Editorial comments:

Changes to be made by the Author(s):

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.

We carefully checked all the manuscript.

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

The summary has been edited.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

The reference style has been corrected.

4. 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: FlowJo software, TreeStar, StemSPAN SFEM, Eppendorf, etc.

Commercial language has been removed from the protocol. But, we still referenced to StemSpan SFEM in the table of material because we strongly recommend the use of this medium. Following suggestion of reviewer 3, now we emphasized that the medium used for the analysis is critical for TMRM staining. We also added a new panel (Figure 3A) showing the difference of TMRM staining in HSC cultured in SFEM or in PBS.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly.

The protocol has been revised as suggested.

6. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

The centrifuge speeds have been converted.

7. The Protocol should contain only action items that direct the reader to do something.

We moved comment in note section and the protocol has been revised following directions.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

The protocol has been revised as suggested.

9. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

We reviewed the protocol trying to better answer this question.

10. 1.2: How do you prepare the same?

We added the non-commercial formula of this buffer in the note.

11. 2.4: Do you perform this in sterile area?

Sterile condition are not necessary for this protocol. We specify this adding a note in the protocol.

12. 5: Please include gating strategy for FACS?

The description of gating strategy has been included in the protocol.

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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.

The filmable content has been highlighted.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Explicit permission to republish figurer has been attached.

15. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

In this version, all figures are described in representative results section.

16. 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 has been revised as suggested.

17. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

The table has been removed and uploaded as asked.

18. 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. Please do not abbreviate journal titles.

The references have been updated.

19. Please alphabetically sort the materials table

The table has been revised.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The author provided in the manuscript the standardized method for accurate quantification of mitochondrial membrane potential (ΔΨmt), which would be of great value for those interested in stem cell biology and metabolism. Thus the Reviewer consider that the manuscript is basically suitable for publication in JoVE if the authors change the minor points shown below.

Minor Concerns:

- While all the procedures described seem technically sound, inhibition of dye efflux activity to visualize mitochondrial activity is, as the authors cited, already reported from other group (1). The author should further emphasize the technical advantage or difference over the previous report in the Discussion section.

We thank the reviewer to give us the possibility to better highlight this point. We improved discussion section to emphasize it.

- Figure 2A looks not necessary for this paper.

We totally agree with the reviewer's suggestion. This panel was removed in the revised version.

(1) de Almeida MJ, Luchsinger LL, Corrigan DJ, Williams LJ, Snoeck HW. Dye-Independent Methods Reveal Elevated Mitochondrial Mass in Hematopoietic Stem Cells. Cell Stem Cell. 2017;21(6):725-9.e4.

Reviewer #2:

Manuscript Summary:
In this manuscript authors describe/explain a protocol for the measurement of mitochondria membrane potential (as an indirect measurement of mitochondria activity) in hematopoietic stem and progenitor cells using flow cytometry. The manuscript is timely as it has recently been shown that efflux activity of HSCs can affect the amount of staining and therefore give mispleading results. This will have implication to researchers working in the field of haematopoiesis, leukaemia and mitochondrial function/metabolism and is therefore of significant importance.

We thank the reviewer for the positive comments.

Major Concerns:
I have no major concerns regarding this manuscript. The authors have relevant experience in sorting different haematopoietic cell populations for the bone marrow of mice (evidenced by high impact factor publications) and measurement of mitochondria activity using flow cytometry. Therefore they are well placed to guide the field in this area.

We thank the reviewer for the positive comment.

Minor Concerns:
While the authors discuss that researchers should use mitochondrial dyes with caution as many (all?) are affected by the mitochondrial membrane potential. Can the authors also comment on the use of reagents that are used to measure mitochondrial ROS (i.e. MitoSOX)? Would it be informative to use them in addition to TMRM (and would Verapamil be required)?

We thank the reviewer for pointing out that all dyes, affected by $\Delta\Psi m$, should be used in presence of Verapamil for HSC investigation. We insert this comment in the discussion section.

Also, can authors further discuss/speculate about the possibility to combine measurement of mitochondrial function/activity and frequency/volume using flow cytometry, as this would be very useful for the field. Can TMRM be used with subsequent mitochondrial dye (that measure mitochondrial volume) measurements when the membrane potential has been disrupted/levelled (i.e. FCCP treatment) to remove the effect of the membrane potential. That is, can membrane potential be measured in one tube (TMRM), and mitochondrial volume in another (i.e. MitoTracker + FCCP)?

We strongly recommend the use of 3D volume analysis based on fluorescent markers in the assessment of mitochondrial mass. We agree, however, that the experimental setting suggested by the reviewer could be used for the analysis of mitochondrial mass by flow cytometry, and have noted this possibility in the discussion section.

Reviewer #3:

Manuscript Summary:
The paper by Morganti et al. describes a protocol to measure the mitochondrial membrane potential of HSCs and progenitor cells in culture. The paper is well written and detail-oriented. Below are a few concerns and minor recommendations that if addressed would strengthen the content and the importance of this manuscript.

We thank the reviewer for the positive comment and for the below suggestions.

Major Concerns:

While the authors stress the importance of Verapimil to reliably evaluate the mitochondrial activity in murine HSCs, it will be informative to discuss whether this protocol is applicable for measuring mitochondrial membrane potential in cultured murine HSCs (on the cultures that can maintain such HSCs) as well as human HSCs. Does inclusion of Verapimil alter significantly the results compared to those measured in the absence of Verapimil in cultured murine HSCs or human HSCs? Does the inclusion of Verapimil result in a greater degree of mitochondrial membrane potential in HSCs when compared to more committed progenitors and differentiated cells in those cultures? There are several publications that the authors can include in this manuscript to discuss these points.

As commended by the reviewer, several groups analyzed the mitochondrial membrane potential in human HSC or cultured HSC[1,2]. However the addition of Verapamil was still not commonly used before the publishing of recent papers[3,4]. Since human HSCs express efflux pumps[5,6], we can speculate that human HSC could show the same effect observed in murine HSCs.

(1) Vannini, N. et al. Specification of haematopoietic stem cell fate via modulation of mitochondrial activity. Nat Commun. 7 13125, doi:10.1038/ncomms13125, (2016)

(2) Papa,L., Zimran, E., Djedaini, M., Ge,Y., Ozbek, U., Sebra, R., et al. Ex vivo human HSC expansion requires coordination of cellular reprogramming with mitochondrial remodeling and p53 activation. Blood Advances. 2:2766-2779. doi.org/10.1182/bloodadvances.2018024273. (2018)

(3) de Almeida MJ, Luchsinger LL, Corrigan DJ, Williams LJ, Snoeck HW. Dye-Independent Methods Reveal Elevated Mitochondrial Mass in Hematopoietic Stem Cells. Cell Stem Cell. 2017;21(6):725-9.e4.

(4) Bonora, M., Ito, K., Morganti, C., Pinton, P. & Ito, K. Membrane-potential compensation reveals mitochondrial volume expansion during HSC commitment. *Exp Hematol.* **68** 30-37 e31, doi:10.1016/j.exphem.2018.10.012, (2018).

(5) Chaudhary PM, Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell. 1991 Jul 12;66(1):85-94.

(6) Moitra K. Overcoming Multidrug Resistance in Cancer Stem Cells. Biomed Res Int. 2015;2015:635745. doi: 10.1155/2015/635745.

Although this paper should not be focused on the interpretation of the data and results, deliberating about

the mitochondria membrane potential in HSCs and comparing that with more differentiated cells becomes important and it is relevant to the use of Verapimil, which is the main focus of this protocol/paper. In this context, in the introduction part, the authors cite Vannini et al (2016). A recent study from the same group of investigators (2019) indicates that the inclusion of verapamil alters only slightly the measurement of mitochondrial membrane potential measured in the absence of verapamil. It would be meaningful for the authors to discuss these points. Indeed, this is critical since the authors (line 309-309) emphasize the importance of this protocol as a reliable procedure for assessing mitochondrial activity.

We thank the reviewer for bringing up this widely-debated point. The main aim of this report is to provide detailed guidelines for a standard protocol in order to clarify the discrepancies currently found between results in the literature. Indeed, different groups have recently shown HSCs as exhibiting both higher or lower mitochondrial membrane potentials in comparison to more mature populations.

In the study of Vannini *et al*., 2019, the authors stated that "verapamil treatment only mildly affected Nicotinamide riboside (NR)-induced ΔΨm decrease thus indicating that ABC-transporter-mediated dye efflux in HSCs does not explain the NR-induced reduction in TMTM staining". They did not show an effect by verapamil in different BM populations, including untreated HSC populations, in contrast to the results from Snoeck's[1] and our group[2]. Nevertheless, we have added a reference to the Vannini paper to produce a complete picture of the current state of research.

(1) de Almeida MJ, Luchsinger LL, Corrigan DJ, Williams LJ, Snoeck HW. Dye-Independent Methods Reveal Elevated Mitochondrial Mass in Hematopoietic Stem Cells. Cell Stem Cell. 2017;21(6):725-9.e4.

(2) Bonora, M., Ito, K., Morganti, C., Pinton, P. & Ito, K. Membrane-potential compensation reveals mitochondrial volume expansion during HSC commitment. *Exp Hematol.* **68** 30-37 e31, doi:10.1016/j.exphem.2018.10.012, (2018).

Minor Concerns:

The authors should provide the number of bone marrow cells used for the detection of HSCs and TMRM staining (line 172) as the cell number might influence the TMRM dye uptake.

The number of cells has been added in the protocol.

The authors need to discuss whether TMRM staining is time sensitive and whether such prolonged incubation with TMRM (1hr) is not toxic and does not affect the integrity of the mitochondria and their function.

TMRM dye has the capacity to equilibrate in all the different compartments (mitochondria, cytosol, and extracellular), and once it reaches equilibrium it is stable in controlled temperature conditions. TMRM manufacturer protocols recommend the use of [20 nM] TMRM for 30 min at 37°C. We suggest the use of a low dose of TMRM [2nM] for 1hr at 37°C. We performed TMRM staining of mitochondria numerous times under these conditions in a previous study (Bonora *et al*., 2018, Experimental Hematology) and never observed any change in mitochondrial morphology. So while we we cannot claim to have ruled out all possible toxic effects, we also feel confident in hypothecizing that TMRM staining at this duration is non-toxic, and does affect the integrity of mitochondria or their function. In addition, another group is known to have stained HSCs with TMRM for 1 hr at a higher dose [200nM] in two different studies (Vannini, 2016 and Vannini, 2019) without any toxic effects.

The authors need to deliberate on whether measuring mitochondrial membrane potential by TMRM staining can be affected by staining the cells contained in media with cytokines as opposed to FBS/PBS solution.

We thank the reviewer for this comment. We reported (and have added a new panel in Figure 3 to illustrate) that TMRM staining in HSCs is affected by medium conditions. In our new Figure 3A, we show that TMRM profiles can change in HSCs, if the staining is performed in PBS rather than SFEM. The reason for these changes should will require further exploration, but since serum-free expansion medium (SFEM) is a well-established HSC culture condition, we strongly recommend performing TMRM analysis in SFEM rather than PBS.

There are a couple of types in lines 207, 252 that need to be corrected

Thank for the notice, we corrected them.