**Authors’ Response Letter**

We thank the editor and the reviewers for the very helpful comments. In response, we have added new discussions, references and revisions to address the concerns. **All the changes have been tracked in the manuscript**. We believe that the revisions considerably strengthen the manuscript. Below we provide a detailed point-by-point response to each comment.

Detailed Point-by-Point Response:

**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 (55099\_R0\_062316.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.

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.

Authors’ Response:

We have proofread the manuscript throughout and made necessary corrections.

2. Please provide an email address for each author.

Authors’ Response:

We have included the email addresses for both authors.

3. How are the mice sacrificed?

Authors’ Response:

The mice are euthanized by isoflurane inhalation, followed by cervical dislocation. It is described in 4.3. We have added “as described in 4.3” in Sections 2.1.3 and 3.1.3.

4. Formatting:

-Please use italics for Latin phrases (ex vivo, in vivo, etc.).

Authors’ Response:

We have used italics for all the Latin phrases.

-PFA is toxic and requires a caution statement.

Authors’ Response:

We have added “caution: personal protection equipment required” in the texts 4.1, and added notes ”Personal protection equipment required. This product may release formaldehyde gas, a chemical known to cause cancer” in the Table of Materials and Equipment.

5. Grammar: 6.1.1 – “Triton TX-100”

Authors’ Response:

We have corrected it to “Triton X-100”.

6. Additional detail is required:

-2.1.1 – How is initial acclimation and training performed (ie length of time, rewards, speed etc.)? For the third day, what speed and incline is used?

Authors’ Response:

We have revised the texts in 2.1.1 and 2.1.2 to be clearer on the initial acclimation and training on the first two days and the speed and incline on the third day.

-4.6 – How are tissues extracted? Please describe or provide a citation.

Authors’ Response:

We have cited the following references on brain and muscle dissection, and added new descriptions in Section 4.6:

1. [Nat Protoc.](http://www.ncbi.nlm.nih.gov/pubmed/23928501) 2013 Sep;8(9):1680-93. doi: 10.1038/nprot.2013.107. Epub 2013 Aug 8. isolation and expansion of human and mouse brain microvascular endothelial cells. [Navone SE](http://www.ncbi.nlm.nih.gov/pubmed/?term=Navone%20SE%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Marfia G](http://www.ncbi.nlm.nih.gov/pubmed/?term=Marfia%20G%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Invernici G](http://www.ncbi.nlm.nih.gov/pubmed/?term=Invernici%20G%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Cristini S](http://www.ncbi.nlm.nih.gov/pubmed/?term=Cristini%20S%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Nava S](http://www.ncbi.nlm.nih.gov/pubmed/?term=Nava%20S%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Balbi S](http://www.ncbi.nlm.nih.gov/pubmed/?term=Balbi%20S%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Sangiorgi S](http://www.ncbi.nlm.nih.gov/pubmed/?term=Sangiorgi%20S%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Ciusani E](http://www.ncbi.nlm.nih.gov/pubmed/?term=Ciusani%20E%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Bosutti A](http://www.ncbi.nlm.nih.gov/pubmed/?term=Bosutti%20A%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Alessandri G](http://www.ncbi.nlm.nih.gov/pubmed/?term=Alessandri%20G%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Slevin M](http://www.ncbi.nlm.nih.gov/pubmed/?term=Slevin%20M%5BAuthor%5D&cauthor=true&cauthor_uid=23928501), [Parati EA](http://www.ncbi.nlm.nih.gov/pubmed/?term=Parati%20EA%5BAuthor%5D&cauthor=true&cauthor_uid=23928501)
2. Nat Protoc. 2015 Oct;10(10):1612-24. doi: 10.1038/nprot.2015.110. Epub 2015 Sep 24. Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting. Liu L, Cheung TH, Charville GW, Rando TA.

-5.1.4 – Is a cryostat used for cutting? Are tissues oriented a certain way?

Authors’ Response:

We used the cryostat Leica CM 1850UV machine for cutting, and have added the information in the texts (5.1.4) and in the Table of Materials and Equipment. The orientation of the tissue is described in 5.1.2: cross section for the vastus lateralis muscle and sagittal section for the hemi-brain.

-5.3.1 – What wavelengths are used?

Authors’ Response:

For GFP, the emission wavelength is 525 nm; for DAPI, the emission wavelength is 490 nm. We have added the information in 5.3.1 and in the legends of Fig 1.

-5.3.2 – Is ImageJ used here? Please indicate the software in the materials table.

Authors’ Response:

No. ImageJ is not used here in 5.3.2, but is used in 6.2.3 for quantification. We have added the software in the Table of Materials and Equipment.

-6.2.2 – What is the composition of the sample buffer? Please include in the materials table is used as purchased. Please also include a citation for how to perform the western blotting, as this is a multistep process.

Authors’ Response:

We used the following sample buffer: 2X Laemmli Sample Buffer (Bio-Rad Laboratories; Cat #: 161-0737), and have included the information in 6.2.2 and the Table of Materials and Equipment.

We have added the following references for western blotting analyses in Section 6.2.2:

1. J Vis Exp. 2010 Oct 14;(44). pii: 2359. doi: 10.3791/2359. Western blotting: sample preparation to detection. Eslami A and Lujan J.
2. Methods Enzymol. 2009;452:181-97. doi: 10.1016/S0076-6879(08)03612-4. Monitoring autophagic degradation of p62/SQSTM1. Bjørkøy G1, Lamark T, Pankiv S, Øvervatn A, Brech A and Johansen T.

7. Branding: 5.1.2 – Please reduce the number of mentions of OCT to 1 in this step.

Authors’ Response:

We have revised the texts and mentioned “OCT” only once.

8. Results: Please define the error bars in Figure 1C (SD, SEM, etc.).

Authors’ Response:

We have defined the error bars as the standard error of the mean (s.e.m.), by adding “Results represent mean±s.e.m.” in Fig 1C legends.

9. Discussion: Please discuss any modifications/troubleshooting that can be performed as well as the future applications of the protocol.

Authors’ Response:

Modifications/troubleshooting is included in the discussion section. We have discussed the future applications in the last paragraph and cited two recent references as examples (Lo Verso et al. 2014; Kuramoto et al. 2016). The usage of treadmill or running wheel in autophagy regulation is relatively new, and we believe will be utilized in more studies in the near future.

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

In the manuscript the authors described two different way to induce autophagy through fiscal exercise: forced run on the treadmill and spontaneous run on the wheel. Beside the technical aspects related to the physiology of the experiments, the authors clarify the methods to monitor autophagy flux through histology and biochemistry.

The major issue in the manuscript is that these methods are not new. Several papers reported how autophagy is induced through physical exercise, in mice and humans as well. Surprisedly, the authors didn't comment or compare their methods with the set up reported in the other papers. A more deep critical analysis of the literature is needed.

Authors’ Response:

To address the reviewer’s comments, we have added the following texts in the discussion:

“Recently we and others have identified exercise as an effective, faster and safer autophagy inducer *in vivo*. Both forced exercise by treadmill and voluntary exercise by running wheel have been used to analyze the effects of exercise on autophagy activation, with variations in exercise duration and intensity. For example, an hour of treadmill running (starting at speed of 10 m/min to a maximum of 40 m/min) induces abundant LC3 lipidation in skeletal muscle, and longer-term voluntary wheel running for 3 months or 4-5 weeks also increases basal autophagy and enhances the expression of autophagy proteins in skeletal muscle.”

We also cited the following references to discuss and compare the published methods on autophagy induction by exercise.

1. Autophagy. 2011 Dec;7(12):1415-23. Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles. Grumati P, Coletto L, Schiavinato A, Castagnaro S, Bertaggia E, Sandri M, Bonaldo P.
2. FASEB J. 2013 Oct;27(10):4184-93. doi: 10.1096/fj.13-228486. Epub 2013 Jun 27. Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. Lira VA1, Okutsu M, Zhang M, Greene NP, Laker RC, Breen DS, Hoehn KL, Yan Z.
3. Autophagy. 2014;10(11):1883-94. doi: 10.4161/auto.32154. Epub 2014 Oct 30. Autophagy is not required to sustain exercise and PRKAA1/AMPK activity but is important to prevent mitochondrial damage during physical activity. Lo Verso F, Carnio S, Vainshtein A, Sandri M.

**Reviewer #2:**

*Manuscript Summary:*

This manuscript is nice. The authors described the exercise pathway for mice and the autophagy detective methods in detail.

*Major Concerns:*

1. About the definition of "aerobic exercise" and the amount of exercise for mice, the authors should explain why the exercise conditions described in this manuscript were used in the research, and why the exercise condition is belonging to aerobic exercise. The different exercise amount maybe causes different autophagy level.

Authors’ Response:

Based on a time course of treadmill exercise in one of our literatures (He C, et al. 2012), we conclude that the exercise conditions described in this manuscript induce the maximal level of autophagy in mouse skeletal muscle, which is also validated by others (Lo Verso et al. 2014). Thus, we used these conditions for autophagy induction in this protocol. Treadmill can be used for either aerobic or anaerobic exercise in mice, which is determined by different exercise duration and intensity. Specifically, aerobic exercise is induced by prolonged running with gradual increases in speed (references listed below), whereas the anaerobic condition is obtained by a short duration of high intensity exercise, which increases muscle mass but does not necessarily enhance exercise endurance (references listed below).

References on aerobic exercise:

* Kregel KC, Allen DL, Booth FW, Fleshner MR, Henrikson EJ, Musch TI, O'Leary DS, Parks CM, Poole DC, Ra'anan AW, Sheriff DD, Sturek MS, Toth LA. Resource book for the design of animal exercise protocols. American Physiological Society; 2006. Exercise Protocols Using Rats and Mice.
* Lightfoot J.T., Turner M.J., Debated K.S. & Kleeberg S.R. Interstrain variation in murine aerobic capacity. Med Sci Sports Exerc. 2001: 33, 5.
* Rezende EL, Chappell MA, Gomes FR, Malisch JL, Garland T., Jr Maximal metabolic rates during voluntary exercise, forced exercise, and cold exposure in house mice selectively bred for high wheel-running. J. Exp. Biol. 2005;208:2447–2458.

References on anaerobic exercise:

* Eur J Appl Physiol. 2002 Jun;87(2):141-4. Epub 2002 Apr 12. Effects of sprint exercise on oxidative stress in skeletal muscle and liver. Kayatekin BM, Gönenç S, Açikgöz O, Uysal N, Dayi A.
* J Appl Physiol. 1998 Jun;84(6):1852-7. Effects of high-intensity intermittent swimming on glucose transport in rat epitrochlearis muscle. Kawanaka K, Tabata I, Tanaka A, Higuchi M.

Moreover, the running speed reported in this protocol has been positively correlated with oxygen consumption capacity, by indirect methods to assess the aerobic capacity (Fernando, P., A. Bonen, and L. Hoffman-Goetz. Predicting submaximal oxygen consumption during treadmill running mice. Can. J. Physiol. Pharmacol. 71: 854–857, 1993). Therefore, the exercise protocol described here belongs to aerobic exercise. We have added the above texts and references in Discussion.

Additionally, white muscle and red muscle maybe have different responses to the same exercise condition. There are lot of questions are not clear as so far. So, I suggest the authors to remind the readers that the name or the part of the muscles should be defined in their research focused on skeletal muscle. With the same reason, I suggest the author to define the name or the part of skeletal muscle and brain in the two graphs in this manuscript. "Skeletal muscle" and "brain" is ambiguous.

Authors’ Response:

We agree with the reviewer that glycolytic and oxidative muscles may have different levels of autophagy in response to exercise. Thus, we have specified in the texts and figure legends that the muscle group used for autophagy analyses is “vastus lateralis”, and that the brain images taken are in the region of the frontal cerebral cortex, as this is the main region of the brain where we detected clear exercise-induced autophagy.

*Minor Concerns:*

1. Line 295 in Page 8: "adverse side effects" should be revised as "adverse effects" or "side effects".

Authors’ Response:

We have corrected it to “adverse effects”.

2. Line 166 in Page 5. In preparing the tissue for western blot analysis, why the tissues were collected after cold PBS perfusion? This step maybe causes the increment of autophagy. Autophagy is a physiological process, so, any changes of physiological condition may cause the activation of autophagy. I think the tissues for Western blot should be collected on ice directly after the mice were euthanized.

Authors’ Response:

We would like to thank the reviewer to point it out. We have mistakenly included the step in this protocol. This step (perfusion with ice-cold PBS) is used only if we need to perform immunostaining and western blotting analyses on tissues of the same mouse. We have now corrected it in Sections 4.2 and 4.8.

3. Fig 1A, Could the authors supply the GFP-LC3 images with DAPI staining?

Authors’ Response:

We have replaced the images with the new ones with DAPI staining in Fig 1A.

4. Fig 2A, the picture of Western blot is not very clear. Could the authors supply a lighter exposure picture of actin?

Authors’ Response:

We have replaced the original actin image with a lighter exposure one in Fig 2A.