) is placed near the outlet of the cooler.

(C) Photo of the chamber.

a toothpick) under the dorsal longitudinal (DL) and dorsal oblique (DO) muscle to lift them (Zarin et al., 2019), and then sever with fine scissors (Figure 3D). The severed muscles will contract by themselves, exposing the underlying sensory neurons (Figures 3B and 3C).

10. Place the experimental chamber housing the fillet prep in the electrophysiology rig.
    a. Place the flow guides (previously made) on either side of the fillet preparation (Figure 4).
    b. Place the saline inlet and aspiration/outlet tubes at both ends of the experimental chamber. Connect the outlet tube to a vacuum.
    c. Place the cooler outlet tube upstream of the fillet prep; the length of the outlet tube should be approximately 2 cm.
11. Prepare for electrophysiological recordings.
    a. Make a patch pipette with a tip diameter of 5–10 micrometers by pulling borosilicate glass (1.0 mm out diameter, 0.75 mm inner diameter) using a programmable electrode puller (Brown et al., 2008). The program for P-97 consists of 3 lines, and each line setting is Heat 285, Pull 0, Velocity 45, Time 150, Pressure 500.
    b. Fill the pipette with saline filtered through a 0.45 micron syringe filter.
    c. Mount the pipette on an electrode holder containing an Ag-AgCl wire and a suction tube attachment and connect to an amplifier head-stage fixed on a micromanipulator.
    d. Connect the electrode holder to a 5 mL syringe with a polyethylene tube.

Note: Although we used *GAL4*$^{19\text{-}12}$ in the linked study, CIII neurons can be visualized by fluorescence microscopy in larvae expressing *UAS-mCD8::GFP* under the control of *GAL4*$^{19\text{-}12}$, *GAL4*^{nompC}, or any other *GAL4* driver targeting neurons of interest (e.g., Figure 3C).

e. While applying positive pressure with a syringe, bring the electrode close to the target cell body on the body wall (Methods video S1).
f. When the tip touches the target cell body, slowly apply negative pressure to draw the cell body into the pipette (Methods video S1).
g. Record spiking activity. Here, we used a Multiclamp 700A amplifier under voltage-clamp mode held at 0 mV, filtering out high frequency noise of the current signal with a 3 kHz low-pass filter. The output signals of the amplifier were digitized at a sample frequency of 10 kHz using an A/D converter (Micro1401-4). The data were acquired to a PC running Spike2 software.

12. Record spiking under chilling. Two different methods can be used to apply cold stimulus while recording spiking activity from a CIII neuron. One approach (a) is to switch perfusates from the saline held at room temperature (approximately 22°C–25°C) to one pre-chilled to the desired temperature (fast-stimulation protocol). The other method (b) is to directly control the perfusate by sending a command voltage waveform to the temperature control device (slow-stimulation protocol).
    a. For the room-temperature condition, the preparation should be continuously superfused with saline at room temperature.
    b. For Fast-Stimulation Protocol:
        i. Set the Warner Instrument CL-100 temperature controller to the target temperature in advance to chill saline to desired stimulus temperature.
        ii. Turn the valve of the three-way stopcock within 0.2 s the saline temperature in the test chamber will sharply drop.
    c. For Slow-Stimulation Protocol:
        i. Set the CL-100 temperature controller to the desired stimulus temperature.

Note: The graphical sequence editor function of Spike2 software allows the user to input the amplitude potential of the rectangular output waveform and the speed of potential change (V/sec) to create a commanded direct waveform of temperature change. The command output can be directly fed to the temperature controller (CL-100) as a voltage signal from DAC output of the data acquisition interface (A/D converter).

Cold-tolerance assay by cold plate

Timing: 4–14 days (depending on the life stage of populations entering the assay)

By modifying the previously described cold-plate behavior assay (Himmel et al., 2020; Patel and Cox, 2017), one can deliver cold-shocks to large batches of larvae as part of a cold-tolerance assay (Figure 5), a method which assesses the ability of larvae to survive cold shocks. In the linked experimental paper, we studied the ability of wandering 3rd instar larvae to survive 1-h cold shocks at 0°C, following real and fictive cold acclimation.

13. Prechill the Peltier device by setting the temperature controller to the target temperature (troubleshooting 3).
14. Collect living larvae of your desired life stage and wash them.
    a. Using a paint brush, gently collect larvae from the dish and place them in the well of a 9-well glass plate. Use tap water and a glass pipette to gently wash residual food off of the larvae.
    b. Transfer the larvae to a dry Kimwipe (or other equivalent paper wipe) to dry off excess water. Leave them on the now moistened wipe as you prepare the aluminum shim arena.

Note: Some of the sensory neurons which control cold sensing are also touch sensors (Turner et al., 2016). Use single, deliberate motions with each brush movement to transport larvae from one position to another. It is currently unknown if and how repeated touches may affect

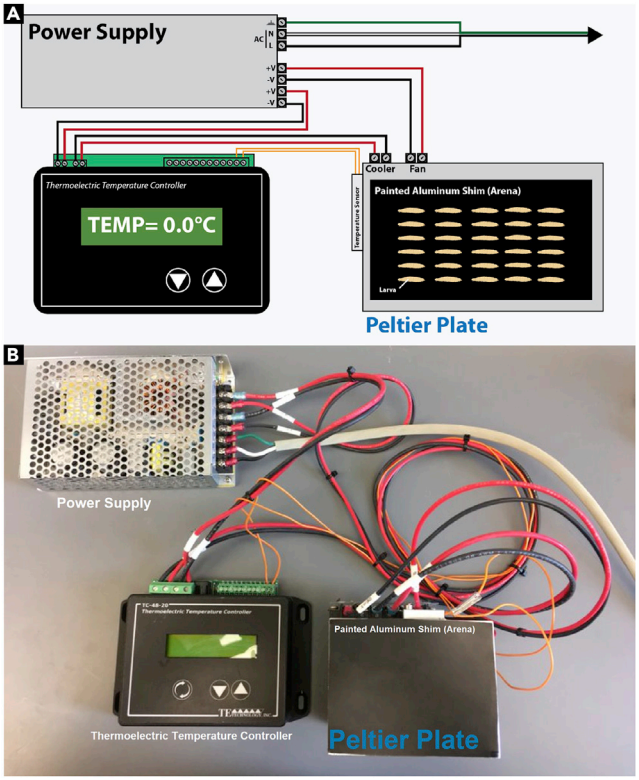

Figure 5. Wiring diagram and cold shock setup

The cold shock apparatus for use in the cold-tolerance assay is made up of a TE Technology thermoelectric cooling device (TC-48-20), a TE Technology Peltier plate (cold plate; CP-031), and a TE Technology power source plugged into a standard wall outlet. Larvae are placed on a thin aluminum shim, which is set on top of the Peltier plate for the length of the cold shock.

(A) Schematic with wiring instructions.

(B) Photo of complete apparatus.

cold acclimation. We therefore advise you to discard larvae which you drop or deliver additional, accidental touches to.

15. Quickly transfer larvae to the aluminum shim arena. Larvae can be placed in the center of the plate and allowed to locomote out to roughly equal distances from one another. Approximately 30 larvae can fit on the plate, but the final number of larvae and biological replicates (individual test populations from different dishes) will depend upon your experimental design.

Note: At cold temperatures larval locomotion will cease and will not resume until after chilling has ceased and larvae begin to warm.

16. Transfer the aluminum shim arena to the prechilled cold plate (Peltier device).
17. Set a timer for the length of time desired for the cold shock (e.g., 1 h).
18. While the larvae are being cold shocked, prepare fresh fly vials to house larvae post cold shock. Each vial should contain equal amounts of food from the same food batch (troubleshooting 4).
19. After the cold shock, move larvae one by one into their new fly vial, gently depositing them on the wall of the vial. Maintain the vials at room temperature and monitor them (Troubleshooting 5).
20. If you began with wandering 3rd instar larvae, approximately 4 days later adults should emerge from pupal casings. Count the number of adults which emerge and compare to the initial number of larvae.
    a. The survival rate is calculated by # survivor adults/# larvae initially shocked.

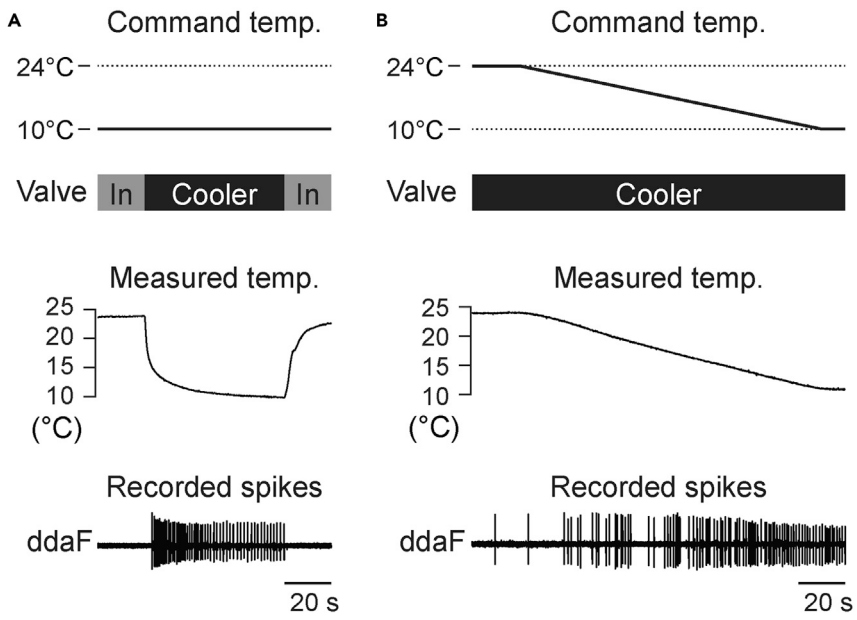

Figure 6. Fast and slow temperature stimulation results

(A and B) show from the top: the command waveform sent to the temperature controller; the path of the saline as determined by the valve; the actual temperature change; and the spiking activity of the recorded neuron.

(A) For the fast-stimulation protocol, the solution in the cooler was chilled in advance to the desired temperature by lowering the temperature setting. Then, the three-way stopcock (Valve) was manually switched from direct injection (In) to the cooler path (Cooler) to deliver the chilled saline.

(B) In the slow-stimulation protocol, the saline path is fixed to the cooler and the saline temperature directly follows the command signal that was sent to the temperature controller from a PC.

EXPECTED OUTCOMES

For electrophysiological experiments, CIII neurons should be largely silent at room temperature (approximately 22°C–25°C) and should show spiking activity during both chilling and steady state cold (Figure 6). For cold tolerance, survival rates can be simply presented as the average proportion or percentage of animals which survive to adulthood. Survival rates will depend on the strain and species in question; in our study, baseline survival rates in non-cold acclimated *Drosophila melanogaster* larvae fell between approximately 25-75%, depending on the strain; thus, one must carefully control for genetic background when comparing across genotypes (Himmel et al., 2021). This method might also be used to determine the lower lethal temperature or lethal time at low temperature (see (Andersen et al., 2015)).

LIMITATIONS

With respect to the OptoBox, the primary issue is the use of blue light, as blue light flashes may have circadian effects in *Drosophila*, and the circadian rhythm is likely a component of cold acclimation (Poikela et al., 2021). This, however, can be appropriately controlled for. For example, in the linked experimental paper we also assessed animals raised in the OptoBox without optogenetic stimulation, and animals which had inactive channelrhodopsins (due to lack of dietary ATR) yet received optogenetic stimulation. Alternatively, the OptoBox can be modified for use of other LED wavelengths (e.g., red), which could thereafter be used in conjunction with red-shifted channelrhodopsins, such as CsChrimson (a Chrimson variant) (Klapoetke et al., 2014).

For our electrophysiological approach, the fast- and slow-stimulation protocol each has drawbacks with respect to control over the rate of temperature change. For the fast-stimulation protocol, although this method can provide the fastest temperature stimulus, it is very challenging to precisely control the rate of change in temperature because the temperature change is primarily affected by

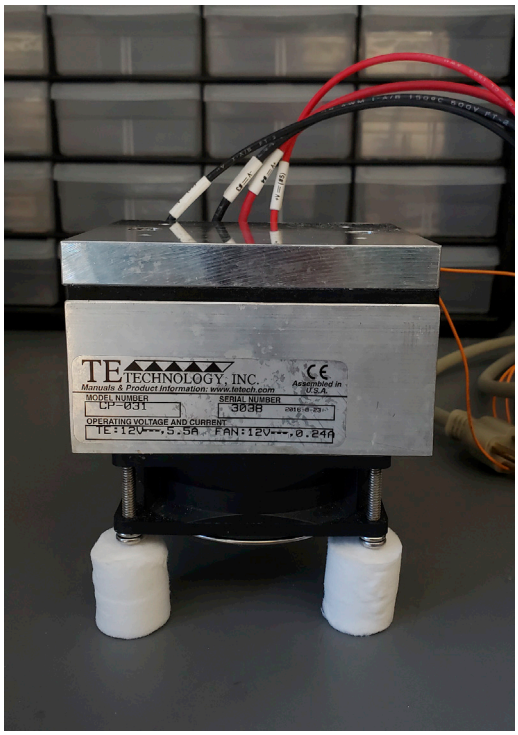

Figure 7. Solution for elevating Peltier plate with *Drosophila* vial plugs to stabilize temperature at extreme cold

the perfusion rate and the volume of fluid in the experimental chamber. The distance from the thermometer probe to the inlet tube and the recorded neurons varies from experiment to experiment because the probe must be placed outside of the fillet. Therefore, it is unlikely that the measured temperature value is exactly the same as the temperature experienced by the sensory neurons. In addition, since the temperature is measured by the change in electrical resistance caused by the temperature change of the sensor probe itself, a time lag of the probe temperature change is unavoidable. The faster the liquid temperature changes, the more pronounced the lag in thermometer sensitivity becomes. This inevitably makes it impossible to measure the actual temperature change accurately. With our protocol, the average rate of measured temperature drop is -3.3 $\pm$ 0.86 °C/s (mean $\pm$ SD, N = 65) ranging from -1.8 to -5.2 °C/s at the onset of the 10°C stimulation.

For the slow-stimulation protocol, there is a limit to how fast the temperature change can be controlled. With the materials described herein, this method can only be applied to slow temperature changes of less than ~0.15°C/s, depending on the perfusion rate.

The cold-tolerance assay by cold plate is relatively straightforward in its approach, can assay cold tolerance in large populations of larvae, and uses equipment with a dual use (in behavioral analyses). However, compared to methods where individual larvae are chilled in sealed containers which are themselves thermocoupled (for example, see (Strachan et al., 2011)), this method has a major disadvantage: larvae are exposed to air circulating in the room for the extent of the assay. As such, ambient temperature, humidity, and other components of the environment might have effects on larval temperature. It is thus recommended that this assay is only used in rooms which are already tightly temperature and humidity controlled.

TROUBLESHOOTING

Problem 1

If you simply pour the food from one dish/container into another, you may find that the food solidifies very quickly and unevenly (step 1).

Potential solution

We have found that the best method is to use an electric pipette controller to quickly and evenly dispense food as you nutate the dish around in your hand. Start dispensing in the center of the dish, and nutate/rotate the dish around in a spiral pattern. This will lead to the food cooling evenly and over the entire base of the dish.

Problem 2

Depending upon humidity and temperature control in the room used, dishes may be prone to drying out over the course of rearing due to the low volume of food. Anecdotally, this leads to failure of larvae to develop, and likely has many effects on larval development. A low volume of food is maintained so that larvae receive the full dose of optogenetic light for the length of their development, so increased food volume is not a viable solution (step 5).

Potential solution

Ideally one will perform these experiments in temperature and humidity-controlled areas which do not dry out plates. If plates do dry out, one fix is to fill the cap of a conical tube with water and house it in the rearing or OptoBox compartment alongside the dish. In our hands, this was sufficient to avoid food drying.

Problem 3

These TE Technology cold plates make use of a bottom-mounted fan attached to a heat sink. When set on a countertop, the fan is directly touching the countertop and has minimal airflow. At extremely cold temperatures the plate may have difficulty maintaining a constant temperature due to this lack of airflow (step 13).

Potential solution

Elevating the cold plate will increase air flow and stabilize the temperature. One can use standard *Drosophila* vial caps to elevate the plate (Figure 7), although anything which is stable and tall enough to increase airflow will suffice.

Problem 4

In the associated experimental paper, we studied wandering third-instar larvae, and housed 10 larvae per vial post cold shock. This often led to the food growing mold, perhaps due to low to non-existent feeding and food turnover by larvae (step 18).

Potential solution

If you are using wandering third instar larvae you can instead fill vials with 10 mL of 2%–3% agar. As animals are post-feeding, they do not require actual food, and the agar will allow a substrate to prevent the animals from desiccating. Alternatively, Methylparaben/Nipagin can be added to fly food as an antifungal.

Problem 5

Larvae which do not survive the cold shock will often not survive to the pupal stage; this will be visibly obvious as the larvae will turn black. We have hypothesized this can contribute to mold growth, although we have not tested this hypothesis (step 19).

Potential solution

Remove dead larvae from the vial, as long as doing so will not disrupt the rest of the population.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Daniel N. Cox (dcox18@gsu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The code for control of LEDs by the Arduino microcontroller is available on the Cox Lab GitHub page (https://github.com/CoxLabGSU/OptoBox). An archived version of record is available on Zenodo (https://doi.org/10.5281/zenodo.6581788).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101510.

ACKNOWLEDGMENTS

We thank Dr. Bing Ye and his laboratory, who provided the original parts list and schematic for their optogenetic rig (Kaneko et al., 2017), which greatly informed the final construction of the OptoBox. We also thank J.G. Donaldson for assistance in final Optobox layout and assembly. This work is supported by NIH R01 NS115209-01 (to D.N.C.). N.J.H. was supported by NIH F31 NS117087-01, a GSU Brains & Behavior Fellowship, and a Kenneth W and Georganne F Honeycutt Fellowship. K.J.D. was supported by a GSU 2CI Neurogenomics Fellowship.

AUTHOR CONTRIBUTIONS

Writing – Original Draft, N.J.H., A.S., and K.J.D.; Writing – Review & Editing, N.J.H., A.S., K.J.D., and D.N.C.; Visualization, N.J.H., A.S., and K.J.D.; Designed the OptoBox, N.J.H. and K.J.D.; Built the OptoBox, K.J.D.; Designed OptoBox protocol, N.J.H. and K.J.D.; Designed electrophysiology protocol, A.S.; Designed cold-tolerance assay, N.J.H.; Supervision, D.N.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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