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Studying mitochondrial structure and function in Drosophila ovaries

--Manuscript Draft--

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Abstract:	Analysis of mitochondrial structure-function relationship is required for a thorough understanding of the regulatory mechanisms of mitochondrial functionality. Fluorescence microscopy is an indispensable tool for direct assessment of mitochondrial structure and function in live cells towards studying mitochondrial structure-function relationship that is primarily modulated by molecules governing fission and fusion events between mitochondria. This paper describes and demonstrates specific methods for studying mitochondrial structure and function in live as well as in fixed tissue in the model organism Drosophila melanogaster. The tissue of choice here is Drosophila ovary that can be isolated and made amenable for live confocal microscopy ex vivo. Furthermore, the paper describes how to genetically manipulate the mitochondrial fission protein, Drp1, in Drosophila ovaries towards studying the involvement of Drp1 driven mitochondrial fission in modulating mitochondrial structure-function relationship. The broad use of such methods is demonstrated in obtaining already published as well as novel data. The described methods can be further extended towards understanding the direct impact of nutrients and/or growth factors on the mitochondrial properties ex vivo. Given that mitochondrial dysregulation underlies the etiology of various diseases, the described innovative methods developed in a genetically tractable model organism, Drosophila, are anticipated to contribute significantly in the understanding of the mechanistic details of mitochondrial structure-function relationship and developing mitochondria directed therapeutic strategies.
Author Comments:	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
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

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08-06-2016
To,
The Editor
The Journal of Visualized Experiments

Dear Dr. Upponi,
Thank you for sharing the reviewers' comments on our manuscript entitled "Studying mitochondrial structure and function in *Drosophila* ovaries" (54989_R1_060716), authored by Danitra J Parker, Aida Moran and myself.

We are happy to submit the revisions to our manuscript and find that the quality of the manuscript has indeed improved substantially after the major revisions. In our revised manuscript, we have incorporated the editorial changes as well as those recommended by the 3 reviewers. Our substantial revisions of the manuscript includes addition of two new figures and a video along with their relevant texts, addition of relevant information as sought by the reviewers, and restructuring of portions of the manuscript for clarification sake. Please find our point by point response to the editorial and reviewers's comments in the rebuttal letter provided along with other manuscript documents.

Thank you very much for the invitation to submit this method article to the JoVE and we sincerely appreciate the extra time that you provided for the thorough revisions of the manuscript. Please let me know if you have any questions.

Sincerely,

……..
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TITLE:
Studying mitochondrial structure and function in *Drosophila* ovaries

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KEYWORDS:
Mitochondria, *Drosophila*, Live tissue, Confocal microscopy, Ovary, Drp1

SHORT ABSTRACT:
Analysis of the mitochondrial structure-function relationship is required for a thorough understanding of the regulatory mechanisms of mitochondrial functionality. Specific methods for studying mitochondrial structure and function in live and fixed *Drosophila* ovaries are described and demonstrated in this paper.

LONG ABSTRACT:
Analysis of the mitochondrial structure-function relationship is required for a thorough understanding of the regulatory mechanisms of mitochondrial functionality. Fluorescence microscopy is an indispensable tool for the direct assessment of mitochondrial structure and function in live cells and for studying the mitochondrial structure-function relationship, which is primarily modulated by the molecules governing fission and fusion events between mitochondria. This paper describes and demonstrates specific methods for studying mitochondrial structure and function in live as well as in fixed tissue in the model organism *Drosophila melanogaster*. The tissue of choice here is the *Drosophila* ovary, which can be isolated and made amenable for *ex vivo* live confocal microscopy. Furthermore, the paper describes how to genetically manipulate the mitochondrial fission protein, Drp1, in *Drosophila* ovaries to study the involvement of Drp1-driven mitochondrial fission in modulating the mitochondrial structure-function relationship. The broad use of such methods is demonstrated in

already-published as well as in novel data. The described methods can be further extended towards understanding the direct impact of nutrients and/or growth factors on the mitochondrial properties *ex vivo*. Given that mitochondrial dysregulation underlies the etiology of various diseases, the described innovative methods developed in a genetically tractable model organism, *Drosophila*, are anticipated to contribute significantly to the understanding of the mechanistic details of the mitochondrial structure-function relationship and to the development of mitochondria-directed therapeutic strategies.

INTRODUCTION

Mitochondria are classically described as the cellular powerhouse, since they are the main seats of energy production in differentiated cells. Moreover, mitochondria play a critical role in metabolism, heat generation, lipid modification, calcium and redox homeostasis, the orchestration of cell signaling processes, etc[1]. Mitochondria also play an active role in the induction of cell death[2], as well as in cell cycle regulation[3]. Such multi-functionality raises the following fundamental questions: a) how do mitochondria perform all these functions simultaneously and b) are there specific mitochondrial pools or subzones that are specialized for distinct functions? In this context, it is important to note that the multifunctional mitochondria are dynamic in their shape, size, and structure within individual cells and that the steady-state shape of mitochondria can vary between cell types. Decades of research from various laboratories suggest that the alteration of mitochondrial shape, size, and structure, collectively called mitochondrial dynamics, is crucial for maintaining various mitochondrial functions[4,5,6]. These findings raise the possibility that mitochondria may accomplish their multi-functionality by virtue of their structural dynamism.

Extensive efforts are underway to understand the mitochondrial structure-function relationship. The dynamism of mitochondrial structure is primarily maintained by their ability to undergo fission and fusion events with each other. Fission of large mitochondria converts them into smaller mitochondrial elements, while fusion between two smaller mitochondria merges them into a larger mitochondrial element[7]. Moreover, transient fusion of two mitochondria may occur to allow the mixing of their contents. The fission and fusion events of the inner and outer mitochondrial membranes are carefully governed by specific sets of proteins. The core fission machinery is composed of dynamin-related protein 1 (Drp1), which is recruited from the cytosol to the mitochondria by its interaction with certain *bona fide* mitochondrial proteins (*e.g.,* Fis1 or Mff1), while Drp1 function can also be regulated by other proteins on the mitochondrial surface[4]. Although Drp1 operates on the outer membrane, its fission abilities impact the inner membrane as well. The orchestration of the fission of outer and inner mitochondrial membranes is not well understood. On the other hand, fusion of the inner membrane is governed at the core by the activities of Opa1, while mitofusins govern the fusion of the outer-membrane[5]. The balance of the counteracting fission and fusion events of mitochondria dictate the steady-state mitochondrial shape in a cell. For example, repression of mitochondrial fission would result in complete and unopposed fusion, while the over-activity of mitochondrial fission would result in fragmentation of mitochondria[3].

The study of the mitochondrial structure-function relationship primarily involves two complimentary approaches: a) analyses of the cellular and organismal phenotypes after genetic manipulation of mitochondrial fission/fusion proteins and b) direct assessments of mitochondrial

structure and function. It is noteworthy that genetic analyses may not always reveal the direct functionality of the molecule at hand (in this case, mitochondrial fission/fusion proteins), as the phenotypes may arise due to secondary effects. Therefore, it is of the utmost importance to develop and use tools to study mitochondrial structure and function directly. Any assessment of mitochondrial structure involves various microscopy tools. Use of fluorescence microscopy of live cells has greatly advanced the studies of mitochondrial dynamics, since mitochondrial dynamism can be monitored both qualitatively and quantitatively using the appropriate fluorescence microscopy tools and techniques[8]. Fluorescence microscopy-based tools have been developed to study mitochondrial structure and function in live and fixed *Drosophila melanogaster* tissues, elucidating the significance of mitochondrial dynamism *in vivo*[9]. These and related methods are described here, with the goal of studying mitochondrial structure and function in the *Drosophila* ovary.

The *Drosophila* ovary consists of germline and somatic lineages, which arise from their respective adult stem cells that reside in the germarium[10,11]. Sixteen syncytial germ cells (GCs) get encapsulated by somatic follicle cells (FCs) to form individual egg chambers that emerge out of the germarium (Figure 1). One of the 16 GCs get committed to become an oocyte, and the remaining 15 GCs develop into nurse cells that support the growth of the oocyte chamber, facilitating the maturation of the egg before it is laid. The majority of the FCs undergo 9 rounds of mitotic divisions before they exit the mitotic cell cycle to terminally differentiate into a patterned epithelial cell layer consisting of anterior follicle cells (AFCs), posterior follicle cells (PFCs), and main body cells (MBCs). The consecutive egg chambers are connected by stalk cells, which are differentiated cells that are also derived from the FCs early in development. Mitochondrial shape regulated by the mitochondrial fission protein Drp1 is actively involved in the process of differentiation during the normal development of the *Drosophila* ovarian FC layer[9,12]. The methods used in these studies to identify the involvement of Drp1 in *Drosophila* follicle cell layer development are described here.

PROTOCOL:

1. Preparation of *Drosophila* (The tools required are depicted in Figure 2A)

1.1. For any of the experiments described, collect *Drosophila* (maintained at room temperature, or 25 ºC) within 5 days of eclosion and place them in a vial filled with 5-7 mL of *Drosophila* food (see Materials Table), with no more than 25 flies in each vial; maintain a female:male ratio of 2:1.

1.2. Sprinkle a small amount of granulated yeast to stimulate *Drosophila* egg production. Perform experimental manipulation within 2-4 days.

2. Dissection of *Drosophila* ovaries (The tools required are depicted in Figure 2A)

2.1. Warm insect dissecting medium (see Materials Table) to room temperature, 25 °C. Fill three wells of an eight-well glass dissecting dish, with 200 µL of medium in each well.

2.2. Anesthetize *Drosophila* with CO_2 by placing the needle of the blow gun under the vial plug. Place them on a fly pad. Using a dissecting microscope, sort out 5 females and place them in the first well of the dissecting dish. Handle one *Drosophila* at a time when performing live microscopy.

2.3. While looking through the eyepiece of the dissection microscope, sever the thorax from the abdomen using two pairs of forceps. Using the forceps, carefully transfer the abdomens to the second well of the dish.

2.4. Use one pair of forceps to hold the abdomen at the posterior end, and slowly push the ovaries out (along with the other abdominal contents) with the other pair of forceps. Should this attempt fail, carefully remove the abdominal exoskeleton by inserting the forceps into the anterior end to release the ovaries.

2.5. Using the forceps, hold an individual ovary by the opaque posterior end (*i.e.*, the yolk-filled, late-stage eggs) and move it carefully to the third well of the dish for teasing to process it for live microscopy (step 3) or for fixing to perform immunostaining (step 7).

2.6. Carefully tease the protective sheath from around the ovaries by sweeping a teasing needle lightly from the posterior to the anterior end of each ovary while holding it by the posterior end with a pair of forceps.
NOTE: To minimize damage during teasing, bend the needle tip and zoom in on each ovary by increasing the magnification of the microscope (Figure 2A). Teasing should be effective enough to break the sheath, but it should also be carefully done to preserve the integrity of the ovarioles.

3. Preparation for live-tissue microscopy
NOTE: The tools required are depicted in Figure 2A.
3.1. Before *Drosophila* dissection, prepare polyL-Lysine coated chambers. To this end, place two 20-µL drops of polyL-Lysine (0.1 mg/mL) on the coverglass (14 mm, No. 0) of a glass-bottomed petri dish (35 mm) at a reasonable distance from each other in order to prevent the merging of the drops. Air-dry the plates for 1 h at 37 ºC and mark the edges of the white polyL-Lysine film on the underside of the plate with an erasable marker.
NOTE: The polyL-Lysine-coated chambers can be stored at 4 °C for a week. If using a previously-coated chamber, bring it to room temperature before starting the *Drosophila* dissection.

3.2. Set the scanning parameters on the confocal microscope to make sure that the sample can be imaged immediately after the completion of the dissection and mounting, as below.

3.3. Place one dissected and teased ovary (following step 2 and stained, if required, following step 5) on a marked polyL-Lysine-coated region and spread the ovary with the teasing needle to separate the ovarioles. Place a 10-µL drop of insect dissecting medium on top of the ovary, making sure to cover the entire polyL-Lysine-coated area. Cover the petri dish.

3.4. Immediately perform confocal microscopy of the mounted sample at room temperature.
NOTE: Only one *Drosophila* should be dissected, processed, and imaged at a time. Also, microscopy should not be performed at 37 °C, which will mimic a heat shock environment for *Drosophila* tissue.

4. Fluorescence Loss In Photobleaching (FLIP) assay to assess mitochondrial matrix continuity

NOTE: Mitochondrial matrix continuity in a fused mitochondrial structure is established after the complete fusion of the mitochondrial inner and outer membranes following a progression through the intermediate steps. Fission of mitochondria may follow the same steps but in the reverse direction (Figure 3A). FLIP is a time-lapse microscopy-based semi-quantitative method that can be used to assess mitochondrial matrix continuity in the final fused state of *ex vivo* mitochondria (steps 3 and 4 in Figure 3A) in live *Drosophila* ovaries[9]. The FLIP assay is performed as a small region of interest (ROI) of the mitochondria expressing a fluorescent molecule in the mitochondrial matrix that is photobleached at regular intervals (FLIP ROI in Figure 3A). As a result, any surrounding mitochondrial region that is continuous with the FLIP ROI (experimental ROI in Figure 3A) will lose signal due to the exchange of molecules in the continuous mitochondrial matrix. The FLIP experiments demonstrated here are performed on transgenic *Drosophila* expressing mitoYFP, which contains the mitochondrial targeting sequence of the human cytochrome oxidase VIII subunit tagged with YFP to target it to the mitochondrial matrix in a freely-diffusible form. A similar experiment can also be performed with the mito pUASP-mito-GFP transgene, as reported previously[9]. A similar FLIP protocol may be used with a probe targeted to the mitochondrial inter-membrane space to be able to detect the continuity resulting from the fusion of the outer but not the inner mitochondrial membranes (step 2 in Figure 3A).

4.1. Open the image acquisition software on the confocal microscope and set the appropriate scanning parameters in the "Acquisition" tab (Table 1). Check the "Time series," "Bleaching," and "Regions" boxes to open the individual tabs. Put the appropriate acquisition parameter values in each tab (Table 1).

NOTE: The pinhole should be left open, as this experiment is designed to monitor overall signal from the whole mitochondrial population in individual cells.

4.2. Use the eyepiece to quickly locate the field of interest in the mounted live tissue.

NOTE: Select the ovarioles that are well spread on the glass-bottomed dish, since confocal microscopy cannot be performed on floating ovarioles.

4.3. Click "live" to acquire a live image of the selected field of interest. Click "stop" to stop live scanning.

4.4. If necessary, adjust the acquisition parameters such that the detected fluorescent signal is below the saturation levels (indicated by the absence of red pixels when the "range indicator" option is checked), with the defined background as set by adjusting the offset values.

4.5. Draw a small ROI using the Bezier drawing tool from the "Regions" tab to demarcate the photobleaching zone on the image acquired by live scanning.

NOTE: The size of the ROI should be around 20-50% of the total fluorescent mitochondrial signal within the cell.

4.6. Perform the image acquisition by clicking on "start experiment."

4.7. Quantify the fluorescence intensity using the proprietary or the open-source software (see Materials Table). Record the mean signal from the ROI where the repetitive bleaching is targeted (FLIP); the ROIs where bleaching has not been performed in the same cell (Experimental); the ROI from another unbleached cell in the same field of view, for assessing overall bleaching during the experimental period (Bleaching); and the ROI on the background area (Background). Subtract the mean background signal obtained from the mean signal in the other ROIs. Normalize the fluorescent signal with the initial pre-bleach signal for the respective cell.

4.8. Plot the normalized data using any standard plotting software.

5. Live staining with fluorescent mitochondrial dyes

NOTE: Steady-state mitochondrial structure and potential can be assessed using dyes that specifically incorporate into mitochondria in live cells and tissues. Live *Drosophila* ovaries can be stained *ex vivo* with fluorescent mitochondrial stains to visualize the mitochondria, to assess mitochondrial reactive oxygen species (mito-ROS) production, and to assess mitochondrial potential per unit mass. This can be accomplished by co-staining with the mitochondrial potentiometric dye tetramethylrhodamine ethyl ester (TMRE) and a compatible live mitochondrial stain representing the mitochondrial mass (see Materials Table for the specific dyes).

5.1. Dilute the stock of the stains in warm insect dissecting medium to the final working concentrations: mitochondrial stain, 250 nM; TMRE, 50 nM; and mito-ROS stain, 5 μM.

5.2. After dissection and teasing the ovaries following step 2, place the ovaries into 200 μL of any particular staining solution in a well of a dissection dish. Incubate them for 10 min with the dish covered by a suitable box wrapped with aluminum foil to protect it from light. Wash the stained ovaries by moving them carefully with forceps into 3 consecutive wells containing medium without stain.

5.3. For co-staining with TMRE and the compatible overall mitochondrial stain, follow the above protocol to stain first with TMRE and then immediately with the overall mitochondrial stain (without any wash steps in between).

5.4. Mount the ovaries on a polyL-Lysine coated glass-bottomed dish following step 3 and prepare for confocal microscopy with the appropriate scanning parameters (Table 1), following steps 4.2-4.4.

NOTE: The signal from the incorporated dyes did not last when attempting to mount the stained samples in mounting medium.

5.5. Check the Z-sectioning box to open the tab. Turn the focus wheel towards the bottom of the sample while it is being live-scanned and click on "set first" to define the bottommost Z-section. Do the same while moving the focus wheel towards the other direction to define the topmost section.

5.6. Perform the image acquisition by clicking on "start experiment."

5.7. Quantify the background-corrected fluorescent intensity from the ROIs for the background signal and the individual cells (as in step 4.7) and plot the data using any plotting software.

6. Generation of Drp1 null mosaics

NOTE: The clonal strategy used here introduces green fluorescent protein (GFP)-negative Drp1 null clones in the background of a GFP-positive, phenotypically wild-type background that is genotypically heterozygous for the Drp1 null mutation[9]. Heat shock-induced flippase-flippase recognition target (FLP-FRT)-mediated site-specific mitotic recombination creates homozygous clones of the functionally null *drpKG03815* allele. The genotype of *Drosophila* carrying the Drp1 mutant is *drpKG03815* FRT40A/CYO, whereas the genotype carrying the heat shock-induced FLP (hsFLP) and UbiGFP clonal marker is hsflp; ubiquitin nls-GFP (UbiGFP) FRT 40A/CyO. The genotype of the selected offspring of the cross between the above genotypes is hsFLP/+; *drpKG03815*FRT40A/UbiGFPFRT40A.

6.1. Synchronize the *Drosophila* for virgin collection by moving them into new vials of fresh food every 2 to 3 days. Monitor the pupariating vials daily in order to collect emerging virgin females.

6.2. Collect red-eyed, curly-wing virgin females from the Drp1 mutant genotype every day, once in the morning and once in the evening, and place them into a separate vial. In parallel, collect male *Drosophila* with dark red eyes and straight wings carrying hsflp and UbiGFP within 5 days of eclosion.

6.3. Set up a cross by adding the males to the virgin females with a female:male ratio of 2:1.

6.4. Sprinkle a small amount of granulated yeast to stimulate egg production.
NOTE: Carefully move the *Drosophila* to a fresh vial of media every 2 to 3 days to increase the amount of progeny and to reduce vial crowding.

6.5. Anesthetize, sort, and collect straight-winged, red-eyed female progeny within 5 days of eclosion.

6.6. For the heat shock, place the collected *Drosophila* into empty vials with a small amount of granulated yeast and a small, soft wipe (to absorb the moisture during the heat shock). Place the vial with the *Drosophila* in a water bath at 38 ºC for 1 h, to generate primarily follicle cell clones, and at 37 ºC for 1 h twice a day (allowing at least 5 h between the 2 heat shocks) for 2 consecutive days, to generate both germline and follicle cell clones.
NOTE: Make sure that the vial is completely submerged in the water up to the level of the plug.

6.7. The heat shock may make the *Drosophila* immobile. Allow the heat-shocked *Drosophila* to recover for 1 h at room temperature when they become mobile again.

6.8. Add the males back to the females in the same proportion as in the cross, and move them to fresh vials with medium sprinkled with a small amount of granulated yeast. Maintain the *Drosophila* for at least 5 days.

6.9. Dissect the ovaries as necessary for a live or fixed experiment.
NOTE: Ovaries isolated from the parental *Drosophila* expressing UbiGFP should be used as negative controls to confirm the efficient induction of GFP-negative clones by the heat shock.

7. Co-immunostaining for Cyclin E and mitochondria

NOTE: To detect *Drosophila* Cyclin E (dCyclinE), we have used a commercially-obtained antibody raised specifically against dCyclinE[9] (see Materials Table). As a mitochondrial marker, we used an antibody against ATP-B (a subunit of the mitochondrial ATP synthase complex)[9].

7.1. Warm 4% paraformaldehyde (PFA) to room temperature, 25 °C. **Caution!** Paraformaldehyde is toxic.
NOTE: After opening the ampule, PFA should be stored at 4 °C and used within 7 days. This is because storage of PFA may allow oxidation to methanol, which would dramatically alter mitochondrial membranes during fixation, even if present in trace amounts.

7.2. Immediately after the dissection, fix the dissected ovaries by placing them in 200 µL of fresh PFA in a well of a glass dissecting dish (without teasing). Keep the dish in a fume hood for 15 min. Wash the fixed ovaries by moving them carefully with the forceps into 3 consecutive wells, each containing 200 µL of 1X phosphate-buffered saline (PBS).
NOTE: The experiment can be stopped here and the samples can be left at 4 ºC for 1 day.

7.3. Tease the ovaries more thoroughly in PBS (similar to step 2.6) to carefully remove the protective fibrous sheath that may hinder antigen access by the antibody.

7.4. Permeabilize the teased ovaries by placing them in a microfuge tube with 500 µL of freshly-made 0.5% PBS-Triton-X100 (PBS-TX) and incubate it for 30 min while rocking at 25 rpms.
NOTE: During the rocking, place the tubes parallel to the rocking movement; placing them perpendicularly may allow the tissue to stick to the cap of the tubes, thus resulting in tissue loss.

7.5. To remove the PBS-TX, place the microfuge tubes in a rack to allow the tissue to settle down at the bottom of the tubes. Inspect them visually and tap the tubes, if necessary, to bring any floating tissues down to the bottom. Aspirate the solution carefully from the top, making sure that the tissue at the bottom of the tubes remains undisturbed.

7.6. Block the ovaries by adding 200 µL of 2% bovine serum albumin (BSA) dissolved in 0.5% PBS-TX, and incubate it on the rocker at room temperature for 1 h. Remove the blocking agent by following step 7.5.

7.7. Add primary antibodies in 200 µL of fresh blocking agent: anti-rabbit dCyclinE antibody (1:100) and anti-mouse ATP-B antibody (1:100). Incubate the tissues on the rocker for 2 h at room temperature.

7.8. Wash the ovaries with PBS-TX 3 times for 15 min each, following step 7.5.

7.9. Add 200 µL of the appropriate secondary antibodies in fresh PBS-TX: anti-mouse-CY3 (1:1,000) and anti-rabbit-CY5 (1:500). Incubate on the rocker for 1 h at room temperature.

7.10. Wash the ovaries with PBS-TX 3 times for 15 min each, following step 7.5.

7.11. To stain the DNA, add Hoechst (1:1,000 dilution) to the final PBS-TX wash.

7.12. Finally, leave the tissue in 500 µL of 1X PBS.

7.13. Using a 1-mL micropipette, remove the immunostained ovaries from the microfuge tube into a fresh well of a glass dissecting dish containing 200 µL of PBS.

7.14. Add one drop of glycerol-based mounting medium to a glass slide and add the immunostained ovaries one by one to the mounting medium.
NOTE: Make sure that the ovaries are indeed transferred to the mounting medium. Failing to do so will lead to tissue loss.

7.15. While looking through the dissection microscope, gently pluck the transparent ovarioles (younger stages) from the opaque mature egg chambers using the teasing needle while holding the opaque portion of the ovary with the forceps. Remove the mature egg chambers from the mounting medium.

7.16. Place a coverglass (22 mm, No. 1) on the slide and press lightly to ensure that the mounting medium is spread uniformly underneath.
NOTE: Pressing on the coverglass also ensures the proper alignment of the tissue along the coverglass. Failing to do so optimally may allow the smaller stages to float in the mounting medium, preventing optimal microscopy of those tissues.

7.17. Air-dry the samples for 15 min and seal the edges of the coverglass carefully with nail polish.

7.18. Perform confocal microscopy as per experimental need (Table 1).

REPRESENTATIVE RESULTS:
The described methods can be used to study mitochondrial structure and function in live and fixed *Drosophila* ovaries (Figure 2B). Provided are some examples of anticipated results obtained with the described methods.

Dissection of the *Drosophila* ovary: When dissected further, the severed abdomens (Figure 3B) from the whole *Drosophila* (Figure 3A) should release the abdominal contents, including 2 ovaries from each individual *Drosophila*. The intact ovaries appear as oval-shaped, white structures (arrows in Figure 3C and D) that ideally should remain attached to each other through the oviductal stalk. The ovarioles in each ovary should be held together by the fibrous sheath (# in magnified portion of Figure 3C), which is removed by careful teasing (thick arrow in Figure 3D; * indicating a badly-teased ovary with detached ovarioles) to expose the ovarioles for staining and microscopy. The released ovaries with oviductal stalks torn during the release of the

abdominal contents can also be used, provided they are not otherwise damaged. Any damaged ovaries (* in Figure 3C) and other abdominal contents (arrowhead in Figure 3C) should be discarded.

Live microscopy of the *Drosophila* ovary: Here, we demonstrate the FLIP technique and the loading of mitochondrial structural and functional dyes in *Drosophila* ovaries.

The FLIP technique, previously applied to assess mitochondrial continuity in the *Drosophila* follicle cells[9], has been demonstrated here in the ovarian nurse cells of transgenic *Drosophila* expressing the mitochondrial matrix probe mito-YFP under a constitutive squash promoter. The FLIP protocol involving iterative photobleaching and intermittent recovery periods in the FLIP ROI allows for a loss of signal in the FLIP ROI, as well as in the surrounding regions if they are in continuity with the FLIP ROI (Figure 4A). FLIP targeted to one of the mitochondrial clouds associated with the nurse cells leads to a greater than 50% loss of the fluorescent signal from the blue and white ROIs surrounding the red FLIP ROI within 200 s (Figure 4B and C); the signal from the green ROI is used for background correction. The fluorescent signal from the yellow ROI in the other untargeted nurse cells remains constant throughout this time frame, signifying minimal overall bleaching during the time course of the experiment (Figure 4B, and C). Also, see Video 1. This result indicates that the mitochondria in nurse cells have fused their outer and inner membranes, exemplified in steps 3 and 4 (Figure 4A), while mitochondria in steps 1 and 2 are not expected to show any loss of fluorescence in this time frame.

It has been previously demonstrated that a semi-quantitative measure of mitochondrial potential per unit mitochondrial mass can be assessed by co-staining with the potentiometric dye TMRE and the overall mitochondrial stain that reflects mitochondrial mass[13]. Here, the same technique was used to demonstrate the assessment of mitochondrial potential per unit mass in live *Drosophila* ovarian tissue. Confocal microscopy of live *Drosophila* ovaries stained with TMRE demonstrates a decrease in signal with the depth of the tissue (Figure 5A). This loss of fluorescent signal due to increasing scatter in greater tissue depth is expected to occur with any kind of fluorophore with a given working distance of the objective in use. Therefore, it is important to discern the maximal depth of the tissue within which the fluorophore can be detected during live tissue imaging. For example, live *Drosophila* ovaries stained with the overall mitochondrial stain (Mito) can be imaged using the described microscope setup (Table 1) within a range of 20 µm from the coverslip, while the maximum imageable depth decreases with increasing egg chamber size (Figure 5B). The optimized microscope settings for the mitochondrial stains detect positive signals in the appropriate detection channels, while the settings to examine any undue signal cross-talk or fluorophore cross-excitation expectedly detected minimal or no signal (Figure 6A). Co-staining of TMRE with the overall mitochondrial stain can be used for both qualitative (Figure 6B) and quantitative (Figure 6C and D) assessments of mitochondrial potential per unit mass in live *Drosophila* ovaries. For example, a) a qualitative analysis demonstrates that the *Drosophila* germarium exhibits higher mitochondrial potential per unit mass in stage 2b where the somatic follicle cells have arisen to encapsulate the germline cells (arrow heads in Figure 6B) and b) semi-quantitative analyses demonstrate the difference in mitochondrial potential per unit mass between (stretch) AFCs and the MBCs, as analyzed from a stage 9 egg chamber (Figure 6C). Note that the quantification has been performed from 2 different optical slices for AFCs and MBCs, depending on their plane of focus.

The use of the mito-ROS probe that has been successfully used in *Drosophila*[14] is demonstrated in live *Drosophila* ovarian epithelial follicle cell layers, where functional null clones of the mitochondrial fission protein Drp1were introduced. Most, but not all, of the live GFP-negative Drp1 null follicle cell clones exhibit elevated staining for mito-ROS (Figure 7A and B). Note that although a distinct mitochondrial signal could not be detected in this tissue, the concentration of the mito-ROS stain could be detected in the nuclear region in the advanced post-mitotic follicle cells, which are significantly larger than the mitotic follicle cells (* in Figure 7C). This may occur due to the interaction of nuclear DNA and the fluorescent oxidation product of the mito-ROS dye, which is a derivative of ethidium[15].

Detection of mitochondrial Cyclin E in fixed *Drosophila* ovarian follicle cells: It has been recently demonstrated that mitochondria can regulate the cell cycle molecule Cyclin E by recruiting it to the mitochondrial surface in mammalian cells and the *Drosophila* follicle cell layer[12]. Specifically, the repression of Drp1 enhances the mitochondrial Cyclin E pool in mammalian cells and the *Drosophila* follicle cell layer[12]. Here, an example of mitochondrial localization of Cyclin E in the *Drosophila* follicle cells is demonstrated using the co-immunostaining protocol described. Note the elevated levels of dCyclinE in the GFP-negative Drp1 null clones where the dCyclinE signal overlaps with that of the mitochondrial ATP-B signal in the germarium (Figure 8A), although the signal cannot be resolved into distinct mitochondrial elements with the limited resolution of confocal microscopy. Also, in post-mitotic MBCs, the GFP-negative Drp1 null clones have both nuclear and cytosolic dCyclinE signals, with the latter significantly overlapping with the ATP-B signal, whereas the wildtype GFP-positive cells only have a nuclear dCyclinE signal (Figure 8B).

FIGURE LEGENDS

Figure 1. Development of the *Drosophila* egg chambers. Cartoon depicting a *Drosophila* ovariole consisting of a chain of developing egg chambers, each composed of an oocyte and the nurse cells (germline) encapsulated by the follicle cell layer (somatic). The egg chambers develop from the germarium and develop through stages where the mitotic follicle cells become post-mitotic.

Figure 2. Experimental plan and preparation. (A) Tools used in the methods described: A. Fly vial; B. Insect dissecting medium; C. Paraformaldehyde; D. Fly brush; E. Dissecting dish; F. Cover glass; G. Microfuge tube. H. Glass-bottomed dish; I. Glass slide; J. 1,000-µL micropipette; K. 200-µL micropipette; L. 2.5-µL micropipette; M. Teasing needle with a bent tip (tip is magnified); N. Thick forceps; O. Thin forceps. **(B).** A flow chart schematic representing the methods described.

Figure 3. Dissection of *Drosophila* ovaries. (A) Anesthetized whole *Drosophila*. **(B)** Severed *Drosophila* abdomens with or without (*) the abdominal contents. **(C)** Released *Drosophila* abdominal contents, including the ovaries (arrows and *) and other contents (arrowheads); magnification of the boxed region on the right highlighting the anterior (Ant) and posterior (Post) ends of the pair of ovaries held by the oviductal stalk, where the ovarioles are held by the fibrous sheath (#). **(D)** Teased *Drosophila* ovary (thick arrow marking appropriately teased and * marking badly teased) in comparison to unteased ovary (thin arrow).

Figure 4. Live-tissue FLIP assay. (A). Schematic representing the steps of mitochondrial fission/fusion, which can be assayed by an appropriate mitochondrial continuity assay using the FLIP method; in this case, a mito-YFP probe in the mitochondrial matrix allows for the assessment of mitochondrial matrix continuity. **(B).** Time-lapse *ex vivo* microscopy of a live transgenic *Drosophila* egg chamber expressing mito-YFP; only select time points (t) are shown. See Video 1 for all the time points. **(C)** Quantitation of the fluorescent signal from the respective colored ROIs in (B) are expressed after background correction and normalization. The arrowheads indicate the reiterative photobleaching time points, whereas the time interval between two consecutive bleaching events is the recovery time. Scale bar: 10 µm. The images were acquired with the parameters described in Table 1.

Figure 5. Live mitochondrial staining and microscopy of the *Drosophila* ovariole. (A) Individual optical slices of a live *Drosophila* ovariole stained with TMRE; the image was acquired with the parameters described in Table 1. Z denotes the distance from the coverslip in µm. **(B)** A live *Drosophila* ovariole loaded with the overall mitochondrial stain (Mito). The X-Z image of the red line in the X-Y image is shown, where B is the bottom and T is the top of the acquired image. The distance from the bottom to the blue line is 20 µm. Scale bar: 20 µm. The confocal images were acquired with the parameters described in Table 1.

Figure 6. Co-staining of live *Drosophila* ovarioles with TMRE and the overall mitochondrial stain. (A) Single optical slice of a live *Drosophila* ovariole co-stained with TMRE and the overall mitochondrial stain (Mito); * indicates an experimental artifact described in the Discussion section. **(B)** Maximum-intensity projection of 3 consecutive optical slices of the germarium in (A); arrowheads indicate the region where the TMRE fluorescence is increased. **(C)** Single optical slice of post-mitotic egg chamber co-stained with TMRE and the overall mitochondrial stain. The top and bottom panels show the plane of focus for AFCs and MBCs, respectively. **(D)**. Box plot showing mean fluorescent intensity of TMRE and the overall mitochondrial stain quantified from individual AFCs and MBCs in the boxed regions in (C). The whiskers of the box plot indicate the maximum and minimum values for each group, excluding the outliers. **(E)** Box plot showing the ratio of fluorescent signal from TMRE and the overall mitochondrial stain in individual cells from (D). The whiskers of the box plot indicate the maximum and minimum values for each group, excluding the outliers. Scale bar: 20 µm. The confocal images were acquired with the parameters described in Table 1.

Figure 7. Staining of live Drp1 null mosaic *Drosophila* ovarioles with the mito-ROS dye. (A) Single optical slice of ovarioles stained with the mito-ROS dye. **(B)** Maximum-intensity projection of 2 consecutive optical slices of an individual egg chamber with follicle cells in the mitotic stage stained with the mito-ROS dye. **(C)** Single optical slice of an individual egg chamber with follicle cells in the post-mitotic stage stained with the mito-ROS dye; * indicates a nuclear signal. The lack of UbiGFP marks the Drp1 null clones. Scale bar: 20 µm. The confocal images were acquired with the parameters described in Table 1.

Figure 8. Co-immunostaining of fixed Drp1 null mosaic *Drosophila* ovarioles with dCyclinE and ATP-B antibodies. (A) Maximum-intensity projection of 3 consecutive optical slices of co-immunostained germarium. **(B)** Maximum-intensity projection of 3 consecutive optical slices of co-immunostained post-mitotic MBCs. The outlines demarcate the UbiGFP-negative Drp1 null

clones. Scale bar: 10 µm. The confocal images were acquired with the parameters described in Table 1.

Figure 9. Examples of potential damage and artifacts. (A) Germarium of UbiGFP-expressing ovariole fixed and stained with the DNA dye Hoechst showing undue gaps (*). **(B)** Egg chamber of UbiGFP-expressing ovariole fixed and stained with the DNA dye Hoechst showing nicks/tears (*). **(C)** Live ovariole with UbiGFP-negative Drp1 null clones stained with the mito-ROS dye showing a deformed chamber (*) and a damaged egg chamber, allowing partial staining of the germline (**); # denotes a possibly real stain of an undamaged egg chamber in the same ovariole. **(D)** Live ovariole stained with the mito-ROS dye showing an overall damaged chamber with intense artifactual staining of the germline. * indicates damage-induced flattened ovarioles with a loss of UbiGFP signal, thus giving rise to artifactual GFP-negative clones. Scale bar: 20 µm. The confocal images were acquired with the parameters described in Table 1.

Video 1. Live-tissue FLIP assay. The complete time course of the photobleaching-based FLIP assay described in Figure 5.

Table1: Confocal microscope settings for the acquisition of the presented representative images.

DISCUSSION:

Critical steps within the protocol.

Photobleaching: Preventing undue photobleaching of fluorescent samples is absolutely necessary to performing efficient confocal microscopy. Therefore, the time used to locate samples through the eyepiece or to set image acquisition parameters through the live scanning mode should be minimized to minimize photobleaching.

Tissue damage: Since mitochondria are considered to be the sensors of cellular health, it is extremely important to ensure that the data obtained using the described methods are physiologically relevant and do not reflect the undue damage caused by the procedural dissection of the *Drosophila* ovaries. The dissection should be performed as quickly as possible, but also with extreme precaution to minimizing tissue damage. The average time for the dissection and teasing of 5 *Drosophila* ovaries is 5 to 7 min (in our hands). Effort must be taken to identify egg chambers showing damaged tissue that will be excluded from the analyses. Tissue damage due to dissection may include undue gaps (* in Figure 9A, as identified by the lack of signal), nicks/tears (* in Figure 9B, as identified by the lack of signal), or deformation of the egg chambers (* in Figure 9C). However, any meaningful chamber deformation arising from the experimental manipulation should be carefully evaluated. Although no coverslip is used on top of the live tissue in the protocol described, flattening of the ovarioles may occur with undue pressure applied to the tissue while teasing or mounting (* in Figure 9D). The flattening of ovarioles has not been observed with fixed samples, since the protocol described involves the fixing of tissues immediately after dissection, with teasing occurring thereafter. Any isolated egg chamber without any signatures of the aforementioned damage may be considered normal. Mechanical damage potentially resulting from dissection may also lead to the loss of GFP, yielding false GFP-negative clones[16], and can also give rise to enhanced dye incorporation. Given that the germline cells residing inside the egg chambers are not normally permeable to the

live mitochondrial dyes (Figures 6 and 7), the enhanced mitochondrial staining of the *Drosophila* germline cells may result from an increase in the permeability of the damaged/dying tissue; such an artefact occurs with the mito-ROS dye (** in Figure 9C and the whole ovariole in Figure 9D), as well as with other live mitochondrial stains (not shown). The loss of UbiGFP in Figure 9D (*) could also result from damage that caused the flattening of the egg chambers. Although other egg chambers in the damaged ovariole may remain authentic and artifact free (# in Figure 9C), it is best to exclude the whole ovariole from the analyses. Similar damage can also allow augmented access of antigens to the antibodies during the immunostaining procedure, resulting in a significant increase of immunostaining in the damaged zones. Therefore, the egg chambers with enhanced live or immunostaining in comparison to the rest of the population should be excluded from the analyses.

Time sensitivity: In the case of live *ex vivo* microscopy, the data should be obtained within 15 min of tissue mounting; otherwise, the experimental results may be affected by alterations of physiology due to the ensuing death of the tissue. Sometimes, depending upon the laser power and other scanning parameters, the 10 µL of mounting medium may evaporate sooner than 15 min. The detection of the mitochondrial Cyclin E pool by co-immunostaining requires the minimization of dissection time (likely due to the transient nature of the mitochondrial Cyclin E pool that is maintained by active mitochondrial respiration[12]). An appreciable mitochondrial Cyclin E pool was also not observed with permeabilization times less than 30 min.

FLIP: The movement of any egg chamber under examination during the FLIP time course may lead to artifactual analyses and thus must be excluded. The use of any live mitochondrial dyes or TMRE is not recommended in a FLIP experiment, since incorporation of the foreign dye may alter the mitochondrial continuity and thus lead to artifactual results.

Drosophila crossing strategy: The cross reciprocal to the one described here (where males carrying the Drp1 mutant allele are used) does not yield many offspring, likely due to an unidentified impact of Drp1 repression in the heterozygous male parents.

Interpretation of clonal data: Any GF-negative clones detected in the control ovaries isolated from the parental *Drosophila* expressing UbiGFP would indicate artifactual false-negative clones, likely generated due to GFP loss caused by damage during the dissection[16]. Nonetheless, the number of GFP-negative clones in the heat-shocked progeny of the cross should be significantly higher than any false-negative clones seen in the controls. The clones generated in the *Drosophila* follicle cell layer by the described mitotic recombination-based strategy can arise from the mitotic follicle stem cells (stem cell clones) or from the mitotic non-stem follicle cells (transient clones). For stem cell clones, the experimental analyses should be performed only 10 days after the heat shock, when the egg chambers with the transient clones have already been laid. For transient clones, the analyses should be performed within 6 days after the heat shock, with the follicle cell clones of the ovarioles without any follicle cell clone in the germarium.

Modifications and troubleshooting.

While assessing mitochondrial potential per unit mass using the described method, one has to be aware of potential artifacts arising out of the microscope optics or mitochondrial dye combinations. Any region showing a weakened signal from the overall mitochondrial stain and a

comparatively elevated TMRE signal (Figure 5A, *) may arise due to fluorescence resonance energy transfer (FRET) between the dyes[8] or due to different optical properties of the red (TMRE) and green (overall mitochondrial dye) fluorescent lights, given a fixed pinhole size in the acquisition setup. The FRET-related artifact could be avoided by reducing the dye concentrations, if possible, while quantitative analyses can be performed on projections of 2-3 consecutive optical slices to avoid the optical artifact. Also, if a fluorescent signal is detected in the crosstalk and cross-excitation channels, the laser and detector settings must be readjusted to minimize the signal in these channels, which is expected to reduce the fluorescent signal in the optimal channels as well.

Laser-induced damaged from the reiterative photobleaching may cause mitochondrial fragmentation and thus cause mitochondrial discontinuity, preventing a successful FLIP. Suspected fragmentation of mitochondria during the execution of the FLIP protocol can be identified by acquiring higher-resolution images with a reduced pinhole (and enhancing the detector gain). If fragmentation of mitochondria is visibly noticed during the execution of the FLIP protocol, the power of the bleaching laser must be reduced and/or the interval between consecutive bleaches must be increased. If there is overall bleaching during the time course of the FLIP experiment (signal from the yellow ROI in Figure 3B and C), the scanning laser must be readjusted to a level that allows minimal or no overall photobleaching.

To avoid the potential artifact due to damage-induced loss of GFP, GFP-positive clones can be introduced using the MARCM strategy, following the described heat-shock protocol. Here, the appropriate *Drosophila* to be used for the cross is virgin female drpKG03815 FRT40A/CYO X male Gal80 FRT 40A/CyO; tub-Gal4, UAS-CD8GFP/TM6[9]. The straight-wing progeny should be used for clone generation using heat shock (as described in step 6).

Limitations of the technique.

A) While the follicle cells residing on the surface of the live *Drosophila* egg chambers incorporate the mitochondrial staining dyes, the germline cells inside the egg chamber fail to do so; the germline of the younger stages stain weakly (Figures 5 and 6). **B)** The live *ex vivo* tissue microscopy msut be performed within 15 min of completion of the staining on ovaries isolated from individual *Drosophila*, one at a time. Since experimental groups should be processed and imaged on the same day, the total time taken to obtain statistically-significant data from live *ex vivo* tissue microscopy from various experimental groups is reasonably longer than those obtained from fixed tissues. **C)** 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[17,15], 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/clones cannot be ruled out and must be investigated further. Note that the positive signal from the mito-ROS stain may not just reflect enhanced superoxide but also the enhanced mitochondrial or cellular oxidative environment[15]. **D)** The overall mitochondrial stain cannot be used for the GFP-negative or GFP-positive clonal experiments, since the fluorescent spectrum of this mitochondrial stain and GFP are indistinguishable. **E)** The FLIP experiment designed to assay mitochondrial matrix continuity would require image acquisition with an open pinhole that encompasses the resolution of the mitochondrial structures.

Significance of the technique with respect to existing/alternative methods.
Studying the mitochondrial structure-function relationship is crucial for a thorough understanding of the regulatory mechanisms of mitochondrial functionality. Because mitochondrial defects significantly contribute to various diseases, including diabetes, cancer, Parkinson's disease, a detailed understanding of the mitochondrial *modus operandi* holds the promise of facilitating the development of mitochondria-directed therapeutic strategies. Live-cell microscopy is an indispensable tool for studying the mitochondrial structure-function relationship. However, most of these studies have been restricted to isolated cells. The study of mitochondrial structure and function has been extended to live *Drosophila* tissue in an *ex vivo* setup[9]. The described protocol of this and related studies will lead to an understanding of mitochondrial structure and function in live *Drosophila* ovaries, *ex vivo*, allowing an understanding of mitochondria in a physiological context. This *ex vivo* approach has significant advantages over *in vitro* studies, since the regulation of the metabolic and energetic properties of mitochondria in a given cell is largely dependent upon the nutrient availability and appropriate signaling in the physiological context of the cells.

Genetic manipulation of mitochondrial structure by repressing the mitochondrial fission protein Drp1 deregulates the cell cycle modulator Cyclin E and affects the development of the *Drosophila* ovarian follicle cell layer[9,12]. Here, the protocol includes a description of how to introduce functionally-null clones of Drp1 in the *Drosophila* ovaries to study the mitochondrial structure-function relationship. A novel pool of mitochondrial Cyclin E on mammalian cells, as well as the *Drosophila* follicle cell layer[12], has been successfully detected, which likely underlies the reported Cyclin E regulation by mitochondria[18]. Here, the manuscript demonstrates a method for detecting the novel mitochondrial Cyclin E pool by co-immunostaining fixed *Drosophila* ovaries for dCyclinE and a mitochondrial marker (ATP-B).

Future applications or directions after mastering this technique.
Given that *Drosophila melanogaster* is a powerful genetic model system, our described methods are anticipated to contribute significantly to the understanding of the genetic basis of the mitochondrial structure-function relationship in health and disease. *Drosophila* has been widely used to tease out the genetic interactions leading to tumorigenesis[19]. The capability of the generation of clones in the *Drosophila* epithelial follicle cell layer allows the study of the interaction of mitochondria and oncogenes or tumor suppressors in individual tumorigenic clones in an *in vivo* and *ex vivo* setup. The described methods can be used to study the regulation of the mitochondrial structure-function relationship on ovaries isolated from appropriate mutant *Drosophila.* The described methods can be further extended to study the direct impact of various nutrient and growth factor signaling on mitochondrial structure and function through the exogenous addition of the appropriate nutrients and/or growth factors in the *ex vivo* imaging medium. The described methods can be appropriately modified and employed in other isolated *Drosophila* tissues, like the larval imaginal discs, which have been widely used to study signal transduction pathways in diseases like cancer[20,21].

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DISCLOSURES:
The authors have no competing financial interests.

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Figure

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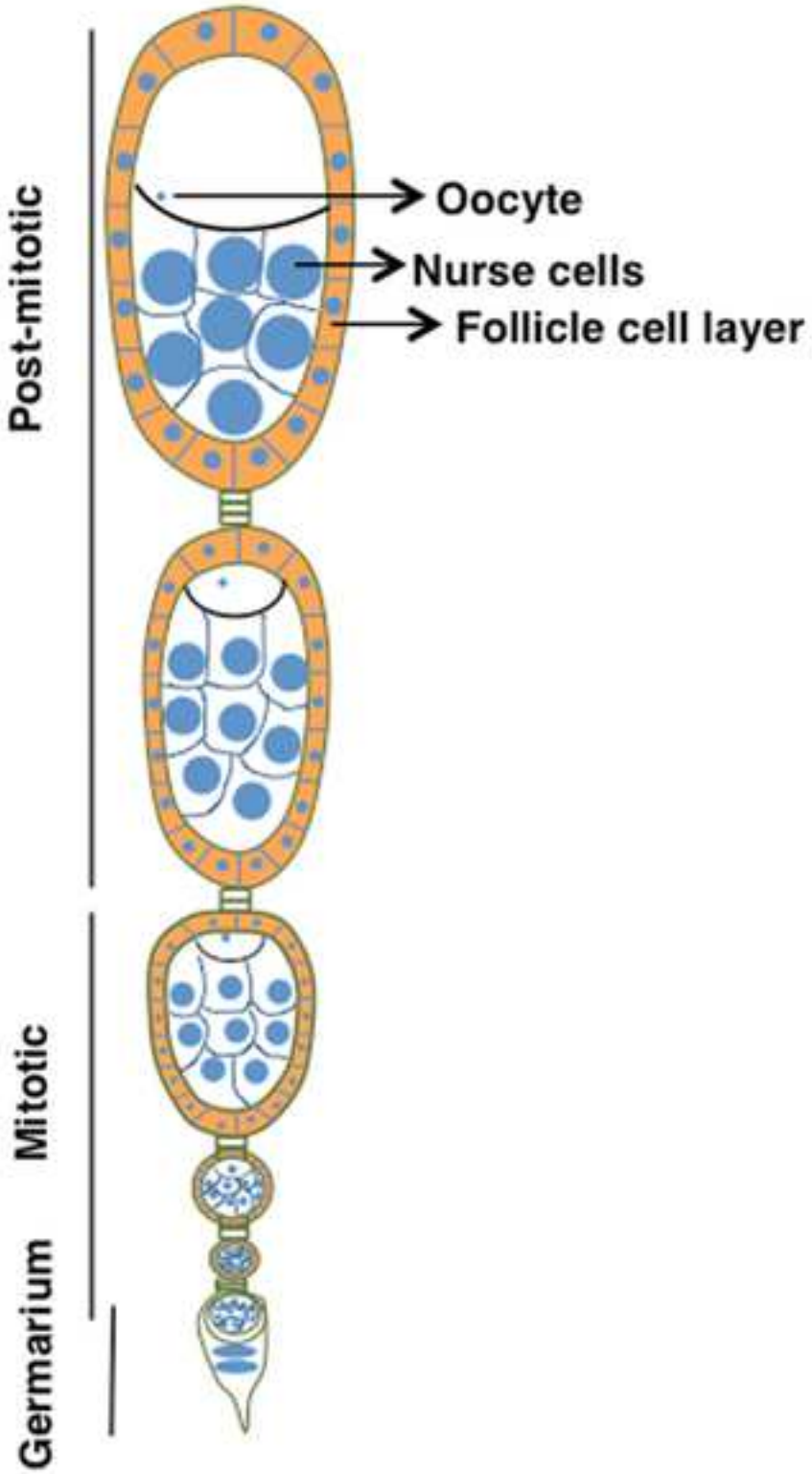
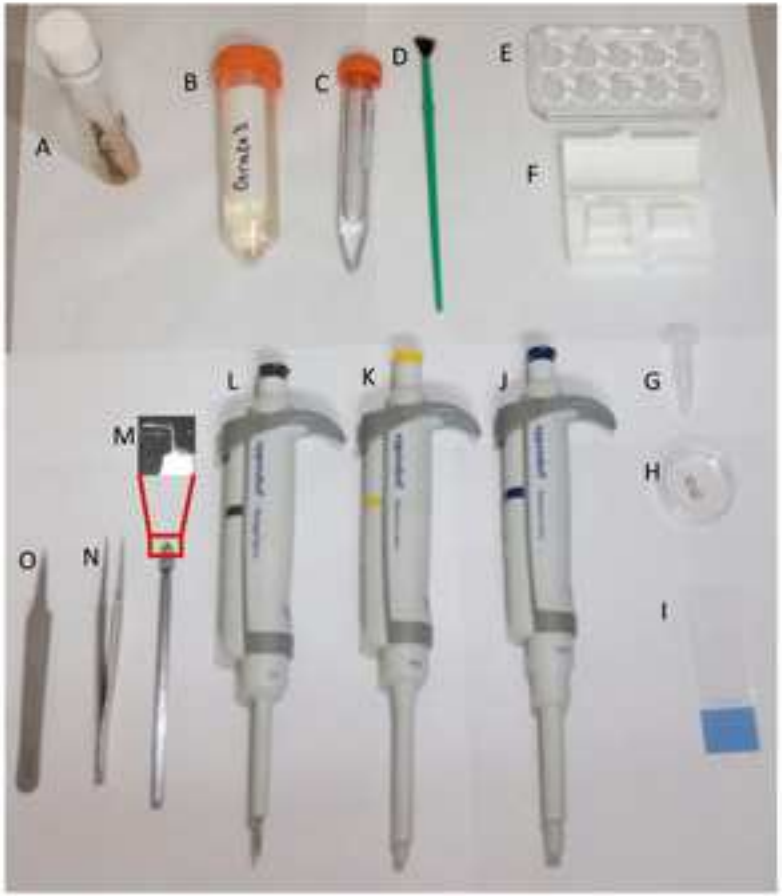


Figure 1

A



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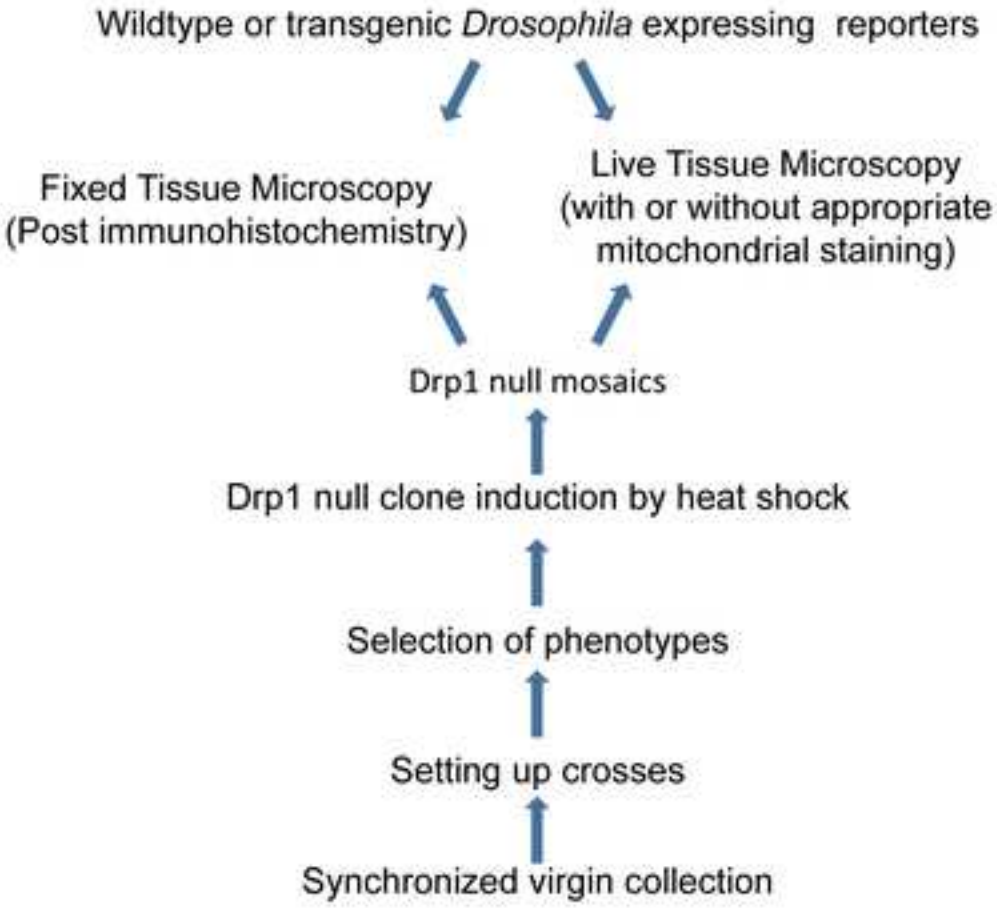

Figure 2

Figure

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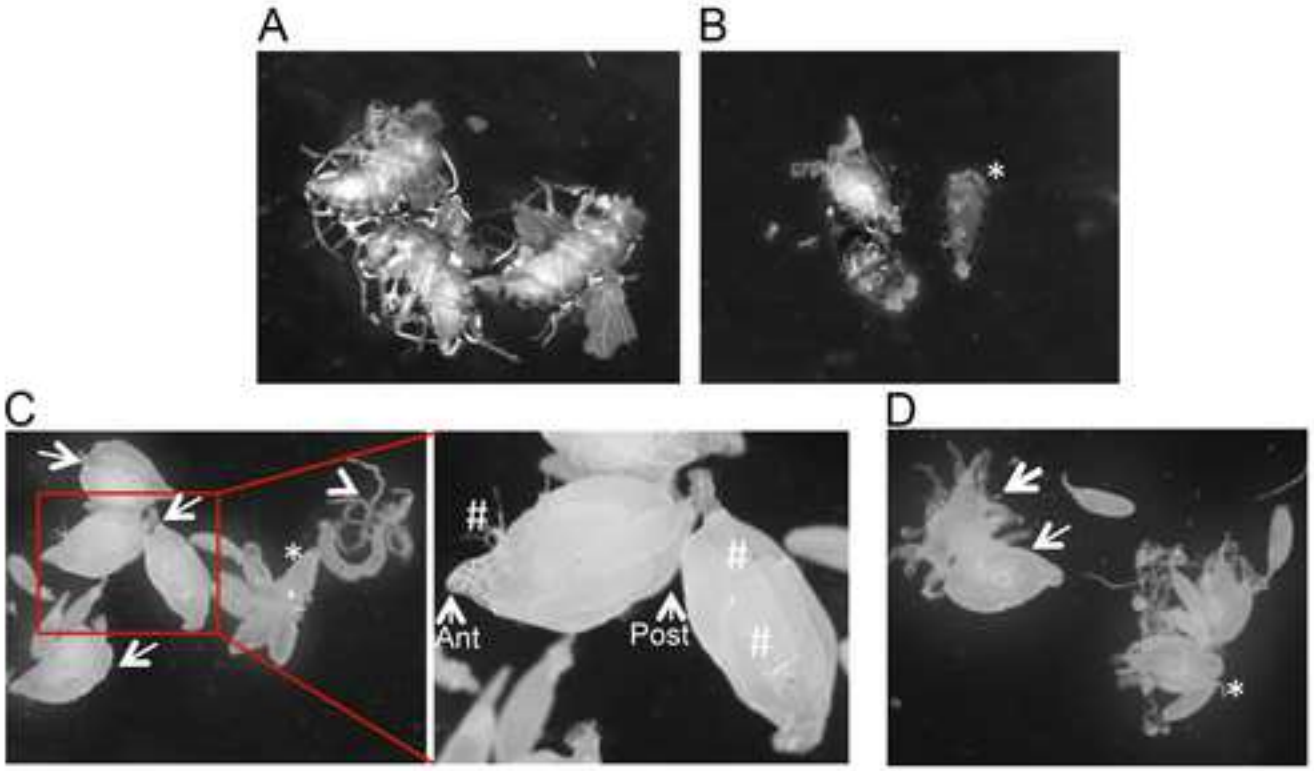

Figure 3

Figure

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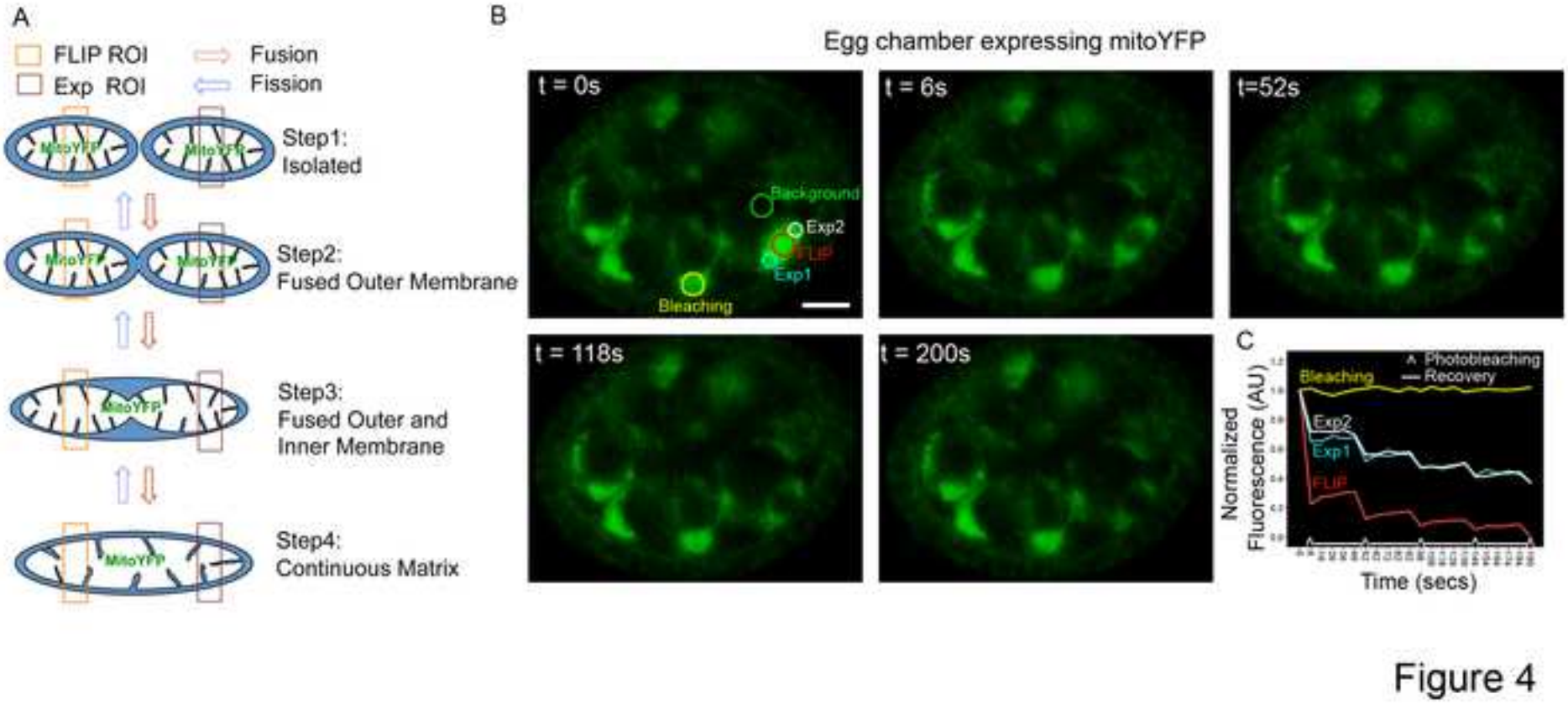

Figure 4

Figure

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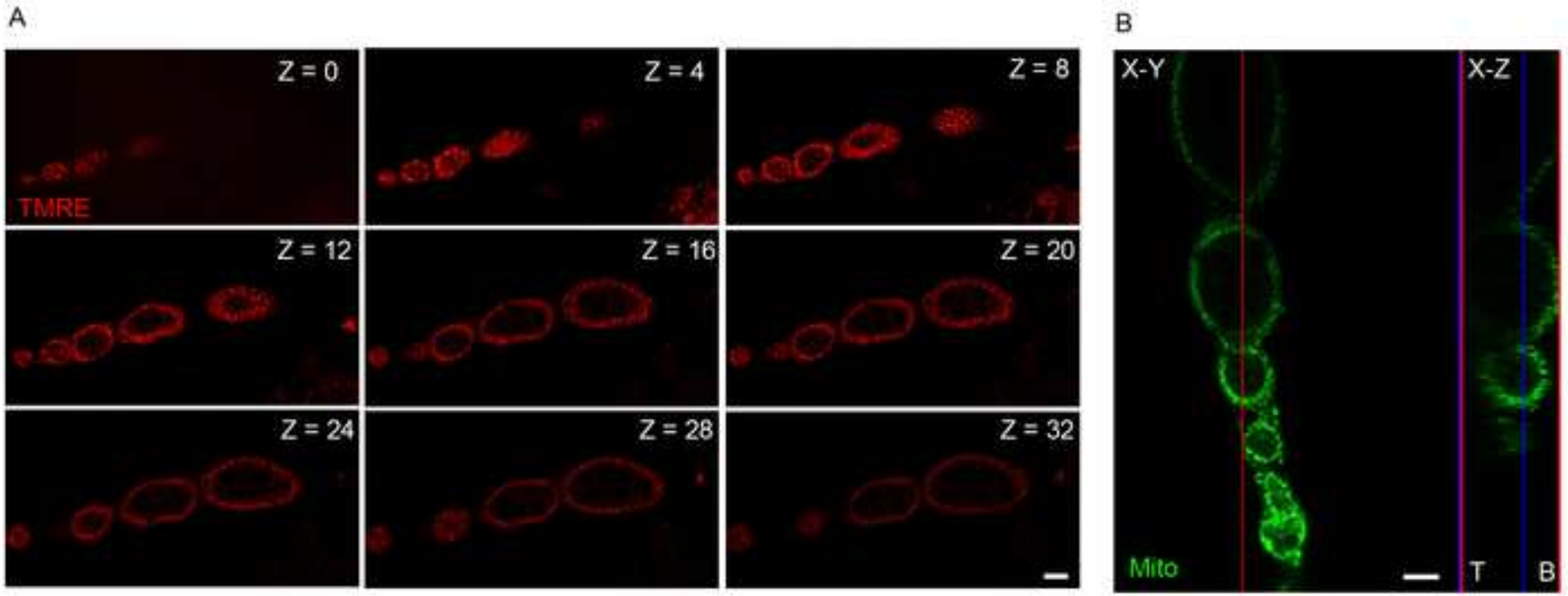

Figure 5

Figure

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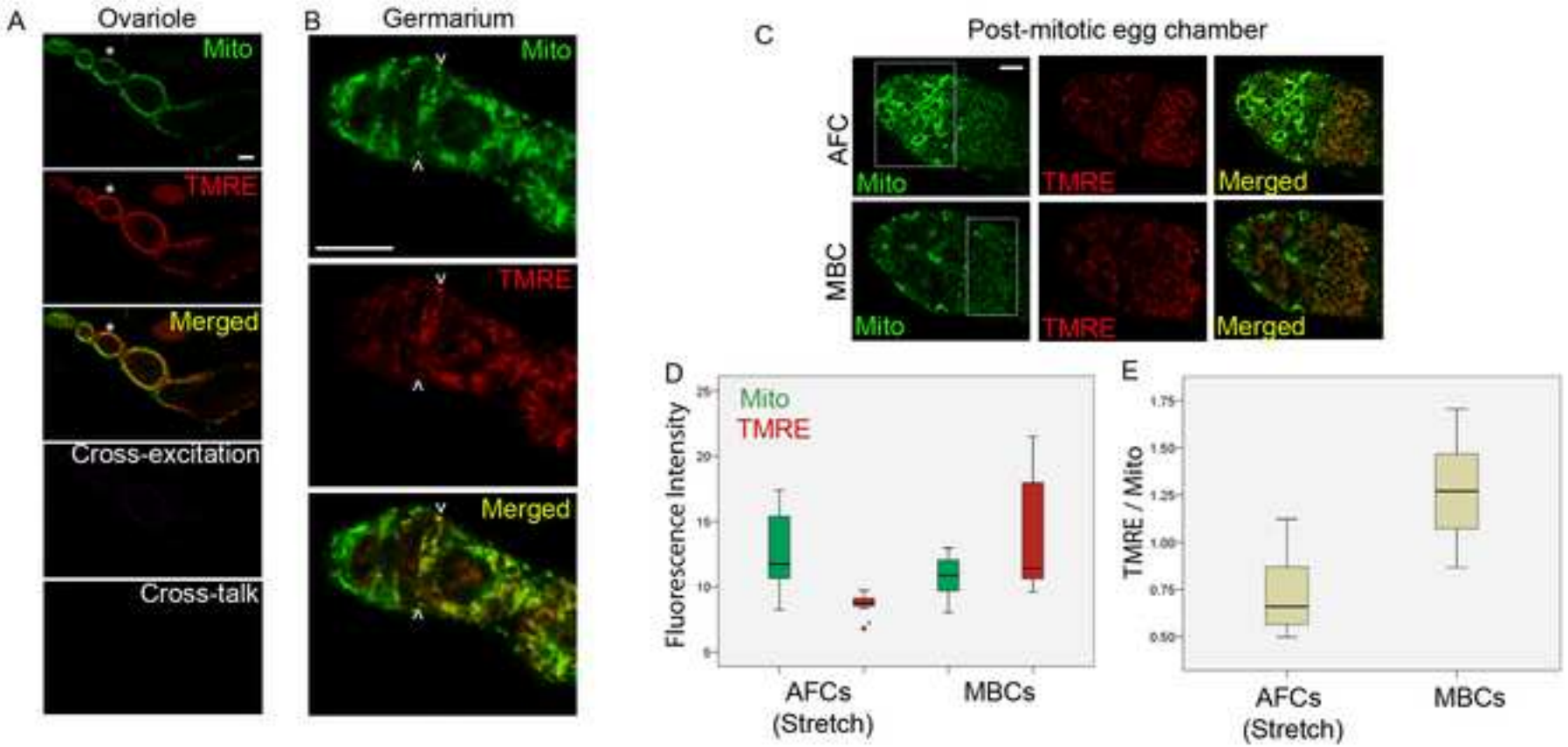

Figure 6

Figure

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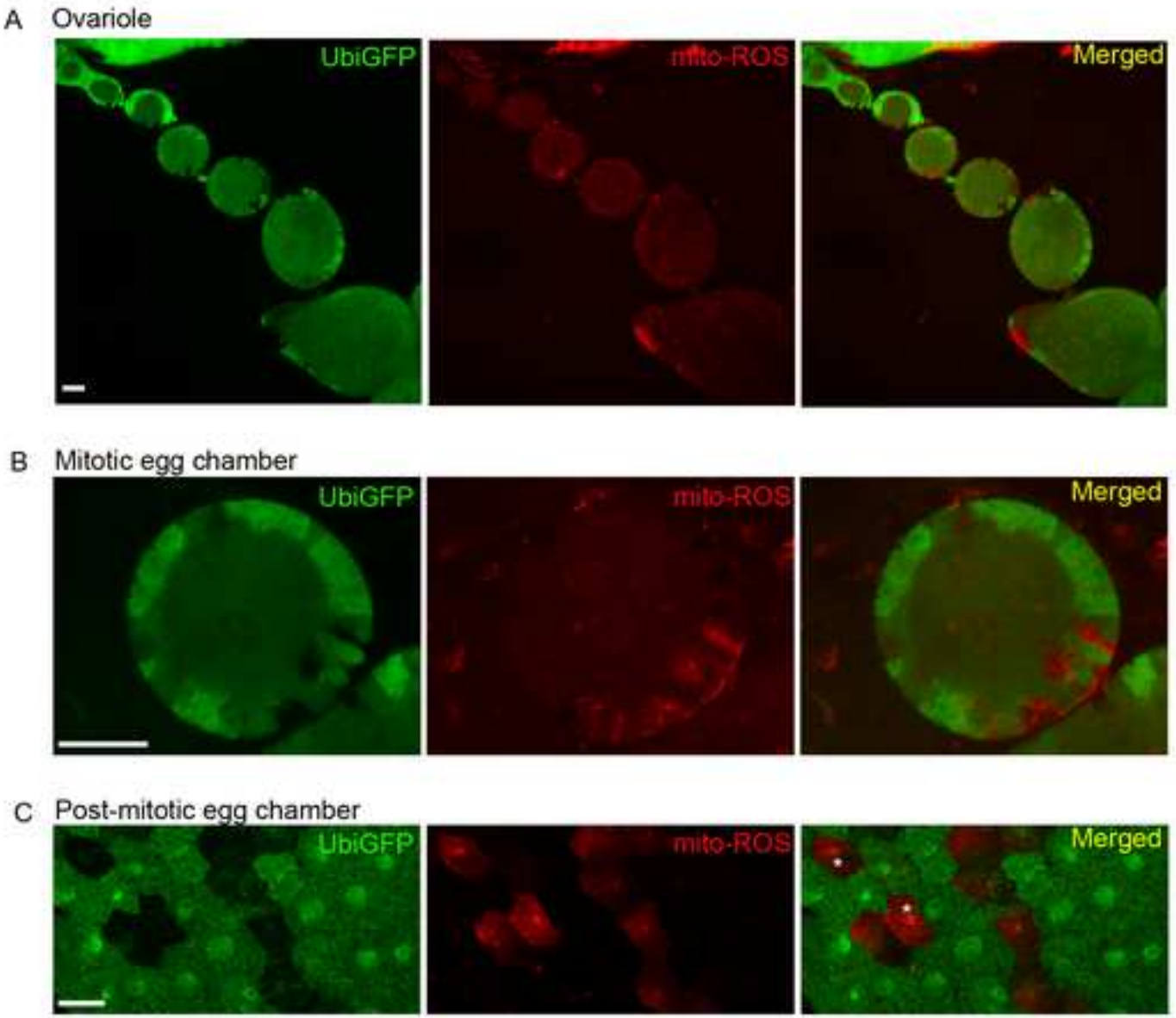

Figure 7

Figure

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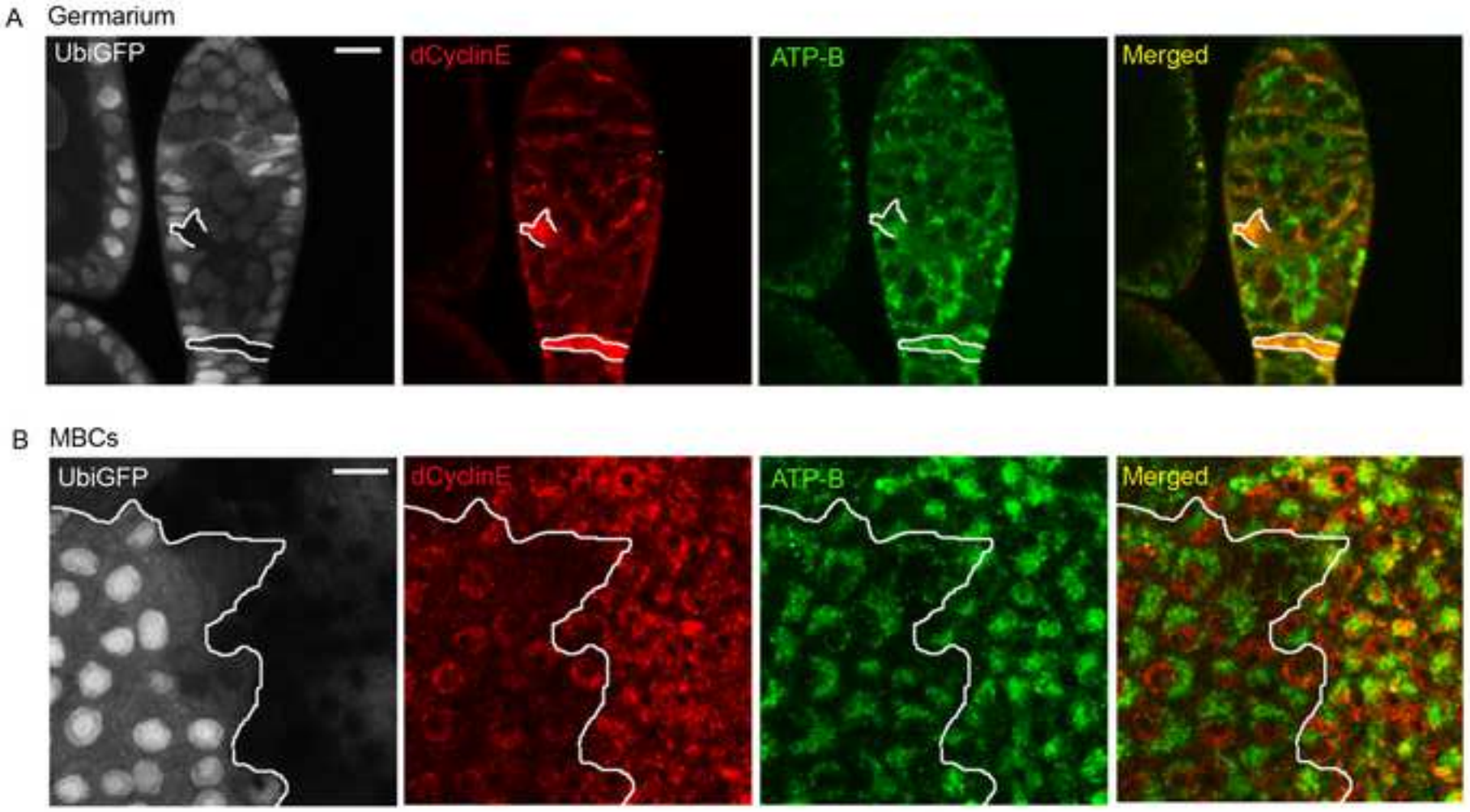

Figure 8

Figure

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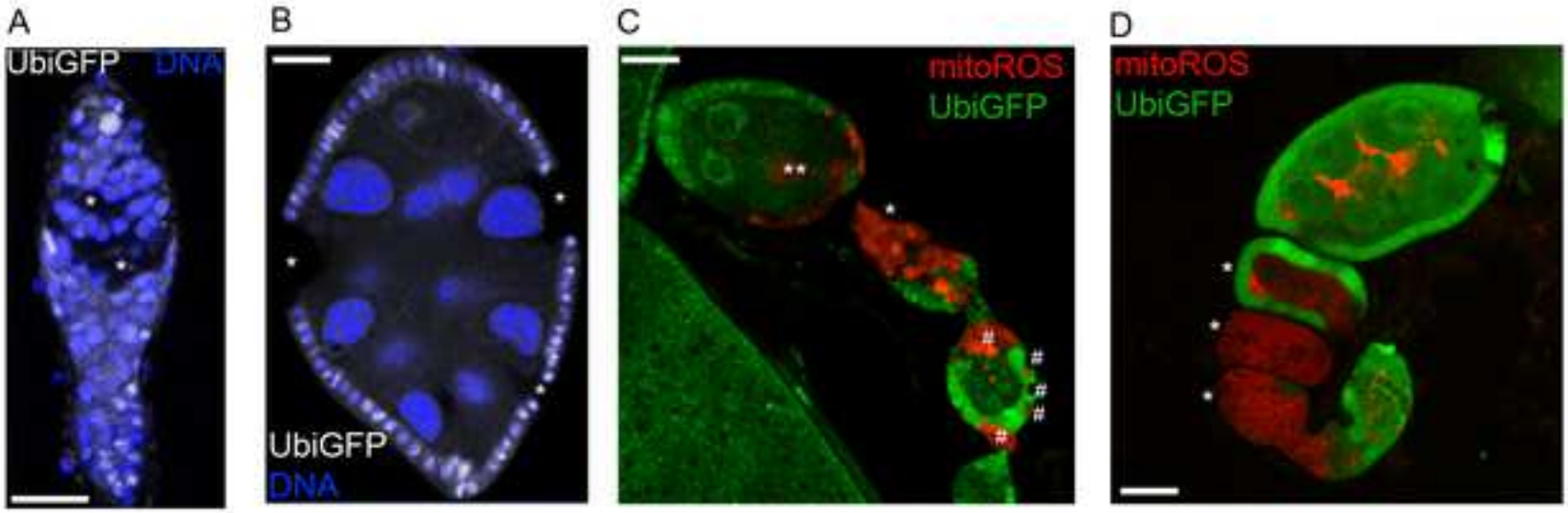

Figure 9

Table 1

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Table1: Confocal microscope settings for acquisition of presented representative images

	Parameters	Fig3A	Fig4A	Fig4B	Fig5A	Fig5B	Fig5C	Fig6A	Fig6b	Fig7A	Fig7B
Acquisition											
	Objective	Plan-Apochromat 63×, 1.0 NA water immersion	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil
	Scan mode	Frame	Frame	Frame	Frame	Frame	Frame	Frame	Frame	Frame	Frame
	Frame size	X:512 Y:512	X:512 Y:512	X:512 Y:512	X:512 Y:512	X:512 Y:512	X:512 Y:512	X:512 Y:512	X:512 Y:512	X:512 Y:512	X:512 Y:513
	Speed	7	8	8	8	8	8	8	7	7	7
	Pixel dwell	3.55µs	2.55µs	2.55µs	2.55µs	2.55µs	2.55µs	2.55µs	3.15µs	3.15µs	3.15µs
	Scan Time	1.97 s	781.96 ms	1.56 s	1.56 s	3.13 s	1.56 s	4.69 s	3.87 s	15.49 s	7.75 s
	Averaging Number	1	1	1	1	1	1	1	1	2	1
	Bit Depth	8 bit	8 bit	8 bit	8 bit	8 bit	8 bit	8 bit	8 bit	8 bit	8 bit
	Direction	Uni	Bi	Bi	Bi	Uni	Bi	Uni	Uni	Uni	Uni
	Zoom	2	0.5	0.5	0.5	2	0.5	0.5	1.9	2.2	2.5
Channels											
	Laser	Gr:514nm (488)nm	Rd:555nm	Gr:488nm Rd:555nm	Gr:488nm Rd:555nm	Gr:488nm Rd:555nm	Gr:488nm Rd:555nm	Gr:488nm Rd:555nm	Gr:488nm Rd:555nm	Gr:639nm Rd:555nm Wt:488nm	Gr:639nm Rd:555nm Wt:488nm
	Pinhole (Optical Slice)	Gr:15 µm	Rd:2.0 µm	Gr:2.0 µm Rd:2.0 µm	Gr:2.4 µm Rd:2.4 µm	Gr:1.7 µm Rd:1.7 µm	Gr:1.2 µm Rd:1.2 µm	Gr:2.3 µm Rd:2.3 µm	Gr:1.5 µm Rd:1.5 µm	Gr:2.0 µm Rd:2.1 µm	Gr:1.5µm Rd:1.5µm
	Gain (Detector)	Gr:700	Rd:600	Gr:700 Rd:600	Gr:700 Rd:600 Ct:600 Ce:700	Gr:700 Rd:700	Gr:700 Rd:600	Gr:700 Rd:733	Gr:700 Rd:733	Gr:700 Rd:600 Wt:544	Gr:700 Rd:705 Wt:700
	Power (Laser)	Gr:100%	Rd:1.0%	Gr: 1.0% Rd:1.0%	Gr: 2.0% Rd:1.0%	Gr: 2.0% Rd:1.0%	Gr: 2.0% Rd:1.0%	Gr: 1.0% Rd:9.5%	Gr: 1.0% Rd:9.8%	Gr:15.0% Rd:1.0% Wt:3.5%	Gr:17.1% Rd:20.0% Wt:2.2%
Z Sectioning											
	Z Interval	2	2	2	2	2	2	2	2	1	2
Light Path											
	Switch Track	Frame	line	line	line	line	line	line	line	Frame	Frame
	Filters	Gr:SP555	Rd:LP560	Gr: SP555 Rd:LP560	Gr:SP555 Rd:LP560 Ct:LP560 Ce:SP555	Gr:SP555 Rd:LP560	Gr:SP555 Rd:LP560	Gr: SP555 Rd:LP560	Gr:SP555 Rd:LP560	Gr:None Rd:None White:None	Gr:BP555-610+LP650 Rd:BP420-475+VP500-16 Wt::BP55-610 + LP650
Time Series											
	Interval Time	10 secs									
Photobleaching											
	Laser Power	488nm, 100%									
	Repeat Bleach after # scans	5									
	Iterations	50									

Footnotes: Green: Gr Rd: Red White:Wt Cross Talk: Ct Cross Excitation: Ce BL: Blue

	Parameters	Figure 9A	Figure 9B	Image 9C	Figure 7C	Image 9D
Acquisition						
	Objective	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil	Plan-Apochromat 40x/1.4 Oil
	Scan mode	Frame	Frame	Frame	Frame	Frame
	Frame size	X:512 Y:512	X:512 Y:512	X:512 Y:512	X:512 Y:512	X:512 Y:512
	Speed	8	7	8	8	8
	Pixel dwell	2.55µs	3.15µs	2.55µs	2.55µs	2.55µs
	Scan Time	6.25 sec	3.87	6.25 sec	6.25 sec	3.13 sec
	Averaging Number	1	1	1	1	1
	Bit Depth	8 bit	8 bit	8 bit	8 bit	8 bit
	Direction	Uni	Uni	Uni	Uni	Uni
	Zoom	1.3	1	1.0	1.0	1.0
Channels						
	Laser	Wt:488nm Bl:405nm	Wt:488 Bl:405	Gr:488nm Rd:555nm	Gr:488nm Rd:555nm	Gr:488nm Rd:555nm
	Pinhole (Optical Slice)	Wt:79µm Bl: 81µm	Wt:79 Bl:81	Gr: 36µm Rd: 36µm	Gr: 79µm Rd: 79µm	Gr: 90µm Rd: 90µm
	Gain (Detector)	Wt:700 Bl:700	Wt:700 Bl:639	Gr:701 Rd:700	Gr:701 Rd:700	Gr:701 Rd:733
	Power (Laser)	Wt:1.5% Bl: 2.8%	Wt:5.0% Bl:3.5%	Gr: 0.3% Rd: 0.5%	Gr: 0.3% Rd: 2%	Gr: 1.0% Rd: 2.0%
Z Sectioning						
	Z Interval	2		0.46		
Light Path						
	Switch Track	Frame	Frame	Line	Line	Line
	Filters	Wt:SP555 Bl: BP420-475	Wt:SP555 Bl:SP490	Gr: 415-735 Rd: 415-735	Gr: 415-735 Rd: 415-735	Gr: SP555 Rd: LP560
	Footnotes: Green: Gr Rd: Red White:Wt Bl: Blue Cross Talk: Ct Cross Excitation: Ce					

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment
JoVE_Materials-Rev.xls

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Grace's Media (Insect Dissecting Medium)	Fisher Scientific	30611031-2	
41 Paraformaldehyde AQ	Electronic Microscopy Sciences	50-259-99	
Mitotracker Green (overall mitochondrial stain)	Life Technologies	m7514	Reconstitute and Aliquot
Tetramethylrhodamine ethyl ester perchlorate	Sigma Aldrich	87917-25MG	Reconstitute and Aliquot
MitoSox (Mito-Ros stain)	Life Technologies	m36008	Reconstitute and Aliquot
PolyLysine	MP Biomedicals	ICN15017625	
Fly Vials	Fisher Scientific	AS-515	
Fly Conicals	Fisher Scientific	AS-355	
Fly Vial Flugs	Fisher Scientific	AS273	
Fly Conical Flugs	Fisher Scientific	AS 277	
Jazzmix Drosophila food (Drosophila food)	Fisher Scientific	AS153	
Bovine Serum Albumin	Sigma Aldrich	A9647-50G	
Cyclin E Antibody (d-300)	Santa Cruz	sc- 33748	
ATPB antibody [3D5] - Mitochondrial Marker	AbCam	ab14730	
Cy3 AffiniPure Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	115-165-146	
Cy5 AffiniPure Goat Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	111-175-144	
Hoechst	Fisher Scientific	H3570	
VectaShield	Fisher Scientific	H100	
Azer Scientific EverMark Select Microscope Slides	Fisher Scientific	22-026-252	
Microscope Cover Glass	Fisher Scientific	12-542-B	

Mat Tek Corp Glass Bottom Mircrowell Dish	Fisher Scientific	P35G-0-14-C
Active Dried Yeast	Fisher Scientific	ICN10140001
Confocal Microscope	Carl Zeiss	LSM 700
Dumont #5 Forceps	Fine Science Technologies	11251-20
Moria Nickel Plated Pin Holder	Fine Science Technologies	26016-12
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Blowgun	Fly stuff	54-104
Blowgun needle	Flystuff	54-119
Dissecting Microscope	Carl Zeiss	Stemi 2000
Analyses software	Carl Zeiss	Zen
Analyses software	Open source	Image J
Research Macro Zoom Microscope	Olympus	MVX10
QICAM Fast 1394 Cooled Digital Camera, 12-bit, Mono	QImaging	QIC-F-M-12-C
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Author(s): Danitra Parker, Alda Moran, Kasturi Mitra

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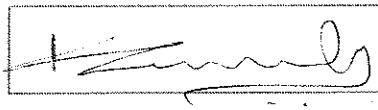
Signature:	 Date: 05-11-2016

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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.

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Authors’ response: All the figures are original.

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Authors' response: DOIs are already included.

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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