Dear Review Editor Amit Krishnan,

Thank you very much for the review of our manuscript entitled: “Flow cytometric analysis of multiple mitochondrial parameters in human induced pluripotent stem cells and their neural and glial derivatives.” (JoVE63116). We sincerely appreciate all valuable comments and suggestions, which helped us to improve the quality of the article

We have modified the manuscript according to the changes indicated. And provided answers to the questions raised by the reviewers. We believe that the comments have resulted in a much better manuscript, and we hope it is now finally acceptable for publication in your journal.

We enclose a point-by-point response to the reviewers’ comments typed in bold in blue with the original reviewer comments typed in regular typeface.

Yours sincerely,
Kristina Xiao Liang
Xiao.Liang@uib.no

Klinisk institutt 1, Universitetet i Bergen
Postboks 7804, 5020 BERGEN
post@k1.uib.no
+47 55 58 54 31

Responses for the comments:

Comments from editor,

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

Response: We have invited an English Native Speaking professor to revise our manuscript to improve the English language. We have thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please provide an email address for each author.

Response: We have provided an email address for each author.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have revised the text to avoid the use of any personal pronouns.

4. Line 24: Repeat term mitochondrial volume.

Response: We have deleted the repeated texts.

5. Please revise the following lines to avoid previously published work: 46-51,65-67,103-106,108-111,352-354,389-390,428-429,440-441, 450-454. Please refer to the iThenticate report attached.

Response: We have revised the texts to avoid previously published work.

6. Please define all abbreviations upon first use. For example, TFAM, TMRE, TOMM20, DPBS, SF, FGF, FBS, DMEM, BDNF, GDNF, PFA, RT, etc.

Response: We have defined all abbreviations upon first use.

7. Please provide references (wherever appropriate) for lines 56-63, 66-83.

Response: We have provided references for lines 56-63 and 66-83.

8. JoVE cannot publish manuscripts containing commercial language. 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.

Response: We have removed all commercial language from your manuscript and used generic terms instead. For example: "Geltrex" into "matrix", "Essential 8" into "iPSCs culture medium", deleted "StemPro", "BD Accuri" into "Flow Cytometer", etc.

9. 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.
Response: We have adjusted the numbering of the Protocol to follow the JoVE Instructions for Authors.

10. Please include all safety procedures and use of hoods, etc.
Response: We have included all safety procedures and use of hoods, etc.

11. Please add more details to your protocol steps. 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.
Response: We have added some more details to our protocol steps.

12. 1.1.1: What was the final volume of the solution? What was the volume in each aliquot? Please specify.
Response: We have added the final volume of the solution and specified the volume in each aliquot.

13. 1.1.2: How many total wells were used? Please specify.
Response: We have specified the number of the wells in step 1.1.2.

14. 1.2: How much media was prepared? Please specify.
Response: We have moved the step of preparation of E8 medium into the supplemental files, where we have described the medium preparation.

15. 1.3.3: How much DPBS was used for rinsing(should all 4ML) and what was the DPSB concentration? Please specify.
Response: We used 4 mL DPBS with a concentration of 1X. We have specified this information.

16. 1.3.4: At which step were the iPSCs inoculated in the plate? Please describe iPSC inoculation in detail along with growth and colony formation.
Response: We have described such details as suggested by reviewer.

17. 1.3.6: How much pressure was applied? Please specify.
Response: We have specified how much pressure we applied.

18. 1.3.7 Note: What does the split ratio stand for? Please mention.
Response: We have mentioned what the split ratio stands for in the steps.

19. 1.4.2: What is the concentration of NIM used? Please specify.
Response: We have described the concentration of NIM used in the supplemental files.

20. 1.4.3: Please describe the microscope settings and parameters.
Response: We have added Figure S1 to describe the microscope settings and parameters.

21. 1.6.2: How much DPBS was added? Please specify.
Response: We have added the amount of DPBS.

22. 1.6.7: What was the solution used for re-suspension? Please mention.
Response: We have described solution used for re-suspension.

23. 1.6.8: What was the volume of PLO and laminin added? At what step were they added, please specify.
Response: We have described the volume of PLO and laminin used and the steps when they were added.

24. 1.6.4, 1.7.4: What was the water bath temperature? Please specify.
Response: We have described the water bath temperature.

25. 1.8.1.2: How much PDL was used to coat each well? Please specify.
Response: We have specified the amount of PDL used to coat in each well.

26. 2.1, 2.2, 2.4: Please mention the volume of PBS, PFA, blocking solution.
Response: We have described the volume of PBS, PFA, blocking solution.

27. 2.3: What was the concentration of primary antibody used?
Response: We have described the concentration of primary antibody used in the Table of Materials.

28. 3.15, 4.8, 4.12: Please provide all instrument settings and parameters.
Response: We have provided the all-instrument settings and parameters in Figure S1 and S2.

29. 4: Please provide volume used for PFA, methanol, block buffer, PBS, primary antibodies, staining solution, secondary antibody.
Response: We have provided the volume used for PFA, methanol, block buffer, PBS, primary antibodies, staining solution, secondary antibody in the manuscript.

30. 5: Please include all the button clicks, command lines, etc. in the software and instrument. If using long scripts and long command, please include as supplementary file. We need actions to show how the software and instrument

are used.
Response: We have provided all the button clicks, command lines, etc. in the software and instrument in Figure S1 and S2.

31. Please include a single line space between all the steps and ensure that the highlight is up to 3 pages 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.
Response: We have highlighted the essential steps of the protocol for the video and ensure that the highlight is up to 3 pages of the Protocol.

32. Please remove the titles and Figure Legends from the uploaded figures. Please provide this information in the Figure Legends section after the Representative Results in the manuscript.
Response: We have removed the titles and Figure Legends from the uploaded figures and provided this information in the Figure Legends section after the Representative Results.

33. All figures should be uploaded separately to your Editorial Manager account.
Response: We have uploaded all figures separately to the Editorial Manager account.

34. Please do not abbreviate journal names in references.
Response: We have changed into the full journal names in references.

35. Please arrange the Table of Materials alphabetically.
Response: We have arranged the Table of Materials alphabetically.

36. Figure 3/4: Please include scale bars in all the images of the panel.
Response: We have included the scale bars in all the images of the panel.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript "Flow cytometric analysis of multiple……" provides an interesting perspective on the entire process from generating specific neuronal cell types to analysing mitochondrial function. The protocols presented are comprehensive and interesting but are set out in a way that are difficult to follow in a linear fashion. This is complicated by poor grammar and stylistic issues, and lack of explanation of some parameters before they are used in the protocol. Everything seems to be there, it is just really difficult to follow. A longer, clearer, and more detailed introduction would be helpful to

communicate the specific issues, or splitting the protocol in two parts; namely generation of the specific cell types followed by analysis of mitochondrial function.

Summary
"mitochondrial volume" is repeated.
Response: We have deleted the repeated text.

Abstract
1) Neurodegenerative diseases and changes in mitochondrial volume are not a conjunction. Consider splitting this sentence for clarity.
Response: We have splitted this sentence into two in order to clarity.

2) Be careful to define mitochondrial membrane potential as distinct from membrane potential. This is important because the plasma membrane also has a potential that is also detected by staining with TMRE, the author refers to the acronym MMP to acknowledge this later, and is also why FCCP is used later as a control.
Response: We agree with the review that the mitochondrial membrane potential is distinct from other membrane potential such as plasma membrane. We have defined the mitochondrial membrane potential in our paper.

3) "are often the feature of these processes" should be " are often a feature of these processes"
Response: We have revised this sentence.

4) The part of the sentence " …and ROS levels and fixed cells" would be better defined using a semi colon "and ROS levels; and fixed cells"
Response: We have revised this sentence.

5) The abstract gives an indication that using TMRE and MTG measure ROS. This is clearly not possible but Mitox-Red is not mentioned until the discussion.
Response: We have described using MitoSox Red for ROS measurement in the abstract.

6) A similar issue arises with the description of TFAM and TOMM20, which suggests that these are used to measure MRC. Only much later is there any indication that these do not measure the MRC, and that the protocol uses a group of antibodies to detect components of each subunit of the electron transport chain. Using specific examples in the abstract therefore may confuse the message. One way around this is to refer to "outer membrane"
Response: To avoid the confusion of the MRC subunit measurement and TFAM assessment. We have revised the sentences in abstract into

“Double staining with antibodies against MRC complex subunits together with translocase of outer mitochondrial membrane 20 (TOMM20) permits assessment of the MRC subunit expressions. Since the amount of TFAM is proportional to mtDNA copy number, measurement of TFAM per TOMM20 gives an indirect measurement of mtDNA per mitochondrial volume.”

Introduction
The use of appropriate commas to delineate lists vs conjunctions is pervasive
1) It is important to be accurate and careful with grammar and intent. For example, Mitochondria may be responsible for producing 80% of total ATP, but they are responsible for producing 100% of the ATP by oxidative phosphorylation.
Response: In order to be accurate and careful with the grammar and intent, we have revised the sentences in abstract into “Mitochondria re responsible for energy supply by producing adenosine triphosphate (ATP) via oxidative phosphorylation and act as metabolic intermediator for biosynthesis and metabolism.”

2) Remove any superfluous definite article "the", such as "the regulation of cell death" or "the production of reactive oxygen species"
Response: We have removed the superfluous definite article "the".

3) Delete "findings" on line 58
Response: We have deleted "findings" on line 58.

4) Line 59. "the ability to measure these, and other' mitochondrial functions is therefore of greet use (this allows future implications of measuring actual respiration as an important function)
Response: We have revised this sentence.

5) Line 60. The statement that animal models do not replicate faithfully human disease is a bit strong and would be best tempered with "some". For example, "Failure of animal models to faithfully replicate some human disease" or "failure of animal models to faithfully replicate human neurodegenerative disease.
Response: We have revised this sentence.

6) Line 74. "multiple" should be replaced with "different types of"
Response: We have replaced "multiple" with "different types of".

Protocol
Part 1 - Subheading 1
1) Thaw a vial (replace "the" with "a")

2) Geltrex (Gibco) (add vendor - if specific)
3) Dilute 1:100 (delete "into")
4) 1:100 is 1% of a stock, and explanation is repetitive unless qualified by v/v or w/v.
5) Store "at" -20oC instead of "in" -20oC
6) Similarly use "at" 4oC
7) "wells" should not be plural for "a 6 wells plate" as the plate is singular
8) "Coated plate" is not a noun and should have a small "c"
9) "recommended to use in 3 days" is not correct English
10) "To avoid gel dry out" should be "To avoid the gel drying out"
Response: We have revised accordingly as the reviewer suggested.

Subheading 2
11) Preparation "of" Essential 8. This does not make sense as a step.
Response: We have removed this step.

Subheading 3
12) Use " at RT or in an incubator at 37^{o}C"
Response: We have changed into " at RT or in an incubator at 37^{o}C".

13) "6 well" not "6 wells"
Response: We have changed into " 6-well plate".

14) There are too many references to "Table of Materials" to provide a continuous protocol.
Response: We have removed some unnecessary references to "Table of Materials"

15) Step 5. "aspirate the gentle dissociation medium" is confusing. Is the gentle dissociation medium the 0.5 mM EDTA.
Response: We mean the EDTA is a dissociation medium that is "gentler" on cells and avoid the potentially damaging. We have revised this into "5 mM EDTA" to avoid confusion.

16) "Gentle shake before putting in the incubator" should be "Shake gently before ..."
Response: We have revised into "Shake gently before ..."

Subheading 4
17) "Preparation" should be "Prepare"
18) Add "(NIM)" after Neural Induction Medium in step 1 as it is not defined before in step 2.
19) Replace Neural Induction Medium with NIM in Step 3.
20) "Observe" is not noun. Use lower case "o".

Response: We have made corresponding changes to these contents based on the reviewers' suggestions.

Part 3

21) MitoSox Red appears out of nowhere.

Response: We have made MitoSox Red in the manuscript.

22) Step 15. Which dye is excited off which laser in this combination.

Response: We have described the filter related to each dye in section 3.15, as described in the paper "3.15 Analyze on Flow Cytometer (with a 3 blue and 1 red laser configuration). MTG is detected in filter 1 (FL1) by use of a 530/30 bandpass filter, while TMRE is detected in filter 2 (FL2) using the bandpass filter 585/40. MitoSox Red is detected in filter 3 (FL3) by use of a 510/580 bandpass filter."

Part 4

23) Wash in PBS "once or twice" is not very scientific

Response: We have clarified the washing step.

24) Primary antibodies "NDUFB10, SDHA, and CoxIV come out of nowhere.

Response: We have added the description of "NDUFB10, SDHA, and Cox IV" in section 4.6.

25) Were all of the antibodies conjugated with alexa 488and mixed together in the same tube at the same time.

Response: All the sample were stained in individual tubes with single staining. Then they were measured individually for their median fluorescence intensity, referred as the total level of the protein expression (after substrate with the MFI of negative control). The specific level is calculated with total level per mitochondrial volume measurement of TOMM20, for example, specific TFAM = total TFAM/TOMM20.

26) What were the second antibodies conjugated to. Were they alexa 488 as they were all detected in the 530/30 filter. What is the reason for for using 4 antibodies here. Does this raise the overall fluorescence, or average out the indirect measure of volume.

Response: We have descripted the detailed information of second antibodies in Tables of Materials. The NDUFB10, SDHA, and COX IV are all conjugated with Alexa 488 using second antibodies and detected in the 530/30 filter. The TFAM and TOMM20 are conjugated with Alexa 488 and in the 530/30 filter.

Part 5

27) The staining protocols do not mention the IgG control or single colour controls, or which is which.

Response: We have added the detailed description of the IgG control in Table of Materials.

Representation of results

28) The author becomes lax, using "mito-volume".

29) Line 340 MRC complex subunit expression should be "expression levels of MRC complex subunits"

30) "TFAM" should be "TFAM;"

Response: We have made corresponding changes to these contents based on the reviewers' suggestions.

Reviewer #2:

Manuscript Summary:

Authors report a novel approach to measure multiple mitochondrial function parameters based on flow cytometry and double staining with two fluorescent reporters or two antibodies, which allows detection of changes in mitochondrial volume, mitochondrial volume, membrane potential, reactive oxygen species level, mitochondrial respiratory chain and mitochondrial DNA.

The present manuscript developed a flow cytometry-based approach measuring multiple mitochondrial parameters in various cell types. This approach has a certain novelty in understanding mitochondrial changes in different diseases. The results were convincing, and the protocols were described clearly. My only concern is how consistent they will be when compared to the commonly used methods. It might be more solid if the author could include some traditional methods and compare those parameters measured by different systems.

Response: We thank the reviewers for the positive response of our manuscript and agree that it may be more reliable, including some traditional methods and comparing the parameters measured by different systems. In our manuscript, we have discussed the comparison of our methods with the traditional microscope-based analysis in the last paragraph of discussion section, as described in the paper "Compared with other microscopy-based assays, flow cytometry has the advantages of speed and reproducibility when analyzing large numbers of cells. In the analysis of microscope photos, the bias of researchers will distort the results to a certain extent, which is not a problem when using flow cytometry. In addition, Flow cytometry analysis requires less than one million cells, and analysis of one sample only takes a few minutes, which means that dozens of samples can be analyzed in one to two hours. This technique can also be applied to a wide variety of cell types including those from other neurodegenerative diseases and should therefore be useful for understanding mechanisms."