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.
Protocol

Photo-labeling neurons in the Drosophila brain

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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 pLpPNs — 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 NaHCO₃ and 0.24 g of NaH₂PO₄; 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 CaCl₂ solution and 8 mL of 1 M MgCl₂ 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 polyethersulfone 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 10X 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     |
| NaHCO₃      | 4 mM                | 0.67 g     |
| NaHPO₄      | 1 mM                | 0.24 g     |
| MgCl₂       | 4 mM                | 8 mL       |
| CaCl₂       | 2 mM                | 4 mL       |
| ddH₂O       | -                   | 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 CO₂ 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 PLEPNs 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 |
|---------------------|--------|------------|
| Antibodies          | 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)
### Materials and Equipment

**Reagents or Resources**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | The Jackson Laboratory | AB_2336990 |
| VECTASHIELD mounting medium | Vector Laboratories | H-1000 |
| 1% paraformaldehyde | Electron Microscopy Sciences | 15710 |
| 10x phosphate buffered saline | Sigma-Aldrich | PS493 |
| Triton X-100 | Sigma-Aldrich | T8787 |
| Collagenase | Sigma-Aldrich | C5138 |
| MgCl2 solution (1 M in H2O) | Sigma-Aldrich | 63069 |
| CaCl2 solution (1 M in H2O) | Sigma-Aldrich | 21115 |
| NaOH solution (10 M in H2O) | Sigma-Aldrich | 72068 |
| NaCl | Sigma-Aldrich | S7653 |
| KCl | Sigma-Aldrich | S5405 |
| HEPES | Sigma-Aldrich | H3375 |
| Trehalose | Sigma-Aldrich | T0167 |
| Sucrose | Sigma-Aldrich | S1888 |
| NaHCO3 | Sigma-Aldrich | S5761 |
| Na2PO4 | Sigma-Aldrich | S5011 |

**Experimental models: organisms/strains**

| D. melanogaster: y1w1118;; R19H07-GAL4attP2; Bloomington Drosophila Stock Center | BDSC: 48867 |
| D. melanogaster: y1w1118;; R20G07-GAL4attP2; Bloomington Drosophila Stock Center | BDSC: 48613 |
| D. melanogaster: y1w1118;; R13F02-GAL4AD; R85D07-GAL4DBD (Aso et al., 2014) | N/A |
| D. melanogaster: yw; UAS-C3PA-GFP (unknown); (Aso et al., 2014) | N/A |
| D. melanogaster: y1w1118;; UAS-C3PA-GFP (unknown); (Aso et al., 2014) | N/A |
| D. melanogaster: y1w1118;; UAS-C3PA-GFP (unknown); (Aso et al., 2014) | N/A |
| D. melanogaster: y1w1118;; UAS-SPA-GFP (unknown); (Aso et al., 2014) | N/A |
| D. melanogaster: yw; MB247-DsRed (unknown); (Riemensperger et al., 2005) | N/A |

**Other**

| 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 x 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 |

### 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 x 512 | 512 x 512 | 512 x 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:
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).
c. Acquire the image sequence (Figure 1C).

Immunolabel the brain for confocal imaging

Timed: 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 µL 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).
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 μL 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 μL 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 μL 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 μL 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 μL 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.
24. Tune the laser to 925 nm and image the sample using the 60× 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):
   a. 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× digital zoom).
   b. Acquire a series of scans that span across the volume of the region of interest.
   c. 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):
   a. Tune the laser to 710 nm.
   b. Configure the imaging software using the parameters listed for photo-labeling groups of neurons (see the materials and equipment section).
   c. 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.
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 immu-
    nolabel 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 fluores-
cence. 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 plpPN (white arrow) — among other neu-
rons 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 presynap-
tic 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 plpPNs (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 plpPNs 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-la-
bel 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 µL 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 if 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 os-
molarity is above the optimal range, add Milli-Q system-treated water until the osmolarity drops
back to the optimal range.
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).

**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.

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**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.

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