THE
EMBO
JOURNAL

A NOVEL APPROACH TO MEASURE MITOCHONDRIAL RESPIRATION IN PREVIOUSLY FROZEN BIOLOGICAL SAMPLES

Rebeca Acin-Perez, Ilan Y. Benador, Anton Petcherski, Michaela Veliova, Gloria A. Benavides, Sylviane Lagarrigue, Arianne Caudal, Laurent Vergnes, Anne Murphy, Georgios Karamanlidis, Rong Tian, Karen Reue, Jonathan Wanagat, Harold Sacks, Francesca Amati, Victor M. Darley-Usmar, Marc Liesa, Ajit Divakaruni, Linsey Stiles and Orian S. Shirihai

Review timeline:

Submission date:	25th November 2019
Editorial Decision:	20th December 2019
Revision received:	30th January 2020
Editorial Decision:	1st March 2020
Revision received:	11th March 2020
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Editor: Elisabetta Argenzio

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 20th December 2019

Thank you for submitting your resource article entitled "A novel approach to measure mitochondrial respiration in previously frozen biological samples" [EMBOJ-2019-103889] to The EMBO Journal. Your study has been sent to three referees for evaluation, whose reviews are enclosed below.

As you can see, while referee #1 and #2 find the presented method potentially interesting, they raise several points that have to be addressed before they can support the publication of your work in The EMBO Journal. In light of referee #3's comments, I informed him/her that the journal publishes novel methods that clearly advance the field and asked to reweigh the manuscript. While this referee recognizes the potential value of the manuscript, s/he requests you to modify the title and to address the concerns related to the measurement of oxygen consumption listed in his/her report below.

Given the overall interest of your study, I would like to invite you to revise the manuscript in response to the referee reports. I should note that conclusively addressing these and all the other referees' points is essential for publication in The EMBO Journal.

REFEREE REPORTS

Referee #1:

This manuscript, authored by renowned researchers in the field of mitochondrial biology, examines a proposes a novel approach to measure mitochondrial respiratory chain function by respirometry in frozen tissue homogenates. Hereto forth oxygen consumption was thought to be reliable only in mitochondria isolated from fresh samples. By manipulating substrates, additives and modes of permeabilization the authors showed that informative data can also be obtained from homogenates obtained from a small amount of various frozen tissues. These findings could be of practical relevance in clinical settings allowing storage and transport of frozen tissue rather than, the often problematic immediate workup of fresh tissue.

My main concerns are twofold ;

1-The freezing procedure of tissues was done in an optimized laboratory setting and not in a possibly problematic, clinical environment.
For example ; tissues obtained in the OR/clinic are often transported at room temp to a freezer -20C? -80C? for storage. Not every hospital has access to bedside liquid nitrogen.

It would be interesting to know if/how much respiratory capacity would be preserved if the sample would remain 30min in room temp, than stored in the freezer prior to shipping (dry ice? Ice blocks?). This could also depend on the tissue, liver might be more vulnerable than muscle.

2-Possible pitfalls and drawbacks are sometimes not adequately addressed.
examples
a- How could a defect in PDHc/Krebs cycle defect behave in this setting?
b- How would you identify an isolated CIII defect?
c- Would you deal with a CoQ defect?
d- Could haemoglobin (i.e. RBC contamination in tissue homogenate interfere? for examples with MTDR measuring mitochondrial content?
e- Are there other limitations that should be mentioned?

Minor comments;
Conventional methods are sometimes not accurately related to; for example mitochondrial isolation can easily be done in ~0.2g muscle -fresh or frozen- and without trypsin (the manuscript states 0.5-2g with trypsin).
When/if /how would, conventional methods be needed to verify oxygen consumption results (see former comments) ?

Referee #2:

The manuscript submitted by Acin-Perez and colleagues describes a method to measure oxygen consumption in frozen biological samples. According to the authors, although the integrity of the inner mitochondrial membrane is lost during freezing as we already know, the complexes of the electron transport chain are still intact and functional. Therefore, providing substrates that directly feed the ETC complexes should be enough to elicit electron flux and consume oxygen. Importantly, this approach enables the analysis of maximal uncoupled mitochondrial respiration in previously frozen samples such as patient-derived tissue from biobanks. The underlying idea is logic and simple. Eventually, analysis of (functional) supercomplexes can be carried out both in fresh and frozen samples with minimal problems, and the same should apply to oxygen consumption. Accordingly, while reading this manuscript I was asking why I didn't think of this idea myself. Overall, data are well performed and discussed, and most of the necessary controls are already in place. While data on OCR are strong, the section on mitochondrial quantification using a fluorescent indicator is rather weak and does not add so much to the overall story. Some points need more attention:
• I do not understand the difference in basal OCR in some panels, including 1D, 1G, 3C and 3E. Basal respiration should correspond to state 1 (no substrates, no ADP) and no difference between fresh and frozen samples should be present. Indeed, according to the figures and legends, both substrates and ADP are injected using the first port of seahorse reader. Therefore, traces should begin (i.e. first time point) with comparable OCR. I think this is simply due to erroneous labeling of

the figures. Most likely, substrates (pyr/mal in panel 1A, succ/rot in panel 1D and NADH in panel 1G) are already present at the beginning of the experiment (state 4 is thus measured here), and only ADP is added with the first injection. Please fix this problem and provide control experiments showing state 1 respiration.

• As far as I know, Chance and Williams define state 2 respiration as a condition with high ADP and low substrates. Conversely, figure legend 1 reports "state 2 (substrate without ADP)", that should be instead considered as state 4. Accordingly, respiratory control ratios (RCRs) are defined as state3/state4 (not state3/state2 as shown).

• As an additional control, the authors should test the effect of atractyloside. It should inhibit state 3 respiration in fresh but not in frozen samples.

• The least convincing part of this work is the use of Mitotracker DeepRed to normalize mitochondrial content. I see several problems here. 1) Figure 6A demonstrates that some parameters can impact on MTDR fluorescence, since Ca^{2+} treatment causes an approximately 25% decrease of fluorescence with no apparent explanation. 2) Figure 6B clearly shows that the relationship between protein content and MTDR fluorescent is linear only over a narrow range (up to 10 ug for isolated mitochondria, without considering that the linearity in the case of total homogenate looks questionable). In a real-life situation, it would be difficult to know if MTDR signal is at saturation or not only looking at raw fluorescence. 3) When comparing figure 7F to 7H, MTDR vs protein show very different results. According to MTDR signal, mitochondrial content is twice higher in the POLg sample, i.e. an effect that should be well visible with other techniques. According to Western blot, the increase in POLg is very limited, thus questioning results obtained with MTDR. Overall, I think the main problem here is that we don't know exactly how MTDR works, and it is thus difficult to understand its strengths and limitation. I think this part can be omitted with no impact on the overall conclusions.

Referee #3:

This paper describes measurements of mitochondrial respiratory chain activity from various tissues and biological sources. The aim is purely methodological: to demonstrate that activity is maintained despite freezing and thawing the samples, which obviously saves a lot of material. My major concern is that there is no actual conceptual novelty in this work; its value may be considerable but it is purely methodological. This is more a matter of decision by the Journal's Editors. To my mind this work is not suitable for the EMBO Journal but should be published in a methodological forum.

I have somewhat minor concerns related to the method of measuring oxygen consumption. There is no information of how the actual numbers of O_2 consumption were obtained. What was the temperature (oxygen solubility is highly temperature dependent)? How was the oxygen concentration determined? How did the authors control for oxygen diffusion into the microplate wells? How was the zero oxygen point established?

1st Revision - authors' response — 30th January 2020

Referee #1:

This manuscript, authored by renowned researchers in the field of mitochondrial biology, examines a proposes a novel approach to measure mitochondrial respiratory chain function by respirometry in frozen tissue homogenates. Hereto forth oxygen consumption was thought to be reliable only in mitochondria isolated from fresh samples. By manipulating substrates, additives and modes of permeabilization the authors showed that informative data can also be obtained from homogenates obtained from a small amount of various frozen tissues. These findings could be of practical relevance in clinical settings allowing storage and transport of frozen tissue rather than, the often problematic immediate workup of fresh tissue.

My main concerns are twofold ;

1-The freezing procedure of tissues was done in an optimized laboratory setting and not in a possibly problematic, clinical environment.

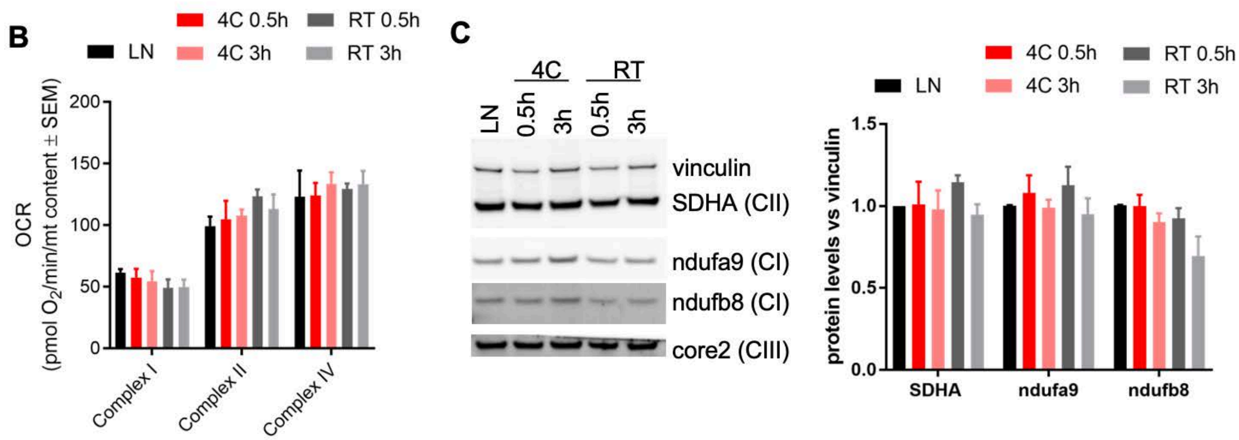
For example ; tissues obtained in the OR/clinic are often transported at room temp to a freezer -20C? -80C? for storage. Not every hospital has access to bedside liquid nitrogen.

It would be interesting to know if/how much respiratory capacity would be preserved if the sample would remain 30min in room temp, than stored in the freezer prior to shipping (dry ice? Ice blocks?). This could also depend on the tissue, liver might be more vulnerable than muscle.

We agree with some of the reviewer concerns. In Figure 7 of the paper we have shown data from human samples (retroperitoneal fat from pheochromocytoma patients) obtained from the clinic and stored at -80 °C for 2 years. We are currently running an extended human study involving fat samples obtained from : i) periadrenal, ii) superclavicular, iii) para-/epicardial, and iv) subcutaneous depots. In this study, samples are collected in a clinical setting from the OR. Samples are collected from the patient and placed in ice cold PBS from 1-3 hours. Upon completion of the surgery, we pick up the samples and flash freeze them before storing them at -80 °C. We have successfully tested these samples for frozen respirometry. However, the reviewer is right in his/her concern about how much of the original activity is preserved, if the samples are kept in PBS at 4 °C or room temperature for an extended amount of time. To address that question, we have now run an experiment in mouse liver samples (3 independent samples), where we have collected samples from the same liver and processed them under different conditions:

i) ice cold PBS for 30 min and freeze (4 °C 0.5h),
ii) ice cold PBS for 3 hrs and freeze (4 °C 3h),
iii) PBS at RT for 30 mins and freeze (RT 0.5h),
iv) PBS at RT for 3 hrs and freeze (RT 3h) and
v) freeze (LN)

The freezing step is done in liquid nitrogen for all the samples. Samples are then stored at -80 °C for at least 2 days before assessing how much of the respiratory capacity is preserved. The results below show that respiratory capacity is preserved when the samples are placed in ice cold PBS (the current procedure that we are following when collecting samples for the OR). When samples are stored at RT for extended amounts of time, Complex IV activity is fully preserved but Complex I activity is slightly decreased while Complex II is slightly increased, possible due to supercomplex rearrangements and/or respirasome instability. These results have been incorporated in Figure S6 (panels B and C) of the revised version of the manuscript.



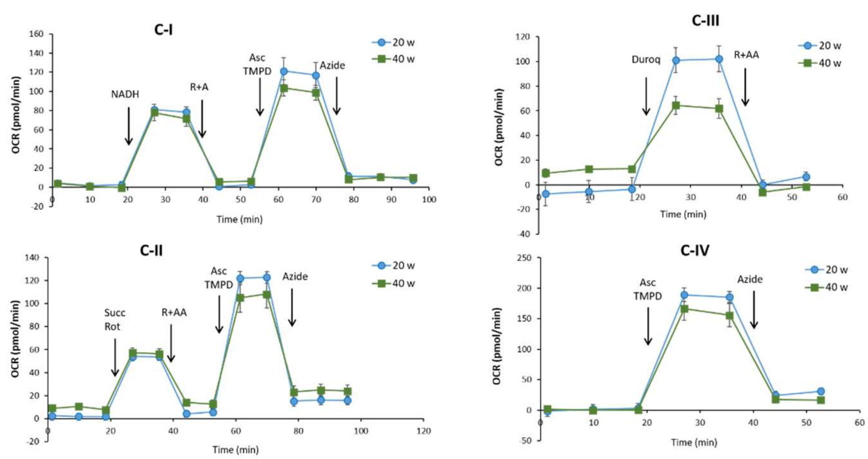
2-Possible pitfalls and drawbacks are sometimes not adequately addressed. examples

a- How could a defect in PDHc/Krebs cycle defect behave in this setting?

We would not expect to observe defects of the TCA cycle, since we are adding exogenous NADH and succinate, both of which bypass TCA cycle and donate electrons directly to Complex I and Complex II, respectively. The limitation is related to the fact that were are measuring electron transport chain function capacity, rather than the integration of fuel oxidation and ETC activity, as previously discussed

b- How would you identify an isolated CIII defect?

We have now incorporated an additional assay to obtain an independent measure of complex III using duroquinol. Here, we are presenting the results of such measurements using heart homogenates from young and old mice.

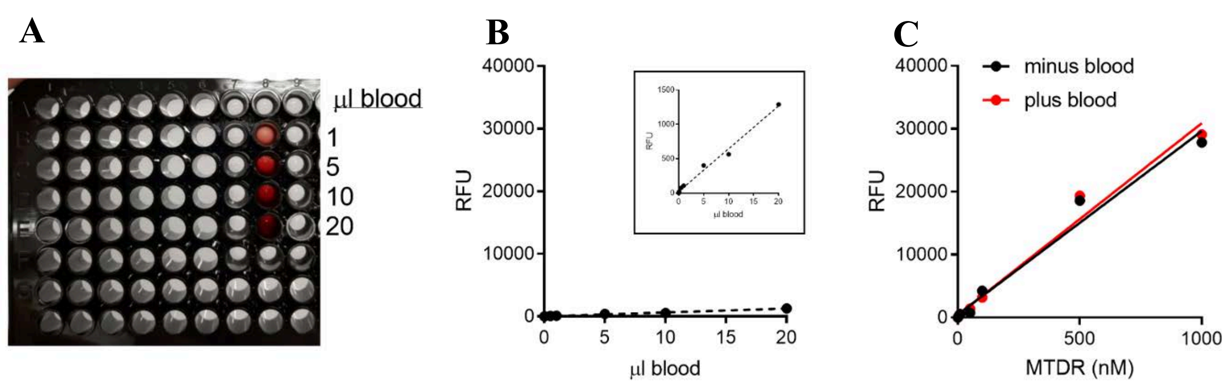


c- Would you deal with a CoQ defect?

We have not tested CoQ deficient samples but we assumed that those defects will be picked up by our analysis because the RIFS assay, unlike the spectrophotometrical approaches, does not add any exogenous CoQ. Samples in RIFS are using their endogenous pool of CoQ that is mobilized when ETC is provided with either NADH or Succinate to Complex I and II, respectively.

d- Could haemoglobin (i.e. RBC contamination in tissue homogenate interfere? for examples with MTDR measuring mitochondrial content?

Once the tissue is collected from the subject, it is placed in PBS before freezing and most of the blood is washed out; therefore, we do not expect to have significant amounts of hemoglobin that would to interfere with MTDR mitochondrial content reading. Nevertheless, we have run an experiment where we include serial dilutions of MTDR, blood and blood plus MTDR. The results below show that RBC at levels that are found in tissues does not interfere with MTDR fluorescence. Hemoglobin levels in human blood is 12-17 g/dl. We added different volumes of human blood to a plate (0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 20 µl) and run the same protocol that we use to measure MTDR fluorescence. We observed that at higher concentrations, RBCs a limited amount of background (panels A and insert of panel B). However, when we prepare the lysates from tissues, we do not expect the contamination observed using 5-20 µl of blood. We also measured fluorescence at increasing concentrations of MTDR and MTDR plus 1 µl of blood and observed similar results (panel C). Note we have used the same Y scale axis in B and C for comparative purposes. These results are now part of the new Figure S5 (panels E and F).



e- Are there other limitations that should be mentioned?

We have tried RIFS in a variety of different tissues in mouse and human, cell lines and blood cells. The protocol is very straightforward in almost all cases with the exception of tissues that have a lot of fiber and collagen such as skeletal muscle or tissues that are very rich in membranes such as brain. In the latter cases, an optimization step was required that included either enzymatic digestion (skeletal muscle) or additional membrane permeabilization (brain) for the reagents and electron donors, particularly NADH, to be accessible to mitochondria.

Minor comments;

Conventional methods are sometimes not accurately related to; for example mitochondrial isolation can easily be done in ~0.2g muscle -fresh or frozen- and without trypsin (the manuscript states 0.5-2g with trypsin).

When/if /how would, conventional methods be needed to verify oxygen consumption results (see former comments)?

We now show lysates obtained from 20mg of tissue, an amount that would not be possible to isolate mitochondria. We agree that trypsin can be avoided to isolate mitochondria from muscle in traditional protocols, as stated by the reviewer. However, using collagenase Type II (0.25 mg/ml final concentration) in such a small amount of tissue to obtain lysates facilitates the access of NADH and the different fuels to mitochondria, as mentioned in the previous point.

Referee #2:

The manuscript submitted by Acin-Perez and colleagues describes a method to measure oxygen consumption in frozen biological samples. According to the authors, although the integrity of the inner mitochondrial membrane is lost during freezing as we already know, the complexes of the electron transport chain are still intact and functional. Therefore, providing substrates that directly feed the ETC complexes should be enough to elicit electron flux and consume oxygen. Importantly, this approach enables the analysis of maximal uncoupled mitochondrial respiration in previously frozen samples such as patient-derived tissue from biobanks. The underlying idea is logic and simple. Eventually, analysis of (functional) supercomplexes can be carried out both in fresh and frozen samples with minimal problems, and the same should apply to oxygen consumption. Accordingly, while reading this manuscript I was asking why I didn't think of this idea myself. Overall, data are well performed and discussed, and most of the necessary controls are already in place. While data on OCR are strong, the section on mitochondrial quantification using a fluorescent indicator is rather weak and does not add so much to the overall story. Some points need more attention:

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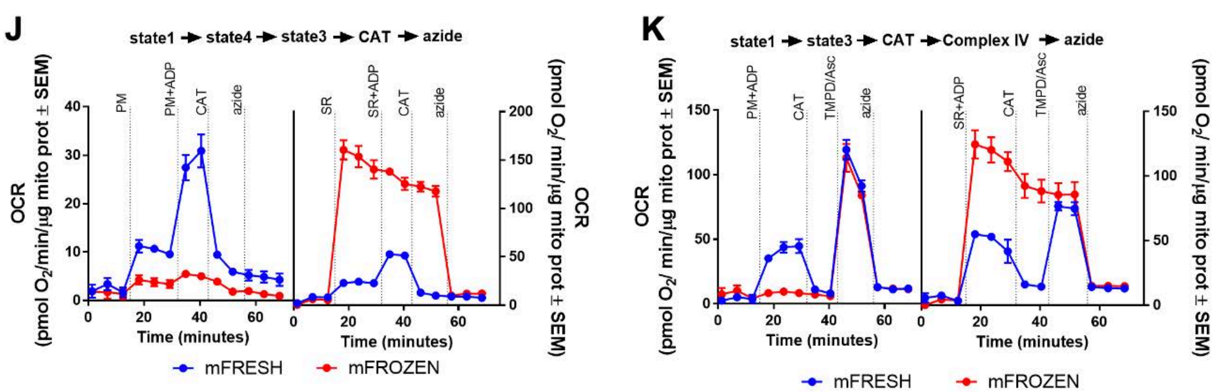
• As far as I know, Chance and Williams define state 2 respiration as a condition with high ADP and low substrates. Conversely, figure legend 1 reports "state 2 (substrate without ADP)", that should be instead considered as state 4. Accordingly, respiratory control ratios (RCRs) are defined as state3/state4 (not state3/state2 as shown).

The reviewer is right on the nomenclature by Britton and Chance. The confusion is that we used the nomenclature used by Nichols and Ferguson in Bioenergetics book, as it was more intuitive in which state 1 is mitochondria alone, state 2 with substrates, state 3 with ADP and state 4 ADP exhaustion or 4o adding oligomycin. We will correct the nomenclature according to Britton and Chance to avoid confusions. Thus, the assumption of the reviewer on the cause for the differences in initial OCR is correct.

• As an additional control, the authors should test the effect of atractyloside. It should inhibit state 3 respiration in fresh but not in frozen samples.

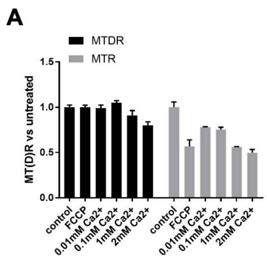
The reviewer is also right in his/her assumptions on how the samples were placed in the seahorse plate: samples are in the presence of substrates (no ADP) in the well and ADP is injected in port A for all the conditions. We have now corrected the labeling in the different planels of Figure 1. In addition, we have performed an experiment comparing fresh versus frozen using liver mitochondria and liver homogenates. We have started the assay with samples in the absence of substrates (state 1); then injected substrates in port A (state 4); substrates plus ADP in port B (state 3); atractyloside (ANT inhibitor) in port C and azide in port D. The results are presented in the figure below and in panels J and K on the new Figure 1.



• The least convincing part of this work is the use of Mitotracker DeepRed to normalize mitochondrial content. I see several problems here.

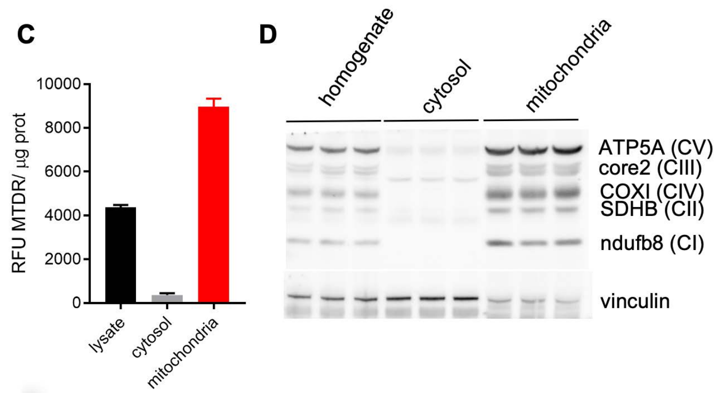
1) Figure 6A demonstrates that some parameters can impact on MTDR fluorescence, since Ca2+ treatment causes an approximately 25% decrease of fluorescence with no apparent explanation.

The calcium concentration used in this assay is in the high range (2mM) so presumably, mitochondrial swelling and membrane rupture occurs. Note that for mitochondrial swelling assays to measure the permeability transition pore, we normally use 0.4mM. We have now repeated the experiment with lower calcium concentrations and showed that MTDR fluorescence is not affected. We have now substituted the panel A in Figure 6 for a new panel including the new data



2) Figure 6B clearly shows that the relationship between protein content and MTDR fluorescent is linear only over a narrow range (up to 10 ug for isolated mitochondria, without considering that the linearity in the case of total homogenate looks questionable). In a real-life situation, it would be difficult to know if MTDR signal is at saturation or not only looking at raw fluorescence.

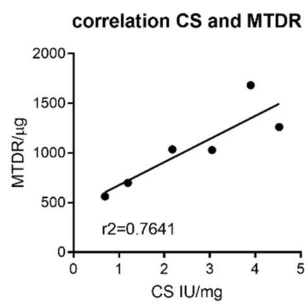
We agree with the reviewer that it might difficult to predict in real-life situations how much sample needs to be added in order to not reach the saturation limit in the assay. We have tested different protein amounts depending on the sample source and found that, if we use the same protein amount in micrograms that we use for the Seahorse assays, we are within the linear range. Every fluorescence and colorimetric assay has the challenge of using sample amounts that read in the linear range of the assay. As additional controls, a standard curve using MTDR as well as dilutions of the original sample can be used to measure mitochondrial content. These results are now part of the new Figure S5 (panels C and D).



3) When comparing figure 7F to 7H, MTDR vs protein show very different results. According to MTDR signal, mitochondrial content is twice higher in the POLg sample, i.e. an effect that should be well visible with other techniques. According to Western blot, the increase in POLg is very limited, thus questioning results obtained with MTDR. Overall, I think the main problem here is that we don't know exactly how MTDR works, and it is thus difficult to understand its strengths and limitation. I think this part can be omitted with no impact on the overall conclusions.

We agree with the reviewer that we do not exactly understand the mechanisms by which MTDR stains mitochondria. However, we still think that the information that it provides is very useful when assessing mitochondrial content.

There is not a single method for measuring mitochondrial mass that is encompassing the fact that the various components of mitochondria are expanding equally. One has to select a surrogate marker for mitochondrial mass and each selection brings with it a potential error that can be addressed in a thorough analysis of the various components of mitochondria. Among the most commonly used are mtDNA/nDNA ratio, western blot analysis and citrate synthase (CS) activity. However, the one, which is suitable for relatively higher throughput, is a fluorescent-based analysis by either high throughput imaging or plate reader. Keeping in mind that mitochondrial mass measurement is not the subject of this publication, our approach was to use the 3 most approachable methods for mitochondrial mass measurements and describe their limitations. We include data with CS activity, WB and fluorescence analysis of MTDR in a plate reader. We believe that MTDR offers an alternative that many readers will consider and thus it is important to examine it and outline its limitations. To compare the methods, we have included in the revised version a comparison of data obtained from CS vs MTDR. The comparison demonstrate that at least in the range of 2-30µg of lysate, the 2 approaches are linearly correlated



In some models of mitochondrial pathology, the cell tries to compensate for the energy deficiency through increased mitochondrial biogenesis (Garone and Viscomi, 2018; Sanchis-Gomar et al., 2014; Uittenbogaard and Chiaramello, 2014; Wredenberg et al., 2002). When bioenergetics analysis is performed in isolated mitochondria, the activity per protein and per mitochondrial content (MTDR) follows the same trend, as expected. However, when the mitochondrial activity is measured in homogenates, the lysate also contains other organelles and the cytosol in addition to mitochondria, all contributing to protein levels. Nevertheless, some lysates might contain more mitochondria than others. By including the measurement of mitochondrial mass, we can refer the mitochondrial activity in the lysates as activity per cell (per protein) or per mitochondria (per MTDR). Under some pathological conditions, increasing mitochondrial mass allows the cell to compensate for bioenergetic deficiencies by having more, albeit dysfunctional, mitochondria. To that point, measuring mitochondrial content/mass is very important. In the POLgMT model, mtDNA accumulates mutations with age that can affect mitochondrially encoded protein turnover without affecting nuclearly encoded protein turn over or levels. Moreover, steady state levels of some proteins do not reflect the abundance of mitochondria or the assembled complexes since they still can be detected in rho0 cells lacking mtDNA.

Referee #3:

This paper describes measurements of mitochondrial respiratory chain activity from various tissues and biological sources. The aim is purely methodological: to demonstrate that activity is maintained despite freezing and thawing the samples, which obviously saves a lot of material. My major concern is that there is no actual conceptual novelty in this work; its value may be considerable but it is purely methodological. This is more a matter of decision by the Journal's Editors. To my mind this work is not suitable for the EMBO Journal but should be published in a methodological forum.

We thank the referee for the comment. This manuscript is submitted to EMBO J Resources format, which is a forum in which EMBO is inviting methodological papers.

I have somewhat minor concerns related to the method of measuring oxygen consumption. There is no information of how the actual numbers of O2 consumption were obtained. What was the temperature (oxygen solubility is highly temperature dependent)? How was the oxygen concentration determined? How did the authors control for oxygen diffusion into the microplate wells? How was the zero oxygen point established?

The method developed in this study is to be used with any platform of oxygen consumption measurement, such as Seahorse XF, Oroboros, Oxygraph and others. We did not get into the details of how the instrument is working as we did not make any changes to the instrument used for oxygen tension measurements. To demonstrate the development described here, we are using the seahorse platform for oxygen consumption measurements. In their website, publications and patents, Seahorse (Agilent) is providing information about the way the measurements are obtained and how the XF instrument takes into account the temperature (37C) and the oxygen solubility at that temperature to convert the mV into nmol of oxygen that we are using as a readout.

2nd Editorial Decision 1st March 2020

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees, whose comments are shown below.

As you will see, they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept the manuscript, there are a few editorial issues concerning text and figures that I need you to address.

REFEREE REPORTS

Referee #1:

Clearly the authors made a significant effort to address all concerns of the reviwers. The revision is satisfactory and I have no further comments.

Referee #2:

The authors successfully addressed my original concerns. I think the paper is now improved and ready to be published

2nd Revision - authors' response 11th March 2020

The Authors have made the requested editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Orian S Shirihai, Rebeca Acin-Perez and Linsey Stiles

Journal Submitted to: EMBO J

Manuscript Number: EMBOJ-2019-104073R

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Samples size was chosen based on previous experience measuring respirometry using seahorse techniques, including biological as well as technical replicates. The same sample size was used for the three independent approaches to measure mitochondrial mass.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Animals were only used for tissue lysate preparation and mitochondrial isolation. The number of animals covered the independent number of experiments performed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There were no samples excluded from the analysis in this study. For validation purposes in our study, we used mouse, zebra fish and human samples previoulsy generated. Exclusion /inclusion criteria were described in previous studies quoted in the paper
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	In the validation experiments, we run the samples blind and then allocate them by group when assessing the results.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical data and number of biological and technical replicates are indicated in every figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Comparisons between groups were made by one-way or two-way analysis of variance (ANOVA). Correction for multiple comparisons was made by Tukey or Sidak tests when appropriate. Pairwise comparisons were made by two-sided Student's t-test. Differences were considered statistically significant at P < 0.05. In the figures, asterisks denote statistical significance (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).
Is there an estimate of variation within each group of data?	Graphs indicate the SEM of the independent experiments subjected to statistical analysis.

Is the variance similar between the groups that are being statistically compared?	Generally, when comparing SEM, the variance was minimal.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	mtOXPHOS cocktail (for combined ATP5B, core2, COXI and SDHB detection-ab110413), ndufa9 (ab188373), UCP1 (ab10983) and β-actin (ab8227) from Abcam; ndufb8 (459210) and SDHA (459200) from Thermo-Fisher Biotechnology; core2 (14742-1-AP) from Proteintech, TOM20 (Sc-11415) from Santa Cruz Biotechnology and vinculin (V9131) from Sigma-Aldrich.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	MICE: Tissues were collected from 12-week-old male C57BL6/J mice (Jackson lab, Bar Harbor, ME). Animals were fed standard chow (mouse diet 9F, PMI Nutrition International, Brentwood, MO) and maintained under controlled conditions (19–22°C and a 14:10 hr light-dark cycle) until euthanasia. ZEBRA FISH: Zebrafish (Danio rerio, Oregon AB) were housed at the Zebrafish facility of the School of Biology and Medicine, maintained at 28.5 °C and on a 14:10 h light dark. Embryos were staged by hours (h) or days (d) post fertilization according to previous publication (Kimmel et al., 1995). Young male zebrafish (1 year old) or old male zebrafish (2 year old) were used.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All mouse procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the NIH, and were approved by the Animal Subjects Committee of the University of California, Los Angeles. Zebrafish husbandry and well fare was approved by the Service de la consommation et des affaires vétérinaires (SCAV) of the Canton of Vaud
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	University of California, Los Angeles Institutional Review Board (IRB#13-001332-AM-00005) or the University of Alabama at Birmingham Institutional Review Board (Protocol #X110718014)
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All human study procedures were approved by the University of California, Los Angeles Institutional Review Board (IRB#13-001332-AM-00005) or the University of Alabama at Birmingham Institutional Review Board (Protocol #X110718014) for the isolation of cells from human blood from healthy subjects. Tumor adjacent retroperitoneal fat biopsies were obtained from pheochromocytoma patients or control patients with neoplasms as described previously (Vergnes
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We are providing additional experimental results in the Supplementary figures
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A