**REBUTTAL LETTER**

**Editorial comments:**

•**NOTE: Please download this version of the Microsoft word document (File name: 54989\_R1\_060716) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.**                    
  
•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•Formatting:  
-“While looking through the eye piece of the dissection microscope” does not need to be repeated at each step, stating it at the first step is fine. (2.3, 2.4, 2.5, 2.6)

**Authors’ response:** Recommended corrections have been performed in the revised manuscript.

-All figure legends should have a title and a brief description.

**Authors’ response:** Recommended corrections have been performed for Figure 1 legend in the revised manuscript. All others already follow this style.

-2.2 – Please use a subscript for CO2.

**Authors’ response:** Recommended correction has been performed in the revised manuscript.

-in vitro should be italicized.  
**Authors’ response:** Recommended correction has been performed in the revised manuscript.

•Length exceeds 2.75 pg of highlighted material. We suggest unhighlighting section 6 or 7, although any section consisting of relatively standard methods could be unhighlighted. Please make sure the final highlighted regions are continuous.

**Authors’ response:** Recommended corrections have been performed in the revised manuscript. The highlighted portions are within the required limit and flow like a continuous description.

•Grammar:  
-Please copyedit the manuscript for numerous typographical errors, especially extra spaces and errors in punctuation.

**Authors’ response:** Recommended corrections have been performed in the revised manuscript.

-Short abstract – Should be “are described and demonstrated” instead of “have been described”.

**Authors’ response:** Recommended correction has been performed in the revised manuscript.

-2.7 “Place teased ovaries immediately for live microscopy…” Place them where?

**Authors’ response:** Recommended correction has been performed in the revised manuscript. Now this line is modified and moved to 3.3 to improve the flow of the manuscript.

-Line 188 – “Similar experiment can also be performed”

**Authors’ response:** We failed to identify the recommendation here.

-Please remove all instances of “your” or “you”.

**Authors’ response:** Recommended corrections have been performed in the revised manuscript.  
  
•Additional detail is required:  
-Line 321: “on a rocker at medium to low speed” – how fast is this?

**Authors’ response:** Recommended correction has been performed in the revised manuscript.

-7.13 – Which steps? The step number is incomplete.

**Authors’ response:** Recommended corrections have been performed in the revised manuscript.  
  
•Branding should be removed:  
-“mitoSox” line 226

**Authors’ response:** Recommended corrections performed in the revised manuscript: MitoSox has been replaced by “mito-ROS stain” and MTGr with “overall mitochondrial stain”, with reference to the Materials table.

-Please remove all trademark symbols from the materials table.

**Authors’ response:** Recommended correction has been performed in the revised manuscript  
  
•Results: Figure 5 D and E – are error bars SD or SE?

**Authors’ response:** These are not error bars, but whiskers of the box plots where the whiskers indicate maximum and minimum values for each group, excluding the outliers. This has now been added to the respective figure legends (Now Figure 6).

•Discussion: Please discuss alternative techniques in more detail in the significance section.

**Authors’ response:** We have provided the alternative techniques in the Modifications and Troubleshooting section, as appropriate. The significance section as worded “Significance of the technique with respect to existing/alternative methods” means significance of the proposed method over the existing or alternative methods. To us, the wording does not mean alternative strategies should be included here. If you can clarify further that will help us.

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.      \

**Authors’ response:** All the figures are original.

•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

**Authors’ response:** DOIs are already included.                  

•IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

**Authors’ response:** We have done this to our best possible abilities.

•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission  
**Authors’ response:** Please see below for our line by line response to the reviewers’ comments.

**Reviewers' comments:**

**Reviewer #1:**  
*Manuscript Summary:*  
In this paper, the authors describe the methods for imaging mitochondria in the Drosophila follicle epithelium as well as analyzing mitochondrial structure and function in this tissue. This protocol utilizes several novel methods developed by the authors, so the detailed descriptions (and, presumably, the video that would accompany the final version) provided in this paper would be very useful to other practitioners in the field. However, I would recommend that the authors address several items before publication:

**Authors’ response:** We thank the reviewer for the encouraging comments on our manuscript. Based on the reviewers’ comments we have done substantial changes to the description of all the sections of the manuscript. We sincerely thank the reviewers since we found that these new changes have, indeed, improved the quality of the manuscript. We have also reorganized various sections to improve clarity and flow of the manuscript. We have also included the editorial corrections required for publication in JoVE (as mentioned in a separate section above). Please note, we had to use generic terms for the live mitochondrial stains based on editorial recommendations: MitoSox has been replaced by “mito-ROS stain” and MTGr with “overall mitochondrial stain”, with reference to the Materials table where the exact names of the dyes are mentioned. Please see below for detailed response to the valuable comments provided by the reviewer.

1. On Line 216, the authors mention the use of open source software to quantify fluorescence intensity. **As there are many open source options, it would be helpful if the authors could provide an example of the open source software they would recommend**. Likewise, there are many ways to perform background subtraction (mentioned on Line 219 and Line 455) and it would be helpful if the authors described the type of background subtraction they would recommend for these applications.

**Authors’ response:** According to the editorial recommendations mention of any name of the software used is possible in the main text. This information is available in the Methods table as per editorial recommendation. In our revised manuscript, we have clarified our recommended method for background correction using mean fluorescence intensities in section 4.7 (Lines :228-234)**.**

2. On Lines 500-501, the authors note that egg chambers with signs of physical damage should be excluded from analysis. Dissections and mounting can produce different types of physical damage, such as tears to the follicle epithelium, flattening of the follicles, separating of follicles from the rest of the ovariole, etc. It would be helpful if the authors provided more specifics on what types of damage are most common, indicated if any type(s) are particularly problematic, and added an image of a damaged ovariole. Lastly, the authors should add a reference to and brief discussion of Haack, et al, Biology Open 2 (12): 1313-20, which describes artifacts that can arise in the follicle epithelium due to physical damage. Since physical damage of the type described in this paper can both create "false clones" and may increase ROS, the authors should discuss the best ways to avoid this pitfall when assaying for ROS (as in Fig. 6) and in related studies.

**Authors’ response:** Following the reviewer’s suggestion, we have included exemplifying images and the relevant text describing different kinds of damage that can be associated with the procedural dissection of the *Drosophila* ovaries (Figure 9 and its legend and text in Lines: 566-594). In this above section, we have also added the relevant reference suggested by the reviewer and provided examples of potential false clones from our experimental demonstrations.

3. On Lines 417-419, the authors statement that "the Drosophila germarium exhibits higher TMRE signal in the stage where the somatic follicle cells have arisen" is ambiguous. Are they referring to Region 2b, where cells of the FSC lineage are first produced and encapsulate the germline or to a later stage where the prefollicle cells become more committed to the follicle cell fate? The precise region where prefollicle cells commit to the main body follicle cell fate has not been well-established. Thus, it would be better to refer to the intended stage by the name of the region (e.g. region 2b or region 3, etc.) in this case. In addition, the authors should add arrows or lines to the images in Fig 5B so the reader can more easily see what cells they are referring to.

**Authors’ response:** Following the reviewer’s suggestion, we have included the stage information (Lines: 445-448) in the text and arrow in the figure (now Figure 6B). The increase in TMRE incorporation is consistently observed in Region2b where induction of FasIII immunostaining occurs indicating the appearance of the first differentiated follicle cells. However, given the nature of our manuscript is description of methods, we refrain from drawing any detailed conclusion.

4. In Figure 6, why is the ROS staining lower in the mutant cells of the third and fourth follicles

compared to the mutant cells in other follicles? For others who would like to use this protocol to replicate this experiment, it would be useful to know whether this variability in staining is biological (i.e. some mutant follicle cells have higher ROS levels than others) or technical (i.e. the staining is expected to be uniform but does not appear so in this image because of an issue such as uneven dye penetration or an inability to capture the signal from all mutant cells in a single focal plane). **Also, the follicle epithelium of the first budded follicle is not continuous around the entire follicle. Is this physical damage or a mutant phenotype?**

**Authors’ response:** We noted that the mito-ROS stain was not very stable in the *ex vivo* *Drosophila* tissue likely due to the transient nature of the ROS analyte it detects, which may underlie the lack of detection of signal from all the Drp1 null clones. However, any biological relevance of the variability in mito-ROS staining in the Drp1 null cells cannot be ruled out and needs to be investigated further (Lines 658-662). In the referred figure which is now Figure 7A, the absence of UbiGFP corresponds to the Drp1-null clones and we did not observe any signs of physical damage.

5. The FLIP experiment described in Figure 3 is confusing. The diagram in Fig. 3a suggests that the mitochondria progress unidirectionally through 4 different mitochondrial states. Is that accurate or can they interconvert back an d forth between these different states? Why is the ROI over just one mitochondrion in the top row but at the junction between two mitochondria in the other rows? Does the data in Fig 3c correspond to mitochondria that are in the final state (continuous matrix) only? What would the data look like if the mitochondria were in the other three states? Can the FLIP differentiate between each of the four states or just between any of the first three vs the fourth? Also, it is difficult to see the colors of the different circles in Fig. 3b against the green background.

**Authors’ response:** To clarify the reviewer’s concern, the description of the rationale, steps and interpretation of the FLIP experiment and the relevant figure has been substantially improved (Protocol 4, now Figure 4 and its legend, Lines: 414 to 427; 603-606; 635-643, 666-668). We have also added a video exemplifying an anticipated outcome of the protocol (Video1). Mitochondrial matrix continuity in a fused mitochondrial structure is established after complete fusion of the mitochondrial inner and outer membranes following a progression through the intermediate steps, while fission of mitochondria may follow the same steps but in the opposite direction. The intermediate steps may exist but cannot be detected with the probe targeted to the mitochondrial matrix. The proposed protocol FLIP performed with the probe targeted to the mitochondrial matrix detects only the fused states of steps 3 and 4 and not in step 1 and 2. A FLIP protocol similar to what has been proposed may be used with a probe targeted to the mitochondrial inter membrane space to be able to detect its continuity resulting out of fusion of the outer but not the inner mitochondrial membranes (Step2). The changes made to the figure are: a) maintained the FLIP ROI at the same position before and after fusion in Figure 4A; b) added an experimental ROI in Figure 4A; c) indicated the steps of fusion and fission in Figure 4A; d) added further description to the ROIs of Figure 4B and C; e) indicated bleaching and recovery time frames in Figure 4C.

6. In the Protocol step 2.1, the authors refer to both "insect dissection medium" and Grace's medium. Are these the same?

**Authors’ response:** Yes, they are same. We apologize for the confusion. Now we have maintained “insect dissection medium” throughout the manuscript.

7. In the Protocol step 3.1, what diameter coverslip is used?

**Authors’ response:** This information has now been added in the relevant sections (Lines: 162 and 386).

8. In Figure 5b, the images appear to have an artifact in which sharp changes in the contrast of neighboring pixels traces out a grid of squares across the image. This is particularly apparent in the MTGr image where a grid of squares is visible at the posterior of Region 3, just anterior to the forming stalk region. What is the cause of this artifact?

**Authors’ response:** We are extremely sorry that we could not identify any potential artifact that the reviewer is referring to in the figure, now Figure 6b. Therefore, we could not take care of this issue.   
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
N/A

**Reviewer #2:**  
*Manuscript Summary:*  
The manuscript reported a protocol to study mitochondrial structure and function in live and fixed Drosophila ovary with the following major steps: 1) Dissection of Drosophila ovaries, 2) Preparation for live tissue microscopy, 3) Fluorescence Loss In Photobleaching Assay, 4) Staining with MTGr, TMRE or MitoSOX Staining, 5) Generation of Drp1 null mosaics, 6) Co-immunostaining for Cyclin E and mitochondria. As what the authors already mentioned, this protocol would be used to study the regulation of mitochondrial structure-function relationship between controls and mutant Drosophila ovaries, as well as various nutrients and growth factor signaling on mitochondrial structure and function.

**Authors’ response:** We thank the reviewer for the encouraging comments on our manuscript. Based on the reviewers’ comments we have done substantial changes to the description of all the sections of the manuscript. We sincerely thank the reviewers since we found that these new changes have, indeed, improved the quality of the manuscript. We have also reorganized various sections to improve clarity and flow of the manuscript. We have also included the editorial corrections required for publication in JoVE (as mentioned in a separate section above). Please note, we had to use generic terms for the live mitochondrial stains based on editorial recommendations: MitoSox has been replaced by “mito-ROS stain” and MTGr with “overall mitochondrial stain”, with reference to the Materials table where the exact names of the dyes are mentioned. Please see below for detailed response to the valuable comments provided by the reviewer.

*Major Concerns:*  
The major concerns for me is the potential artifacts, although the authors already touched this in the troubleshooting section, it would be much better if the authors could set up some standards for telling the real and potential artifacts.

**Authors’ response:** Now we have provided new figures (Figure 1 and 9 and their legends) and added detailed description in the Result as well as Discussion to section to set up standards and describe potential artifacts. Figure 1 describes the steps of *Drosophila* dissection, including identification of ideal or damage ovaries (Lines 402-411). Figure 9 describes artifactual results that may arise from potential damage to the *Drosophila* ovaries during the procedural dissection (Lines 566-594.). Figure5A also indicates potential artifact that has been discussed with its trouble shooting (Lines 625-631). We have also expanded the Discussion section (outlined based on editorial requirements) to include details of experimental artifacts, trouble shooting, limitation and alternative strategies.

*Minor Concerns:*  
Since the authors wrote that "the live ex vivo tissue microscopy has to be performed within 15 minutes on ovaries isolated form individual Drosophila at a time", it will be nice if the authors could have a better way on how to prepare for this if there are various experimental groups. And also, it would be much clearer if the authors add the time control in each step of the protocol.  
For Drosophila egg chamber in Figure 1, I would suggest the authors make individual nurse cells, instead of the cluster ones.

**Authors’ response:** We had this point covered in the limitations section and have now expanded that according to the reviewer’s suggestion (Lines 653-658). All the protocols have the incubation times mentioned in their description. The time taken for the *Drosophila* dissection is subjective. However, we have now included the average time taken by us for the dissection and teasing of *Drosophila* ovaries in the Discussion section (Lines 570-572). We have also modified igure 1 according to the reviewer’s suggestion.

*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #3:**  
*Manuscript Summary:*  
Studying the structure-function relationship of mitochondria can help assess aspects of mitochondrial dysfunction, as it relates to genetic or physiological defects. This article describes a method for examining Drosophila mitochondria, derived from ovariole tissue, via fluorescence microscopy. The article describes the dissection of ovariole tissue, the fixation and staining of ovariole tissue, microscopy of the tissue, and generation of mutant clones within the developing ovary. Of these various aspects, the only one that realistically benefits from the visual medium of JoVE is the dissection of ovariole tissue; generation of mutant clones, and fixation, staining, and microscopy are such standard procedures, with little novelty here in those aspects. The manuscript also includes a significant amount of what appears to be new data on mitochondria in Drp1 null mosaic clones, which seems distinctly out of place. I cannot recommend this manuscript for publication.

**Authors’ response:** We are sorry to find that the reviewer finds our manuscript not well presented for publication in JoVE. We would like to stress upon the fact that the novelty of our manuscript is the live tissue microscopy of the *Drosophila* ovaries towards studying mitochondrial structure-function on a single cell level as published in Mitra et.al, JCB, 2012. We have also included the fixed immunostaining to describe the identification of the novel mitochondrial Cyclin E as requested in the editorial invitation for this manuscript, based on our publication (Parker et.al., JCS, 2015). Nonetheless, our description of the other relatively more standard techniques like *Drosophila* dissection, generation of clones in the *Drosophila* ovary etc are unavoidable steps towards demonstrating the successful microscopy method for studying mitochondrial structure function in live and fixed tissue. Nonetheless, based on the reviewers’ comments we have done substantial changes to the description of all the sections of the manuscript. We sincerely thank the reviewers since we found that these new changes have, indeed, improved the quality of the manuscript. We have also reorganized various sections to improve clarity and flow of the manuscript. We have also included the editorial corrections required for publication in JoVE (as mentioned in a separate section above). Please note, we had to use generic terms for the live mitochondrial stains based on editorial recommendations: MitoSox has been replaced by “mito-ROS stain” and MTGr with “overall mitochondrial stain”, with reference to the Materials table where the exact names of the dyes are mentioned. Please see below for detailed response to the valuable comments provided by the reviewer.

*Major Concerns:*  
1. The procedures that might have the most general interest are the least fully articulated. For example, the dissection of ovaries, which would most benefit from a visual presentation, does not adequately describe the process. In step 2.4, the ovaries are forced out of the abdomen using forceps, but no care is given at all to how one distinguishes the ovaries from the other material that would inevitably come along during the procedure.

**Authors’ response:** To address the reviewer’s concerns, now we have added Figure 1 with its legend and the relevant text to describe the steps of *Drosophila* dissection (Lines 402-411). In this section we have covered how to distinguish the *Drosophila* ovaries from the other abdominal contents that are released along with the ovaries (Figure 1C).

2. Likewise, the photobleaching assay seems like it could be useful and novel, but how and when it should be used or how best to apply it and analyze the data doesn't seem adequately explored. The analysis of this sort of data (rather than the acquisition) seems like the challenging part, but is barely given any treatment at all.

**Authors’ response:** Indeed, the photobleaching protocol is an important and novel aspect of our manuscript.To address the reviewer’s concerns, we have substantially expanded and improved the description of the FLIP protocol in the Protocol, Results as well as Discussion sections (Protocol 4, Lines 414-427, 603-606, 635-643, 666-668) as well as the relevant figure (now Figure 4 and its legend). We have also added a video exemplifying an anticipated outcome of the protocol (Video1). The description now includes a) clarification of the mitochondrial structures that can be identified with the described protocol (steps 3 and 4 of Figure 4A); b) the limitations and problems of the technique being lack of resolution of the mitochondrial structures and possible laser induced fragmentation of mitochondria during the execution of the FLIP that can be avoided by reducing the laser power for the directed photobleaching. Our protocol already covered the image analyses for FLIP, which has now been expanded for clarification purpose. We would like to stress that the image quantification in this case is relatively simple as it only involves standard quantification of fluorescent signal. FLIP is a semi-quantitative technique therefore cannot yield quantitative results like Fluorescence Recovery of Photobleaching (FRAP) where the fluorescence signal can be used to obtain diffusion coefficients after appropriate mathematical operations. In the revised manuscript we have mentioned about the semi-quantitaive nature of the FLIP assay (Lines 188-190).

3. I am confused by the amount of space and emphasis given to presentation of data (Figures 4-7), rather than methodology. It almost appears the authors have some random data they'd like to present but haven't published (or been able to publish?) elsewhere and are trying to publish it via a methodology paper. That doesn't seem to fit with the mission or purpose of JoVE.

**Authors’ response:** Here our focus was to demonstrate novel techniques used for studying mitochondrial structure-function in *Drosophila* ovaries. The data that appears random to the reviewer has been chosen from our various experimental efforts behind our two published papers (Mitra et. al., JCB, 2012 and Parker et. al., JCS, 2015): **a)** the FLIP data has been demonstrated in *Drosophila* follicle cells in the JCB paper and here we demonstrate it on the germline nurse cells to prove broader applicability of the technique; **b)** the staining with any mitochondrial dye follows the same protocol that was used in the JCB paper for TMRE loading; **c)** we have previously published co-staining of TMRE and mitotracker green in mammalian cells (Mitra et.al, PNAS, 2009), which we applied here on the *Drosophila* ovary and demonstrated that the quantification of the ratio of the fluorescence signal can yield different values for different cell types; **d)** successful mitoSOX staining has been already reported by others in *Drosophila* (ref 14 in the manuscript). Here we used mitoSOX staining of the Drp1 null clones to demonstrate detection of mitoSOX in cells with perturbed mitochondrial structure, thus maintaining our focus on the study of mitochondrial structure-function; e) the immunostaining protocol describes the already published data on the novel mitochondrial Cyclin E pool (Parker et.al., JCS, 2015) but in other cell types of the same lineage as published.

In the revised manuscript, we have refrained from any interpretation of the data presented (Lines 398-473) to rectify any wrong impression to the reviewer that any unpublishable sketchy data has been presented here. As other readers, we rightfully keep in mind the data presented in methods/protocol papers are to be considered only as ‘proof of principal’ and not ‘research findings’ so as to be able to cite them appropriately.

*Minor Concerns:*  
1. Manuscript is riddled with typos, repeated words ("Staining with MTGr, TMRE or MitoSOX"), and references to steps that don't exist (step 5.3 has a reference to non-existent step 2.8; and step 7.6 to non-existent step 2.9). This sloppy lack of editing is frustrating and detracts from the manuscript.

**Authors’ response:** We sincerely apologize for all the typographical errors. Now we have corrected them in the revised manuscript.

2. Figure 3 figure legend is insufficient to describe the figure. Four circles are outlined in the top left panel of 3B but none are described in the figure legend.

**Authors’ response:** In the legend the ROIs are mentioned as ‘colored ROIs’. Now we have included description of the colored ROIs in the figure and its legend (Now figure 4) as well as in the relevant text (Lines 419-422).   
  
*Additional Comments to Authors:*  
N/A