**Editorial comments:**

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55002\_R1\_060716.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.  
  
  
1. Please include a space between all numbers and their corresponding units: Table 1, Figure 2/4, etc. It should be 8 h, not 8h.

*Response: A space between all numbers and their corresponding units has been included.*

2. Length exceeds 2.75 pg of highlighted material and must be reduced accordingly. We suggest removing highlighting from food preparation steps.

*Response: Highlighting has been removed from food preparation steps as suggested. The length of current highlighted material is within 2.75 pg.*  
  
3. Grammar:   
-Title should be: Drosophila Preparation and Longitudinal Imaging of Heart Function in vivo….

*Response: Title has been revised to be* *“Drosophila Preparation and Longitudinal Imaging of Heart Function in vivo Using Optical Coherence Microscopy” as suggested.*

-Line 134 – “Xenopus embryo is”

*Response: “Xenopus embryo is” has been revised to “The Xenopus embryo is” in line 134.*

-3.2.8 – “tube to track the development and imaging”

*Response: We have replaced “tube to track the development and imaging” with “Label the tube for longitudinal study through the next developmental stages”.*  
  
4. Additional detail is required:  
-3.2.3, 3.2.4, 3.2.7 – How are these data acquired? What actions are performed to do so?

*Response: The actions performed to acquire the data have been explained in 3.2.3, 3.2.4, and 3.2.7.*

-3.2.5 – How is the beam blocked?

*Response: We have added the method of blocking the imaging beam in 3.2.5.*

-3.3.1.5 – Are these the same parameters as in section 3.2? Please specify.

*Response: The parameters of the data acquisition software in 3.3.1.5 are the same as in section 3.2, and have been specified in this section.*

-How are images analyzed? Please include a section/step at the end of the protocol on how the images are analyzed using Matlab and ImageJ. Citations can be provided in lieu of detail, and this should not be highlighted for filming.

*Response: We have included a new section at the end of the protocol to explain how images are analyzed using Matlab and ImageJ. A citation has also been referred. This new section was not highlighted for filming.*  
  
5. Results: Please provide a scale bar for Figure 1A.

*Response: A scale bar has been added in Figure 1A as suggested.*

6. Discussion: Please discuss any modifications/troubleshooting that can be performed.

*Response: We have discussed the possible modifications and troubleshooting in the discussion section in page 13.*

**Reviewers' comments:**

**Reviewer #1:**  
*Manuscript Summary:*  
The authors present in details the cardiac imaging experiments of Drosophila in vivo using optical coherence microscopy (OCM). Drosophila is a very useful animal model for heart function studies and OCM is a powerful imaging method with a highly suitable imaging scale for the Drosophila cardiac development. Thus, the approach presented in this manuscript is of great significance for the further related investigations. The procedures of sample preparation and imaging protocol are very well described in the manuscript, and I think the representative data and discussions are clearly shown with a proper amount of information. Below I have some minor comments that the authors are suggested to address.  
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
Minor Comments  
1. For the Protocol step 1.1, I suggest the authors to add at least a reference to their previous publication where the OCM system was described with details.

*Response: We appreciate the reviewer’s suggestion and have added a reference to one of our previous publications where the OCM system was described with details.*

2. Looks like the protocol steps 2.1.3, 2.1.4 and 2.1.5 are for two experiments, one optogenetic pacing and one obesity related heart dysfunction. Therefore, the authors might want to consider combining 2.1.4 and 2.1.5 into one step so the whole structure is clearer.

*Response: We thank for the reviewer’s suggestion and have combined 2.1.4 and 2.1.5 into one step as suggested.*

3. After the step 3.2.3, the authors are suggested to point out what the background noise data are used for. Also, in the step 3.2.3, if this is for background noise removal, what is the reason of acquiring 100 B-scans?

*Response: The purpose of acquiring the background noise data has been specified in the step 3.2.3, and the reason of acquiring 100 B-scans has been explained in the note after 3.2.3. The background noise data acquisition is used for background noise subtraction in the data analysis step, and acquiring 100 B-scans is for convenience of data acquisition, 3 of which can be used for the background subtraction.*

4. In the step 3.2.4, since the imaging parameters have the x-direction scanning distance, it is suggested to attribute this as "repeated B-scan" or "M-B mode" instead of "M-mode", or at least add "2D" in the phrase to avoid confusion or misunderstanding.

*Response: We thank the reviewer for this suggestion. To avoid confusion, we have added “transverse” and “2D + time” in the note of 3.2 where the M-mode appeared for the first time in the protocol to make the “M-mode” easily understood.*

5. The same comment as #4 for 3.3 (Note) and the step 3.3.2.4.

*Response: We have included “2D” before “M-mode” for 3.3 (Note) and the step 3.3.2.4 to avoid the confusion as suggested.*

6. For the step 3.4.2, it will be better to specify the anesthetic used for the fly and to provide an example of the dose based on the size of the fly and vial.

*Response: We appreciate the reviewer’s suggestion. We have included the size of the vial used in the step 3.4.1. The dose of the anesthetic used for the fly has been specified based on the size of the fly and vial in 3.4.2.*

7. Table 1 was not mentioned in the manuscript. The authors are suggested to decide where in the manuscript it is referred to.

*Response: We have included Table 1 in the representative results section.*  
  
*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #2:**  
*Manuscript Summary:*  
The manuscript describes the experimental protocols using Drosophilae as an experimental model with optical coherence microscopy imaging. From the OCT results, the morphological changes of drosophila heart can be observed and the parameters for evaluation of heart functions can be acquired. This manuscript is well-written and it will be helpful for readers from the related research communities. Also, this manuscript is almost ready for publication. There is one minor question.  
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
1. In this study, the author used OCM for the study on drosophila beating behavior and also briefly compare the difference between OCT and OCM. However, for the readers or potential users, they may not have ideas of how to choose the suitable OCM system for similar experiments. Can the authors comment on the resolution and the imaging speed of OCT system when using OCM/OCT to visualize the heart structures and observe the beating behaviors of larva or adult flies?

*Response: We thank for the reviewer’s suggestion. The imaging speed and resolution of OCT/OCM system for visualizing the heart structures and functions in Drosophila has been commented and suggested in the discussion section.*

*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #3:**  
*Manuscript Summary:*  
The manuscript described the experimental protocols for preparation of Drosophila and optical imaging of the heartbeat with the OCM system throughout the life cycle of the specimen. Overall, the protocol is well written, while there are some small issues need to be addressed before publication.  
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
1. The description of the OCM system used in the protocol and the parameters needed to be considered when selecting an OCM system should be put in the section 'Preparation of OCM system for optical imaging of Drosophila', instead of in the discussion.

*Response: We thank the reviewer for the suggestion of moving the description of the OCM system and the parameters to the protocol. The method for selecting an OCM system has been described in the section 1 in protocol, including the key parameters. Since the parameters of different OCM systems may vary even with the same optical components, it would be helpful to discuss our OCM system and its parameters for performing Drosophila heart imaging as an example in the discussion section.*

2. Since this is a method protocol paper, the author should mention the definition of M-mode in OCT and the reason to select it in the section 3.2.

*Response: We appreciate the reviewer’s suggestion. The M-mode image has been defined as “transverse image in 2D + time”, and the reason to select it was explained in the note of section 3.2.*

3. In section 3.2.3, '0.3V ' and '0V' is selected to use, while before that there is no description about the scanning system, do these parameters stay the same when people use different scanner? If not, what's the point to use such specific numbers?

*Response: We thank the reviewer for pointing out this problem. The voltages may vary with the scanner used and have been replaced with the scanning ranges to cover in the fruit fly.*

4. In section 3.2.4, when you acquire M-mode data, why still 0.3 V in the x-transverse direction? Doesn't M-mode mean time-Z data instead of B-mode (X-Z data)?

*Response: M-mode means 2D B-mode (X-Z data) as described in 3.2.4. The voltage in the x-transverse direction is supposed to be the same with 3.2.3 in the background acquisition to cover the same distance in fruit fly to conduct background subtraction.*

5. Please move the scale bar to one place not blocking the image especially in Fig.3.

*Response: We thank the reviewer’s suggestion. The scale bar has been modified in Fig. 3 for not blocking the image.*

6. In Fig.4, so only the plus error is shown? If yes, there should not be a bottom cap then.

*Response: In Fig. 4, only the plus error bar was shown. We have included both the plus and the minus error bars in the current figure.*

*Additional Comments to Authors:*  
N/A