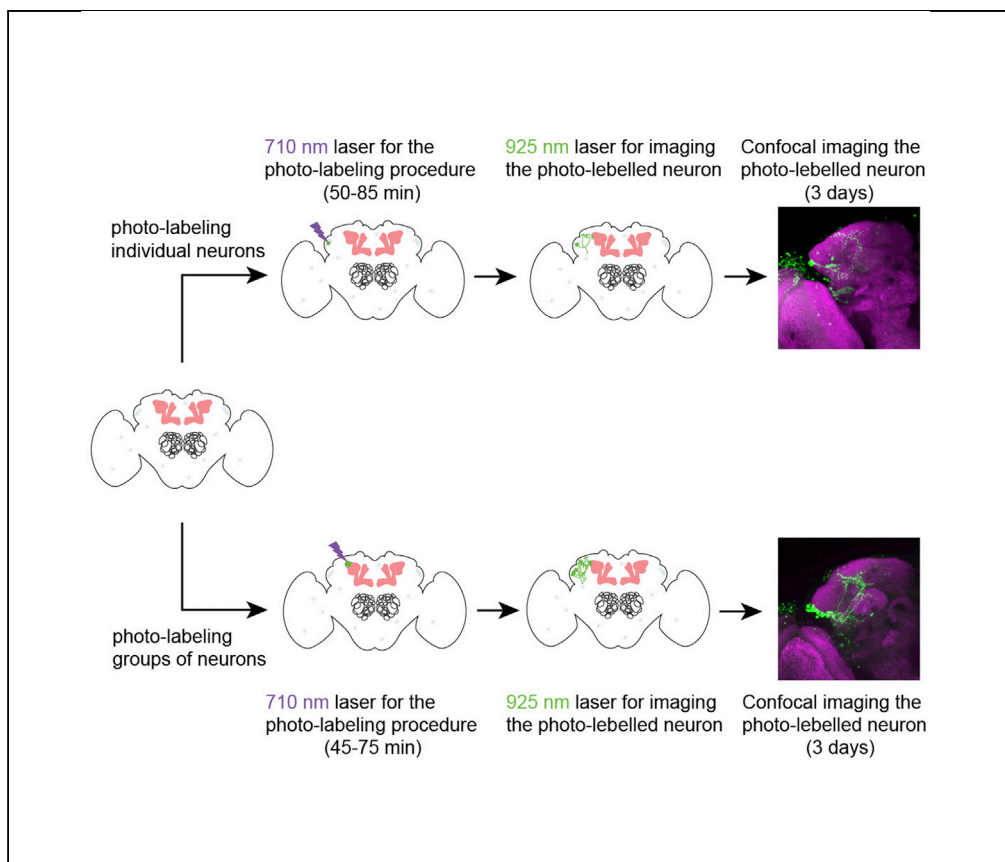


## Protocol

# Photo-labeling neurons in the *Drosophila* brain



Many genetically encoded tools, including large collections of GAL4 transgenic lines, can be used to visualize neurons of the *Drosophila melanogaster* brain. However, identifying transgenic lines that are expressed sparsely enough to label individual neurons, or groups of neurons that innervate a particular brain region, remains technically challenging. Here, we provide a detailed procedure in which we used broadly expressed transgenic lines and two-photon microscopy to photo-label neurons with specificity, thereby permitting their morphological characterization.

Jinzhi Li, Kaitlyn  
Elizabeth Ellis,  
Sophie Jeanne  
Cécile Caron

sophie.caron@utah.edu

### HIGHLIGHTS

Photo-labeling individual and groups of neurons in the *Drosophila* brain

Visualizing the complete morphology of individual neurons

Visualizing neurons innervating a common brain area

Li et al., STAR Protocols 2,  
100381  
March 19, 2021 © 2021 The  
Author(s).  
[https://doi.org/10.1016/  
j.xpro.2021.100381](https://doi.org/10.1016/j.xpro.2021.100381)



## Protocol

Photo-labeling neurons in the *Drosophila* brainJinzhi Li,<sup>1,2</sup> Kaitlyn Elizabeth Ellis,<sup>1</sup> and Sophie Jeanne Cécile Caron<sup>1,3,\*</sup><sup>1</sup>School of Biological Sciences, University of Utah, Salt Lake City, UT 84112, USA<sup>2</sup>Technical contact<sup>3</sup>Lead contact\*Correspondence: [sophie.caron@utah.edu](mailto:sophie.caron@utah.edu)  
<https://doi.org/10.1016/j.xpro.2021.100381>

## SUMMARY

Many genetically encoded tools, including large collections of GAL4 transgenic lines, can be used to visualize neurons of the *Drosophila melanogaster* brain. However, identifying transgenic lines that are expressed sparsely enough to label individual neurons, or groups of neurons that innervate a particular brain region, remains technically challenging. Here, we provide a detailed procedure in which we used broadly expressed transgenic lines and two-photon microscopy to photo-label neurons with specificity, thereby permitting their morphological characterization.

For complete details on the use and execution of this protocol, please refer to Li et al. (2020).

## BEFORE YOU BEGIN

## Experimental model and subject details

Flies (*Drosophila melanogaster*) were raised on standard cornmeal agar medium and maintained in an incubator set at 25°C, 60% humidity with a 12 h light/12 h dark cycle. Crosses were set up and reared under the same conditions. Both male and female flies were used in all experiments. Details of genotypes used in this study and their sources are described in the [key resources table](#).

## Generate the transgenic flies necessary for the photo-labeling procedure

1. This procedure requires *Drosophila* specimens that carry all the transgenes necessary to target the expression of a photo-activatable form of the green fluorescent protein (PA-GFP) — for instance SPA or C3PA (Datta et al., 2008; Ruta et al., 2010) — to the neurons of interest. Any of the available binary expression systems can be used (Lai and Lee, 2006; Potter and Luo, 2011). We used the GAL4/UAS binary expression system to drive expression of a combination of both SPA and C3PA. In particular, we used the *R19H07-GAL4* transgene — which is expressed in a large number of neurons, including the  $\text{PLP}$ PNs — as well as one copy of the *UAS-SPA* transgene and four copies of the *UAS-C3PA* transgene (Li et al., 2020; Ruta et al., 2010).

**Note:** We found that increasing the number of copies of the *UAS-C3PA* and *UAS-SPA* transgenes yields better results, whereas increasing the number of copies of the *GAL4* transgene has little to no effect.

**Note:** This protocol can be used to photo-label neurons in different *Drosophila* species. Here we used *Drosophila melanogaster* but species for which similar transgenes are available, for instance *Drosophila sechellia* and *Drosophila simulans*, can also be used.

**Note:** For better results, use adult flies that are 1 to 3 days old.



### Prepare the *Drosophila* adult hemolymph-like saline

⌚ Timing: 30 min

2. Weigh the appropriate amount of each of the following chemicals: 12.62 g of NaCl, 0.75 g of KCl, 2.38 g of HEPES, 3.78 g of trehalose, 6.85 g of sucrose, 0.67 g of NaHCO<sub>3</sub> and 0.24 g of NaH<sub>2</sub>PO<sub>4</sub>; place all the chemicals in a 2 L bottle.
3. Add 1,750 mL of deionized, filtered water (preferably water treated by the Milli-Q system) to the bottle containing the chemicals; completely dissolve the chemicals until the saline solution is clear and homogeneous.
4. Add 4 mL of a 1 M CaCl<sub>2</sub> solution and 8 mL of 1 M MgCl<sub>2</sub> solution to the saline solution; mix well.
5. Add 310 μL of a 10 M NaOH solution, one drop at a time, to the saline solution; mix well.
6. Add 238 mL of deionized, filtered water to the saline solution.
7. Filter the saline through a sterile membrane filter (in this case a polyestersulfone membrane with 0.22 μm pore size).
8. Verify that the osmolarity and pH of the saline solution are within the optimal range; the osmolarity should be between 265 and 275 mOsm and the pH should be 7.3.

**Note:** The amount used and the final concentration of each chemical is listed in the following table:

**Note:** The pH of the *Drosophila* adult hemolymph-like saline can be adjusted using NaOH (if it is too acidic) or HCl (if it is too basic). The osmolarity of the saline can be adjusted by adding trehalose (if it is too low) or by adding Milli-Q water (if it is too high) (See [problem 1](#) in the [troubleshooting](#) section).

**Note:** The *Drosophila* adult hemolymph-like saline can be stored up to one month at 4°C.

### Prepare the solutions used for the immuno-staining protocol

⌚ Timing: 15 min

9. Make a phosphate buffered saline solution (PBS) by diluting 10 mL of the 10× phosphate buffered saline stock solution in 90 mL of deionized, filtered water.

**Note:** Store the diluted PBS solution at room temperature (22°C–28°C).

10. Make the 2% paraformaldehyde fixing solution (2% PFA) by diluting 125 μL of the 16% paraformaldehyde stock solution in 875 μL of PBS.

Reagent	Final concentration	Amount
NaCl	108 mM	12.62 g
KCl	5 mM	0.75 g
HEPES	5 mM	2.38 g
Trehalose	5 mM	3.78 g
Sucrose	10 mM	6.85 g
NaHCO <sub>3</sub>	4 mM	0.67 g
NaHPO <sub>4</sub>	1 mM	0.24 g
MgCl <sub>2</sub>	4 mM	8 mL
CaCl <sub>2</sub>	2 mM	4 mL
ddH <sub>2</sub> O	-	Up to 2 L

△ **CRITICAL:** For optimal results, make the 2% PFA dilution from EM grade 16% PFA stock solution on the same day when it is used. Store the solution at 4°C.

△ **CRITICAL:** PFA is carcinogenic and highly volatile. Avoid skin contact by wearing gloves and preparing the 2% PFA solution under a fume hood.

11. Make the 0.3% Triton X-100 PBS solution (PBST):
  - a. In a 15 mL tube, mix 1.5 mL of Triton X-100 with 13.5 mL of PBS; gently shake the tube until the Triton X-100 is completely dissolved.
  - b. In a separate 15 mL tube, mix 0.45 mL of the solution made in step 11a with 14.55 mL of PBS.
12. Make the stocker blocker solution (5% PBST-NGS) in a 15 mL tube by diluting 0.3 mL of normal goat serum (NGS) in 5.7 mL of PBST.

**Note:** The 0.3% PBST solution can be stored at room temperature (22°C–28°C) for up to 2 weeks. The stocker blocker solution can be stored at 4°C for up to 2 weeks.

### Prepare the brain sample to be photo-labeled

⌚ **Timing:** 5–10 min

13. Prepare the materials necessary for dissecting the brain:
  - a. Place a small piece of non-sticky material (here we cut a small square piece of SYLGARD from a plate, the bottom of which is entirely coated with SYLGARD.) Then place the cut SYLGARD piece at the bottom of a 10 mm Petri dish; prepare two such dishes.
  - b. Fill the dish with *Drosophila* adult hemolymph-like saline.
14. Prepare the brain for the photo-labeling procedure:
  - a. Briefly anesthetize a fly using CO<sub>2</sub> or ice; dissect the fly in one of the dishes prepared in step 13a by removing its brain from the head case; remove as much of the trachea and connective tissues as possible without damaging the brain.
  - b. Use a Pasteur pipette to transfer the dissected brain to the other dish, which should be clean; use two tungsten wires to pin the brain down on the SYLGARD surface (see [key resources table](#)).

**Note:** The brain should be oriented such that the neurons of interest are as close as possible to the objective lens. For instance, the <sub>PLP</sub>PNs are located on the posterior side of the brain, thus we oriented the brain with its anterior side facing the SYLGARD surface.

**Note:** Depending on the age of the specimen, it might be tricky to remove all the trachea and connective tissues without damaging the brain (step 14a). To facilitate the dissection of older specimens, briefly incubate the dissected brain in a 2 mg/mL collagenase solution — for about 30 s to 60 s — following step 14a. Rapidly rinse the brain after this treatment using the *Drosophila* adult hemolymph-like saline.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Antibodies</i>		
Mouse monoclonal anti-nc82	Developmental Studies Hybridoma Bank	AB_2314866
Alexa Fluor 633 goat polyclonal anti-mouse IgG	Thermo Fisher	A21052

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Normal goat serum (NGS)	The Jackson Laboratory	AB_2336990
VECTASHIELD mounting medium	Vector Laboratories	H-1000
16% paraformaldehyde	Electron Microscopy Sciences	15710
10x phosphate buffered saline	Sigma-Aldrich	P5493
Triton X-100	Sigma-Aldrich	T8787
Collagenase	Sigma-Aldrich	C5138
MgCl <sub>2</sub> solution (1 M in H <sub>2</sub> O)	Sigma-Aldrich	63069
CaCl <sub>2</sub> solution (1 M in H <sub>2</sub> O)	Sigma-Aldrich	21115
NaOH solution (10 M in H <sub>2</sub> O)	Sigma-Aldrich	72068
NaCl	Sigma-Aldrich	S7653
KCl	Sigma-Aldrich	P5405
HEPES	Sigma-Aldrich	H3375
Trehalose	Sigma-Aldrich	T0167
Sucrose	Sigma-Aldrich	S1888
NaHCO <sub>3</sub>	Sigma-Aldrich	S5761
NaH <sub>2</sub> PO <sub>4</sub>	Sigma-Aldrich	S5011
<b>Experimental models: organisms/strains</b>		
<i>D. melanogaster</i> : y <sup>1</sup> w <sup>1118</sup> ; R19H07-GAL4 <sup>attP2</sup> ;	Bloomington Drosophila Stock Center	BDSC: 48867
<i>D. melanogaster</i> : y <sup>1</sup> w <sup>1118</sup> ; R20G07-GAL4 <sup>attP2</sup> ;	Bloomington Drosophila Stock Center	BDSC: 48613
<i>D. melanogaster</i> : y <sup>1</sup> w <sup>1118</sup> ; R13F02-GAL4 <sup>AD</sup> <sup>VK00027</sup> , R85D07-GAL4 <sup>DBD</sup> <sup>attP2</sup> ;	(Aso et al., 2014)	N/A
<i>D. melanogaster</i> : yw, UAS-C3PA-GFP <sup>unknown</sup> ;;	(Aso et al., 2014)	N/A
<i>D. melanogaster</i> : y <sup>1</sup> w <sup>1118</sup> , UAS-C3PA-GFP <sup>attP40</sup> ;;	(Aso et al., 2014)	N/A
<i>D. melanogaster</i> : y <sup>1</sup> w <sup>1118</sup> ; UAS-C3PA-GFP <sup>attP2</sup> ;	(Aso et al., 2014)	N/A
<i>D. melanogaster</i> : y <sup>1</sup> w <sup>1118</sup> ; UAS-C3PA-GFP <sup>VK00005</sup> ;	(Aso et al., 2014)	N/A
<i>D. melanogaster</i> : y <sup>1</sup> w <sup>1118</sup> , UAS-SPA-GFP <sup>attP40</sup> ;;	(Aso et al., 2014)	N/A
<i>D. melanogaster</i> : yw, MB247-DsRed <sup>unknown</sup> ;;	(Riemensperger et al., 2005)	N/A
<b>Other</b>		
CrystalCap ALS HT 0.3–0.4 mm CryoLoop	Hampton Research	HR8208
CrystalWand Magnetic	Hampton Research	HR4-729
Dumont #55 forceps	Fine Science Tools	11295-51
1 L bottle top filter	Corning	431174
Filter receiver and storage bottle	Thermo Fisher Scientific	FB12566515
Polystyrene Petri-dish, 35 mm × 10 mm	Thermo Fisher Scientific	FB0875711YZ
Tungsten 99.95% CS	California Fine Wire Company	100211
SYLGARD 184	Electron Microscopy Sciences	24236-10
Pockels cells	Conotopics	N/A
Ultrafast Chameleon Ti:sapphire laser	Coherent	N/A
Ultima two-photon microscope	Bruker	N/A

**MATERIALS AND EQUIPMENT**

Imaging parameters for live scan and photo-labeling procedure

Parameter	Live scan	Photo-labeling individual neurons	Photo-labeling groups of neurons
Objective lens	60x	60x	60x
Image resolution	512 × 512	512 × 512	512 × 512
Pixel size	0.39 μm	0.012 μm	0.078 μm
Pixel dwell time	4 μs	2 μs	2 μs
Average frame	8	4	4
Laser wavelength	925 nm	710 nm	710 nm
Laser power	1–14 mW	5–30 mW	5–30 mW

**Note:** The laser power was measured by first removing the objective lens from the turret and then placing the measuring device directly below the objective lens socket.

### STEP-BY-STEP METHOD DETAILS

#### Photo-label individual neurons

⌚ Timing: 50–85 min

This protocol can be used to photo-label individual neurons.

**Note:** This protocol is similar to procedures that have been used in other studies. (Alpert et al., 2020; Aso et al., 2014; Caron et al., 2013; Datta et al., 2008; Frank et al., 2015; Li et al., 2020; Ruta et al., 2010)

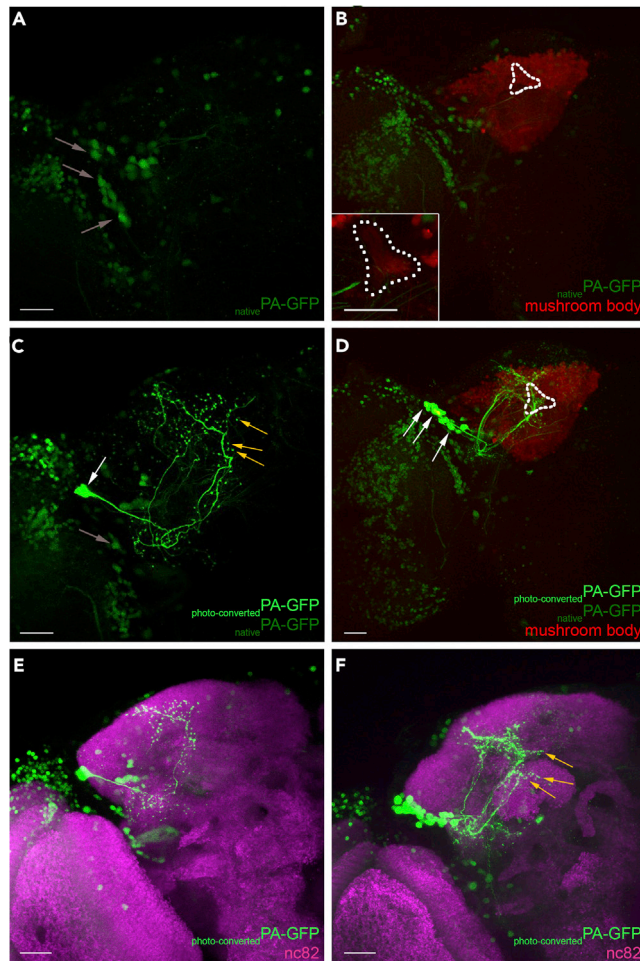
1. Configure the imaging software using the live scan parameters outlined in the [materials and equipment](#) section.

**Note:** We used an Ultima two-photon microscope (Bruker) equipped with an ultrafast Chameleon Ti: Sapphire laser (Coherent) modulated by Pockels Cells (Conotopics) for both photo-labeling and image acquisition. The exact instrumental configuration described here may be different from instruments made by other manufacturers. For the imaging software, we used Prairie View (Bruker).

2. Tune the laser to 925 nm and image the sample using the 60× objective lens, such that the cell body of the neuron of interest can be located and visualized based on the dim baseline fluorescence of PA-GFP ([Figure 1A](#)).
3. Define a region of interest (we used the ROI function that is available with the Prairie View software) around or within the cell body of the targeted neuron ([Figure 2](#)):
  - a. In live scan mode, image the sample at the highest magnification, such that the cell body of the neuron of interest occupies most of the imaging window (we used 32× digital zoom).
  - b. Select a z plane in which the PA-GFP signal is the strongest; acquire a single scan at that plane; on the acquired scan, define a 1.0 μm by 1.0 μm region of interest in the center of, or around, the cell body.
  - c. Stop the live scan mode.
4. Set up the photo-labeling cycle ([Figure 3](#)):
  - a. Tune the laser to 710 nm.
  - b. Configure the imaging software using the parameters listed for photo-labeling individual neurons (see the [materials and equipment](#) section).
  - c. Perform a timed sequence acquisition of the region of interest defined in step 3b; set the number of repetitions in the sequence to 70 and the period — the resting time between each repetition — to 10 s.

⚠ **CRITICAL:** To obtain optimal results, it is important to photo-convert a sufficient number of PA-GFP molecules in the neuron of interest, while preventing the neuron from being damaged. The laser power required for photo-labeling individual neurons varies from sample to sample and should be adjusted accordingly. For example, we used a total laser power value that was within the range of 11 to 22 mW.

5. Run the photo-labeling cycle.
6. Wait 15 min after the completion of the photo-labeling cycle to allow the sample to recover; repeat the photo-labeling cycle once or twice, depending on the results but always allow for a 15-min recovery period in between cycles.
7. Image the photo-labeled neuron:



**Figure 1. Photo-labeling individual neurons or groups of neurons**

(A) Under a broadly expressed transgenic line in the *Drosophila* brain, many neurons express the native form of PA-GFP (gray arrows).

(B) In addition to the native form of PA-GFP expression driven by the same transgenic line as in (A), A red fluorescent protein, DsRed, is expressed in the mushroom body to visualize its dorsal accessory calyx (white dashed outline); (inset) the dorsal accessory calyx is outlined by white dashed lines and targeted for the photo-labeling procedure.

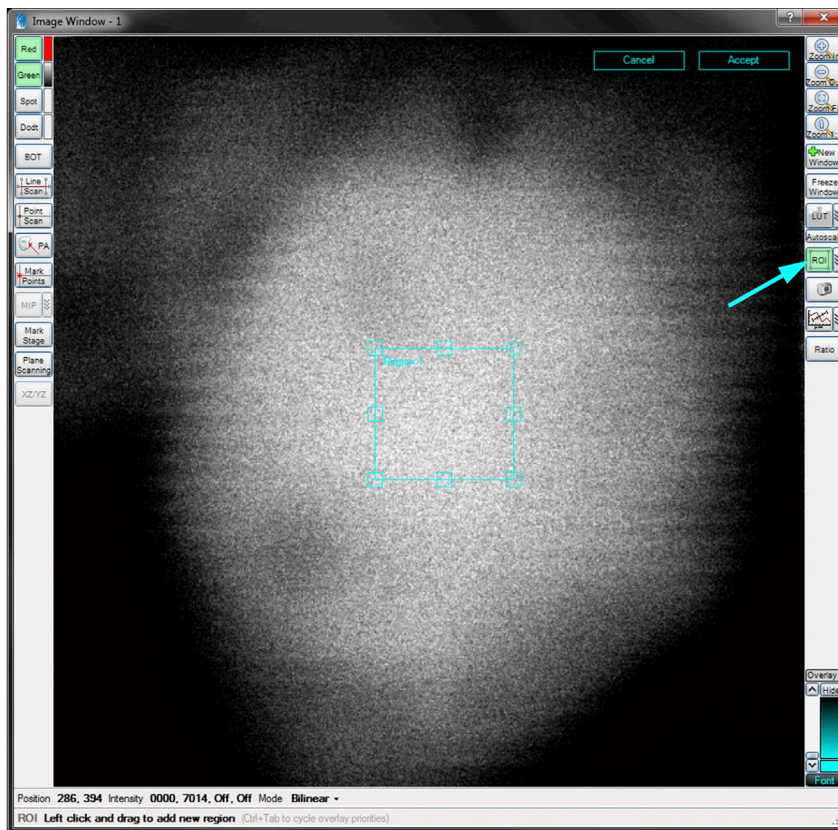
(C) A single  $P_{LP}PN$  was photo-labeled by targeting its soma (white arrow) and imaged using two-photon microscopy. The entire morphology of the photo-labeled neuron is clearly distinguishable from other neurons that also express PA-GFP, but were not photo-labeled (gray arrow). The morphological features of the photo-labeled neuron — such as its axonal projections and presynaptic boutons (yellow arrows) are clearly visible.

(D) A group of  $P_{LP}PN$ s (white arrows) was photo-labeled by targeting the dorsal accessory calyx of the mushroom body (white dashed outline) and imaged using two-photon microscopy.

(E) The sample with the photo-labeled  $P_{LP}PN$  in (C) was immuno-stained using the nc82 antibody (magenta) and imaged using confocal microscopy.

(F) The sample with a group of photo-labeled  $P_{LP}PN$ s in (D) was immuno-stained using the nc82 antibody (magenta) and imaged using confocal microscopy. The morphological features of the photo-labeled neurons, including their dendritic branches, axonal projection, and presynaptic boutons (yellow arrows), were visible once the photo-labeling procedure was complete. The following genotype was used in this figure: *yw/yw; MB247-DsRed<sup>unknown</sup>, UAS-C3PA-GFP<sup>unknown</sup>/UAS-C3PA-GFP<sup>attP40</sup>, UAS-C3PA-GFP<sup>attP2</sup>, UAS-C3PA-GFP<sup>VK00005</sup>, UAS-C3PA-GFP<sup>VK00027</sup>/R19H07-GAL4<sup>attP2</sup>*;,. Scale bar, 20  $\mu$ m in all panels. Figure reprinted with permission from Li et al. (2020).

- a. Tune the laser to 925 nm.
- b. Configure the imaging software using the listed parameters for the live scan mode, such that the entire brain volume encompassing the photo-labeled neuron can be imaged (see [materials and equipment](#) section).



**Figure 2. Defining a region of interest (ROI) for the photo-labeling procedure on the targeted neuron**

First, image the targeted neuron (white) at the highest magnification possible, until its soma almost occupies the entire imaging window. Then select the ROI function tab (cyan arrow) on the imaging software, then create a small area (cyan square) on the center of the soma of the targeted neuron. This small area (cyan square) is the region targeted for the photo-labeling procedure.

- c. Acquire the image sequence (Figure 1C).

### Immunolabel the brain for confocal imaging

⌚ Timing: 3 days

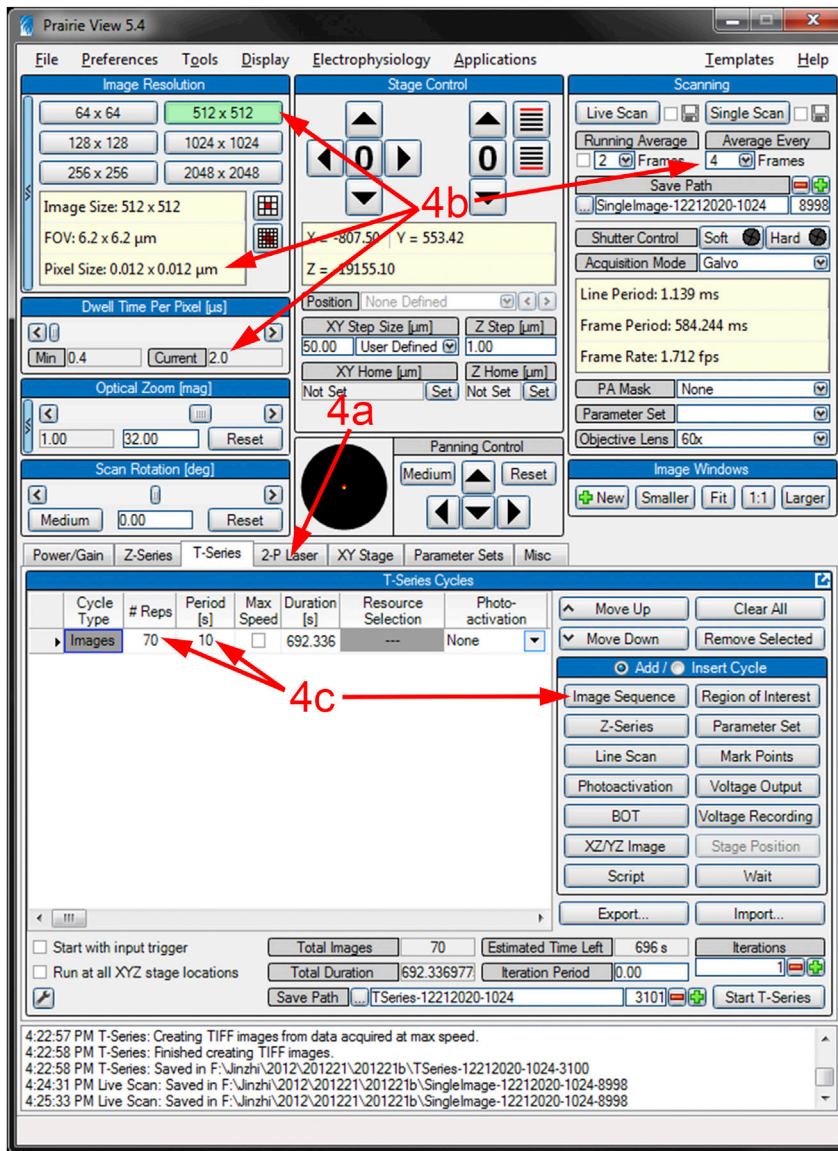
This procedure can be used to immuno-stain samples in which individual neurons were successfully photo-labeled.

**Note:** The time required for the following steps may be different from other experiments that also require fixing a *Drosophila* brain for confocal imaging. We do the following for fixing a brain sample for imaging photo-labeled neurons under the confocal microscope.

**Note:** Each row of the culture plate is numbered, from 1 to 12, and each column is labeled with a letter, from A to F. We refer to each well by its respective number and letter. For example, the first well in the first row is referred to as "well 1A."

8. **Day 1.** In a 72-well culture plate add 12  $\mu$ L 2% PFA solution in well 1A.
9. Transfer the brain sample from the imaging dish to well 1A; incubate for 45 min at room temperature (22°C–28°C).





**Figure 3. Setting up the photo-labeling cycle for photo-labeling an individual neuron on the imaging software user interface**

See step 4 under [photo-label individual neurons](#) for setting up the parameters for the photo-labeling procedure.

**Note:** Use a CryoLoop to avoid damaging the brain while transferring it between wells.

**Note:** For optimal results, do not store more than one brain in the same well. Each column can be used for treating a brain.

**Note:** To prevent the brain from dehydrating, keep it completely submerged in the solution at all times; to keep the humidity high in the culture plate, place a damp piece of tissue paper, such as KimWipes, in the plate, but not over the sample, and keep the plate covered in between steps.

10. Add 12  $\mu$ L of PBST in well 2A to 6A.

11. Wash the brain 5 times by sequentially transferring the brain between wells:

- a. Wash the brain for 1 min for the first 3 washes (well 2A, 3A, and 4A).
- b. Wash the brain for 15 min for the last 2 washes (well 5A and 6A).
12. Meanwhile, add 12  $\mu$ L of stocker blocker solution to well 7A; once the last wash is completed, transfer the brain to well 7A; incubate for 30 min at room temperature (22°C–28°C).
13. Meanwhile, prepare the primary antibody solution by diluting the mouse anti-nc82 antibody in the stocker blocker solution (1:20).

**Note:** For optimal results, make the primary antibody solution on the same day when it is used. Store the solution at 4°C.

14. Add 12  $\mu$ L of the primary antibody solution to well 8A and transfer the brain to this well when step 12 is complete.
15. Cover the culture plate and seal it with parafilm; incubate overnight (at least 24 h) at 4°C.

**Note:** For optimal results, incubate the brain in the primary antibody solution for 24 h.

16. **Day 2.** Using a vacuum line, remove any remaining solution from wells 2A to 5A; refill these wells with 12  $\mu$ L PBST.
17. Wash the brain 4 times by sequentially transferring the brain between wells:
  - a. Wash the brain for 1 min for the first 2 washes (well 2A and 3A).
  - b. Wash the brain for 15 min for the last 2 washes (well 4A and 5A).
18. Meanwhile, make the secondary antibody solution by diluting the Alexa Fluor 633 goat-anti-mouse antibody in stocker blocker solution (1:500); store the solution at 4°C in the dark until it is used.

**Note:** For optimal results, make the secondary antibody solution on the same day when it is used. Store the solution at 4°C in dark.

19. Add 12  $\mu$ L of the secondary antibody solution to well 9A; incubate overnight (at least 24 h) at 4°C in the dark.
20. **Day 3.** Wash the sample by repeating steps 16 and 17.
21. Mount the brain on a glass microscope slide:
  - a. Stack two reinforcement stickers on top of each other and place the stack in the center of a glass microscope slide.
  - b. Add a small drop of VECTASHIELD mounting medium to the center of the stack of reinforcement stickers.
  - c. Transfer the brain to the mounting medium and place it in the desired orientation.
  - d. Place a round, glass coverslip on top of the stack of reinforcement stickers.
  - e. Seal the edge of the coverslip to the slide with nail polish. Keep the slide in the dark until the nail polish hardens.

**Note:** When placing the coverslip on top of the stack of reinforcement stickers, avoid creating large air bubbles within the mounting medium, as they may displace the brain.

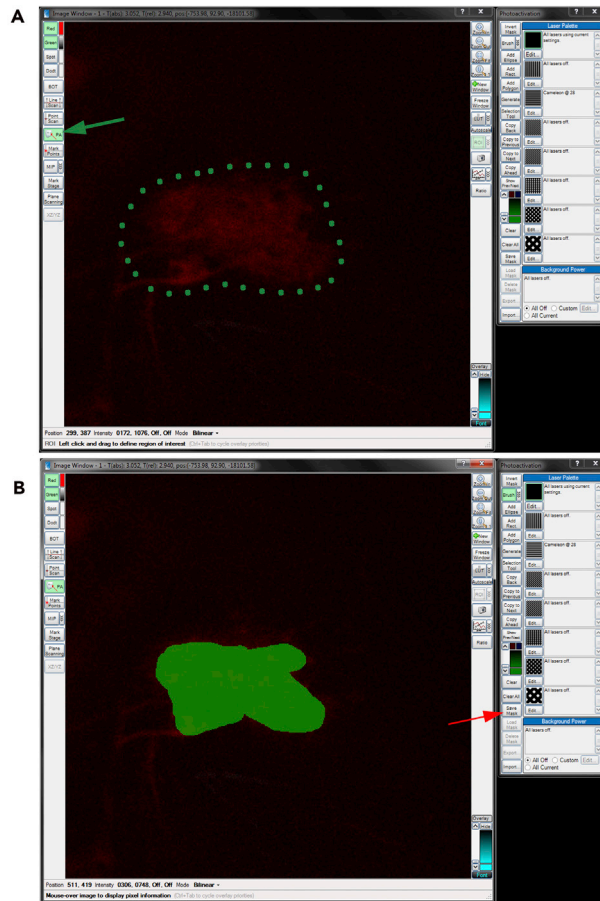
22. Image the brain with the photo-labeled neuron under a confocal microscope ([Figure 1E](#)).

### Photo-label a group of neurons

⌚ Timing: 45–75 min

This protocol can be used to photo-label a group of neurons that project to a common brain area.

23. Configure the imaging software on the two-photon microscope using the live scan parameters outlined in the [materials and equipment](#) section.

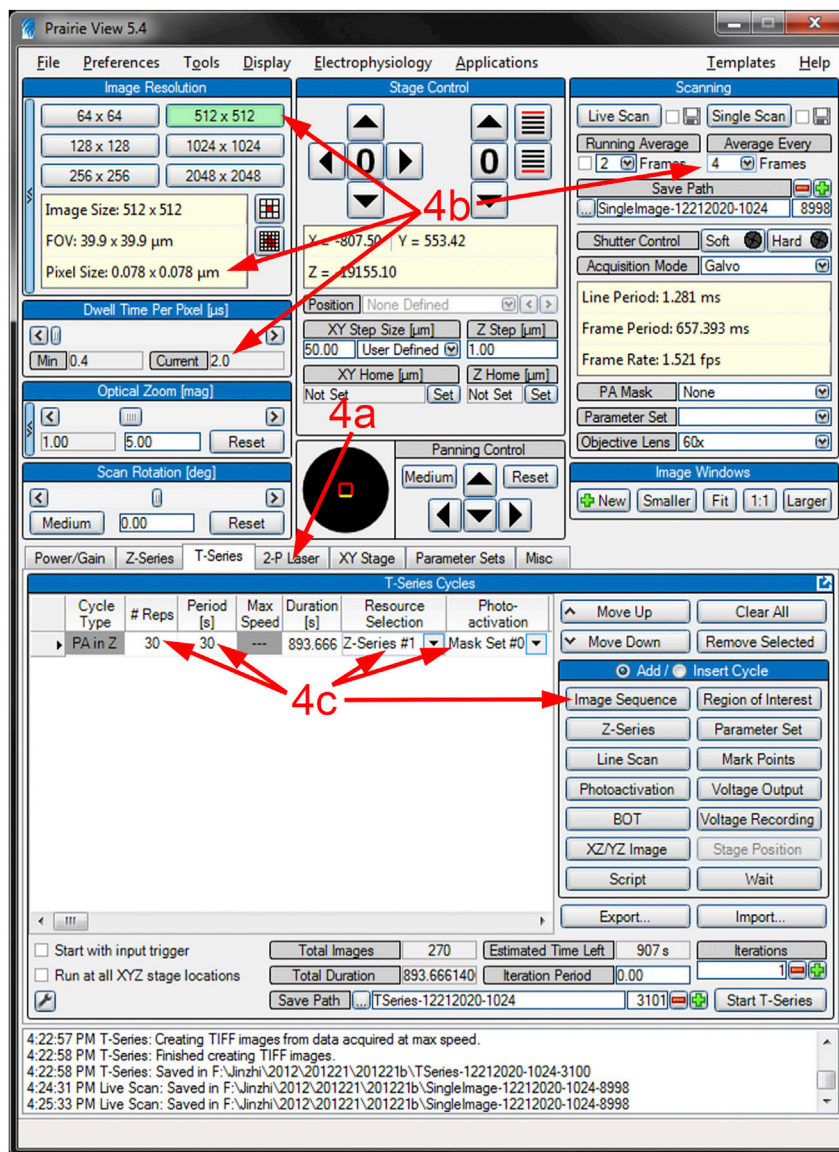


**Figure 4. Defining the region of interest (ROI) for the photo-labeling procedure on the targeted brain region**  
 (A) After taking a z series of the brain region targeted for the photo-labeling procedure (green dashed outline, here only showing a single plane of the z series), select the PA mask function tab on the imaging software (green arrow).  
 (B) On each plane of the z series, define an area of the targeted brain region (green) for the photo-labeling procedure. Save the mask (red arrow) after a ROI is defined in every plane of the z series.

24. Tune the laser to 925 nm and image the sample using the 60 $\times$  objective lens, such that the brain area of interest can be located and visualized (Figure 1B).

**Note:** The brain area of interest is visualized by expression of an independent fluorophore.

25. Define a region of interest around or within the brain region of interest (Figure 4):
- In live scan mode, image the sample at the highest magnification such that the brain region of interest occupies most of the imaging window (we used 5 $\times$  digital zoom).
  - Acquire a series of scans that span across the volume of the region of interest.
  - Define the area within the series of scan that is to be photo-labeled (we used the “PA Mask” function that is available with the Prairie View software).
26. Set up the photo-labeling cycle (Figure 5):
- Tune the laser to 710 nm.
  - Configure the imaging software using the parameters listed for photo-labeling groups of neurons (see the materials and equipment section).
  - Perform a timed sequence acquisition of the region of interest defined in step 25; set the number of repetitions in the sequence to 30 and the period — the resting time between each repetition — to 30 s.



**Figure 5. Setting up the photo-labeling cycle for photo-labeling neuronal groups on the imaging software user interface**

See step 26 under [photo-label a group of neuron](#) for setting up the parameters for the photo-labeling procedure.

**Note:** For optimal results, use a z series that is comprised of 5 to 10 planes. The distance between each plane should be at least 2 μm but not more than 5 μm.

△ **CRITICAL:** As when photo-labeling individual neurons, it is important to photo-convert a sufficient number of PA-GFP molecules in the neurons of interest, while avoiding causing damage to these neurons. The laser power required for photo-labeling groups of neurons varies from sample to sample and should be adjusted accordingly.

27. Run the photo-labeling cycle.
28. Wait for 15 min after the completion of the photo-labeling cycle to allow the sample to recover; repeat the photo-labeling cycle once or twice, depending on the results. Always allow for a 15-min recovery period in between cycles.

29. Image the photo-labeled group of neurons.
  - a. Tune the laser to 925 nm.
  - b. Configure the imaging software using the listed parameters for the live scan mode, such that the entire brain volume encompassing the photo-labeled group of neurons can be imaged (see the [materials and equipment](#) section).
  - c. Acquire the image sequence ([Figure 1D](#)).
30. The brain sample can be recovered and immuno-stained by following instructions in the [immunolabel the brain for confocal imaging](#) section ([Figure 1F](#)).

## EXPECTED OUTCOMES

When collecting images of the photo-labeled neuron using two-photon microscopy, make sure that its entire morphology of the selected neuron is clearly distinguishable from the background fluorescence. Morphological features — such as dendritic branches, axonal projections, and presynaptic boutons — should be clearly visible.

[Figure 1C](#) shows a photo-labeled neuron — in this case a  $p_{LP}PN$  (white arrow) — among other neurons that also express PA-GFP but are not photo-labeled (gray arrows). The entire morphology of this photo-labeled neuron is clearly visible, including its dendrites, axonal projection, and presynaptic boutons ([Figure 1C](#), yellow arrows). After imaging the neuron using two-photon microscopy, this sample was immuno-stained and imaged a second time using confocal microscopy ([Figure 1E](#)).

[Figure 1D](#) shows a group of photo-labeled neurons — here 13  $p_{LP}PN$ s (white arrows) — among other neurons that also express PA-GFP but are not photo-labeled. These neurons were recovered after photo-labeling the dorsal accessory calyx of the mushroom body ([Figure 1D](#), white dashed outline), a region in which  $p_{LP}PN$ s extend some of their axonal projections. After imaging the group of neurons using two-photon microscopy, the sample was immuno-stained and imaged a second time using confocal microscopy ([Figure 1F](#)).

## LIMITATIONS

Although this protocol can be used to visualize most, if not all, neurons of the *Drosophila* brain, it has a few limitations. Successful photo-labeling largely depends on the number of PA-GFP molecules that are expressed in the neuron(s) of interest. This protocol is not suitable for transgenic lines that have very low expression levels. The position of the neuron(s) of interest within the brain can also affect results: neurons located in the center of the brain are typically more difficult to photo-label than neurons located in more superficial areas.

## TROUBLESHOOTING

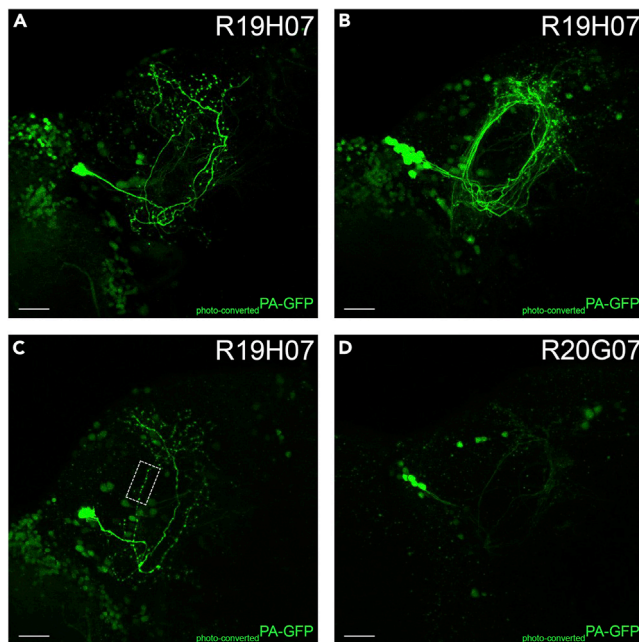
### Problem 1

The pH and the osmolarity of the *Drosophila* adult hemolymph-like saline are not within the optimal range (step 8 of the [prepare the Drosophila adult hemolymph-like saline](#) section).

### Potential solution

If the pH of the saline is below 7.3, add 20  $\mu$ L of the 10 M NaOH solution, one drop at a time, and measure the pH again. Repeat until the pH reaches around 7.3. If the pH of the saline is above 7.3, add Milli-Q system-treated water until the pH drops back around 7.3.

If the osmolarity is below the optimal range (265–275 mOsm), add 1–2 mL of 2 M trehalose solution and measure the osmolarity again. Repeat until the osmolarity reaches the optimal range. If the osmolarity is above the optimal range, add Milli-Q system-treated water until the osmolarity drops back to the optimal range.



**Figure 6. Examples of successful and unsuccessful photo-labeling**

(A and B) A successfully photo-labeled single posterior lateral protocerebrum projection neuron ( $p_{LP}PN$ ) (A) and  $p_{LP}PN$  population (B). The photo-labeled neurons are distinguishable from other neurons that also express photoactivatable GFP (PA-GFP) but are not photo-labeled.

(C) A photo-labeled  $p_{LP}PN$  is dying due to damage incurred during the photo-labeling cycle, as evidenced by the degradation of the neuronal branches (white dashed box).

(D) Photo-labeled  $p_{LP}PN$  populations driven by a weak transgenic line, as evidenced by neuronal branches that are barely visible after photo-labeling. The following genotypes were used in this figure: (A) *yw/yw; MB247-DsRed<sup>unknown</sup>, UAS-C3PA-GFP<sup>unknown</sup>/UAS-SPA-GFP<sup>attP40</sup>; UAS-C3PA-GFP<sup>attP2</sup>, UAS-C3PA-GFP<sup>VK00005</sup>, UAS-C3PA-GFP<sup>VK00027</sup> / R19H07-GAL4<sup>attP2</sup>*; (B-C) *UAS-C3PA-GFP<sup>unknown</sup>/UAS-SPA-GFP<sup>attP40</sup>; R19H07-GAL4<sup>attP2</sup>/R13F02-GAL4<sup>VK00027</sup>, R85D07-GAL4<sup>DBD</sup><sup>attP2</sup>*; (D) *UAS-C3PA-GFP<sup>unknown</sup>/CyO; R20G07-GAL4<sup>attP2</sup>/MKRS*. Scale bar, 20  $\mu$ m in all panels.

## Problem 2

The cell body of the neuron of interest cannot be located before starting the photo-labeling procedure (step 2 of the [photo-label individual neurons](#) section).

### Potential solution

If the cell body of the neuron of interest is located in the center of the brain, it may be difficult to image. A brief photo-labeling cycle that targets the dendritic branches or axonal projection of the neuron of interest can be used to facilitate the identification of its cell body. This brief photo-labeling cycle will lead to the photo-conversion of a small number of PA-GFP molecules within the neuron of interest, which will rapidly diffuse and increase its brightness.

## Problem 3

After performing several photo-labeling cycles, the targeted neuron, or group of neurons, is not clearly visible (step 7 and 29; [Figure 6D](#)).

### Potential solution

The targeted neuron could be dimly photo-labeled even after performing several photo-labeling cycles. This outcome most likely results from a low number of photo-converted PA-GFP molecules within the targeted neurons. The first solution is to increase the expression level of the PA-GFP molecules, namely by using different transgenic lines — either by selecting a promoter that expresses at higher levels in the neuron(s) of interest or by adding more copies of the *PA-GFP* transgenes.

Alternatively, increasing the laser power that is used during the photo-labeling cycle, or increasing the number of photo-labeling cycles, can yield larger amounts of photo-converted PA-GFP molecules.

#### **Problem 4**

The targeted neuron is damaged and its axonal projection shows signs of degeneration (step 7 and 29; [Figure 6C](#)).

#### **Potential solution**

Signs of neuronal degeneration — such as fragmentation of the axonal projection — are detectable when the targeted neuron is injured ([Figure 6C](#)). Such damage is irreversible. Exposure to high levels of laser power (>30 mW) is the most likely cause for neuronal degeneration. Reducing the laser power that is used during photo-conversion of PA-GFP should yield better results.

#### **Problem 5**

Additional neurons are photo-labeled after running the photo-labeling procedure on the targeted neuron or brain region of interest (step 7 and 29).

#### **Potential solution**

Additional photo-labeled neurons primarily result from areas outside of the defined region of interest (ROI) also being photo-labeled. When defining the ROI for photo-labeling an individual neuron (step 3 of the [photo-label individual neurons](#) section), make sure the ROI sits well within the targeted neuron, make sure the ROI sits well within the targeted neuron ([Figure 2](#)). When defining the ROI for a particular brain region of interest (step 3 under the [photo-label a group of neurons](#) section), Make sure the ROI covers the region of interest only. In addition, set the laser power for the background (areas outside the defined ROI) to zero.

## **RESOURCE AVAILABILITY**

### **Lead contact**

Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Sophie Caron ([sophie.caron@utah.edu](mailto:sophie.caron@utah.edu)).

### **Materials availability**

This study did not generate new reagents or transgenic lines. The transgenic lines used in this protocol are available upon request.

### **Data and code availability**

This study did not generate datasets or codes.

## **ACKNOWLEDGMENTS**

We thank the Caron laboratory members for comments on the manuscript; Adam Lin for the standard cornmeal agar medium; and Shannon Torstrom, Hayley Smihula, and Adam Weinbrom for assistance with general laboratory concerns. This work has been funded in part by grants from the National Institute for Neurological Disorders and Stroke (R01 NS 106018 and R01 NS 1079790). Further financial support was provided by the Georges S. and Dolores Eccles Foundation (S.J.C.C.), the Dale A. Stringfellow Fellowship (J.L.), and the Eunice Kennedy Shriver National Institute of Child Health & Human Development of the National Institutes of Health under award number T32HD007491 (K.E.E.).

## **AUTHOR CONTRIBUTIONS**

J.L., K.E.E., and S.J.C.C. wrote the manuscript. J.L., K.E.E., and S.J.C.C. conceived the protocol and designed the experiments. J.L. performed the experiments and analyzed the data. S.J.C.C. supervised the research.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

### REFERENCES

- Alpert, M., Frank, D., Kaspi, E., Flourakis, M., Zaharieva, E., Allada, R., Para, A., and Gallio, M. (2020). A circuit encoding absolute cold temperature in *Drosophila*. *Curr. Biol.* 30, 2275–2288.e5.
- Aso, Y., Hattori, D., Yu, Y., Johnston, R.M., Iyer, N.A., Ngo, T.T.B., Dionne, H., Abbott, L.F., Axel, R., Tanimoto, H., and Rubin, G.M. (2014). The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife* 3, e04577.
- Caron, S.J.C., Ruta, V., Abbott, L.F., and Axel, R. (2013). Random convergence of olfactory inputs in the *Drosophila* mushroom body. *Nature* 497, 113–117.
- Datta, S.R., Vasconcelos, M.L., Ruta, V., Luo, S., Wong, A., Demir, E., Flores, J., Balonze, K., Dickson, B.J., and Axel, R. (2008). The *Drosophila* pheromone cVA activates a sexually dimorphic neural circuit. *Nature* 452, 473–477.
- Frank, D.D., Jouandet, G.C., Kearney, P.J., MacPherson, L.J., and Gallio, M. (2015). Temperature representation in the *Drosophila* brain. *Nature* 519, 358–361.
- Lai, S.L., and Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in *Drosophila*. *Nat. Neurosci.* 9, 703–709.
- Li, J., Mahoney, B.D., Jacob, M.S., and Caron, S.J.C. (2020). Visual input into the *Drosophila melanogaster* mushroom body. *Cell Rep.* 32, 108138.
- Potter, C.J., and Luo, L. (2011). Using the Q system in *Drosophila melanogaster*. *Nat. Protoc.* 6, 1105–1120.
- Riemensperger, T., Völler, T., Stock, P., Buchner, E., and Fiala, A. (2005). Punishment prediction by dopaminergic neurons in *Drosophila*. *Curr. Biol.* 15, 1953–1960.
- Ruta, V., Datta, S.R., Vasconcelos, M.L., Freeland, J., Looger, L.L., and Axel, R. (2010). A dimorphic pheromone circuit in *Drosophila* from sensory input to descending output. *Nature* 468, 686–690.