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Flow cytometric analysis of mitochondrial reactive oxygen species in murine hematopoietic stem and progenitor cells and MLL-AF9 driven leukemia

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TITLE:

Flow Cytometric Analysis of Mitochondrial Reactive Oxygen Species in Murine Hematopoietic Stem and Progenitor Cells and MLL-AF9 Driven Leukemia

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AML, HSCs, ROS, superoxides, mitochondria, flow cytometer

SUMMARY:

We describe a method for using multiparameter flow cytometry to detect mitochondrial reactive oxygen species (ROS) in murine healthy hematopoietic stem and progenitor cells (HSPCs) and leukemia cells from a mouse model of acute myeloid leukemia (AML) driven by MLL-AF9.

ABSTRACT:

We present a flow cytometric approach for analyzing mitochondrial ROS in various live bone marrow (BM)-derived stem and progenitor cell populations from healthy mice as well as mice with AML driven by MLL-AF9. Specifically, we describe a two-step cell staining process, whereby healthy or leukemia BM cells are first stained with a fluorogenic dye that detects mitochondrial superoxides, followed by staining with fluorochrome-linked monoclonal antibodies that are used to distinguish various healthy and malignant hematopoietic progenitor populations. We also provide a strategy for acquiring and analyzing the samples by flow cytometry. The entire protocol can be carried out in a timeframe as short as 3-4 h. We also highlight the key variables to consider as well as the advantages and limitations of monitoring ROS production in the mitochondrial compartment of live hematopoietic and leukemia stem and progenitor subpopulations using fluorogenic dyes by flow cytometry. Furthermore, we present data that mitochondrial ROS abundance varies among distinct healthy HSPC sub-populations and leukemia progenitors and discuss the possible applications of this technique in hematologic research.

INTRODUCTION:

Reactive Oxygen Species (ROS) are highly reactive molecules derived from molecular oxygen. The most well-defined cellular location of ROS production is the mitochondria, where electrons that pass through the electron transport chain (ETC) during oxidative phosphorylation (OXPHOS) are absorbed by molecular oxygen leading to the formation of a specific type of ROS called superoxides[1]. Through the actions of a series of enzymes, called superoxide dismutases or SODs,

superoxides are converted into hydrogen peroxides, which are subsequently neutralized into water by enzymes such as catalase or glutathione peroxidases (GPX). Perturbations in ROS-regulatory mechanisms can lead to the excess production of ROS, often referred to as oxidative stress, which have harmful and potentially lethal cellular consequences such as macromolecule damage (i.e., DNA, protein, lipids). Moreover, oxidative stress is related to several pathologies, such as diabetes, inflammatory diseases, aging and tumors[2-4]. To maintain redox homeostasis and prevent oxidative stress, cells possess a variety of ROS-regulating mechanisms[5].

Physiological levels of certain ROS are necessary for proper embryonic and adult hematopoiesis[6]. However, excess ROS is associated with DNA damage, cellular differentiation and exhaustion of the hematopoietic stem and progenitor pool. There is also evidence that alterations in redox biology may differ between leukemia and healthy cells. For example, ROS levels tend to be higher in acute myeloid leukemia (AML) cells relative to their healthy counterparts and other studies have suggested that leukemia stem cells maintain a low steady-state level of ROS for survival[7,8]. Importantly, strategies for therapeutically capitalizing on these redox differences have shown promise in several human cancer settings[9,10]. Therefore, assays that allow for the assessment of ROS levels in mouse models may improve our understanding of how these species contribute to cellular physiology and disease pathogenesis as well as potentially provide a platform for assessing the effectiveness of novel redox-targeting anti-cancer therapies.

PROTOCOL:

All of the animal procedures described in this protocol have been approved by the Institutional Animal Care and Use committee (IACUC) at Fox Chase Cancer Center.

NOTE: The protocol workflow is divided into 4 parts as presented in **Figure 1** and the required reagents are listed in the **Table of Materials**.

1. Bone marrow (BM) isolation

NOTE: MLL-AF9 leukemia mice were generated as described previously[11].

1.1. Recover mono-nuclear bone marrow cells, as described previously[12-15], from wild type C57.Bl6 mice (which express the CD45.2 congenic marker) as well as from C57.Bl6-SJL mice (which express the CD45.1 congenic marker) that have been transplanted with MLL-AF9-expressing leukemia cells ($CD45.2^{+}$).

NOTE: BM can be recovered from mice either by crushing[12,13] or by flushing bones[14,15]. For the experiments presented here, BM was recovered from both healthy and leukemia mice via flushing.

2. Mitochondrial ROS fluorogenic dye staining

2.1. Once mono-nuclear bone marrow cells have been recovered from healthy and/or leukemia mice, stain an aliquot of cells with Trypan Blue and count using a hemocytometer to determine

the starting number of total BM cells.

2.2. Centrifuge the cells at 300 x *g* for 5 min. Aspirate the supernatant and resuspend the pellet in F-PBS (PBS supplemented with 2% fetal bovine serum and a 1% Penicillin/Streptomycin cocktail) to a concentration of $2x10^6$ cells/mL.

2.3. Aliquot 200 µL of cell suspension per tube into 9 single-color control tubes labeled as follows: No stain, B220-Cy5-PE, cKit-Cy7-APC, Sca1-PacBlue, CD150-APC (for healthy HSPCs only), CD45.2-APC (for leukemia cells only), CD34-FITC, Mitochondrial ROS dye and Live/dead cell stain.

NOTE (Optional): A positive control for the induction of mitochondrial ROS can be prepared by treating $2x10^5$ cells in 200 µL with 20 µM of Menadione Sodium Bisulfite (MSB) for 1 h at 37 ˚C in a 5% CO_2 incubator. A second control to reverse the MSB-mediated induction of mitochondrial ROS can be prepared by treating $2x10^5$ cells in 200 µL with 20 µM of MSB plus 100 µM N-acetyl-L-cysteine (NAC) for 1 h at 37 ˚C in a 5% CO_2 incubator.

2.4. Aliquot the remaining cells in a tube (experimental tube) and centrifuge at 300 x *g* for 5 min.

2.5. Resuspend the cells in F-PBS with a live/dead cell stain according to the manufacturer's instructions. Incubate on ice for 30 min. Be sure to add live/dead stain to the single-color control tube.

2.6. Add 1.0 mL of room temperature (RT) F-PBS to both single-color and experimental tubes stained with the live/dead dye. Centrifuge 5 min at 300 x *g* at RT.

2.7. Resuspend 50 µg of the mitochondrial ROS dye in 13 µL of dimethyl sulfoxide (DMSO) to obtain a 5 mM stock solution.

2.8. Dilute mitochondrial ROS dye to a final concentration of 5 µM in RT F-PBS with or without Verapamil (50 µM).

2.9. Aspirate off the wash of the live/dead cell stain. Add 200 µL of mitochondrial ROS dye stain containing Verapamil to each experimental tube as well as the mitochondrial ROS dye single-color control tube.

2.10. Vortex to mix and incubate for 10 min at 37 ˚C in the dark.

2.11. Add 1.0 mL of RT F-PBS to the mitochondrial ROS-stained single-color control and experimental tubes. Centrifuge 5 min at 300 x *g* at RT.

2.12. Aspirate off the supernatant and wash the cells with an additional 1.0 mL of RT F-PBS. Centrifuge 5 min at 300 x *g* at RT.

3. Lineage antibody staining

3.1. Prepare the antibody cocktails listed in **Table 1**.

NOTE: These antibodies cocktails have been optimized previously[14-16].

3.2. Aspirate the supernatant from the final mitochondrial ROS dye wash of the experimental tubes containing healthy BM and add 200 µL of antibody cocktail #1 to each tube. Vortex to mix. Also prepare the single-color control tubes. Incubate for 60 min on ice in the dark.

3.3. Aspirate the supernatant from the final mitochondrial ROS dye wash of the experimental tubes containing leukemia BM and add 200 µL of the antibody cocktail #2 to each tube. Vortex to mix. Incubate for 60 min on ice in the dark.

3.4. Wash with 1.0 mL of cold F-PBS and centrifuge 5 min at 300 x *g* at RT.

3.5. Resuspend cells in 500 µL of cold F-PBS and filter the cells in a flow cytometer tube using a 40 µm filter to exclude aggregates.

4. Flow cytometry acquisition and analysis

NOTE: Several hematopoietic stem and progenitor subsets are rare, such as long-term hematopoietic stem cells. Thus, ideally 3-5 million events should be collected for each experimental tube during flow cytometry acquisition for sufficient analysis of mitochondrial ROS in the various HSPC subsets.

4.1. Use the no-stain control tube to set the forward (FSC-A) and side (SSC-A) scatter plots based on the size and complexity of the cell population analyzed.

4.2. Use the no-stain and single-color control tubes to compensate the flow cytometer.

4.3. Gate out extraneous debris from the forward and side scatter plot (**Figure 2A,B**, first panel from the left).

4.4. Gate out doublets using a double discriminator such as the forward discriminator (**Figure 2A,B**, second panel from the left).

4.5. Follow the gating strategy proposed in **Figure 2A,B** to select live cells, lineage low cells and the various HSPC and leukemia subsets as presented in **Figure 2A,B**.

4.6. For each population of interest, analyze the median fluorescence intensity (MFI) of the TRPE channel (x-axis) in a histogram plot to evaluate differences in the mitochondrial ROS signal (Figure 3A-C, left panels). Levels of mitochondrial ROS can be evaluated at the single-cell level by comparing mitochondrial ROS staining versus specific lineage markers in a scatter plot.

REPRESENTATIVE RESULTS:

Presented is a method for analyzing ROS in the mitochondria of multiple healthy and MLL-AF9-expressing leukemia progenitor populations. **Figure 1** displays a schematic view of the protocol workflow, which consists of 4 major steps: 1) BM isolation from mice; 2) Staining BM cells with a fluorogenic dye that recognizes mitochondrial ROS, particularly superoxides; 3) Surface marker antibody staining to delineate various healthy and leukemia hematopoietic populations; and 4) Flow cytometry acquisition and analysis.

Figure 2A,B depicts a representative gating strategy for analyzing various hematopoietic stem and progenitor populations in healthy and leukemia BM. An FSC/SSC plot is applied to eliminate debris and an FSC-area (FSC-A) versus FSC-height (FSC-H) plot is used to exclude doublets and aggregates. A panel of lineage surface markers (**Table 1** and step 3.1) are combined to exclude a variety of mature hematopoietic populations such as lymphocytes, erythrocytes, granulocytes and monocytes/macrophages (i.e., Lineage low or Lin^{low}). The lineage cocktail also includes a CD48 antibody and therefore all subsets of lineage low cells are also $CD48^-$. Sca-1 and c-Kit cell surface expression is used to distinguish a heterogeneous mixture of HSPCs called $CD48^-$ LSKs (Lin^{low}, $Sca\text{-}1^+$, $c\text{-}Kit^+$, $CD48^-$) from myeloid progenitors (Lin^{low}, $Sca\text{-}1^-$, $c\text{-}Kit^+$, $CD48^-$). To further distinguish various HSPC subsets, the SLAM marker, CD150 as well as CD34 are also added[17-21]. **Figure 2B** also depicts a representative gating strategy for cKit high-expressing leukemia progenitors (Lin^{low}, $c\text{-}Kit^{high}$; $Sca\text{-}1^-$), which are enriched for leukemia initiating cells (LICs)[11] as well as leukemia cells expressing intermediate-low expression of cKit ($cKit^{Int\text{-}low}$).

A comparison of mitochondrial ROS staining between healthy $CD48^-$ LSK and myeloid progenitors shows that myeloid progenitors display significantly higher levels of mitochondrial ROS staining (**Figure 3A**). Moreover, $cKit^{high}$ leukemia progenitors display significantly higher levels of mitochondrial ROS staining compared to $CD48^-$ LSK, myeloid progenitors or $cKit^{int\text{-}low}$ leukemia cells (**Figure 3A**). $cKit^{Int\text{-}low}$ leukemia cells also displayed significantly higher mitochondrial ROS staining compared to $CD48^-$ LSK cells but not to myeloid progenitors (**Figure 3A**). LSKs further sub-divided by CD150 expression showed that mitochondrial ROS staining did not significantly vary in $CD48^-$ LSK cells sub-divided by CD150 high ($CD150^{High}$), intermediate ($CD150^{Int}$) or no ($CD150^{Neg}$) expression (**Figure 3B**). However, steady-state mitochondrial ROS staining of $cKit^{high}$ or $cKit^{Int\text{-}low}$ leukemia cells was found to be significantly higher than CD48- LSKs-$CD150^{High}$, -$CD150^{Int}$ or -$CD150^{Neg}$ cells (**Figure 3B**). LSK cells expressing low to no levels of CD34 are enriched for long-term reconstituting hematopoietic stem cells, particularly CD48- LSK cells that are $CD34^-$ and $CD150^{high}$ [20,21]. However, subdividing $CD48^-$ LSK cells by CD150 and CD34 expression did not reveal any significant differences in mitochondrial ROS staining amongst these six HSPC subsets (**Figure 3C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of the protocol work-flow. Step 1) BM isolation from healthy and MLL-AF9 leukemic mice; Step 2) cellular staining using a mitochondrial ROS dye (mROS); Step 3) cellular staining with fluorochrome-linked monoclonal antibodies to discriminate hematopoietic stem and progenitor cells (HSPCs) population in healthy and leukemic mice; Step 4) Flow Cytometry acquisition and analysis of mitochondrial ROS in several HSPC populations.

Figure 2: Flow cytometry gating strategies for healthy and MLL-AF9-expressing bone marrow cells. (**A**) BM cells isolated from healthy mice were stained with a live/dead dye (QDot), mitochondrial ROS dye (TRPE). BM from healthy mice was subsequently stained with antibodies recognizing lineage markers plus CD48 (Cy5-PE), c-Kit (Cy7-APC), Sca1 (PacBlue), CD34 (FITC), CD150 (APC). (**B**) In addition to live/dead cell and mitochondrial ROS stains, BM from leukemia mice were also stained with antibodies recognizing lineage markers plus CD48 (Cy5-PE), c-Kit (Cy7-APC), Sca1 (PacBlue) and CD45.2 (APC), which is applied to discriminate between MLL-AF9 leukemia cells from healthy recipient BM cells (CD45.1).

Figure 3: Mitochondrial ROS levels in healthy and MLL-AF9 Bone Marrow. Left panels are representative histograms of mitochondrial ROS levels of the indicated populations and the respective right panels represent bar graph analyses of the MFI of mitochondrial ROS for the indicated populations (n = 4). (**A**) Comparison of mitochondrial ROS levels in healthy $CD48^-$ LSK, myeloid progenitors as well as MLL-AF9 Lin^{low} c-Kit^{High} and MLL-AF9 Lin^{low} c-$Kit^{Int\text{-}Low}$ cells. (**B**) Comparison of mitochondrial ROS levels in healthy $CD48^-$ LSK cells based on their CD150 expression versus MLL-AF9 Lin^{low} c-Kit^{High} and MLL-AF9 Lin^{low} c-$Kit^{Int\text{-}Low}$ cells. **C.** Comparison of mitochondrial ROS levels in healthy $CD48^-$ LSK cells based on their CD150 and CD34 expression. (* $p \leq 0.05$; ** $p < 0,01$*** $p < 0.001$; **** $p < 0.0001$).

Figure 4: Changes in mitochondrial ROS levels using pro- and anti-oxidant compounds. Histograms of mitochondrial ROS levels in healthy and MLL-AF9-expressing BM cells treated with 20 µM of menadione sodium bisulfite (MSB) for 1 h at 37 ˚C in a 5% CO_2 incubator (positive Control) or with 20 µM of MSB in combination with 100 µM N-acetyl-L-cysteine (NAC) for 1 h at 37 ˚C in a 5% CO_2 incubator.

Figure 5: Optimization of the mitochondrial ROS and lineage-recognizing antibody stains. (**A**) Comparison of mitochondrial ROS (mROS) levels in MLL-AF9 expressing leukemia cells using 1 or 5 µM for either 10 or 30 min. (**B**) Comparison of the MFI of the CD34 channel (FITC) in healthy LSKs stained for 20, 60 or 90 min with the antibody cocktail #1 (n=4, * $p \leq 0.05$; ** $p < 0,01$). (**C**) Comparison of antibodies staining before or after incubation for 30 min at 37 ˚C in a 5% CO_2 incubator.

Figure 6: Order of mitochondrial ROS and lineage marker antibody staining. (**A**) Dot plots of the indicated HSPCs populations obtained by using different orders of staining. (**B**) Mitochondrial ROS levels evaluated in the LSK and Myeloid Progenitors compartments obtained used different order of staining (Red = antibody staining for 1 h at 4 ˚C followed by mitochondrial ROS staining for 10 min at 37 ˚C; Blue = mitochondrial ROS staining for 10 min at 37 ˚C followed by antibody staining 1h at 4 ˚C). (**C**) Quantification of the MFI of mitochondrial ROS (mROS) staining using different orders of staining in the indicated populations (n = 4, * $p \leq 0.05$).

Figure 7: Impact of verapamil treatment on mitochondrial ROS dye staining in healthy and MLL-AF9-expressing BM cells. (**A**) Dot plots of the indicated HSPC populations in samples treated with or without 50 µM Verapamil for 10 min at 37 ˚C in a 5% CO_2 incubator. (**B**) Histograms of

mitochondrial ROS and mitoMASS Green dye staining in the indicated HSPC populations in the presence (blue) or absence (red) of 50 µM Verapamil. (**C-E**) Quantification of mitochondrial ROS levels in the indicated healthy (C & D) and leukemia (E) cell populations in BM samples treated with or without 50 µM Verapamil during mitochondrial ROS staining (n = 4, * p≤0.05).

Table 1: Antibody cocktails. List of antibody cocktails prepared in Step 3.1. to identify various hematopoietic sub-populations within healthy and leukemia bone marrow.

DISCUSSION:

Fluorogenic dyes that have been developed for the detection of ROS are frequently evaluated in fixed cells by microscopy or in live cells by flow cytometry[22]. Flow cytometric evaluation of mitochondrial ROS in BM cells using mitochondrial ROS fluorogenic dyes has two major advantages: 1) It is a fast and simple technique that is suitable for live cell analysis and 2) it allows for distinguishing and analyzing rare populations at the single-cell level in the BM using surface marker staining. The step-by-step protocol presented here has been developed to study the *ex vivo* redox status of hematopoietic stem and progenitor populations from both healthy mice as well as a mouse model of AML driven by MLL-AF9 using flow cytometry. There are several key technical variables that need to be considered during the execution of this protocol.

First, the use of pro- and anti-oxidant controls allows the user to establish a baseline for increases in mitochondrial ROS staining as well as the specificity of the stain. For the protocol presented here, the pro-oxidant MSB was utilized as a positive control for a detectable induction of mitochondrial ROS staining in both healthy and leukemia BM cells (**Figure 4**). The anti-oxidant NAC can be used as an additional control, as it largely reverses the mitochondrial ROS staining induced by MSB (**Figure 4**).

Second, the mitochondrial ROS fluorogenic dye employed in this study is recommended to be used at a concentration of 5 µM for 10 min. However, the manufacturer also suggests that the concentration and time of staining may vary between cell types. In this study, mitochondrial ROS staining was compared at both 1 µM and 5 µM for either 10 or 30 min. The analysis revealed that a concentration of 5 µM for either 10 to 30 min is sufficient to detect MSB-mediated changes in mitochondrial ROS as well as those reversed by NAC treatment (**Figure 5A**). Since there was no quantitative difference between 10 and 30 min, a staining time of 10 min was selected for this study to minimize the length of the assay.

Third, CD34 antibody incubation times vary within the literature from 20 to 90 min[23,24]. To optimize the protocol presented here, murine bone marrow cells were incubated with the antibody cocktail #1 (Step 3.1) for 20, 60 or 90 min. As shown in **Figure 5B**, significantly stronger CD34 staining was observed on cells stained for 60 or 90 min compared with the 20 min stain. However, a significant difference in CD34 staining was not observed between antibody incubation times of 60 and 90 min (**Figure 5B**) and thus a 60 min antibody stain is recommended for the presented protocol.

Fourth, in the presented protocol, both healthy and leukemia cells are first stained with the mitochondrial ROS fluorogenic dye, washed and then stained with fluorochrome-linked lineage antibodies. Although a direct assessment of combining the mitochondrial ROS stain with lineage antibodies was not conducted in this study, an evaluation of fluorochrome-linked lineage antibody staining was assayed under mitochondrial ROS staining conditions for 10, 20 and 30 min at 37 °C. This analysis revealed that 30 min of incubation at 37 °C substantially alters lineage surface marker staining (**Figure 5C**). Additionally, mitochondrial ROS staining was evaluated by first, staining healthy BM cells with fluorochrome-linked lineage antibodies followed by staining with the mitochondrial ROS fluorogenic dye as well as *vice versa* order of operations. Staining cells first with lineage antibodies followed by mitochondrial ROS staining resulted in lower mitochondrial ROS signals compared to the *vice versa* staining protocol (**Figure 6A,B**) – including significant differences for $CD48^{-}$ LSK $CD150^{high}$, $CD48^{-}$ LSK $CD150^{Int}$ CD34 and myeloid progenitor populations (**Figure 6C**).

Fifth, recent studies show that different murine healthy HSPC populations possess distinct abilities to efflux several mitochondrial staining fluorogenic probes such as those used to assess mitochondrial mass (hereafter referred to as mitoMASS green) or potential[25,26]. Therefore, the impact of the efflux pump inhibitor verapamil on the staining of healthy and leukemia progenitors with the mitochondrial ROS fluorogenic dye was also assessed. Simultaneous staining of HSPCs with mitochondrial ROS did not alter lineage marker antibody staining (**Figure 7A**), however, verapamil did significantly improve mitochondrial ROS staining signals in a variety of healthy and leukemia progenitor populations (**Figure 7B,C**). Notably, the magnitude of improved mitochondrial ROS staining by verapamil was not as great as that seen with the mitoMASS green fluorogenic dye (**Figure 7B**).

Sixth, in the protocol presented here, BM cells were recovered by removing the bone tips (epiphysis) followed by flushing of the bones. However, the epiphysis contains hematopoietic cells that could potentially be lost by bone flushing. As an alternative, BM cells can be extracted by mashing bones with a mortar and pestle as previously described[12,13].

The protocol presented here provides a foundation for the use of fluorogenic dyes to measure intracellular redox biology in live cells extracted from living organisms. However, it is important to note that no single fluorogenic dye can be assumed to be definitively specific and therefore additional studies with alternative methods should be conducted to verify any findings. Furthermore, the results of this study suggest that leukemia cell populations enriched for LICs (i.e., Lin^{low} $cKit^{high}$) display higher levels of mitochondrial ROS than healthy myeloid progenitors or other HSPC populations. However, the Lin^{low} $cKit^{high}$ leukemia cell population evaluated here has been shown to be heterogeneous and can be further sub-divided by other lineage markers[11,27]. Furthermore, recent studies show that LICs possess a distinct metabolic phenotype[28]. Therefore, future studies assessing mitochondrial ROS in parallel with metabolic assays or probes as well as additional lineage marker antibodies will be informative.

This straightforward protocol allows for the measurement of mitochondrial ROS levels in living hematopoietic cells and may provide a basis for studying the redox biology of healthy and

diseased stem and progenitor cells as well as for assessing the effectiveness of redox-targeting therapies.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

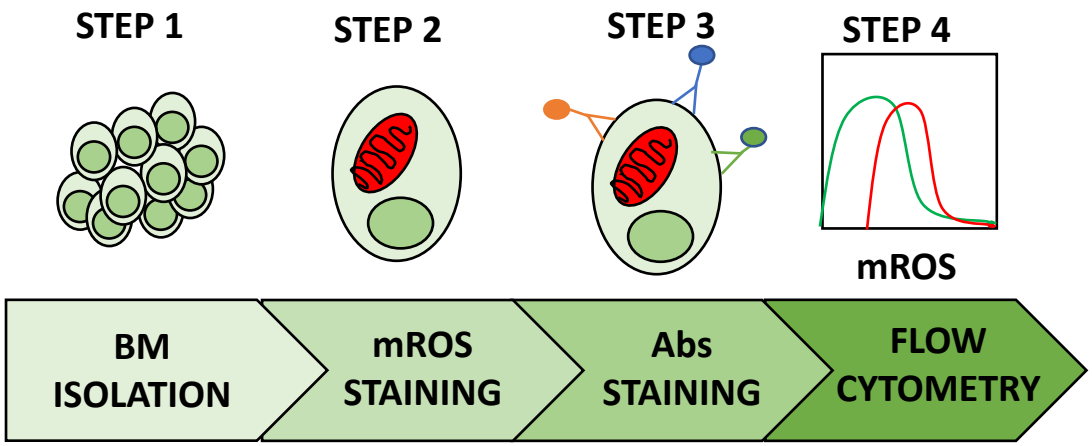


Figure 2

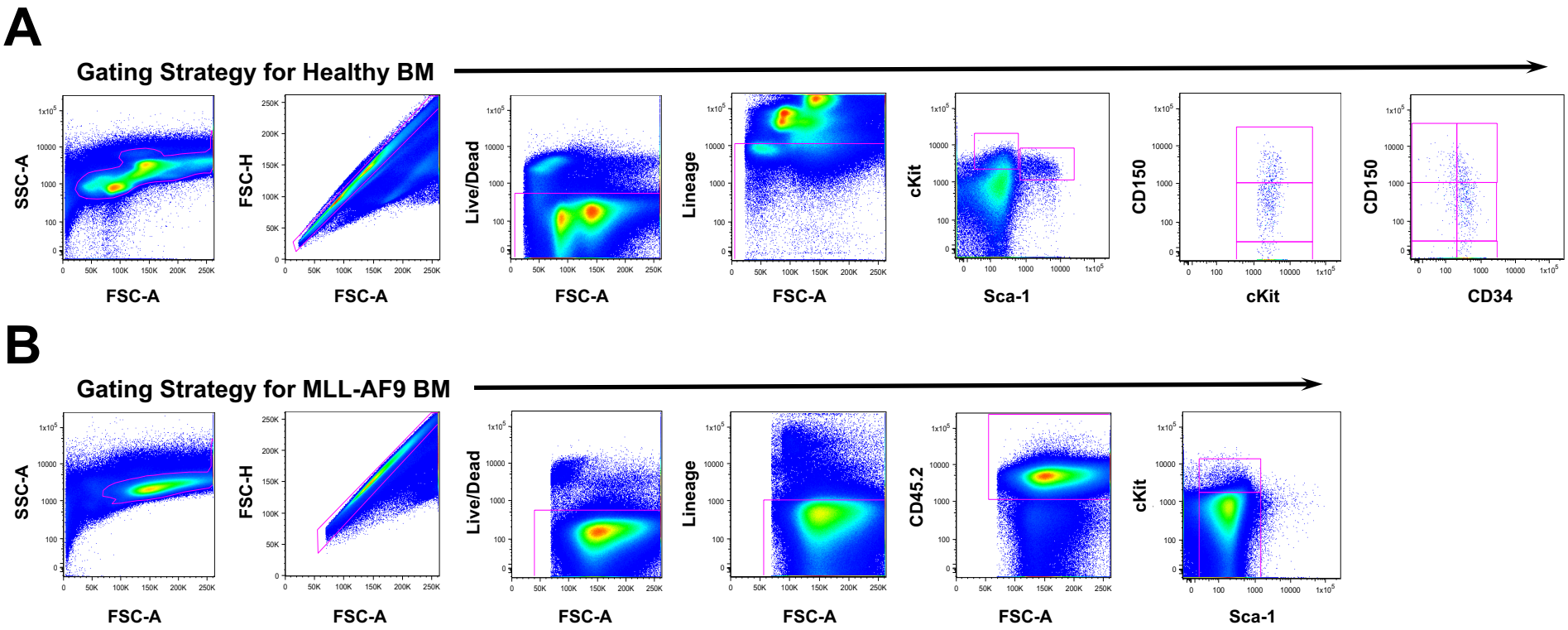


Figure 3

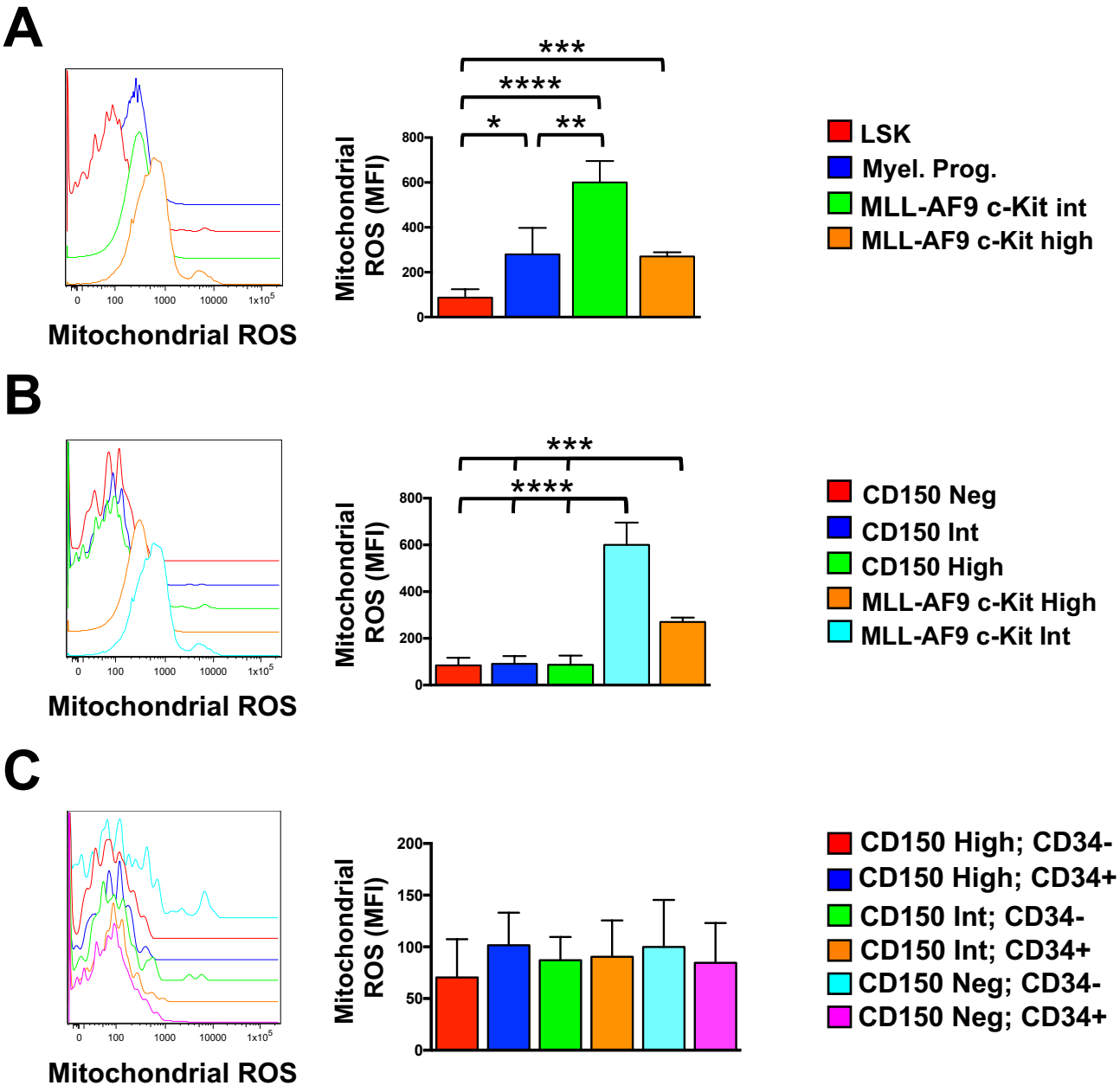


Figure 4

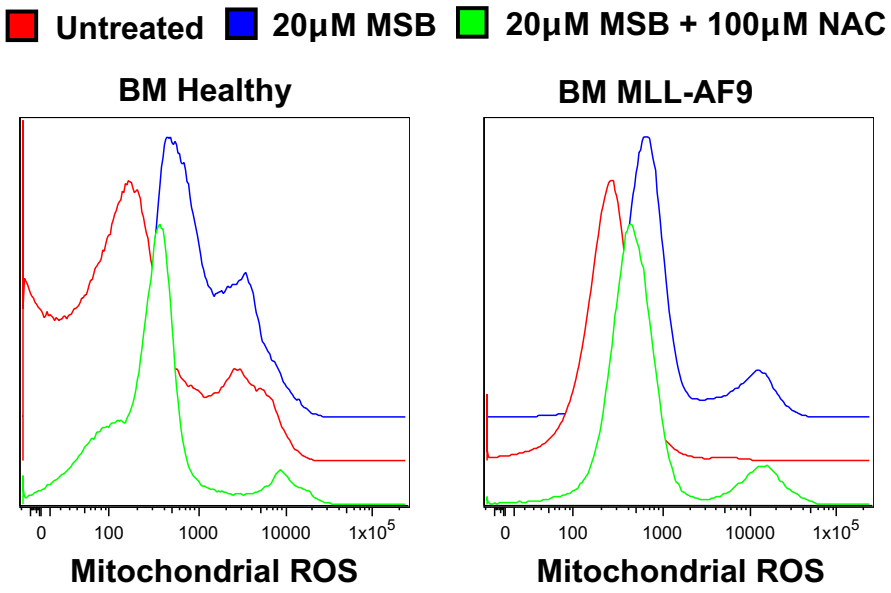


Figure 5

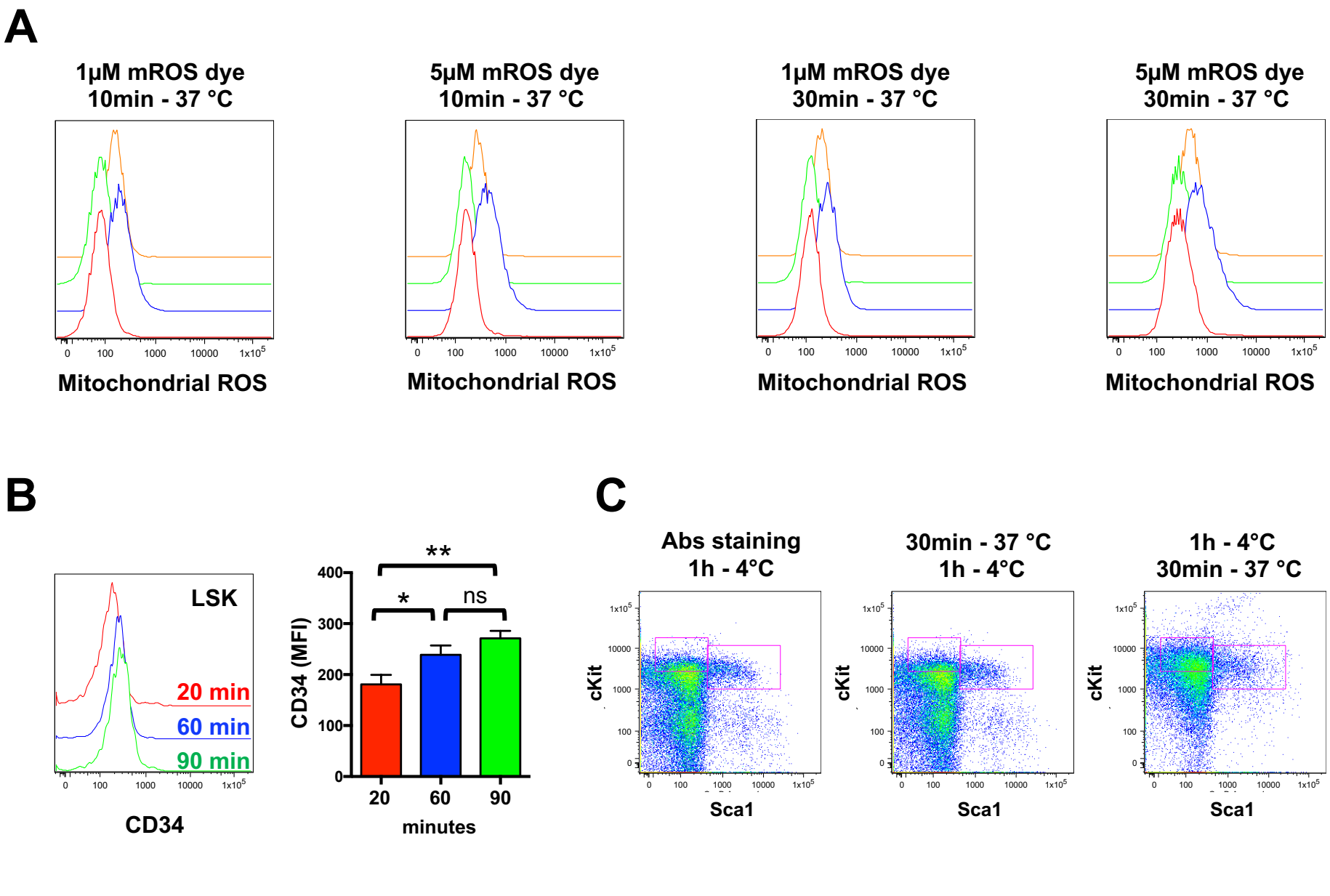


Figure 6

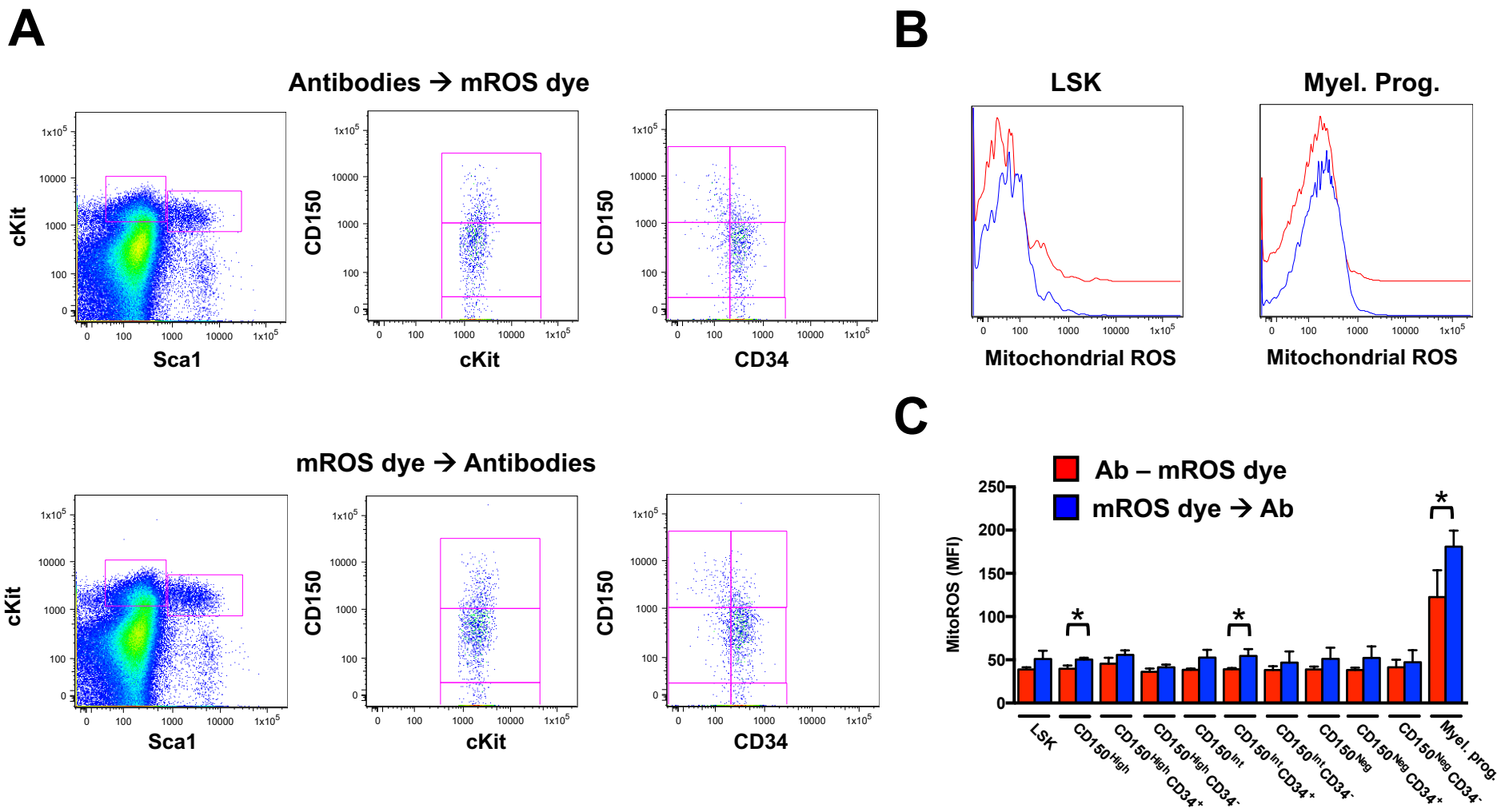
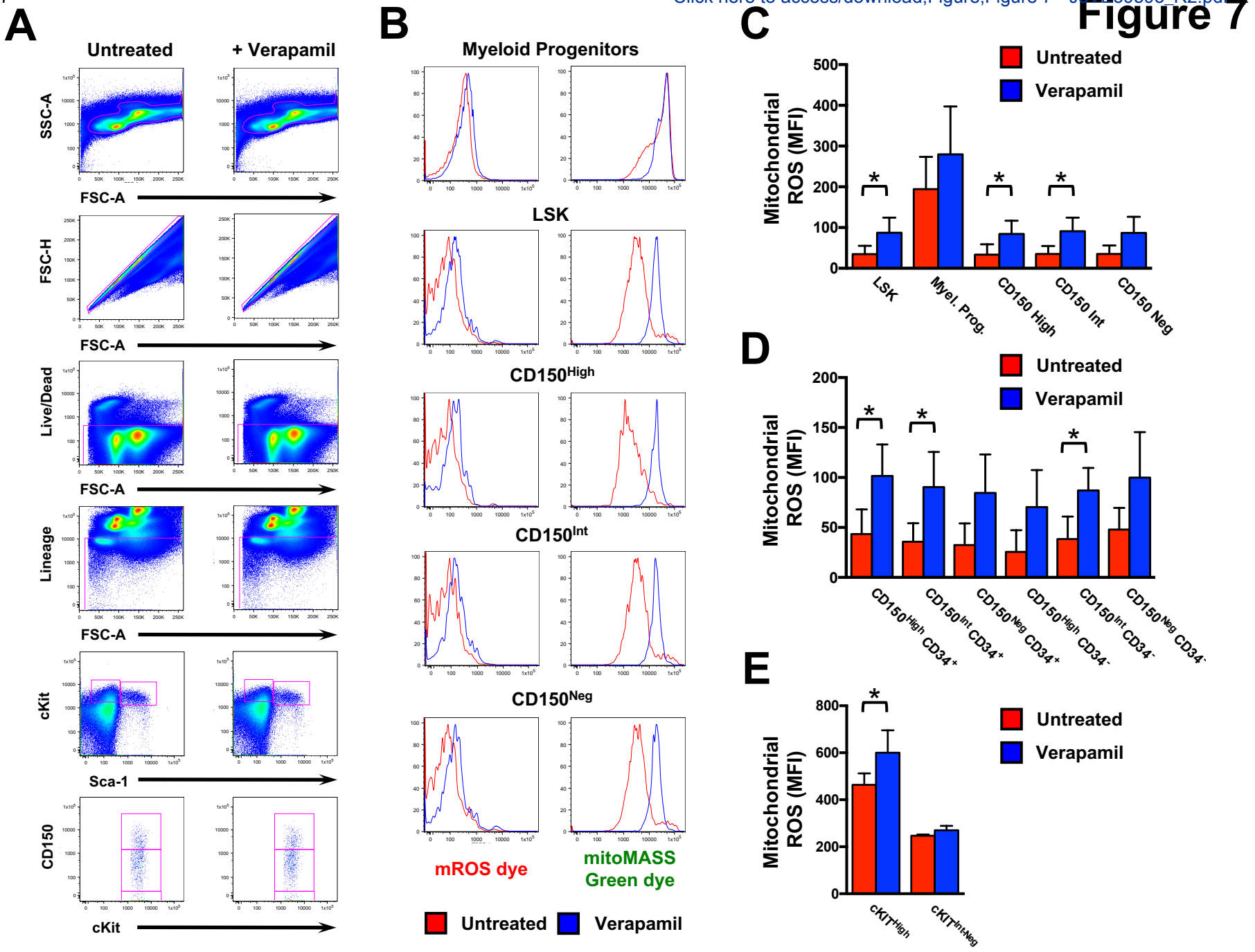


Figure 7



Antibody cocktail #1 (for healthy mice)	
Antibody	**µL/mouse**
CD3-Cy5-PE	5
CD4-Cy5-PE	2
CD8-Cy5-PE	2
CD19-Cy5-PE	2
B220-Cy5-PE	2
Gr1-Cy5-PE	2
CD48-Cy5-PE	2
Ter119-Cy5-PE	0.5
cKit (CD117)-Cy7-APC	2
Sca-1-PacBlue	1
CD150-APC	2
CD34-FITC	2.5
F-PBS	175

Antibody cocktail #2 (for leukemia mice)	
Antibody	**µL/mouse**
CD3-Cy5-PE	5
CD4-Cy5-PE	2
CD8-Cy5-PE	2
CD19-Cy5-PE	2
B220-Cy5-PE	2
CD48-Cy5-PE	2
Ter119-Cy5-PE	0.5
CD45.2-APC	2
cKit (CD117)-Cy7-APC	2
Sca-1-PacBlue	1
F-PBS	179.5

Name of Material/ Equipment	Company
Heat inactivated FBS	VWR Seradigm LIFE SCIENCE
Penicillin Streptomycin	Corning
PBS	Fisher Scientific
15 mL conical tube	BD falcon
50 mL conical tube	BD falcon
40 µm cell strainers	Fisher Scientific
RBC Lysis Buffer	Fisher Scientific
Menadione sodium Bisulfite	Sigma aldrich
NAC	Sigma aldrich
CD3 PE-Cy5 clone 145-2c11	Biolegend
CD4 PE-Cy5 clone RM4-5	eBioscience
CD8 PE-Cy5 clone 53-6.7	eBioscience
CD19 PE-Cy5 clone 6D5	Biolegend
B220 PE-Cy5 clone RA3-6B2	Biolegend
Gr1 PE-Cy5 clone RB6-8C5	Biolegend
Ter119 PE-Cy5 clone Ter-119	Biolegend
CD48 PE-Cy5 clone HM48-1	Biolegend
CD117 APC-Cy7 clone 2B8	Biolegend
Sca1 peacific Blue clone D7	Biolegend
CD150 APC clone TC15-12F12.2	Biolegend
CD34 FITC clone RAM34	BD Bioscience
CD45.2 APC clone 104	Biolegend

MitoSOX Red	ThermoFisher Scientific
Mitotracker Green	ThermoFisher Scientific
Live/dead Yellow Dye	ThermoFisher Scientific

Catalog Number	Comments/Description
97068-085	Media
30-002-CI	Media
BP399-20	Buffer
352096	Tissue Culture Supplies
352098	Tissue Culture Supplies
22-363-547	Tissue Culture Supplies
50-112-9751	Tissue Culture Supplies
M5750	Pro-oxidant
A7250	Anti-oxidant
100310	Antibody
15-0041-81	Antibody
15-0081-81	Antibody
115510	Antibody
103210	Antibody
108410	Antibody
116210	Antibody
103420	Antibody
105825	Antibody
108120	Antibody
115909	Antibody
553733	Antibody
1098313	Antibody

M36008	Dye
M7514	Dye
L34967	Dye

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Author(s): Daniela Di Marcantonio and Stephen M. Sykes

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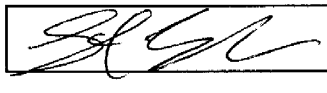
Signature:	
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Point-by-point response to Reviewer Comments: We thank the reviewers for their appreciation of our work and for their insightful comments. We have addressed each of the reviewer's concerns and critiques in the point-by-point rebuttal below. These suggestions have been very helpful and have resulted in a much-improved and stronger manuscript. The original comments of the reviewers are in blue, responses in black.

Reviewer #1:

1. As mentioned by the authors, the most important procedure in the manuscript must be a reproducible method of "two-step" MitoSOX staining process. In some study, ROS levels in HSC are assessed Vice Versa or even after its sorting (Mantel CR et al., Cell, 2015, PMID: 26073944). Authors must explain in Introduction why two-step process is needed, and discuss more extensively why this sequential order, but not Vice Versa, is important in Discussion.

Response: Given the additional suggestions of all Reviewers, we have introduced several changes to the original protocol. Due to these changes and reassessments, we have actually eliminated the "critical" need to perform MitoSOX staining prior to antibody staining. The specific observations that contributed to the change in our statements are as follows:

1. First, Reviewer #2 recommended that we incorporate a CD34 antibody to further delineate LSK, CD150 populations. Because staining times for the anti-mouse CD34 vary greatly within the literature from 20 to 90 minutes (refs 23 & 24 of the revised manuscript), we first tested three anti-CD34 staining incubation times: 20, 60 and 90 minutes. From this analysis, we found that both 60 and 90 minutes provided a significantly stronger signal than 20 minutes. However, we did not observe a significant difference in staining between 60 and 90 minutes. These results are presented in Figure 5B of the revised manuscript. Based on these observations, we employed a 60 minute incubation time for antibody staining for all analyses presented in this study.
2. Second, although we have not directly incubated cells with MitoSOX and antibodies simultaneously, we have observed that incubating antibody-stained cells for 30 minutes or longer at 37 degrees centigrade in MitoSOX staining buffer substantially diminishes antibody staining. These results are presented in Figure 5C of the revised manuscript. Given that our revised antibody staining time is 60 minutes, we deduced that simultaneous staining of the antibodies with MitoSOX would not be ideal.
3. Third, since our revised staining protocol we revisited the order of antibody-MitoSOX stains. As we show in Figure 6 of the revised manuscript, we do observe a slight qualitative increase in MitoSOX staining if we stain bone marrow cells with MitoSOX followed by surface antibodies rather than vice versa – this difference is also statistically significant in certain populations (Figure 6C). Based on these cumulative observations, we have revised the language of the manuscript.

2. Preparation of cell Suspension from murine bone marrow was already published in JoVE several years ago (Frascoli M et al., PMID: 22805770). Authors should focus on or emphasize their unique point.

Response: Thank you for the suggestion. We have condensed the protocol by removing the procedural steps of the bone marrow harvest with a direct reference to Frascoli et al., PMID: 22805770. The changes are located in lines 86-89 of the revised manuscript.

3. Many of researchers in the field may not be familiar with CD48-CasB (Line 117).

Response: Our apologies for the typo – that should have been PacBlue not CasB. These mistakes have been corrected.

4. Please add NOTE for this antibody. One of the interesting tips in the staining is the different dosage of the antibody. CD3:CD4:Ter119:CD48=5:2:0.5:1. It is suggested to add a comment for this tip.

Response: The antibody staining conditions (e.g. volumes, concentrations and ratios) were developed by Demetrios Kalaitzidis (Kalaitzidis and Neel, PMID: 19020663 and Kalaitzidis et al., PMID: 22958934). The references are cited in a NOTE on lines 157 of the revised manuscript.

5. Fig. 1B: As shown in the panel, MLL-AF9 leukemic cells must specifically be enriched in c-Kit+ Sca-1- fractions. But, this enrichment is not striking though a significant change is found in FS-A/SS-A can be found. Is this because these mice are at the early stage of leukemia? Perhaps, MLL-AF9 BM from later stage should be better representation?

Response: We thank the Reviewer for the suggestion. The MLL-AF9 leukemia cells used in these studies were generated in the C57.Bl6 strain and therefore express the CD45.2 congenic marker and subsequently transplanted into the C57.Bl6-BoyJ strain (from Jackson laboratories), which express the CD45.1 congenic marker. We have now repeated our analyses with an antibody that specifically recognizes the CD45.2 version of mouse CD45 to distinguish leukemia cells from healthy recipient cells. Additionally, per the Reviewer's suggestion, we also repeated the central results with mice that had frank leukemia. These revised results are presented in Figure 2 of the revised manuscript.

6. Fig. 3: Positive and negative control for MitoSOX should be included. The MitoSox levels of other fractions (c-Kit+Sca1+, and MPPs) from MLL-AF9 mice should also be shown as control, especially as non-AML cells can be found in those mice.

Response: We thank the Reviewer for this very important suggestion and in response, we have included both a positive control (treatment of healthy or leukemia BM with the pro-oxidant Menadione Sodium Bisulfite or MSB) as well as an additional control for reversing ROS-induced changes in MitoSOX signal (the anti-oxidant N-acetyl-L-cysteine or NAC in combination with MSB). The results can be found in Figure 4 of the revised manuscript. After

repeating our analyses, the vast majority of healthy cells were undetectable in leukemia mice due to the high disease burden as seen in the scatter plots presented in Figure 2.

Minor point:
Line 75: "mice" should be removed.

Response: Our apologies for this error, which has now been corrected.

Line 174: Abbreviation for Forward scatter is typically FSC (Line 174).

Response: Again, our apologies for the error, which has now been corrected throughout the manuscript and figures.

Line 181: When a term "LT-HSC" is used, a comparison must be "ST-HSC". Authors are recommended to use "HSC" or the exact surface markers (Line 181).

Response: In response to this helpful suggestion, we have removed references to various HSC populations as different labs utilize different surface markers for defining HSPC populations. We now refer to various HSPC populations based on their surface marker composition.

Reviewer #2:

1. The visable protocol optimisations provided in this study can be summarized into the following points:
1: The sequential staining order of MitoSOX followed by fluorescent antibodies is important.
2: The authors optimise the MitoROX staining time in the MLL-AF9 cells to 10 minutes. This is in fact the same time specified by the company for microscopy staining with MitoSOX. However, clearly great variation in optimal staining times have been reported for different cell types so it is difficult to say if this is a shortening of time. Antibody staining time was reduced from the standard 30mins-1hr to 20 minutes.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2225540/pdf/nihms38100.pdf
3: The authors also carry out antibody stainings at room temp as opposed to 4 degrees or on ice. The authors therefore report a total shortening of the staining protocol from approximately 1 hr to 30 minutes.
a. On lines 236-240, the authors state that the sequential staining of ROS followed by antibody staining as a critical consideration but it is not clear why. Have they also tested antibody staining for 30 mins @ 4 degrees followed by 30 mins/10 mins of MitoSOX staining at 37 degrees? Have they also tested adding the antibodies to the MitoSOX stain buffer and staining together for 30mins - 1hr at 37 degrees? How much of a difference does this overall shortening of the stain protocol from 1hr to 30 minutes make?

Response: We thank the Reviewer for these important questions. Based on combined suggestions of all Reviewers, we have modified the original protocol, such as adding:

1. A marker to distinguish leukemia cells from healthy cells (Reviewer #1)

2. A CD34 stain (Reviewer #2)
3. A Live/dead stain (Reviewer #3)
4. Verapamil to block any efflux pumps (Reviewers #2 & 4)

Therefore, we have incorporated these additional invaluable steps in addressing each of the Reviewer's queries below:

1. Because staining times for the anti-mouse CD34 vary greatly within the literature from 20 to 90 minutes (refs 23 & 24 of the revised manuscript), we first tested three anti-CD34 staining incubation times: 20, 60 and 90 minutes. From this analysis, we found that both 60 and 90 minutes provided a significantly stronger signal than 20 minutes. However, we did not observe a significant difference in staining between 60 and 90 minutes. These results are presented in Figure 5B of the revised manuscript. Based on these observations, we employed a 60 minute incubation time for antibody staining for all analyses presented in this study.
2. For optimization of MitoSOX concentration and time, we tested either 1uM or 5uM of MitoSOX either for 10 or 30 minutes at 37 degrees centigrade. We found that 1uM of MitoSOX (regardless of incubation time), yielded a low MFI in cells challenged with the pro-oxidant menadione sodium bisulfite (MSB), however, this induction was not altered by the addition of the anti-oxidant N-aceytl-L-Cysteine (NAC). In contrast, we found that at 5uM, MitoSOX signals increased in cells exposed to MSB and this induction was partially reversible by NAC. However, we did not observe a significant difference in MitoSOX signal between and 10 minute and 30 minutes incubation times. These results are presented in Figure 5A of the revised manuscript. Based on these findings, we employed the shorter incubation time to obtain a sufficient MitoSOX signal in the shortest amount of time.
3. While we have not directly incubated cells with MitoSOX and antibodies simultaneously, we have observed that incubating antibody-stained cells for 30 minutes or longer at 37 degrees centigrade in MitoSOX staining buffer substantially diminishes antibody staining. These results are presented in Figure 5C and discussed in lines 336-340 of the revised manuscript. Given that our revised antibody staining time is 60 minutes, we deduced that simultaneous staining of the antibodies with MitoSOX would not be ideal.
4. Lastly, since our revised staining protocol includes a live/dead cell stain, a mouse CD34 antibody and verapamil, we revisited the order of antibody-MitoSOX stains. As we show in Figure 6 of the revised manuscript, we do observe a slight qualitative increase in MitoSOX staining if we stain bone marrow cells with MitoSOX followed by surface antibodies rather than vice versa – this difference is also statistically significant in certain populations (Figure 6C). Based on these observations, we have revised the language of the manuscript.

b. On lines 242 - 244 the authors state that ideal durations and concentrations of MitoSOX should be optimized for different tissues/cells. The authors could add this data from their murine HSPC/AML cells to a supplemental figure.

Response: Per the request of the Reviewer, we have included the data optimizing the time and concentration of MitoSOX in MLL-AF9 progenitors as Figure 5A of the revised manuscript.

2. Are the authors concerned however by the recent reports that the cationic MitoTracker dyes (also provided by ThermoFischer) and TMRM are effluxed through drug efflux pumps, resulting in a reversal of views presented in the field as to the mitochondrial mass and activity differences between HSPC populations. Have the authors tested to see if MitoSOX (also a cationic dye) is effluxed from the cells. One could imagine that HSCs having high expression of these efflux pumps therefore efflux out MitoSOX to a higher extent than other HSPC or AML populations, compromising the accuracy of the data.
https://doi.org/10.1016/j.stem.2017.11.002
https://doi.org/10.1016/j.exphem.2018.10.012

Response: We thank the Reviewer for this excellent suggestion, which was also raised by Reviewer 4. In response, we have repeated our staining analyses of both healthy and leukemia mice in the presence or absence of verapamil. As a positive control for verapamil (based on the de Almeida et al., 2017), we also assessed how verapamil impacted Mitotracker Green staining in various hematopoietic stem and progenitor cell (HSPC) populations. Consistent with published studies, Verapamil treatment enhanced Mitotracker Green staining in various HSPC populations: Greater than 10-fold in healthy CD150 High-expressing lineage low, cKit+, Sca-1+ (LSK) cells, 5-fold in LSK cells. However, verapamil only mildly increased (~1.3-fold) Mitotracker Green staining in healthy myeloid progenitors. We also found that verapamil treatment strengthened the MitoSOX median fluorescence intensity (MFI) signal in LSK cells (~2.5-fold increase) and various LSK subsets divided by CD150 and CD34 staining (~2.0-2.6-fold increase). Whereas verapamil mildly increased the MitoSOX MFI in healthy myeloid progenitors (1.4-fold increase) and leukemia progenitors (1.3-fold). Given that verapamil strengthened the MitoSOX signal, we have presented the data comparing MitoSOX signal in leukemia progenitors versus various healthy HSPC with verapamil in Figure 3. Additionally, we have included the data comparing various healthy and leukemia cell populations with and without verapamil in Figure 7 and discussed the introduction of verapamil in lines 137-142 and 347-356 of the revised manuscript. We have also cited the above referenced manuscripts.

3. Also, in a recent paper by Hao et al, using a genetically encoded NADH/NAD+ reporter called "SONAR" in a murine MLL-AF9 AML model, the authors identify metabolic heterogeneity amongst the bulk MLL-AF9 population which can be related back to leukaemia initiating potential, tendency to perform symmetric vs asymmetric divisions, homing capacity and localization within different niches. The authors nicely show that MLL-AF9 SONAR high cells (glycolytic) are highly enriched for leukaemia initiating cells, that these cells have a preference for symmetric divisions, home better and engraft in endosteal niches. In contrast the SONAR mid and low cells (higher in TCA/respiration activity) have poor leukaemia initiating capacity and home to a lesser degree. While here a higher level of MitoROS in MLL-AF9 progenitors would agree with the Hao paper that the non-leukaemia initiating progenitors would have higher levels or respiration, I worry that the authors here have underestimated the heterogeneity within their MLL-AF9 cells as seen by Hao et al, also ignoring the potential for aberrant C-KIT

expression during transformation and have failed to functionally test whether their stated MLL-AF9 progenitors are indeed the progenitors, not LICs or a mixed bag. Have these sorted populations from murine MLL-AF9 mice been tested functionally by colony assays, LTC-ICs or in vivo engraftment studies? Vannini et al., showed using the mitochondrial membrane potential dye TMRM, that even within FACs phenotypically defined LT-HSC, based on TMRM levels these can be functionally divided into LT-engrafting and non-engrafting HSC. Therefore, to functionally determine/confirm the identity of these cells would be important. Could the authors also perform a mitochondrial activity stain or a seahorse to support the idea that these cells have higher levels of respiration? Have the authors checked that the sorted cells indeed have an MLL-AF9 mutation? Would an MLL-AF9 model with a fluorescent reporter not provide better assurance that MLL-AF9 positive cells have indeed been gated for?

https://doi.org/10.1016/j.cmet.2018.11.013
https://dx.doi.org/10.1038%2Fncomms13125

Response: First, based on the Reviewer's suggestion as well as that of Reviewer #1, we have added an additional antibody to our staining protocol to specifically gate on leukemia cells. The MLL-AF9 leukemia cells used in these studies were generated in the C57.Bl6 strain and therefore express the CD45.2 congenic marker and subsequently transplanted into the C57.Bl6-BoyJ strain (from Jackson laboratories), which express the CD45.1 congenic marker. We have now repeated our analyses with an antibody that specifically recognizes the CD45.2 version of mouse CD45 to distinguish leukemia cells from healthy recipient cells. These revised results are presented in Figure 2 of the revised manuscript.

Second, all of the additional questions raised by the Reviewer are insightful, very interesting and experiments we are in the process of carrying out. While these studies are beyond the scope of this method-focused paper, we have addressed some of these possibilities in the discussion (lines 361-372) in order to convey that the cKit high population of the MLL-AF9 model is a heterogeneous and further studies are needed to determine the functionality of leukemia cells with distinct MitoSOX signals.

4. Have the results been independently tested by another method of mitochondrial ROS quantification? Does another exist? Can this be done with electron resonance spectroscopy?

Response: We are in the process of utilizing independent assays to determine the redox potential of different organelles in distinct healthy and malignant populations, however, we believe that these analyses are beyond the scope of this method-focused paper.

5. On lines 245-246 the authors state "This protocol can also be modified to use alternative ROS-targeting fluorogenic dyes for measuring redox status in healthy and malignant hematopoietic cells." Have they indeed tested these optimisations on other ROS dyes such as CellROX or also for dyes such as TMRM, JC-1/9 or MitoTracker?

Response: We have indeed validated this protocol using other dyes. As shown in Figure 7B, we have used to similar strategy to stain various healthy populations with Mitotracker Green.

Minor points:
1. Fig 2A- Replace SLAM marker contour plot with a scatter plot.

Response: We have replaced all contour plots throughout the manuscript with scatter plots.

2: In Fig 3A and on lines 201-202, CD48- CD150+ cells are referred to LT-HSC. Can these cells not be better viewed as bulk HSC, with a CD34 stain further delineating LT-HSC from ST-HSC?

Response: We thank the reviewer for another excellent suggestion. First, we have elected, in part based on the suggestion of Reviewer #1, to refer all HSPC population based on their surface marker expression rather than labels such as LT-HSCs. Second, per the recommendation, we have included CD34 staining to further delineate the LSK, CD150 populations. These data have been included in Figures 2 and 5B of the revised manuscript.

3: In Fig3 would be good to see the scatter plots for the ROS stains to see what the clouds look like.

Response: We have inserted text (lines 196-198) recommending that users can analyze MitoSOX levels in scatter plots versus various lineage markers.

4: The authors use menadione sodium bisulfite as a positive control to induce ROS. Have they also tried using NAC as a negative control. Should this not be proposed in the article as a good second control to bring along?

Response: We thank the Reviewer for this very important suggestion, which was also raised by Reviewer #1. In response, we have included both a positive control (treatment of healthy or leukemia BM with the pro-oxidant Menadione Sodium Bisulfite or MSB) as well as an additional control for reversing ROS-induced changes in MitoSOX signal (the anti-oxidant N-acetyl-L-cysteine or NAC in combination with MSB). The results can be found in Figure 4 of the revised manuscript.

Reviewer #3:

1. Flushing rather than crushing the bones to release the marrow is by no means the standard in the field. There are differences in the microenvironment between the epiphysis and the diaphysis. By cutting the epiphysis and flushing out the remaining marrow, HSCs and leukemic cells from one particular setting will be overlooked. Since the oxidative state of cells can be influenced by their surroundings, it seems important to analyze all cells and not bias oneself to a particular microenvironment. If crushing using a mortar and pestle is considered too harsh, a gentler approach is to crush the bones in a petri dish using the blunt end of a 50mL conical tube.

Response: We absolutely agree with the Reviewer that alternative methods for extracting bone marrow could reveal differences in MitoSOX staining depending on the location of healthy or leukemia hematopoietic stem and progenitors in the bone. Therefore, we have included this point in the protocol (lines 91-93) and discussion (lines 357-360) sections of the revised manuscript.

2. Figure 2, demonstrating the gating strategy does not show a viability dye. It is mentioned in the methods sections that one can be added. As inclusion of dead cells would significantly increase the risk of introducing artifacts in the analysis, the use of a viability dye should be strongly recommended, and the results should be demonstrated in the gating strategy.

Response: We thank the reviewer for this suggestion and we have now included a live/dead cell stain in all of our presented findings. Additionally, we have removed the live/dead staining as an additional note and included it as a mandatory component of the staining protocol.

Reviewer #4:

My only concern is that they use mitosox dye to compare mitochondrial ROS between populations of HSC and progenitors and no inhibitor of efflux pumps. This because it has been recently been shown that Mitotracker green (similar to Mitosox) which is used to measure mitochondrial content, undergoes efflux from HSCs, leading to artifactually low fluorescence, that is unless inhibitors to efflux pumps are used 1. It is a very simple experiment and would be worth trying to inhibit efflux pumps using - verapamil in combination with mitosox

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):725-729.e724.

Response: We thank the Reviewer for this excellent suggestion, which was also made by Reviewer 2. In response, we have repeated our staining analyses of both healthy and leukemia mice in the presence or absence of verapamil. As a positive control for verapamil based on the referenced citation, we also assessed how verapamil impacted Mitotracker Green staining in various hematopoietic stem and progenitor cell (HSPC) populations. Consistent with published studies Verapamil treatment enhanced Mitotracker Green staining in various HSPC population: Greater than 10-fold in healthy CD150 High-expressing lineage low, cKit+, Sca-1+ (LSK) cells, 5-fold in LSK cells. However, verapamil only mildly increased (~1.3-fold) Mitotracker Green staining in healthy myeloid progenitors. We also found that verapamil treatment, strengthened the MitoSOX median fluorescence intensity (MFI) signal in LSK (~2.5-fold increase) and various LSK subsets divided by CD150 and CD34 staining (~2.0-2.6-fold increase). Whereas verapamil mildly increased the MitoSOX MFI in healthy myeloid progenitors (1.4-fold increase) and leukemia progenitors (1.3-fold). Given that verapamil strengthened the MitoSOX signal, we have presented the data comparing MitoSOX signal in leukemia progenitors versus various healthy HSPC with verapamil in Figure 3. Additionally, we have included the data comparing various healthy and leukemia cell populations with and without verapamil in Figure 7 and discussed the introduction of verapamil in lines 137-142 and 347-356 of the revised manuscript. We have also cited the above referenced manuscript as well as Bonora et al., PMID: 30395909.

Point-by-point response to Editorial Comments: We thank the editorial team for their comments. We have addressed each of the editorial comments in the point-by-point rebuttal below. The original comments are in blue, responses in black.

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

Response: The revised manuscript has been proofread thoroughly.

2. 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]."

Response: Not applicable.

3. JoVE cannot publish manuscripts containing commercial language. This includes company names before an instrument or reagent. 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. Examples of commercial language in your manuscript include MitoSOX, etc.

Response: We edited the revised manuscript to remove all commercial names including MitoSOX, which has been replaced with the term "mitoROS dye" or "mitoROS fluorogenic dye"

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

Response: We have revised the text to remove the use of any personal pronouns throughout the entire manuscript

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
(line 107-115)

Response: All bullets and dashes have been removed from the manuscript and the revised manuscript now adheres to the numbering recommended in the JoVE instructions for authors.

6. Figure 1: Please add a short description of the figure in Figure Legend.

Response: A Figure Legend has been added to Figure 1 on lines 241-246 of the revised manuscript.

7. Please do not abbreviate journal titles for all references.

Response: All journal titles have their full names in the references in the revised manuscript.

8. Please do not highlight any steps describing euthanasia or anesthesia.

Response: Based on the suggestions of Reviewer #1 and #3, we have replaced the protocol steps describing the recovery of bone marrow from mice with references 12-15, and therefore, we no longer have any steps throughout the manuscript that refer to euthanasia or anesthesia.

9. 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

Response: Based on the recommendations of the Reviewers, the discussion has been extensively revised and includes details regarding the above mentioned a) – e) points.

JoVE Submission JoVE59593R2

Below is our responses to the Editorial requests made for JoVE Submission 59593_R2

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

Response: We have utilized Microsoft Word Spelling and Grammar check to check grammar and spelling. We have also had three separate individuals review the manuscript for spelling and grammar errors. All changes have been incorporated and can be found under track changes.

2. 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. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Response: The Table associated with Step 3.1. has been removed from the revised manuscript and has been included as a excel file called Table 1. We have included a description of Table 1 in the representative results section of the revised manuscript.

3. Please use h, min, s for time units.

Response: The requested changes have been made and the locations of these changes can be found as track changes.

4. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. 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. Examples of commercial language in your manuscript include mitoROS, etc.

Response: Our apologies for the oversight. Throughout the text of the manuscript, we have replaced "mitoROS" with "mitochondrial ROS". Locations of these changes can be found as track changes. Additionally, "mitoROS" has been replaced with mitochondrial ROS in the Figures, with the exception of Figure 1, 5A (top labels), 6A & C and 7A, where "mitoROS" has been replaced with "mROS".

5. Step 1.1: Please write this step in the imperative tense.

Response: Step 1.1 has been corrected to now be in the imperative tense.

6. For references, if there are more than 6 authors, list only the first author then et al.

<u>Response</u>: We have adjust references 14, 15 and 28 to only list the first author followed by et al.