We would like to thank the reviewers for their insights and thoughtful comments. We have made every possible effort to address all of reviewers’ comments, as outlined in detail below. Hence, we believe that the revised manuscript is significantly improved with respect to focus and depth. Below is my response to their comments.

**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

The manuscript is thoroughly revised and checked for spelling and grammatical errors.

**2. Please revise lines 60-63 and 98-101 to avoid previously published text.**

Revised

**3. Affiliations: Please note that numbering follows the order of authors. First author gets 1, next author with different affiliation gets 2, etc., following from first to last.**

Revised

**4. Abstract: Please do not include references here.**

References are now removed from the abstract

**5. Please spell out each abbreviation the first time it is used.**

Spelled out on the first time

**6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.**

Revised

**7. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.**

Revised

**8. 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.**

Revised

**9. 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. However, notes should be used sparingly**, **and actions should be described in the imperative tense wherever possible.**

Revised

**10. Lines 86-95: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please move the discussion about the protocol to the Discussion.**

Revised as per the suggestion

**12. Line 162: Please reference Figure 2 in this step so the readers know what wells A1, B4, C3 and D6 refer to.**

New figure showing wells A1, B4, C3 and D6 is incorporated

**13. Please include single-line spaces between all paragraphs, headings, steps, etc.**

Single-line spaces are included now

**14. 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. 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.**

Highlighted in yellow

**16. 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.**

Highlighted in yellow

**17**. **Figure 1: Please use SI abbreviations for all units (µL, h) instead of µl and hr. Please include a space between all numerical values and their corresponding units (4 °C, 30 °C, 100 µL).**

Corrected

**18. Figure 2: Please define the acronyms (WT, MT, COMP, etc.).**

Defined in the figure legend

**19. Figures 3 and 4: Please define error bars in the figure legend. Please include space between all numerical values and their corresponding units (10 mM, 1 µM).**

Corrected

**20. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. 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

Discussion is revised to include all critical steps in the protocol

**21. Please combine the future application paragraph with Discussion section.**

Combined.

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

JoVE endnote style has been used  
  
**Reviewers' comments:**  
  
Reviewer #1:  
  
Manuscript Summary:  
This manuscript reports the use of "Seahorse bioenergetics assay" for studying perturbations in mitochondrial functions in C. albicans. The authors make use of this assay to flesh out information on the respiratory and glycolytic ability of a mitochondrial mutant.

1. **While the assay looks promising, the authors need to perform a few more control experiments to prove the efficacy of their protocol.**

We have performed additional experiments and a different assay medium to confirm the efficiency of the protocol. By using RPM1 1640 medium as suggested by the reviewer, we found better responses to the mitochondrial inhibitors.

1. **The authors do not mention which mitochondrial mutant they have used in this study. They should mention which gene has been mutated and what functions are affected in this null mutant. At least two more mutants should be included in their study to prove the statistical significance of their protocol.**

We have included the mutant name (*mam33Δ/Δ*) in this manuscript and provided its description. Since this is a method paper, analyzing more mutants is beyond the scope of this paper.

1. **Additionally, the authors should keep the naming of the strains consistent in the study. They write "mutant" and then also use "MT" as another abbreviation for the mutant in the same figure 3. Same goes for the complementary strain too**.

In the revised paper, the mutant and the complementary strain names are kept consistently throughout.

1. **Adherence assays in Candida also make use of RPMI 1640 routinely. The authors should check the efficiency of their protocol with this medium also.**

Thanks for the pointing out about RPMI 1640. When we used RPMI, we found RPMI 1640 worked better in terms of the efficiency of oxphos inhibitors. In the revised submission, we employed RPMI 1640 instead of DMEM.

1. **The inhibitors of mitochondrial function did not work in their assay. The authors should give some more explanation to this. If the routinely used inhibitors do not work in this assay, the assay does not have any therapeutic potential.**

In the revised manuscript, we employed RPMI and found the inhibitors of mitochondrial function worked efficiently.

1. **Any other drug/compound that has been shown to affect mitochondrial function can also be included in the study.**

We have now also investigated and included SHAM-an inhibitor of alternate oxidase (AOX) and KCN- an inhibitor of complex IV in the revised manuscript.

1. **Authors should mention the troubleshooting attempted while the assay was performed.**

We mentioned the inefficiency of DMEM for mitochondrial inhibitors in the revised manuscript.

1. **Line no 89 "the ability to" should be "the ability of".**

Corrected

1. **Line no 108 "filter add store" should be "filter and store"**

Corrected

1. **Line 216 : change 'are shown' to 'showed'**

Deleted

1. **Line 218 the sentence should ne phrased as " to obtain and optimal OCR, titration of**

Changed to “To obtain optimal OCR

1. **the WT …..was performed with cell numbers ranging from….per well"**

Corrected

1. **Line 269 : instead of 'peturbate', 'perturbs' should be used.**

Corrected

1. **Line 288 : use word 'analyse/assess' instead of 'interrogate'**

Corrected

1. **Line 293: meaning of the statement is not clear.**

Removed

1. **Kindly rephrase the statement written in "future application".**

Revised

1. **Figure 1: Analysis should be Analyze**

Corrected

Reviewer #2:  
  
Manuscript Summary:  
This manuscript uses real-time extracellular flux assay to investigate mitochondrial respiration and glycolytic function in C. albicans. The authors present a simple protocol that may be used to any Candida spp. strain. The method is interesting and can be useful to study new antifungals or gene manipulation.  
  
I have a few comments:

1. **line 69: Authors report "Recent studies have indicated...drugs". No studies were cited. Please add references.**

Now included.

1. **Please, define all abbreviation (e.g., WT, MT) in the manuscript.**

Abbreviations are defined now.

1. **Both "g" and "RPM" are units used for centrifugation steps. It is better to use g, which refers to the acceleration applied to your samples. RPM is not as useful a unit, because the force varies with the radius of your machine (the bigger the radius, the more acceleration is applied to your samples for the same RPM). In this way, please, change rpm to g in the manuscript.**

RPM is most commonly used in the shakers to grow microbial cultures

**4.** **It seemed to me that there is an appropriate equipment for this assay. If this is true, please, clarify in the manuscript**.

Instrument is clarified in the revised paper

1. **Also, improve the legends of figure 2 and table 1. For example: Fig. 2: Layout of wells and group assignment "as visualized from Seahorse X24 software" ???. Also, define abbreviations.**

Corrected to “Fig. 2. Layout of wells and group assignment as visualized in the Seahorse XF24 software” “Table 1. Protocol commands for the assay.  
  
6**. In the last page, there are three lines highlighted in yellow. I am not sure what they mean**.

This was an inadvertent error. We have corrected it.  
  
Reviewer #3:

Manuscript Summary:  
1. **The manuscript by Venkatesh, Chauhan, Suzuki, and Chauhan details a method for utilizing the Seahorse platform to assess OCR and ECAR readouts for mitochondrial respiration and glycolytic function in C. albicans strains. While the goal is aimed at detailing this system, the lack of strain details provides only tangential conclusions to be made by the reader. This system, however, presents the opportunity to rapidly and reproducible assess the responsibility of specific genetic mutations to affect overall respiration within multiple strain backgrounds.**

The details of the mutant and complementary strains are detailed in the revised manuscript

2. **The abstract describes how antifungal tolerance is impacted by mitochondrial function but there is little to no description of this in the Introduction. A paragraph should be added to include how mitochondrial function is tied to antifungal tolerance or resistance.**

Importance of mitochondria on antifungal tolerance is highlighted in the introduction (lines 78-80). A more in-depth description on this topic is beyond the scope of this paper.

3. **Another major question is why this protocol is needed. There are a number of yeast-specific protocols for assaying mitochondrial function.**

Standardized methods to understand mitochondrial function in pathogenic fungi are poorly developed. The method presented herein is an effort toprovide a protocol to measure the basal oxygen consumption rate (OCR), a measure of mitochondrial respiration, and extracellular acidification rates (ECAR), a measure of glycolytic function in *C. albicans* strains. The method described herein can be applied to any *Candida* *spp.* strains without the need to purify mitochondria from the intact fungal cells. Furthermore, this protocol can also be customized to screen for inhibitors of mitochondrial function in *C. albicans* strains.

4. **Line 89-90: This sentence is floating. What does this adaptation measure? Is it OCR, ECAR, or something else?**

Corrected

5**. Lines 86-95: The starting cell number for the assay should be known for this protocol. Is it known that cell density has no impact on respiration rates? I would expect the opposite to be true based on previous reports:** <https://www.sciencedirect.com/science/article/pii/S1550413112002380>.

The starting cell number is critical for the assay. In fact, we titrated the optimal C. albicans cell number for this assay. The importance of starting cell number is described line 248-252.

6. **The first two sentences of the first paragraph are describing methods written in the protocol. I would suggest omitting these sentences and describing the purpose of the experiment in the context in which it was performed.**

Corrected

7. **This first paragraph also fails to state that the experiment was performed before moving into analysis of results. There's a major disconnect there.**

Corrected

8. **The mutant used in this study is not named and must in order for the experiments described here to be reported on any way. This is particular important for a methods paper.**

Mutant name is now included and detailed in the revised paper

9**. From Figure 3 it is not clear what part of the curve is used for area under the curve (AUC) measurements in 3A or 3B and why. My assumption would be that after the final injection, AUC would be calculated but this does not represent the given data. More clarification is needed.**

New figures are included, which is much more clear and detailed in the data analysis part.

**10. Additionally, the complemented strain appears significantly different across the curve from the WT in 3A, which it should phenocopy. No statement is given on why this difference exists and if there's a significant different between the two.**

New data more focused on the protocol and the method

**11. The complemented strain is more similar to WT in 3B but is still significantly different across most of the assay. There's no comment on this and no difference is suggested from the bar graph. This requires much more explanation.**

In our revised manuscript, we have addressed these concerns by optimizing the experiments and providing new data

**12. Finally, the text states that the experiment is flawed based on the optimal OCR range. This should be redone so the data lies within an acceptable range of detection.**

We have redone the experiments and found the optimum OCR range and cell number, corresponding changes are done

13. **The protocol numbering system is not consistent with the JoVe format.**

Corrected

14. **All steps requiring use of the machine would benefit from images as the location of these ports and method used for injection are not obvious from reading.**

Corresponding figures are now incorporated in the revised manuscript

15. **An additional section, Step 9, should be included for data analysis.**

The revised manuscript contains data analysis section.

16. **There's a lot of concerns regarding grammar across the manuscript.**

The manuscript is thoroughly revised and checked for spelling and grammatical errors.

17. **There are too many places where this is problematic to list but a selection are below. These grammatical issues are present in Figure 1 as well.**

Figure 1 is corrected

18**. Step 2. Part 1.1 is a massive run-on sentence and needs to be rewritten in its entirety.  
Please clarify that all of Step 3 Parts 1.2-1,4 should be performed in the hood as only 1.2 is currently noted as such.**

All the step 3, 1.2-1.4 should be performed in the hood. Corresponding changes have been made in the revised manuscript as below

NOTE: Perform all the below steps in a laminar hood.

* 1. Dissolve Poly-D Lysine in tissue culture grade water to make 50 μg/mL final concentration. Mix it well and aliquot into a 1.5 mL microcentrifuge tubes and store at -20 °C for the long term. You need 50 μL per well, and for 24 wells, it requires 1.2 mL. Therefore, aliquot at least 1.3 mL per microcentrifuge tube.
  2. Add 50 μL per well and incubate at room temperature with the lid covered for 1-2 h.
  3. Aspirate the solution and rinse one time with 500 μL sterile tissue culture grade water.
  4. Open the lid and allow the wells to air dry. Use the plate on the same day or store at 4 °C for a maximum of 2-3 days.

19. **Step 4 Part 1.1 needs clarification. Turn what "upside down". What is it "it" in the next phrase, etc.,**

The sentence is now re-phrased.

1. **Line 29: This sentence is a little awkward. I'd suggest changing "..is gaining more importance in studying changes.." to "..is gaining more importance to the study of changes.."**

Corrected

21. **References in the abstract is unusual and should be saved for the body of the paper**.

References from the abstract are removed

22. **Line 63: "drug to drug" should be either "drug-drug" or "interactions between drugs".**

Corrected

23. **Line 66: stating conservation of targets for antifungals between fungi and humans could be stated more explicitly here.**

Line 75 explicitly states challenges in discovery of new targets due to the fact that fungi, like humans, are eukaryotes.

**24. Line 71: There are a number of more recent references that can be used here that are more broad in scope as well. I'd include this review (PMID 25088819) and this recent paper (PMID 29719235).**

Thank you for the suggestion. These references are now added into the text.

25. **Line 86: "a" should be inserted between "in" and "Seahorse"**

Corrected

1. **Line 89: "to" should be "for"**

Corrected

1. **Step 6, Part 1.3 - grammar.**

Corrected

1. **Step 8, Part 1.2: Wording is awkward, consecutive "by"s**

The entire protocol is rewritten and is clear now

1. **Step 8, Part 1.3 "form" should be "from"**

Modified the sentence

1. **The "O" in the temperature is a zero and not give as an "O"**

Corrected

1. **The Experimental Materials table is missing information. Please provide the company name and catalog number**

Provided