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Abstract:	Progressive neurodegenerative diseases like Alzheimer's disease or Parkinson's disease are an increasing threat to human health worldwide. Although mammalian models have provided important insights into the underlying mechanisms of pathogenicity, the complexity of mammalian systems together with the high costs are limiting their use. Therefore the simpler, but well-established Drosophila model provides an alternative for investigating the molecular pathways that are affected in these diseases. Besides behavioral deficits, neurodegenerative diseases are characterized by histological phenotypes like neuronal death and axonopathy. To quantify neuronal degeneration and to determine how it is affected by genetic and environmental factors, we are using a histological approach that is based on measuring vacuoles in adult fly heads. To minimize effects of systematic error and to directly compare sections from control and experimental flies in one preparation, we are using the "collar" method for paraffin sections. Neurodegeneration is then assessed by measuring the size and/or number of vacuoles that have developed in the fly brain. This can either be done by focusing on a specific region of interest or by analyzing the entire brain due to obtaining serial sections that span the complete head. Therefore, this method allows one to not only measure severe degeneration but also relatively mild phenotypes that are only detectable in a few sections, as occur during normal aging.
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Cover Letter

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Dear Sephora Zaman,

Please find enclosed our revised manuscript entitled "**Mass histology to quantify neurodegeneration in *Drosophila*"**. We have addressed the editorial and reviewer comments and this is described in detail in the response to reviewers file. Changes in the text are indicated by the track changes option. I hope that our manuscript is now suited for publication in JoVE.

Sincerely,

Doris Kretzschmar

Title: Mass histology to quantify neurodegeneration in *Drosophila*

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Short abstract:

Drosophila is widely used as a model system to study neurodegeneration. This protocol describes a method by which degeneration, as determined by vacuole formation in the brain, can be quantified. It also minimizes effects due to the experimental procedure by processing and sectioning control and experimental flies as one sample.

Long Abstract:

Progressive neurodegenerative diseases like Alzheimer's disease or Parkinson's disease are an increasing threat to human health worldwide. Although mammalian models have provided important insights into the underlying mechanisms of pathogenicity, the complexity of mammalian systems together with their high costs are limiting their use. Therefore, the simple but well-established *Drosophila* model provides an alternative for investigating the molecular pathways that are affected in these diseases. Besides behavioral deficits, neurodegenerative diseases are characterized by histological phenotypes such as neuronal death and axonopathy. To quantify neuronal degeneration and to determine how it is affected by genetic and environmental factors, we use a histological approach that is based on measuring the vacuoles in adult fly heads. To minimize the effects of systematic error and to directly compare sections from control and experimental flies in one preparation, we use the "collar" method for paraffin sections. Neurodegeneration is then assessed by measuring the size and/or number of vacuoles that have developed in the fly brain. This can either be done by focusing on a specific region of interest or by analyzing the entire brain by obtaining serial sections that span the complete head. Therefore, this method allows one to measure not only severe degeneration but also relatively mild phenotypes that are only detectable in a few sections, as occurs during normal aging.

Introduction:

With the increase in life expectancy, neurodegenerative diseases like Alzheimer's or Parkinson's have become an increasing health threat for the general population. According to the National Institutes of Health, 115 million people worldwide are predicted to be affected by dementia in 2050. Although significant progress has been made in identifying genes and risk factors involved in at least some of these diseases, for many of them, the underlying molecular mechanisms are still unknown or not well understood.

Simple invertebrate model organisms like *Caenorhabditis elegans* and *Drosophila melanogaster* offer a variety of experimental advantages to study the mechanisms of neurodegenerative diseases, including a short life cycle, large number of progeny, and the availability of well-established and sometimes unique genetic and molecular methods[1-12]. Furthermore, these organisms are amenable to unbiased interaction screens that can identify factors contributing to these diseases by their aggravating or ameliorating effects on neurodegenerative phenotypes.

Analyzing such genetic interactions and assessing aging effects requires quantitative protocols to detect neurodegeneration and to measure its severity. This assessment can be done relatively easily when measuring behavioral aspects in *Drosophila*, such as olfactory learning, negative geotaxis, or fast phototaxis, which provide a numeric performance value[13-21]. It is also possible to determine the effects on neuronal survival by counting neurons. However, this is only possible when focusing on a specific population that is clearly identifiable, like the dopaminergic neurons that are affected in Parkinson's disease, and even then, the results have been controversial[22-24].

The protocol described here uses the collar method to perform paraffin serial sections, a method that was originally developed by Heisenberg and Böhl, who used it to isolate anatomical brain mutants in *Drosophila*[25]. The use of the collar method has subsequently been adapted, including in cryosections, vibratome sections, and plastic sections[26-28]. Here, this method is employed to obtain serial sections of the entire fly head, which can then be used to measure the vacuoles that develop in flies with neurodegenerative phenotypes[16,21,29-32]. These measurements can be done in specific brain areas or can cover the entire brain; the latter approach allows one to identify even weak degenerative phenotypes, as observed during aging. Finally, when using the collars, up to 20 flies can be processed as one preparation, which is not only less time-consuming, but also allows for the analysis of control and experimental flies in the same preparation, minimizing artifacts due to slight changes in the preparation.

Protocol:

1. Fixing the Head on Collars and Embedding in Paraffin

Note: All of the steps in the fixation process should be done in a fume hood. Methylbenzoate, while not posing a health risk, has a highly distinct odor, which can be overwhelming if not handled in a fume hood.

1.1   Before anesthetizing the flies, make up 50 mL of Carnoy solution by adding 15 mL of chloroform and 5 mL of glacial acetic acid to 30 mL of 99% ethanol (do not mix the chloroform and acetic acid). Pour it in a glass container with a flat bottom, such as a crystalizing dish, to ensure that the collars can lay flat and are completely covered by the solution.

1.2   Anesthetize the flies with CO_2 or ether.

1.3   Thread flies (up to 20 with most collars) by their necks into the collars using forceps. Remember to align all of the heads in the same orientation, as seen in Figure 1A, and be gentle to ensure that no damage occurs to the head or eyes.

1.4   Include *sine oculis* flies (arrows, Figure 1A) at random positions so that the order of the flies can be easily identified in the sections. In addition, if the experimental flies have a light or white eye color, thread some red-eyed flies, such as wild type, between them to ensure that sufficient pigment is present to stain the slide. Record the order of the flies on a protocol sheet together with the collar number if using more than one collar.

1.5   Once a collar has been finished, place it in the prepared Carnoy solution for 3.5-4 h.

1.6   Dump out the Carnoy solution into the appropriate disposal canister and begin the ethanol washes. Make sure to pour slowly so as not to disturb the placement of the collars in the container.

1.7   Wash the collars for 30 min in 99% ethanol twice.

1.8   Wash the collars in 100% ethanol for 1 h. Be sure to change the washes on time to prevent over-dehydration.

1.9   Put the collars in methylbenzoate overnight at room temperature. Seal the container with parafilm to prevent the evaporation of the methyl benzoate.

1.10   Pour the methylbenozate into the proper disposable container in the fume hood. Add a previously-prepared mixture of 1:1 low melting point (56-57 °C) paraffin wax and methylbenzoate. From this point on, the collars need to be kept in an incubator at 65 °C to make sure that the paraffin does not harden.

1.11   Pour out the methylbenozate and paraffin mixture into the proper disposable container, and pour molten pure paraffin wax, kept at 65 °C, onto the collars.

1.12   Change the paraffin after 30 min and repeat this at least five times. At least 6 to 8 washes should be performed.

1.13   Once the washes are complete, place the collars into a rubber ice cube tray with slots approximately the size of the collars. Pour molten paraffin over them until completely covered and allow it to harden overnight (try to avoid air bubbles).

1.14   Remove the paraffin blocks containing the collars from the ice cube tray. Separate the paraffin block from the collar using a razor blade, gently breaking off the collar. The heads will be in the paraffin block while the bodies will stay in the collar. The blocks can be kept at room temperature.

1.15   To clean the collars, soak them in a deparafinization agent at 65 °C to remove the paraffin, clean with light scrubbing, and wash in ethanol before reusing.

2. Sectioning and Mounting

2.1   Warm a heating plate to 50 °C. Place the object holders (or metal mounting blocks) and razor blades on the plate and let them warm up.

2.2   Depending on the desired orientation for sectioning, attach the paraffin block either with the heads towards the side (for horizontal sections) or facing upwards (for frontal sections) to a heated mounting block (briefly melting the block at the contact side). Remove the block from the heating plate and allow it to cool for at least 10 min to ensure that the paraffin is hardened enough for a proper seal onto the mounting block. Keep the row of heads aligned in parallel with the surface of the block as much as possible to prevent uneven sections.

2.3   Take a razor blade and trim the excess paraffin away from the fly heads so that only a small row with the embedded heads remains (the razor blade can be warmed up for easier trimming). Make sure not to trim too much so that the paraffin does not break during sectioning (more trimming can be done during sectioning).

2.4   Place the mounting block into the object holder of the microtome and ensure that the alignment of the row of heads is as parallel as possible to the edge of the blade.

2.5   Prepare microscope slides by covering them with a thin layer of poly-lysine solution and let them dry for 5 min. Cover them with water shortly before use.

2.6   Cut 7-μm sections and transfer the ribbon of sections to the slide floating on the water.
Note: To obtain the entire brain for horizontal sections, we collect the ribbon from when starting to cut into the eye until the head has been completely cut (cutting from the proboscis into the brain). More than one slide may be needed for the entire head.

2.7   Place the slide on a heat plate at 37 °C and allow the ribbon to expand for about 1 min.

2.8   Remove excess water (by pouring it off or using a tissue) and dry the slides overnight.

2.9   Remove the paraffin wax from the slide by placing the slides in a tall, vertical slide-staining jar filled with a deparafinization agent (completely covering the sections). Perform three washes for 30 min to 1 h each.

3. Photographing and Analyzing the Sections

3.1   Allow the prepared slides to dry for 1-2 days. Then, examine them on a fluorescence microscope under blue light.

3.2   Use a lower magnification to determine the orientation of the flies and to find the region of interest if focusing on a specific region.

Note: For the *sws* flies (see Figure 2), we find the section that contains the great commissure and take an image (usually at 40X magnification). When analyzing the entire brain (as in Figure 3), we scroll through all the sections from a head and either photograph the section with the most severe phenotype or all sections that show vacuoles.

3.3   For a double-blind analysis, take and number images without knowing the genotype and record the collar number and position of the head in the row to identify them later.

3.4   Once the images have been taken, analyze them using an imaging software.

3.5   Count the number of vacuoles per section or per head. To measure the vacuole size, open the images in a software program and select the vacuoles with a selection tool. Determine the amount of pixels in the selected vacuoles.

3.6   For conversion to μm^2, take a photo of a stage micrometer calibration slide at the magnification used for acquiring photos. Determine the amount of pixels in 100 μm^2 to calculate a conversion factor.

3.7   Convert the total pixel number into μm^2 by dividing the number of pixels by the conversion factor calculated in the above step.

Representative Results:

Using the described method results in serial sections stained by the eye pigment[33] that encompass the entire fly head. A part of this is shown in **Figure 1B**, where the sections from an individual head are shown from top to bottom. The sections from different flies are seen left to right in this example. To facilitate orientation and identification of the flies, an eyeless fly (*sine oculis*) is inserted as a marker at position three (arrow, **Figure 1B**).

        To quantify neurodegeneration, we measure the formation of vacuoles that can be detected in these sections. Vacuoles are defined as round, dark spots that are within the green fluorescent neuropil (arrowheads in **Figure 2** and **3**) or cortex and that are visible in at least two consecutive sections of the fly brain. Quantifying

neurodegeneration by measuring vacuoles can either be done by focusing on a specific brain region or by analyzing the entire brain. Limiting the analysis to a specific region of the brain is useful in cases where a mutation only affects a specific region, like the olfactory lobes in the *futsch^olk* mutant[34], but it can also be used when there is severe degeneration in all or many regions of the brain. An example for the latter is the *swiss cheese (sws)* mutant (**Figure 2**), where measuring all vacuoles would be too time consuming. We therefore took only one image and, to ensure that the measurements were always done at the same level, we took all images at the level of the great commissure (gc, **Figure 2A**), which is only contained in one or two sections. Whereas we did not detect vacuole formation in 1-day-old *sws^1* flies (data not shown), a loss-of-function allele[35], some vacuoles were detectable in 7-day-old *sws^1* flies (arrowheads, **Figure 2A**). Aging the flies to 14 days (**Figure 2B**) and 21 days (**Figure 2C**) further increased this phenotype, showing its progressive nature. Counting the number of vacuoles in the deutocerebral neuropil (dn) using the described method confirmed a significant increase in the number of vacuoles with age. Also, the combined area encompassed by vacuoles was significantly increased with aging (**Figure 2D**).

However, not all mutants show such a severe phenotype as *sws*, and in those cases, differences in degeneration are difficult to determine when focusing on a small area. Similarly, the degeneration that occurs during aging is quite mild (**Figure 3A-C**) and therefore, we analyzed the entire brain when quantifying this phenotype. Determining the sum of all vacuoles in the brain revealed a significant increase with age, and this was also the case when measuring the combined area of these vacuoles (**Figure 3D**).

Figure Legends:

Figure 1. Paraffin serial sections.
A) Using the collar method, experimental and control flies can be processed as one sample by threading them onto one collar. Eyeless *sine oculis* flies are inserted for orientation (arrows). **B)** Schematic showing the orientation of the fly heads in the collar. **C)** In this image, sections from different fly heads are oriented left to right on the slide. From top to bottom on the slide, serial sections from the same fly head can be seen. In this case, a *sine oculis* fly was inserted at position three (arrow). The sections are stained by the fluorescent eye pigment that washes over the sections after cutting. Scale bar in A = 5 mm and in C = 0.5 mm.

Figure 2. Progressive neurodegeneration in the *swiss-cheese* mutant.
Paraffin head section from 7-day- **(A)**, 14-day- **(B)** and 21-day- **(C)** old *sws^1* flies. The arrowheads point to vacuoles that have developed with aging. The age-related degeneration is quantified by counting the number of vacuoles and measuring their combined area **(D)**. The SEM and number of analyzed flies is indicated. The scale bar = 25 μm. ***p < 0.001.

Figure 3. Neurodegeneration occurs with age.
Paraffin head section from 10-day- **(A)**, 30-day- **(B)** and 60-day- **(C)** old wild-type flies. The arrowheads point to vacuoles that have developed in aged flies. The age-related

degeneration is quantified by counting the number of vacuoles and measuring their combined area **(D)**. The SEM and number of analyzed flies is indicated. The scale bar = 25 µm. ***p < 0.001.

Discussion:

The described method provides a means to quantify neurodegeneration in the brain of *Drosophila*. While other methods, like counting a specific cell type, can be used to identify neurodegeneration, the advantage of this method is that it can be applied more generally. Counting cells requires that these cells can be reliably identified using either a specific antibody or the expression of a cell-specific marker, which is not always available. Furthermore, it has been shown that dramatically different results can be obtained with that method[24], presumably due to the conditions used for labelling and for detection. Another method to detect degenerating cells is the use of cell death markers, like anti-activated caspase 3. However this only identifies cells actively undergoing cell death, and after the cells have died, they are no longer detectable. Another advantage of the method proposed here is that no staining is required due to the autofluorescence caused by the eye pigment, which saves time and minimizes artifacts caused by changes in the staining conditions. It is important to note that, when using this method, the flies' eyes have enough pigment to stain the slide. If the eye color is too light, adding some wild-type flies to the collar would be advisable to ensure sufficient and even staining across the slide. An additional advantage of this method is that multiple areas can be examined, even in the same head. Although we do not show the data, we have used these sections to examine neurodegeneration in the retina and glial cell loss in the lamina cortex[30,36]. As described here, this method provides horizontal sections, but by turning the paraffin block by 90° when melting it onto the object holder, one can also obtain frontal sections. Thus, this method is a versatile procedure that allows for the timely and efficient measurement of neurodegeneration in the fly brain. Because the formation of vacuoles has been observed in many fly models of human neurodegenerative diseases, including models for Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and diseases caused by polyglutamine-repeats[16,37-40], this method can be used to quantify neurodegenerative phenotypes in a variety of disease models.

Overall, this protocol is simple and easily completed once the initial setup of the equipment is carried out. Some notes to keep in mind are to carefully time the ethanol washes to avoid over-dehydration of the heads, to carefully trim the paraffin blocks so as not to lose sections or heads, and to not over-expand the ribbon on the water when the slide is on the heat block. If the ribbon is allowed to expand too far, tearing of the fly heads can result and the order of the heads in the ribbon can be lost. In addition, being blind to the genotype while doing the analyses is essential to avoid bias. This is best achieved by having one person preparing the slides and keeping the records while another person is taking the pictures and doing the measurements. One of the limitations of this method is that degeneration of only a few cells will be very difficult to detect. In that case, a specific stain of the affected cell population would be more informative. In addition, this method does not allow one to distinguish between different

kinds of cell death, which requires more specific methods, such as a TUNEL staining to determine apoptotic cell death. Lastly, this method cannot differentiate between cell death and axonal degeneration, which would also be detectable as vacuoles in the neuropil.

As shown in our results, this method can be used to address degeneration in specific brain areas or in the entire brain. In our experience, it is useful to analyze only a specific area when the phenotype is quite strong, even when all brain regions are affected. This significantly reduces the work load and does not affect the outcome. We originally analyzed several areas in the *sws* mutant and observed very similar results in the progression of the phenotype when comparing only one area and when analyzing all areas (data not shown). However, it should be noted that a clearly-identifiable region should be chosen to avoid artifacts due to the analysis of different areas or areas at different levels.

In contrast, in cases where the phenotype is relatively mild, it is better to analyze the entire brain, because the likelihood of finding vacuoles in a specific region is low. For example, this is the case when determining age-related neurodegeneration, as shown in Figure 3, which results in only 4-5 vacuoles in the entire brain in 60-day-old flies. When counting vacuoles in the entire brain, one has to take into account that small vacuoles will only show up in one section, whereas larger ones will extend over several sections. Concerning the latter, the close proximity of adjacent sections when using this method (Figure 1) provides another advantage, because it is relatively easily to determine whether the same vacuole is present in several sections.

In conclusion, this protocol can prove useful for studying many *Drosophila* models of different neurodegenerative diseases. Identifying interacting proteins that ameliorate or aggravate the degenerative phenotype can provide crucial insights about the underlying mechanisms that cause or modify diseases such as Alzheimer's and Parkinson's. In this publication, we use this method to detect degeneration that is progressive, where the animals develop normally but show increasing degeneration during aging. Additionally, this method can also be adapted to determine degeneration that is caused by developmental defects, which should already be present in newly-eclosed flies.

## Disclosures:

The authors have nothing to disclose.

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40   Li, Y. *et al.* A Drosophila model for TDP-43 proteinopathy. *Proc Natl Acad Sci U S A* **107**, 3169-3174, doi:10.1073/pnas.0913602107 (2010).

Excel Spreadsheet- Table of Materials/Equipment
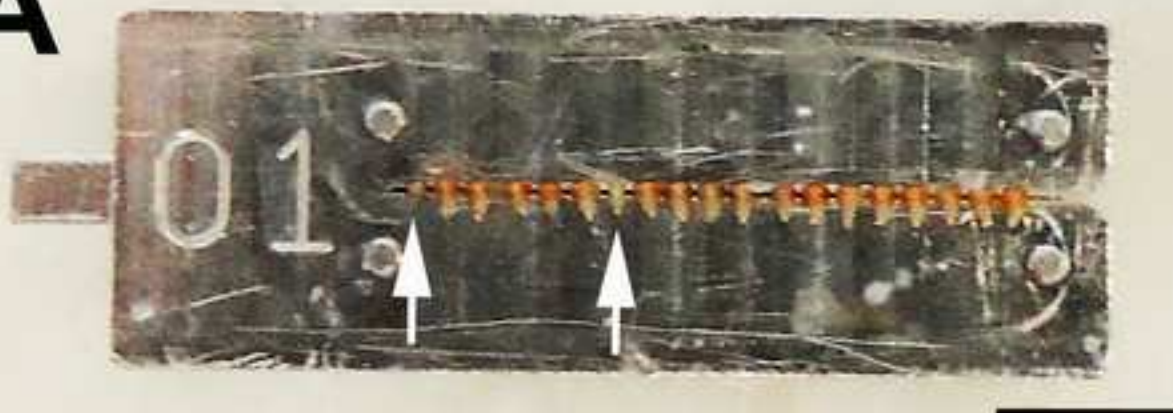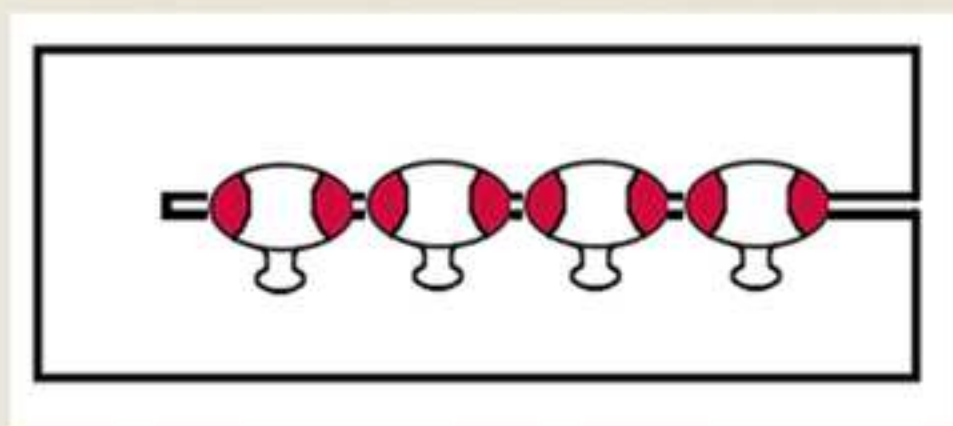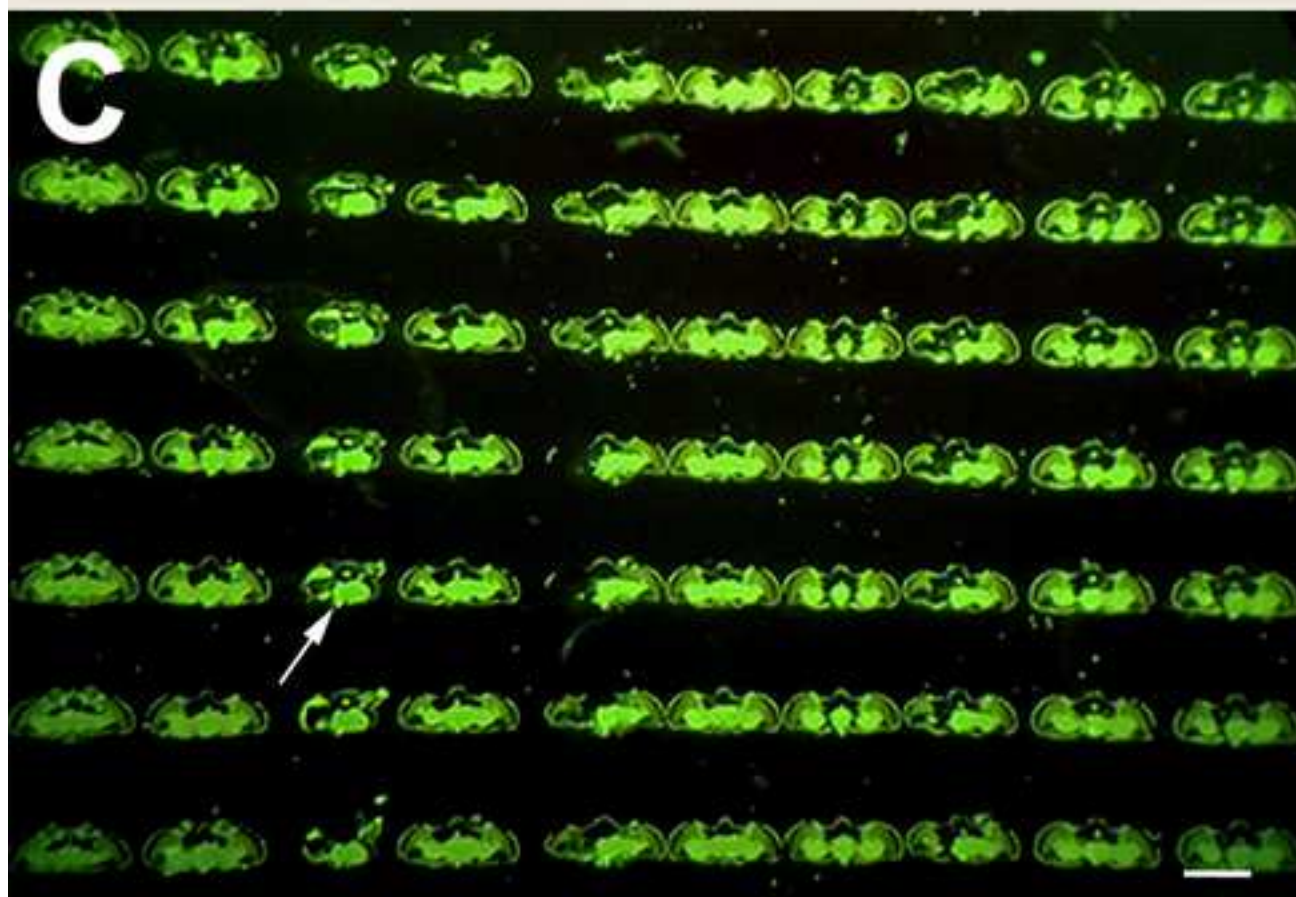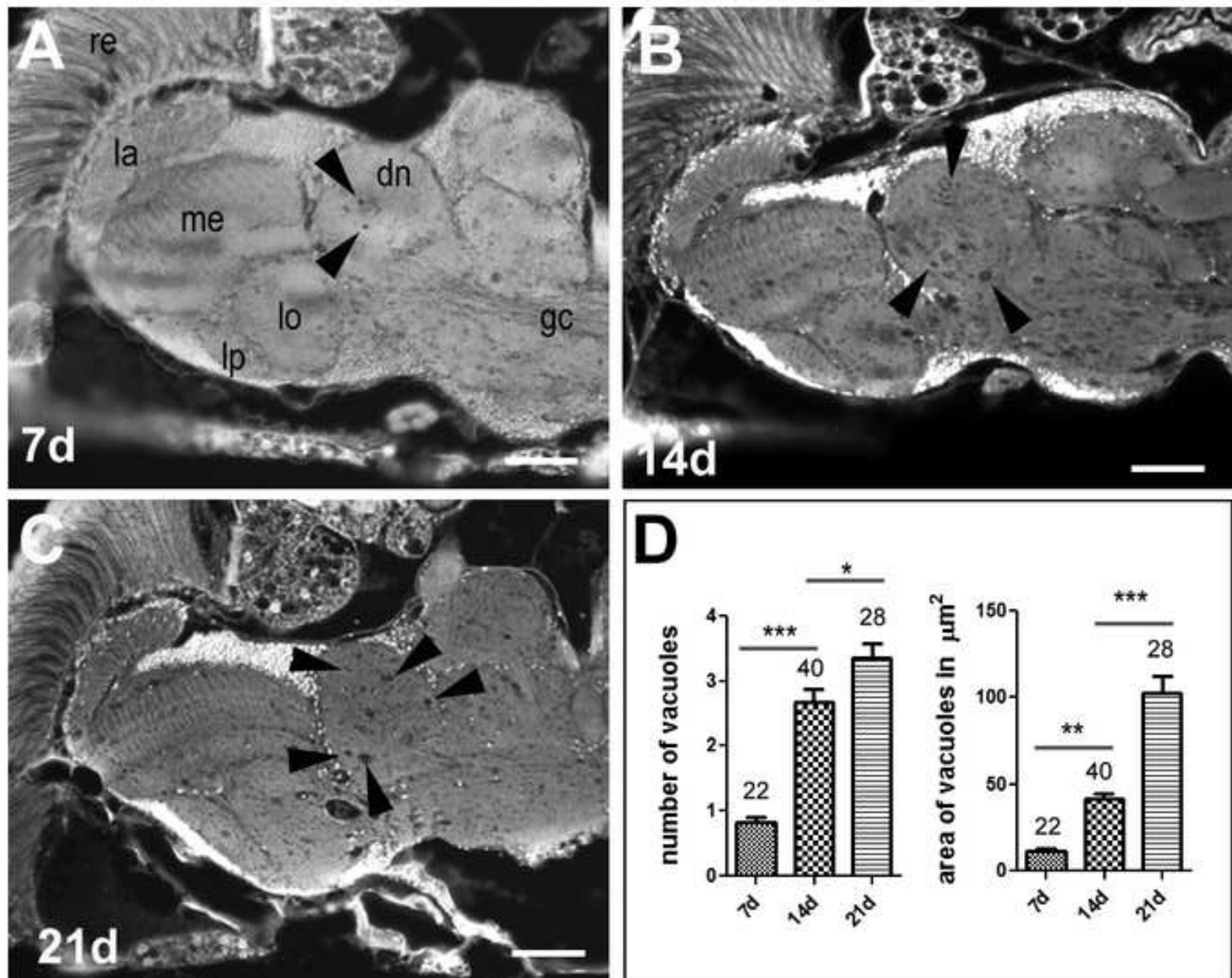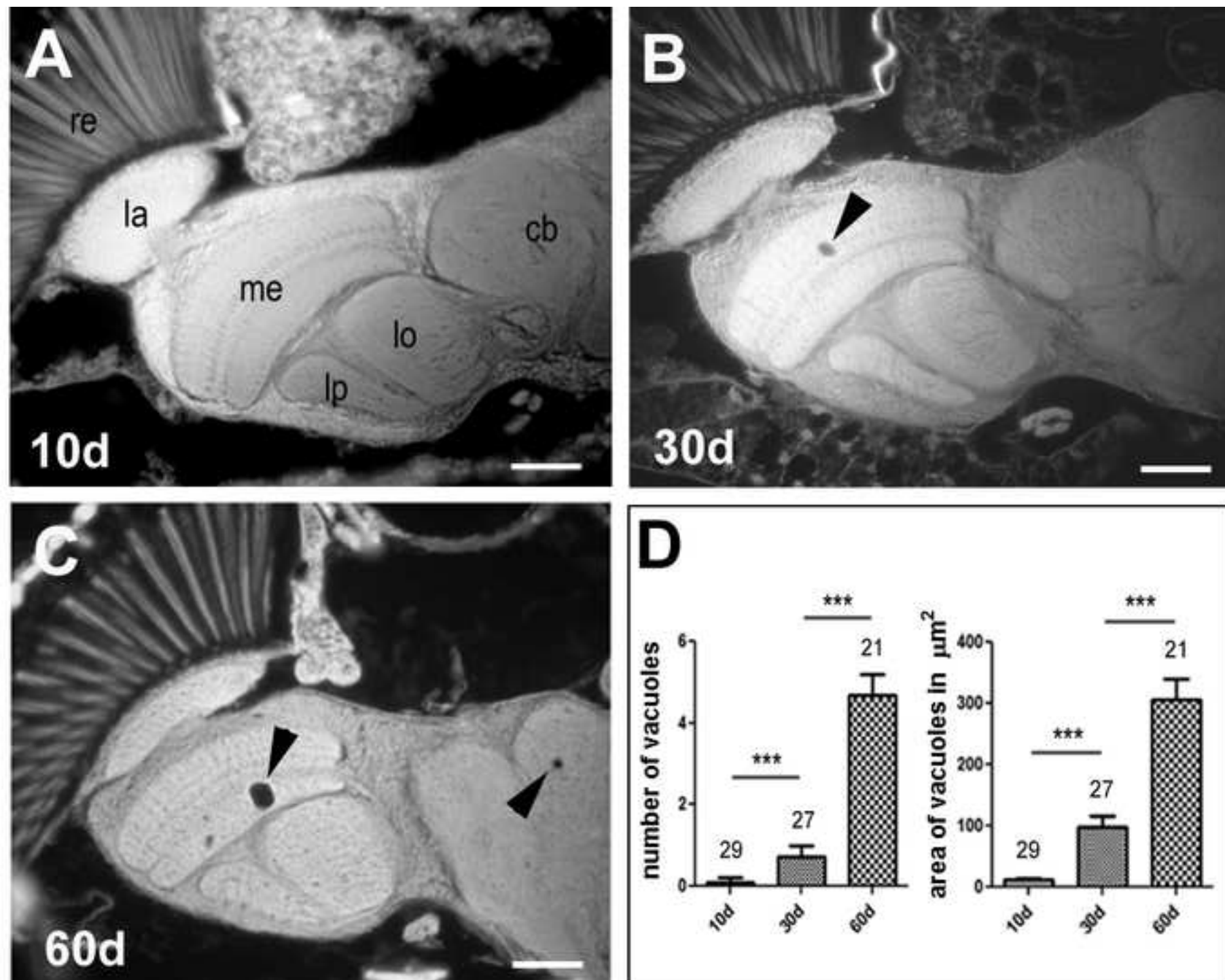
Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Name of the Reagent/Equipment	Company	Catalog Number	
Collar	Genesee Scientific	TS 48-100	We are using custom made collars that are made from one piece of m
Rubber ice cube tray for embedding	Household store		The size can be made to fit by glueing in additional walls
Crystallizing dish	Fisher Scientific company	08-762-3	
Ether	Fisher Scientific Company	E138-1	
Ethanol	Decon Laboratories Inc.	2701	
Choloroform	Fisher Scientific Company	C298-500	
Glacial Acetic Acid	Fisher Scientific Company	A38-212	
Methylbenzoate Use in fume hood	Fisher Scientific Company	M205-500	Distinct Odor
Low Melting Point Paraffin Wax	Fisher Scientific Company	T565	Make sure to keep extra melted in a 65°C waterbath
Microtome	Leica Biosystems	Reichert Jung 2040 Autocut	

Microscope Slide	Fisher Scientific Company	12-550D	
Microscope Cover Glass	Fisher Scientific Company	12-545-M	
SafeClear	Fisher Scientific Company	314-629	Three different containers for washes
Vertical Staining Jar with Cover	Ted Pella Inc.	432-1	
Permount	Fisher Scientific Company	SP15-500	
Poly-L-lysine Solution	Sigma Life Science	P8290-500	

metal instead of layers as the ones by Genesee. A discription to make collars can be found at http://flybrain.neurobio.arizona.edu/Flybrain

/html/atlas/fluorescent/index.html

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Author(s): | Elizabeth Sunderhaus and Doris Kretzschmar

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CORRESPONDING AUTHOR:

Name: Doris Kretzschmar

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Institution: Oregon Health and Science University

Article Title: Mass histology to quantify neurodegeneration in Drosophila

Signature: _D. Kre_   Date: 7/13/2016

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Response to reviews:

Editorial

Grammar:
-Please copyedit the manuscript for numerous minor errors, particularly in the figure legends. Such editing is required prior to acceptance.
-Line 210: "After cutting, sections from different flies are oriented left to right are sections from different flies"
-Line 217: vacuoles that have developedwith aging
-Line 230: in the brainof
-Line 237: cell death markers"
-Line 251: loose sections
-Line 253: loosing the order, additon
-Line 264 - diffent
-Line 272 – this methods
-Line 278 – This techniques

The manuscript has been proof-read and errors corrected

Formatting:
-Please include spaces between all paragraphs.

Spaces have been included

-References – Please abbreviate all journal titles.

References have been cited using the endnote style tool provided on the JoVE website

Additional detail is required:
-1.3 what are you using to thread the flies? Forceps?

The use of forceps has been added

-1.14 so only the heads are supposed to be fixed in 1.13? They should not be "completely covered" as stated? Or do we slice away the solid paraffin until we have removed all but the heads? When/how are heads separated from the fly body?

The entire flies in the collars are fixed but after the paraffin hardens the paraffin block is broken off from the collar, separating the heads, which are in the paraffin from the bodies, which remain in the collar. The text has been changed to clarify this.

-2.9 how much deparafinization wash do you typically use?

Covering the sections has been added

Results: Please add scale bars to Figures 1A,B, 2B,C, and 3B,C.

Scale bars have been added

Discussion: Please discuss the limitations of the technique.

Limitations are discussed in the discussion

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A new version of the agreement with open access has been downloaded

Reviewers' comments:

Reviewer #1:
The authors, fail to refer to the critical paper that originally demonstrated this method (Heisenberg and K. Böhl 1979) and all the follow up papers listed below. They should all be listed and discussed before they start their demonstration.
-Han, Meller, and Davis (1996) The Drosophila Brain Revisited by Enhancer Detection J Neurobiol. 31(1):88-102.
-Strausfeld, Sinakevitch and Vilinsky (2003) The mushroom bodies of Drosophila melanogaster: an immunocytological and golgi study of Kenyon cell organization in the calyces and lobes. Microsc res Tech. 62(2):151-6
-Lin et al (2015) Automated in situ brain imaging for mapping the Drosophila connectome. J Neurogenet. 29(4):157-68. doi: 10.3109

We have added these references.

Reviewer #2:

Major Concerns:
-I am confused about the use of the term "spongiform lesions", which to my knowledge are associated with prion diseases, instead of the more generalized term "vacuole". How do the authors define spongiform lesions, and why was this term chosen, especially since some (if not all) of the references cited as using this method refer in their text to observed lesions as "vacuoles"? It would be useful, particularly for those weighing whether this method will be applicable to their disease model, if the authors added a line or two about the disease models to date in which such lesions have been observed, any insights regarding the nature of the lesions observed, and, at a more practical level, what qualifies a dark spot as a lesion (minimal size? Darkness? Shape?).

My understanding is that spongiform lesions is a general clinical term for "holes". However, we have changed it to vacuoles. We have added a sentence and several references that vacuoles also occur in other disease models and that this method can be used to quantify degeneration in these models.

-Figure 1A. This figure is a bit blurry such that it is hard to tell how the heads are oriented relative to the collar — add a schematic, a side view, and/or a sharper photo

We have added a schematic as figure 1B

-Materials: The authors list Genesee Scientific as the source for fly collars, but add that they are using a custom-made collar. What, if anything, is the difference, and are the two equivalent? If not, can they list the source of their custom collar?

We have added a sentence about our collars and also provide a link to a website that describes the dimensions and material to make collars

Minor Concerns:
-Line 87. More specifics about the container to be used (size, shape, lid needed, material?)

This has been added to the protocol and to the materials table

-Item 3.4. Specify imaging software used

We were told by the journal that we were not allowed to mention a specific program

-Overall: Reads well, but many spelling and a few grammatical errors (4-5 on most pages of the text). "Commissure."

The manuscript has been proof-read and errors corrected

Reviewer #3:

Major Concerns:
the text need significant attention see below
Line 20. Degeneration or neuronal death? Do you know that the neurons were there earlier and now gone? (degeneration), or simply missing due developmental reasons and or necrosis? sws

may be a true degenerative mutant although strictly speaking you would need to track the neurons in the same animal as it ages.

We now discuss that this methods does not distinguish between death and degeneration and we have added a sentence in the discussion that this method can also be used to determine degeneration that is caused during development and present in newly eclosed flies. I also want to point out that this is not an analysis of swiss-cheese but sws is only used as an example to show how this method can be used to quantify degeneration.

Minor Concerns:
Line 94 protocal?
Line 95 a collar as been finished?
Line 112. What happens if the collars stay in paraffin in the oven overnight or 6-10 hrs??
Line 125. Trimming does not necessarily need warming up the blocks.it can be done with the hardened blocks mounted and trimmed with the razor blade. Perhaps this can be listed as alternative?
Line 141. These landmarks for cutting are useful in which head orientation?
Line 176. For the eye pigment method shouldn't it be mentioned that flies need to have pigmented eyes? Or at least a few flies per collar (ie controls) need to have pigmented eyes? Method not appropriate for white eyed flies….
Line 183. Sentence starting with Only…is very poorly constructed and contains double negatives…and 3 "only"s!
Line 210. Sentence : After cutting …makes no sense
Line 230. space after brain….
Line 237. markers NOT markes
Line 242 apostrophe in wrong orientation!
Line 257-Line 284. Largely redundant discussion. Needs to be reorganized as to be more succinct per point and less repetitive

The manuscript has been proof-read and errors corrected