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Dissection and Lipid Droplets Staining of the Oenocytes in Drosophila Larvae

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Dear Editor,

We are submitting the revised manuscript of "Dissection and Lipid Droplets Staining of the Oenocytes in *Drosophila* Larvae" (JoVE60606).

Thank you for giving us the opportunity to revise the above manuscript. And we thank you for the very professional and constructive comments. We have carefully read all the comments and responded to every point. Together with the revised manuscript, we provided a point-by-point response to the comments. All the changes made in the revised manuscript are highlighted in red.

We believe that we have made an adequate revision on the original submission, and hope that the revised manuscript is now acceptable for publication in *JoVE*. We look forward to hearing from you soon.

Sincerely,

Renjie Jiao, PhD
Sino-French Hoffmann Institute, School of Basic Medical Sciences, Guangzhou Medical University

TITLE:

Dissection and Lipid Droplet Staining of Oenocytes in *Drosophila* Larvae

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KEYWORDS:

fat metabolism, lipid droplets, oenocytes, dissection, starvation, BODIPY493/503, staining, *Drosophila*, larvae

SUMMARY:

Presented here are detailed methods for the dissection and lipid droplet staining of oenocytes in *Drosophila* larvae using BODIPY 493/503, a lipid droplet-specific fluorescent dye.

ABSTRACT:

Lipids are essential for animal development and physiological homeostasis. Dysregulation of lipid metabolism results in various developmental defects and diseases, such as obesity and fatty liver. Usually, lipids are stored in lipid droplets, which are the multifunctional lipid storage organelles in cells. Lipid droplets vary in size and number in different tissues and under different conditions. It has been reported that lipid droplets are tightly controlled through regulation of its biogenesis and degradation. In *Drosophila melanogaster*, the oenocyte is an important tissue for lipid metabolism and has been recently identified as a human liver analogue regarding lipid mobilization in response to stress. However, the mechanisms underlying the regulation of lipid droplet metabolism in oenocytes remain elusive. To solve this problem, it is of utmost importance to develop a reliable and sensitive method to directly visualize lipid droplet dynamic changes in oenocytes during development and under stressful conditions. Taking advantage of the lipophilic BODIPY493/503, a lipid droplet-specific fluorescent dye, described here is a detailed protocol for the dissection and subsequent lipid

droplet staining in the oenocytes of *Drosophila* larvae in response to starvation. This allows for qualitative analysis of lipid droplet dynamics under various conditions by confocal microscopy. Furthermore, this rapid and highly reproducible method can also be used in genetic screens for indentifying novel genetic factors involving lipid droplet metabolism in oenocytes and other tissues.

INTRODUCTION:

Lipids are essential for cell survival. In addition to their traditional role as integral components of cellular membrane systems, lipids also play crucial functions in energy supply and signaling transduction throughout the life cycles of individual animals[1]. Thus, lipid metabolism must conform to strict regulations to maintain physiological hemostasis in cells. It is known that dysregulation of lipid metabolism results in various diseases, such as diabetes and fatty liver. Despite the great importance of lipid metabolism in animal health, the mechanisms underlying lipid metabolism regulation remain largely unknown.

Drosophila has been extensively used for years since professor Thomas H. Morgan started to use them in studies involving genetics and other basic biological questions[2]. In the last few decades, emerging evidence has shown that *Drosophila* is an excellent model organism in the study of many lipid metabolism-associated diseases, such as obesity[1,3]. In particular, *Drosophila* shares highly conserved metabolic genes with humans and possess similar relevant tissues/organs and cell types for lipid metabolism.

For example, the fat body of *Drosophila*, which is responsible for triacylglyceride storage, functions analogous to human adipose tissue. Recently, a cluster of specialized hepatocyte-like cells (i.e., oenocytes), which have been reported to be a functional analog to the human liver, have been shown to be involved in fatty acid and hydrocarbon metabolism in fruit flies[4,5]. Similar to the case in mammal livers, oenocytes respond to starvation by activating lipid droplet formation in both larval and adult *Drosophila*, resulting in lipid droplet accumulation in oenocytes[4,6-8]. Anatomically, oenocytes are tightly attached to the basal internal surface of the lateral epidermis in clusters of approximately six cells per abdominal hemisegment, which makes it impracticable to isolate oenocyte clusters from the epidermis. Thus, oenocytes must be attached to the epidermis during dissection and staining.

Lipids are stored in the form of lipid droplets, which are organelles with single layer membranes in cells[9]. Lipid droplets exist in almost all cell types across different species[10]. Lipid droplet dynamics, including its size and number, change in response to environmental stressors. This is regarded as a reflection of metabolic status in response to stress, such as aging and starvation[7,8]. Therefore, it is of great importance to develop a feasible and reliable method to qualitatively determine lipid droplet dynamics in oenocytes during development and under stressful conditions. In particular, in the third instar larvae, oenocytes contain few or no detectable lipid droplets under fed conditions, but they do contain numerous large lipid droplets after nutrition deprivation[4]. To verify the effectiveness of this method, it is suggested to perform lipid droplet staining in the oenocytes under starved conditions.

Currently, several lipophilic dyes are available for lipid droplets staining, such as the nonfluorescent dyes Sudan Black and Oil Red O and fluorescent dyes Nile Red and BODIPY493/503[11]. Sudan Black and Oil Red O are commonly used for tissue cholesteryl esters and triacylglycerols and can be easily detected by light microscopy. However, relatively high background staining and relatively low resolution are two limiting factors for its applications in qualitative analysis of lipid droplet dynamics. To overcome the limitations of nonfluorescent dyes, Nile Red and BODIPY493/503 are utilized as ideal substitutes for lipid droplet staining. It has been reported that Nile Red can also detect some unesterified cholesterol, which makes BODIPY493/503 a more specific dye for cellular lipid droplets, to some extent[12-14].

Above all, to fulfill a need for rapid and sensitive analysis of lipid droplets in oenocytes, this protocol presents a feasible and highly reproducible method of fixative-based lipid droplet-specific staining using BODIPY493/503 as the stain dye. In this report, oenocytes are dissected, and BODIPY493/503 is used for lipid droplet staining in the oenocytes, in which lipid droplets are detected by confocal microscopy. The ease and affordability of this procedure make it ideal for modification and further use in other applications, such as flow cytometry.

PROTOCOL:

1. Egg laying

1.1. Prepare the standard cornmeal food for egg laying.

NOTE: For the recipe and cooking procedure for standard cornmeal food used here, see the previously published details[15].

1.2. Prepare fresh yeast paste by adding 6 mL of distilled water to 4 g of active dried yeast in a 50 mL centrifugal tube. Use a spatula to mix and make a paste.

1.3. Make egg-laying bottles by filling the cornmeal food into the bottles and spread approximately 1 g of yeast paste onto the surface of the cornmeal food with a spatula.

1.4. Place the flies of desired genotypes in an egg-laying bottle and place it in an incubator with a constant temperature of 25 °C and humidity of 60%.

NOTE: Ideal crosses usually consist of 150 virgin flies and at least 75 males. Keeping flies in dark by placing a lightproof box over the egg-laying bottles will increase the reproduction rate.

1.5. Before egg collection, let flies lay eggs for 1 h to allow removal of all old eggs that remain stored in the female oviducts.

1.6. Let flies lay eggs in a new egg-laying bottle for 1 h and remove the adults from the bottle.

NOTE: Control the egg-laying time to minimize the developmental range in order to obtain

larvae within a precisely controlled developmental stage.

1.7. Allow the eggs to develop for 84 h into the third instar larvae in the incubator at 25 °C with a 12 h/12 h light/dark cycle.

2. Starvation treatment for the larvae

NOTE: As mentioned above, in the third instar larvae, there are few or no detectable lipid droplets in the oenocytes under normal feeding conditions, but numerous large lipid droplets can be induced in the oenocytes under stress conditions, such as starvation. To further verify this method, it is necessary to pretreat these larvae to induce lipid droplet biogenesis in oenocytes. Here, a starvation time course of 12h, 24 h, and 36 h was chosen as the paradigm. In particular, a short period of starvation (e.g., 3 h) is sufficient to induce detectable lipid droplets in oenocytes. Starvation duration may vary according to specific experimental goals and settings.

2.1. Make chambers for starvation and control treatments.

2.1.1. For starvation treatment chambers: place a filter paper of appropriate size in a 6 cm Petri dish and pipette 1 mL of PBS onto the filter paper.

2.1.2. For control treatment chambers: place 5 mL of Bloomington standard cornmeal food in the Petri dish.

2.2. Use a spatula to gently dig up the top layer of food containing larvae that are still burrowing in the food and transfer them to a Petri dish filled with 5 mL of PBS. Gently stir the larvae in PBS to remove any food contamination from the larvae and make it as clean as possible.

2.3. Use a small paintbrush to collect 40 third instar larvae of the same approximate size. Sort them randomly into a starvation or control chamber, with 20 larvae each.

2.4. Place the chambers in the incubator at 25 °C with 60% humidity and allow development for 12 h, 24 h, and 36 h of treatment.

NOTE: For the larvae in the starvation chamber, add 1 mL of PBS every 12 h to avoid larvae dehydration.

3. Dissection of oenocytes

3.1. Use a small paintbrush to pick larvae of the appropriate age (12 h, 24 h, or 36 h after treatment), then transfer them into a new Petri dish filled with 5 mL of ice-cold PBS to wash.

NOTE: Repeat step 2.2 when dealing with larvae in a control chamber to remove any food

contamination.

3.2. Fill a dissection plate with ice-cold PBS and use forceps to gently transfer larvae into the dissection plate. Put the dissection plate under a stereo microscope for the following dissection step.

NOTE: The ice-cold temperature will help slow movements of the larvae and facilitate dissection.

3.3. Turn the larvae ventral side up and dorsal side down and gently hold in place using forceps. Secure the larvae to the dissection plate by placing a dissection pin though the pharynx at the anterior end and another pin through the spiracle at the posterior end.

NOTE: The dorsal side is most easily identified by presence of the dorsal trunks of the trachea.

3.4. Use Vannas spring scissors to incise (longitudinally) through the epidermis from the posterior to anterior end.

3.5. Grasp the edges of the middle part of the incised epidermis with forceps, pull gently to the side, and secure with pins so that the epidermis is pinned flat on the dissection plate.

3.6. Remove the internal tissue of the epidermis using forceps.

NOTE: Caution must be taken when removing the tracheal branches to avoid damage to the oenocytes, which are localized in the internal surface of epidermis.

3.7. With forceps, retrieve the dissection pins and transfer the epidermis into a 1.5 mL microcentrifuge tube filled with PBS on ice.

3.8. Continue to dissect other larvae following the procedure described above.

4. Lipid droplet staining

4.1. Incubate the dissected epidermis in the fixation buffer for 30 min at room temperature (RT) on a rotator.

NOTE: The fixation buffer contains 4% paraformaldehyde (PFA) in PBS.

4.2. Remove the fixation buffer, followed by a quick wash. To perform a quick wash, add 1 mL of PBS at RT into the tube after the removal of PFA, gently resuspend the tissues, and discard the PBS.

CAUTION: The fixation buffer contains PFA, which is harmful to human health. It is important to properly dispose the fixation buffer as hazardous waste.

4.3. Wash the samples 3x for 5 min each with PBS to wash out all possible PFA residue.

4.4. Incubate the epidermis with BODIPY 493/503 (1 µg/mL; see **Table of Materials**) for 30 min at RT on a rotator.

NOTE: From this step onwards, wrap the microcentrifuge tube with a piece of aluminum foil to protect samples from light and minimize the possible photo-bleaching.

4.5. Remove the BODIPY493/503 staining solution and wash the samples 3x for 10 min each with PBS to completely remove residual dyes.

5. Mounting and imaging

5.1. Place 6 µL of mounting medium on a clean microscope slide.

NOTE: Mounting medium is used for longer detection time based on its antifade properties.

5.2. Use forceps to pick up one epidermis and gently remove the residual PBS with a wipe.

5.3. Place the epidermis in the mounting medium and adjust its orientation so that its internal surface containing the oenocytes is on the bottom and its external surface is on the top.

5.4. Gently place a coverslip onto the epidermis.

NOTE: Remove any extra mounting medium leaking from under the coverslip with a wipe, if necessary. To facilitate imaging, gently push down on the coverslip with forceps so that when observing the slide through the microscope, the oenocyte cluster can be easily imaged in one single plane. Alternatively, it is practicable to cut the epidermis into two semi-epidermis through the middle line to avoid rolling-up of the whole epidermis when mounting the tissues.

5.5. Apply clear nail polish around the edges of the coverslip to seal.

5.6. Put the slides in a lightproof sample box and allow the nail polish to dry at RT, which may take 5–10 min.

5.7. Proceed with microscopic analysis. Take images using a confocal microscope (magnification of 40x with optimized GFP or FITC filter settings, excitation = 488 nm, emission = 503 nm) to acquire clean and sensitive signals with minimized background.

REPRESENTATIVE RESULTS:

Successful execution of this procedure should result in clear lipid droplets staining that reveals the number and size of lipid droplets in the oenocytes. **Figure 1A,A',A''** shows that there are few detectable lipid droplets (green dots) in the oenocytes of normal feeding larvae during

different developmental stages. **Figure 1B,B',B''** shows increased lipid droplets (green dots) amount in the oenocytes in response to 12 h (B), 24 h (B'), and 36 h (B'') periods of starvation. It should be noted that after 96 h, the fed larvae (A) seemed to show some lipid droplet accumulation in the oenocytes, which may have been due to their fast growth rates. Researchers should pay increased attention when dealing with the larvae during this stage.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative images of lipid droplet staining. Images are shown over the time course of development and starvation in the oenocytes of *Drosophila* larvae using BODIPY 493/503. (**A,A',A''**) Lipid droplet staining (green dots) of representative images of oenocytes cluster in fed larvae. (**B,B',B''**) Lipid droplet staining (green dots) in representative images of oenocytes clusters after 12 h (B), 24 h (B'), and 36 h (B'') periods of starvation, starting from larvae with an age of 84 h. All images were taken in the same magnification. Scale bar = 20 µm.

DISCUSSION:

Among those outlined above, there are several critical steps in this protocol, with the egg laying time period being one of these. As a lipid-mobilizing tissue, the oenocyte is highly sensitive to nutrition status[6,8]. Prolonged egg-laying time periods may result in crowed larvae and increased food competition, leading to inaccurate results. The 1 h egg-laying time period used in this protocol allows the larvae to develop without nutrition competition. A much larger number of larvae that can result from longer egg-laying time periods may also affect the lipid droplet amounts and patterns (e.g., size, number, and morphology) in oenocytes.

Additionally, prolonged egg-laying time periods also results in a larval population with a wide variation of developmental stages. In this context, researchers should be careful when using mutants that affect embryonic or larval development, since their influence on lipid droplet patterns may be influenced by secondary effects of developmental defects. The time course procedure suggests that lipid droplets are rarely detectable in the fed condition oenocytes during development from early third instar larvae (84 h) to late third instar larvae (120 h). In addition, unlike the amber pigmentation of oenocytes in adults, larval oenocytes are colorless[5]. Thus, increased attention should be paid during oenocyte dissection to avoid potential damage to oenocytes, especially when removing the surrounding tissues (i.e., tracheal branches). Furthermore, lipid droplets are single layer membrane organelles, which are sensitive to detergents such as Triton X-100[9]. Thus, it is important to make sure that buffers used in this protocol do not contain detergents.

As mentioned above, there are several dyes used to determine the lipid droplet amount and pattern changes in *Drosophila* and other species. Among these, BODIPY 493/503 may be most suitable for lipid droplet-specific staining compared to other fluorescent (e.g., Nile Red) or nonfluorescent dyes (e.g., Oil Red O); although, more novel and advanced dyes are being developed. For example, LipidSpot 488 and its derivates are a series of newly developed fluorescent dyes with minimal background staining of cellular membranes and other organelles.

They allows rapid staining of lipid droplets in both live and fixed cells, with no washing step required[16,17]. A limitation of this lipid droplet staining protocol is that it is not optimal for quantitatively measuring fat reserves in cells, even though it works well for qualitative assessment of lipid droplet size, number, and morphology.

In addition to the lipid droplet staining in oenocytes, this method may also be applied to other tissues in larvae (i.e, muscle, fat body, and gut tissue) with minor modifications during tissue dissection and sectioning[7]. Moreover, this method also works well for lipid droplet staining of adult tissues[7]. A modified version of this method may be extended to broader applications in combination with immunohistochemistry staining with antibodies. In this case, fixed tissues should be permeated with saponin (0.1% for 30 min at RT) instead of traditional detergents before incubating with antibodies, which facilitates the crossing of antibodies across plasma membranes and maintenance of lipid droplet membrane integrity[8,18].

In summary, this protocol provides a feasible method for researchers to investigate whether certain genetic or environmental manipulations cause qualitative changes in lipid droplet amounts and patterns (i.e., size, number, and morphology) without the need for difficult operations and expensive equipment and materials.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure

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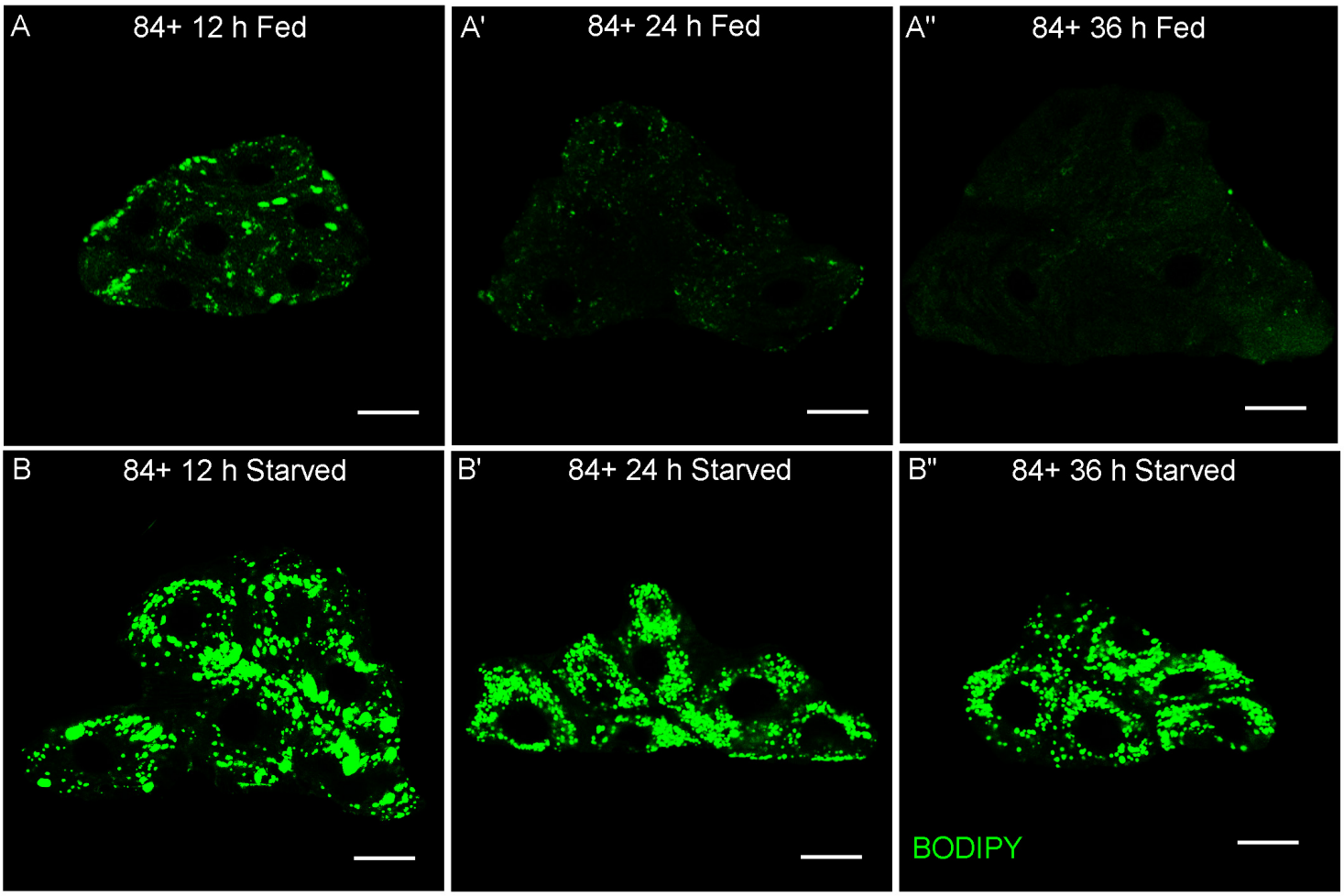

Name of Material/Equipment	Company	Catalog Number	Comments/Description
50 mL centrifuge tube	Corning	430829	50 mL
6 cm Petri dish	Thermo Fisher	150326	6 cm
Agar			For fly food
Aluminum foil	N/A	N/A	Protect smaple from light
BODIPY 493/503	Invitrogen	D3922	Lipid droplet staining dye
Confocal microscope	Leica	Leica TSC SP5	Confocal imaging
Corn syrup			For fly food
Cornmeal			For fly food
Coverslip	Citoglas	10212424C	20 × 20 mm, 0.13-0.17 thick
Dissection pin	N/A	N/A	
Dissection plate	N/A	N/A	
Filter paper	N/A	N/A	Diameter: 11 cm
Fixation buffer	N/A	N/A	4% Paraformaldehyde (PFA) in 1xPBS
Forcep	Dumont	11252-30	#5
Incubator	Jiangnan	SPX-380	For fly culture
Microcentrifuge tube	Axygen	MCT-150-C	1.5 ml
Microscopy slide	Citoglas	10127105P-G	
Mounting medium	VECTASHIELD Antifade Mounting Medium	H-1000	Antifade mounting medium
Nail polish	PanEra	AAPR419	Seal the coverslip
Paintbrush	N/A	N/A	
PBS	N/A	N/A	1xPBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4,1.8 mM KH2PO4, pH 7.4)
Rotator	Kylin-Bell Lab Instruments	WH-986	
Scissor	Smartdata Medical	SR81	Vannas spring scissor
Soy flour			For fly food
Spatula	N/A	N/A	
Standard cornmeal food	N/A	N/A	Accoding to Bloomington standard cornmeal food recipe
Stereo microscope	Leica	Leica S6E	For tissue dissection

Wipe paper	N/A	N/A	
Yeast			For fly food
yw	Kept as lab stock	N/A	*Drosophila*

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1, Line 110, Please replace this with a superscripted citation and list this in the table of materials.

Authors' Response: Thanks, we have made the revisions.

2, Line 117, Do you do this under a dissection microscope?

Authors' Response: Yes.

3, Line 188, Specify the type of scissor. Presumably a small dissection scissors or similar should be used.

Authors' Response: We used Vannas spring scissor, which is commonly used in ophthalmic and neurosurgical procedures. We have described it in the Table of Materials.

4, Line 192, Unclear what is meant. Please clarify.

Authors' Response: We have re-write this part to clarify.

5, Line 194, Specify which tissues.

Authors' Response: From the dissected epidermis.

6, Line 199, How is it handled? Mention tools used.

Authors' Response: With forceps

7, Line 254, Mention emission and excitation settings. Also mention magnification and lens NA.

Authors' Response: 40 x objective

8, Line 255, Specify wavelength ranges.

Authors' Response: with excitation/emission of 488/503 nm

9, Line 267, Please provide a common color-scale bar for A,A',A'', B, B',B'' to show that the figures have been window-leveled or scaled uniformly.

Authors' Response: Thanks for the suggestion. We have made the revisions.

10, Line 268, Please use arrows to point to example droplets.

Authors' Response: Thanks for the suggestion. The green signals indicate the lipid droplets in this image. And what we focused is the increased lipid droplet accumulation in the starved condition(B-B'). We have added more sentences to describe the results in the text.