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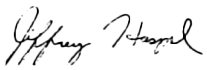
Dear Dr. Weldon:

We are pleased to submit our revised protocol for your consideration, which is entitled "*Measuring Diurnal Rhythms in Autophagic and Proteasomal Flux*". Please also convey our thanks to our three Reviewers who have helped us to improve this work. We are attaching revised files as well as a point-by-point response the Reviewer's comments (below). We look forward to your feedback and appreciate your consideration.

Sincerely,



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Response to Reviewers' comments:
Reviewer #1:
**Please, consult the statistician and confirm that the sample number and data normality support the use of a parametric test. Give also detailed instructions about the sample numbers with information when to apply a parametric or a non-parametric test.*
Response: Parametric tests have traditionally been used for determining the statistical significance of autophagic flux and represents the accepted standard. To our knowledge, non-parametric tests are never employed in this type of readout. Additional information on statistical treatment can be found in two of our prior publications[1,2]. Regardless of the statistical test used, the protocol for executing our technique is the same.

**Please, indicate in western blots, where the right band should be. The antibodies appear rather unspecific and also the positive controls show numerous bands.*
Response: We thank the Reviewer and have emended the figure legend to Figure 2a to clarify this as follows: "Note that under leupeptin-treated conditions, p62 runs as ladder ranging from the monomeric form at about 50 kDa to SDS-stable complexes at about 250 kDa". Regarding the positive controls, p62 tends to naturally form SDS-stable complexes. The purified Lys48-Ub protein used as a standard in Fig. 2B comes from the manufactures as a heteromeric mixture, so a ladder is expected.

**Strictly speaking, the levels of p62(/SQSTM1?) do not indicate autophagy flux. This must be discussed in more detail.*
Response: Macroautophagic flux is defined as the difference in p62 content between leupeptin treated and sham treated samples as described at length in our prior publication, which focuses on the logic of our measurement[1]. This protocol is focused on how to use a turnover assay to observe circadian rhythms in both autophagic and proteasomal activity.

Minor Concerns:
**It remains obscure why some text is highlighted with yellow color.*
Response: This was do at the request of the Journal editor so they can estimate how much video recording will be needed to document the protocol.

**An Excel sheet may not get properly opened with all program versions and it contains links to some other files.*
Response: We thank the Reviewer and have corrected this.

Reviewer #2:
1. Did the authors investigate taking blood samples from the mice in order to correlate the "in vivo" flux with secreted protein levels? This would certainly increase the usability of the protocol for many researchers for whom clinical relevance is a major concern. This point is nothing more than something to think about rather than a requirement for acceptance of the protocol in the journal.

Response: We thank the Reviewer and agree with their assessment. We have indeed used this technique on blood samples but have not yet published this. As such we confined ourselves to outlining published applications of our technique.

2. It would be appropriate for the authors to move the table of Reagents and Materials from its current location on line 271 to before section 2. Also, this will eliminate the need to incorporate this information in the description of the protocol as done for e.g. in Section 2.2 (Line 101) or as an appendix.
Response: We thank the Reviewer and have moved the table accordingly.

3. On Line 146, the use of decant is ambiguous. It may suggest a removal of the 7 mL of HB added in 4.2 (Line 131) with a subsequent addition of another 7 mL aliquot before homogenization. Alternatively, it appears that the authors mean to transfer the liver sample and the 7 mL of HB in which it is immersed into the Dounce homogenizer. If the latter is what the authors mean, a replacement of "decant" with "transfer".
Response: The term “decant” was replaced with “transfer” to provide a more straightforward description.

4.On Line 281, the authors state that the "It is best for a single operator to perform all tissue dissections since different individuals dissect at different speeds, but if this is infeasible it may be worthwhile to increase the time between protease inhibitor injection and tissue harvest from 2 hours to 3 or 4 hours (provided this is done consistently across time points). I find it somewhat impractical to recommend a single operator for a 24-hour experiment especially one that requires the operator to be present every 2 hours for the entire of the study. Did the authors evaluate the variation between single and multiple-operator to determine the impact of having multiple people work on the study?
Response: The Reviewer is quite correct that these experiments are arduous and are easier to execute with more than one operator. We do tend to employ single operators for most experiments but recognize this is a tall order. We therefore described the use of multiple operators in further detail in the discussion section. The main thing is to perform training experiments to ensure all operators use consistent dissecting techniques prior to attempting a full time series experiment, and to carefully record any run-time issues for future troubleshooting.

5. The discussion, while appropriate, lacks a description of common problems the authors encountered and which other scientists will likely encounter in the conduction of an study of this scale.
Response: We thank the Reviewer and have emended the discussion section accordingly.

Reviewer #3:

The protocol described by Ryzhikov et al. is aimed at measuring biological rhythms in protein catabolism via autophagy and the proteasome in the liver with an in vivo mouse model. The described methodology is quite straightforward and well explained although a big number of mice is required (one mouse per time point for each condition) to run such an experimental protocol which makes it less practical. The figure legends should be expanded to be more self explanatory, especially for Figure 2 for which it would be also important to explain why the time points reported in figures 2C & 2D (Zeitgeber Time axis), are not the same as those shown for the western blots presented in figures 2A & 2B, respectively.
Response: We thank the Reviewer and apologize for this typo. We have corrected the X-axes for Figures 2C and 2D. We have also included sample lab notes to help readers become better acquainted with this technique. Regarding the numbers of mice needed to perform this kind of experiment, this is indeed a limitation of our technique which we have now highlighted in the discussion section.

REFERENCES

1. Haspel, J. *et al.* Characterization of macroautophagic flux in vivo using a leupeptin-based assay. *Autophagy.* **7** (6), 629-642, (2011).
2. Ryzhikov, M. *et al.* Diurnal Rhythms Spatially and Temporally Organize Autophagy. *Cell Rep.* **26** 1-13, (2019).