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Protocol

Cell Ablation Techniques for the Larval *Drosophila* Neuromuscular System

James Ashley[1] and Robert A. Carrillo[1,2,3]

[1]*Department of Molecular Genetics and Cell Biology,* [2]*Neuroscience Institute, University of Chicago, Chicago, Illinois 60637, USA*

Tissue development requires local and long-distance communication between cells. Cell ablation experiments have provided critical insights into the functions of specific cell types and the tissue surrounding the dead cells. In the *Drosophila* neuromuscular system, ablation of motor neurons and muscles has revealed the roles of the ablated cells in axon pathfinding and circuit wiring. For example, when muscles are denervated due to laser ablation of their motor neuron inputs, they receive ectopic innervation from neighboring motor neurons. Here, we describe two methods of specific cell ablation. The first is a genetic ablation approach that uses *GAL4* (ideally expressed in a small subset of cells) to drive expression of cell death genes *reaper* and *head involution defective*. The second method relies on reactive oxygen species produced by light activation of the *Arabidopsis*-derived Singlet Oxygen Generator, miniSOG2, expressed in a subset of cells. For the latter, the precision stems from both the *GAL4* and the restricting of the blue-light stimulation area.

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MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Antibodies

Goat anti-horseradish peroxidase (HRP) Alexa 647 (Jackson ImmunoResearch 123-605-021)

This antibody cross-reacts with several neuronal cell surface molecules (Jan and Jan 1982), such that even brief incubations can easily label all neurons.

Goat anti-mouse Alexa 568 (Thermo Fisher A11031)

Mouse anti-Dlg (Developmental Studies Hybridoma bank, 4F3)

Fixative of choice

Fly food in a vial (e.g., Genesee, 32-116)

Variety of food does not matter, we use Cornmeal, Molasses, and Yeast Medium from Bloomington Drosophila *Stock Center (https://bdsc.indiana.edu/information/recipes/molassesfood.html).*

[3]Correspondence: robertcarrillo@uchicago.edu

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Fly strains
GAL4 strain (e.g., A8-*GAL4*) (Venkatasubramanian et al. 2019)
GAL80 strain (optional; see note below Step 1)
GAL80^{ts} strain (optional; see note below Step 1; for general temporal control, we use tubP-*GAL80^{ts}*, Bloomington *Drosophila* Stock Center 7016)
UAS-*hid*,UAS-*rpr* strain (Zhou et al. 1997)
UAS-*miniSOG2*-T2A-*His::GFP* strain

This strain has low-level expression in epithelial cells, salivary glands, and some neurons, but not enough for ablation (Makhijani et al. 2017).

UAS-reporter strain (e.g., 10XUAS-*mCD8::GFP*, Bloomington *Drosophila* Stock Center 32184)
Grape agar plates <R>
Yeast paste

Combine active dry yeast with an approximately equal volume of H_2O in a 50-mL tube and mix thoroughly with a laboratory spatula. Prepare ~25% of the tube volume and store at 4°C with the cap loosened. Yeast will expand violently if not careful. The consistency should resemble stiff peanut butter; if too liquid, add more yeast; if too dry, add more H_2O.

Equipment

Compound microscope (with a 488-nm X-Cite XYLIS LED source, a 20× objective, and a 470/30 filter cube)

Use the 20× objective X-Cite XYLIS LED source to generate ~3 W/cm^2 using 100% power and the 470/30 filter cube.

Coverslips (25-mm × 25-mm, #1.5)
Forceps (Dumont #3; Fine Science Tools 11231-30)
Glass slides (25-mm × 75-mm)
Laboratory tape
Paint brush, small, 3/0 (any art supply store)

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METHOD

Cell ablation can be achieved by genetic means (Steps 1–4) or using light (Steps 5–11).

Genetic Means of Cell Ablation

With genetic cell ablation, cell death proteins are expressed soon after the GAL4 *is induced, and the cells die shortly thereafter. Therefore, it is important to determine the expression pattern and timing of the* GAL4 *line of interest.*

1. Begin by verifying the expression pattern and expression timing of the specific *GAL4* line of interest.

   While the expression patterns of many GAL4 *lines have been characterized, a large subset of* GAL4 *lines lacks spatiotemporal resolution.*

   i. To determine when *GAL4* is expressed in the strain of interest, set up a reporter cross (e.g., collect males from the *GAL4* line and cross them to virgin females from a UAS-*GFP* line) and dissect progeny at different stages (embryos and larvae) (see Protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** [Ashley and Carrillo 2024a]). Fix and examine the different stages under a fluorescent microscope to assess expression timing.

   ii. If it is difficult to find a *GAL4* driver that only expresses in the cells of interest, consider limiting GAL4 activity with GAL80, which is a GAL4 inhibitor. GAL80 is expressed under known promoters in a partially overlapping set of cells to inhibit GAL4 activity; the remain-

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ing GAL4 activity is now in a smaller subset of cells. Follow Step 1.i to visualize the expression pattern of *GAL4* in the *GAL4/GAL80* line of interest.

For example, if GAL4 *in the strain of interest expresses in motor neurons of interest, but is also expressed in sensory neurons, express* GAL80 *under a sensory neuron promoter, and when the two elements are combined, the only active GAL4 would be in motor neurons.*

iii. Consider the timing of *GAL4* expression, which can also be critical. For example, the death of stem cells can affect multiple cell lineages, and not just the cells of interest. There are systems available to restrict expression to specific timing, such as the TARGET system. The TARGET system includes ubiquitous expression of a temperature-sensitive GAL80, tubP-*GAL80*^{ts}, which inhibits GAL4 at 18°C and ceases to inhibit at 29°C (McGuire et al. 2003). As above, follow Step 1.i to visualize *GAL4* expression in this line.

2. Build a cell ablation line.

   i. Obtain the UAS-*rpr*,UAS-*hid* strain, which contains both UAS elements on the second chromosome.

   Here, we are ablating neurons, and it was reported that overexpression of the cell death genes hid *or* rpr *alone was not as effective as simultaneous overexpression of both (Zhou et al. 1997).*

   ii. Using standard *Drosophila* techniques, cross a 10XUAS-*mCD8::GFP* strain into the UAS-*rpr*, UAS-*hid* background to easily visualize the cells and confirm their ablation.

   *The 10XUAS-*mCD8::GFP *strain expresses a membrane-tethered GFP protein, which allows for easy visualization of neurons and cell bodies.*

3. Cross the ablation line to the *GAL4* of choice as follows: Cross males from the UAS-*hid*,UAS-*rpr* ablation line described in Step 2 to virgin females from the A8-*GAL4* line.

   *The A8-*GAL4 *line expresses* GAL4 *in a small subset of motor neurons. In our hands, the result of this cross was a complete loss of these specific neurons in the larval nerve cord (Fig. 1A; Wang et al. 2021).*

4. Examine morphological changes by dissecting larvae (see Protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** [Ashley and Carrillo 2024a]) and fixing and labeling the dissected larvae with antibodies that label the cells of interest (see Protocol: **Immunohistochemistry and Morphometric Analysis of *Drosophila* Larval Body Wall Neuromuscular Junction Preparations** [Ashley and Carrillo 2024b]).

   In our example, we used antibodies against horseradish peroxidase (HRP) to label the presynaptic neuron and Discs large (Dlg) to label the postsynaptic muscle area. Through morphometric analysis (see Protocol: ***Immunohistochemistry and Morphometric Analysis of* Drosophila *Larval Body Wall Neuromuscular Junction Preparations*** *[Ashley and Carrillo 2024b]) of the larval neuromuscular system, in which each larval muscle receives input from two motor neurons, we found that if one of the neurons is ablated (using A8-*GAL4 *driving* rpr *and* hid*), the remaining neuron showed neuromuscular junction (NMJ) expansion (Wang et al. 2021, 2023).*

Light-Induced Cell Ablation

Here, the phototoxic gene miniSOG2*, which generates oxygen free radicals in the presence of blue light, is under the control of UAS, and its overexpression is driven by a specific* GAL4 *line. Targeted cell death occurs when animals are exposed to intense blue light.*

5. Cross the *GAL4* line of choice (e.g., A8-*GAL4*) to the UAS-*miniSOG2-T2A-His::GFP* line (Fig. 1B). Set up these crosses at 25°C in an embryo collection chamber (see Protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** [Ashley and Carrillo 2024a]).

6. Build an illumination chamber depending on the stage of the animal.

   i. For late embryos or early first-instar larvae, place two pieces of laboratory tape on a glass slide, ~15 mm apart, such that they will hold up and provide a bridge for a 22-mm coverslip (Fig. 1C, left). This gap will be sufficient to protect the samples from being crushed when a coverslip is placed on top.

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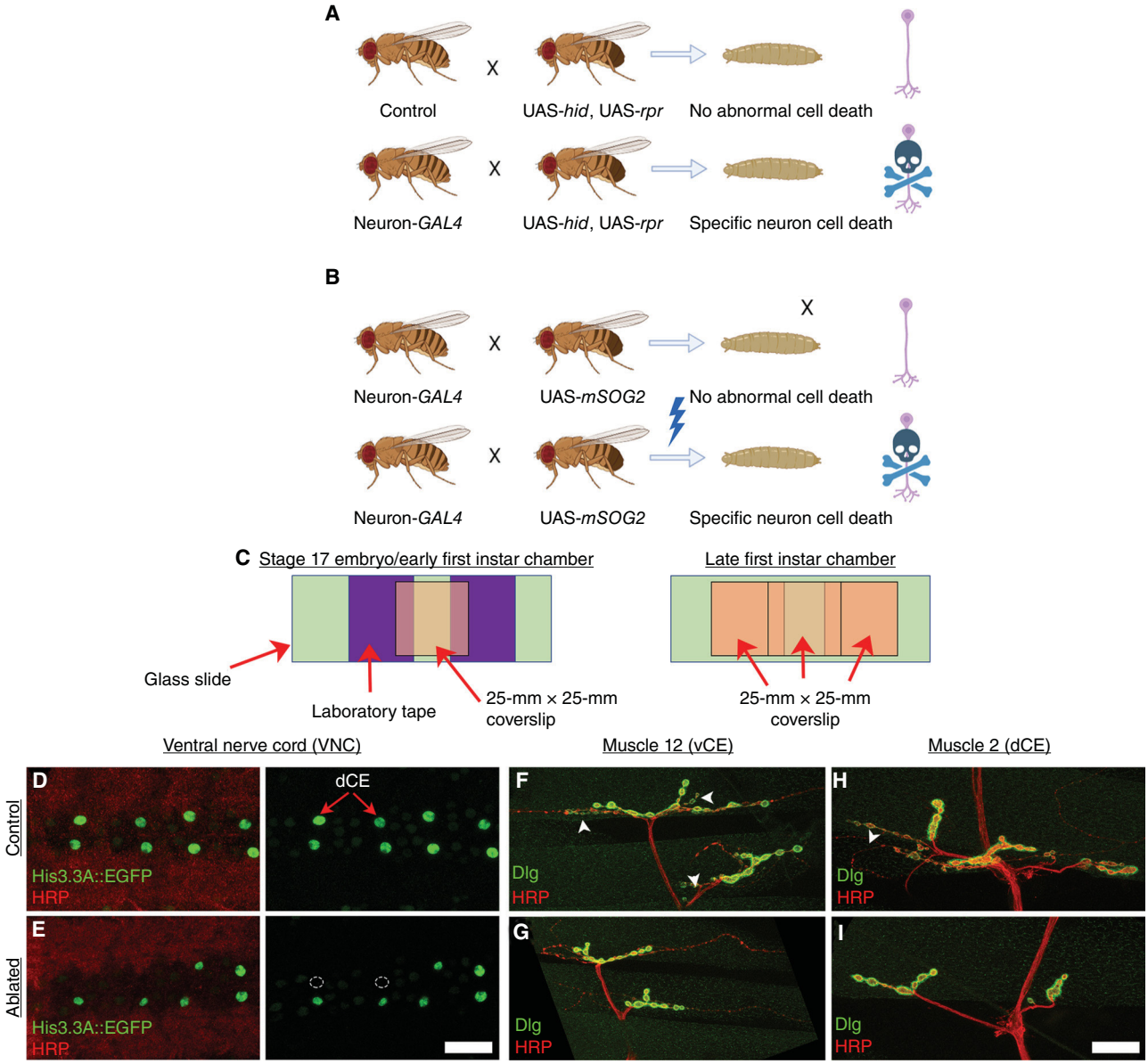

FIGURE 1. Targeted cell ablation. (*A*,*B*) Cartoon representations of crosses resulting in targeted cell death. (*A*) Genetic cell ablation in which cell death genes UAS-*hid* and UAS-*rpr* are overexpressed, and any neuron that expresses *GAL4* will be killed. (*B*) Triggered cell ablation in which the phototoxic gene UAS-*miniSOG2*, which generates oxygen free radicals in the presence of blue light, is overexpressed by a specific *GAL4*. Targeted cell death occurs only when animals are exposed to intense blue light (made with Biorender.com). (*C*, *left*) Light-exposure chamber for late stage 17 embryos/early first-instar larvae made with laboratory tape to elevate the coverslip. (*Right*) Late first-instar chamber made with #1.5 coverslips to elevate the middle coverslip higher. (*D–I*) Controls (*D*,*F*,*H*) and larvae overexpressing UAS-*miniSOG2-T2A-His-GFP* under the control of A8-*GAL4* (Is motor neuron driver) (*E*,*G*,*I*). (*D*,*E*) Dorsal view of ventral nerve cords (VNCs) labeled with antibodies against horseradish peroxidase (HRP, red) and GFP (green). Note the absence of dorsal common exciter cell bodies (dashed circles) in *E*. (*F–I*) Muscle 12 and muscle 13 neuromuscular junctions (*F*,*G*) and muscle 2 neuromuscular junctions (*H*,*I*) labeled with antibodies against Discs large (Dlg) (green) and HRP (red). Arrowheads in *F* and *H* denote Is boutons in controls. Note the absence of Is boutons in *G* and *I*. (dCE) Dorsal common exciter, (vCE) ventral common exciter. Scale bar, *D–I*, 20 µm.

ii. For larger late first-instar and later larvae, place two #1.5 coverslips on a glass slide, building a bridge for the center coverslip (Fig. 1C, right).

7. Collect embryos or larvae. For the embryo collection chambers set up in Step 5, change grape agar plates with yeast paste after 2 h as in Protocol: ***Drosophila* Late Embryonic through Late Larval**

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Stage Body Wall Dissection: Dissection Tools and Techniques (Ashley and Carrillo 2024a). Incubate for ~22–24 h at 25°C to obtain late stage 17 embryos or ~48 h to collect late first-instar larvae.

8. Place multiple embryos or larvae on the appropriate illumination chamber using forceps, and gently place a coverslip on top. Animals do not need to be anesthetized, as the gap is small enough to hold them tightly.
9. Transfer the illumination chamber to the stage of the compound microscope.
10. Using the transmitted light source, bring each embryo or larva into focus and then turn on the 488-nm light for 3 min at room temperature.

    In our case, we used the 20× objective on our microscope with the light source at 100%. Higher magnification would further concentrate the light, but with larvae, it was difficult to fully immobilize them, and the lower magnification allowed for illumination of a larger area.

11. Carefully remove the coverslip using #3 forceps and place embryos/larvae into a fresh vial of food using a soft 3/0 paint brush.

    i. Collect larvae from the food vial 48–120 h after blue-light exposure.

       If samples were exposed as embryos and the goal is to examine third-instar morphology, collect larvae at ~120 h, or until the wandering third-instar stage.

    ii. Dissect larvae (see protocol: ***Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques** [Ashley and Carrillo 2024a]).

    iii. Prepare samples with fixation and antibody labeling (see **Protocol: Immunohistochemistry and Morphometric Analysis of *Drosophila* Larval Body Wall Neuromuscular Junction Preparations** [Ashley and Carrillo 2024b]).

       *As the UAS-*miniSOG2-T2A-His::GFP *line also expresses GFP-tagged histones, there is no need for antibodies to score cell loss. The loss of GFP can be used as a proxy to score cell death.*

    iv. Examine samples on a fluorescent microscope with at least a 40× objective.

       As light was applied during embryonic and early larval stages, ablated cells will be obvious by the end of larval development by lack of GFP expression. We examined larval ventral nerve cords and found an ~50% loss of A8-expressing neurons per animal (Fig. 1D,E).

    v. After confirming cell death, examine adjacent cells/neurons for phenotypes using specific markers, as above.

       This is completely dependent on the question being asked. Here, we labeled NMJs with and without Is motor neurons ablated (Fig. 1F–I).

DISCUSSION

The ablation protocol can be used to kill cells of interest at specific developmental stages to determine whether and how the loss of those cells affects development of neighboring cells. We described how to control spatiotemporal expression and activity of *GAL4* to limit expression to the cells of interest. In previous studies, we and others examined pairs of motor neurons that innervate the same muscle. After ablating one of the neurons, immunohistochemistry with antibodies against HRP and Dlg revealed that the adjacent motor neuron increases its axon terminal size (Aponte-Santiago et al. 2020; Wang et al. 2021, 2023; Han et al. 2022). A similar approach can be applied to other cells of interest; however, the phenotypic analysis will depend on the research question.

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RECIPES

Grape Agar Plates

Reagent	Quantity
Agar	12 g
H_2O	300 mL
Grape juice (Welch's or equivalent)	100 mL
Sucrose	5.35 g

1. Combine H_2O and agar together in a microwave-safe flask, and heat until just boiling. Use caution not to boil over.
2. Stop the microwave until the bubbles subside.
3. Restart the microwave and heat until bubbles rise, watching carefully to stop before boiling over.
4. Again, stop the microwave until bubbles subside.
5. Repeat Steps 3 and 4 until the bubbles become large and the agar is dissolved.
6. Meanwhile, heat the grape juice and sugar together until the sugar is dissolved.
7. Combine the two solutions and pour into the lids of 35-mm Falcon Petri plates (see Protocol: *Drosophila* Late Embryonic through Late Larval Stage Body Wall Dissection: Dissection Tools and Techniques [Ashley and Carrillo 2024 Cold Spring Harb Protoc doi:10.1101/pdb.prot108499]).

   Pour such that there is a convex meniscus over the edge of the lid. This fills about 50 35-mm Petri dish lids.
8. After cooling, store in a lidded container at 4°C until ready to use. These are stable up to 2 wk, but discard if agar begins to dry and pull away from the sides.

   Warm to room temperature before use in the egg-laying chamber.

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REFERENCES

Aponte-Santiago NA, Ormerod KG, Akbergenova Y, Littleton JT. 2020. Synaptic plasticity induced by differential manipulation of tonic and phasic motoneurons in *Drosophila*. *J Neurosci* **40:** 6270–6288. doi:10.1523/JNEUROSCI.0925-20.2020

Ashley J, Carrillo RA. 2024a. *Drosophila* late embryonic through late larval stage body wall dissection: dissection tools and techniques. *Cold Spring Harb Protoc* doi:10.1101/pdb.prot108499

Ashley J, Carrillo RA. 2024b. Immunohistochemistry and morphometric analysis of *Drosophila* larval body wall neuromuscular junction preparations. *Cold Spring Harb Protoc* doi:10.1101/pdb.prot108500

Han Y, Chien C, Goel P, He K, Pinales C, Buser C, Dickman D. 2022. Botulinum neurotoxin accurately separates tonic vs. phasic transmission and reveals heterosynaptic plasticity rules in *Drosophila*. *eLife* **11:** e77924. doi:10.7554/eLife.77924.sa2

Jan LY, Jan YN. 1982. Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and in grasshopper embryos. *Proc Natl Acad Sci* **79:** 2700–2704. doi:10.1073/pnas.79.8.2700

Makhijani K, To TL, Ruiz-Gonzalez R, Lafaye C, Royant A, Shu X. 2017. Precision optogenetic tool for selective single- and multiple-cell ablation in a live animal model system. *Cell Chem Biol* **24:** 110–119. doi:10.1016/j.chembiol.2016.12.010

McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL. 2003. Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science* **302:** 1765–1768. doi:10.1126/science.1089035

Venkatasubramanian L, Guo Z, Xu S, Tan L, Xiao Q, Nagarkar-Jaiswal S, Mann RS. 2019. Stereotyped terminal axon branching of leg motor neurons mediated by IgSF proteins DIP-α and Dpr10. *eLife* **8:** e42692. doi:10.7554/eLife.42692

Wang Y, Lobb-Rabe M, Ashley J, Anand V, Carrillo RA. 2021. Structural and functional synaptic plasticity induced by convergent synapse loss in the *Drosophila* neuromuscular circuit. *J Neurosci* **41:** 1401–1417. doi:10.1523/JNEUROSCI.1492-20.2020

Wang Y, Zhang R, Huang S, Valverde PTT, Lobb-Rabe M, Ashley J, Venkatasubramanian L, Carrillo RA. 2023. Glial Draper signaling triggers

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cross-neuron plasticity in bystander neurons after neuronal cell death in *Drosophila*. *Nat Commun* **14:** 4452. doi:10.1038/s41467-023-40142-y

Zhou L, Schnitzler A, Agapite J, Schwartz LM, Steller H, Nambu JR. 1997. Cooperative functions of the reaper and head involution defective genes in the programmed cell death of *Drosophila* central nervous system midline cells. *Proc Natl Acad Sci* **94:** 5131–5136. doi:10.1073/pnas.94.10.5131

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