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The colorimetric assay of citrate synthase activity in Drosophila melanogaster

--Manuscript Draft--

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Dear Dr. Steindel,

We thank you for the insightful comments that we believe have improved our manuscript significantly. We have submitted a revised manuscript, a rebuttal letter, and other related documents. Below are our point-by-point responses to your comments.

Best regards,

Ping Wei, MD, PhD
Shanghai Diabetes Institute, China

Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response:
We have proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please revise lines 29-30, 33-34, 44-45, 50-51, 69-72, 125-127, 146-147, 181-184, 192-194 to avoid previously published text.
Response:
In the revised version, we deleted the sentences in lines 29-30, 33-34, 44-45, 50-51, 125-127, 146-147, 192-194 in the formal version. We also revised the sentences in lines 69-72 and 181-184 in the formal version.

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5. Please provide an institutional email address for each author if possible.

Response:

None of the authors have an institutional email address.

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Response:

We have removed all the commercial language form the manuscript.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Response:

We have revised the protocol following the instruction.

8. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

Response:

We have revised the highlightedsteps following the instruction.

9. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response:

Thank you. We have revised the protocol following the instruction.

10. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Response:

Thank you. We have revised the protocol following the instruction.

11. Discussion: Please remove the subheadings within the Discussion part and combine information for the two protocols (i.e., merge critical steps, modifications, etc. of both protocols).

Response:

Thank you. We have revised the protocol following the instruction.

12. References: Please do not abbreviate journal titles.

Response:

Thank you. We have revised the references following the instruction.

13. Table of Equipment and Materials: Please revise the Table of Materials to include the name, company, and catalog number of all relevant supplies, reagents, equipment and software. Please sort the items in alphabetical order according to the name of material/equipment.

Response:

Thank you. We have revised the references following the instruction.

TITLE:

A Colorimetric Assay of Citrate Synthase Activity in *Drosophila melanogaster*

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KEYWORDS:

mitochondrial mass, citrate synthase activity, colorimetric assay, *Drosophila melanogaster*, mitochondrial disfunction, metabolic studies

SUMMARY:

We present a protocol for a colorimetric assay of citrate synthase activity for quantification of intact mitochondrial mass in *Drosophila* tissue homogenates.

ABSTRACT:

Mitochondria play the most prominent roles in cellular metabolism by producing ATP through oxidative phosphorylation and regulating a variety of physiological processes. Mitochondrial dysfunction is a primary cause of a number of metabolic and neurodegenerative diseases. Intact mitochondria are critical for their proper functioning. The enzyme citrate synthase is localized in the mitochondrial matrix and thus can be used as a quantitative enzyme marker of intact mitochondrial mass. Given that many molecules and pathways that have important functions in mitochondria are highly conserved between humans and *Drosophila*, and that an array of powerful genetic tools are available in *Drosophila*, *Drosophila* serves as a good model system for studying mitochondrial function. Here, we present a protocol for fast and simple measurement of citrate synthase activity in tissue homogenate from adult flies without isolating mitochondria. This protocol is also suitable for measuring citrate synthase activity in larvae, cultured cells, and mammalian tissues.

INTRODUCTION:

Mitochondria are best known as the power-producing organelles in most eukaryotic organisms, which produce the energy currency, ATP, through the tricarboxylic acid cycle (i.e., Krebs cycle) and oxidative phosphorylation. Mitochondria are also found to play important roles in a lot of other physiological processes, such as regulation of apoptosis[1], Ca^{2+} homeostasis[2,3], reactive oxidation species (ROS) generation[4], and endoplasmic reticulum (ER)-stress response[5]. Mitochondrial dysfunction can affect any organ in the body at any age and is a primary cause of metabolic, aging-related[6], and neurodegenerative diseases[7]. Intact mitochondria are mechanistically related to mitochondrial function. Thus, proper quantification of intact mitochondrial mass is very important for evaluating mitochondrial function[8]. Citrate synthase, a rate-limiting enzyme in the first step of the tricarboxylic acid cycle[9], is localized in the mitochondrial matrix within eukaryotic cells, and thus can be used as a quantitative marker for the presence of intact mitochondrial mass[9,10]. Citrate synthase activity can also be used as a normalization factor for intact mitochondrial proteins[11,12].

The fruit fly, *Drosophila melanogaster*, is an excellent model system for studying mitochondrial function, as many molecules and pathways that play pivotal roles in mitochondria are evolutionarily conserved from *Drosophila* to humans[13-15]. Here, we present a protocol for a fast and simple method for measurement of citrate synthase activity by a colorimetric assay in *Drosophila* tissue homogenates[16] in a 96 well plate format. In the citrate synthase activity assay, citrate synthase in *Drosophila* tissue homogenate catalyzes the reaction of oxaloacetate with acetyl coenzyme A (acetyl CoA) to form the citrate CoA-SH and H^+. CoA-SH subsequently reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to generate a colored product, 2-nitro-5-thiobenzoate (TNB), which can be easily measured spectrophotometrically at 412 nm. Citrate synthase activity can be reflected by the rate of color production.

PROTOCOL:

1. Colorimetric citrate synthase activity assay for *D. melanogaster*[16]

1.1. Collect ten adult flies for each sample. Collect at least triplicate samples for each genotype.

1.2. Prepare 500 µL of ice-cold extraction buffer containing 20 mM HEPES (pH = 7.2), 1 mM EDTA, and 0.1% triton X-100 in a 1.5 mL test tube for each sample.

1.3. Anesthetize adult flies with CO_2 on an anesthesia pad and isolate the desired tissues. To isolate the adult fly thoraxes, for example, fix the fly thoraxes by a pair of forceps, and then isolate the fly abdomens by cutting along the border of the thorax and abdomen using a pair of scissors. Collect the fly thoraxes for the muscle citrate synthase activity assay.

NOTE: Determine the fresh weight of tissue at this step if using it to normalize citrate synthase activity.

1.4. Transfer 10 adult fly thoraxes to 100 µL of ice-cold extraction buffer immediately and

homogenize with a pellet on ice. To keep the samples ice-cold, homogenize the samples for 5–10 s on ice, then have the samples sit on ice for 5 s, and repeat until all the tissues in the tube are homogenized completely.

NOTE: Tape the tube, check the homogenates to make sure that there are no clots in the homogenates and that the tissues in the tube are homogenized completely. All sample treatments should be performed on ice.

1.5. Take 10 µL of each homogenized sample in a new tube for protein content measurement. Keep the samples for protein measurement on ice.

NOTE: The protein samples can be frozen and stored at -80 °C for later analysis. The protein concentrations serve as an internal parameter for normalization of citrate synthase activities.

1.6. Add 400 µL of freshly prepared ice-cold extraction buffer (20 mM Tris-HCl (pH = 8.0), 0.1 mM DTNB, 0.3 mM acetyl CoA, 1 mM oxaloacetic acid) to each remaining sample to make a total volume of 500 µL. Mix well by gently pipetting up and down at least 5x, avoiding bubble formation. For each reaction, add 1 µL of diluted cell lysate to 150 µL of the reaction solution. Mix thoroughly and immediately by gently pipetting, avoiding bubble formation. Measure the absorbance at 412 nm every 10–30 s for 4 min at 25 °C using a plate reader capable of measuring absorbance at 412 nm at minimum intervals of 10 s.

NOTE: Change the tip before pipetting a different reagent and avoid forming bubbles in the wells. Incomplete mixing of the reagents may cause variations in the measurements. The citrate synthase activity assay should be done at room temperature immediately after the samples are collected, as this assay is used to quantify intact mitochondrial mass.

1.7. Plot data as optical density (OD) absorbance (abs) (Y-axis) versus time (in minutes) (X-axis). Then calculate the slope for the linear portion of the curve, where the reaction rate is

$$\frac{\Delta OD}{\Delta T} = \frac{OD1 - OD2}{T1 - T2}$$

Divide the value by the sample protein concentration to normalize the citrate synthase activity. The citrate synthase activity is calculated as

$$\frac{reaction\ rate}{protein\ (mg)} \times sample\ dilution = citrate\ synthase\ activity\ (\Delta abs/min/mg\ protein)$$

NOTE: The sample protein concentration can be measured by a protein concentration assay kit.

REPRESENTATIVE RESULTS:

Figure 1 presents an example of the kinetic curves for the OD absorbance at 412 nm over time obtained using the citrate synthase activity colorimetric assay to measure the *Drosophila* thorax tissue homogenates of different genotypes. It is well known that PGC-1α is a master regulator of mitochondrial biogenesis. PGC-1α is functionally conserved between *Drosophila* and humans. *Drosophila* RNF34 (dRNF34) is an E3 ubiquitin ligase for *Drosophila* PGC-1α, dPGC-1, and promotes

dPGC-1 protein degradation[17]. Transmission electron microscopy and mitochondrial DNA qPCR have shown that knockdown of dRNF34 in *Drosophila* muscle increases mitochondrial content, which is suppressed by knockdown of dPGC-1[17]. Based on these results we hypothesized that knockdown of dRNF34 in *Drosophila* muscle would increase mitochondrial citrate synthase activity, which should be reversed by knockdown of dPGC-1. Indeed, using the colorimetric assay of citrate synthase activity described here, we found that knockdown of dRNF34 in *Drosophila* muscle increased mitochondrial citrate synthase activity, which was reversed by knockdown of dPGC-1. Specifically using the method described here, an initial linear enzymatic rate was established for each assay (**Figure 1**). The trend lines with their formulas and coefficient of determination (R^2) are shown in the graph (**Figure 1**). The slopes of the trend lines represent the maximal reaction rates, which are equivalents of the maximal citrate synthase activities of the different genotypes. The slopes for the different genotypes are different (**Figure 1**). The coefficient of determination is closer to 1; the formulas of the trend lines are more reliable. The protein concentration normalized maximal citrate synthase activities of different genotypes were calculated from the **Figure 1** data (**Figure 2**). The maximal citrate synthase activity of fly thoraxes with muscle-specific dRNF34 knockdown increased, which was reversed by muscle-specific dPGC-1 knockdown (**Figure 2**).

[**Figure 1** here]

[**Figure 2** here]

FIGURE LEGENDS:

Figure 1. An example of the kinetic curves for the colorimetric assay of citrate synthase activity. The adult fly thorax homogenates of (**a**) 24B-Gal4>+ (**b**) 24B-Gal4>dRNF34RNAi(II), and (**c**) 24B-Gal4>dRNF34RNAi(II),dPGC-1RNAi were subjected to the colorimetric assay of citrate synthase activity. The horizontal axes represent reaction times, and the vertical axes represent OD absorbencies at 412 nm. A linear enzymatic rate was established for each genotype. The trend lines were drawn and the formulas and R^2 of the trend lines are shown in the graph.

Figure 2. The maximal citrate synthase activities calculated from the kinetic curves and normalized by protein concentration. The maximal citrate synthase activity of fly thoraxes with muscle-specific dRNF34 knockdown increased, which was reversed by muscle-specific dPGC-1 knockdown. All the data are represented as means ± SEM (*$p < 0.01$, by ANOVA test, and Tukey's test for multiple comparisons, n = 3, 10 thoraxes per replicate). This figure is modified from Wei et al.[17].

DISCUSSION:

Metabolic studies using *Drosophila* as a model must take into consideration the genetic background, diet, and stock maintenance of the flies[18]. To avoid the effects of different genetic backgrounds on the measurement of citrate synthase activity, different strains of *Drosophila* should be backcrossed to the control strain for 10 generations. The genetic background of all *Drosophila* strains used in our experiments is w^{1118}, so we used w^{1118} as a control. Generally, we think w^{1118} is a good control, as it is the genetic background for most *Drosophila* strains and is

much easier to backcross than Canton-S. The diet and stock maintenance must be exactly the same for all the experimental groups. In our experiments, flies are normally maintained at 25 °C and 50–60% relative humidity. The sample preparation step should also be performed with caution. The crosses that are set up for the experiment should be in a similar and appropriate scale. To set up a cross, 3–4 female virgin adult flies are kept with 2–3 male adult flies in a 4 cm diameter, 15 cm high vial supplied with fresh food. The food recipe can vary (e.g., high fat diet, normal diet, high sugar diet), depending on the experimental design. Two days later, the parental generation of flies is transferred to a new vial with fresh food. Caring for the larvae hatched in the food includes adding some water if necessary. When the filial generation of flies starts to hatch, the vials are flipped every 24 h, the flies of the desired genotype are collected in a new vial for the experiment, and the hatch date of the flies is marked on each vial. Approximately 20-30 collected flies of the same genotype, gender, and age are kept in a vial with food. The collected flies must be transferred to fresh vials with appropriate food every two days until the experiments are performed. To avoid the effect of age on citrate synthase activity, the flies of the different groups tested should hatch on the same day. In addition, the gender of the flies should be matched. Usually the body size of females is larger than that of males, thus the protein concentration of female bodies is higher than that of male bodies.

The citrate synthase activity assay can also be used for measuring the citrate synthase activity in larvae. To this end, each sample can consist of five wandering third instar larvae. The third instar larvae can be distinguished by a dark orange ring at the tip of their posterior spiracles, which is lacking or weakly present in the second instar larvae.

In step 6, the dilution ratios of samples may vary for different samples. For the samples that are first measured, several dilution ratios have to be tried to determine an appropriate dilution ratio to establish a linear enzymatic rate in the assay. The total measuring time for the maximal enzyme activity that establishes a linear enzymatic rate in the assay varies depending on the enzyme-substrate ratio. If the substrate is extremely overdosed compared to the enzyme, then the enzyme activity or the reaction rate reaches maximal, which allows the establishment of a linear enzymatic rate in the assay. As the reaction goes on, the amount of the substrate decreases, and the enzyme activity or the reaction rate slows down. If a linear enzymatic rate cannot be plotted, which means that the substrate is not overdosed compared to the enzyme, further dilute the tissue homogenates with reaction solution or shorten the interval time for the 412 nm readings until a linear enzymatic rate plot is established. The interval time for the 412 nm reading should be no more than 30 s. The duration for the 412 nm reading is based on the reaction rate and time. The spectrophotometer reading should stop when no further color change is observed for all reactions.

The citrate synthase activity can be normalized by the sample protein concentration, sample fresh weight, or cell number. The sample fresh weight can be measured before homogenizing as in step 3. The cell number can also be used to normalize the lysate of cultured cells, but the cells must be completely lysed. DNA content is not a good option for an internal control, as the DNA extraction procedure may introduce variations in the samples, particularly for samples containing a small amount of DNA.

If no color change is observed after the reaction, several different troubleshooting steps can be taken:
1. Check the sample preparation step, making sure to handle the samples properly, keep the samples on ice, and avoid multiple freeze-thaw cycles.
2. Try to use a higher protein concentration, because some samples might have much lower expression levels of citrate synthase that are undetectable by the citrate synthase activity assay.
3. Note that some commercially available kits for citrate synthase activity assay cannot be used for *Drosophila*, because these kits determine mitochondrial citrate synthase activity based on immunocapture of mammalian citrate synthase and the mammalian citrate synthase antibody may not recognize the *Drosophila* citrate synthase.

Likewise, if there is a discrepancy between samples:
1. Make sure there are no bubbles in the wells.
2. Examine whether this is caused by poor pipetting technique.

In contrast to measuring citrate synthase activity by a colorimetric assay in purified mitochondria, which requires almost 200 flies for the purification of mitochondria of each genotype[19], we present a protocol for a fast and simple assay for measurement of citrate synthase activity by a colorimetric method in tissue homogenate or in whole cell extracts[16]. For each sample, only ten flies hatched on the same day are needed; for triplicate samples of each genotype, 30 flies are needed. The protocol described is applicable not only to *Drosophila* but also to other systems, such as cultured cells and mammalian tissues.

Besides the citrate synthase activity assay, other methods can be used to quantify mitochondrial mass. Compared to other commonly used quantitative methods of mitochondria, such as measurement of mitochondrial DNA content by qPCR and quantification of mitochondrial proteins by Western blotting, which detect all the mitochondria regardless of their function, the citrate synthase activity assay is used to quantify the presence of intact mitochondrial mass. Unlike the mitochondrial respiration assay, which needs mitochondrial purification and specialized assay equipment[16], the citrate synthase activity assay allows researchers to carry out rapid measurements by spectrophotometry with simple sample preparation, such as whole cell extract or tissue homogenate, and no need for mitochondrial isolation. Thus, the citrate synthase activity assay is a rapid and economical assay for quantification of intact mitochondrial mass.

DISCLOSURES:
The authors declare no conflicts of interest.

ACKNOWLEDGMENTS:
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Figure 1

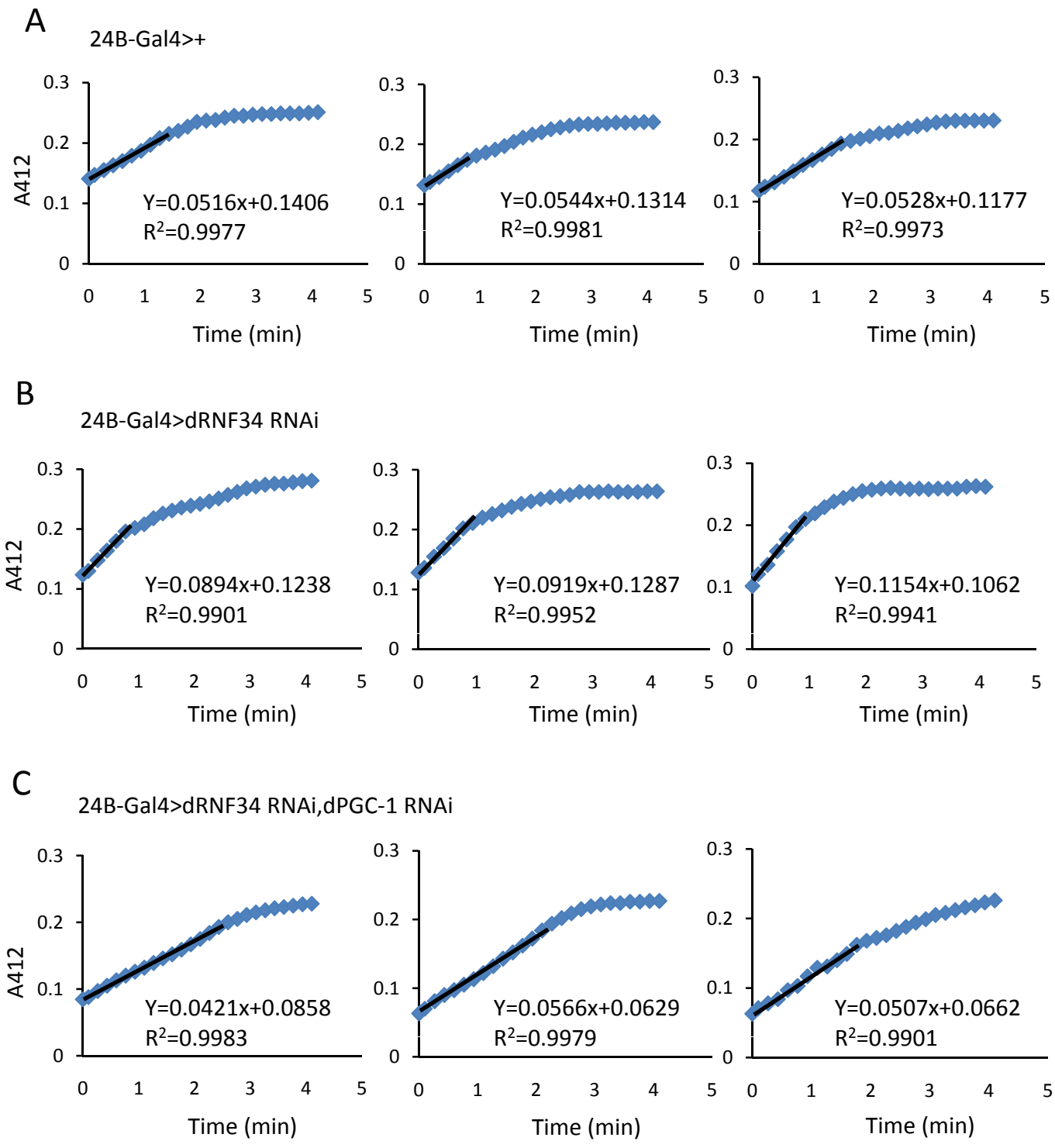
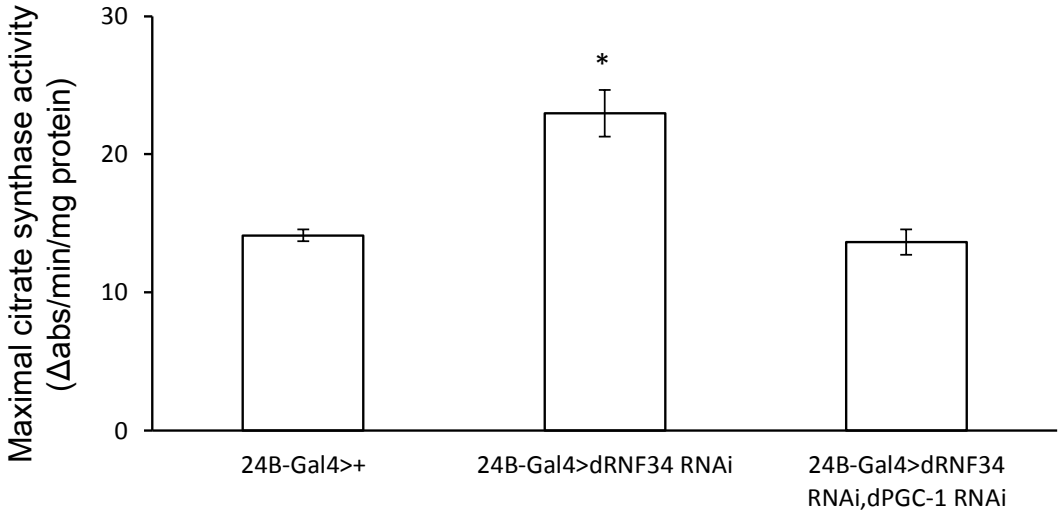


Figure 2
Figure 2



Name of Material/Equipment	Company	Catalog Number
2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)	Sigma-Aldrich	V900477
2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIZMA Base)	Sigma-Aldrich	V900483
Acetyl-CoA	Sigma-Aldrich	A2181
Dithio-bis-nitrobenzoic acid (DTNB)	Sigma-Aldrich	D8130
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	V900106
Oxaloacetate	Sigma-Aldrich	O4126
Pellet pestle	Sangon	F619072
Pellet pestle motor	Tiangen	OSE-Y10
Plate reader	BioTek	Eon
Protein BCA Assay kit	Beyotime	P0010S
Scissors	WPI	14124
Triton X-100	Sangon	A110694-0100

MS ID: JoVE5945

We thank the reviewers for their insightful comments that we believe have improved our manuscript significantly. Below are our point-by-point responses to the reviewers' comments.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this manuscript entitled "Methods for measuring triglyceride level and citrate synthase activity in Drosophila" as a visual methods paper, Wei et al., present two protocols for measuring triacylglyceride (TAG) levels and mitochondrial citrate synthase (CS) in Drosophila. While these protocols are of utmost importance for the fly community, the authors' methodology does not sound like another alternative to existing visualized methods for TAG assays. On the other hand, the protocol for citrate synthase measurements can be a very useful method. However, in both protocols there are certain major concerns that need to be addressed, which can improve the over all quality of their manuscript.

Major Concerns:
1. This manuscript is written in a very confusing way to begin with. The writing sounds as if the authors are presenting protocols to measure TAGs using TLC based on the lines 35, 36, 59-60. But that does not seem to be the case as the authors perform a colorimetric assay. Secondly, the mitochondrial section is very abrupt. These are two completely different protocols together with a completely different sample preparation methods. There is no common ground between the two methods. For e.g., I would have appreciated if the authors developed one method of preparing the fly lysates in such a way that both TAG and CS could be measured from the same lysate. The authors may want to either build on this or just discuss only their citrate synthase protocol in greater detail.
Response:
We agree. We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*.

2. With regard to the TAG assays, what the community would like to see and learn the most is measuring TAGs from hemolymph, especially from the adults. The authors may want to expand their TAG assays to hemolymph, which will be a valuable contribution to their manuscript.
Response:
We agree. But we have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*.

3. How did the authors come up with the number of flies/larvae to be 5? Perhaps here, the authors could demonstrate or at least discuss that less than 5 flies may not be sufficient for downstream assays. Moreover, what is the upper limit for the number of flies.

Response:

Thank you for your suggestion. But we have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*.

4. In the sample preparation section for TAGs, the authors keep mentioning that the samples can be stored at -80oC for measuring TAGs at a later time points (for protein and TAG). In an ideal scenario, the assays must be done soon after the lysates are prepared to avoid protein and TAG loss over freeze-thaw cycles. Thus, the authors must discuss this point in a different way and perhaps add a note suggesting that the samples can be frozen in case they cannot be measured right away.

Response:

Thank you for your suggestion. But we have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*.

5. The TAG measurement steps (entire section 1.3) are written in a misleading way. The ideal order of the experimental method must be samples in the wells first and then followed by the addition of TAG working reagent. This will ensure that the reactions start uniformly across all the wells of the 96-well plate. Additionally, taking 1ul of samples is too less in volume and may lead to erroneous results.

Response:

We agree. But we have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthaseactivity in *Drosophila*.

6. The authors did not include a section which discusses how to measure the levels of free glycerol, in which case the TAG reagent is excluded from the samples.

Response:

Thank you for your suggestion. But we have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*.

7. Lastly, the authors must discuss, as a note, that TAGs can also be normalized per fly in case the protein levels change due to genetic manipulations.

Response:

We agree. But we have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*.

Citrate synthase (CS) section:

1. To begin with the preparation of adult flies for both TAG and CS assays, the authors' method of rinsing the adult flies twice and returning to the anesthesia pad sounds a bit weird. In my opinion, the flies do not carry food if maintained well. Hence, rinsing can be avoided and the flies sorted on anesthesia pad can directly be snap frozen for downstream lysis.

Response:

We agree. We have deleted the step of rinsing the adult flies twice and returning to the anesthesia pad.

2. The authors must discuss whether CS activity can be normalized by DNA content.

Response:

Thanks for the suggestion. We added in the note of the step 7 as follows.

"Practically DNA content is not a good option for an internal control, as the DNA extraction procedure may introduce variations in samples particularly for samples containing small amount of DNA." See lines 161-163.

3. Representative figures section: I totally fail to understand the figures.

A) What was the rationale to knockdown dRNF34?

Response:

We have added the rationale to knockdown dRNF34 in the figure section, which is as follows.

"It is well known that PGC-1α is a master regulator of mitochondria biogenesis. PGC-1α is functionally conserved between *Drosophila* and human. *Drosophila* RNF34 (dRNF34) is an E3 ubiquitin ligase for *Drosophila* PGC-1α, dPGC-1, and promotes dPGC-1 protein degradation[17]. Transmission electron microscopy and mitochondrial DNA q-PCR have shown that knockdown of dRNF34 in *Drosophila* muscle increases mitochondrial content, which is suppressed by knockdown of dPGC-1[17]. Based on the results we hypothesized that knockdown of dRNF34 in *Drosophila* muscle might increase mitochondrial citrate synthase activity, which should be reversed by knockdown of dPGC-1." See lines 168-175.

B) How were the abdomens dissected/isolated?

Response:

We revised the step 3 as follows.

"Anesthetize adult flies with CO_2 on an anesthesia pad and isolate the desired tissues. To isolate the adult fly thoraxes, for example, fix the fly thoraxes by a pair of forceps, and then isolate the fly abdomens by cutting along the border of thorax and abdomen using a pair of scissors. Collect the fly thoraxes for the muscle citrate synthase activity assay." See lines 103-106.

C) If a HFD experiment has been done, where are the controls? The authors must show that HFD induced an increase in TAG levels and then show the effects of knocking down any gene.

I understand the figures were modified versions of the authors' previous publication, however it does not mean that they do not represent their data well for the current publication.

Over all, the authors must emphasize the use of their methods in simpler ways. From the abstract stand point, it sounds like a general methods paper. While on the other hand, the results represent TAG and CS levels upon knocking down a gene, which makes it sound like a research paper without providing proper controls nor background.

Response:

Thanks for the suggestion. But we have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*. We have added the background of dRNF34 to explain the rationale to knockdown dRNF34 in the figure section as follows.

"It is well known that PGC-1α is a master regulator of mitochondria biogenesis. PGC-1α is functionally conserved between *Drosophila* and human. *Drosophila* RNF34 (dRNF34) is an E3 ubiquitin ligase for *Drosophila* PGC-1α, dPGC-1, and promotes dPGC-1 protein degradation[17]. Transmission electron microscopy and mitochondrial DNA q-PCR have shown that knockdown of dRNF34 in *Drosophila* muscle increases mitochondrial content, which is suppressed by knockdown of dPGC-1[17]. Based on the results we hypothesized that knockdown of dRNF34 in *Drosophila* muscle might increase mitochondrial citrate synthase activity, which should be reversed by knockdown of dPGC-1." See lines 168-175.

We also revised the figures and the figure legends. We hope that the reviewer is satisfied with the revised version.

Minor Concerns:

Some sentences in the abstract repeat in the introduction section.

Response:

We revised the abstract and introduction and deleted the repeated sentences.

Reviewer #2:

Manuscript Summary:

The manuscript by Wei and colleagues documents two user friendly, high-through, and colorimetric assay based methods to quantify triglyceride level and citrate synthase activity in Drosophila. In general, description of two methods is reasonably clear and easy to read. Authors concentrate on methods to measure triglyceride level and citrate synthase activity in larvae and adult flies, thus this protocol provide basic information for readers interested in metabolic regulation in Drosophila. I realize this protocol potentially useful (particularly Citrate synthase activity assay). However, it needs some clarifications and improvements, prior to publication.

Major Concerns:

1. For Triglyceride measurement. Here is a similar protocol in terms of high fat diet feeding and high throughput-measurement of Triglyceride in Drosophila published on 9/13/2018 from JoVE (https://www.jove.com/video/56029/high-fat-diet-feeding-high-throughput-triacylgl

yceride-assay). Authors need to clarify the advantage of this submitted TAG measurement protocol and compare the similarities and differences between two protocols in the introduction and discussion part.

Response:

Thank you for your reminding the similar protocol. We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*.

2. For two protocols, the authors should describe and discuss the possible effect of the genetic background on those measurements. Is it preferable to backcross the strains to a reference control genotype? This step is a requisite in most flies' metabolic experiments. Even though white gene may impact on metabolism in Drosophila, Is w1118 still a good control, since this is a classical background for genetic tools? Is it same as Canton-S for those measurements?

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*. We discussed the possible effect of the genetic background on mitochondrial citrate synthase measurement as follows.

"We think that it is preferable to backcross the strains to a reference control genotype for *Drosophila* metabolic experiments since genetic backgrounds do affect many metabolic phonotypes. The genetic background of all *Drosophila* strains used in our experiments is w^{1118}, so we used w^{1118} as a control. Generally, we think w^{1118} is a good control, as it is the genetic background for most of *Drosophila* strains and is much easier for backcrossing than Canton-S." See lines 76-80.

3. For materials and figures part, I am not satisfy with this current version. An article published in JOVE should give sufficient technical and methodological information, which is missing in the current version. For example, in material part, authors should provide all of fly strains' information, fly maintain conditions (Temperature, humidity, light/dark condition, adult fly's age for experiment, etc.). Since the physiological status may impact on results, the population density of flies needs to be further clarified. How many males and how many females are used to generate healthy offspring for the experiments in what kind of fly bottles or vials (volume)? Authors should further provide protocol to describe how to get physiology and aged matched larvae and adult flies to conduct experiments. Authors should provide details information of food recipes for both of normal food and HFD, and please provide information in terms of how many nutrients does each food component provided as well. Please provide all of the information of the chemical reagents that used in this protocol.

Response:

Thanks for the suggestion. We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*. We discuss all other issues as follows.

"Flies are normally maintained at 25 °C and 50-60% relative humidity, which may vary depending on experiments. To set up a cross, put 3-4 female virgin adult flies with 2-3 male adult flies in a 4 cm-diameter, 15 cm-high vial supplied with fresh food.

The food recipe varies, such as high fat diet, normal diet, high sugar diet and so on, depending on the design of experiments. Two days later, transfer the parental generation of flies to a new vial with fresh food. Take care of the larvae hatched in the food by adding some water if necessary. When the filial generation of flies start to come out, flip the vials every 24 hours, collect the flies of desired genotype in a new vial for experiment and mark the hatch date of the flies on each vial. Take care of the collected flies by transferring them to fresh vials with appropriate food every two days until performing experiments. To avoid the effect of age on citrate synthase activities, the flies of different groups should be hatched on the same day. In addition, the gender of flies should be matched as well. Usually the body size of females is larger than that of males, thus the protein concentration of female bodies is higher than that of male bodies." See lines 81-93.

4. Authors should re-draw all figures and re-write figure legends. In both of two figures, authors missed all UAS-RNAi control. Authors should clearly label control group and experimental group in figures, and show significant difference between them. Authors also need to clearly describe the statistic method they used for each figure. If there are multiple groups need to compare, authors must use ANOVA analysis, rather than t-test.

Response:

Thanks for the suggestions. We agree. We re-drew all figures, re-wrote all figure legends, and used ANOVA analysis, for which please refer to Figure 1 and Figure 2 and their legends. For the control groups, we used 24B-GAL4 > w^{1118} as control. We agree that it would be better to have UAS-RNAi controls as well, but we think that our rescue experiments are alternatives to UAS-RNAi controls. In our rescue experiments, we showed that the dRNF34 RNAi phenotype was rescued by dPGC-1 RNAi, which was consistent with our published biochemical data showing that the ubiquitin E3 ligase dRNF34 ubiquitinates dPGC-1 protein and promotes its degradation. Thus, we think that our data of dRNF34 RNAi phenotypes are reliable.

Minor Concerns:

1. For step 1.1 and 2.1, if authors recommend that the age of flies is important for measurement, the method of "hatched same sample group on the same day" seems like not sufficient to get the same age of flies to test. Authors should clarify whether need to collect the same group flies within the same day after flies' eclosion. In addition, apparently the gender of flies is critical for metabolic process measurement. Please clarify are there any different steps when conduct experiments in male and female flies. It would be great, if authors could provide extra information or notes about it.

Response:

Thanks for the suggestions. We agree. We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activity in *Drosophila*. We revised protocol as follows.

"When the filial generation of flies start to come out, flip the vials every 24 hours, collect the flies of desired genotype in a new vial for experiment and mark the hatch date of the flies on each vial. Take care of the collected flies by transferring them to fresh vials with appropriate food every two days until performing experiments. To

avoid the effect of age on citrate synthase activities, the flies of different groups should be hatched on the same day. In addition, the gender of flies should be matched as well. Usually the body size of females is larger than that of males, thus the protein concentration of female bodies is higher than that of male bodies." See lines 86-93.

2. For step 1.1, since the third-instar larval stage in Drosophila is a period characterized by dynamic modifications in both of developmental and behavior (https://www.sciencedirect.com/science/article/pii/S0896627303003969), (https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1006583), (https://www.sciencedirect.com/science/article/pii/S2211124716312505#bib26), authors need to clarify which stage of third-instar larval they recommend to use in this protocol (early stage of third-instar larval or late stage of third-instar larval?), and to clarify whether there is a different to use different stages of third-instar larval in this protocol. If so, please provide extra protocol to describe how to synchronize larval in the same stage, since only hatched them in the same day can't properly synchronize the stage third-instar larval.

Response:

Thank you for your suggestions, but we have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in *Drosophila*.

3. For step 1.1.3 and step 2.3, please describe how to confirm "remove all traces of food that might be attached to the body of flies". Please clarify how to conduct "dried flies by CO2" and how to confirm dried out those flies.

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in Drosophila. We have deleted the step of rinsing the adult flies twice and returning to the anesthesia pad in theprotocol for measuring mitochondrial citrate synthase (CS) activity in Drosophila, as this step is not necessary for measuring CS activity.

4. For step 1.2.1 and step 2.4 Author need to describe how to "homogenize completely", and how is "sufficient homogenization" confirmed.

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in *Drosophila*.

We wrote the note for step 4 (step 2.4 in the former version) about how to confirm "sufficient homogenization", which is as follow.

"Tape the tube and check homogenates, and make sure there are no clots in the homogenates and the tissues in the tube are homogenized completely." See lines 113-114.

5. For step 1.2.1. Authors need to clarify the difference between "freeze simples in liquid nitrogen" and "directly add pre-cold PBST". Presumably the purpose of freezing the flies at -80 C is to euthanizes them and for long term storage. If the flies be stored at a specific temperature and analyzed (measurement of triglyceride level

and citrate synthase activity) later, please clarify how long those simples could be stored at liquid nitrogen, those information are important for readers when they replicate this protocol.

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in *Drosophila*.

6. For Step 1.2.2. Please clarify how long those protein simples could be stored at -80 C?

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in *Drosophila*.

7. For Step 1.2.3, "Heat the remaining supernatant at 70 °C for 10 min", please provide more information about "heat", how to heat? Whether step need to vortex simples during the heating?

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in *Drosophila*.

8. "The heat-inactivated samples can be frozen and stored at −80 °C for later measurement", please clarify how long can be stored?

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in *Drosophila*.

9. For step 1.3.1. "Store the Triglyceride Working Reagent in an amber bottle." Please clarify is there a specific temperature for keep this "Triglyceride working reagent"? And how long this solution could be stored?

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in *Drosophila*.

10. For step 1.3.1 and step 2.6, please clarify how to "mix well" your simples and how it confirmed?

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in *Drosophila*.

For the issue of “mix well”, we revised the protocol in step 6 (step 2.6 in the former version) as follows.

"mix well by gently pipetting up and down at least five times and avoid forming bubbles." See lines 123-124.

“Incomplete mixing of reagents may cause variation of measurements.” See line 129.

11. For citrate synthase activity assay, please clarify whether this protocol could use for larvae Drosophila, if it is possible, please clarify which tissue (or the whole larvae) could be used in this protocol.

Response:
Thanks for the suggestion. We revised this as follows.
“The citrate synthase activity assay can also be used for measuring the citrate synthase activity in larvae.” See lines 94-95.

12. For citrate synthase activity assay, Step 2.6. Authors need to describe the detail protocol how to "adjust the duration and interval time that based on the reaction rate time for spectrophotometer reading".
Response:
Thanks for the suggestion. We revised step 6 (step 2.6 in the former version) as follows.
“The dilution rate varies for different samples. For the samples that are first measured, several dilution ratios have to be tried to determine an appropriate dilution ratio to establish a linear enzymatic rate in the assay. The total measuring time for the maximal enzyme activity which establishes a linear enzymatic rate in the assay varies depending on the enzyme-substrate ratio. If compared to the enzyme, the substrate is extremely overdosed, then the enzyme activity or the reaction rate reaches maximal, which allows to establish a linear enzymatic rate in the assay. As the reaction goes on, the substrate becomes less and less and the enzyme activity or the reaction rate slows down. If a linear enzymatic rate can't be plotted, which means that the substrate is not overdosed compared to the enzyme, further dilute the tissue homogenates with reaction solution or shorten the interval time for the 412 nm reading until establishing a linear enzymatic rate plot. The interval time for the 412 nm reading should be no more than 30 s. The duration for the 412 nm reading is based on the reaction rate and time, and stop spectrophotometer reading when no color change is observed for all reactions.” See lines 130-142.

Reviewer #3:
Manuscript Summary:
The authors describe a protocol for measuring TAG content in Drosophila. In addition, they explain a protocol for measuring citrate synthase activity.

Major Concerns:
I don't see why those two protocols are better than those published and why the two measurements should be combined. The efficacy of the protocols has not been proven, since the only "data" the authors show is data copied from another publication. They also do not describe what has been done in detail. They do not compare to other protocols or give any improvements to other protocols. They discuss limitations of the protocols but don't give solutions to them besides saying that one should use other assays- so why publishing those not new protocols again? In addition, these protocols are not very complex and thus I do not see why you would need an additional methods paper for them. I don't think that will help anyone. In addition, the paper lacks explanations for basically all assumptions they make.
Response: We revised the manuscript. We deleted the method for measuring

triglyceride level and focus on the method for measuring mitochondrial citrate synthase (CS) activity in *Drosophila*. We hope that the reviewer 3 is satisfied with the revised version.

Reviewer #4:

Manuscript Summary:

In this manuscript, the authors describe two colorimetric assays for quantifying the triglycerides level and citrate synthase activity in Drosophila. For both assays, they have presented the detailed procedures, highlighted the most important steps, and discussed the pros and cons. Overall, this manuscript is clearly written. However, several points listed below need to be further addressed.

Major Concerns:

1. In 2.6, citrate synthase activity is measured by recording absorbance at 412 nm every 10s for 4 min using spectrophotometer. We would expect to see a kinetic curve for the absorbance over time.

a. What does the typical curve look like? I strongly suggest the authors to provide a Figure to illustrate the results.

Response:

Thanks for the suggestion. We added in Figure 1 the kinetic curves for the absorbance at 412 nm over time.

b. If the kinetics does not follow the Michaelis-Menten equation, what does that mean and how to troubleshooting? During the 4 min recording, how long will the maximal enzyme activity will typically remain?

Response:

Thanks for the suggestion. We addressed the questions in the note of step 6, which is as follows.

“The dilution rate varies for different samples. For the samples that are first measured, several dilution ratios have to be tried to determine an appropriate dilution ratio to establish a linear enzymatic rate in the assay. The total measuring time for the maximal enzyme activity which establishes a linear enzymatic rate in the assay varies depending on the enzyme-substrate ratio. If compared to the enzyme, the substrate is extremely overdosed, then the enzyme activity or the reaction rate reaches maximal, which allows to establish a linear enzymatic rate in the assay. As the reaction goes on, the substrate becomes less and less and the enzyme activity or the reaction rate slows down. If a linear enzymatic rate can't be plotted, which means that the substrate is not overdosed compared to the enzyme, further dilute the tissue homogenates with reaction solution or shorten the interval time for the 412 nm reading until establishing a linear enzymatic rate plot. The interval time for the 412 nm reading should be no more than 30 s. The duration for the 412 nm reading is based on the reaction rate and time, and stop spectrophotometer reading when no color change is observed for all reactions.” See lines 130-142.

2. The language, especially the Introduction part needs further editing.

Response:

Thanks for the suggestion. We revised it, and we hope that the reviewer is satisfied with the revised version.

Minor Concerns:

1. Method 1, the samples can be frozen at −80 °C for later measurement. Will the reliability of the data be affected by the frozen? How long can the samples be frozen?

Response:

We have deleted the method for measuring triglyceride level and focus on the method for measuring mitochondrial citrate synthase activityin *Drosophila*.

2. The authors could discuss whether these methods are applicable to other systems? Such as culture cell and mammalian tissues.

Response:

We addressed this issue in the discussion as follow.

“The assay can be used for cultured cells and mammalian tissues as well..” See lines 97-98.

3. Figure 2, annotation of Y-axis should be "maximal citrate synthase activity"

Response:

We corrected it.

Reviewer #5:

Manuscript Summary:

The manuscript presented nicely describes two protocols to assess mitochondrial activity in a low-cost simplified way. This would be of interest to the fly community. Your protocol nicely breaks down the major steps of the protocol with enough detail. Other than the minor concerns listed below, I question why two different protocols are being combined into one protocol paper. If you expanded on their connection and on how these two pieces of data could be used together it may make the protocols more cohesive. Otherwise, they read as two distinct experiments.

Response:

We agree. We have deleted the method for measuring triglyceride level as suggested by some reviewers and focus on the method for measuring citrate synthase activity in the revised version.

Minor Concerns:

1. You do not compare data from your method to those already existing. Is there published reference data that you could discuss?

Response:

Thanks for the suggestion. We added in the discussion section as follows.

"In contrast to measuring citrate synthase activity by colorimetric assay in purified mitochondria, which needs almost 200 flies for each sample of a genotype for

purification of mitochondria[19], we present a protocol for a fast and simple assay for measurement of citrate synthase activity by a colorimetric method in tissue homogenate or in whole cell extract[16]. For each sample, only ten flies hatched on the same day are needed, for triplicate samples of each genotype, 30 flies are needed." See lines 242-247.

2. The significance is explained in terms of simplicity over other systems as well as caveats of measuring in flies. Expand upon why creating a protocol to obtain this data is significant in general.

Response:

Thanks for the suggestion. We addressed this issue in the introduction section as follows.

"Citrate synthase is localized in the mitochondrial matrix within eukaryotic cells, which is a rate-limiting enzyme in the first step of the tricarboxylic acid cycle[9], and thus it can be used as a quantitative marker for the presence of intact mitochondrial mass[9,10]. Citrate synthase activity can also be used as a normalization factor for proteins of intact mitochondria[11,12]." See lines 56-60.

3. Choose one sample (adult or larvae) and mention applicability to other life stages in discussion.

Response:

Thanks for the suggestion. We added in the revised manuscript that the citrate synthase activity assay is applicable not only to *Drosophila* (adult and larvae) but also to other systems, such as cultured cells and mammalian tissues. See lines 94-95, 97-98, 247-248.

Response to Editorial comments:

1. There are many long paragraphs as 'Notes' within the protocol. Please either make these into numbered protocol steps (in the imperative; no more than 4 sentences per step) or move them to the Introduction, Results, or Discussion as appropriate.

Response: Thanks for the suggestion. We have moved the long paragraphs as 'Notes' to the discussion section.

2. The second activity equation (reaction rate*1000/13.6*path length) looks like it should produce units of µmol/min/L (i.e, µM/min); it is also unclear how this is 'activity per enzyme activity unit'. Please clarify.

Response: Thanks for your comment. Some commercial kits recommend the citrate synthase activity of samples to be expressed as enzyme activity units per microliter of sample (U/ml) or enzyme activity units per microgram of protein (U/µg). One unit of citrate synthase is the amount of enzyme that will generate 1.0 mol CoA per minute at pH 7.2 at 25 ℃. 1000/13.6*path length is only a constant, which does not affect the relative relationships of samples. In this protocol, we only calculated the relative citrate synthase activities by the first activity equation, which is in step 7. Therefore, in the revised version, we deleted the second activity equation in the note of step 7, which may confuse readers.

3. The linear fits in Figure 1 are confusing-it looks like they're only fit to the first few points (i.e., the linear/steady-state portion of the graph), but extend well beyond those points. Can you clarify this; e.g., by shortening the lines?

Response: We agree. Thanks for the suggestion. We shortened the lines in Figure 1.

From: chshou@sibs.ac.cn <chshou@sibs.ac.cn>
Sent: Tuesday, November 27, 2018 5:51 PM
To: wei_ping_cmu <wei_ping_cmu@hotmail.com>
Subject: 回复: Re: ABBS-2018-154

魏平，您好！
感谢您的来信。我们同意您合理使用该论文中的 Fig2 E 和 Fig4 A 并加以引用和说明。书已经通过印刷品的方式寄出，请到收发室查询。
谢谢
Sincerely

Caihua Shou
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https://academic.oup.com/abbs
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ABBS Editorial Office
021-54920955

2018-11-28
From: wei ping
Date: 2018-11-28 09:12
To: chshou@sibs.ac.cn
Subject: Re: ABBS-2018-154

寿老师:
您好！

我收到 JoVE 杂志的约稿，写一篇关于在果蝇组织中测甘油三酯和 CS 活性的方法的文章，我想在文章中引用我最近在贵刊发表的

RNF34 modulates the mitochondrial biogenesis and exercise capacity in muscle and lipid metabolism through ubiquitination of PGC-1 in *Drosophila*

论文中的 Fig2 E 和 Fig4 A, 稍作修改， 以作为该方法说明的例证。当然，在该 JoVE 杂志方法文章中会注明该两幅图引自贵刊我的上述文章。希望得到贵刊的允许。谢谢！

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上海市黄浦区重庆南路 280 号 西 5 号楼侧楼 102 室
谢谢！

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Author(s): Ping Wei, Wen Xue, and Jiwu Wang

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