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Improving the accuracy of flow cytometric assessment of ΔΨm in hematopoietic stem and progenitor cells through the inhibition of efflux pumps

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TITLE:

Improving the Accuracy of Flow Cytometric Assessment of Mitochondrial Membrane Potential in Hematopoietic Stem and Progenitor Cells through the Inhibition of Efflux Pumps

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mitochondrial membrane potential, hematopoietic stem cell, TMRM, verapamil, flow cytometry, efflux pumps, multi drug resistance

SUMMARY:

Xenobiotic efflux pumps are highly active in hematopoietic stem and progenitor cells (HSPCs) and cause extrusion of TMRM, a mitochondrial membrane potential fluorescent dye. Here, we present a protocol to accurately measure mitochondrial membrane potential in HSPCs by TMRM in the presence of Verapamil, an efflux pump inhibitor.

ABSTRACT:

As cellular metabolism is a key regulator of hematopoietic stem cell (HSC) self-renewal, the various roles played by the mitochondria in hematopoietic homeostasis have been extensively studied by HSC researchers. Mitochondrial activity levels are reflected in their membrane potentials (ΔΨm), which can be measured by cell-permeant cationic dyes such as TMRM (tetramethylrhodamine, methyl ester). The ability of efflux pumps to extrude these dyes from cells can limit their usefulness, however. The resulting measurement bias is particularly critical when assessing HSCs, as xenobiotic transporters exhibit higher levels of expression and activity in HSCs than in differentiated cells. Here, we describe a protocol utilizing Verapamil, an efflux pump inhibitor, to accurately measure ΔΨm across multiple bone marrow populations. The resulting inhibition of pump activity is shown to increase TMRM intensity in hematopoietic stem

and progenitor cells (HSPCs), while leaving it relatively unchanged in mature fractions. This highlights the close attention to dye-efflux activity that is required when ΔΨm-dependent dyes are used, and as written and visualized, this protocol can be used to accurately compare either different populations within the bone marrow, or the same population across different experimental models.

INTRODUCTION:

Hematopoietic stem cells (HSCs) are self-renewing, multi-potent, and capable of giving rise to all the cells of the blood[1,2]. Cellular metabolism is a key regulator of HSC maintenance, along with transcriptional factors, intrinsic signals and the microenvironment[3-5]. The proper control of mitochondrial function and quality is therefore critical to HSC maintenance[6,7].

Mitochondrial membrane potential (ΔΨm) is a key parameter in the assessment of mitochondria as it directly reflects their functionality, which derives from the equilibrium of proton pumping activity in the electron transport chain and the proton flow through F_1/F_O ATP synthase. These are both required (depending on gene expression and substrate availability) for the oxygen-dependent phosphorylation of ADP to ATP[8,9]. Taking advantage of the electronegativity of the mitochondrial compartment, various potentiometric dyes have been developed to measure ΔΨm. One of them is tetramethylrhodamine methyl ester perchlorate (TMRM), which has been extensively used to measure ΔΨm by flow cytometry in a variety of cells[10], including hematopoietic stem and progenitor cells[11].

Mitochondrial dyes must be used with some caution in HSCs, however, because the high activity of the xenobiotic efflux pumps of these cells can result in dye extrusion[12]. Indeed, the extrusion of mitochondrial dyes such as Rhodamine 123 has allowed researchers to isolate HSCs[13] or identify HSC "side populations" by exploiting the differential extrusion of the dyes Hoechst Blue and Hoechst Red[14,15]. It has also been shown that Fumitremorgin C, a specific blocker of the ATP-binding cassette sub-family G member 2 (ABCG2) transporter, does not affect the staining pattern of MitoTracker in HSPCs[16]. After the publication of these results, multiple studies were performed using mitochondrial dyes in the absence of xenobiotic efflux pump inhibitors, leading to the widespread impression that HSCs have only a small number of mitochondria with low ΔΨm[16-18].

Recently, it was demonstrated, however, that Verapamil, a wide spectrum inhibitor of efflux pumps, significantly modifies the staining pattern of the mitochondrial dye MitoTracker Green[19]. This discrepancy is likely due to the fact that Fumitremorgin C is highly selective for Abcg2, while HSCs also express other transporters such as Abcb1a (which is only weakly sensitive to Fumitremorgin C)[19]. We have also reported that other mitochondrial dyes, such as TMRM, Nonyl acridine orange, and Mitotracker Orange (MTO) exhibit the same patterns as Mitotracker Green. More importantly, we have observed that the flow cytometric patterns of HSPCs reflect their ΔΨm in addition to mitochondrial mass[11].

The intake of TMRM dye strictly depends on the negative charge of mitochondria, but the resulting accumulation of dye is in constant balance between its intake and clearance by efflux

pumps[20]. The difference in xenobiotic efflux pump expression between HSCs and mature cell populations affects this balance and can lead to biased results. The use of dedicated inhibitors such as Verapamil should be considered in the analysis of ΔΨm by potentiometric dyes. Here we describe a modified protocol for accurate ΔΨm measurement by TMRM-based flow cytometry which corrects for xenobiotic transporter activity through the use of dedicated inhibitors.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the Albert Einstein College of Medicine.

1. Preparation of solutions

1.1. Staining Buffer (phosphate-buffered saline (PBS) + 2% fetal bovine serum (FBS)): Add 10 mL of FBS in 500 mL of a sterile PBS solution.

NOTE: This solution can be stored at 4 °C for at least one month in sterile condition. Before starting the following procedures, put an aliquot of this solution (50 mL) on ice.

1.2. ACK (ammonium-chloride-potassium) lysing buffer: Place an aliquot of ACK lysing buffer (1 mL) on ice before starting the procedure.

NOTE: To prepare the same non-commercial buffer, dissolve 8.02 g of NH_4Cl, 1 g of $KHCO_3$ and 37.2 mg of Na_2EDTA in 1 L of H_2O. Adjust the pH to 7.2-7.4. Store for up to 6 months at room temperature.

1.3. Culture medium: Add 50 ng/mL stem cell factor (SCF) and 50 ng/mL thrombopoietin (TPO) to serum-free medium for culture and expansion of hematopoietic cells (see **Table of Material** for commercial medium recommended).

1.4. TMRM stock solution: Prepare TMRM (1 µM) solution by dissolving 5 µg of TMRM powder in 10 mL of ethanol. Store this solution at −20 °C protected from the light.

1.5. Verapamil stock solution: Prepare Verapamil (50 mM) solution by diluting 24 mg of Verapamil in 1 mL of ethanol. Store this solution at −20 °C.

1.6. FCCP stock solution: Prepare carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP) (1 M) solution by dissolving 254 mg in 1 mL of ethanol. Store this solution at −20 °C protected from the light.

2. Bone marrow isolation

2.1. Euthanize the mouse by CO_2 inhalation following institutional guidelines and spray the mice with 70% ethanol.

NOTE: This step prevents contamination of the cells of interest without compromising experimental results.

2.2. Using a pair of forceps and sharp scissors, make a small snip in the ventral skin of the mouse and stretch the skin.

2.3. Extract the femur and tibia while taking care not to dislodge the heads of the femur as they contain a large amount of bone marrow cells. Place the removed bones in a 6-well plate filled with 1.5 mL of staining buffer.

NOTE: This procedure does not require sterile area.

2.4. Remove the muscles from the bones and cut the ends of the bones allowing the exit of the bone marrow (**Figure 1A**). Place the cleaned bones in new wells with 1.5 mL of staining buffer.

NOTE: Muscles and other tissues must be carefully removed to avoid syringe clogging in the next step.

2.5. Flush out the bone marrow using a 3 mL syringe with a 25G needle.

NOTE: Continue to flush the bone marrow until the bone becomes white (**Figure 1B**).

2.6. Collect all cells in a 1.5 mL tube, centrifuge for 5 min at 180 x *g* then discard the supernatant (**Figure 1C**).

2.7. Resuspend the pellet in 300 µL of ice cold ACK lysing buffer very carefully, put on ice for 1 min and immediately inactive lysis by adding 1 mL of staining buffer. Centrifuge for 5 min at 180 x *g*.

NOTE: The cells pellet appears white (**Figure 1D**). The total number of cells isolated is about 2-4 x 10^7.

2.8. Resuspend the pellets in 1 mL of staining buffer and filter using a cell-strainer cap (12 x 75 mm, 5 mL of capacity) with a 35 µm nylon mesh incorporated to obtain mononuclear cells. After blocking, keep the sample on ice.

3. Immunostaining for detection of HSC

3.1. Prepare Lineage (Lin) cocktail. In 400 µL of staining buffer, add 4 µL of the following biotinilated antibodies against CD3e, CD4, CD8, B220, CD11b, Gr1, Ter119, CD19, Nk1.1, IgM and Il7Ra to obtain a final dilution 1:100.

3.2. Prepare fluorophores conjugated-antibodies (Abs). In 400 µL of staining buffer, add 4 µL of the following Abs to obtain a final dilution 1:100. Streptavidin-Pacific Blue, Sca1-PE/Cy7, c-kit-

APC/Cy7, CD48-APC, CD150-PerCP/Cy5.5 and CD34-FITC. Keep in ice, protected from light.

3.3. Centrifuge the sample for 5 min at 180 x *g*, then discard the supernatant.

3.4. Add 400 µL of Lin cocktail solution to the cells pellet. Add 4 µL of CD135-biotinilated Ab. Vortex quickly to mix and incubate for 30 min in ice.

3.5. Wash the sample adding 3 mL of staining buffer, spin down for 5 min at 180 x *g* and discard the supernatant.

3.6. Add 400 µL of Abs solution. Vortex quickly to mix and incubate for 30 min on ice.

3.7. Wash the samples with 3 mL of staining buffer, spin down for 5 min at 180 x *g* and discard the supernatant.

4. TMRM staining

4.1. Prepare TMRM staining solution. Add 2.2 µL of TMRM stock solution and 1.1 µL of Verapamil (2 nM and 50 µM as final concentration, respectively) in 1.1 mL of serum-free medium for culture and expansion of hematopoietic cells (see **Table of Material** for commercial medium recommended) with TPO and SCF.

NOTE: This is the most important step of the protocol. Verapamil addition is necessary to block the efflux pumps which are highly expressed in the HSCs and can extrude TMRM.

4.2. Resuspend the bone marrow in 1 mL of TMRM staining solution, vortex quickly and incubate for 1 h at 37 °C.

NOTE: TMRM staining must not be washed out. PE-dedicated compensation control is also resuspended in 100 µL of TMRM staining solution, and subjected to incubation for 1 h at 37 °C after quick vortex.

4.3. Filter the sample using a cell-strainer cap (12 x 75 mm, 5 mL of capacity) with a 35 µm nylon mesh incorporated to avoid clogging of the flow cytometer.

NOTE: TMRM staining increases the possibility of clog formation in the sample. Filtration of the sample just before flow assays is recommended.

4.4. Add 1 µL of 4',6-diamidino-2-phenylindole (DAPI) to exclude dead cells by flow cytometry.

5. Acquisition by flow cytometer

5.1. Run bone marrow sample and acquire at least 1 x 10^6 events.

5.2. Set up the gating strategy to identify the different hematopoietic populations (**Figure 2**).

5.2.1. Display live bone marrow mono-nuclear cells (BM-MNCs), $DAPI^-$ fraction, in plot for Pacific Blue to identify $CD135^-Lin^-$ (Lin^-) and Lin^+ fractions.

5.2.2. Plot Lin^- fraction for APC/Cy7 (c-kit) versus PE/Cy7 (Sca-1) to identify multipotent progenitors (MPP) fraction, as c-kit^+ and Sca-1^+.

5.2.3. Plot MPP fraction for APC (CD48) versus PerCP/Cy5.5 (CD150) to identify HSC fraction, as $CD150^+$ and $CD48^-$.

5.2.4. Display HSC fraction for FITC (CD34) to divide $CD34^-$-HSC and $CD34^+$-HSC.

5.3. Acquire the TMRM intensity (PE channel) in each population.

5.4. After acquisition, add to the sample 1 µL of FCCP to obtain the final concentration of 1 mM and incubate at 37 °C for 5 min, then acquire 1 x 10^6 events.

NOTE: FCCP is a mitochondrial uncoupler which is used to dissipate the ΔΨm. It is used as experimental control to confirm that TMRM staining works correctly. After the administration of FCCP, the TMRM intensity should drastically be decreased (**Figure 3A**).

5.5. Analyze data normalizing the average intensity of PE of each population by the intensity of PE of all BM-MNCs.

REPRESENTATIVE RESULTS:

The protocol described above enables the easy isolation of BM-MNCs from a mouse model. **Figure 1** summarizes the main steps of the protocol: bone isolation, flushing out of the bone marrow, red blood cell lysis, and antibody staining followed by TMRM staining to measure mitochondrial membrane potential in a specific hematopoietic population.

BM-MNCs contain several cell populations, including HSCs. The antibody cocktails used in this protocol are well-established in the purification of HSCs ($CD34^-$ and $CD34^+$), multipotent progenitor cells (MPPs), Lin^- as well as Lin^+ cells, respectively [21]. The gating strategy for isolating these fractions is shown in **Figure 2**.

After the identification of populations of interest, TMRM intensity, which should appear as a bright signal, was assessed. TMRM staining in serum-free expansion medium (SFEM) is highly recommended, as TMRM profiles in HSPCs can undergo alteration when staining is performed in PBS +2% FBS (**Figure 3A**).

Figure 3C shows the average intensity of each population, which is normalized by the intensity of BM-MNCs. HSCs express high activity levels of xenobiotic efflux pumps capable of extruding TMRM dye[20], and indeed, we found TMRM profiles in HSPCs were changed in the presence of

Verapamil (**Figure 3B,C**). Similar results were obtained by other inhibitors such as Cyclosporin H (**Figure 3C**). Thus, the accurate amount of TMRM loaded in the mitochondria by ΔΨm can be measured after inhibition of the efflux pumps by Verapamil or Cyclosporin H (**Figure 3C**).

Finally, FCCP can be used to verify the accuracy of TMRM staining. FCCP depolarizes mitochondria, resulting in a reduction in TMRM intensity (**Figure 3D**). This approach can also be used to determine the background intensity of the staining and/or as a negative control.

FIGURE LEGENDS:

Figure 1: Protocol flowchart. Graphical summary of the procedure to isolate and stain BM-MNCs to determine ΔΨm. Critical steps are highlighted by picture inserts (**A-D**). Femurs and tibias from adult C57BL/6 mice were isolated and their ends are removed (**A**). Long bones as in A after flush out (**B**). Isolated BM-MNCs before (**C**) and after (**D**) ACK lysis.

Figure 2: Gating setup. Schematic representation of gating strategy to identify the different hematopoietic populations, including $CD34^-$-HSC and $CD34^+$-HSC, MPP, Lin^- and Lin^+ cells. The panels were modified from Bonora, M. et al[11].

Figure 3: Flow cytometry analysis of mitochondrial membrane potential. (**A**) Representative distribution of ΔΨm in HSCs stained with TMRM in PBS+2%FBS (green) or in serum-free expansion medium (SFEM) (red). (**B, C**) Representative distribution of ΔΨm in $CD34^-$-HSC and Lin^- cells (**B**) and quantification of ΔΨm in $CD34^-$-HSC, $CD34^+$-HSC, MPP, Lin^- and Lin^+ cells (**C**) stained with TMRM in presence or absence of efflux pump inhibitors. TMRM intensity of each population was normalized by the TMRM intensity of own BM-MNCs (modified from Bonora, M. et al[11]). (**D**) Representative histogram of TMRM intensity distribution in HSCs before (pink) and after (light blue) FCCP addition.

DISCUSSION:

Mitochondrial membrane potential measurement is a cornerstone of the analysis and assessment of mitochondria, which are critical to the metabolic state of the cell. Here, we describe a protocol for the analysis of ΔΨm by TMRM staining. TMRM is a cell-permeant fluorescent dye which accumulates in active mitochondria due to ΔΨm, and its respective levels remain in equilibrium between the extracellular, cytoplasmic and mitochondrial compartments[10]. This protocol can be adapted for various dyes, including tetramethylrhodamine, ethyl ester (TMRE) and JC-1. Appropriate staining conditions are critical to achieving accurate results. These include: protection from light exposure, proper incubation time, maintenance of constant temperature (37 °C) and extracellular concentration. When TMRM dye levels have not yet reached perfect equilibrium, unexpected changes in staining intensity can be detected during data acquisition in the flow cytometry process. Therefore the recommended staining period for TMRM is 1 h rather than 30 min (as mentioned in one TMRM manufacturer's protocol). It is also suggested that the sample should be acquired at least two times within 5-min in order to compare the stability of the dye.

Another critical step of the protocol is the setting up of an efficient color match among antibodies

and dye. Hematopoietic populations can be detected by flow cytometry using well-established surface markers[21].The gating strategy described here (**Figure 2**) is compatible with TMRM staining, and allows for the measurement of its intensity at different differentiation stages simultaneously. TMRM matches the PE channel, but its intensity is usually much weaker than that of antibodies conjugated with PE (data not shown). As this can affect color compensation, causing unexpected shifts in the spectra of some populations, the sample with TMRM staining should be used as a compensation control for the PE channel.

The major advantage of the current protocol is that it enables the removal of the effect of xenobiotic efflux pumps, whose efficient extrusion of TMRM dye modifies both its equilibrium across the plasma membrane and its mitochondrial accumulation. This is a critical step in the accurate assessment of HSC mitochondrial function by dye absorption. As HSCs express more active efflux pumps than committed cells[19], Verapamil or similar drugs, such as Cyclosporine H, a Ca^{2+}-independent multidrug resistance inhibitor[22] (**Figure 3B**-**C**), should be used in the staining procedure for HSCs to more accurately assess levels of mitochondrial activity. Because this critical issue was pointed out only recently[11,19] and is still under discussion[23], the main aim of this report is to provide an easy and detailed protocol which will help to standardize the procedure for obtaining reproducible data and reduce the apparent discrepancies between the results of different research groups. The protocol here described shows in detail each step, including doses, timing and specific media. For instance, TMRM profiles in HSPCs can differ depending on their medium (**Figure 3A**). A detailed analysis of the mechanisms involved will require further exploration, but since serum-free expansion medium (SFEM) is a well-established method for HSC culture, SFEM rather than PBS is strongly recommended when performing TMRM staining for HSCs. Further, the accurate assessment of mitochondrial membrane potential demonstrated here through the inhibition of efflux pumps highlights possible future applications of Verapamil, as well as other dyes (e.g. MitoTracker, MitoSOX), in the investigation of HSCs.

Importantly, TMRM intensity as shown by flow cytometry may not precisely reflect mitochondrial volume. Flow cytometry measures total fluorescence intensity per cell, but the distribution of mitochondrial dyes to mitochondria depends on ΔΨm. It is therefore difficult to discern whether the critical contribution to a TMRM reading derives from ΔΨm or mitochondrial volume[11]. We have confirmed that MTO, one of the dyes most frequently used to measure mitochondrial mass, is affected by both ΔΨm and efflux pump activity. We have also observed that Verapamil changes the intensity profile of MTO in HSPC populations, but have found no correlation between MTO intensity and mitochondrial volume[11]. Combined measurement of mitochondrial volume and ΔΨm should be used to normalize data and eliminate the effects of membrane potential, and further exploration of mitochondrial content should be carried out using ΔΨm-independent techniques.

Such techniques would include 3D volume analysis based on fluorescent markers (e.g., immunostaining of mitochondrial proteins), electron microscopy[11], and quantitation of mitochondrial/nuclear DNA ratios. The use of mitochondrial dyes (i.e., TMRM) in combination with efflux system inhibitors will doubtless prove of great benefit in the elucidation of mitochondrial biology through flow cytometry.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

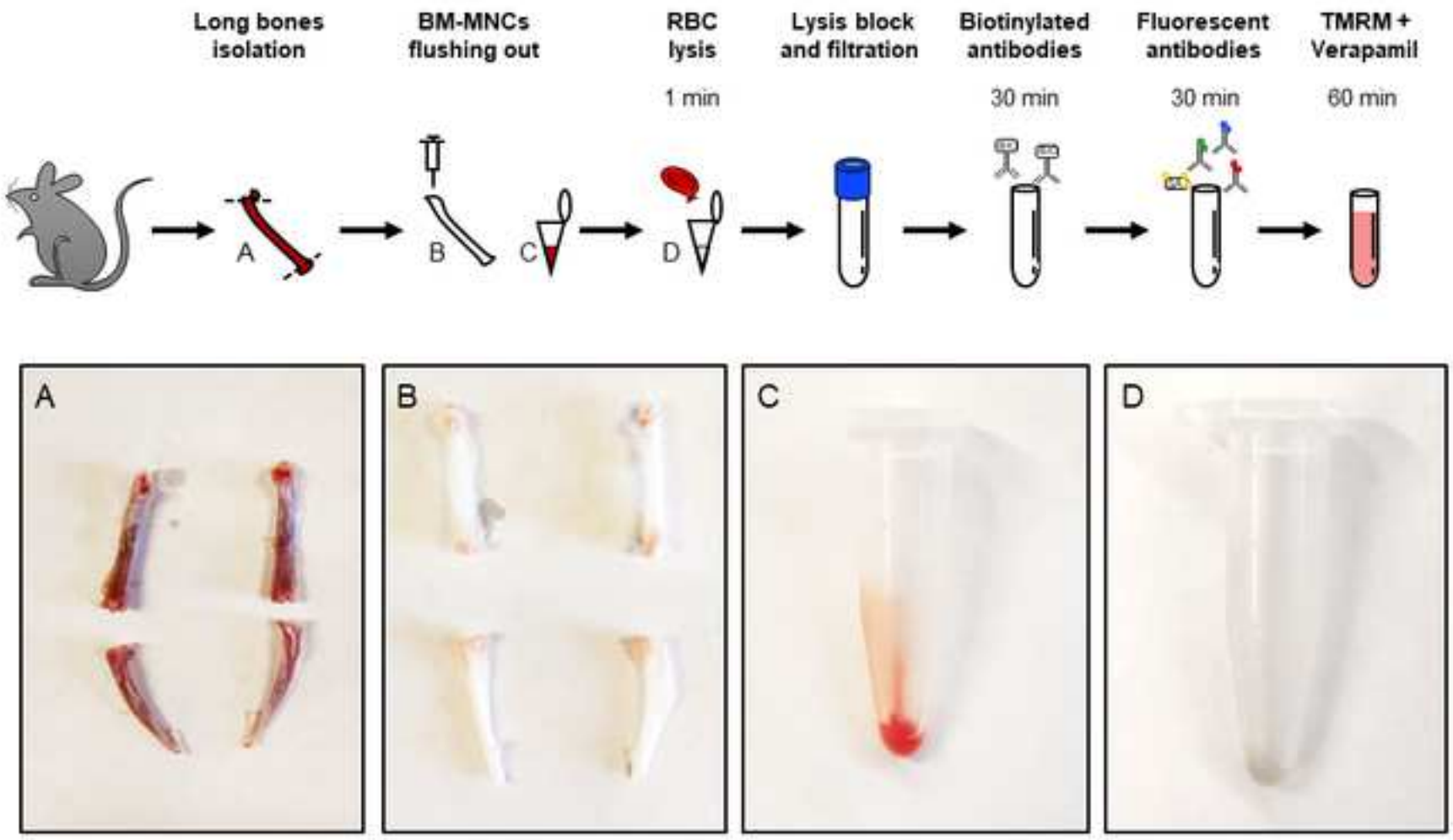

Figure 2

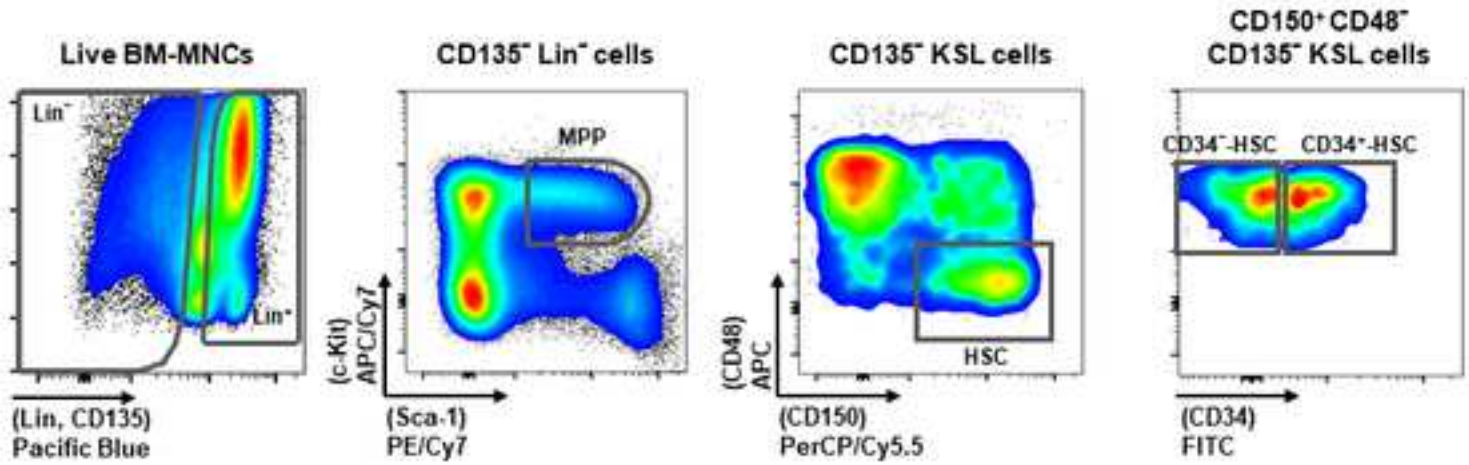

Figure 3

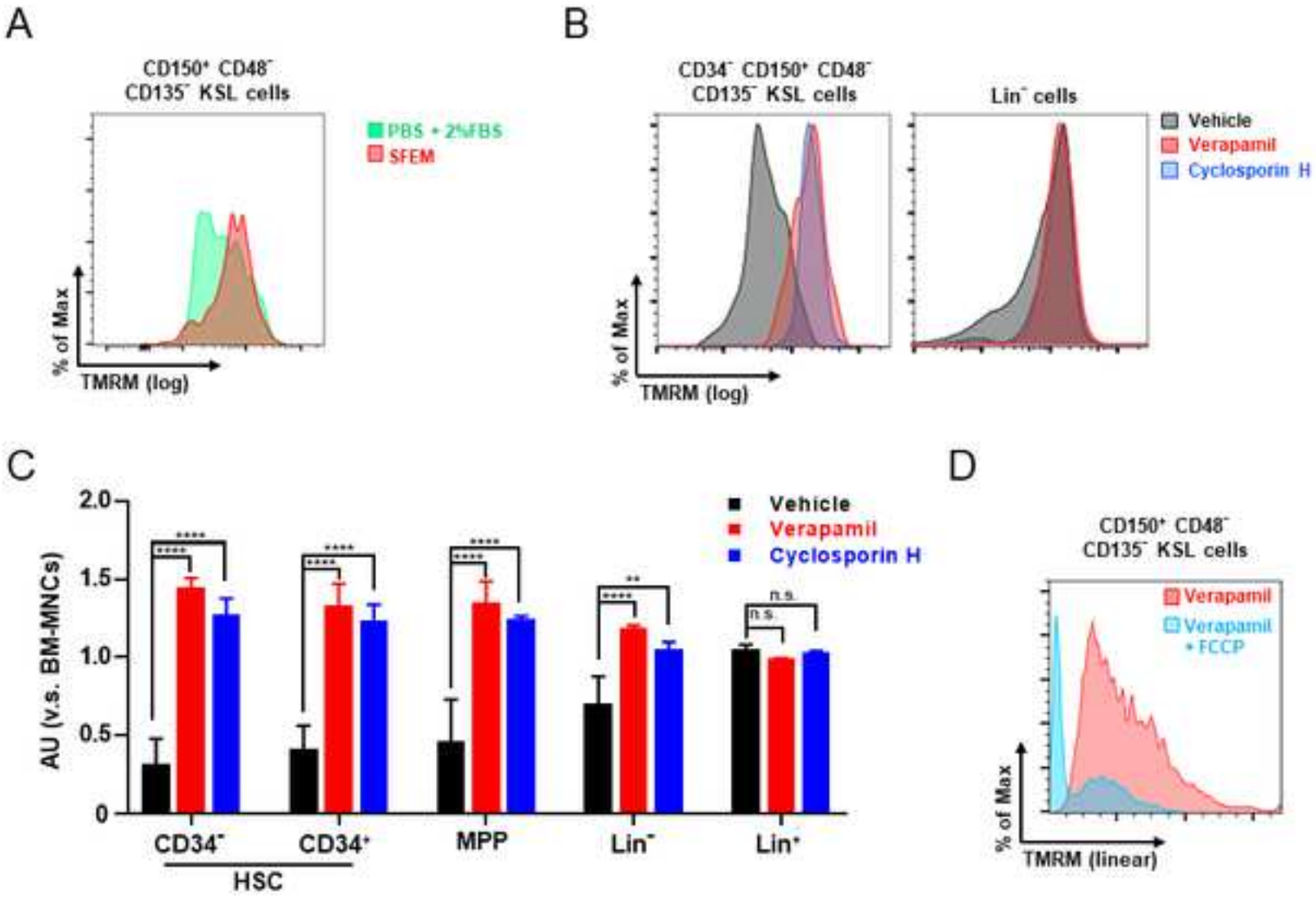

TABLE OF MATERIALS:

Name of Material/ Equipment	Company	Catalog Number
ACK lysing buffer	Life Technologies	A1049201
B220-biotin	BD Bioscience	553086
CD3e-biotin	Life Technologies	13-0031-85
CD4-biotin	Fischer Scientific	BDB553782
CD8-biotin	Life Technologies	13-0081-85
CD11b-biotin	BD Bioscience	553309
CD19-biotin	BD Bioscience	553784
CD34-FITC	eBioscience	11-0341-85
CD48-APC	eBioscience	17-0481-82
CD135-biotin	eBioscience	13-1351-82
CD150-PerCP/Cy5.5	Biolegend	115922
c-kit-APC/Cy7	Biolegend	105826
Cyclosporin H	Millipore Sigma	SML1575-1MG
DAPI solution (1mg/mL)	Life Technologies	62248
Fetal Bovine Serum (FBS)	Denville	FB5001-H
FCCP	Millipore Sigma	C2920-10MG
Gr1-biotin	Biolegend	108404
IgM-biotin	Life Technologies	13-5790-85
Il7Rα-biotin	eBioscience	13-1271-85
Nk1.1-biotin	Fischer Scientific	BDB553163
Phosphate buffered saline (PBS)	Life Technologies	10010023
Sca-1-PE/Cy7	eBioscience	25-5981-81
SCF murine	PEPROTECH	250-03-10UG
StemSpan SFEM medium	STEMCELL technologies	9605
Streptavidin-Pacific Blue	eBioscience	48-4317-82
Ter119-biotin	Fischer Scientific	BDB553672
TMRM	Millipore Sigma	T5428-25MG
TPO	PEPROTECH	315-14-10UG
Verapamil hydrochloride	Millipore Sigma	V4629-1G

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Title of Article:	Improving the accuracy of flow cytometric assessment of mitochondrial membrane potential in hematopoietic stem and progenitor cells through the inhibition of efflux pumps
Author(s):	Claudia Morganti, Massimo Bonora, Keisuke Ito

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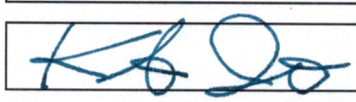
Signature:	
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Editorial comments:

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.

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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.

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