Protocol

A protocol to detect neurodegeneration in *Drosophila melanogaster* whole-brain mounts using advanced microscopy

*Drosophila melanogaster* is an excellent model organism to study neurodegeneration. Assessing evident neurodegeneration within the fly brain involves the laborious preparation of thin-sectioned H&E-stained heads to visualize brain vacuole degeneration. Here, we present an advanced microscopy-based protocol, without the need for sectioning, to detect vacuole degeneration within whole fly brains by applying commonly used stains to reveal the brain parenchyma. This approach preserves the whole-brain architecture and enables rapid, reproducible, and quantitative analyses of vacuole-like degeneration associated with specific brain regions.
Protocol

A protocol to detect neurodegeneration in *Drosophila melanogaster* whole-brain mounts using advanced microscopy

Joseph A. Behnke, 1,4,* Changtian Ye, 1 Kenneth H. Moberg, 1 and James Q. Zheng1,2,3,5,*

1Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322, USA
2Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322, USA
3Center for Neurodegenerative Diseases, Emory University School of Medicine, Atlanta, GA 30322, USA
4Technical contact
5Lead contact
*Correspondence: joseph.aaron.behnke@emory.edu (J.A.B.), james.zheng@emory.edu (J.Q.Z.)
https://doi.org/10.1016/j.xpro.2021.100689

SUMMARY

*Drosophila melanogaster* is an excellent model organism to study neurodegeneration. Assessing evident neurodegeneration within the fly brain involves the laborious preparation of thin-sectioned H&E-stained heads to visualize brain vacuole degeneration. Here, we present an advanced microscopy-based protocol, without the need for sectioning, to detect vacuole degeneration within whole fly brains by applying commonly used stains to reveal the brain parenchyma. This approach preserves the whole-brain architecture and enables rapid, reproducible, and quantitative analyses of vacuole-like degeneration associated with specific brain regions.

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

BEFORE YOU BEGIN

© Timing: 0.5 h

Frank brain atrophy can be measured in the fly brain by detecting the presence of vacuoles (Heisenberg and Böhl 1979, Sunderhaus and Kretzschmar 2016). Traditionally, this is done using 4 or 7 μm thick coronal sections from paraffin embedded fly heads (Wittmann, Wszolek et al. 2001, Sunderhaus and Kretzschmar 2016). Sections can be stained with hematoxylin and eosin (H&E) and imaged with standard white light microscopy or left unstained and imaged under a standard epifluorescent microscope using blue light which shows the autofluorescence of brain parenchyma (Wittmann, Wszolek et al. 2001, Sunderhaus and Kretzschmar 2016). The degree of vacuolization (both frequency and area) increases in aged *Drosophila*, which is further exacerbated in mutants that express proteins implicated in neurodegenerative diseases (Wittmann, Wszolek et al. 2001, Sunderhaus and Kretzschmar 2016). Vacuolization is also seen following head trauma exposure (Saikumar, Byrns et al. 2020, Behnke, Ye et al. 2021).

The following protocol describes standard fly fixation, dissection and whole-brain staining techniques combined with advanced microscopy (confocal or two-photon) which can be used with any wild-type or transgenic fly lines (for Young [3–5 d] and Aged [5 wk] male and female flies). Common mechanisms of neurodegeneration involve the destabilization and disassembly of the actin cytoskeleton found within the neuropil containing axons and dendrites (Kommaddi, Das et al. 2018, Wang,
Simon et al. 2019). As such, the essential stains within this protocol involve a phalloidin stain, which detects filamentous actin-rich neuropil, and a nuclear stain, such as DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride). This method preserves the 3-D cytoarchitecture of the brain and does not require the use of thin-cut sections.

1. Prepare buffers ahead of time.
2. Use freshly prepared formaldehyde that is less than 1 month old.

△ CRITICAL: If using transgenic fly strains with fluorescently tagged proteins of interest, use non-overlapping fluorophore-based nuclear and phalloidin stains.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) (1:1000) | Thermo Fisher Scientific | Thermo Fisher Scientific Cat# D1306; RRID:AB_2629482 |
| Alexa Fluor™ 594 Phalloidin (1:100) | Thermo Fisher Scientific | Thermo Fisher Scientific Cat# A12381; RRID:AB_2315633 |
| Mouse monoclonal 4F3 discs large (DLG) antibody (1:100) | DSHB | DSHB Cat# 4F3 anti-discs large; RRID:AB_528203 |
| Alex Fluor™ 594 Goat a-mouse (1-400) | Thermo Fisher Scientific | Thermo Fisher Scientific Cat# A11032; RRID:AB_2534091 |
| Alexa Fluor™ 488 Phalloidin (1:100) | Thermo Fisher Scientific | Thermo Fisher Scientific Cat# A12379; RRID:AB_2315147 |
| Chemicals, peptides, and recombinant proteins |        |            |
| SlowFade™ Gold Antifade Mountant | Thermo Fisher Scientific | S36937 |
| Clear nail polish | N/A | N/A |
| 10X Concentrate phosphate buffered saline | Sigma-Aldrich | PS493 |
| Triton™ X-100 | Sigma-Aldrich | X100 |
| Pierce™ 16% Formaldehyde (w/v), Methanol-free | Thermo Fisher Scientific | 28908 |
| Experimental models: organisms/strains |        |            |
| D. melanogaster: nSyb-Gal4 | Teri Ngo (Rubin Lab) (Pfeiffer, Truman et al. 2012) | N/A |
| D. melanogaster: Uas-hTau | Bloomington Drosophila Stock Center | BDSC# 51363 |
| D. melanogaster: w1118 | Bloomington Drosophila Stock Center | BDSC# 5905 |
| Software and algorithms |        |            |
| ImageJ (Fiji) | http://fijisc | RRID: SCR_002285 |
| Other |        |            |
| SecureSeal™ imaging spacer | Thermo Fisher Scientific | GBL654002 |
| Superfrost™ Plus Microscope Slides | Fisherbrand | 22-037-246 |
| Chemglass Life Sciences Coverslip, square (18 x 18 mm), #1.5 thickness (0.16–0.19 mm) | Chemglass Life Sciences | CLS17641818 |
| Red Sable Brush, Size 5#0 | Electron Microscopy Sciences | 66100-50 |
| FV1000MPE multiphoton laser scanning microscope | Olympus | N/A |
| Nikon C2 laser-scanning confocal system | Nikon | N/A |
| Dumont #5 Forceps | Fine Science Tools | 11251-20 |
| 35 mm x 10 mm Petri dishes | Coming | CLS430588-500EA |
| Sylgard 184™ | Electron Microscopy Sciences | 24236-10 |
MATERIALS AND EQUIPMENT

0.5% PBS Triton X-100 (0.5% PBS-T)

| Reagent             | Final concentration | Amount  |
|---------------------|---------------------|---------|
| Triton X-100        | 0.5% (v/v)          | 0.5 mL  |
| 10x PBS             | 1x                  | 10 mL   |
| ddH₂O               | n/a                 | 89.5 mL |
| Total               | n/a                 | 100 mL  |

0.008% PBS Triton X-100 (0.008% PBS-T)

| Reagent                | Final concentration | Amount  |
|------------------------|---------------------|---------|
| 0.5% PBS Triton X-100  | 0.008% (v/v)        | 1.6 mL  |
| 10x PBS                | 1x                  | 10 mL   |
| ddH₂O                  | n/a                 | 88.4 mL |
| Total                  | n/a                 | 100 mL  |

Storage: Store 0.5% PBS-T and 0.008% PBS-T at 20°C–23°C. Buffers are stable for at least 2–3 months.

4% PFA in 0.5% PBS Triton X-100 (0.5% PBS-T)

| Reagent                        | Final concentration | Amount  |
|--------------------------------|---------------------|---------|
| 16% Formaldehyde (PFA)         | 4% (v/v)            | 2.5 mL  |
| Triton X-100                   | 0.5% (v/v)          | 0.05 mL |
| 10x PBS                        | 1x                  | 1 mL    |
| ddH₂O                          | n/a                 | 6.45 mL |
| Total                          | n/a                 | 10 mL   |

Note: Unused 4% PFA can be stored at 4°C for 1–2 months.

CRITICAL: Paraformaldehyde is toxic and is a known carcinogen. Prepare the 4% PFA solution within a fume hood while wearing gloves.

STEP-BY-STEP METHOD DETAILS

Whole-body tissue fixation and dissection: Day 1

Timing: 4 h + 5 min per brain dissection

Whole flies are fixed using paraformaldehyde and then their brains are subsequently dissected.

1. Quickly incapacitate flies using CO₂
2. Transfer flies from the same genotype/condition to their own prelabelled 2 mL Eppendorf tube; avoid using more than 15–20 flies per tube
3. Apply 1.8 mL of 4% paraformaldehyde (PFA) in phosphate-buffered saline containing 0.5% triton X-100 (0.5% PBS-T) and invert closed tube containing fixative and flies several times over to adequately immerse flies

CRITICAL: Ensure that flies sink within fixative solution by periodically checking after beginning fixation incubation.

4. Incubate tubes sideways on a nutator, rocker or shaker at 20°C–23°C for 3 h
5. Wash flies 4 times with 1.8 mL of 0.5% PBS-T, 15 min each with nutation
a. To wash, slightly angle tube and aspirate the top layer of liquid with a P-200 pipette, then replace with fresh 0.5% PBS-T

6. Dissect fly brains in 0.008% PBS-T and transfer to 600 µL Eppendorf containing 0.008% PBS-T
   a. Several helpful resources exist for aiding in fly brain dissections
      i. Videos:
         https://youtu.be/dc9tFpXv0m0 (HHMI 2019)
         https://www.jove.com/t/55128 (Tito et al., 2016)
      ii. Text: Immunohistochemistry in Drosophila (Helfrich-Förster 2007)

Optional: Fly brains can be freshly dissected and subsequently fixed in 4% PFA for 20 m at 20°C–23°C with nutation. Either dissection strategy works well. The decision to bulk fix flies prior to dissection or dissect fresh brains prior to fixation is largely based on timing preferences i.e. if all of the flies need to be processed at the same time, then bulk fixation is the preferred method.

Note: Be sure to remove as much of the air sac as possible, which can otherwise result in floating brains.

△ CRITICAL: Take care when replacing solutions within the Eppendorf containing fly brains, otherwise aspiration of fly brains may happen. To minimize this possibility, allow brains to sink following replacement of solution, and place the pipette away from the brains while leaving a residual amount (~50 µL) of solution each time.

△ CRITICAL: Whole-fixed fly brains are a little more brittle than fresh ones, be careful while dissecting to minimize inadvertent tissue damage.

Pause point: Prior to brain dissections, fixed whole flies can be stored in 1 X PBS at 4°C for up to several days. Dissected brains can also be stored in 1 X PBS at 4°C for several days.

Whole-brain staining and mounting: Day 1–day 2

⊙ Timing: 15 min preparation + 16–24 h incubation

For standard staining to detect frank neuronal atrophy, only phalloidin and DAPI staining are required. Additional immunohistochemistry can be performed on the same brains for the study of additional proteins of interest. If additional immunohistochemistry is performed, do so prior to incubation with phalloidin and DAPI.

7. Prepare phalloidin and DAPI cocktail in 0.5% PBS-T (580 µL per 600 µL Eppendorf)
   a. DAPI is used at a 1:1000 concentration
   b. Alexa Fluor conjugated phalloidin is used at a 1:100 concentration
8. Apply 400 µL phalloidin + DAPI cocktail (0.5% PBS-T) per Eppendorf tube of fly brains and incubate tubes upright 16–24 h at 4°C with nutation
9. Following 16–24 h incubation, wash fly brains 4 times with 580 µL 0.5% PBS-T, 15 min each with nutation at 20°C–23°C.
10. Following the final wash in 0.5% PBS-T, wash fly brains in 580 µL 1 X PBS for 30 min with nutation at 20°C–23°C to remove residual detergent
11. Mount fly brains anterior side up on Superfrost Plus microscope slides with SecureSeal™ imaging spacer (Figure 1)
   a. Prior to securing SecureSeal™ imaging spacer onto microscope slide, cut a V-shaped wedge into the spacer to allow for air bubbles to escape, and then remove one adhesive layer of the spacer and apply it to the slide
b. Add 10 μL of SlowFade™ Gold Antifade Mountant to the microscope slide, followed by transfer of brains in 1× PBS using a P-200 pipette with the tip cut.

c. Using a brush, position fly brains at a 45° angle with anterior side facing up.

d. Aspirate residual mountant plus 1× PBS and allow brains to adhere for 10–15 min.

e. Add 30 μL of mountant on the top portion of the imaging spacer, slightly angle the cover slip, and gently place over the spacer and well containing the fly brains. Allow the mountant to perfuse throughout the well and in between the coverslip and spacer. Avoid air bubbles.

f. Seal perimeter of coverslip with nail polish and allow nail polish to dry before storing or imaging.

**Optional:** Any combination of fluorophore tagged phalloidin and nuclear stains can be used. If performing additional immunohistochemistry, the same fluorophore for phalloidin and nuclear stains can be used depending on the number of available detectable microscope channels.

**Optional:** If additional immunohistochemistry is performed, additional steps including blocking in normal serum is required. If additional antibody staining is performed, longer incubation steps are needed for sufficient antibody penetration. Additional methods can be found in *(FlyLight 2015)*.
Pause point: Stained fly brains can be stored for an extended period of time in 1 X PBS at 4°C for several days before mounting onto microscope slides. Slides can be stored at 4°C but should be placed horizontally to avoid movement of brains within the spacer and kept covered to minimize light exposure.

Whole-brain imaging: Day 3

© Timing: 5–10 min per brain

12. Perform two-photon imaging of the vacuoles in the Drosophila whole-brain scanning at a resolution of 1024 X 1024 with a Z-step of 1 μm. Using these parameters, an entire Drosophila brain corresponds to ~150–180 Z-sections. Total image acquisition typically takes 8–10 min per brain.

Note: Drosophila whole-brain imaging is performed using an Olympus Fluoview two-photon system fitted with a water immersion two-photon objective XLPLN25XWMP, which has a working distance of 2 mm and N.A. of 1.05. We set the IR laser at 780 nm wavelength to simultaneously excite the DAPI and Alexa Fluor 594 phalloidin, which will be collected by two non-descanned detectors with specific filters for FITC (fluorescein isothiocyanate) and Alexa Fluor 594, respectively. Here, DAPI fluorescence is collected by FITC channel since DAPI emission spectrum overlaps with FITC. If performing additional antibody staining, DAPI and Alexa Fluor 488 phalloidin can be detected using the FITC, and an additional antibody of interest can be detected using the red channel.

Note: Maintain consistent laser strength and detection settings across all samples being compared. The adult Drosophila brain is less than 200 μm thick (A-P) after mounting. Set scanning depth to cover the entirety of the brain (this should be adjusted for each individual brain sample). If signal strength is low within the deeper portion of the tissue, a depth compensation protocol within the imaging software may be utilized to increase signal intensity. A laser strength between 2–3% is typically sufficient within our imaging setup.

Note: While standard laser scanning confocal imaging can be performed, it does generate weaker signals in the deeper layers of the brain due to deflection and absorption of the excitation laser reaching the deeper layers and the loss of emission signals. Therefore, one needs to compensate by increasing the laser intensity for deeper layers and gain of the detector. Most of the current confocal systems have a built-in function for the depth-compensation imaging routine as well. We find that a two-photon (2P) laser can sufficiently penetrate the whole Drosophila brain and generates similar levels of fluorescence throughout the whole brain. Therefore, 2P imaging is recommended for this work. It should also be noted that the standard FITC/TRITC filter set can be used with the caveats that there will be some bleed-through of DAPI signals to the TRITC channel as well as the less than optimal collection of Alexa Fluor 594 fluorescence.

Note: 2P sectioning of the whole brain can be done at increased Z-resolution by reducing the Z-step.

Pause point: Mounted slides can be stored (4°C) for several days to weeks before significant deterioration of signal strength. Protect from light to minimize signal loss.

Whole-brain vacuole analysis in image J: Day 4

© Timing: 5–10 min per brain

13. Import stack of images into Fiji (Image J)
14. Using the wand (tracing) tool within Fiji, isolate vacuoles at their largest area and assign them as regions of interest (ROIs). Adjust tolerance as needed within the tool to best select each vacuole. Within the ROI manager, save each set of ROIs per brain as its own “.zip” file. Upon completion of the stack, select all ROIs per given brain and perform Measure (with Area selected within Set Measurements) and save Results as “.csv”. Import measurement data within preferred stats package.

**Note:** The primary criteria for determining pathogenic vacuoles includes hole location and shape. For location, we refer to the arrangement of physiologically normal holes which often appear in a stereotypical location, which is almost always symmetrical in nature. For assessing shape, we examine whether the hole is oval or spheroid in appearance, which is indicative of pathogenic vacuole, or tubular in nature, which corresponds to air sac conduit. Examining sequential frames of the stack is important for determining the overall shape of holes of interest. Holes that appear on the surface of the brain, which assume a crater-like appearance, do not have a defined enclosure and as such are not considered pathogenic vacuoles. Please refer to Figure 1C for location of the most prominent physiologically normal holes found within the fly midbrain. For a more comprehensive reference of physically normal holes, please refer to the Virtual Fly Brain Resource (Milyaev, Osumi-Sutherland et al. 2011): https://v2.virtualflybrain.org/org.geppetto.frontend/geppetto?id=FBbt_00045046&amp;i=VFB_00101567

**EXPECTED OUTCOMES**

The presented protocol describes a procedure to measure frank neurodegeneration within the fly brain by detecting the presence of vacuoles. The fly brain architecture consists of a cortical cell body rind that envelops a synaptically-dense neuropil (Ito, Shinomiya et al. 2014). Vacuoles are determined based on regions devoid of phalloidin (mostly within the actin-rich neuropil) and DAPI (nuclei of the cell body rind) signal in regions typically occupied by brain matter. Within the neuropil region, vacuoles correspond to the loss of a neuropil-specific marker, discs large (DLG), which is the fly homolog of post-synaptic density-95 (PSD-95) (Parnas, Haghighi et al. 2001), demonstrating that this is a neuron-specific loss of tissue (Figure 2). This strategy incorporates existing standard fly fixation, dissection and whole-brain staining techniques combined with advanced microscopy (confocal or two-photon) which can be used with any wild-type or transgenic fly lines. Validation of this method demonstrates that it can be used to discriminate neurodegeneration found in models of aging and tauopathy (Figure 2), and neurodegeneration secondary to head trauma (Behnke, Ye et al. 2021). Although our lab uses two-photon microscopy to image vacuoles, confocal can be used in its place with a modest decrease in signal intensity within deeper regions of the brain (Figure 3). Representative Z-stacks from healthy and vacuole-laden brains are found in Methods videos S1 and S2, respectively.

**LIMITATIONS**

This protocol enables detection of neurodegenerative vacuoles within whole-mount fly brains using confocal or two-photon microscopy. Vacuole size is measured in terms of area, by selecting the image slice per given vacuole with the largest diameter. Although this strategy provides consistent results, especially given that vacuoles are often spherical or spheroid in shape, it does ignore the 3-D nature of vacuoles. Future work will take our existing strategy of preserving the whole fly brain and apply volumetric analyses on vacuoles to provide further characterization and measurement of vacuole size.

**TROUBLESHOOTING**

**Problem 1**

Weak Signal (steps 1–3)
Figure 2. Detecting neurodegeneration in Drosophila whole-brain mounts

(A) Representative max projection of whole-brain mount stained with DAPI (blue) and phalloidin (magenta) and imaged using two-photon microscopy. White scale bar = 100 μm.

(B) Representative montage of Z-stack showing range of acquired brain slices. White scale bar = 100 μm.

(C) Validation of vacuoles showing representative brain slice from young (3–5 day old) nSyb-GAL4, aged (5-week-old) nSyb-GAL4 and nSyb>hTau flies. Green outline corresponds to physiologically normal holes. Yellow outline corresponds to pathological degenerative vacuoles. Yellow scale bar = 50 μm.

(D) Shaded region from (C) showing representative vacuoles (yellow arrows) and physiologically normal hole (green arrow). Gray scale bar = 24 μm. Vacuoles correspond to loss of neuropil. Co-labeling with neuropil-specific marker, discs large (magenta; DLG, a post-synaptic density 95 homolog) reveals region devoid of DAPI and phalloidin (gray) is found within region of neuropil.

(E and F) Boxplot quantification of (E) vacuole number and (F) area from young and aged nSyb-GAL4 and young and aged nSyb>hTau flies. Mann–Whitney U test between young and aged groups and between aged nSyb-GAL4 and nSyb>hTau, with Bonferroni correction. Boxplots in (E and F) contain individually plotted values with whiskers corresponding to the maximum 1.5 interquartile range.
**Potential solution**
Weak staining signal can be attributed to several different processes, including tissue fixation, staining and image acquisition. Insufficiently fixed tissue will not maintain its physical structure, nor will it properly react to tissue stains. For properly fixed tissue that still exhibits weak signal, consider increasing phalloidin and/or DAPI concentrations. Additionally, consider increasing the incubation time from 16–24 h–48 h. Weak signal during the image acquisition stage can be improved through increasing laser strength and/or detection settings.

**Problem 2**
Poor Tissue Quality (step 1)

**Potential solution**
Ensure proper fixation methods and make sure flies sink during fixation, otherwise, floating flies will not be properly preserved. While dissecting fixed flies, poorly preserved tissue will readily break during dissection handling.

**Problem 3**
Brains sticking to pipet tips when aspirating (steps 1 and 2)

---

**Figure 3. Comparison of vacuole imaging using confocal and two-photon microscopy**
(A–C) Anterior and (A’–C’) middle regions of the same representative brain imaged under (A, A’) 5% confocal laser power, (B, B’) 5%–10% z-corrected confocal laser power, and (C,C’) 3% two-photon laser power. Whole-brain mounts stained with DAPI to detect nuclei and phalloidin to detect actin (brain parenchyma). Yellow arrows indicate the presence of vacuoles. Scale bar = 100 μm.
Potential solution
Fly brains have a tendency to stick to surfaces, including pipette tips. This can result in loss of tissue while moving brains between Eppendorf tubes. To minimize this risk, coat pipette tips in solution containing detergent, such as that used for routine wash steps. Additionally, cut P200 pipet tips with a blade to create a bigger opening and minimize shear stress.

Problem 4
Floating Fly Brains (steps 1 and 2)

Potential solution
Ensure removal of air sacs from the fly brain, which is most pronounced on the posterior medial surface of the fly brain. Otherwise, air trapped within the remnant air sac can cause the brain to float and has increased risk for being inadvertently aspirated during washes.

Problem 5
High Background Signal (step 3)

Potential solution
High non-specific background signal can arise most commonly from overfixation or remnant pigmented tissue, such as the compound eye. Additionally, insufficient washing may contribute to background signal. To minimize background signal, fix tissue accordingly, and remove pigmented tissue, such as the compound eye. Perform sufficient washing, both in the number of washes and volume of washes (>300 µL in 600 µL Eppendorf tube) especially when performing additional antibody staining.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, James Zheng, PhD (james.zheng@emory.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The published article includes all datasets/code generated or analyzed during this study.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100689.

ACKNOWLEDGMENTS
This work was supported in part by research grants from the National Institutes of Health to J.Q.Z. (GM083889, R01MH104632) and to K.H.M. (MH107305). We would like to thank Teri Ngo (Rubin Lab) for providing fly stocks.

AUTHOR CONTRIBUTIONS
J.A.B. and J.Q.Z. designed the research; J.A.B. and C.Y. performed the research; J.A.B., C.Y., and J.Q.Z. analyzed the data; J.Q.Z. and K.H.M. supervised the research; and J.A.B., C.Y., and J.Q.Z. wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.
REFERENCES

Behnke, J.A., Ye, C., Setty, A., Moberg, K.H., and Zheng, J.Q. (2021). Repetitive mild head trauma induces activity mediated lifelong brain deficits in a novel Drosophila model. Sci. Rep. 11, 9738.

FlyLight. (2015). Double label IHC for adult Drosophila CNS. https://www.janelia.org/sites/default/files/Project%20Teams/Fly%20Light/FL%20Protocol%20-%20Adult%20IHC%20-%20Double%20Label.pdf.

Hesenberg, M., and Böhl, K. (1979). Isolation of anatomical brain mutants of Drosophila by histological means. Zeitschrift für Naturforschung C 34, 143–147.

Helfrich-Förster, C. (2007). Immunohistochemistry in Drosophila. Circadian Rhythms (Springer), pp. 533–547.

HHMI (2019). Drosophila adult brain dissection. YouTube: https://youtu.be/dc9tFpXv0m0.

Ito, K., Shinomiya, K., Ito, M., Armstrong, J.D., Boyan, G., Hartenstein, V., Harzsch, S., Hesenberg, M., Homberg, U., and Jenett, A. (2014). A systematic nomenclature for the insect brain. Neuron 81, 755–765.

Kommaddi, R.P., Das, D., Karunakaran, S., Nanguneri, S., Bapat, D., Ray, A., Shaw, E., Bennett, D.A., Nair, D., and Ravindranath, V. (2018). Aβ mediates F-actin disassembly in dendritic spines leading to cognitive deficits in Alzheimer’s disease. J. Neurosci. 38, 1085–1099.

Milyaev, N., Osumi-Sutherland, D., Reeve, S., Burton, N., Baldock, R.A., and Armstrong, J.D. (2011). The Virtual Fly Brain browser and query interface. Bioinformatics 28, 411–415.

Parnas, D., Haghighi, A.P., Fetter, R.D., Kim, S.W., and Goodman, C.S. (2001). Regulation of postsynaptic structure and protein localization by the Rho-type guanine nucleotide exchange factor dPix. Neuron 32, 415–424.

Pfeiffer, B.D., Truman, J.W., and Rubin, G.M. (2012). Using translational enhancers to increase transgene expression in Drosophila. Proc. Natl. Acad. Sci. U S A 109, 6626–6631.

Saikumar, J., Byrns, C.N., Hemphill, M., Meaney, D.F., and Bonini, N.M. (2020). Dynamic neural and glial responses of a head-specific model for traumatic brain injury in Drosophila. Proc. Natl. Acad. Sci. U S A 117, 17269–17277.

Sunderhaus, E.R., and Kretzschmar, D. (2016). Mass histology to quantify neurodegeneration in Drosophila. J. Vis. Exp. https://doi.org/10.3791/54809.

Tito, A.J., Cheema, S., Jiang, M., and Zhang, S. (2016). A simple one-step dissection protocol for whole-mount preparation of adult drosophila brains. J. Vis. Exp. https://doi.org/10.3791/55128.

Wang, G., Simon, D.J., Wu, Z., Belsky, D.M., Heller, E., O’Rourke, M.K., Hertz, N.T., Molina, H., Zhong, G., and Tessier-Lavigne, M. (2019). Structural plasticity of actin-spectrin membrane skeleton and functional role of actin and spectrin in axon degeneration. Elife 8, e38730.

Wittmann, C.W., Wszolek, M.F., Shulman, J.M., Salvaterra, P.M., Lewis, J., Hutton, M., and Feany, M.B. (2001). Tauopathy in Drosophila: neurodegeneration without neurofibrillary tangles. Science 293, 711–714.