August 6th, 2018

**Dear**

**Editor-in-Chief**

**Journal of Visualized Experiments (JoVE)**

**Re: Manuscript No. JoVE58696**

I would like to submit the revised version of the manuscript entitled “NON-INVASIVE ASSESSMENT OF DORSIFLEXOR MUSCLE FUNCTION IN MICE” by Frederico Gerlinger Romero et al, to be re-evaluated for publication in Journal of Visualized Experiments (JoVE). We have considered the reviewers comments and recommendations, as well as performed several modifications to improve the manuscript. The modifications in the text are included in the revised version of the manuscript highlighted in red font. We are also submitting the letter of responses to the reviewers, addressing all the comments point-by-point and indicating where in the text the modifications were made. We do appreciate the valuable suggestions that were made and hope to have adequately addressed all the raised points. Once again, we thank the opportunity to have our manuscript evaluated by Journal of Visualized Experiments (JoVE).



Sincerely yours,

Frederico Gerlinger-Romero PhD

Associate Research Fellow

School of Exercise and Nutrition Sciences, Faculty of Health

**Answers to Reviewers' comments**

We are very grateful for the opportunity to have our manuscript evaluated by Journal of Visualized Experiments (JoVE) referees. We thank the reviewers for careful and critical discussion of our manuscript and helpful comments. We have made all the requested changes. We have addressed each criticism below and have made corresponding changes in the attached manuscript. A ‘redlined version’ of the revised manuscript has also been uploaded for convenience. We believe that the protocol reads more clearly due to the suggested revisions.

**EDITORIAL COMMENTS:**

Changes to be made by the Author(s) regarding the manuscript:

**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.**  
*A: We have revised the text accordingly.*

**2. Please note that both Standard Access and Open Access are checked in the uploaded ALA. Please select one type only. Note that in the Questionnaire Responses Open Access is selected. Please be consistent.**

*A: The correction was made in this document.*

**3. Figure 1: Please increase the resolution of this figure. Currently the text is difficult to read. Probably expand panels A and B and arrange them vertically.**

*A: We have revised the figure.*

**4. Figure 3: Please explain the red line and green line in the figure legend.**

*A: We have revised the figure legend.*

**5. Figure 5: Please line up the panels better and ensure that each panel has the same dimension if possible.**

*A: We have revised the panels in this figure legend.*

**6. Please revise the protocol to be a numbered list: step 1 followed by 1.1, followed by 1.1.1, etc.**

*A: The corrections were made.*

**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. Please move the discussion about the protocol to the Discussion.**

*A: We have revised the text*

**8. Please include single-line spaces between all paragraphs, headings, steps, etc.***A: The corrections were made.*

**9. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.**

*A: we have highlighted the essential steps on the manuscript (in yellow).*

**10. 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. Please do not highlight any steps describing anesthetization and euthanasia.***A: The highlight steps were made on the manuscript.*

**11. 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.**

*A: The highlight steps were made on the manuscript.*

**12. References: Please do not abbreviate journal titles.**

*A: We have revised the references.*

**13. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.**

*A: The correction was made in the Table of Equipment and Materials.*

**REVIEWERS' COMMENTS:**

**Reviewer#1:**

**1. The authors stabilized the leg of the mice by clamping the knee. While, a needle was reported to be used to stabilize the limb of the animal (Lovering et al., 2011). It would benefit from providing a more detailed discussion of the differences and/or advantages between these two procedures.**

*A: Lovering et al has shown a similar procedure using a custom-made apparatus that required to use a thin pin through the proximal tibia to achieve optimal stabilization. However, we have used a different step-up without any intervention on the hindlimb. Besides, we feel the clamp is able to avoid any unwanted inflammation that a trans-osseous pin might create, while still allowing accurate assessment of muscle contractility. This is now addressed in the revised text (lines 170-175).*

**2. Line 176, As mentioned by the authors that the optimized amperage for stimulation may be different among mice, so is there a process to determine the specific amperage for each assessed animal before stop the Instant Stim? If so, it should be described in the Protocol Section.**

*A: Thank you. This is now discussed in the revised text as recommended (lines 139-142).*

**3. A close-up figure or diagram of the custom-made electrodes and the position where the electrodes put on the mice leg should be provided to further aid other investigates to better perform this method. I think this figure or diagram is necessary since the video that will describe this step may be not clear enough.**

A: Based on the reviewer’s suggestion we have included a close up of the electrodes (inset in Fig 2)

**4. The authors provided various figures to describe the normal contractility of the wildtype mice. As discussed, a normalization step is required for accurate evaluation of muscle contractility. In practice, researchers often compare muscle contractility between non-treated and treated muscle, or between wild-type and genetic modified mice, of which, the muscle masses or fiber areas are usually not comparable. A detailed discussion of 'Normalization' should be provided. As for measurement of force from different time points using the same animal, how to normalize without sacrificing the mice? In addition, dorsiflexion involves contraction of three types of muscle, of which the cross sectional area should be used to generate the 'specific force'?**

*A: The reviewer raises an interesting question. We have mentioned that is important to select the appropriate method based on the aim of the study because all different procedures have some advantages and disadvantages. This procedure can be used to track and monitoring the hindlimb [absolute] force production over different time points. Using the first measurement as a reference would allow monitoring relative muscle function over disease progression or with treatment. The notion of measuring the “percent loss” after injury and the “percent recovery” over time is not new (e.g., PMCIDs 28056487; 25920768; 24947322). If the normalization is required torque can be normalized to body weight, muscle mass of age-matched mice sacrificed at the same time points, or terminal experiments can be performed. We and others have shown the majority of torque produced by the dorsiflexors is from the TA (PMID: 14672973), and we have shown previously that this model results in injury to this muscle (PMID: 24066899; 16205165; 14522817; 17466731). This has been clarified in the revised text (lines 229-231; 273-274).*

**Reviewer#2:**

**Major Concerns:**

**1. The Key point of the protocol is to correctly place the electrode. I'd strongly recommend adding a carton diagram in either Figure 2 or 3 to help readers better understand the placement of the electrode.**

*A: We thank the reviewer for this suggestion and we have made some modifications on this figure. The description of electrode placement has been expanded in the manuscript (item 4) and additional details will also will be on the video.*

**2. Section 2.2, the authors set the "Run time" to 2 min. This needs justification. Have they tested different run time?**

A: *We have described this time based on the previous experiments where 2 minutes was long enough to achieve the optimal voltage. However, if this time is not enough to achieve the optimal voltage it can be either changed to a longer time or the instant slim can be started again as many time as needed. This has been clarified in the revised text (lines 139-142).*

**3. Section 2.5, the authors suggest "RANGE" knob to 10 mA. However, later on, the author indicated that for WT mice, the range should be less than 2mA. This needs clarification.**  
*A: We have described this procedure using a commercial apparatus and it can be used for a range of different experiments. The range on the high-power bi-phase stimulator can be used in 10 mA, 100 mA, 1 A, 20 V and 80 V. The RANGE (left bottom) knob has to be on 10 mA and during the optimizing of the electrodes the fine adjustment should be achieved using the right next bottom labeled ADJUST which means by using this bottom the voltage can be adjusted between 0 and the maximum 10 mA. We have added the corrected description on the article (line 152-153; 191).*

**4. The authors mentioned about two other commonly used mouse muscle function assays, ex vivo and in situ. These protocols have been described in several JoVE papers. I suggest to cite these papers (see below).**

**Hakim CH, Wasala NB, Duan D (2013) Evaluation of muscle function of the extensor digitorum longus muscle ex vivo and tibialis anterior muscle in situ in mice. J Vis Exp: e50183  
Moorwood C, Liu M, Tian Z, Barton ER (2013) Isometric and eccentric force generation assessment of skeletal muscles isolated from murine models of muscular dystrophies. J Vis Exp: e50036**

*A: We thank the reviewer for these suggestions. We have added these two references.*

**Minor Concerns:**

**1. The authors stated that their protocol can reduce the number of mice. This may not necessarily true for some applications. For example, the total absolute force of mdx mice are not altered. Only specific force can be used to distinguish the difference between normal and affected.**

*A: We have clarified this in the text. We know that absolute force is not reduced in mdx and the difference can only be detected if force is normalized to muscle mass. However, if one is studying muscle injury and recovery, this can be done in the absence of killing animals at each time point to obtain muscle mass. The “percent loss” in the force production (torque) we are interested in, not normalized force. As mentioned above, the notion of measuring the “percent loss” after injury and the “percent recovery” over time is not new (e.g., PMCIDs 28056487; 25920768; 24947322); (lines 273 and 274).*

**2. The authors provided an example using the right leg. It should be mentioned that this approach can be used for both limbs.**

*A: We thank the reviewer for bringing this to our attention and we have also included this information (lines 162 and 163).*