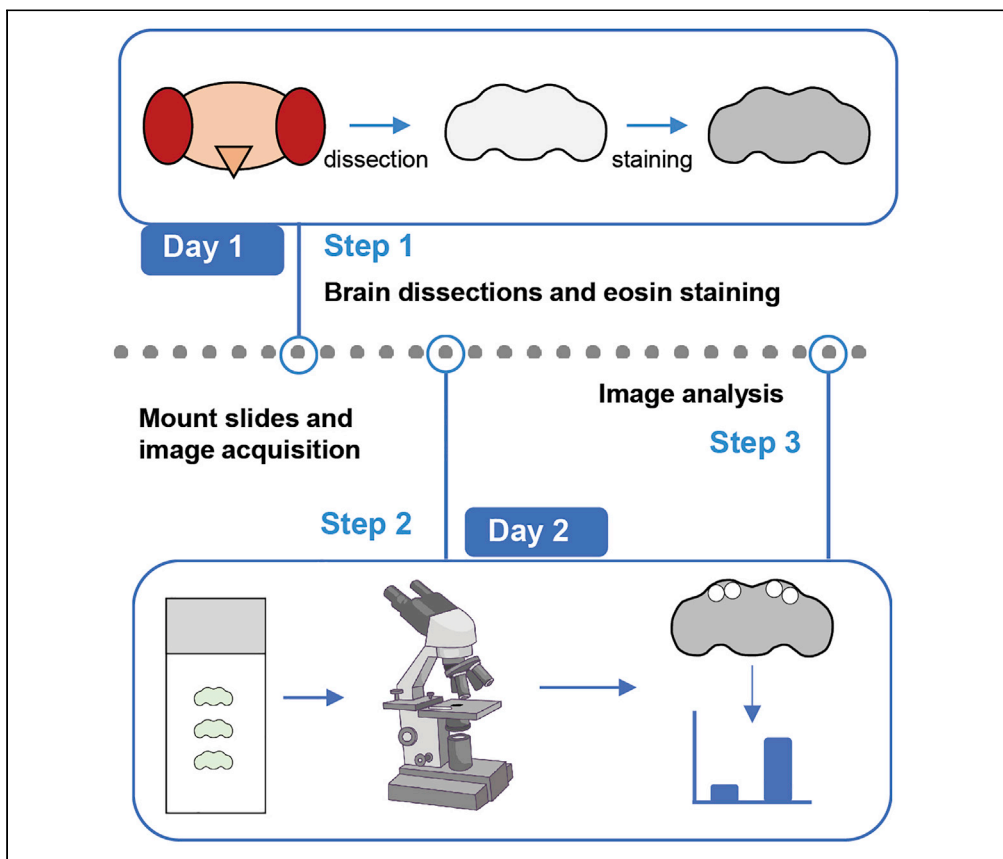


Protocol

Eosin whole-brain mount staining to analyze neurodegeneration in a fly model of Alzheimer's disease



In the fly brain, neurodegeneration is detected by the presence of vacuoles using conventional hematoxylin and eosin (H&E) or phalloidin staining, which are lengthy and expensive processes. Here, we present a faster and cost-effective 2-day protocol to visualize vacuoles in a fly model of Alzheimer disease. We describe eosin staining in the whole-brain mount, followed by confocal microscopy and image analysis with an open source Fiji plugin. This protocol can be applied to visualize different modules in the fly brain.

Suzana Ulian-Benitez, Abigail Strausbaugh, Hjelmstad, Guilherme Oliveira Barbosa, Pejmun Haghighi

suzanaub@gmail.com (S.U.-B.)
phaghighi@buckinstitute.org (P.H.)

Highlights

Efficient and cost-effective protocol to analyze neurodegeneration in *Drosophila*

Details of eosin whole-brain mount staining to assess vacuoles in the fly brain

Eosin whole-brain mount can also be used to assess the morphology of brain modules

Development of an open source Fiji plugin to assist image analysis

Ulian-Benitez et al., STAR Protocols 3, 101377
June 17, 2022 © 2022 The Authors.
<https://doi.org/10.1016/j.xpro.2022.101377>

Protocol

Eosin whole-brain mount staining to analyze neurodegeneration in a fly model of Alzheimer's disease

Suzana Ulian-Benitez,^{1,4,*} Abigail Strausbaugh Hjelmstad,^{1,3} Guilherme Oliveira Barbosa,² and Pejmun Haghighi^{1,5,*}

¹Buck Institute for Research on Aging, Novato, CA 94945, USA

²University California San Francisco, San Francisco, CA 94158, USA

³Present address: Duke University, Durham, NC 27708, USA

⁴Technical contact

⁵Lead contact

*Correspondence: suzanaub@gmail.com (S.U.-B.), phaghighi@buckinstitute.org (P.H.)
<https://doi.org/10.1016/j.xpro.2022.101377>

SUMMARY

In the fly brain, neurodegeneration is detected by the presence of vacuoles using conventional hematoxylin and eosin (H&E) or phalloidin staining, which are lengthy and expensive processes. Here, we present a faster and cost-effective 2-day protocol to visualize vacuoles in a fly model of Alzheimer disease. We describe eosin staining in the whole-brain mount, followed by confocal microscopy and image analysis with an open source Fiji plugin. This protocol can be applied to visualize different modules in the fly brain.

BEFORE YOU BEGIN

Age-dependent loss of neurons is a common phenotype associated with Alzheimer's related toxicity in a variety of in vivo models from flies to vertebrates. Conventionally, in the fly model of Alzheimer's disease, the degree of this neuronal loss is quantified by assessing the number and size of vacuoles that appear in the brain tissue following hematoxylin and eosin (H&E) staining (Figure 1A) (Hegde et al., 2014; Loewen and Ganetzky, 2018). While the exact nature of these vacuoles remains unclear, it is widely accepted that they closely reflect the extent of neurodegeneration in disease models (Bulus et al., 2020; Cook et al., 2012; Coombe and Heisenberg, 1986; Da Cruz et al., 2008; Heisenberg and Böhl, 1979; Herman et al., 1971; Hill et al., 2020; Petersen et al., 2013; Ray et al., 2017; Sekiya et al., 2017). *Drosophila melanogaster* has been a particularly robust model for studying the genes and molecules involved neuronal death, where the assessment of vacuoles in the brain has been a routine technique for measuring the degree of neurodegeneration (Bolkan and Kretschmar, 2014; Coelho et al., 2018; Iijima et al., 2008; Maksoud et al., 2019; Ray et al., 2017; Sunderhaus and Kretschmar, 2016; Wittmann et al., 2001).

To overcome the lengthy steps of processing, sectioning, and staining for conventional H&E (Figure 1D), we have developed an easy and less expensive alternative protocol, which allows for the visualization of vacuoles (Figures 2) and other brain structures (Figure 3) in the fly brain by doing eosin staining in the whole-brain preparations followed by confocal microscopy. Although here we report a protocol for a fly model of Alzheimer's disease, this method can be extended to other contexts in which the flies undergo neurodegeneration.

Fly pushing

© Timing: 2 months



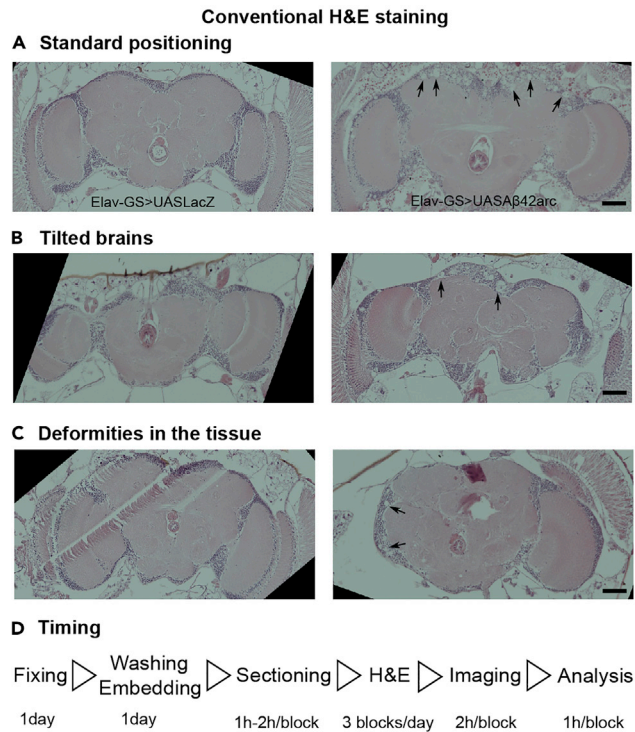


Figure 1. Conventional H&E staining

(A) Representative images of Elav-GS>UASLacZ and Elav-GS>UASA β 42arc brains in standard positioning. Central brain and both optic lobes can be visualized in the same section. Arrows point to vacuoles. Scale bar: 50 μ m.
 (B) Representative images of tilted brains. Arrows point to vacuoles. Scale bar: 50 μ m.
 (C) Representative images of deformities in the brains. Arrows point to vacuoles. Scale bar: 50 μ m.
 (D) Timing estimate for a conventional H&E experiment.

In this protocol, a human familial arctic mutant of A β ₁₋₄₂ (E22G) that is more prone to aggregation is overexpressed in all neurons with Elav-GeneSwitch (Elav-GS) driver during adult stages by adding the RU-486 hormone to their food (Osterwalder et al., 2001). The experimental flies are kept in a fly incubator at 25°C under a 12 h light/dark cycle.

1. Collect virgin female flies from the genotype Elav-GS and males from the genotypes UASLacZ and UASA β 42arc.
2. Set up 2 independent G0 crosses in normal fly food as described: 20 females of Elav-GS and 10 males of UASLacZ and 20 females of Elav-GS and 10 males of UASA β 42arc.
3. Let them mate for 48 h at 25°C and then remove the flies from the vials.
4. Keep the vials at 25°C and wait 10–12 days until F1 offspring emerge.
5. Select F1 females Elav-GS>UASLacZ and Elav-GS>UASA β 42arc and place them in a vial containing RU-486 food. This is day 0.

Note: If it is desirable to work with mated females, cross 20 F1 females to a 5 males for 2–3 days in regular fly food, then remove males and place F1 females in a fresh vial containing RU-486 food. This is day 0.

6. Transfer females to a new vial every other day to keep the food fresh until they reach 50 days on RU-486 food.

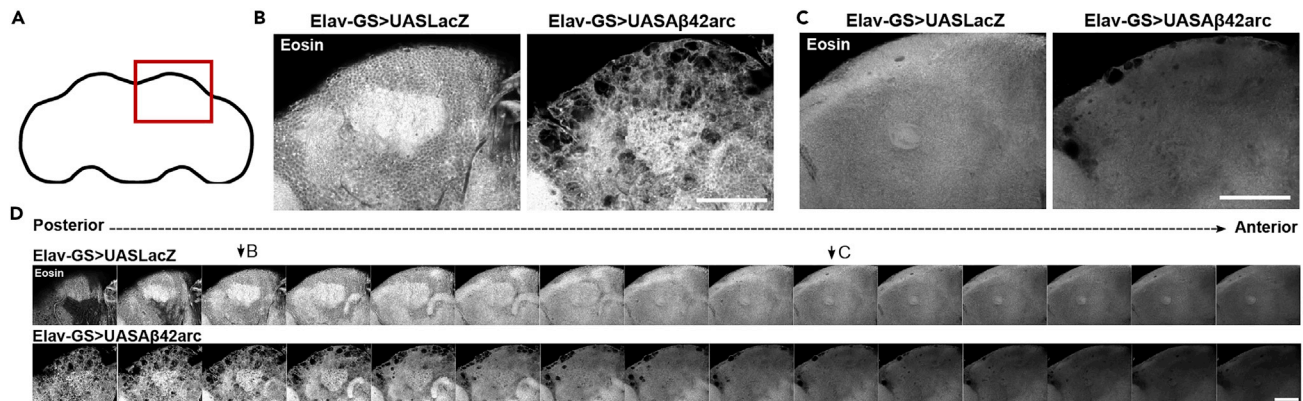


Figure 2. Eosin whole mount staining in adult flies at 50 days on RU-486 food

(A) Scheme of the fly brain, with the area of interest highlighted in red.

(B) Representative images of Elav-GS>UASLacZ and Elav-GS>UASAβ42arc flies of confocal sections in the region of the Calyx of the Mushroom Body, at the dorsal surface of the brain. Scale bar: 50 μm.

(C) Representative images of control and disease flies of confocal sections in the region of the Pedunculus of the Mushroom Body, deeper in the brain. Scale bar: 50 μm.

(D) Serial confocal sections showing from posterior to anterior view of control and disease flies. Scale bar: 50 μm.

Fly food preparation

⌚ Timing: 1 day

This is the protocol to prepare fly food containing RU-486.

7. Add the following reagents into a food processor: brewer's yeast, *Drosophila* agar type II, malt, corn flour, soy flour, molasses, and water (refer to [materials and equipment](#) table).
8. Cook for 1 h and 50 min at 95°C.
9. Inside a hood, add the following reagents into a 15 mL centrifuge tube: methylparaben, ethanol and propionic acid. Add RU-486.
10. Turn off heat of the food processor but continue mixing as the food cools to 75°C.
11. When the food is cooled to 75°C, add the previous mix with methylparaben, ethanol, propionic acid, and RU-486 into the food processor and mix thoroughly.

⚠ **CRITICAL:** Preservative and RU-486 should be added when food is cooled to a minimum of 75°C, otherwise it can make the reagents ineffective.

Note: If the food goes past below 75°C it will start to solidify while still in the food processor. It is important to keep it at this temperature while mixing the preservative and RU and then start dispensing the food into vials right away.

12. Dispense food into vials (10–12 mL per vial) manually using a serological pipette.

Alternatives: If preparing bigger batches of food, an automatic food dispenser can be used.

13. Let the food cool down until there are no more water drops and cap the vials.

⚠ **CRITICAL:** The vials should be dried before capping, otherwise water drops may remain in the vials, and they will trap the flies.

⚠ **CRITICAL:** Fly food should be kept at 4°C for no longer than 4 weeks to prevent it from drying out and to ensure the RU-486 is effective.

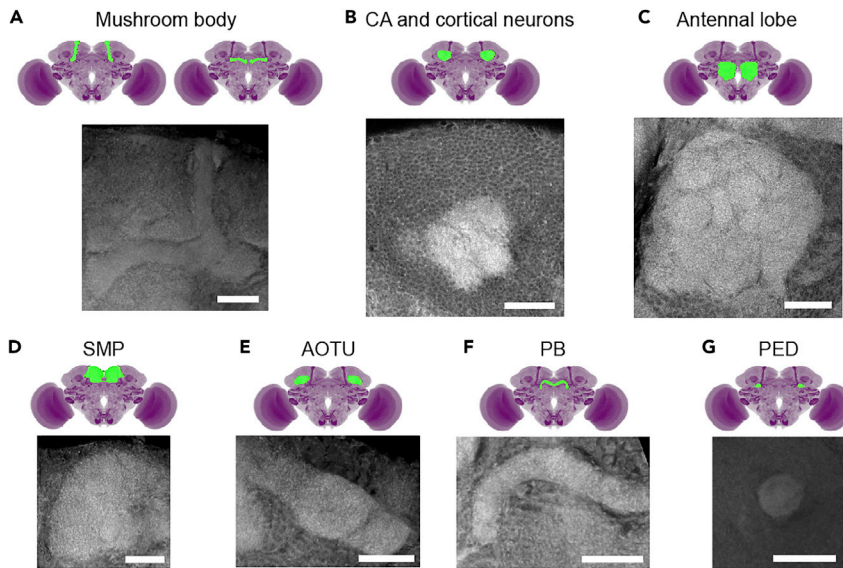


Figure 3. Eosin whole mount staining in the fly brain highlights brain modules

(A) Mushroom body.

(B) Calyx of Mushroom Body (CA) and cortical neurons.

(C) Antennal lobe.

(D) Superior Medial Protocerebrum (SMP).

(E) Anterior optic tubercle (AOTU).

(F) Protocerebral Bridge (PB).

(G) Pedunculus of the Mushroom Body (PED). Source of fly brain images: Virtual Fly Brain (<https://v2.virtualflybrain.org/org.geppetto.frontend/geppetto>). Scale bar: 25 μ m.

Prepare buffers

⌚ Timing: 30 min

Prepare buffers for brain dissections and washes.

14. Prepare the wash buffer 0.1% of Triton-X-100 in PBS (0.1%PBT).

15. Prepare the fixative 4% of paraformaldehyde (PFA) in PBS.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Brewer's yeast	Genesee Scientific	Cat#62-107
Drosophila agar type II	Apex	Cat#66-104
Malt	Genesee Scientific	Cat#62-110
Corn flour	Honeyville Grain	Cat#003-331-0001
Soy flour	Genesee Scientific	Cat#62-115
Molasses	Genesee Scientific	Cat#62-117
Methylparaben	VWR	Cat#IC10234101
Ethanol	Koptec	Cat#V1001
Propionic acid	Acros	Cat#14930-0025
RU-486 (Mifepristone)	Cayman	Cat#10006317
Triton X-100	Thermo Fisher Scientific	Cat#85111

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Paraformaldehyde (PFA) 20%	Electron Microscopy Sciences	Cat#15713
10× PBS Buffer pH 7.4	Invitrogen	Cat#AM9624
Eosin Y stain	StatLab	Cat#STE0150
VECTASHIELD	Vector Laboratories	Cat#H-1000
Experimental models: Organisms/strains		
Adult females of <i>D. melanogaster</i> : Elav-GeneSwitch	Osterwalder et al., PNAS, 2001	N/A
Adult males of <i>D. melanogaster</i> : UASLacZ: w [*] ; P{UAS-lacZ.Exel}2	Bloomington Drosophila Stock Center	BDSC#8529 FlyBase: FBst0008529
Adult males of <i>D. melanogaster</i> : UASβ42arc: w ¹¹¹⁸ ; P{UAS- APP.β42.E693G.VTR}8	Bloomington Drosophila Stock Center	BDSC#33774 FlyBase: FBst0033774
Software and algorithms		
ImageJ/Fiji	(Schindelin et al., 2012)	N/A
CheeseMaker Tool	This work https://doi.org/10.5281/ZENODO.5819097	https://github.com/gbarbosabio/CheeseMaker
Other		
Tweezers #5	Fine Science Tools	Cat#11295-10
15 mm Netwell Insert with 74 μm Mesh Size Polyester Membrane	Corning	Cat#3477
Slide "PTFE" Printed Slides 12 well 5 mm well diameter	Electron Microscopy Sciences	Cat#63425-05
Cover slip #1	VWR	Cat#48404-453
Food processor	HotmixPRO Gastro	N/A
ZEISS Confocal LSM700 Upright	ZEISS	N/A

MATERIALS AND EQUIPMENT

Fly food (1 L)	Amount
Brewer's yeast	18 g
Drosophila agar type II	13.8 g
Malt	75 g
Corn flour	80 g
Soy flour	10 g
Molasses	22 g
Methylparaben	2 g
70% ethanol	7.2 mL
Propionic acid	6.25 mL
RU-486 (200 μM)	85.3 mg
ddH ₂ O	1 L
Total	Approx. 1 L

Note: Store fly food at 4°C for up to 4 weeks prior to use.

0.1% PBT	Final concentration	Amount
Triton-X-100	0.1% (v/v)	50 μL
10× PBS	1×	5 mL
ddH ₂ O	n/a	44.45 mL
Total	n/a	50 mL

Note: Wash buffer can be kept at 4°C for up to 4 weeks.

4% PFA in PBS	Final concentration	Amount
20% PFA	4% (v/v)	10 mL
10× PBS	1×	5 mL
ddH ₂ O	n/a	39.5 mL
Total	n/a	50 mL

Note: 4% PFA can be prepared in small microcentrifuge tubes of 1.5 mL and kept frozen at –20°C for up to 6 months. When in use, thaw one aliquot at a time.

△ **CRITICAL:** Paraformaldehyde is a toxic reagent. Avoid skin contact and breathing it by preparing PFA solution in a fume hood wearing protective equipment. PFA may cause cancer and may irritate the airways.

STEP-BY-STEP METHOD DETAILS

Whole-brain dissections and washes

⌚ **Timing:** 1 h 15 min per 5 brains

Heads are first fixed in PFA, the brains are dissected in PFA and later washed in PBT.

1. Anesthetize 50-day old F1 females in a pad with CO₂.

Note: Process different genotypes separately.

2. Briefly wash flies in 70% ethanol in a petri dish to remove waxy coating and place them in a drop of ice-cold PBS in a dissecting chamber.
3. Decapitate flies and remove the proboscis while still in PBS, and then transfer the heads to a drop of ice-cold 4% PFA in the dissecting chamber.
4. Fix heads for 15 min in this drop of 4% PFA.
5. Dissect brain in a new drop of ice-cold 4% PFA in the dissecting chamber.

△ **CRITICAL:** It is important that the brains are not over fixed in PFA as it may result in higher levels of background fluorescence. To avoid this, dissect a few brains at a time (suggestion max. 5 brains). An alternative is to start dissecting the brains in 4% PFA right after the heads are transferred to 4% PFA and then leave them in the drop until 15 minutes has elapsed, and then proceed to the washing step.

6. Place brains in a 74 μm mesh netwell inside the 12-well plate (Figure 4) containing washing buffer.

Note: From step 6 onwards, the protocol can be performed in the plate with the netwells (Figure 4C). Brains can be added to the netwell and only the netwell is transferred from one well to the other. This avoids the need to wash brains in microcentrifuge tubes and pipette buffers in and out, reducing the chances of losing brains that get stuck on the tips of the pipettes.

Alternatives: In the absence of the netwells and plate, a microcentrifuge tube can be used, and solutions can be added and removed from the tube with a pipette.

7. Wash brains 2 times in 0.1% PBT for 30 min each at room temperature with gentle agitation.
8. Wash brains 1 time in PBS for 30 min at room temperature with gentle agitation.

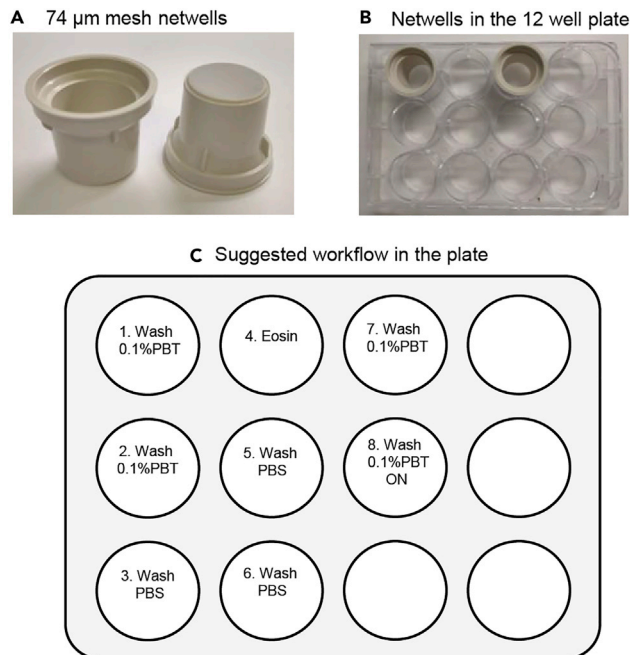


Figure 4. Plate and netwells for staining and washes
(A–C) Suggested netwells (A) and plate (B) for brain washes and suggested workflow (C).

Eosin staining and mounting

⌚ Timing: 16 h

Eosin staining of fixed and washed fly brains, followed by mounting brains on a slide.

9. Add 2 mL of Eosin into a clean well and transfer the netwell with the brains to the eosin. Stain brains for 3 min at room temperature and gentle agitation.

⚠ **CRITICAL:** Eosin is toxic and flammable. Wear protective equipment, and keep away from heat, sparks, open flames, and hot surfaces.

Note: The same 2 mL of eosin can be used to stain several samples within 1 hour. The eosin is diluted in ethanol, and if the ethanol is left outside for long periods of time it evaporates concentrating the eosin which can lead to staining variability.

10. Wash brains 2 times in PBS for 30 min each at room temperature with gentle agitation.
11. Wash brains 1 time in 0.1% PBT for 10 min at room temperature with gentle agitation.
12. Wash brain 1 time in 0.1% PBT overnight at 4°C with gentle agitation.

⚠ **CRITICAL:** it is important to wash overnight. A shorter wash will not be enough to remove all the excess of eosin.

13. Place the brains on a printed slide in a drop of VECTASHIELD.

Note: Brains can be transferred from the netwell/plate to the slide by aspirating them with a pipette and dispensing them on the slides. Under the scope pick one brain at a time with the

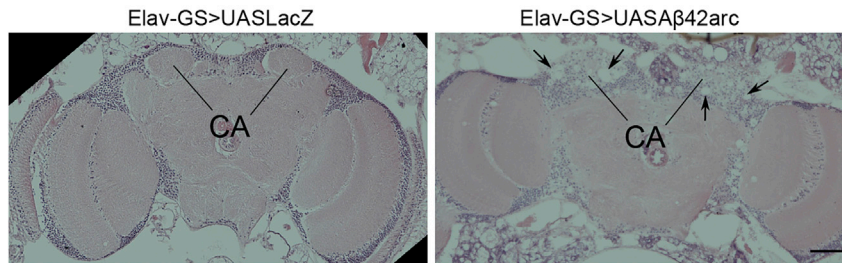


Figure 5. CA region of the fly brain in *Elav-GS>UASLacZ* and *Elav-GS>UASAβ42arc* flies.
Arrows point to vacuoles. Scale bar: 50 μm .

help of a tweezer by lifting it from underneath and place it in a drop of mounting media in the desired well.

Note: It is suggested that the brains are placed on a printed slide so the coverslip does not squeeze the brains.

Alternatives: As an alternative to the printed slides, bridges on the slides can be made with 2–3 layers of transparent tape or coverslips.

Note: Orient the brains in the desired way. In this protocol, the back of the brain is facing the coverslip, so to start imaging from back region close to the Calyx of the Mushroom Body (CA) region (Figure 2B).

14. Add coverslip and seal with nail polish.

▣ **Pause point:** mounted slides can be kept covered at 4°C for at least 4 months prior to imaging.

Imaging

⌚ **Timing:** 2 min per brain

15. Localize CA area in the brain in the confocal microscope (Figures 2B).
16. Image brains using the 488 nm laser and acquire the images with 1024 × 1024 pixels and 8-bit resolution (for optimal software readout). Other parameters such as laser power, averaging, zoom may vary.
17. Use 40× magnification to better visualize the vacuoles and other brain structures.

Note: Depending on the microscope and on the tissue the imaging settings may vary. Adjustment to the images can be done later in Fiji (ex. Convert to 8-bit, reduce background).

Note: Using this fly strain, we noticed that the vacuoles are concentrated in the areas where the Mushroom Body Kenyon cells are located, at the Calyx (CA), close to the surface of the back of the brain (Figures 2 and 5). Thus, we took a single snapshot at the CA region to perform vacuoles analysis (Figures 6 and 7).

⚠ **CRITICAL:** If using a confocal microscope, it is important to orient the brains properly on the slide while mounting because the laser does not have much penetrance. The deeper it gets, the lower the fluorescence intensity will be, meaning only the first half of the

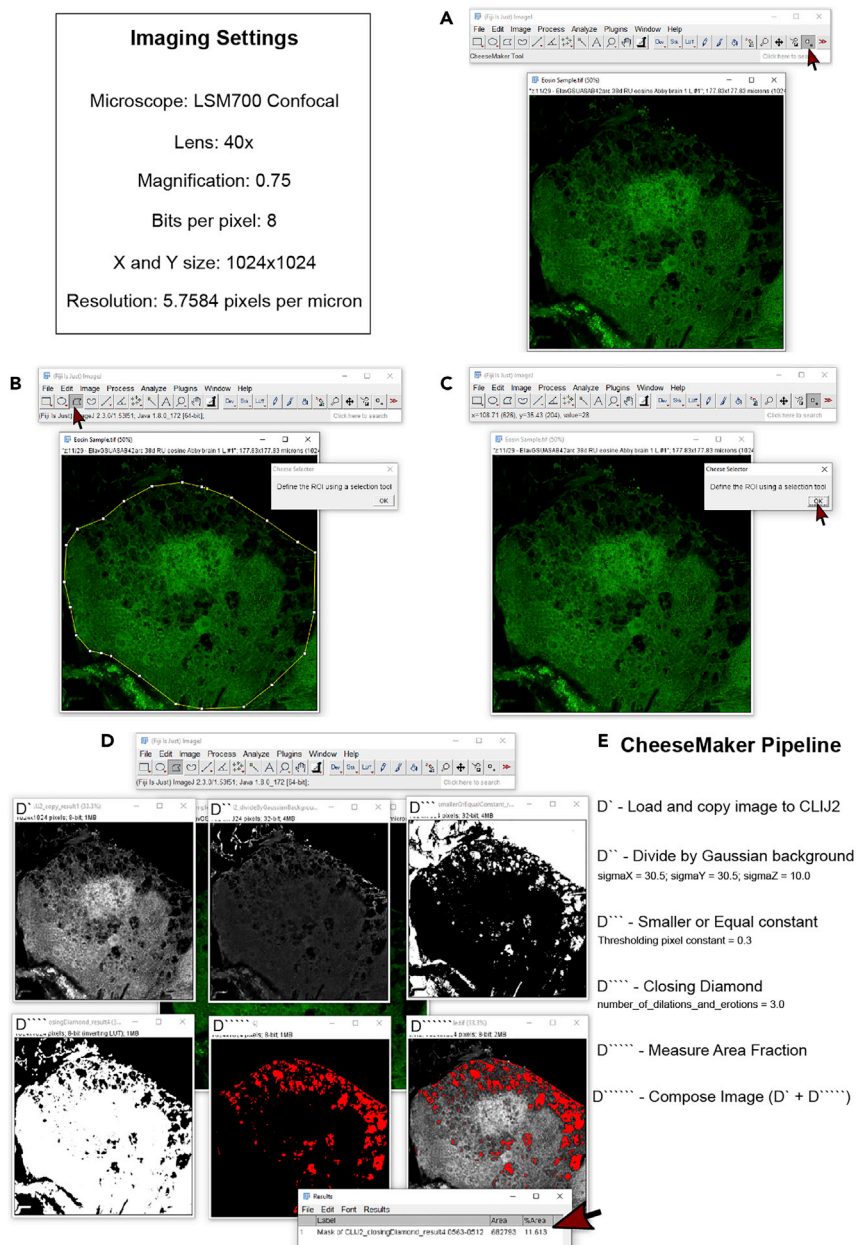


Figure 6. Image analysis using CheeseMaker tool in Fiji

- (A) Open image in Fiji after CheeseMaker tool installation and click on CheeseMaker Tool button (red arrow).
- (B) Select an ROI tool (red arrow) and draw the ROI in the image.
- (C) Hit ok in the Cheese Selector window (red arrow).
- (D) CheeseMaker will process the images and give the Area Fraction in the Results window (red arrow).
- (E) Pipeline of image analysis in Fiji.

brain will be good to be imaged (Figure 2D). Thus, using this approach, the CA region is closer to the coverslip and a single snapshot can be taken without any problems of laser penetration. However, if desirable to acquire a z-stack, other settings can be used to acquire images deeper in the tissue, such as laser strength, signal detection, z-correction and gain.

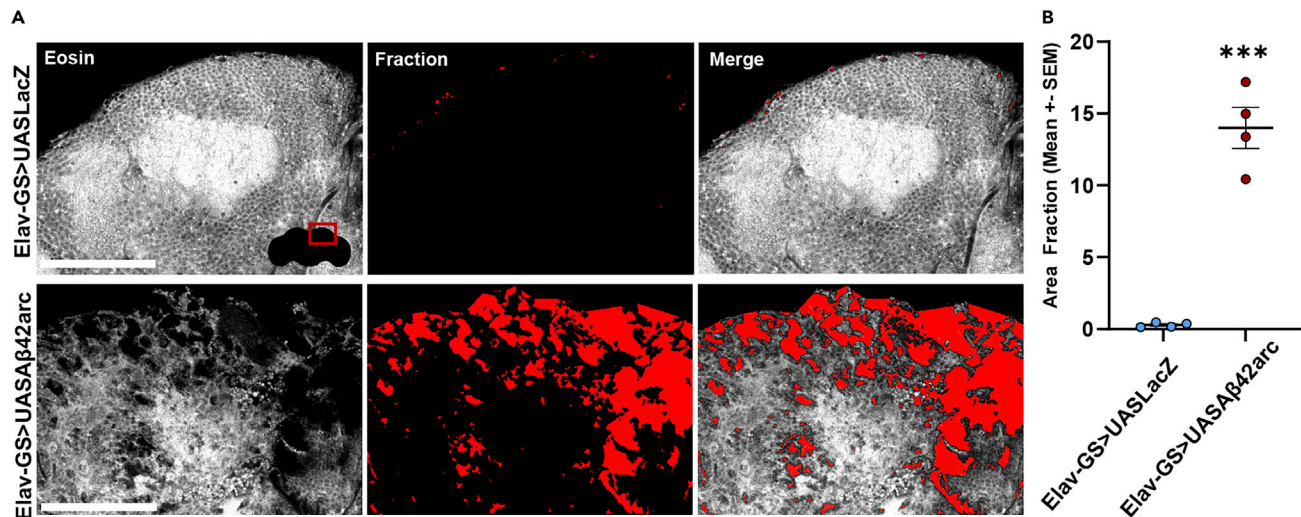


Figure 7. Image quantification with Fiji and CheeseMaker Tool

(A) Representative images of control and disease brains after 50 days on RU-486 food. First panel shows eosin staining, second shows area fraction quantified by CheeseMaker tool, and third one is a merge. Scale bar: 50 μ m.

(B) Graph showing quantification of area fraction (%) (mean \pm standard error of the mean). T-test was applied (Elav-GS>UASLacZ: n = 4; Elav-GS>UAS β 42arc n = 4; p < 0.0001).

Install Fiji/CheeseMaker Tool

⌚ Timing: 5 min

We have developed a plugin in ImageJ/Fiji to assist in the image analysis. CheeseMaker is a non-analytical ImageJ/Fiji tool to quantify the relative area occupied by the non-eosin-stained tissue (vacuoles) on a manually selected area of interest of a single confocal section of the *Drosophila* brain stained with eosin (Figure 6).

18. Download and Install Fiji: <https://imagej.net/software/fiji/downloads>.
19. Update Fiji and install plugins:
 - a. Click in the main menu: Help>Update...>Manage Update Sites.
 - b. In this window click the checkboxes of the following plugins:
 - i. 3D ImageJ Suite.
 - ii. BoneJ.
 - iii. clij.
 - iv. clij2.
 - v. clij-assistant.
 - vi. clij-assistant-extensions.
 - vii. IJPB-plugins.
 - c. Click the "Close" button on the Manage Update Sites window.
 - d. Click the "Apply changes" button on ImageJ Updater window.
20. Download the CheeseMaker Tool from the GitHub repository.
 - a. Open GitHub link: <https://github.com/gbarbosabio/CheeseMaker>.
 - b. Click green "Code" button and click "Download ZIP" button.
 - c. Download compressed file: "CheeseMaker-main". Decompress downloaded file.
 - d. Open Fiji, click, Plugin>Install...
 - i. Find decompressed folder CheeseMaker-main.
 - ii. Click file "CheeseMaker.ijm" to open.

- iii. In the window "Save Plugin, Macro or Script", move up one level to the folder "Fiji.App", click on the folder "macros", then "toolsets", and finally click "Save". To complete installation, close Fiji and reopen.
- e. To activate the CheeseMaker tool in the tool bar, click on the toolbar button at the far right: "More Tools" ">>" and click "CheeseMaker". One new button should appear on the Fiji Tools Bar: "CheeseMaker tool" (Figure 6A).

Image analysis using CheeseMaker Tool

⌚ Timing: 1 min/image

This section describes how to quantify vacuoles using CheeseMaker Tool in a single image (Figure 6).

21. Open image in Fiji.
22. Click on the CheeseMaker Tool button located at the Toolbar (Figure 6A).
23. Click on the image to be analyzed. A window will pop-up saying "Define the ROI using a selection tool" (Figure 6B).
24. Click on the desired selection tool from the Toolbar and define a ROI in the image (Figure 3B).
25. Click "Ok" on the Cheese Selector window (Figure 6C).
26. The CheeseMaker tool will process and analyze the image in the defined ROI and show all the steps in different windows including a Results window (Figure 6D).
27. Save the results and the images of interest.
28. Close all opened images.
29. Open a new image and repeat steps 2 through 6 in this section.

Note: Before using the CheeseMaker tool, make sure images are converted to 8-bit (Image>Type>8 bit) if they were acquired using other bits per pixel settings.

Note: Once the CheeseMaker Tool is activated in ImageJ, the user selects an area of interest, and the tool will segment the selected area into two categories: with and without eosin staining. The segmentation steps involve: 1- Thresholding the pixels which the ratio of staining signal/Gaussian background ($\sigma_{max}=30.5$, $\sigma_{may}=30.5$, $\sigma_{maz}=10.0$) signal are below 0.3 (Figure 6D'''). This will segment pixels which contain no eosin staining or very low signal in relationship to the nearby background. 2- Three rounds of dilation and erosion (Morphological Closing) (Legland et al., 2016) are applied to every thresholded pixel with low eosin staining (Figure 6D'''). This process considers a vacuole 5 nearby pixels in the shape of a cross that are black; it removes isolated non-black pixels and removes any deformities on the edges of the tissue by closing narrow gaps between proximal thresholded areas. CheeseMaker Tool is assisted by a pipeline generated with CLIJ2 (Haase et al., 2019) and estimates the Area Fraction (percentage) occupied by the vacuoles inside a Region of Interest (ROI) (Figure 6D). This tool does not analyze morphological aspects of the vacuoles (such as size, area or shape).

EXPECTED OUTCOMES

In this protocol, we overexpressed A β 42arc in all neurons in adults using the Elav-GS driver. We then stained whole-brain mounts with plain eosin. Eosin binds and stains proteins and vacuoles are recognized by the lack of or very faint eosin stain. Old flies at 50 days on RU-486 food have prominent and numerous vacuoles, and the brains have a spongy appearance (Figure 7). This protocol facilitates vacuole visualization since conventional H&E staining requires prior embedding and sectioning of the samples (Iijima et al., 2008; Maksoud et al., 2019; Sekiya et al., 2017; Sunderhaus and Kretzschmar, 2016), which is time consuming and prone to generate deformities in the tissue and tilted brains (Figures 1B–1D). Moreover, this protocol is faster and cheaper than using phalloidin staining

followed by 2-photon microscopy to visualize vacuoles (Behnke et al., 2021). Moreover, while phalloidin is superior to the conventional H&E staining in terms of speed, it is significantly more expensive.

We generated the CheeseMaker tool to be used in Fiji to facilitate image analysis. This tool processes the images, and measures the area fraction, which appear in the Results window (Figure 6D). It also generates an image in gray scale of the original image with the vacuoles highlighted in red, as well as all the images generated during the image processing (Figure 6D).

Finally, brain modules have been previously studied by using a neuronal driver combined with a UAS-GFP reporter (Chakraborty et al., 2011; Iijima et al., 2008). In the context of diseases, brain modules show neuroanatomical and morphological defects (Chakraborty et al., 2011; Iijima et al., 2008). In this protocol, it is also possible to visualize brain modules with the advantage of using a straightforward staining and having no need of a reporter line, making the genetics easier. The modules are well preserved, and the researcher can assess their anatomy and morphology (Figure 3).

LIMITATIONS

Although this protocol aims to improve the laborious process of conventional H&E staining for vacuole visualization, it has some limitations. First, eosin stain cannot be combined with other staining, as its fluorescence bleeds through several channels. If necessary, a previous protocol has reported the usage of phalloidin staining to visualize vacuoles and that can be used in combination with other markers (Behnke et al., 2021).

Second, confocal microscopy does not have enough depth to penetrate the whole-brain mount to image the whole tissue. We recommend mounting the brains with the surface of interest close to the cover slip to overcome this challenge or to try another more powerful imaging technique such as two-photon microscopy.

Third, the CheeseMaker Tool was developed to process the images and analyze the Area Fraction occupied by the vacuoles in a defined ROI in a single image. Moreover, it does not distinguish between vacuoles and other structures that may lack eosin stain, such as trachea and physiological vacuoles (“holes”). To avoid the later, it is recommended that the image acquisition is at an area with very few or no physiological “holes”, or those can be excluded when defining an ROI for the CheeseMaker analysis.

Fourth, the CheeseMaker tool is an automatic processing, which can fail to detect very small vacuoles or vacuoles that are not completely clear of eosin staining. To adjust this, an extra step needs to be done which consist of adjusting the brightness and contrast to highlight the vacuoles (see [problem 3](#) of [troubleshooting](#) section).

Finally, the CheeseMaker tool does not currently allow manual inclusion or exclusion of vacuoles, but changes to the source code can be done to improve image analysis.

TROUBLESHOOTING

Problem 1

High background levels.

High background levels may arise if eosin is not completely removed from the sample (step 12). This will create increased background levels and poor sample quality with eosin leaking out of the tissue in the mounted slide (Figure 8).

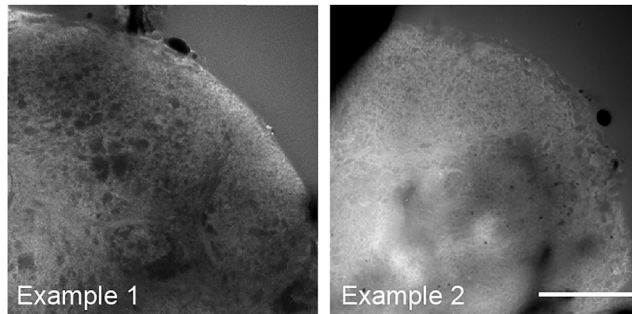


Figure 8. Two representative images of brains that were not completely washed after eosin staining

A background eosin staining can be seen outside the brain outline. Scale bar: 50 μm .

Potential solution

To overcome this problem, increase the number of washes or the time of each wash. Generally, an overnight wash should be enough to remove all the excess stain. If not, increase it until no eosin is seen leaking out from the brain on the mounted slide.

Problem 2

Fragile brains.

Old and sick/diseased flies have fragile brains and are more difficult to dissect without damaging the tissue. Tears in the tissue can result in increased false positives when assessing vacuoles.

Potential solution

To minimize this problem, fix the heads without the proboscis in 4% PFA before brain dissections. A longer incubation may be necessary to improve the fixation. Apply the same fixation time to all the samples of the same experiment as excess fixative can increase background fluorescence. Excess background may be removed while imaging by adjusting the offset levels in the imaging software, or later in Fiji before running the CheeseMaker Tool.

Problem 3

CheeseMaker Tool is not recognizing vacuoles properly.

The CheeseMaker Tool may fail to recognize small vacuoles or vacuoles that are not completely clear of eosin staining, or that have a brighter background (Figure 9; step 26). The CheeseMaker tool considers a vacuole a region in which 5 pixels in a shape of a cross are black. Isolated black pixels will not be considered as a vacuole.

Potential solution

The plugin will fail to recognize vacuoles when the background inside the vacuole is not black (Figure 10C). To solve that, first, it is important to optimize the staining and the imaging protocol to perform the eosin staining as equal as possible throughout the samples. Staining all the samples at the same time with the help of netwells and a plate (Figure 4) can help. Second, image acquisition must be adjusted for each experiment (or even each sample depending on the quality of the staining) to maximize software readout. Third, if the image still has a lot of background, the following settings can be applied before using the CheeseMaker Tool (Figure 10B): with the image open in Fiji, go to LUT>HiLo. Then adjust the brightness and contrast till vacuoles appear mostly in blue, then click apply (Figures 10D–10F). Run the CheeseMaker tool as usual (Figures 10G–10J). Close attention needs to be given if necessary to change background levels. Reducing too much background or too little may result in false positives or false negatives, thus, care must be taken when manipulating the image before analysis.

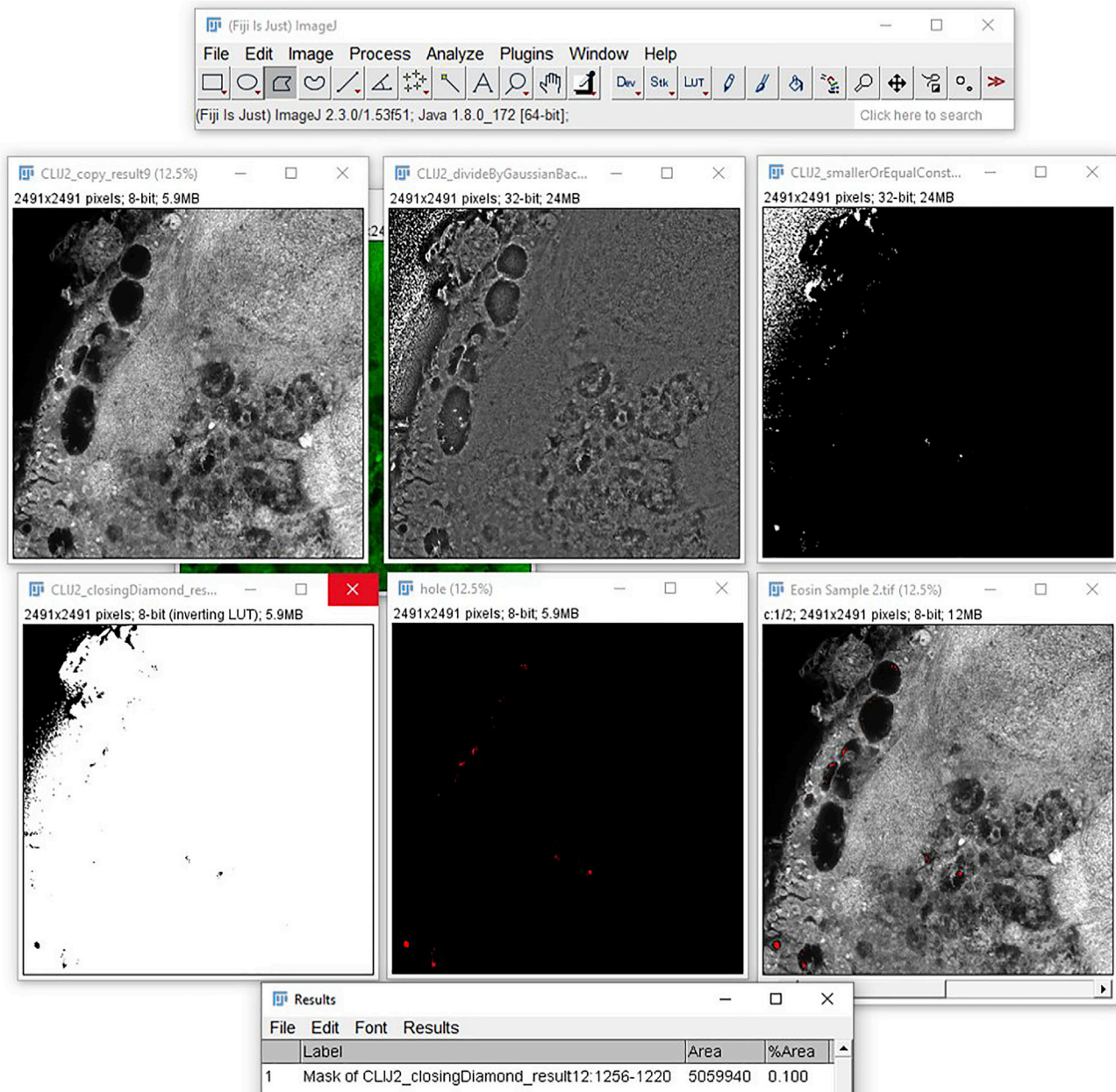


Figure 9. The CheeseMaker Tool not recognizing vacuoles properly

CheeseMaker Tool will process any image using its own pipeline, but it may fail to recognize vacuoles when the background levels inside the vacuoles are too high.

Fourth, a macro could be created by the user using different filters to best represent the vacuoles in the image. Finally, the CheeseMaker Tool source code could be modified by the user to each experiment.

Problem 4

Fiji shows an error message when using the CheeseMaker Tool.

An error message may appear when trying to use the CheeseMaker tool (step 25).

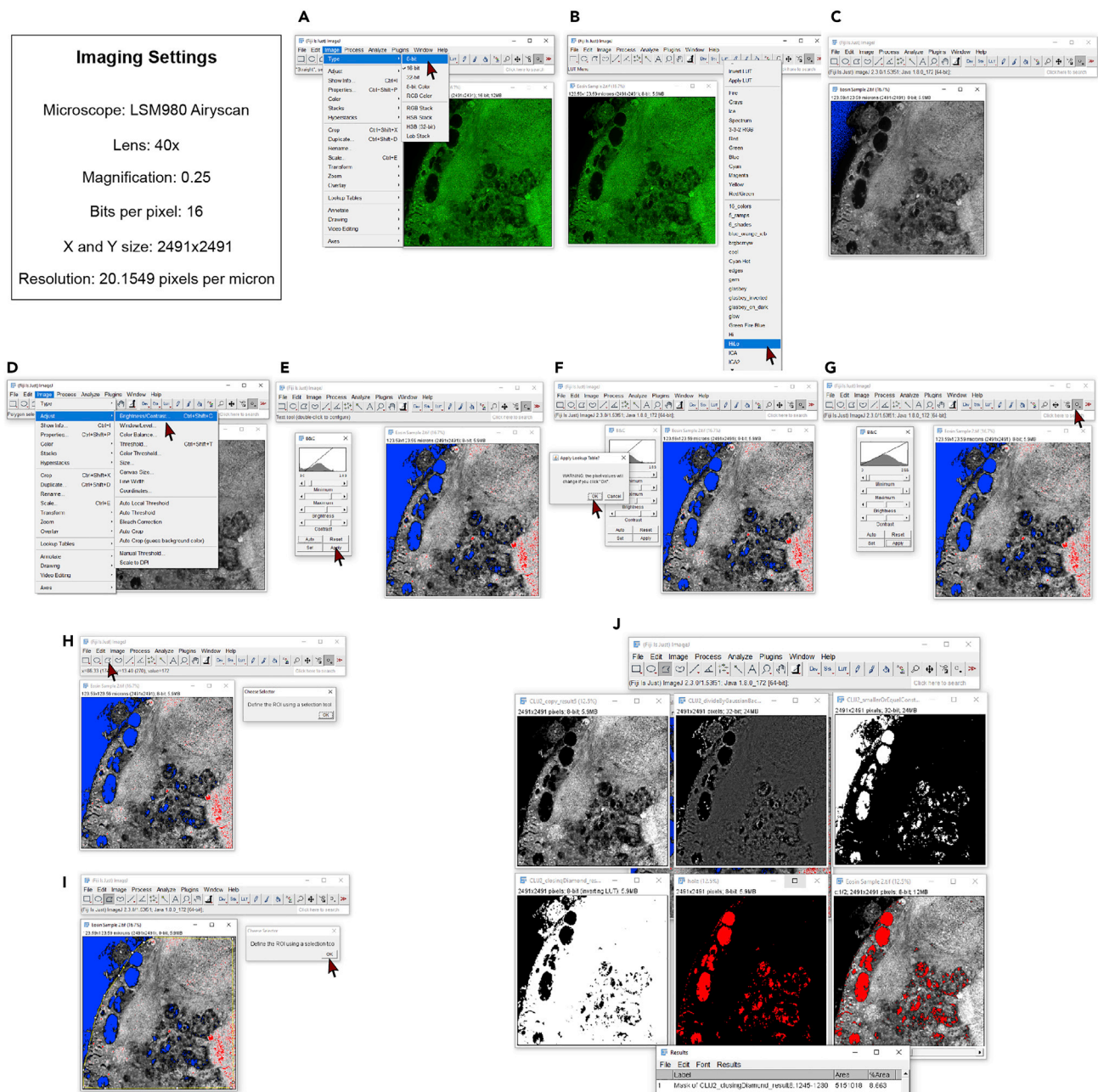


Figure 10. Adjusting background levels to optimize software analysis

- (A) Convert the image to 8-bit.
- (B) Change LUT to HiLo to better visualize the background of the entire image.
- (C) Blue color represents no background. Black color represents low background levels inside the vacuoles.
- (D–F) (D) Adjust the brightness and contrast so that inside the vacuole it becomes blue (E), hit apply and then ok (F).
- (G–J) Run CheeseMaker as usual.

Potential solution

Error messages appear when not all the plugins are installed. Please check that all the following plugins are installed: 3D ImageJ Suite, BoneJ, clij, clij2, clij-assistant, clij-assistant-extensions, IJPB-plugins. If not, install them and restart Fiji.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Pejmun Haghighi (PHaghighi@buckinstitute.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets/code generated during this study are available at <https://github.com/gbarbosabio/CheeseMaker> as well as at Zenodo repository ([Ulían-Benitez et al., 2022](#)).

ACKNOWLEDGMENTS

We thank Glenn Foundation for Medical Research for the fellowship awarded to S.U.B. and NIH for a grant provided to P.H. (R01GM117412). We thank the Morphology Court at the Buck Institute for providing reagents and the confocal microscope ZEISS LSM700 for imaging. We thank Megumi Mori and Jill Farnsworth for testing and validating the Fiji plugin CheeseMaker Tool.

AUTHOR CONTRIBUTIONS

Conceptualization, S.U.B. and G.O.B.; Methodology, S.U.B., A.S.H., and G.O.B.; Investigation, S.U.B.; Project Management, S.U.B.; Software, G.O.B. and S.U.B.; Validation, S.U.B.; Visualization, S.U.B.; Writing - Original Draft, S.U.B.; Writing - Review & Editing, S.U.B., A.S.H., G.O.B., and P.H.; Funding acquisition, S.U.B. and P.H.; Resources, P.H.; Supervision, G.O.B. and P.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Behnke, J.A., Ye, C., Moberg, K.H., and Zheng, J.Q. (2021). A protocol to detect neurodegeneration in *Drosophila melanogaster* whole-brain mounts using advanced microscopy. *STAR Protoc.* 2, 100689. <https://doi.org/10.1016/j.xpro.2021.100689>.
- Bolkan, B.J., and Kretschmar, D. (2014). Loss of tau results in defects in photoreceptor development and progressive neuronal degeneration in *Drosophila*. *Dev. Neurobiol.* 74, 1210–1225. <https://doi.org/10.1002/dneu.22199>.
- Bolus, H., Crocker, K., Boekhoff-Falk, G., and Chtarbanova, S. (2020). Modeling neurodegenerative disorders in *drosophila melanogaster*. *Int. J. Mol. Sci.* 21, 3055. <https://doi.org/10.3390/ijms21093055>.
- Chakraborty, R., Vepuri, V., Mhatre, S.D., Paddock, B.E., Miller, S., Michelson, S.J., Delvadia, R., Desai, A., Vinokur, M., Melicharek, D.J., et al. (2011). Characterization of a *drosophila* Alzheimer's disease model: pharmacological rescue of cognitive defects. *PLoS ONE* 6, e20799. <https://doi.org/10.1371/JOURNAL.PONE.0020799>.
- Coelho, D.S., Schwartz, S., Merino, M.M., Hauert, B., Topfel, B., Tiede, C., Rhiner, C., and Moreno, E. (2018). Culling less fit neurons protects against amyloid- β -induced brain damage and cognitive and motor decline. *Cell Rep.* 25, 3661–3673.e3. <https://doi.org/10.1016/j.celrep.2018.11.098>.
- Cook, M., Mani, P., Wentzell, J.S., and Kretschmar, D. (2012). Increased RhoA prenylation in the loechrig (loe) mutant leads to progressive neurodegeneration. *PLoS ONE* 7, e44440. <https://doi.org/10.1371/journal.pone.0044440>.
- Coombe, P.E., and Heisenberg, M. (1986). The structural brain mutant vacuolar medulla of *drosophila melanogaster* with specific behavioral defects and cell degeneration in the adult. *J. Neurogenet.* 3, 135–158. <https://doi.org/10.3109/01677068609106845>.
- Da Cruz, A.B., Wentzell, J., and Kretschmar, D. (2008). Swiss cheese, a protein involved in progressive neurodegeneration, acts as a noncanonical regulatory subunit for PKA-C3. *J. Neurosci.* 28, 10885–10892. <https://doi.org/10.1523/JNEUROSCI.3015-08.2008>.
- Haase, R., Royer, L.A., Steinbach, P., Schmidt, D., Dibrov, A., Schmidt, U., Weigert, M., Maghelli, N., Tomancak, P., Jug, F., and Myers, E.W. (2019). CLIJ: GPU-accelerated image processing for everyone. *Nat. Methods* 17, 5–6. <https://doi.org/10.1038/s41592-019-0650-1>.
- Hegde, V.R., Vogel, R., and Feany, M.B. (2014). Glia are critical for the neuropathology of complex I deficiency in *Drosophila*. *Hum. Mol. Genet.* 23, 4686–4692. <https://doi.org/10.1093/HMG/DDU188>.
- Heisenberg, H.M., and Böhl, K. (1979). Isolation of anatomical brain mutants of *Drosophila* by histological means. *Zeitschrift Naturforschung C* 34, 143–147. <https://doi.org/10.1515/znc-1979-1-228>.
- Herman, M.M., Miquel, J., and Johnson, M. (1971). Insect brain as a model for the study of aging - age-related changes in *Drosophila Melanogaster*. *Acta Neuropathol.* 19, 167–183. <https://doi.org/10.1007/BF00684595>.
- Hill, C.S., Sreedharan, J., Loreto, A., Menon, D.K., and Coleman, M.P. (2020). Loss of highwire protects against the deleterious effects of traumatic brain injury in *Drosophila melanogaster*. *Front. Neurol.* 11, 401. <https://doi.org/10.3389/fneur.2020.00401>.
- Iijima, K., Chiang, H.C., Hearn, S.A., Hakker, I., Gatt, A., Shenton, C., Granger, L., Leung, A., Iijima-Ando, K., and Zhong, Y. (2008). A β 2 mutants with different aggregation profiles induce distinct pathologies in *Drosophila*. *PLoS ONE* 3, e1703. <https://doi.org/10.1371/journal.pone.0001703>.
- Legland, D., Arganda-Carreras, I., and Andrey, P. (2016). MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ. *Bioinformatics* 32, 3532–3534. <https://doi.org/10.1093/BIOINFORMATICS/BTW413>.
- Loewen, C.A., and Ganetzky, B. (2018). Mitochondrial interactions affecting lifespan and neurodegeneration in a *Drosophila* model of leigh syndrome. *Genetics* 208, 1535–1552. <https://doi.org/10.1534/GENETICS.118.300818>.

- Maksoud, E., Liao, E.H., and Haghghi, A.P. (2019). A neuron-glia trans-signaling cascade mediates LRRK2-induced neurodegeneration. *Cell Rep.* 26, 1774–1786.e4. <https://doi.org/10.1016/j.celrep.2019.01.077>.
- Osterwalder, T., Yoon, K.S., White, B.H., and Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. *Proc. Natl. Acad. Sci.* 98, 12596–12601. <https://doi.org/10.1073/PNAS.221303298>.
- Petersen, A.J., Katzenberger, R.J., and Wassarman, D.A. (2013). The innate immune response transcription factor relish is necessary for neurodegeneration in a *Drosophila* model of ataxia-telangiectasia. *Genetics* 194, 133–142. <https://doi.org/10.1534/GENETICS.113.150854>.
- Ray, A., Speese, S.D., and Logan, M.A. (2017). Glial draper rescues A β toxicity in a *Drosophila* model of Alzheimer's disease. *J. Neurosci.* 37, 11881–11893. <https://doi.org/10.1523/JNEUROSCI.0862-17.2017>.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. <https://doi.org/10.1038/nmeth.2019>.
- Sekiya, M., Maruko-Otake, A., Hearn, S., Sakakibara, Y., Fujisaki, N., Suzuki, E., Ando, K., and Iijima, K.M. (2017). EDEM function in ERAD protects against chronic ER proteinopathy and age-related physiological decline in *Drosophila*. *Dev. Cell* 41, 652–664.e5. <https://doi.org/10.1016/J.DEVCEL.2017.05.019>.
- Sunderhaus, E.R., and Kretschmar, D. (2016). Mass histology to quantify neurodegeneration in *drosophila*. *J. Vis. Exp.* 54809. <https://doi.org/10.3791/54809>.
- Ulian-Benitez, S., Hjelmstad, A.S., Barbosa, G.O., and Haghghi, P. (2022). Gbarbosabio/CheeseMaker: CheeseMaker. <https://doi.org/10.5281/ZENODO.5819097>.
- 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. <https://doi.org/10.1126/SCIENCE.1062382>.