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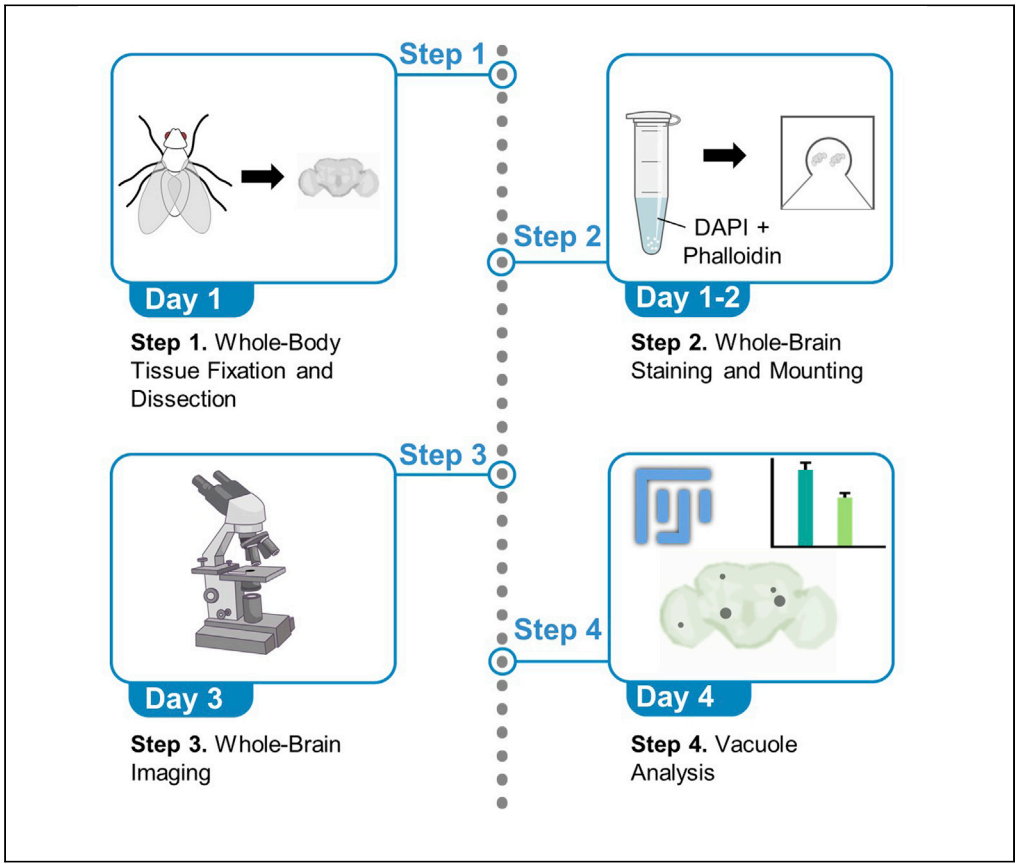
A protocol to detect neurodegeneration in *Drosophila melanogaster* whole-brain mounts using advanced microscopy



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.

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Highlights

Reproducible protocol to measure whole-brain vacuole degeneration within *Drosophila*

Utilizes easily accessible immunofluorescent tissue stains and microscopy methods

Can be performed alongside additional whole-brain immunostaining procedures

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

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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 α-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
10× Concentrate phosphate buffered saline	Sigma-Aldrich	P5493
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://fiji.sc	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 × 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 × 10 mm Petri dishes	Corning	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
10× PBS	1×	10 mL
ddH_2O	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
10× PBS	1×	10 mL
ddH_2O	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
10× PBS	1×	1 mL
ddH_2O	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 CO_2
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× PBS at 4°C for up to several days. Dissected brains can also be stored in 1× 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× 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

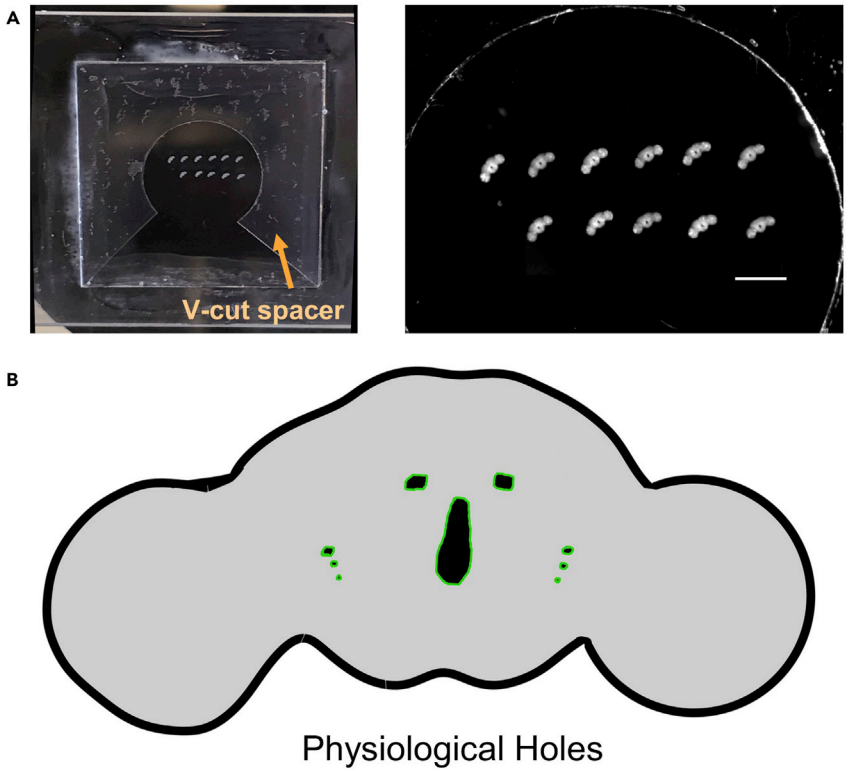

Figure 1. Organization of *Drosophila* whole-brain mounts

(A) Brains are positioned in a uniform fashion at 45° to maximize the field of view for microscopy and minimize space on the microscope slide. Alternatively, for mispositioned brains, most microscope imaging software have a rotation function that can be used to fully capture the entire brain's field of view. Scale bar = 1 mm.

(B) Cartoon schematic of physiologically normal holes. For additional reference, please refer to Virtual Fly Brain Resource (Milyaev, Osumi-Sutherland et al. 2011):https://v2.virtualflybrain.org/org.geppetto.frontend/geppetto?id=FBbt_00045046&i=VFB_00101567

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 × 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 × 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&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)

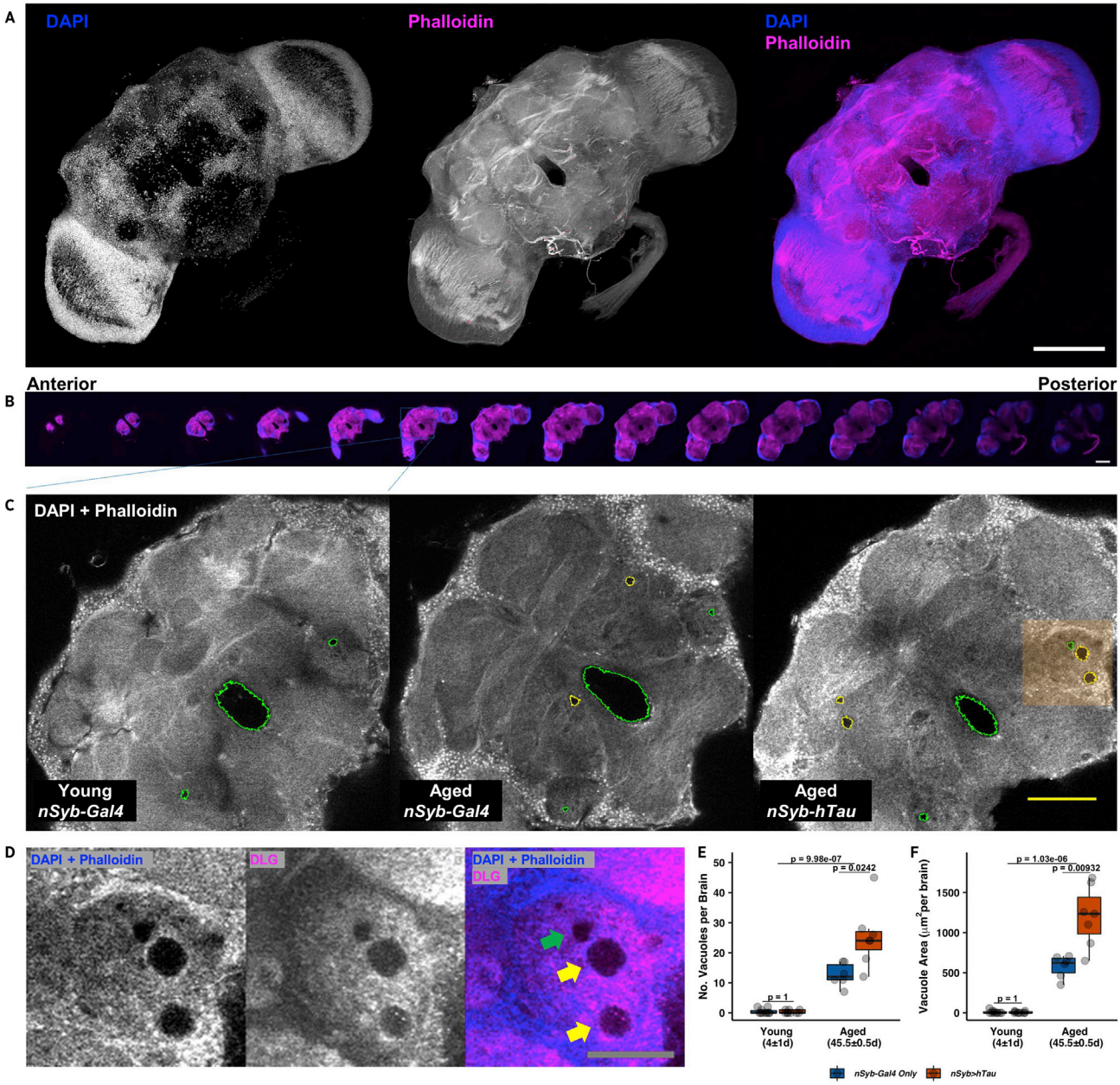

Figure 2. Detecting neurodegeneration in *Drosophila* whole-brain mounts

(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.

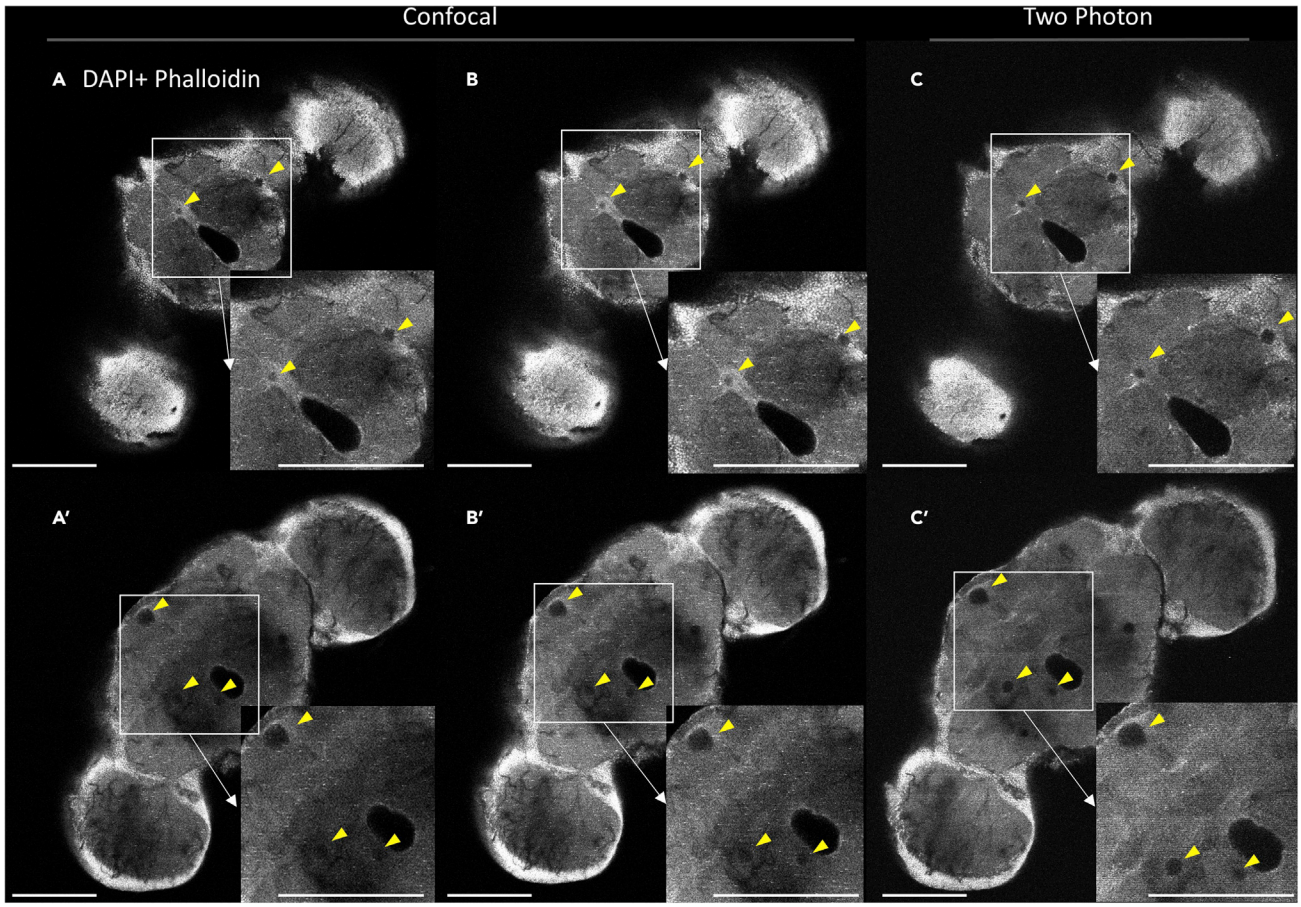

Figure 3. Comparison of vacuole imaging using confocal and two-photon microscopy

(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

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)

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.

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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.

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