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Morphological and functional evaluation of axons and their synapses during axon death in Drosophila melanogaster

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Corresponding Author:	Lukas Jakob Neukomm, PhD University of Lausanne Lausanne, Vaud SWITZERLAND
Corresponding Author's Institution:	University of Lausanne
Corresponding Author E-Mail:	lukas.neukomm@unil.ch
Order of Authors:	Maria Paglione
	Arnau Llobet Rosell
	Jean-Yves Chatton
	Lukas Jakob Neukomm, PhD
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UNIL | Université de Lausanne
Lukas J. Neukomm, PhD
SNSF Assistant Professor
Rue du Bugnon 9
CH-1005 Lausanne

Vineeta Bajaj, PhD
JoVE Review Editor
1 Alewife Center, Suite 200
Cambridge MA 02140
USA

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Manuscript resubmission to the *Journal of Visualized Experiments (JoVE)*

Dear Dr. Bajaj,

Following your invitation and a first round of peer review, we would like to re-submit our manuscript entitled "Morphological and functional evaluation of axons and their synapses during axon death in *Drosophila melanogaster*" for consideration in *JoVE*.

We greatly appreciated the positive feedback provided by the editor as well as all three reviewers. Their suggestions helped us to greatly improve the quality of our manuscript. As you will see in our rebuttal letter, we aimed to implement most of the suggestions brought up by the editor and reviewers. Here, we would like to highlight the most important changes:

- We changed the sequence of our experimental procedures from "1. Brain, 2. Wing, 3. Optogenetics" new to "1. Wing, 2. Brain, 3. Optogenetics", as suggested by reviewer #3. This also means that we exchanged "Functional" and "Morphological" in the title of our manuscript.
- Our protocol section consists now solely of action steps, as requested by the editor. The "non action steps" text has been relocated to and implemented in the discussion, which underwent substantial editing.
- The crosses performed in our experiments have been added as schematics in all result figures, as suggested by the editor and several reviewers. This should facilitate the understanding of the underlying genetics in our experiments.

Importantly, the revised manuscript still covers the three most important and state-of-the-art axon death assays in the fly, and our major novel finding, where over-expression of *dnmnat* (*dnmnat^{OE}*) results in functionally preserved axons and synapses for at least one week. Our article should therefore be of broad interest to the readership of *JoVE*.

Thank you in advance for considering our submission. Please feel free to contact me directly if you require any additional information.

I look forward to hearing from you soon.

Best regards,

Lukas J. Neukomm

Faculté de biologie et de médecine | FBM
Département des neurosciences fondamentales | DNF

Tél.+41 21 692 52 88 | lukas.neukomm@unil.ch

TITLE:

Morphological and Functional Evaluation of Axons and their Synapses during Axon Death in *Drosophila melanogaster*

AUTHORS AND AFFILIATIONS:

Maria Paglione[1*], Arnau Llobet Rosell[1*], Jean-Yves Chatton[1] Lukas J Neukomm[1]

[1]Department of Fundamental Neurosciences, University of Lausanne, Lausanne VD, Switzerland

* These authors contributed equally.

Corresponding Author:
Lukas J Neukomm (lukas.neukomm@unil.ch)

Email Addresses of Co-authors:
Maria Paglione (maria.paglione@unil.ch)
Arnau Llobet Rosell (arnau.llobetrosell@unil.ch)
Jean-Yves Chatton (jean-yves.chatton@unil.ch)

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Drosophila, Neurobiology, Wallerian Degeneration, injury-induced axon degeneration, axon death, genetics, optogenetics, behavior, microscopy, fluorescence

SUMMARY:

Here, we provide protocols to perform three simple injury-induced axon degeneration (axon death) assays in *Drosophila melanogaster* to evaluate the morphological and functional preservation of severed axons and their synapses.

ABSTRACT:

Axon degeneration is a shared feature in neurodegenerative disease and when nervous systems are challenged by mechanical or chemical forces. However, our understanding of the molecular mechanisms underlying axon degeneration remains limited. Injury-induced axon degeneration serves as a simple model to study how severed axons execute their own disassembly (axon death). Over recent years, an evolutionarily conserved axon death signaling cascade has been identified from flies to mammals, which is required for the separated axon to degenerate after injury. Conversely, attenuated axon death signaling results in morphological and functional preservation of severed axons and their synapses. Here, we present three simple and recently developed protocols that allow for the observation of axonal morphology, or axonal and synaptic function of severed axons that have been cut-off from the neuronal cell body, in the fruit fly *Drosophila*. Morphology can be observed in the wing, where a partial injury results in axon death side-by-side of uninjured control axons within the same nerve bundle. Alternatively, axonal morphology can also be observed in the brain, where the whole nerve bundle undergoes axon death triggered by antennal ablation. Functional preservation of severed axons and their synapses can be assessed by a simple optogenetic approach coupled with a post-synaptic

grooming behavior. We present examples using a *highwire* loss-of-function mutation and by over-expressing *dnmnat*, both capable of delaying axon death for weeks to months. Importantly, these protocols can be used beyond injury; they facilitate the characterization of neuronal maintenance factors, axonal transport, and axonal mitochondria.

INTRODUCTION:

The morphological integrity of neurons is essential for sustained nervous system function throughout life. The vast majority of the neuronal volume is taken by axons[1,2]; thus life-long maintenance of particularly long axons is a major biological and bioenergetic challenge for the nervous system. Multiple axonal-intrinsic and glial-extrinsic support mechanisms have been identified, ensuring life-long axonal survival. Their impairment results in axon degeneration[3], which is a common feature of nervous systems being challenged in disease, and by mechanical or chemical forces[4,5]. However, the underlying molecular mechanisms of axon degeneration remain poorly understood in any context, making the development of efficacious treatments to block axon loss challenging. The development of effective therapies against these neurological conditions is important, as they create an enormous burden in our society[6].

Injury-induced axon degeneration serves as a simple model to study how severed axons execute their own disassembly. Discovered by and named after Augustus Waller in 1850, Wallerian degeneration (WD) is an umbrella term that comprises two distinct, molecularly separable processes[7]. First, after axonal injury, axons separated from their cell bodies actively execute their own self-destruction (axon death) through an evolutionarily conserved axon death signaling cascade within one day after injury[8]. Second, surrounding glia and specialized phagocytes engage and clear the resulting axonal debris within three to five days. The attenuation of axon death signaling results in severed axons that remain preserved for weeks[9-12], while the attenuation of glial engulfment culminates in axonal debris which persists for weeks in vivo[13-15].

Research in flies, mice, rats and zebrafish revealed several evolutionarily conserved and essential mediators of axon death signaling[8]. Axon death mutants contain severed axons and synapses that fail to undergo axon death; they remain morphologically and functionally preserved for weeks, in the absence of cell body support[9,10,12,13,16-23]. The discovery and characterization of these mediators led to the definition of a molecular pathway executing axon death. Importantly, axon death signaling is activated not only when the axon is cut, crushed or stretched[24,25]; it also seems to be a contributor in distinct animal models of neurological conditions (e.g., where axons degenerate in an injury-independent manner[4], yet with a range of beneficial outcomes[4,8]). Therefore, understanding how axon death executes axon degeneration after injury might offer insights beyond a simple injury model; it could also provide targets for therapeutic intervention.

The fruit fly *Drosophila melanogaster* (*Drosophila*) has proven to be an invaluable system for axon death signaling. Research in the fly revealed four essential evolutionarily conserved axon death genes: *highwire (hiw)*[11,14], *dnmnat*[12,26], *dsarm*[10] and *axundead (axed)*[12]. The modification of these mediators – loss-of-function mutations of *hiw*, *dsarm* and *axed*, and over-expression of *dnmnat* – potently blocks axon death for the life span of the fly. While severed wild type axons undergo axon death within 1-day, severed axons and their synapses lacking *hiw*, *dsarm* or *axed* remain

not only morphologically, but also functionally preserved for weeks. Whether functional preservation can also be achieved through high levels of *dnmnat* remains to be determined.

Here, we will present three simple and recently developed protocols to study axon death (e.g., the morphology and function of severed axons and their synapses over time) in the absence of cell body support. We demonstrate how attenuated axon death results in severed axons which are morphologically preserved with a *hiw* loss-of-function mutation ($hiw^{\Delta N}$) and how attenuated axon death results in severed axons and synapses that remain functionally preserved for at least 7 days with over-expression of *dnmnat* ($dnmnat^{OE}$). These protocols allow for the observation of individual axonal and synaptic morphology either in the central, or peripheral nervous system (CNS and PNS, respectively)[13,14], while the functional preservation of severed axons and their synapses in the CNS can be visualized by the use of a simple optogenetic setup combined with grooming as a behavioral readout[12].

PROTOCOL:

1. Observation of axon morphology during axon death in the PNS

1.1. Wing injury: partial injury of axon bundles

1.1.1. Use 5 virgin females and 5 males from the right genotype (**Figure 4A**, P_0 generation) to perform crosses at room temperature (RT). Pass P_0 into new vials every 3-4 days. Collect freshly eclosed adult progeny (F_1 generation) daily and age them for 7 - 14 days.

1.1.2. Anesthetize flies on CO_2 pads. Use micro scissors to cut the anterior wing vein roughly in the middle of the wing (**Figure 1A**). Use one wing for the injury and the other wing as an age-matched uninjured control. Apply one injury per wing, and make sure to get sufficient wings injured (approximately 15 wings).

NOTE: The whole wing can be cut through, but it is sufficient to cut only the anterior wing vein. This is the strongest part of the wing.

1.1.3. Recover the flies in food-containing vials.

1.2. Wing dissection and visualization of axons

1.2.1. Spread 10 µL of halocarbon oil 27 with a pipette along a whole glass slide (**Figure 1B**).

1.2.2. Cut off the injured, as well as, the uninjured control wing at desired time points (e.g., 1- or 7-days post injury). Use micro scissors to cut, and tweezers to grab the wing. Mount maximal 4 wings into halocarbon oil 27 (**Figure 1B**) and cover them with a cover slide.

1.2.3. Image the wing immediately using a spinning disk microscope. Acquire a series of optical sections along the z-axis with 0.33 µm step-size and compress z-stacks into a single file for

subsequent analyses.

NOTE: Do not grab the anterior wing vein where cell bodies and axons are housed. Grab the wing at the center. The tissue in wings is not fixed; keep the time from mounting wings to imaging these under 8 min.

[Place **Figure 1** here]

2. Observation of axon and synapse morphology during axon death in the CNS

2.1. Antennal ablation: injury of whole axon bundles

2.1.1. Use 5 virgin females and 5 males from the right genotype (**Figure 5A**, P_0 generation) to perform crosses at RT. Pass P_0 into new vials every 3-4 days. Collect freshly eclosed adult progeny (F_1 generation) daily and let them age for 7 up to 14 days.

2.1.2. Anesthetize flies on CO_2 pads. Use tweezers to ablate the right 3rd antennal segment for unilateral ablation; or both left and right 3rd antennal segments for bilateral ablation (**Figure 2A-C**). This will remove GFP-labeled neuronal cell bodies, while their axonal projections remain in the CNS.

NOTE: Antennal ablation severs the whole axon bundle. If unilateral ablation is performed, the axon bundle on the contralateral side (the unablated antenna) serves as internal control. Make sure to perform sufficient antennal ablations (approximately 15 animals).

2.1.3. Recover the flies in food-containing vials.

2.2. Brain dissection and visualization of axons

2.2.1. Mix silicone elastomer base (9 mL) and curing agent (1 mL) in a volume ratio of 10:1. Transfer each 5 mL mixture into a 35 mm tissue culture plate, and reduce air introduced by mixing with gentle agitation in the fume hood overnight. The mixture solidifies within 24 h.

NOTE: Dissection plates must be prepared only once and may be used multiple times.

2.2.2. Anesthetize flies on CO_2 pads and decapitate adult heads using two tweezers at desired time points (e.g., 1 or 7 days after antennal ablation). Use one tweezer to grab the neck, and the other tweezer to fix the thorax. Gently pull the neck and head off the thorax.

NOTE: Leave decapitated heads on the CO_2 pad until the desired number is achieved, but make sure to proceed to the next step within 30 min.

2.2.3. Transfer all heads into a 1.5 mL microcentrifuge tube containing 1 mL of fixing solution containing 4% paraformaldehyde (PFA) and 0.1% Triton X-100 in phosphate buffered saline (PBS)

using tweezers that have been dipped into the fixing solution.

NOTE: Fly heads stick well on wet tweezers. It makes it feasible to transfer all heads readily into the microcentrifuge tube.

2.2.4. Fix heads for 20 min with gentle agitation at RT. Put the microcentrifuge tube on ice, heads will gravitate to the bottom of the microcentrifuge tube. Remove the supernatant with a pipette and repeat this procedure with five 2 min washes with 1 mL of washing buffer containing 0.1% Triton X-100 in PBS with rocking at RT, to remove residual fixing solution.

NOTE: Videos on how to dissect adult *Drosophila* brains are readily available[27].

2.2.5. Transfer the heads with a glass pipette into a dissection plate filled with washing buffer. Use one tweezer to grab and pull the proboscis off the head, while holding the head with the other tweezer. This will leave a hole were the proboscis was attached to the exoskeleton.

2.2.6. Use two tweezers to remove the exoskeleton between the hole and each compound eye. This will make it feasible to open the head structure with both tweezers, and to gently scratch out the brain within.

2.2.7. Clean each brain by removing trachea or fat stuck to it (**Figure 2D**, top). Once the brain is cleaned, put it in a new microcentrifuge tube containing 1 mL of washing buffer on ice.

NOTE: Damaged or lost optic lobes will not affect the olfactory lobe in the center of the brain (**Figure 2D**, top).

2.2.8. Replace washing buffer with 1 mL of fixing solution once all brains are collected and accumulated at the bottom of the microcentrifuge tube. Fix brains for 10 min with rocking at RT, followed by five 2 min washes in 1 mL of washing buffer with rocking at RT.

2.2.9. Apply primary antibodies (1:500) in washing buffer overnight with rocking at 4 °C, followed by 10 washes over 2 h using 1 mL of washing buffer with rocking at RT.

2.2.10. Apply secondary antibodies (1:500) in washing buffer 2 h with rocking at RT and wrap microcentrifuge tube in aluminum foil to block light. Keep the microcentrifuge tube covered with aluminum foil for the rest of the procedure. Apply ten washes with 1 mL of washing buffer over 2 h with rocking at RT.

2.2.11. Remove the supernatant and use a single drop of antifade reagent to cover the brains in the microcentrifuge tube. Incubate brains for at least 30 min at 4 °C before preparing them for mounting and imaging.

2.2.12. Prepare a cover slide, stick lab tape on it, and cut out a “T”-like shape from the tape (**Figure 2D**, bottom). The resulting space serves as area where the brain-containing antifade

reagent[28] will be pipetted into, preferably into both chambers.

NOTE: Use a 20-200 µL pipette tip where 3 mm of the tip has been cut off to widen the opening of the pipette. This will make it feasible to pipette the brain-containing antifade reagent. Carefully cover the brains with a cover slide.

2.2.13. Use clay to prepare two small even rolls. Ensure that the clay rolls are not higher than the height of a glass slide. Stick the clay rolls onto the glass slide (**Figure 2D**, bottom). Place the brain-containing cover slide sandwich onto the clay rolls.

NOTE: GFP-labeled axons and their synapses are located in the front of the brain. It is, therefore, easier to image them from the front. However, brains will either face up, or face down on the cover slide sandwich. Clay rolls serve as sandwich holders, and during imaging, the sandwich can be flipped upside down. This will make it feasible to acquire images from the front from every brain.

2.2.14. Acquire a series of optical sections along the z-axis with 1.0 µm step-size using a confocal microscope, and compress z-stacks into a single file for subsequent analyses, to assess the number of axonal projections remaining intact.

[Place **Figure 2** here]

3. Grooming induced by optogenetics as a readout for axon and synapse function

3.1. Optogenetic setup

3.1.1. Perform the optogenetic experiment in a dark room. Ensure that the setup consists of an 850 nm infrared (IR) LED spotlight to illuminate flies in the dark (**Figure 3A**), a flashing 660 nm red LED spotlight to activate neurons expressing CsChrimson, and a monochrome camera with a 700 nm longpass filter, which prevents the recording of red light flashes.

3.1.2. Use a 3D printer to generate a tiny circular behavior chamber with a diameter of 1 cm, cover it with a cover slide, and place an 860 nm emitter coupled to the red LED spotlight next to the chamber (**Figure 3B**).

NOTE: The emitter indicates when the red LED spotlight is on, thus activating the neurons.

3.1.3. Mount the LED spotlights and the camera on top of the chamber (**Figure 3A, C**).

3.1.4. Activate neurons by 10 Hz flashes during 10 s. The duration of activation can be adjusted according to the experimental design.

3.2. Preparing flies for optogenetics

3.2.1. Melt fly food in a microwave. After the food cooled down, before solidification, add 1:100 of 20 mM all trans-retinal in ethanol (EtOH) to a final concentration of 200 µM. Mix well, and pour the food immediately into empty vials.

NOTE: Avoid adding all trans-retinal to hot food, this could result in less efficient optogenetics.

3.2.2. Cool the food in vials in a dark, cold room. Cover vials containing solidified food with plugs or cotton balls. Wrap vials with aluminum foil.

3.2.3. Use 5 virgin females and 5 males (**Figure 6A**, generation P_0) from the right genotype to perform crosses at RT. Pass P_0 into new vials every 3-4 days. Collect freshly eclosed adult progeny (generation F_1) on a daily base and let them age for 7 up to 14 days in aluminum-covered vials containing 200 µM all trans-retinal in fly food.

3.2.4. Collect flies by tapping them from food-containing vials into an empty vial with no food. Cool the vial down in ice-containing water for approximately 30 s. Flies will fall asleep. Put individual flies rapidly into small chambers covered with a cover slide (**Figure 3B**).

NOTE: As soon as flies warm up, they wake up. It is crucial to quickly spread individual flies into single chambers each. Avoid CO_2 pads to anaesthetize flies, this will impact their behavior.

3.2.5. Perform optogenetics to elicit antennal grooming. Here, the protocol consists of the following intervals: 30 s where the red light is absent, followed by 10 s of red-light exposure at 10 Hz. Repeat this procedure three times in total, followed by an additional 30 s interval where the red light is absent[12,29,30].

NOTE: This protocol can be adjusted according to the experimental preference.

3.2.6. Collect individual flies from each chamber on CO_2 pads. Subject them to antennal injury. Ablate both the left and the right 2nd antennal segments (**Figure 2C**). This will remove the cell bodies of Johnston's organ (JO) neurons, while the axonal projections remain in the CNS. Recover the flies in aluminum-covered vials containing 200 µM all trans-retinal.

NOTE: For antennal grooming induced by optogenetics, the sensory neuron cell bodies are housed in the 2nd antennal segment (**Figure 2C**).

3.2.7. At corresponding time points (e.g., 7 days post antennal ablation), subject flies to another grooming assay (go back to step 3.2.4).

[Place **Figure 3** here]

REPRESENTATIVE RESULTS:

Above, we described three methods to study the morphology and function of severed axons and their synapses. The first method allows for high-resolution observation of individual axons in the

PNS. It requires clones generated by the MARCM technique[14,31]. Here, we performed crosses to generate wild type and *highwire* mutant MARCM clones (**Figure 4A**). A simple cut in the middle of the wing induces axon injury of neurons housed distal (e.g., at the outer side of the wing), while proximal neurons (e.g., between the cut site and the thorax) remain uninjured. This approach makes it feasible to observe axon death side-by-side of uninjured control axons in the same nerve bundle (**Figures 1A, 4B**). Here, we used a genetic background resulting in low numbers of GFP-labeled clones (e.g., two in each experiment[14]). We present examples of 1 and 7 days after injury of wild type axons, to provide examples of control axons, axons undergoing axon death, and axonal fragments being cleared by surrounding glia, respectively. In addition, we repeated axonal injury in *highwire* mutants where we analyzed the outcome 7 days after injury.

Uninjured control wings harbor two wild-type clones, thus two GFP-labeled wild-type axons (**Figure 4B**, wild type, uninjured control). One day after cutting the middle of the wing by the use of micro scissors, axon death is induced in GFP-labeled axons where cell bodies are distal to the cut site, while axons from proximally housed cell bodies serve as an internal control within the same nerve bundle (**Figure 4B**, wild type, 1 day post injury). Note the axonal debris trace in the upper part indicated by the arrow. 7 days after axonal injury, GFP-labeled axonal debris is cleared by surrounding glia, while GFP labeled uninjured control axons remain in the nerve bundle (**Figure 4B**, wild type, 7 days post injury, arrow). In contrast, *highwire* mutant axons that have been severed for 7 days remain morphologically preserved, consistent with previous findings[11,14] (**Figure 4B**, *highwire*, 7 days post injury, arrow). These results demonstrate the powerful visual resolution of the *Drosophila* wing. Axon death can be observed side-by-side of uninjured controls in the same nerve bundle. While wild-type axons undergo axon death within 1 day after injury and the resulting debris is cleared within 7 days, axon death deficient *highwire* mutants remain morphologically preserved for 7 days.

[Place **Figure 4** here]

The second method describes how to visualize whole axon bundles projecting into the CNS where they form synapses, which belong to neurons housed in both left and right antennae (**Figures 2A-C**). Here, we performed crosses to generate wild type and *highwire* mutant MARCM clones (**Figure 5A**). Uninjured, GFP-labeled axons and their synapses can be visualized over the course of days to weeks, in the absence of injury (**Figure 5B**, Wild type, uninjured control). Alternatively, animals can be subjected to 3rd antennal segment ablation, and severed GFP-labeled axons and their synapses can be observed during a time course over hours to days. We focused on 7 days after antennal ablation, because at this time point, axons and their synapses have undergone axon death, and resulting debris is cleared by surrounding glia. If unilateral ablation of the right antenna is performed, then the right axon bundle is severed and will disassemble and the resulting debris is fully cleared 7 days after injury (**Figure 5B**, wild type, unilateral ablation, 7 days post injury, arrows), consistent with previous findings[13]. Alternatively, both the right and the left antennae can be ablated, which will sever both axon bundles, and 7 days after injury, axons and their synapses disappeared (**Figure 5B**, wild type, bilateral ablation, 7 days post injury, arrow). In contrast, unilateral ablation of the right antennae in *highwire* mutants results in severed axons that remain preserved 7 days post injury, consistent with previous findings[11,14] (**Figure 5B**,

highwire, unilateral ablation, 7 days post injury, arrow). These results demonstrate that severed wild-type axons undergo axon death and the resulting debris is cleared within 7 days, while axon death deficient *highwire* mutants fail to undergo axon death and remain morphologically preserved for 7 days.

[Place **Figure 5** here]

The third method allows for the observation of functional preservation of severed axons and their synapses in the CNS. It relies on the manipulation of a subset of JO neurons housed in the 2nd antennal segment which are sufficient to induce antennal grooming. Expression of a red-shifted channelrhodopsin (CsChrimson) in JO neurons, combined with dietary supplementation of all trans-retinal, is sufficient to elicit a simple post-synaptic grooming behavior upon red light exposure[12,30]. Here, we performed crosses to generate wild type JO neurons, and JO neurons over-expressing *dnmnat* (*dnmnat*^{OE}) (**Figure 6A**). Wild type flies or flies containing JO neurons with attenuated axon death (*dnmnat*^{OE}), both harbor a potent grooming behavior before injury. However, 7 days post injury (e.g., bilateral ablation of the 2nd antennal segment), grooming fails to be elicited by optogenetics in wild type flies due to injury-induced axon and synapse degeneration, while animals with attenuated axon death continue to groom (**Figure 6B, Movie 1,2**). Attenuated axon death is therefore capable of functionally preserving severed axons and their synapses for 7 days.

[Place **Movie 1 and 2** here]

[Place **Figure 6** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Observation of axon morphology during axon death in the wing. (**A**) Schematic fly wing with two sparsely GFP-labeled sensory neurons, which are also separately indicated below. The site of injury and the field of observation are indicated. (**B**) Schematic setup for wing imaging. Injured and uninjured control wings (grey) are mounted in halocarbon oil 27 (red) on a glass slide (light blue) and covered with a cover slide (black).

Figure 2: Observation of axon and synapse morphology during axon death in the brain. (**A**) Side view of a schematic fly head with GFP-labeled cell bodies, axons and synapses. (**B**) High-magnification front view of GPF-labeled olfactory receptor neurons and their axons and synapses. Cell bodies are housed in the 3rd antennal segment, and their axons project into the CNS. Axons form synapses in a glomerulus in the left olfactory lobe, cross the midline and form synapses in the glomerulus on the contralateral olfactory lobe. (**C**) Examples of fly heads with unilateral antennal ablation. Top: Uninjured control. Middle: Ablation of the 3rd antennal segment. Bottom: Ablation of the 2nd (and thus also 3rd) antennal segment. (**D**) Brain preparation. Top: Schematic dissected fly brain with indicated olfactory lobes and axonal projections in the field of view. Bottom: Schematic setup for brain imaging. Two clay rolls (green) are mounted onto a glass slide (light blue), they carry a cover slide sandwich, which contains fly brains (grey). Brains are

mounted in antifade reagent(purple), surrounded by a lab tape (orange), and covered by two cover slides (black).

Figure 3: Optogenetic setup to induce grooming as a readout for axon and synapse function. (**A**) Illustration of assembled components required for optogenetics. Infrared (IR) LED spotlight, camera and red LED spotlight (from left to right, respectively). The components including a detailed description are listed in the table of materials. (**B**) Top view illustration of a behavior chamber including an IR emitter to indicate red LED spotlight activation. (**C**) Illustration of a single mount setup. A total of three mount setups are required for the two LED spotlights and the camera, respectively.

Figure 4: Approach to study axon death of GFP-labeled sensory neuron axons in the wing. (**A**) Schematic crosses to generate wild type and *highwire* clones in the wing (P_0 and F_1 generation, respectively). Virgin females are on the left, males on the right. See table of materials for genotype details. (**B**) Examples of control and injured GFP-labeled axons. The field of view is indicated in (**Figure 1A**). From left to right: uninjured wild type control axons, wild type axons 1-day post injury, wild type axons 7 days post injury, *highwire* mutant axons 7 days post injury, respectively. Arrows indicate severed axons (scale bar, 5 µm).

Figure 5: Approach to study axon death of GFP-labeled sensory neuron axons in the brain. (**A**) Schematic crosses to generate wild type and *highwire* clones in the brain (P_0 and F_1 generation, respectively). Virgin females are on the left, males on the right. See table of materials for genotype details. (**B**) Examples of control and injured GFP-labeled axons. From left to right: uninjured wild type controls, wild type 7 days post unilateral antennal ablation, wild type 7 days post bilateral antennal ablation, and *highwire* mutants 7 days post unilateral antennal ablation, respectively. Arrows indicate severed axon bundles (scale bar, 10 µm).

Figure 6: Approach to visualize axonal and synaptic function after axotomy. (**A**) Schematic crosses to generate wild type and *dnmnat* over-expressing JO sensory neurons (P_0 and F_1 generation, respectively). Virgin females are on the left, males on the right. See **Table of Materials** for genotype details. (**B**) Individual ethograms of grooming behavior induced by optogenetics. Top: individual ethograms of wild type flies before and 7 days after injury (blue). Bottom: individual ethograms of flies over-expressing *dnmnat* ($dnmnat^{OE}$) in JO neurons before and 7 days after injury (red). Each bin indicates at least 1 grooming behavior within 1 s. The black line indicates the sum of all bins. (**C**) Quantification of grooming behavior. Data is shown as average ± standard deviation, $p > 0.001$ (one-way ANOVA, multiple comparison with Tukey's post hoc test).

Movie 1: Representative wild type grooming behavior elicited by optogenetics before and 7 days after antennal ablation.

Movie 2: Representative grooming behavior elicited by optogenetics in flies over-expressing *dnmnat* in JO neurons before and 7 days after antennal ablation.

DISCUSSION:

The protocols described here allow for the robust and reproducible observation of morphology as well as function of axons and their synapses separated from their cell bodies in *Drosophila*. The wing assay facilitates the observation of axon death side-by-side of uninjured control axons in the PNS[14], while the antennal assay facilitates the observation of whole nerve bundles of GFP-labeled axons and their synapses, to assess both morphology and function in the brain (CNS)[12]. There are critical steps and certain advantages for each approach to study morphology that have to be taken into consideration when designing experiments.

To observe axon morphology in the PNS in the wing, experiments can be readily performed, because of the transparency of the wing: it allows to bypass dissection and immunohistochemistry. However, due to the lack of fixation, the wings have to be imaged immediately after mounting[14]. Currently, two distinct Gal4 drivers are frequently used, either *ok371-Gal4* or *dpr1-Gal4*, and both references offer distinct approaches to quantify degeneration[14,26]. Sparse labeling of a few neurons is recommended, by using "Mosaic Analysis with a Repressible Cell Marker (MARCM)"[14,31], as the resolution of axonal morphology is unprecedented. In contrast, the observation of synapses is not possible in wings, they are located in the ventral nerve cord inside the thorax of the flies. Furthermore, additional axonal markers cannot be visualized by immunohistochemistry: the waxy cuticle makes it impossible for the diffusion of fixatives and antibodies into the underlying tissue.

To observe axon and synapse morphology in the CNS, brain dissections have to be performed. They offer the advantage of visualizing additional axonal and synaptic markers by the use of immunohistochemistry, and synapses can be observed alongside axons in the same field of view[10,13]. A large collection of characterized olfactory receptor neuron (ORN) Gal4 drivers is readily available[32], and frequently, *OR22a-Gal4* is the driver of choice. For antennal ablation, cell bodies of OR22a neurons are housed in the 3rd segment (**Figure 2B**). A fluorescence intensity-based quantification is used to quantify the degeneration of either axons or synapses[13]. Conversely, experiments are time consuming due to brain dissection and antibody staining.

To visualize axonal and synaptic function after axotomy, optogenetics is used to trigger antennal grooming: it serves as a readout for functional preservation of severed axons and their synapses[12]. The grooming circuit and corresponding sensory, inter- and motorneuron Gal4 drivers have been thoroughly described[29,30]. *GMR60E02-Gal4* labels a subset of Johnston's organ (JO) sensory neurons, which are required and sufficient for grooming[29,30]. For antennal ablation, cell bodies of JO neurons are housed in the 2nd antennal segment (**Figure 2B**). An optogenetic setup can readily be built from scratch, or an existing setup adjusted. Importantly, experiments have to be performed in a dark room, and flies thus visualized with an infrared (IR) LED spotlight. When using CsChrimson as a channel, it is crucial to supply the food with all trans-retinal and a red LED spotlight to activate JO neurons[29]. Alternatively, blue light-sensitive channels and a blue LED spotlight, or the TrpA1 channel and temperature can be used for neuronal activation[29,33]. The quantification of grooming behavior has already been described[12,29].

When these assays are used to specifically study axon death, it is important to note that the

phenotype of morphological or functional preservation should be robust over time. There are cases where axon death leads to a consistent yet less pronounced phenotype in morphological preservation[34,35], and whether such a phenotype translates into functional preservation remains to be determined.

Axon death phenotypes have also been observed in neurons during development of *Drosophila* larvae, where nerves were crushed rather than injured[11,23]. Here, we specifically focused on adult *Drosophila* neurons which completed development. In this context, the use of RNA interference[36], or tissue-specific CRISPR/Cas9[37] can readily be implemented. Importantly, the above techniques can be used in an axon death independent context: they facilitate the characterization of neuronal maintenance factors[38], axonal transport[39], age-dependent axonal mitochondria changes[40], and morphology of axonal mitochondria[41].

ACKNOWLEDGMENTS:
We would like to thank the entire Neukomm lab for contributions. This work was supported by a Swiss National Science Foundation (SNSF) Assistant Professor award (grant 176855), the International Foundation for Research in Paraplegia (IRP, grant P180), SNSF Spark (grant 190919) and by support from the University of Lausanne and the Department of Fundamental Neurosciences (État de Vaud) to LJN.

DISCLOSURES:
The authors declare that they have nothing to disclose.

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Figure 1

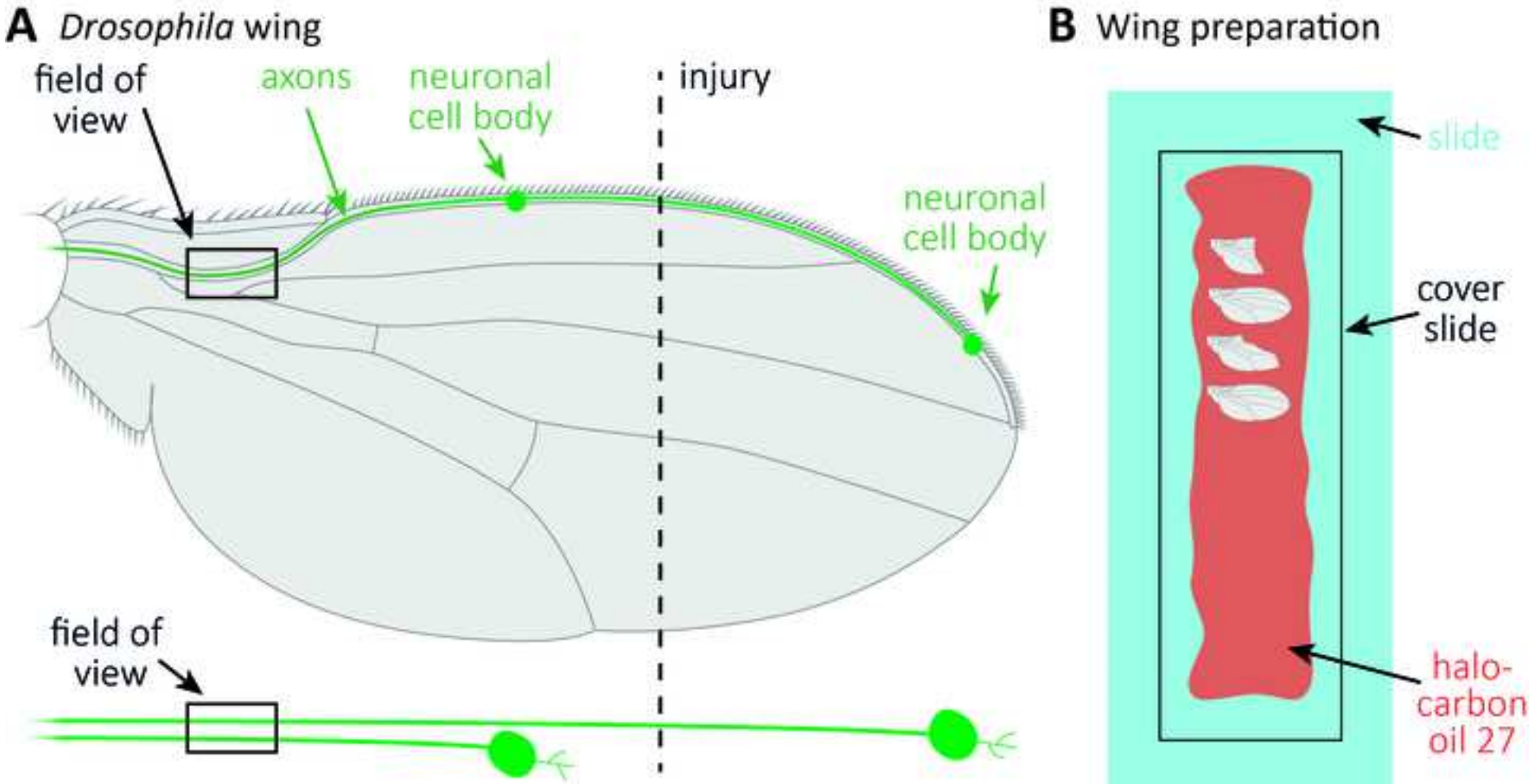

Figure 2

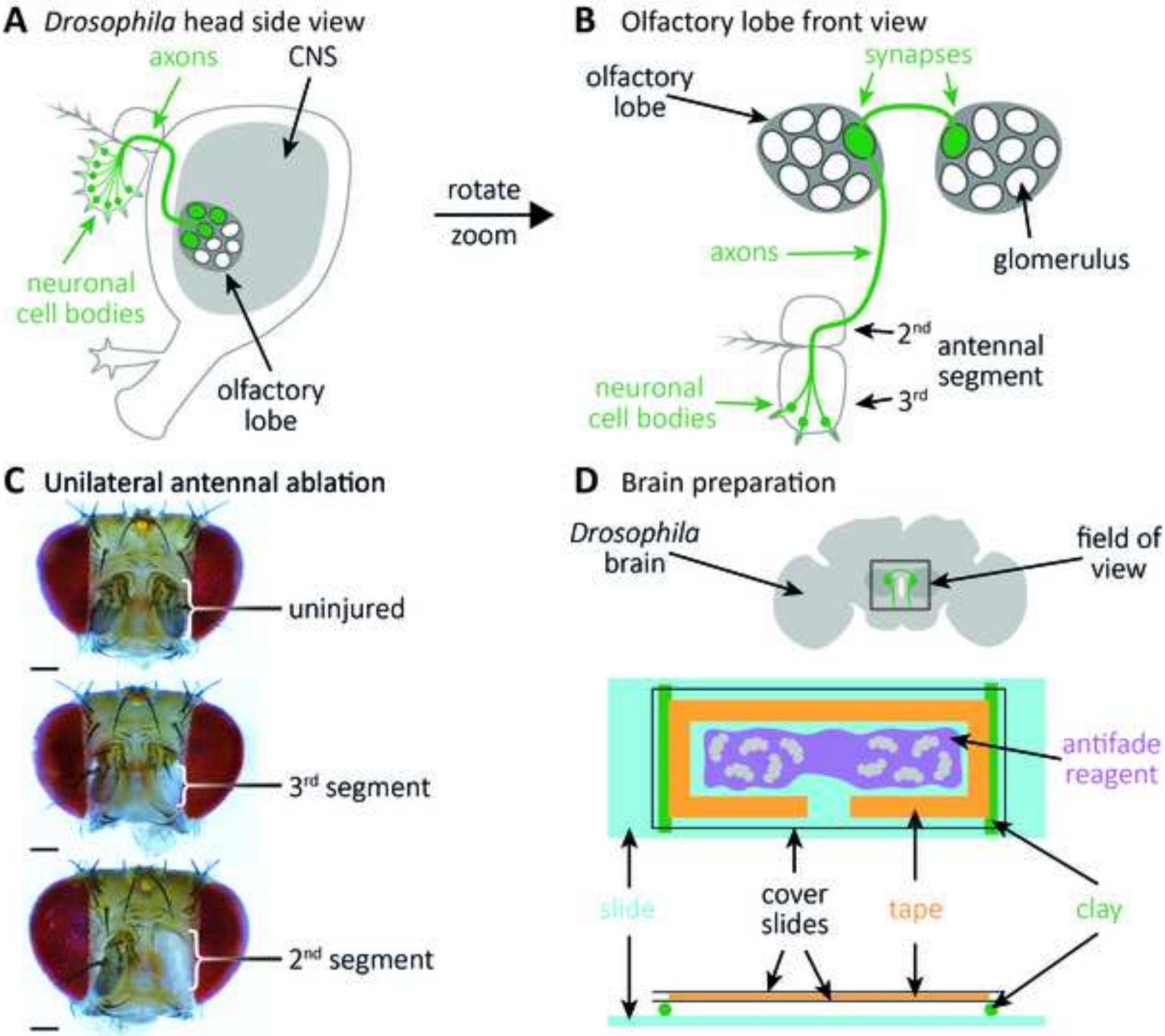

Figure 3

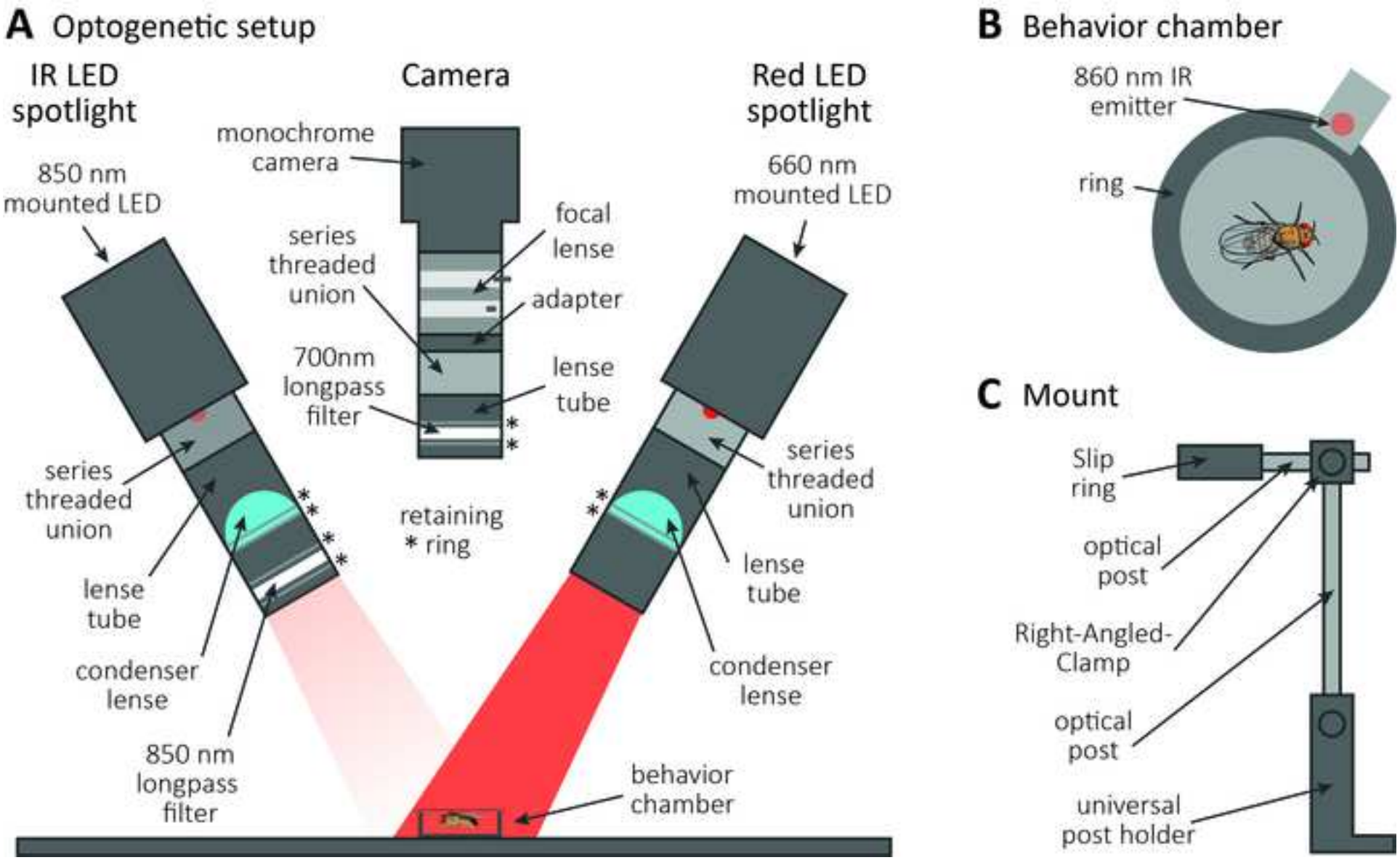

Figure 4

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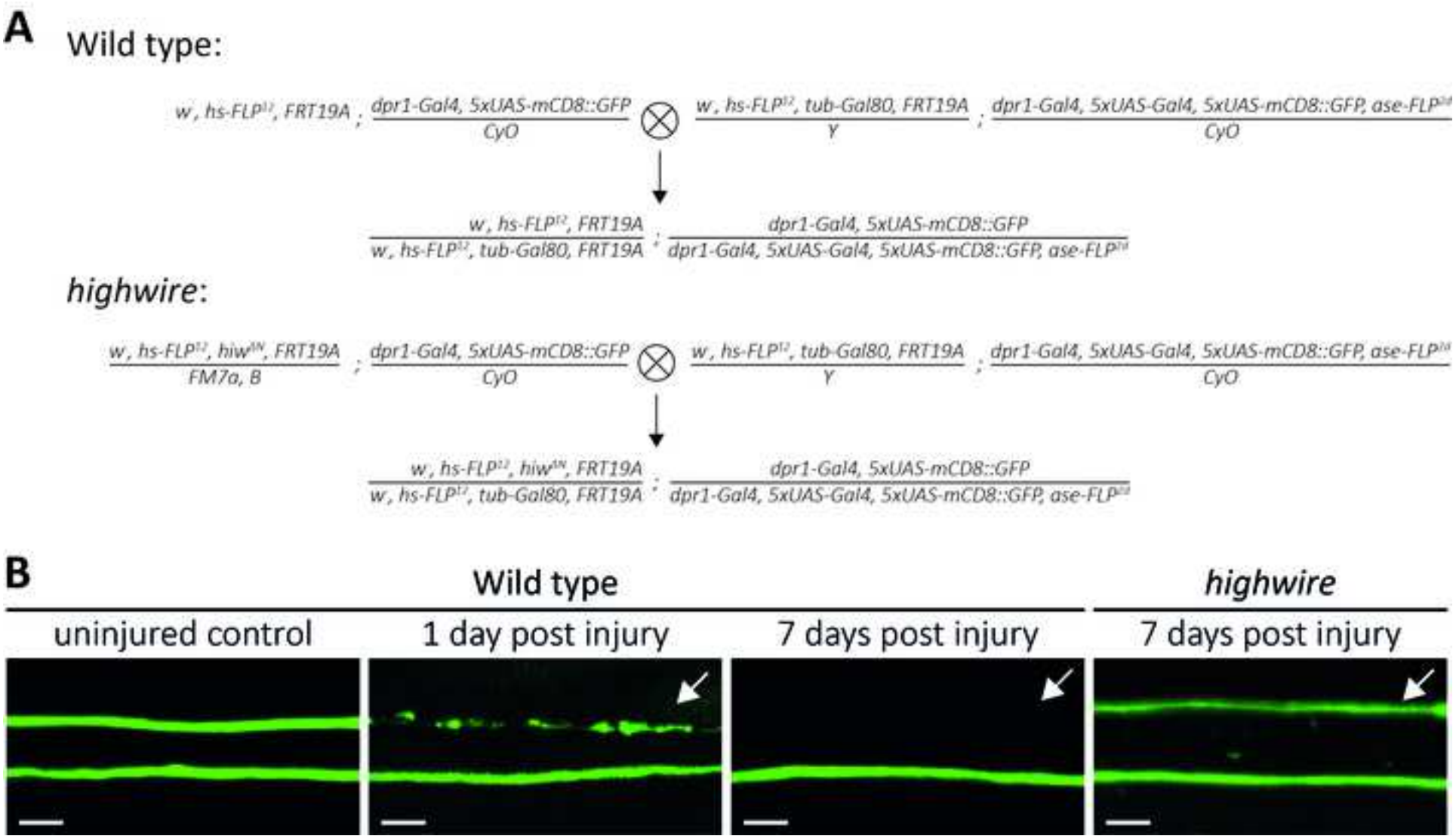

Figure 5

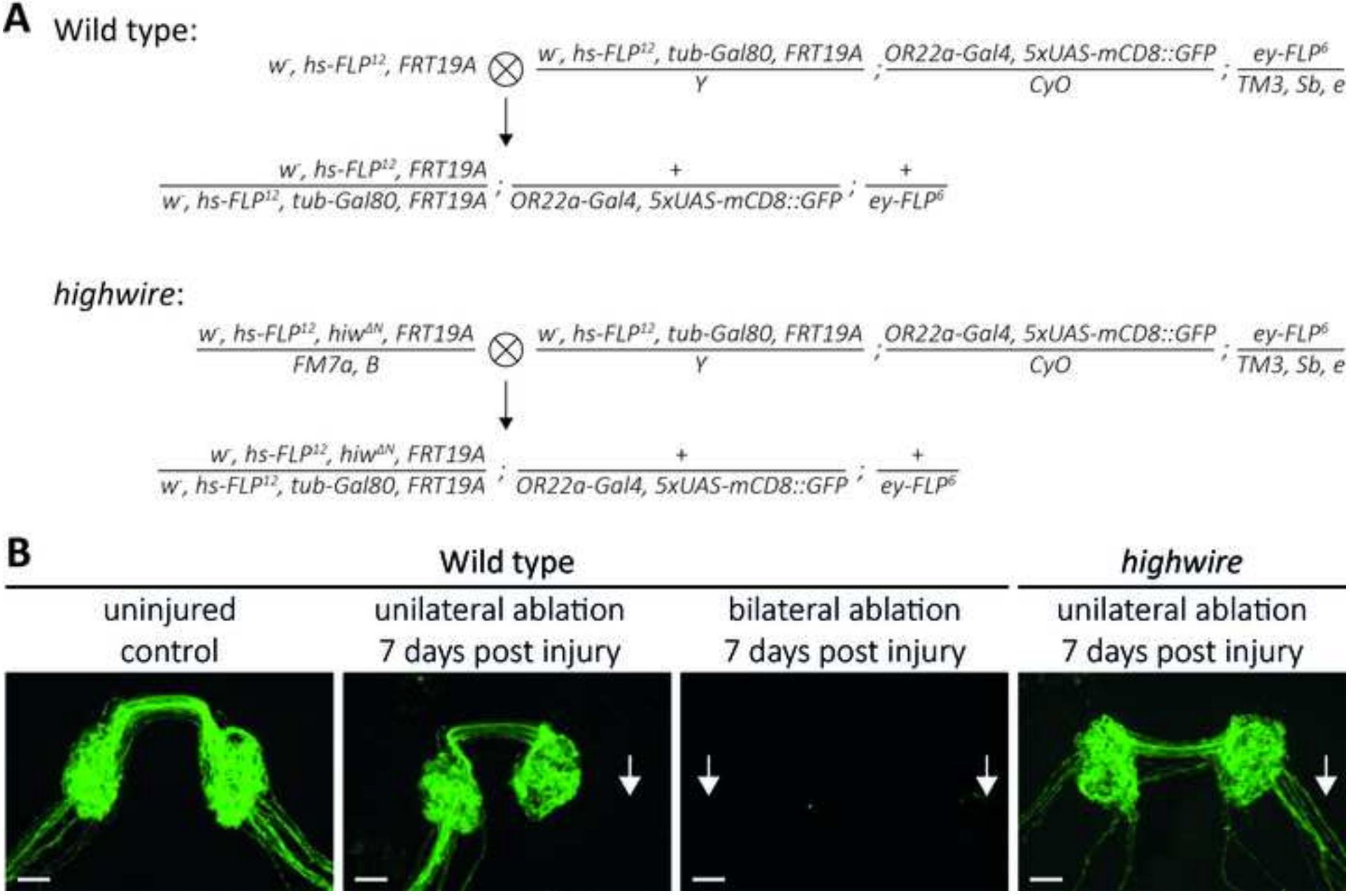

Figure 6

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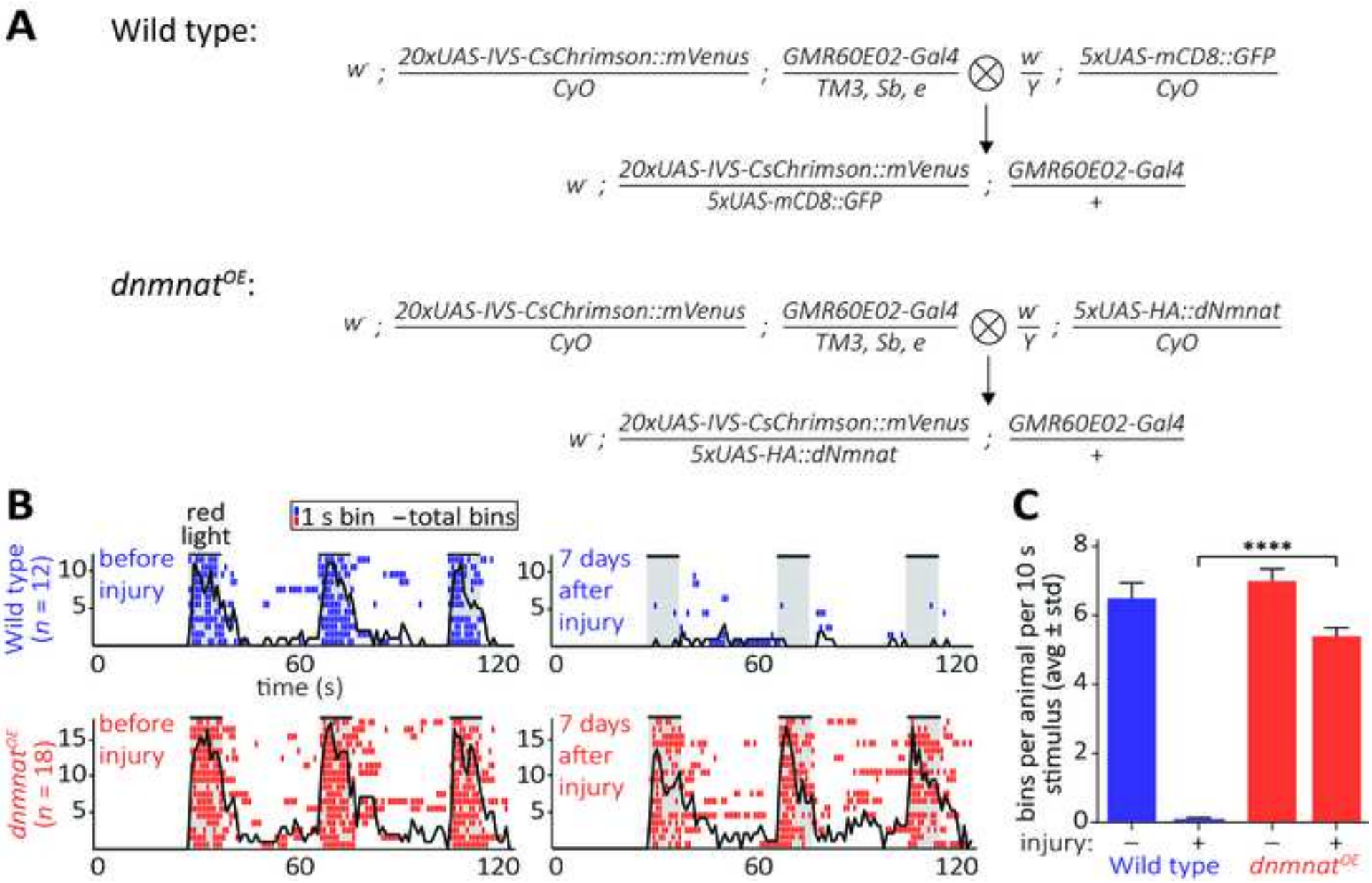

Video 1

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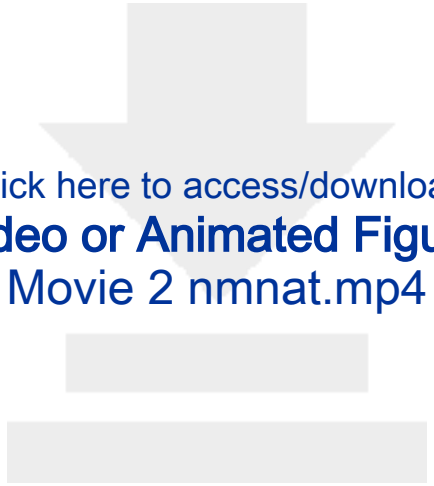
Video or Animated Figure

Movie 1 wild type.mp4

Video 2



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Video or Animated Figure

Movie 2 nmnat.mp4

Table of Material

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Name of Material / Equipment	Company	Catalog Number	Comments/Description
Tweezers (high precision, ultra fine)	EMS	78520-5	Antennal ablation
MicroPoint Scissors (5-mm cutting edge)	EMS	72933-04	Wing injury
1.5 mL microcentrifuge tube	Eppendorf	30120086.0000	
35mm tissue culture dish	Sarstedt	83.3900	
Cover Slips, Thickness 1	Thermo Scientific™	BB02400600A113MNT0	
Superfrost Microscope Slides	Thermo Scientific™	AA00008032E00MNT10	
High-Sensitivity USB 2.0 CMOS Camera, 1280 x 1024, Global Shutter	Thorlabs	DCC1240M	Camera setup
SM1 Retaining Ring for Ø1" Lens Tubes and Mounts	Thorlabs	SM1RR	
25mm 1/1.2" C mount Lens	Tamron	M112FM25	
Adapter with External M27 x 0.5 Threads and Internal SM1 Threads	Thorlabs	SM1A36	
Aspheric Condenser Lens, Ø25 mm, f=20.1 mm, NA=0.60, ARC: 650-1050 nm	Thorlabs	ACL2520U-B	
Ø25.0 mm Premium Longpass Filter, Cut-On Wavelength: 700 nm	Thorlabs	FELH0700	
SM1 (1.035"-40) Coupler, External Threads, 0.5" Long	Thorlabs	SM1T2	
SM1 Lens Tube Without External Threads, 1" Long, Two Retaining Rings Includec	Thorlabs	SM1M10	
850 nm, 900 mW (Min) Mounted LED, 1200 mA	Thorlabs	M850L3	IR LED spotlight
SM1 (1.035"-40) Coupler, External Threads, 0.5" Long	Thorlabs	SM1T2	
SM1 Lens Tube Without External Threads, 2" Long, Two Retaining Rings Includec	Thorlabs	SM1M20	
Aspheric Condenser Lens, Ø25 mm, f=20.1 mm, NA=0.60, ARC: 650-1050 nm	Thorlabs	ACL2520U-B	
Ø25.0 mm Premium Longpass Filter, Cut-On Wavelength: 850 nm	Thorlabs	FELH0850	
SM1 Retaining Ring for Ø1" Lens Tubes and Mounts	Thorlabs	SM1RR	
660 nm, 940 mW (Min) Mounted LED, 1200 mA	Thorlabs	M660L4	Red LED spotlight
Aspheric Condenser Lens, Ø25 mm, f=20.1 mm, NA=0.60, ARC: 650-1050 nm	Thorlabs	ACL2520U-B	
SM1 (1.035"-40) Coupler, External Threads, 0.5" Long	Thorlabs	SM1T2	
SM1 Lens Tube Without External Threads, 2" Long, Two Retaining Rings Includec	Thorlabs	SM1M20	
15 V, 2.4 A Power Supply Unit with 3.5 mm Jack Connector for One K- or T-Cube	Thorlabs	KPS101	LED control
T-Cube LED Driver, 1200 mA Max Drive Current	Thorlabs	LEDD1B	
150 mm x 300 mm x 12.7 mm Aluminum Breadboard, M6 Double-Density Taps	Thorlabs	MB1530/M	Mount base
Ø12.7 mm Universal Post Holder, Spring-Loaded Locking Thumbscrew, L = 75 mn	Thorlabs	UPH75/M	Mount, 3x (IR LED, red LED, cam)
Ø1.20" Slip Ring for SM1 Lens Tubes and C-Mount Extension Tubes, M4 Tap	Thorlabs	SM1RC/M	
Ø12.7 mm Optical Post, SS, M4 Setscrew, M6 Tap, L = 150 mm	Thorlabs	TR150/M	
Ø12.7 mm Optical Post, SS, M4 Setscrew, M6 Tap, L = 40 mm	Thorlabs	TR40/M	
Right-Angle Clamp for Ø1/2" Posts, 5 mm Hex	Thorlabs	RA90/M	
M6 x 1.0 Stainless Steel Cap Screw, 16 mm Long, Pack of 25	Thorlabs	SH6MS16	screws for mount onto base
USB-6001 14-Bit 20 kS/s Multifunction I/O and NI-DAQmx	National Instruments	782604-01	Red LED spotlight controller

20k Ohm 1 Gang Linear Panel Mount Potentiometer	TT Electronics/BI	P230-2EC22BR20K	fintuner for indicator
IR (860nm) emitter, 100 mA radial	Osram	475-1365-ND	Red light indicator
cable	-	-	Misc

Name of Chemical	**Company**	**Catalog Number**	**Comments/Description**
All-trans retinal	Sigma	R2625	
Ethanol absolute	Vwr	20821.296	
Halocarbon Oil 27	Sigma	H8773	
Mowiol	Merk	81381	
Paraformaldehyde	Sigma	F8775	
Phosphate buffered saline (PBS)	Sigma	P5493	
Sylgard 184 silicone elastomer base	Dow Corning Corp	4019862	
Sylgard 184 silicone elastomer curing agent	Dow Corning Corp	4019862	
Triton X-100	Sigma	T8787	

Name of Reagent/Resource	**Company**	**Catalog Number**	**Comments/Description**
Chicken anti-GFP antibodies	Rockland	600-901-215	Antibodies
Goat Dylight anti-Chicken	Abcam	ab96947	

Namne of transgenic fly	**Company**	**Catalog Number**	**Comments/Description**
FM7a, B	BDSC	RRID:BDSC_785	**X chromosome**
FRT19A[hs-neo]	BDSC	RRID:BDSC_1709	
hiw[ΔN]	BDSC	RRID:BDSC_51637	
hs-FLP[12]	BDSC	RRID:BDSC_1929	
tub-Gal80[LL1]	BDSC	RRID:BDSC_5132	
w[1118]	BDSC	RRID:BDSC_3605	
20xUAS-IVS-CsChrimson::mVenus	BDSC	RRID:BDSC_55135	**2nd chromosome**
5xUAS-Gal4[12B]	Kyoto	RRID:Kyoto_108492	
5xUAS-HA::dnmnat	BDSC	RRID:BDSC_39702	
5xUAS-mCD8::GFP[LL5]	BDSC	RRID:BDSC_5134	
ase-FLP[2d]	Freeman laboratory	Neukomm *et al.,* 2014 (PNAS)	
CyO	BDSC	RRID:BDSC_2555	
dpr1-Gal4	BDSC	RRID:BDSC_25083	
OR22a-Gal4	BDSC	RRID:BDSC_9952	
ey-FLP[6]	BDSC	RRID:BDSC_5577	**3rd chromosome**
GMR60E02-Gal4	BDSC	RRID:BDSC_39250	
TM3,Sb,e	BDSC	RRID:BDSC_3644	

JoVE60865 manuscript rebuttal letter

We would like to thank all reviewers and the editor for their positive feedback including comments and valuable suggestions to strengthen our manuscript. We tried to implement most if not all of the suggestions which were brought up. Below, we'll respond point-by-point to each comment.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We've proof read the manuscript multiple times ourselves, and hope there are no errors left.

2. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

We carefully went through our protocol section and substantially changed it. We also ensured that the numbering fits according to the JoVE Instructions for Authors.

3. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example Sylgard 184, Eppendorf tube, chicken anti-GFP, Rockland, goat Dylight® anti-chicken, Abcam, 1:500, Mowiol, national Instrument USB Multifunction I/O Device (NI273 DAQ 6001, etc.

We removed all commercial language from our manuscript. The details of each product can be found in the Table of Materials and Table of Reagents.

4. Please include a single line space between each step, substep, and note in the protocol section.

We updated our manuscript accordingly.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

We tried to adjust our protocol section accordingly.

6. The Protocol should contain only action items that direct the reader to do something. Please move the intro/discussion about the protocol to the Introduction/Discussion section as applicable.

All our texts that were not action items in the protocol section were either rewritten as action items, or relocated and implemented as regular text in the discussion section.

7. Please move all the materials to the table of materials. Please make a separate table for buffer and solutions. Please do not embed Tables from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of a .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text. Please then sort the materials table in alphabetical order.

As already mentioned above under point 3., we carefully went through our protocol section and removed all commercial language, where possible, to the table of materials. We updated and reformatted the table as well where possible.

8. Please include the volume and concentrations of all solutions used in the experiment.

We added missing volumes and concentrations into the protocol section.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please write the actions in a stepwise manner and using complete sentences providing all specific details associated with your experiment. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

We tried to stick to 2-3 actions per step.

10. Please ensure you answer the “how” question, i.e., how is the step performed? This will include discrete experimental steps, button clicks, the knob turns, etc.

We carefully went through all action steps in the protocol section and asked the “how” question in every step.

11. 1.2.1: How are the crosses performed. Please detail all the action.

We now added more details to describe the crosses in the action steps.

12. Line 220: Please detail how to perform MARCM crosses.

We added more details to describe the crosses in the action steps. In addition, we also added the MARCM crosses as schematics in each result figure.

13. Ste 3.1: Please rewrite this part as a numbered action step, describing the action being performed.

We re-wrote this section as action steps. It is important to notice that we do not want to encourage the readers to build precisely such a setup. Each laboratory can use its own optogenetic setup, and adjust it accordingly.

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We understand that we do not exceed the 10-page limit.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. "This figure has been modified from [citation]."

We did not reuse figures from any previous publication.

16. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We significantly expanded on details in the discussion section, making sure that the five points mentioned above are covered to the best of our knowledge.

17. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

We formatted our references by using the JoVE format in our citation manager.

Reviewers' comments:

Reviewer #1:

Manuscript Summary: In the manuscript, "Functional and morphological evaluation of axons and their synapses during axon death in Drosophila melanogaster", Paglione et al describe a set of assays that allow for assessment of neuronal morphology and/or function following neuronal injury. These are some clever and to my knowledge, very robust, assays that have been used in the field to identify and characterize so-called "axon death" genes. It is a very good idea to publish video instructions for these assays as they could be even more broadly used in the neuron degeneration field.

Criticisms: The protocols are overall clear but would profit from some more detail in the instructions (1). Also some of the figures could be a little more adapted to the protocols (2).

Major Concerns:

(1) A major use of these protocols is to characterize new degeneration genes. This would obviously be implemented at the level of the genetic cross, but the crosses are not explained at all. In particular, the images for protocols 1 and 2 involve relatively complicated clonal ("MARCM") experiments that require some genetic knowledge to be carried out, and the reagents for which are not listed. I think it would be helpful to include a simple crossing scheme in the protocol that can be adapted to the study of new genes for people who are not so familiar with fly genetics (maybe involving dsRNA expression driven by one of the listed GAL4 lines). This would also allow the protocol to be adapted more easily to the study of other degenerative conditions (how adaptation to other disease studies can be achieved should also be briefly discussed).

> We thank reviewer #1 for the specific concern and suggestion. We now included in all of our action steps more details covering how to perform MARCM and “regular” crosses. Furthermore, we added a schematic describing our genetic crosses in each result figure. This should make it feasible to adapt crosses according to individual needs. In the discussion section, we also mentioned that either RNAi or CRISPR/Cas9 can readily be implemented in our crosses.

(2) Figure 1 essentially contains a schematic description of the fly olfactory system but no hint as to how to use it in the injury assay. At least it should be indicated where the antenna must be pinched off. A photo would be helpful in my opinion.

> We agree with this suggestion. Figure 2C (formally figure 1) highlights which part of the antennae has to be ablated.

Minor Concerns: (personal opinion) while the optogenetic behavior assay is an awesome way to demonstrate functionality of severed axons, the appropriate setup is not so easy to come by. I remember dimly there is a behavioral assay based on the wing neurons that induces kicking or wing grooming that can be induced just by stroking the wing? That would even allow for simultaneous morphology/function assessment of the same neurons.

> We agree with reviewer #1. We did not intend the idea that a novel optogenetic setup should be built for this. In fact, an existing optogenetic setup can be adjusted accordingly.
>
> The suggestion to use wing stroking as an approach does not work in this context, unfortunately. Stroking is sensed by mechanosensory neurons, which have their dendrites projected into the mechanosensory bristles. Injury would result in cut-off mechanosensory bristles, thus dendrites and cell bodies. Stroking would therefore fail to activate the axon.

Reviewer #2:

This protocol describes two methods for evaluating axon degeneration in Drosophila: antenna removal, and wing injury; as well as an optogenetic method for examining axon function following antenna removal. Both axon damaging methods have been used in multiple studies that made valuable contributions to the axon degeneration field, so a JoVE video is likely to be useful to multiple labs. The optogenetic method is an ingenious way to evaluate the function of detached axons that has the potential to be used more widely.

Although I cannot personally comment on the specific technical details of this protocol, since I don't have experience with these methods, the protocol is clearly written and thorough. The video itself will be most useful for illustrating the dissection procedures—removing antennae, dissecting the brain, cutting the wing, etc—since these procedures are difficult to describe in words. This is already a comprehensive protocol, but the one subject that could be discussed more is image analysis. Not much needs to be said about this, but a brief mention of how images can be scored would be appreciated.

We thank reviewer #2 for the nice and positive feedback. In the action steps, we included a comment indicating that there are already many useful websites out there which provide a detailed visualization of brain dissection. However, we will show how to perform wing injury and antennal ablation in a video recorded by the film crew. We also added references in the discussion containing image analyses information, e.g. how quantification is performed in each distinct approach.

Reviewer #3:

Manuscript Summary: The authors have prepared a manuscript discussing the state of the art of procedures for studying axon destruction in the fruit fly Drosophila melanogaster. Three methods for observing the Wallerian degeneration of axons are described: Antennal ablation and observation of CNS axon destruction, Wing injury and PNS axon ablation and optogenetics analysis of fly grooming behaviour. The manuscript gives a clear description of experimental protocols, which are written in a manner appropriate for an audience with some basic experience in working with Drosophila. Expected results are clearly described, including clear figures illustrating examples of phenotypic differences when axon destruction mutant genes are tested. Useful videos of expected results for the optogenetic assay are provided.

Major Concerns: There are no Major issues with the manuscript, however I have several minor points to address.

Minor Concerns:
Line 98-100: axon death signaling is activated not only when the axon is cut, crushed or stretched24,25, it also seems to be a major contributor in different animal models of neurological conditions, e.g. where axons degenerate in an injury-independent manner4 - This statement is a little bold. Evidence from models of neurodegenerations are not entirely supportive of a of role axon destruction signaling genes in their pathogenesis and progression. Examples of effective and ineffective modification of neurodegenerative phenotypes would be useful for the reader.

We changed the sentence in the following way: “Importantly, axon death signaling is activated not only when the axon is cut, crushed or stretched[24,25], it also seems to be a contributor in distinct animal models of neurological conditions, *e.g.* where

axons degenerate in an injury-independent manner[4], yet with a range of beneficial outcomes[4,8].”

Line 118-120: The Gal4/UAS system combined with the clonal tools, they allow for the observation of individual axonal and synaptic morphology either in the central, or peripheral nervous system (CNS and PNS, respectively)12,13
- Language is a little unclear in the first half of this statement. What are "the clonal tools"?

We added more details to describe the crosses in the action steps. In addition, we also added the MARCM crosses as schematics in each result figure. This should help the reader to understand what genotypes were used in this manuscript.

The authors may consider adding a brief statement and citation for reader interested in mammalian models for axon destruction, for example neuronal culture and rodent nerve cut/crush injury models.

We appreciate this concern. However, we’d like to focus in our article on the tools in *Drosophila*, and how they can be used in a broader context. We therefore would like not to mention mammalian models and the approached used there.

The sequence of experimental procedures might be more logically arranged as 1. Wing Ablation; 2. Antennae ablation; 3. Optogenetic assay of antennae ablation flies.

We fully agree with this suggestion and arranged the experimental procedures accordingly. We also changed the sequence in the discussion. In addition, we changed the title of our article from “Functional and morphological evaluation…” to “Morphological and functional evaluation…”, so that it is in line with the novel arrangement.

Though videos of *Drosophila* brain dissection are available via other sources (for example Janellia Farm website) a video demonstrating the procedure for antennae and wing injury would be helpful additions to the manuscript.

We agree with this suggestion. Instead of a video, we now included Figure 2C highlighting the parts of antennae to be ablated with tweezers. In addition, we also provided a link from Janelia Farm (Section 2.2.) which thoroughly explains how to perform *Drosophila* brain dissections.

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

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CORRESPONDING AUTHOR

Name:	Lukas Neukomm
Department:	Department of Fundamental Neurosciecnes
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