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.