August 5, 2018

Editorial Office

*Journal of Visualized Experiments*

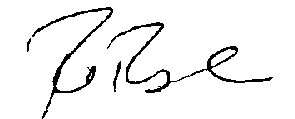
Re: Manuscript entitled “Measurement of energy metabolism in explanted retinal tissue using extracellular flux analysis.”

Dear Colleagues,

Thank you for the valuable feedback on our manuscript. We have addressed all concerns from the three peer reviewers as well as the editorial comments. The point-by-point rebuttal is pasted below.

I appreciate your consideration of this work for publication in *Journal of Visualized Experiments*.

Sincerely,



Rithwick Rajagopal

POINT-BY-POINT REBUTTAL

|  |  |  |
| --- | --- | --- |
| Comment | Rebuttal | Location in Revised Document |
| Editorial Comments |  |  |
| 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. | Thank you. Completed. |  |
| 2. Figures 2-5: Please define error bars in the figure legend. | Completed. | Figure legends 2,3,4,5 |
| 3. Please provide an email address for each author. | Completed. | Cover page |
| 4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™),  registered symbols (®), and company names before an instrument or reagent. Please remove all commercial  language from your manuscript and use generic terms instead. All commercial products should be sufficiently  referenced in the Table of Materials and Reagents. For example: Trizma, Triton, Integra Miltex, etc. | Trademark language removed. | Main protocol. Trade names moved to material list (Excel file). |
| 5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1  should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or  indentations. | Completed. The protocol section was revised to match this formatting. |  |
| 6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). | Completed. | Introduction and discussion was revised to remove personal pronouns. |
| 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. | The term “should be” was identified in several instances within the discussion and all instances were revised.  Imperative tense is used in the revision. | Discussion, lines 3017 and 317. |
| 8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the  actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how”  question, i.e., how is the step performed? Alternatively, add references to published material specifying how to  perform the protocol action. | These points have been addressed in a revised protocol that incorporates several editorial comments. | Protocol, lines 103-224. |
| 9. Lines 98-100: Please move materials information to the Table of Materials. | Completed. | Materials supplement. |
| 10. Line 106: Please specify which powder. What is used to adjust the pH? Please spell out ddH2O. | Both points have been addressed. | Protocol, lines 112-113 |
| 11. Lines 109 and 111: These steps as written are unclear. Please revise. For instance, adding 1 M stock of  what? | These points have been addressed in a revised protocol that incorporates several editorial comments. | Protocol, lines 115-117 |
| 12. Line 120: What is used to adjust the pH? | HCl or NaOH. Text Added | Protocol, line 112 |
| 13. Lines 125-136: Please write the text in the imperative tense in complete sentences. | These points have been addressed in a revised protocol that incorporates several editorial comments. | Protocol, 123-133 |
| 14. Please do not include Note as a separate step. | Completed and revised. | Protocol, line 140-142, 180-183, 186-191 |
| 15. 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.  16. 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. | Completed. | Protocol, highlighted text. |
| 17. 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. | Completed. | Protocol, highlighted text. |
| 18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6  paragraphs with citations:  a) Critical steps within the protocol  b) Any modifications and troubleshooting of the technique  c) Any limitations of the technique  d) The significance with respect to existing methods  e) Any future applications of the technique | The discussion section was modified to incorporate these changes and the changes suggested by reviewer 3. | Discussion, lines 227-264. |
| 19. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I.  Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first  author then et al. | Completed | References |
| 20. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all  references. | Completed | References |
| REVIEWER 1 |  |  |
| 1. Line 106 "Dissolve powder …" . Which powder ? | The protocol revised to clarify this ambiguity. | The protocol now reads: “2.1. Prepare base media by dissolving 8.4 mg of powdered media in double-distilled H20, and adjust pH to 7.4 with either HCl or NaOH, to final volume of 1 L. Filter sterilize this solution with a 0.22 µm tissue culture filter.” |
| 2. Line 111 What is the stock concentration pyruvate? | Corrected | The new protocol reads: “Add 1 M glucose and 100 mM sodium pyruvate to the media to achieve final concentration of 5 mM glucose and 1 mM pyruvate.” |
| 3. The first letter of catalog number for 2-deoxyglucose is illegible. | This has been corrected as well. | The new materials list has the catalog number from Sigma: " D6134" |
| REVIEWER 2 |  |  |
| Though the authors explains their technique very well and but the fact that their method used, doesn't provide  any extra added information about how the Gnat1 gene would be responsible in retinal energy metabolism. | This is an excellent point. We agree that the negative results for the Gnat1 tissues do not provide any additional insight into this gene on retinal bioenergetics. However, the contrast between our measurements and those made by Du et al (JBC 2016) using a custom-made perfusion apparatus point out an important limitation in the use of commercially-available extracellular flux analyzers: that light-induced changes in retinal energy metabolism cannot be reliably recorded. For this reason, we would like to leave the results included – in order to highlight the limitation of this protocol. However the manuscript was modified to provide additional details about why one would expect a change in bioenergetic demand in these animals. | See answer to comment below. |
| Minor concern is that authors should include more of description about Gnat1 gene and how this protein is  associated with retinal metabolism. | We agree, and have expanded our description of this gene in the Representative Results section. | These sentences were added/modified in the Results section:  “Because Gnat1-/- animals lack the machinery to close cyclic-nucleotide gated ion channels in response to light stimuli, their rod photoreceptors remain depolarized even in light14. The subsequent need to maintain potassium efflux would create a large ATP demand, resulting in bioenergetic strain. To determine if such shifts in energy demands would increase oxidative phosphorylation or glycolytic flux, tissues from wild type mice and Gnat1-/- mice were compared using the extracellular flux analyzer.” |
| REVIEWER 3 |  |  |
| There are no major concerns with the manuscript, and only minor concerns with regards to data analysis and  interpretation exist and are listed below. Many of these comments are addressed in "Analysis and interpretation  of microplate-based oxygen consumption and pH data" by Divakaruni...and Jastroch (PMID:25416364). | The paper suggested by this reviewer is highly relevant to the content of this manuscript and to the overall goal of the protocol we describe. The descriptions of the caveats of extracellular flux recording using XF Analyzers is exceptional and was enlightening to read. We have added this reference to our bibliography and have used it as a guide to describe limitations of our own technique. |  |
| (1) Lines 46 & 71: Any references to energy generation or consumption should be avoided, and replaced with  energy transduction or ATP generation/consumption. | This is an important distinction, and we appreciate the correction. In both instances, phrases were modified as suggested. | Introduction |
| (2) Line 54: Although it was previously noted that the retina was the only post-mitotic tissue capable of aerobic  glycolysis, a comment should be made regarding how it is now clear most all tissues sit on a continuum of  oxidative phosphorylation and glycolysis, even many post-mitotic cells & tissues. | This point is also well-taken, and we have made the suggested addendum. | The following phrase was added to the introduction, immediately following the sentence highlighted by this reviewer:  “Since those initial observations, many post-mitotic tissues have been described to engage in varying degrees of glycolysis in additional to oxidative phosphorylation to meet their ATP demands.” |
| (3) Line 245: It is unclear why the authors choose to normalize to total DNA content over protein. A discussion  about the relative merits and drawbacks of each normalization method should be addressed. | We have now added discussion around this choice of normalization to the manuscript. | In the discussion:  “This protocol describes normalization of data to total DNA content, as a proxy for cell number. Such a technique is advantageous because it will account for changes to cell number driven by variation in retinal thickness, punch size, or differences in cellularity between genetically dissimilar samples. However, total protein-based normalization is also a reliable method, and has the advantage of minimizing differences in total mitochondrial mass between retinal samples. |
| (4) Line 263: The drawbacks of measuring the fractional changes from "baselined" data between groups should  be mentioned in addition to the benefits | These points were also added to the discussion. | To the discussion, the following was added:  “Normalization of flux recordings to the baseline, as shown in the representative results, is advantageous because it minimizes variability in retinal punch size between replicates and easily allows interpretation of changes due to pharmacologic interventions. However, reporting of raw values allows for better comparison between different experiments and for experiments performed using different flux recording methods.” |
| (5) Line 314: It is not stated whether the authors have titrated the FCCP concentration to arrive at 1 uM. It is  possible that a rate close to the basal rate of metabolism can be achieved with a different concentration of FCCP  after oligomycin injection? Additionally, if the maximal respiratory rate cannot be measured in the presence of  oligomycin, the authors should note that addition of FCCP without ATP synthase inhibition may create an  unsustainable ATP demand from mitochondrial hydrolysis of ATP by the ATP synthase. | Interestingly, we and others have attempted titration of FCCP to high doses, and used other uncouplers (such as BAM15) without any observable change in retinal OCR. These findings (including titration of the uncoupler) are extensively discussed in Kooragayala et al. IOVS 2013, which is a reference in our bibliography. |  |
| (6) Line 330: It is well established that in 3D cultures CO2 evolved from the TCA cycle can be a dominant  source of medium acidification (addressed in PMID:25416364). This caveat should be addressed in the  discussion of ECAR measurements in retinal explants. | We have addressed this important caveat in the revised version of the discussion.  The reference suggested by this reviewer was also added, as mentioned before. | To the discussion, the following lines were included:  “An important caveat preventing the use of ECAR as a pure proxy for glycolytic rate is that CO2 liberated by the citric acid cycle can be a significant source of acid in cultured tissue17.” |
| (7) The oligomycin-independent respiratory rate in Figure 4 does not appear to reach steady-state (likely due to  the time required for penetration of the drug into the core of the explant). It should be noted that all  measurements should be made at steady-state whenever possible. | We agree that this representative figure appears not to allow oligomycin-independent respiration to reach steady state. However, our group has observed an oligomycin-dependent mitochondrial toxicity that appears to be specific to retinal mitochondria. This is similar to observations of Dr. Anand Swaroop’s group at the National Eye Institute (Kooragayala et al, IOVS, 2013). Therefore, the lack of drop to steady state may be due to progressive toxicity, rather than tissue penetrance. Nevertheless, the point about allowing compounds to reach steady-state is well-taken and has been amended to the revision. | To the discussion:  “All measurements are taken only after retinal metabolism has reached steady-state following injection of test compounds.” |