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Visualizing the Effects of Oxidative Damage on Drosophila Egg Chambers using Live Imaging

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TITLE:

Visualizing the Effects of Oxidative Damage on Drosophila Egg Chambers using Live Imaging

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KEYWORDS:

Drosophila ovaries, hydrogen peroxide, oxidative damage, live imaging, mitochondria, Clueless, ribonucleoprotein particle, bliss particles

SUMMARY:

The objective of this protocol is to use live imaging to visualize the effects of oxidative damage on the localization and dynamics of subcellular structures in *Drosophila* ovaries.

ABSTRACT:

Live imaging of *Drosophila melanogaster* ovaries has been instrumental in understanding a variety of basic cellular processes during development, including ribonucleoprotein particle movement, mRNA localization, organelle movement, and cytoskeletal dynamics. There are several methods for live imaging that have been developed. Due to the fact that each method involves dissecting individual ovarioles placed in media or halocarbon oil, cellular damage due to hypoxia and/or physical manipulation will inevitably occur over time. One downstream effect of hypoxia is to increase oxidative damage in the cells. The purpose of this protocol is to use live imaging to visualize the effects of oxidative damage on the localization and dynamics of subcellular structures in *Drosophila* ovaries after induction of controlled cellular damage. Here, we use hydrogen peroxide to induce cellular oxidative damage and give examples of the effects of such damage on two subcellular structures, mitochondria and Clu bliss particles. However, this method is applicable to any subcellular structure. The limitations are that hydrogen peroxide can only be added to aqueous media and would not work for imaging that uses halocarbon oil. The advantages are that hydrogen peroxide is readily available and inexpensive, acts quickly, its concentrations can be modulated, and oxidative damage is a good approximation of damage caused by hypoxia as well as general tissue damage due to manipulation.

INTRODUCTION:

Multiple different cellular stressors may arise during the experimental culture and manipulation of tissues ex vivo, including heat shock, oxidative stress, osmotic stress, nutritional stress, and toxicity conditions. Live imaging is a powerful tool used to visualize real-time changes in ex vivo tissues after experimental treatment and manipulation. Fine tissue dissections and manipulation take practice, and the amount of time from dissection to imaging can vary depending on

experience. The rationale for developing this method is based on the concern that preparing tissue for live imaging can cause cellular stress during dissection and imaging preparation. This could be particularly problematic for processes sensitive to changes in cellular metabolism and available oxygen levels, such as mitochondrial function. While having a parallel wild type sample is an important control, there is still the possibility that some or all observed changes in subcellular structures could be due to damage or cell stress from dissection and do not reflect normal physiology or the treatment or mutation being studied.

To address this potential problem, we use hydrogen peroxide addition during live imaging in order to induce cellular oxidative damage[1]. The purpose of this method is to induce damage to tissues in order to monitor the effect on subcellular structures. This protocol is useful for two purposes: 1) determining whether changes in subcellular localization of the structure of interest is due to the stress caused by inexperienced dissection and 2) once the researcher is confident with the dissection techniques described to monitor the effect of controlled stress on the structure of interest. Here we show two examples of how increased oxidative damage causes changes in two subcellular structures, mitochondria and Clu bliss particles. To do this, we use the *Drosophila* ovary which is a common model for live imaging studies. The first example examines mitochondrial localization. In our experience, normal mitochondrial localization in female germ cells is highly sensitive to perturbations and can act as a harbinger of cellular stress. Mitochondria in *Drosophila* female germ cells are normally evenly dispersed throughout the cytoplasm[2]. Hydrogen peroxide addition causes the organelles to quickly mislocalize and cluster in a similar manner to various mutations[3-5]. The second example are bliss particles formed by Clueless (Clu). Clu is a ribonucleoprotein that is diffuse throughout the cytoplasm; however, it also forms mitochondria-associated particles under optimal cellular conditions[5]. Because the presence of Clu particles is dependent upon healthy cellular conditions, we have termed them “bliss” particles[3,5,6]. Addition of hydrogen peroxide causes these particles to quickly disperse and become homogeneous in the cytoplasm[5]. In the course of our studies, we have observed changes in localization of both of these subcellular structures, but only after performing live imaging studies could we fully appreciate the effect of cellular stress and oxidative damage on localization and dynamics of mitochondria and bliss particles.

The utility of this protocol as an addition to already established or alternative methods depends on several factors. First, the imaging protocol must be amenable to drug-addition. If the sample is mounted under a coverslip and in halocarbon oil, this method would not be possible[7]. H_2O_2 addition causes a rapid rise in oxidative damage, therefore, this timescale may not be appropriate. Oxidative damage may be regarded as a proxy for hypoxia; however, it may be too harsh or too generalized to function as an appropriate control for damage for certain subcellular components. Finally, for imaging experiments that last hours such as those following a developmental process, H_2O_2 addition may be too strong (for example[8]). Testing a concentration curve may overcome this limitation.

PROTOCOL:

1. Preparation of dissection and imaging media

NOTE: The media best suited for this live imaging experiment contains Schneider's Drosophila media containing 15% heat inactivated fetal bovine serum, 0.6x Pen-Strep, and 200 µg/mL bovine insulin, hereafter referred to as Complete Schneider's media.

1.1. Perform the media preparation under sterile conditions to ensure that it does not become contaminated. The media was developed to support *Drosophila* ovarioles for extended periods of time[9].

1.2. Add 15% heat inactivated fetal bovine serum, 0.6x Pen-Strep, and 200 µg/mL bovine insulin to the Schneider's media.

1.3. Mix the contents well and store at 4 °C overnight.

NOTE: Insulin does not dissolve completely in the Complete Schneider's media, and you will notice a precipitate settle in the bottom of the tube.

1.4. Make aliquots of the media, being sure to leave the precipitate as it will interfere with imaging.

NOTE: This solution may be used within one month if stored in aliquots at 4 °C.

2. Collection of *Drosophila* for dissection

NOTE: Detailed *Drosophila* collection and dissection procedures may also be found in Weil et al.[10] and Parker et al.[11].

2.1. For optimal female germ cell imaging, first prepare a vial containing standard cornmeal fly food and a dab of wet yeast paste that is the consistency of peanut butter. This ensures the female flies are well-fed and will produce all follicle developmental stages for imaging.

2.2. For optimally healthy flies, collect 0-1 day old females and transfer with males into a fly food vial containing wet yeast paste.

NOTE: Make sure the sleeping flies do not contact the yeast paste as they can stick to it.

2.3. Feed the flies 3-7 days, changing the vial and the yeast paste daily.

NOTE: Make sure the yeast paste contacts the fly food so it does not dry out.

3. *Drosophila* ovary dissection

NOTE: It is important to prepare the media solutions fresh because hydrogen peroxide is susceptible to oxidation and TMRE degrades over time.

3.1. Right before dissection, in Complete Schneider's media, prepare a fresh aliquot of 2 µM H_2O_2 solution, a fresh aliquot of 46 nM tetramethylrhodamine, ethyl ester (TMRE), and a fresh aliquot of a 46 nM TMRE solution containing 2 µM H_2O_2.

3.2. For ovary dissection, use two pairs of fine forceps and a pair of electrolytically sharpened tungsten needles[12]. To dissect the ovaries, fill 2-3 wells of a glass bottom dissecting dish (watch glass) with Complete Schneider's that has been warmed to room temperature.

3.3. Anaesthetize the vial of fattened flies with carbon dioxide and segregate the desired number of female flies to be dissected. Place a single fly in the media using forceps.

3.4. Under a dissecting microscope, gently grasp the fly by the thorax using one pair of fine forceps. With the other pair of forceps, grasp the posterior, and gently pull to remove the ovaries.

NOTE: if the ovaries do not come out smoothly using this method, the entire abdomen may also be removed from the fly, and the ovaries can be gently squeezed out of either end of the abdomen using forceps.

3.5. Remove any extraneous cuticle or tissue, then transfer the ovaries to a new well containing fresh media. The ovaries should still be moving from the surrounding muscle sheath.

4. Preparing ovarioles for imaging

4.1. Using sharpened tungsten needles, gently tease the ovarioles apart, taking care to remove the surrounding muscle sheath (**Figure 1**).

4.2. Gently tease away any muscle sheath and nerve fibers attached to the isolated ovarioles (**Figure 2**).

NOTE: If the muscle sheath is not removed, the ovariole will twitch and move, causing problems with image acquisition (**Video 1**).

4.3. If the subcellular structures of interest are endogenously labeled, proceed to Step 4.4. If the structures of interest will be labeled with a fluorescent dye, proceed to Section 5.

4.4. Once the ovarioles have been cleanly dissected, using a micropipette, transfer them in a 100 µL drop of Complete Schneider's imaging media into the glass depression of a glass bottom dish. The individual ovarioles will sink to the bottom of the droplet.

4.5. Proceed to Section 6 for imaging.

NOTE: For imaging mitochondria and Clu bliss particles, no more than five-ten minutes should elapse from the start of dissection to imaging.

5. Staining mitochondria with TMRE

NOTE: Additional detailed procedures on live staining of mitochondria with fluorescent dyes may be found in Parker et al. 2017.

5.1. After step 4.3, transfer the isolated ovarioles in a 100 µL drop of 46 nM TMRE media into the glass depression of a glass bottom dish. The individual ovarioles will sink to the bottom of the droplet.

5.2. Incubate at room temperature for 20 minutes. Place the lid onto the glass-bottom dish and place the dish into a covered box for the duration of the experiment to protect from light.

NOTE: After incubation, samples can be imaged directly with no washes.

5.3. Repeat steps 5.1-5.2 to prepare at least two dishes of TMRE-labeled ovarioles, one to serve as an experimental group and one to serve as a control.

6. Live image acquisition

6.1. Once the ovarioles are mounted, place one of the glass bottom dishes onto the microscope and configure the imaging parameters as necessary. The optimum excitation/emission wavelengths for the TMRE used here are 549 nm/574 nm.

6.2. After locating the desired field of view, acquire still images or brief videos of one or more ovarioles as desired as a record of pre-treatment conditions.

7. Addition of hydrogen peroxide during imaging

7.1. Pause live imaging, remove the lid from the glass bottom dish, and carefully add 100 µL of 2 µM H_2O_2 solution to the dish using a micropipette if imaging endogenously labeled structures. If imaging mitochondria, carefully add 100 µL of 46 nM TMRE with 2 µM H_2O_2 solution to the dish using a micropipette (**Video 2**).

7.1.1. Avoid breaking the surface of the existing media droplet or adding the solution too quickly so as not to displace the ovarioles resting on the bottom of the dish (**Video 2**).

7.2. Replace the dish lid (dish cover), relocate and refocus the desired field of view if necessary, and resume imaging (**Video 3**, **Video 4**, **Figure 3**, **Figure 4**).

7.2.1. Take care to resume imaging as quickly as possible after H_2O_2 addition as the experimental treatment is time-sensitive (**Video 5**).

7.3. Acquire still images or brief videos of one or more ovarioles as desired. This will serve as

a record of post-treatment conditions.

7.4. For the control, place the second glass bottom dish onto the microscope and repeat Section 6.

7.5. Repeat step 7.1, this time adding 100 µL of TMRE-only media to the dish. If imaging endogenously labeled structures, add 100 µL of the Complete Schneider's media-only solution.

7.6. Repeat steps 7.2 and 7.3 to acquire data for the control group.

NOTE: For imaging mitochondria and Clu bliss particles, no more than three-five minutes should elapse from the addition of hydrogen peroxide to imaging.

REPRESENTATIVE RESULTS:

The described protocols can be used to study the effects of hydrogen peroxide during live imaging of *Drosophila* ovaries. As shown in **Figure 3**, **Figure 4**, **Video 3** and Video 4, this procedure provides an effective means to visualize tissue changes and dynamics after experimental treatment in real-time. Importantly, this protocol is specific for the addition of H_2O_2 to ovarioles while imaging; however, it may be adapted for the exogenous addition of other drugs or reagents of interest. In addition, follicles may be labeled with any fluorescent dyes of interest (e.g., tetramethylrhodamine (TMRE), LysoTracker) prior to imaging (**Video 3**). The most critical steps to obtaining clear imaging results are 1) the proper dissection and isolation of single ovarioles with all contractile elements removed (**Figure 1** and **Figure 2**) and 2) the speed at which imaging is restarted after hydrogen peroxide addition. **Video 4** is an illustration of a properly dissected follicle that remains steady throughout the imaging duration as compared to **Video 1**, which illustrates a poorly dissected follicle exiting the field of view during imaging. **Video 5** is an illustration in which the H_2O_2 effects on TMRE-labeled mitochondria have already progressed prior to the start of imaging as a result of too much elapsed time. As compared to **Video 3** in which imaging was restarted immediately after hydrogen peroxide addition (time 0) and intact, dispersed mitochondria are still visible, the mitochondria in **Video 5** have already began to visibly clump and lose their membrane potential upon the restart of imaging. This issue is mostly attributed to disruption of the sample positions during H_2O_2 addition and can be alleviated by following the technique to keep the imaging media and sample position intact (**Video 2**). Of note, in control experiments performed without H_2O_2 added to the media, the mitochondria in follicles remains properly dispersed, and the TMRE dye remains sequestered in the mitochondria.

FIGURE AND TABLE LEGENDS:

Figure 1: Isolation of single ovarioles from *Drosophila* ovaries. (**A**) Cartoon indicating a pair of ovaries, a single ovariole (arrow) with the germarium at the tip (arrowhead) and the two muscle sheaths that surround the ovariole (brown, epithelial) and the ovary (brown, peritoneal). (**B**) Dissected *Drosophila* ovary. (**C**) Subsequent separation of the teased ovary into individual ovarioles (arrow). Scale bar = 100 um.

Figure 2: Removal of nerve fibers and contractile elements from single ovarioles. (**A**) Single

ovariole with remnants of nerve tissue and the muscle sheath still attached (arrow). (**B**) Gentle removal of all remaining tissue attached to ovariole in A (arrow). Scale bar = 100 µm.

Figure 3: H_2O_2 causes mitochondrial mislocalization. (**A-A"**) Live-image stills of a well-fed *clu*^{CA06604} (Clu::GFP flies) follicle. Addition of H_2O_2 causes mitochondria to clump over the duration of imaging. TMRE labeling of mitochondria indicates that mitochondria are initially dispersed at time 0 (**A**), and that mitochondria start to clump after H_2O_2 addition (**A'**) At a later time-point, the TMRE labeling becomes spotty due to mitochondria losing their membrane potential and therefore their ability to sequester the dye (**A"**). White = TMRE (**A-A"**). Scale bars: 10 µm in (**A**) for **A-A"**[5].

Figure 4: H_2O_2 disperses Clu. (**A-A"**) Live-image stills of a well-fed *clu*^{CA06604} follicle. Addition of H_2O_2 causes particles to disperse and come homogeneous in the cytoplasm over the duration of imaging. White = Clu::GFP (**A-A"**). Scale bar: 40 µm in (**A**) for **A-A"**[5].

Video 1: Follicle from a *clu*^{CA06604} female. As described in **Figure 2**, failure to remove nerve fiber contractile elements from single ovarioles will cause marked drifting and movement of the ovarioles during imaging and subsequent inability to analyze imaging data. White = Clu::GFP.

Video 2: Proper addition of H_2O_2 to sample dish. Hydrogen peroxide should be added to the sample dish using an appropriately sized micropipette. Dispensing H_2O_2 without breaking media surface. Care is taken to avoid breaking the surface of the imaging media to minimize sample drift during hydrogen peroxide addition.

Video 3: H_2O_2 addition during imaging of TMRE labeled follicles. Follicle from a *clu*^{CA06604} female stained with TMRE. H_2O_2 causes mitochondrial mislocalization in *Drosophila* follicles. White = TMRE dye. Imaged one frame per 15 s for 20 min, and the video is 10 frames per second[5].

Video 4: H_2O_2 addition during imaging of Clu::GFP follicles. Follicle from a *clu*^{CA06604} female. H_2O_2 disperses Clu particles as described in Figure 4. White = Clu::GFP. Imaged one frame per 15 s for 15 min, and the video is 10 frames per second.[5]

Video 5: Delayed imaging after H_2O_2 addition. Follicle from a *clu*^{CA06604} female. Addition of hydrogen-peroxide to follicles is a time-sensitive treatment. The restart of imaging was delayed in this video as a result of sample displacement during H_2O_2 addition. The mitochondria have already begun to visibly clump and lose their membrane potential upon the restart of imaging at time 0 (as compared to time 0 in Video 3). Failure to resume imaging quickly after treatment will result in inaccurate and unusable results as the early experimental effects will be missed prior time imaging. White = TMRE. Imaged one frame per 15 s for 20 min, and the video is 10 frames per second.

DISCUSSION:

This protocol could be a useful addition as a control for artifacts due to ovary dissection and tissue incubation for any live imaging experiment. The critical steps are similar to those found for

other live imaging protocols. Learning how to dissect whole *Drosophila* ovaries takes practice; however, this skill can usually be learned fairly quickly with the appropriate dissection tools. More difficult to master is removing the muscle enveloping the ovaries and each ovariole[13]. This must be done to ensure muscle contractions do not interfere with image acquisition. If using sharpened tungsten needles to do this does not prove successful, the germarium at the tip of the ovariole can be grasped with forceps and the ovariole pulled from the muscle sheath. However, this technique is problematic if the earliest developmental stages are to be examined because they can become damaged. Another key step is to not dislodge the ovarioles resting on the bottom of the dish when adding H_2O_2. An additional important aspect is shared by all live imaging: the researcher should ensure that the structure of interest is fluorescently well-labeled before treatment. The dishes used here (**Table of Materials**) are commonly used for live imaging; however, any dish or slide with a glass coverslip on the bottom or even a large glass coverslip should work as long as the drop of media can be covered to prevent media evaporation. While we use a particular microscope, any inverted microscope with an objective of sufficient magnification to see the subcellular structure in question and an attached camera that has sufficient resolution and image capture rate should work.

While our laboratory is primarily interested in mitochondrial function, this method could be helpful examining the dynamics and localization of any subcellular structure or organelle, such as the nucleus, cytoskeleton or endoplasmic reticulum. However, this method has limitations. In order to add hydrogen peroxide, the tissue must be in an aqueous media. An alternative method for live imaging is to use halocarbon oil, which has been instrumental in describing many important processes in *Drosophila* ovarioles including the first example of dynamic movement of GFP in a model organism[7,14]. In addition, adding hydrogen peroxide to the media causes wide-spread oxidative damage which may be too general an insult to the tissue to be informative for the cellular process of interest, particularly for longer experiments examining development. Though it may not be feasible to perform experiments which require visualizing the cell over long periods of time due to this rapid, extensive, and likely irreversible oxidative damage, we have seen that the acute hydrogen peroxide treatment we have described is applicable to most stages of oogenesis as we are able to see the same effects in most stages within the imaging time period. Given the low cost and ease of the protocol, it may be a useful control for damage and can be used as a treatment before fixation and antibody labeling as well.

In our hands, H_2O_2 treatment mimics the changes in mitochondrial mislocalization and Clu bliss particle dispersion that we see in various *Drosophila* mutants. It also mimics results we see for new researchers in the lab learning dissection techniques. Therefore, this method clearly revealed that sample preparation and general cellular stress can lead to unexpected and previously unexplained changes to mitochondrial mislocalization and the presence of bliss particles. Moving this technique forward, hydrogen peroxide concentrations could be modulated using a higher or lower concentration. If a cellular effect is seen using a lower concentration, it is possible the stress phenotype may be reversible by replacing the media with Complete Schneider's. Different cell stressors such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP), arsenite or simple heat shock might prove useful for general cellular stress for other subcellular structures. Since live imaging of ex vivo tissues requires manual manipulation and incubation in

different media, this control should be a useful addition to ensure any observations are as close to normal physiology as possible.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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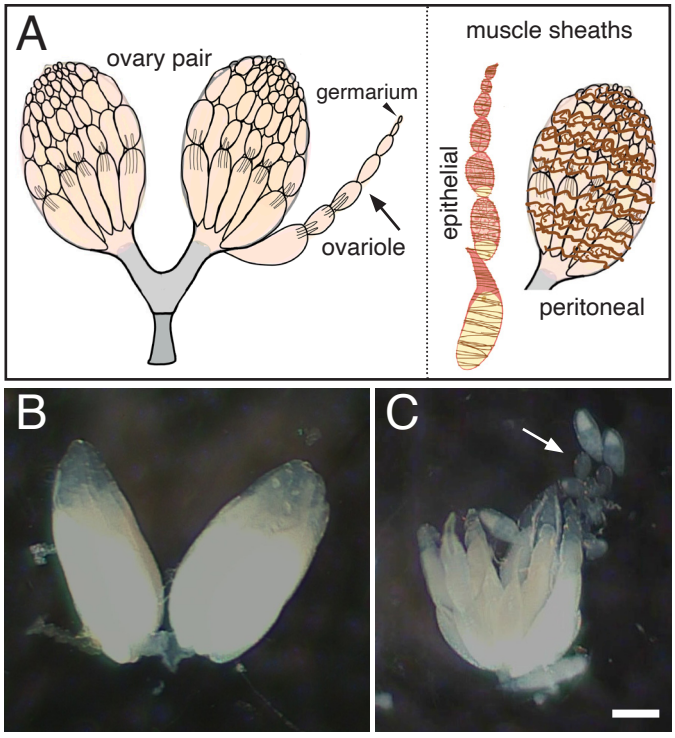

Figure 2

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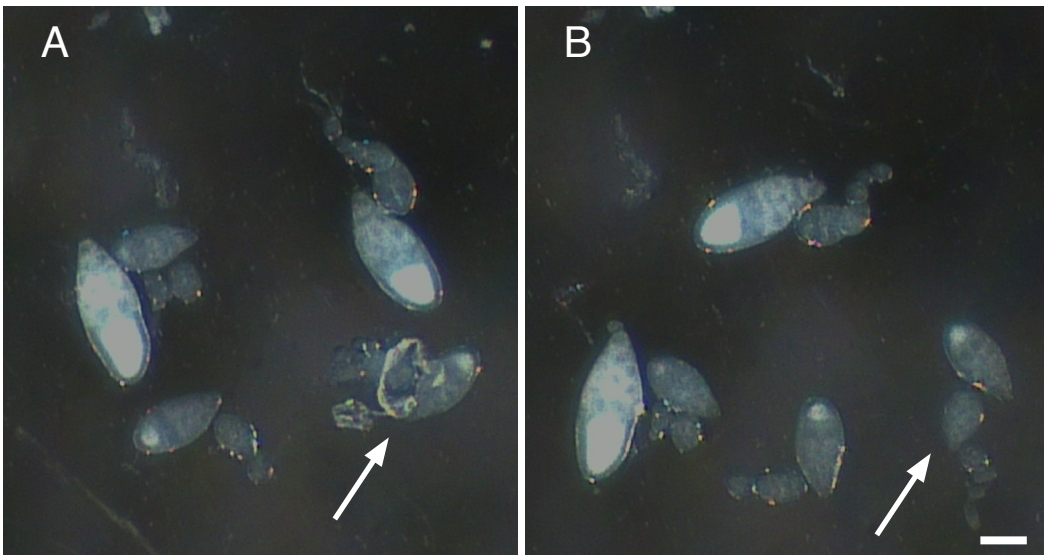

Figure 3

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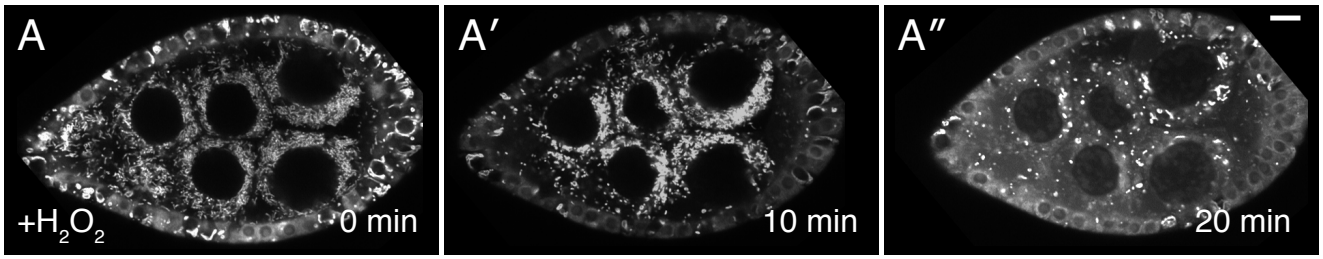
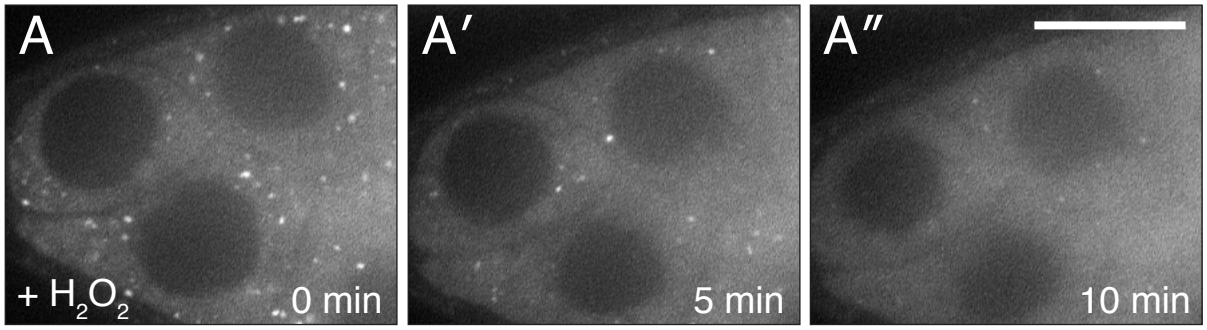


Figure 4

Click here to access/download;Figure;Fig. 4 - Particle dispersion resub.pdf



Video 1

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Video or Animated Figure

Video 1 - Failure to remove contractile elements.mov

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Video or Animated Figure

Video 2 - Proper Addition of H2O2 to Sample Dish.mp4

Video 3

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Video or Animated Figure

Video 3 - H2O2 stresses mitochondria.avi

Video 4

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Video or Animated Figure

Video 4 - H2O2 disperses particles.avi

Video 5

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Video or Animated Figure

Video 5 - Delayed imaging after H2O2 addition.mov

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Active dry yeast	Red Star®		
CO_2 gas			99.9% purity
CO_2 pad			
Dissecting microscope, Nikon SMZ645 model	Nikon		
Dissecting needles - PrecisionGlide needles	BD	305165	B-D 21G1 size
Dissecting needles - PrecisionGlide syringes	BD	309657	Luer-Lok tip, 3 mL size
Dissecting needles - tungsten wire	Electron Microscopy Sciences	73800	
Dumont #5 forceps (2 pairs)	Fine Science Tools	11251-10	
NI-150 High Intensity Illuminator	Nikon Instruments Inc.		
Gibco Fetal Bovine Serum, Heat Inactivated	Fisher Scientific	10082-147	
Gibco Schneider's Drosophila Media	Sigma-Aldrich	21720-024	
Hydrogen peroxide solution, 30% (w/w) in H2O	Sigma-Aldrich	H1009	
Insulin from bovine pancreas	Sigma-Aldrich	I6634	
Spinning disk microscope	Nikon		Equivalent scopes may also be used
Lonza BioWhittaker Antibiotics: Penicillin-Streptomycin mixtures	Fisher Scientific	17-602E	
MatTek Corporation Glass Bottom Dishes, 35 mm	Fisher Scientific	NC9344527	
Micropipettes and tips of appropiate size	Eppendorf		

Microcentrifuge tubes, 1.7 mL	VWR	87003-294	
Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE)	AnaSpec	AS-88061	
w[1118]	Bloomington Drosophila Stock Center	5905	Wild-type flies
y w; clu[CA06604]		Available upon request.	Clu::GFP trap flies

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have done that.

2. 2.2: What concentration of CO2 is used?
The concentration is 99.9% pure CO2. This is a standard technique for putting flies to sleep. This information was added as a comment in the Table of Reagents.

3. 4.3: Incubate at what conditions?
Clarified instructions to denote incubation at room temperature.

4. Figure 1 and 2: Please provide scale bars.
Scale bar has been added to Panel 1C and Figure 1's legend as well as Panel 2B and Figure 2's legend.

Changes to be made by the Author(s) regarding the video:
1. Please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol. You can revise the written protocol to match up to the narration.
The written manuscript has been revised to match the video narrations.

2. Remove the background music
The background music has been removed.

3. Hold on the chapter titles for a second or two longer
The chapter titles have had approximately two seconds added.

4. On the title card at the beginning there is an extra space between "Cell" and "and" on the first line of the institution
These spaces have been corrected.

5. Remove the city, state, and zip code on the institution on the title card
These have been removed.

6. Missed some dissolve transitions at 1:38, 4:07, 5:45
Cross dissolves have been added at these time points.

7. Chapter title screen "glitches" in early at 6:33
This has been fixed.

8. Arrows in the results segment at 6:43 and 7:53 fade in too slow and quickly cut out. Make the fade in quicker, and have them fade out
The arrows now dissolve in and out in 0.3 seconds

Please upload a revised high-resolution video here:
https://www.dropbox.com/request/F2GxM0EJDCuhMAzC7wIq?oref=e

Response to reviewers:

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this manuscript, the authors describe methods to visualize oxidative stress on the Drosophila ovary. They detail the approaches for inducing oxidative stress in the ovary and demonstrate how to visualize the effects of oxidative stress on two different structures: mitochondria and bliss particles. They demonstrate the efficacy of their approach and the protocol is sufficiently detailed to allow for application of this protocol. This approach can be used more broadly to visualize the effects of oxidative stress on other cellular structures. In addition, these approaches can be adapted to examine the effects of other treatments on cellular structures via live imaging.

Major Concerns:
Video 5 appears to resemble the control data more in Video 3 than Video 4. However, the figure legend describes this data as having Clu::GFP in white, as shown in Video 4. Please confirm labeling in the figure legend for this video.
We have corrected the figure legend of Video 5 to reflect that white = TMRE dye, not Clu::GFP.

As figures and videos have been reused with permission from previous work, this previous work should be cited throughout, particularly in the figure legends (Figure 3, Figure 4, Video 3, and Video 4.).
Citations for Sheard et al. *Dev Biol.* 2020 have been added to the figure legends for Figure 3, Figure 4, Video 3 and Video 4.

Minor Concerns:
Line 116 is unclear, it seems that perhaps it should read " if the ovaries do not smoothly remove…"
This is the correct interpretation. For clarity, the sentence has been changed to read “if the ovaries do not come out smoothly using this method…”(Line 141)

Line 145: Please write out what TMRE stands for on first use.
Added tetramethylrhodamine, ethyl ester. (Line 131)

Line 185: perhaps it should say the proper dissection and isolation.
This is correct. Replaced the word “of” with the word “and”. (Line 218)

Line 189-90: It would be helpful to state here which control video we should compare video 5 to.
This has been clarified to reflect that the comparison is between Video 5 and Video 3 in lines 224-229. Video 3 is the control video in which imaging was restarted quickly after

hydrogen peroxide addition. Healthy, intact mitochondria are visible at time 0, and the mitochondria become clumped and lose their membrane potential over the duration of the video. Video 5 is an illustration of a delayed start to imaging after hydrogen peroxide addition (as a result of the sample position shifting after H2O2 was added). The mitochondria in Video 5 are already clumped and have lost membrane potential by the time the sample was re-found on the microscope and imaging was restarted.

Line 290: What does CCCP stand for?
Added carbonyl cyanide m-chlorophenyl hydrazone. (Line 342)

Reviewer #2:
Manuscript Summary:
The video is nicely presented. Images are perfect and narration is very clear. Procedure is well explained. Relevancy to other scenarios is made clear. Given the potential to get imaging artifacts this is a useful method to see presented in detail.

Major Concerns:
None

Minor Concerns:
1. I don't think most people will know what bliss particles are, so suggest a sentence of explanation. Not clear if or how this links to mitochondria.
Added a concise description of why Clu particles are called bliss particles in lines 69-73. While Clu is important for mitochondrial function, Clu particles are used here as an example of a subcellular structure whose localization is sensitive to cell stress.

2. Type line 116: "Note: if the ovaries do smoothly "
This has been clarified. (Line 141)

3. Line 155. Would be useful to state the optimum excitation and emission wavelength for TMRE.
Added the optimum excitation and emission wavelengths as listed on the TMRE product description on the AnaSpec website. (Lines 183-184)

4. Line 172. Several mentions that you have to image quickly. I would like the authors to given some guidelines here - how quick?
We have added a “note” at the end of the protocol mentioning 5-10 minutes from the start of dissection to imaging. (Lines 206-207)

5. Line 210. Mention the arrow in figure 2.
Mentioned the arrow in appropriate place for Fig 2A (Line 254).

6. Line 215. I got a bit confused as to why all the imaging is done in the clu mutant background. I assume that the TMRE fluoresces at a different wavelength and therefore the GFP trap doesn't interfere. But this could be made clearer.

Added (Clu::GFP flies) in parenthesis after *clu*^{CA06604} to denote that this genotype is the Clu:GFP trap flies in the Figure 3 legend, not a *clu* mutant fly. Clu::GFP flies behave as wild type flies. Also added the fly genotypes used to the Table of Reagents. (Line 258)

7. Line 242. When the authors say the particles disperse. I assumed they meant the particles moved further apart. But it looks like the particles either fade or disaggregate. Could the authors clarify?
We have clarified this description in the text. Clu protein forms robust particles under optimal conditions. With stress, the particles disperse, and the Clu protein becomes homogeneous in the cytoplasm. (Lines 70-74)

8. Line 247. It wasn't clear what features the reader should be looking for to say that there is a failure to resume imaging quickly enough. Could the authors be more specific?
This has been clarified in the figure legend (lines 288-291) for Video 5 to denote that the mitochondria are already clumped at time 0 as a result of delayed imaging, as compared to Video 3.

Reviewer #3:
Manuscript Summary:
In this video and manuscript, Sheard and Cox describe a protocol to test oxidative damage on cells during live imaging. In particular, the authors adapt existing live imaging methods to allow treatment of dissected Drosophila ovaries with hydrogen peroxide and monitor the oxidative damage in real-time.

Overall, the written protocol is well-written, and the steps are laid out clearly. The video is very clear, the sound is good, the pace is good, the text is easily readable, and all figures and images are clear. The video does an especially good job of demonstrating trickier parts of the protocol, such as part 3 of the written protocol ("Preparing ovarioles for imaging") and part 6 of the written protocol ("Addition of hydrogen peroxide during imaging"). This protocol should be useful for anyone looking to perform live imaging using the Drosophila ovary, especially if they want to understand impacts of stress on cells with clear readouts (e.g., mitochondria dispersal). Moreover, this method could be used for other drug treatments, although it is limited to those drugs that are soluble in aqueous media.

Major Concerns:
1. In the first section, starting at line 86 "Preparation of dissection and imaging media", I was wondering if the pH of the media is important in this protocol? It is for some stages of oogenesis (e.g., Prasad and Montell, Dev Cell 2007 - reference [7] in this manuscript).
We have not noticed the pH as important for imaging mitochondria or Clu particles.

2. For Section 3 ("Preparing ovarioles for imaging"): In the video the authors mention that this part of the protocol should be done quickly to avoid damage to the egg

chambers. The authors could add a time frame for how long this part of the protocol should ideally take someone who is dissecting and preparing samples for imaging.
This has been added as a note at the end of the protocol. (Lines 161-162)

3. In Section 6 ("Addition of hydrogen peroxide during imaging"), do the authors use a control such as untreated samples, or maybe just addition of water rather than hydrogen peroxide? If so, this could be added to the text.
Added a note at the end of Section 4 to instruct readers to prepare an additional sample to be used as a control (lines 176-178). Also added more steps to Section 6 to clarify the procedure of adding either TMRE-only media or Complete Schneider's media as a control (lines 201-204).

4. In the Discussion, the authors mention that longer experiments may not be suited to the hydrogen peroxide treatment. I was wondering if the authors think there are specific stages of oogenesis that may be better suited to their protocol, or if they think their protocol is applicable to most stages?
We have clarified these points in the Discussion. This treatment is applicable to all stages of oogenesis based on what we have seen during our imaging experiments. (Lines 327-330)

Minor Concerns:
1. The sentence in line 116 is a little awkward and could be reworded ("Note: if the ovaries do smoothly remove using this method, …").
This has been clarified. (Line 141)

2. In Section 3, number 4 (lines 134-136), the authors could add specific endogenously labeled fly lines to the text and/or the Table of Materials - or maybe just refer to collections available at public stock centers.
Added the fly lines used to the Table of Materials.

Reviewer #4:
Manuscript Summary:
This manuscript describes a method for mounting Drosophila ovarioles for live imaging and exposing them to oxidative stress, to uncover what deliberate stress does to the intracellular distribution of organelles. Generally, this protocol is well explained, materials and equipment are carefully listed, the critical steps are highlighted, and the video is well done and full of good practical tips. This will be a great addition to the JoVE collection. I do have some concerns about clarity and completeness (listed below) that should be addressed before publication.

Major Concerns:
1. There is not quite enough morphological information for the reader not well versed with Drosophila oogenesis to fully understand the procedure.
* For one, the reader/viewer may not know what an ovariole is. The reference to Fig. 1, right, might lead the novice to conclude that an ovariole is actually an egg chamber,

since the individual egg chambers are obvious in this image, but not individual ovarioles. A brief explanation of ovarioles might be necessary.
We have added a new panel A to Figure 1 depicting ovaries, ovariole, germarium, and both types of muscle sheaths.

* I really like the emphasis on removing any muscle sheath and nerve fibers and the inclusion of Video 2 that shows the consequences of not removing all motile elements. However, I am concerned that Fig. 2A is not clear enough for the naïve reader to be confident of what nerve fibers and muscle sheaths look like. A better description of what to look for to recognize those structures should be given, so that even somebody not intimately familiar with Drosophila ovaries can recognize them and thus be sure that they have actually been removed.
The muscle sheaths are depicted in a cartoon in Figure 1.

* The discussion talks about "germarium". Without some introduction, this term will confuse many readers.
The germarium is now indicated in panel A, Figure 1.

2. The video and manuscript make a strong point about inadvertent damage to the tissue and its consequences. It should therefore include a control for the addition of the H2O2, otherwise the reader will worry that simply adding new media might cause the redistribution of mitochondria/dissolution of Clu particles. I do not think it is necessary to actually show this, but - if correct - a statement in the text similar to the following would be sufficient: "When in control experiments media without H2O2 is added, the distribution of mitochondria remains unaltered, dispersed throughout the cells, and TRM signal does not fade."
Added a note at the end of Section 4 to instruct readers to prepare an additional sample to be used as a control (lines 161-162), additional steps to Section 6 to clarify the procedure of adding either TMRE-only media or Complete Schneider's media as a control (lines 201-204), and a sentence to the end of the representative results section stating that the mitochondria in the control do not clump (line 231-233).

3. There is a bit of a problem with the logic of how the H2O2 experiment is presented. At times, the manuscript seems to imply that the deliberate stressor of H2O2 allows you to detect whether the dissection damaged the tissue and caused stress. That is, of course, logically not correct as the dissection might already cause some level of stress, but not so much - or a different type - as H2O2 addition. Thus, not seeing the mitochondrial accumulation does not prove there is no significant stress. The manuscript talks around this issue and - to my understanding - tries to make the point that for many novices initial attempts at dissection cause oxidative stress and mitochondrial clumping; this clumping can also be seen by deliberately inducing stress with H2O2. Therefore, one use of this experiment is to deliberately induce stress with H2O2 and compare the phenotypes observed with a control dissection. If the control dissection already shows the clumping, better care must be taken to improve dissection. I believe this is a good and useful point, but it is made in a roundabout may in the

current presentation. I think the impact of the protocol will be improved if the authors make this point more explicitly.
The following has been added to the introduction to clarify how this protocol can be used:
“This protocol is useful for two purposes: 1) determining whether changes in subcellular localization of the structure of interest is due to the stress caused by inexperienced dissection and 2) once the researcher is confident with the dissection techniques described, to monitor the effect of controlled stress on structure subcellular localization.” (Lines 58-62)

4. I was confused by video 5 and its description. I suspect that this video shows one of two things: a) an egg chamber for which the authors deliberately waited a certain amount of time after H2O2 addition before starting to image or b) an egg chamber for which the authors had trouble finding the egg chamber after addition of H2O2 and that by the time they could start imaging, enough time had elapsed to cause mitochondrial relocalization already. The meaning of the video would be much clearer if the authors explained which of those situations applies; if they know how much extra time elapsed that would also be instructive to add.
Video 5 is an illustration of a delay in imaging as a result of the sample being displaced. This point is clarified in the figure legend for Figure 5 (lines 289-291) as well as in the Representative Results (lines 224-231).

Minor Concerns:
Twice in the text the phrase "referenced" is used (lines 100 and 143). I am unclear what it means. Are we supposed to go to the cited papers for detailed methods or do the cited papers have further references in them that will lead to papers with detailed methods? If the former is meant, I suggest using "… may also be found in …"
This sentence has been revised to reflect the suggested change in both lines. (lines 112 and 167)

Line 116: "if the ovaries do smoothly removed using this method" I am confused about this. From the context, I suspect it should read ""if the ovaries are not smoothly removed using this method". Please clarify.
This has been clarified. (Line 141)

Step 3.5: It would be helpful to add "go to section 5", otherwise the reader might believe they also need to do the steps in section 4.
Added Step 3.5 to denote that the reader should skip section 4 and proceed to section 5 if imaging endogenously-labeled structures. (Line 159)

Line 165: "remove the lid" This is the first time "lid" is mentioned. To avoid confusion, please mention earlier that a lid is to be placed on the glass bottom dish; that may not be obvious to the reader. Also, I presume that the expression "dish cover" in line 170 is synonymous with "lid". If so, it is better to stick to using the same term. If the two expressions are not equivalent, I misunderstood the protocol.

Added instructions to place the lid on the glass bottom dish in step 4.3 (line 172). We have also clarified that the phrase “dish cover” as narrated for Step 6.2 is synonymous with “dish lid” (line 240) as per the written protocol.

Fig. 2A: The legend talks about remnants of nerve tissue and the peritoneal sheath. Please point those remnants out in the image itself, e.g. with an arrow/arrowhead. Also, it is confusing that the term "peritoneal sheath" only appears in the legend. It will be less confusing to the naïve reader if either peritoneal sheath is explained in the text or the legend simply talks about "muscle sheath".
We have changed the word “peritoneal” to “muscle”. (Line 254). We have also added panel A to Figure 1 with a cartoon depicting ovaries, ovariole and the two types of muscles (peritoneal sheath and epithelial sheath) that must be removed.

The legend of video 3 contains the sentence "This protocol is amenable … components as well." It appears misplaced to me, and I believe this argument is better made in the main text rather than a legend. If the authors want to expand the legend, I suggest they comment on the fading/spreading of the signal during the course of the movie.
Removed this sentence from the legend.

Legend of video 5: information of imaging rate/playback rate is missing. This video was imaged one frame per 15 s for 20 mins, and the video is 10 frames per second. This information has been added to Figure 5’s legend. (Line 293-294)

Line 259-262: As written, I expect a solution to the problem "this technique is problematic" in the sentence that follows, but the next sentence covers something else. I think the problem is that "problematic" is at the end of the sentence and therefore is stressed. Consider reversing the second sentence to "However, this technique is problematic if the earliest developmental stages are to be examined." Also, it might be useful to explain what these earliest stages are - just the germarium, egg chambers up to stage 4, etc.?
We have revised this sentence to reflect the suggested change. (Line 306-308)

Line 269: It is confusing to talk about the image capture rate of the microscope as that rate might be set by the camera rather than the scope itself (the text implies that an epifluorescence scope with a camera might work). You may want to consider rewording this to avoid such a confusion.
This has been reworded to say:
“While we use a particular microscope, any inverted microscope with an objective of sufficient magnification to see the subcellular structure in question and an attached camera that has sufficient resolution and image capture rate for the desired process should work.” (Line 314-316)

Line 281: "can be used as a treatment for examining fixed tissues as well." Mentioning "treatment" implies that H2O2 is applied to fixed tissue, but I believe that would be pointless. To avoid this confusing, consider "can be used for examining fixed tissues as well."

“Treatment” has been changed to “treatment before fixation and antibody labeling” for clarity. (Line 332)

Line 289: "perhaps the effect of lower concentrations can be reversed upon dilution" I am not entirely sure that what means as lower concentrations do imply that the stock solution is diluted more. Do you mean washing out the H2O2 or diluting it by adding fresh media? This needs to be explained more.
For clarity, this has been replaced with the following:
“Moving this technique forward, hydrogen peroxide concentrations could be modulated using a higher or lower concentration. If a cellular effect is seen using a lower concentration, it is possible the stress phenotype may be reversible by replacing the media with Complete Schneider’s.” (Line 339-342)

The table lists dissecting needles with tungsten tips. It would be helpful to add a source and catalog number. Also, there is a comment in the video that the needles were electrophoretically sharpened. This needs to be explained somewhere, possibly with a citation, otherwise readers will not be able to track this method down.
We have included a citation for how to electrolytically sharpen tungsten needles to Step 2.2. (Line 134) We have also added tungsten wire, syringes, and syringe needles necessary for dissecting needles to the Table of Reagents along with sources and catalog numbers.

The table lists "spinning disk/TIRF/3D-STORM microscope". Could this be replaced with "fluorescence microscope"? Certainly, a scanning confocal would be good enough, and possibly epifluorescence microscopy would be sufficient. The comment "equivalent scopes may also be used" should be in the comments/description column and should be expanded as the reader may not be able to judge what "equivalent" means.
We removed TIRF/3D-STORM from the microscope name and moved “equivalent scopes may also be used” to the Comments/Description column of the Table of Reagents. We have also added the clarifying sentence, “While we use a particular microscope, any inverted microscope with an objective of sufficient magnification to see the subcellular structure in question and an attached camera that has sufficient resolution and image capture rate for the desired process should work.” to the Discussion. (Line 314-316)

In the table, there is a weird dotted line under the microscope entry. Is something missing there?
We are unable to see this line on our version.

The last line of the table is off: there is one only parenthesis, and the entry may be duplicative with the Penicillin-Streptomycin mixture listed earlier.
This is correct. Deleted the duplicate entry on the last row of the table.

The still images at 06:40 in the movie are labelled with "+H2O2 added at time zero". To my eye, the "O" looks like the number zero, not the letter "O". Please double check.

This has been corrected.

And a really minor point for the image sequence around 08:25 when Kelsey Sheard is speaking: There seems to be a mirroring effect where the hand of Ms. Sheard is visible on the back wall. The hand motions are a bit distracting, especially since the viewer will wonder what they are actually seeing there. I am not saying that this should be fixed or reshot, just pointing out a weird quirk of this otherwise professionally shot video.