**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*  
The Access type of your article is listed as "Open" on your Article and Video License Agreement, but as "Standard" on the JoVE submission site. Please ensure that the Access type is the same in both cases.

*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*