**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.

*Thank you – we have carefully proofread the manuscript to correct spelling and grammar.*

2. Figure 2: Please define the “+”/”-“ symbols in the figure legend.

*+ Sign represents with the treatment and – sign represents without the treatment*

*Figure 2 was deleted in the revised version. We think it is enough to state the observation without the figure.*

3. Figure 3: Please line up panels A and B.

*Panel B was removed since the typical CM. spectra at room temperature was shown already in figure 1*

4. Figures 4, 6 and 7: Please define error bars in the figure legend.

*Bars in the figures have been defined as standard error in the legend.*

5. Title: Please revise the title to be less wordy.

*Title is revised* to “**Use of Electron Paramagnetic Resonance (EPR) in Biological Samples at Ambient Temperature and 77 K”**

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

*We have edited the short abstract accordingly.*

7. Please revise the Long Abstract (150-300 words) to include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate. Please focus on the general types of results acquired.

*We have edited the long abstract as requested.*

8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

*SI abbreviations has been used*.

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

*Thank you for noticing this. We have corrected the spacing after numbers.*

10. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

*Statement has been moved*

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

*Text was revised*

12. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and 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. For example: Eppendorf, Critoseal, Norell, Teflon, Bruker, etc.

*All the companies name identified have been removed, and the information included in the Table of Materials and Reagents. We did include the name of the instrument and proprietory software in Data Analysis, as this program is unique to how we calculated nitroxide concentration, however we also included alternative methods since the protocol does not require the specific spectrometer.*

13. 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, dashes, or indentations.

*Numbering was adjusted*

14. Line 121: Please specify the concentration of NaOH solution used.

*Concentration of NaOH was added*

15. Lines 148, 162-169, 214-217, 267-270, 276-279: Please write the text in the imperative tense in complete sentences.

*Text was revised*

16. 1.1.12: Does “KHB buffer + 100 µM DTPA” refer to the KHB containing 100 µM DTPA?

Please specify.

*Yes - it appears now in the manuscript as KHB containing 100 µM DTPA*

17. 1.3.1: Can this step be simply deleted? 1.3.2 referred to the specific steps.

*Step has been deleted*

18. 2.1.1: Please mention how animals are anesthetized and how proper anesthetization is confirmed.

*A description of anesthesia has been added*

19. 2.1.6, 3.1.7, etc.: Please specify the EPR conditions used in this step.

*EPR conditions were added*

20. 2.3.2: Please specify the volume of blood or BALF added.

*Volume was specified*

21. 3.1.1, 3.2.1, 3.3.2: Please refer to specific steps.

*This part has been revised*

22. 3.1.2: Please describe how to homogenize the tissue.

*A description has been added* (3.2.2)

23. 3.1.9: Please specify what is incubated with CMH.

*In the referenced section 3.2 on fresh lung tissue, we have restated in 3.2.9 that the lung homogenate was incubated with CMH in the presence of several inhibitors*

24. 3.2.2, 3.3.3: What is used to cut?

*We used a single edged blade to cut tissue; this has been added to the protocol.*

25. 3.2.7: Please specify the centrifugal speed.

*We added the mini table centrifuge speed (*8,500 *rpm*) (3.1.6)

26. Please include single-line spaces between all paragraphs, headings, steps, etc. After that, 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.

*Text format has been revised* *as requested*

27. Please discuss Figure 3C-D in the protocol.

*Figure 3 has been modified to show the tracing of the CMH radical and the PTFE tubing utilized, and stated in the protocol step 1.3.1*

28. Please discuss Figure 6C-D in the Representative Results.

*Figure 6 has been modified and the results discussed in the representative results section*

29. Line 479: Should it be Figure 5C, D?

*Figure 5 has been modified*

30. Please shorten the figure legends. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

*Figure legends have been revised as instructed*

31. Discussion: Please rephrase/revise to explicitly discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

*Discussion has been edited to discuss critical steps, troubleshooting and limitations.*

32. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis).

*Format has been changed*

33. 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.  
34. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

*References have been revised*

**Reviewers' comments:**  
  
**Reviewer #1:**  
Query 1: In presented manuscript authors describe the methods of detection superoxide using EPR and different hydroxylamine probes. Unfortunately, in manuscript authors show some flaws in logic:  
In abstract authors state that EPR is "direct" and "specific" method for detection of ROS (superoxide in the case of this paper). However, presented in the paper method of superoxide detection is neither direct nor specific. First, authors do not detect superoxide directly but use hydroxylamine reaction with superoxide and detect the product of this reaction - nitroxide. This approach does not have any difference compare to, for example, hydroethidine/fluorescent approach (detection of product of hydroethidine reaction with superoxide by fluorescence). Second, it is not specific method, authors use an addition of SOD to discriminate between superoxide specific and superoxide unspecific oxidation of hydroxylamines to corresponding nitroxides. I highly recommend rewriting manuscript according to logic.

*We have edited the text to more accurately describe the advantage of utilizing EPR for the measurement of oxygen radicals. We acknowledge that EPR is not detecting superoxide itself due to the short half-life time of this radical, but instead, the reaction of superoxide with the EPR probe forms a nitroxide radical which is stable and thus detectable by EPR. We contend that EPR is a direct method to detect free radicals unambiguously, unlike hydroethidine (DHE). DHE requires a two-step reaction to detect O2•− through the formation of a free-radical intermediate followed by HPLC or relative fluorescence to detect the product; the reagent can be oxidized by other species and is subject to auto-oxidation.(* *J. Zielonka, B. Kalyanaraman / Free Radical Biology & Medicine 48 (2010) 983–1001). We have edited the manuscript to improve accuracy.*

Query 2: As was said previously, authors use a SOD addition to elucidate superoxide-based oxidation of hydroxylamine to nitroxide for detection of superoxide production by biological milieus. In contrary, in mitochondria-based experiment authors directly show that addition of SOD "minimally affects" (statistically?) the increase of nitroxide signal (Figure 1C). Therefore, in this experiment authors show that hydroxylamine conversion to nitroxide is not happens due to oxidation by superoxide. Direct electron transfer from hydroxylamine to components of mitochondria should be considered for this experiment. This part of manuscript must be rewritten stating that no actual superoxide detection can be detected in this system but the mitochondrial redox state is detected, according to a logic of the rest of the paper.

*We recognize that the exclusive use of a cell-impermeable SOD, which will not be able to react with cytosolic or mitochondrial superoxide, has raised confusion for this Reviewer, thus we have edited the manuscript to clarify the experiments and conclusions. We have also added supporting data with the cell permeable PEG-SOD to demonstrate the ability to differentiate superoxide generated in the extracellular vs. intracellular compartments using impermeable and permeable SOD. CMH was developed and is widely used as a superoxide-selective probe, though it does have the potential to react with other free radial species, necessitating the inclusion of DTPA as a metal chelator to prevent its reaction with metal ions. PEG-SOD will react with cytosolic but not mitochondrial superoxide. It is well established that mito-TEMPO-H, with the same targeting properties of MitoSOX, accumulates in the mitochondria and thus will react with mitochondrial superoxide* *(Dikalov et al. 2011). . To avoid confusion or inaccuracy, we have described the product as nitroxide when presenting the total CM. signal and mitochondrial probe mito-TEMPO. and reserve superoxide specifically for the SOD inhibitable component. As illustrated in the experiments in Figure 6B using lung tissue, one can also interrogate the source of superoxide with different enzymatic inhibitors.*

Query 3: Authors do not provide any statistical description of data evaluation, statistical analyses has to be described and p values assign for all the data obtained.

*Response 3: The project was designed to demonstrate methods rather than perform statistical analyses between treatment groups, thus though we have not included the statistical analyses and p values to the figures.*

Query 4: Authors must check all mathematical calculations of concentration through out of the paper, especially in following cases:

*We apologize for the errors and appreciate the careful review. We have carefully reviewed the calculations, including the specific examples identified by the Reviewer.*

1) Line 151: MW is wrong.

*We have corrected this mistake.*

2) Lines 168-169: Stock solution concentration is not equal to 10 mM, compare to line 161.

*The CPH and Mito-TEMPO-H concentration were corrected.*

3) Wrong mathematical statement in line 227

*Corrected*

4) There are many inconsistence in actual prepare concentration trough out of paper.  
There are no description of stock solution/buffers preparation procedure for lines 156, 343, 363-364

*Buffers and stocks preparation were added*

Query 5: There is no protocol for lung collection indicated in lines 341, 374, 408.

*A description of lung collection was added*

Query 6: Line 414 - no description of "tissue cell"

*A description was added*  
Query 7: Something wrong with reference numbering, it is starting from 16 in line 275, please, correct.

*Reference numbering started from the introduction*

Query 8: SOD-based control date values must be included in Figures 4, 5, 6, 7

*We have rearranged the presentation of the data and now include the SOD*

*treatments only in Figure 6.*

Query 9: Line 526: CAT1H compound is not described previously, please, give the name and description or reference

*CAT1H name was added*

Query 10: Line 539: Add description of SOD2

*A description was added*

Query 11: Line 508: Which component of CP was used?

*We are not clear on the Reviewer’s question. In the EPR spectra, we detected overlapping CP. signal and ascorbic acid radical signal. The data reported is the CP. component in the spectra. Using the SpinFit, one can distinguish the concentration or the Spins from spectra arising from multiple radicals*

Query 12: In Table at he end of manuscript buffer DMEM is indicated, I was not able to find where his buffer was used.

*DMEM is the cell culture media used for RAW 264.7 cells. DMEM was mentioned in line 176*   
  
**Reviewer #2:**  
Article summary:  
This is an article from Hanan Elajaili et al. describing a protocol using cyclic hydroxylamines and EPR to measure superoxide in vitro and ex vivo. The method is based on the oxidation of a cyclic hydroxylamine by the superoxide anion radical leading to an EPR active nitroxide radical that can be quantify by EPR. The methods is highly sensitive but suffer from a low selectivity as the hydroxylamine can be oxidize by many other species. Therefore, great care and appropriate controls are required.  
Such protocol certainly deserve publication in JoVE but the manuscript contains mistakes that have to be corrected before being suitable for publication.  
  
Major concerns:

*The Reviewer noted multiple inaccuracies in the protocol; we regret these errors and appreciate the careful review that identified them. We have reviewed every calculation to ensure accuracy. Please see notes for the specific problems identified. Based on extensive publications, we propose that EPR is not highly sensitive, as compared to fluorescent probes, but is specific and allows for the quantitative measure of free radicals. The CMH probe is selective, though admittedly not exclusive, for superoxide, with no reactivity for hydrogen peroxide (S. I. Dikalov et al. 2004) It is commonly described as a superoxide selective probe. We have used nitroxide in the y axis to reflect total CMH radical concentration, acknowledging that it may also include other superoxide-derived ROS and not exclusively the superoxide radical itself.*

Query 1: L168. 5 mg of CPH in 1 mL will give a solution of 20 mM and not 10 mM, should all the finding using a wrong concentration of stock solution being corrected or it is a typo in the manuscript ?

*Thank you for noting this mistake, which has been corrected.*

Query 2: L190. 500 μL of KHB + 15 μL of SOD + 12.5 μL of CMH (10 mM) + 40 μL of PMA will not give a final concentration of CMH of 0.25 mM as stated nor 30 U/mL of SOD. Such inaccurate calculation repeats many time in the manuscript, please check and correct everywhere in the manuscript to ensure scientific rigor.

*The step 1.1.1 in the protocol was corrected to read:*

* + 1. *Add KHB containing 100 µM DTPA to each well, and treat in a total volume of 500 µl with the following*

Query 2: How the nitroxide concentration is calculated by EPR? Please give details, is it using a calibration curve based on the double integration or based on the intensity of one peak.

*EPR spectra were simulated using SpinFit, a program incorporated in the software of the specific spectrometer used, and the concentration was obtained using SpinCount. Alternatively a calibration curve of a stable nitroxide such as 4-Hydroxy-TEMPO (TEMPOL) or 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl can be made and the concentration can be obtained by comparing the intensity of the signal from the sample and the standard. These options have been clarified.*

Query 3: L227 at l160 the stock solution is 10 mM, at l227 it is 9.5mM.

*The stock solution has been presented as 9.5 mM rather than rounded up to 10 mM.*

Query 4: L435-438 The sentence is of not very clear, please reformulate. The level of the blank is never indicated, it would be interesting to show the time course of the appearance of the EPR signal with the probe alone under the same condition or at least the level at the same time of measurement.

*The sentences were revised to be clearer. We did not perform the time sweep experiment to monitor the increase in the signal over time. Instead, we present the end point measurements after 50 min incubation time period. The incubation time was based on pilot experiments not included in the protocol to ensure the reaction was complete. The EPR signal with the probe alone under the same condition it has been always used as a control and is now included.*

Query 5: Having graphs showing the concentration of superoxide is misleading. For example, Fig4D shows a concentration of superoxide in the blood of about 25 μM. This is not really the concentration of superoxide in the blood (steady state) but rather the concentration of nitroxide radical accumulated for a certain time from the reaction of the hydroxylamine with superoxide.

*It has been clarified in the figure legend and in the representative results that the concentration reported is the concentration of the nitroxide that accumulated in blood or BALF under the experimental conditions.*

Query 6: The reason for measuring the EPR at 77k is not given. Please explain.

*We evaluate samples both at room temperature and at 77K. A central goal of this JoVE manuscript is to demonstrate a practical method to collect samples and store them at -80 C and collect data at 77K, allowing for a more convenient efficient work flow and ability to transfer samples from other labs to an EPR facility. Another advantage is the ability to test a larger volume of sample. At room temperature, the measurements are performed in EPR capillary tubes while at 77k, the larger volumes frozen in the EPR tubing allow for improved signal to noise, and thus sensitivity.*  
  
Minor Concerns:  
Query 7: L160. Degassed should be replace by deoxygenated.

*Degassed has been replaced by deoxygenated.*

Query 8: L119. The name of DTPA is wrong.

*The spelling of DTPA was corrected*

Query 9: mito-TEMPO-H is some time written as mito-Tempo-H. TEMPO is more commonly used than Tempo.

*We now consistently used mito-TEMPO-H*  
  
Query 10: L267. Correct the time constant.

*The time constant is corrected*

Query 11: L284 100 μL of heparin (concentration?).

We *used 100 µl of 1000 USP units per ml (Sagent Pharmaceuticals, Inc.). Concentration was added and the product was added to the table of materials.*

*Alternatively we used a syringe coated with heparin containing DTPA 100 µM*

Query 12: L324 "load blood" this part is superoxide in BALF.

*Corrected*

Query 13: L405 100 μL of CPH (presumably from the 10 mM solution?) injected at a dose of 20 mg/kg would give a mouse of about 11 g, is it correct?

*To prepare CPH stock solution 5 mg was dissolved in 1ml filtered and deoxygenated 50 mM phosphate buffer which gives 22 mM . 10 mM was a typo. 100 µl of 5 mg/ml of CPH stock was giving to mice with an average body weight of 25 g for 20mg/kg dose. This was stated now in the manuscript*

Query 14: The Figure 3D is not clear, it would be very valuable to replace it.

*3C and 3D have been removed and replaced with a better photo.*

Query 15: In general, the fraction of nitroxide generated from the superoxide is only a fraction of the total signal. One can wonder whether this method is adequate for measuring superoxide? It is also true that measuring superoxide radical is extremely challenging and other techniques have their own drawbacks.

*We agree that the extracellular superoxide was a small portion of the total nitroxide signal, shown by the cell-impermeable SOD1 inhibitable component. We did not directly determine the contribution of intracellular superoxide and other investigators have also demonstrated that CMH is relatively selective for superoxide. That being said, we have adjusted the protocol to reflect the more conservative accurate interpretation. We also have presented a protocol to identify the other species contributing to the total nitroxide signal which most likely generated downstream results of superoxide being generated in the system.*

**Reviewer #3:**  
Manuscript Summary:  
The manuscript entitled "Superoxide Detection in in vitro and in vivo Models Using Spin Probes and Electron Paramagnetic Resonance (EPR) at Ambient Temperature and 77 K" by H. Elajailli et al. present different methods for the detection of ROS using hydroxylamine spin probes and EPR in cells and tissues. The authors have published a large number of articles using these methods and have thus a high expertise regarding their application. However, the manuscript does not satisfy educational standards expected in JoVE because some interpretations are not supported by the presented results.

*We appreciate this comment and have extensively editing the manuscript to refine the interpretations of the data and ensure it meets the educational standards of JoVE.*

Major Concerns:  
Query 1 and 2: The limitations of the technique should be much more clearly stated and appropriate controls added (especially with SOD-PEG). I agree with the statement in the abstract that EPR detection using spin probes has many advantages but that appropriate controls are critical to ensure accurate interpretation of the results. The discussion lists rather clearly the limitations of the technique and precautions for accurate interpretations. On the contrary, the title, abstract, introduction, method, and result sections are much more ambiguous by insisting on superoxide detection and determination of superoxide concentrations. Therefore, unexperienced readers may overestimate the specificity of the detection (as often seen in the literature). Cyclic hydroxylamine spin probes react not only with superoxide but also with many other radicals and metal ions. Depending on the cell or tissue type and condition, the level of nitroxide radical signal due to superoxide may vary. Interferences due to hydroxylamine oxidation by enzymatic processes have been reported (for example, with photosynthetic electron transport chain in Kozuleva et al. Free Radic. Biol. Med. 89 (2015) 1014-1023; with cytochrome c oxidase in Chen et al. Biochim. Biophys. Acta 970 (1988) 90126-90127). Controls with cell-impermeable SOD only reports on extracellular superoxide production and are not sufficient to decide on the origin of the nitroxide signal remaining after SOD treatment. Typically, cell-permeable SOD-PEG controls are required to attribute part of the signal that was not inhibited by SOD to intracellular superoxide using the procedure applied by the authors in a previous study (reference 19). It would be helpful to the reader to give a list of potential interferences with appropriate controls to rule them out as a table in the manuscript.

Query 2: In accordance with the previous remark, the title of the article should be changed to "ROS detection in in vitro and in vivo models...". Also the titles of protocols at lines 172, 219, 247, 280, 282, 307, 327, 338, 340, 373, and 403 and the titles of result sections at lines 419, 449, 462, 465, and 484 should be more accurate and refer to "ROS detection" instead of "superoxide detection", unless specific controls including SOD-PEG are added. The main text should be corrected in a similar way throughout.

*We thank the Reviewer for this important point. While we want to emphasize the established function of CMH as a superoxide selective probe that does not react with hydrogen peroxide, we also do not want to overstate the signal as indicating superoxide without confirming the SOD-inhibitable component of the signal. We have therefore changed the nomenclature to use nitroxide radical rather than superoxide in the y-axis of the figures unless we are representing only the SOD inhibitable signal, and modified this in the text. We recognize that other radicals can react with CMH, and have included the table in the discussion with different controls, as suggested above. We also included the important point raised that enzymatic processes such as cytochrome c oxidase can result in oxidation of hydroxylamine and recommended the researchers to be cautious in Interpreting the results.*

Query 3: Short Abstract (Lines 38-40): Even though the detection of free radicals by EPR is direct, when spin probes are used to detect ROS, it is an indirect method of detection of ROS. The proximity of the two first sentences in the short abstract is thus misleading. I advise they are changed to "The use of different types of spin probes coupled to electron paramagnetic resonance spectroscopy (EPR) allows for the detection of reactive oxygen species (ROS), including superoxide, in specific cellular compartments, provided appropriate controls are performed to ensure accurate interpretation of the data. Storage of samples..."

*Thank you for the suggestion. The abstract has been revised.*

Query 4: Abstract (lines 46-52): As in the short abstract, this section is misleading. EPR is unable to measure directly ROS radicals at physiological levels due to instability and lack of sensitivity. The use of spin probes (or spin traps) is an indirect method of detection of ROS and it should be clearly stated.

*Response 4: We acknowledge that EPR is not detecting superoxide itself due to the short half-life time of this radical, but instead, the reaction of superoxide with the EPR probe forms a nitroxide radical which is stable and thus detectable by EPR. We contend that EPR is a direct method to detect free radicals unambiguously*

Query 5: Abstract (line 56-58): Add SOD-PEG pretreatment to allow for intracellular superoxide detection. It is excessive to talk about "superoxide concentration" when intracellular or intramitochondrial detections are performed without the proper controls that allow exclusion of interfering processes.

*We have added additional data to demonstrate the use of SOD-PEG to designate cytosolic superoxide. It is well established that mito-TEMPO-H, with the same targeting properties of MitoSOX, accumulates in the mitochondria and thus will react with mitochondrial superoxide. Previous study shows that SOD2 overexpression significantly attenuated measurements with mitoTEMPO-H (Dikalov et al. 2011) and this control has been described in the discussion.*

Query 6: Introduction (line 91): Kinetic comparison with spin traps is misleading. Reaction rates of hydroxylamine spin probes with superoxide are in the range of 10^3-10^4 M-1.s-1. Antioxidant enzymes as SOD (k ≈ 10^9 M-1 s-1) will outcompete the spin probe easily, so any variation in the level of antioxidant enzymes will ruin accurate determination of superoxide concentration with spin probes.

*We agree with the Reviewer that the reaction between superoxide and SOD is faster than superoxide with the spin probes, however, high concentrations of the spin probes detect superoxide in biologic settings. These methods can be used to determine the ROS production in specific pathophysiologic conditions associated with altered production or antioxidant defenses as shown in this study, or in experimental conditions designed exclusively to test the production of superoxide from a designated source.*   
Query 7: Introduction (line 103): Additional references of applications of spin probes in vitro should be given here.

*References have been added.*

Query 8:Results (line 428): The attribution of remaining signal to intracellular superoxide is abusive. It certainly is due to intracellular nitroxide but nothing in the experimental procedure support links it to superoxide. If the authors have additional elements to support this attribution (such as additional experiments with SOD-PEG), they should add them in the manuscript. Otherwise, the interpretation must be revised and Figure 1B removed. (Same remark for line 444).

*As discussed above, we have substituted nitroxide for superoxide when indicating total CMH signal, and reserved superoxide only for the SOD or SOD-PEG inhibitable signal.*

Query 9: Results (line 435): The attribution of the signal of mito-TEMPO-H after SOD treatment to mitochondrial superoxide and derivation of superoxide concentration are abusive. It has been shown that the nitroxide derived from mito-TEMPO-H is reduced in the mitochondria (Dikalova et al. Redox Biol. 4 (2015) 355-362), which is likely to interfere with the measurement of absolute superoxide concentrations, especially rate of bioreduction vary between conditions. Moreover, the experimental conditions do not allow exclusion of interference from other ROS. Thus, Figure 1D should be removed.

*Response 9: We have attributed the mito-TEMPO-H signal as mitochondrial superoxide. The probe is designed to accumulate in the mitochondria. Antimycin A (AA)* *a known inhibitor of mitochondrial electron transport chain (mETC) complex III has been used to induce superoxide production in the mitochondria and the mito-TEMPO. signal was increased upon the stimulation with Antimycin A. We based our interpretation based on a previous study that shows that SOD2 overexpression significantly attenuated measurements with mitoTEMPO-H (Dikalov et al. 2011). We agree with the reviewer that the signal after SOD treatment is misleading and have removed this component of the experiment. As discussed in the referenced manuscript* (Dikalova et al. Redox Biol. 4 (2015) 355-362) *the nitroxide signal from mito-TEMPO-H may underestimate the exact superoxide generation in the mitochondria due to the bioreduction of nitroxide radical.*

Query 10: Results (Lines 454-461): It should be made clear that only extracellular superoxide is measured in blood and BALF.

*Since we treated the whole blood and BALF containing cells, CMH will detect both intracellular and extracellular ROS. We removed the SOD treatments in these figures and thus did not present data for extracellular superoxide.*

Query 11: Results (lines 466-473): Do the authors assume the homogenate contains intact cells? If so, they should specify that only extracellular superoxide is measured in fresh lung tissue homogenates using their protocol. Is there any difference in the results when SOD-PEG is used instead of SOD?

*We understand the importance of this question. Upon reflection, we cannot be confident whether the SOD inhibitable signal represents only extracellular superoxide in homogenized and frozen samples since it is also possible that cell membranes are damaged. We note that if this information is desired, this can be more accurately assessed using the in vivo spin probe delivery shown in Figure 7 with permeable or impermeable SOD. To elucidate the origin of the nitroxide signal generated, we also presented a protocol to use scavengers and enzyme inhibitors to identify the enzymatic source of the ROS or define the contribution of different radicals. (Figure 6 and Table)*

Query 12: Results (from line 499): It should be explained here or in the discussion why CPH is preferred over CMH for in vivo experiments.

*It is unknown if CMH can be safely administered to animals while injection of CPH probe has been reported to be non-toxic, thus we selected CPH for in vivo experiments. We have clarified this in the representative results.*

Query 13: Discussion (lines 591-596): The reader should be warned that adaptation of the protocols described here is possible but that a thorough investigation of the identity of ROS involved in the formation of the nitroxide needs to be performed using appropriate antioxidants before attribution of the signal is performed in a new system. Besides, additional controls need to be performed to prove that the level of endogenous antioxidant enzymes (SOD) and the reduction potential of the tissue are similar between conditions when concentrations of superoxide are derived and compared.

*Thank you for these comments. We agree with these points. We expect that alterations in endogenous antioxidant enzymes, and redox state will change the levels of specific ROS under disease conditions and propose that EPR is a useful method to evaluate this problem.*

Query 14:Legend of Figure 1 + Figure 1 (lines 606-611): Reference to total superoxide, intracellular superoxide, and mitochondrial superoxide are abusive as discussed above. Labels of y-axis in Fig. 1B and 1D also are, so they should be removed.

*As discussed several times above, we will improve accuracy and rigor, using nitroxide in the y axis to reflect total CMH radical concentration, and use superoxide to reflect only the SOD inhibitable component.*

Query 15:. Figure 2: The y-axis should be labeled "Nitroxide" and not "Superoxide".

*As discussed above, we agree and have altered the y axis labels.*

Query 16: Figures 4, 5, and 6: The values of the SOD treated samples for each condition should be displayed in the figures.

*figures have been modified to better represent the protocol*

Minor Concerns:  
Query 17: Line 168: check mass of CPH to be added.

*To prepare CPH stock solution 5 mg was dissolved in 1ml filtered and deoxygenated 50 mM phosphate buffer which gives 22 mM . 10 mM was a typo. 100 µl of 5 mg/ml of CPH stock was giving to mice with an average body weight of 25 g for 20mg/kg dose. This was stated now in the manuscript*   
  
Query 18: Protocols (line 186): Control with SOD-PEG should be added

*An additional data regarding the effect of SOD-PEG on the signal was added*

Query 19Protocols (line 199): It is not clear what is done with this buffer sample.

*Response 19: We have clarified that EPR measurements were performed on the buffer.*

Query 20: Protocols (line 201). It is not clear what is done with this cell sample.

*Response 20: We also clarified that EPR measurements were performed on the cells.*

Query 21 : 21. Protocols (line 206): It is not clear whether the sample is that prepared at line 199, at line 201, or both.

*Both and that was clarified in the protocol*

22. Protocols (line 224): SOD treatment is missing here.

*SOD treatment was removed from the figure.*

Query 23:Line 270: "1.28 ms" instead of "1.28 m".

*The typo was corrected*

Query 24: Line 308: PBS contains DTPA, not the syringe.

*Corrected*

Query 25: Line 343: Tris-EDTA buffer containing sucrose does not appear in the reagent list nor in the table of materials.

*Buffers preparation was added*

Query 26: Protocols (line 362): Reagents used for the controls and their preparation must be added to the reagent list.

*: Stocks preparations were added*

Query 27: Line 379: Specify that the weight of each piece of lung tissue must be recorded independently.

*Specified*

Query 28Line 381: It is not clear that the first piece of lung tissue cut at step 3.2.2 is used here and not both.

*Response 28: clarified*

Query 29: Protocols (Lines 381 and 384): What is meant by "mix"? Is homogenization of the tissue performed?

*No homogenization was performed*.

Query 30: Line 386: The tissue weight of the second piece of tissue was already recorded at line 379. *Corrected*

Query 31: Lines 397-401 should be placed in paragraph 3.2.2 at line 377.

*Response 31: step was modified*

query 32 : = Line 405: How is CPH solution for in vivo injection prepared?

*Response 32 :A description and more details about the preparation has been added*

Query 33 : Lines 437-438: Verb is missing in the sentence.

*Thank you the sentence was edited*

Query 34 : Line 479: Give figure number.

*Figure was modified*

Query 35: Lines 481-483: The sentence about frozen tissue should be moved to the next section dedicated to these type of samples (near line 497).

*The sentence moved*   
Query 36: Line 489: A full stop is missing after concentration.

Response 36: *Thank you. The sentence was fixed*.

Query 37: Lines 491-492: Verb is missing in the sentence.

*Response 37: The sentence was edited*

Query 38: Legend of Figure 3 (line 626) + Figure 3: The title of the legend (referring to 77K) does not match part 3B of the figure (room temperature spectra).

*Response 38: figure 3 B was removed to prevent confusion and since a typical CM. spectra at room temperature was presented in figure 1*

Query 39: Lines 635-636: Verb is missing in the sentence.

*The sentence was edited*

Query 40 Legend of Figure 5B (line 646) and Figure 6B (line 652): Explain how superoxide component is derived from the data in the legend.

*Superoxide component was derived from subtracting the signal in presence of SOD from the signal in the absence of SOD. The figures have been modified.*   
Query 41: Figure 5C: CMH is present in all conditions. Please clarify x-axis label to reflect this fact. The value for the first condition in figure 5C does not match the values in Figure 5A. Why? Please clarify the legend.

*different tissues from different mice were used and we observed variability due to the heterogeneity in lung injury with bleomycin. Also the test using different inhibitors was done at 37 C and we are comparing to effect of the inhibitors to the values in figure C not A. new data has been presented in the revised version.*

Query 42 :Reference 12 on line 726 is incomplete.

*Corrected*

Query 43: Authors should consider including heparin (from section 2.1.1), catalase, and the tissue cell in the table of materials. Maybe they should give some details about the characteristics of the EPR spectrometer needed.

*Information was added*

**Reviewer #4:**  
Manuscript Summary:  
This manuscript presents practical methods for detection of superoxide using cyclic hydroxylamine spin probes and EPR in different cellular compartments in vitro and different fluids and lung tissue in vivo, at both room temperature and 77 K. The topic of the article is of great interest, the protocols are well described and allow other researchers to replicate the experiments, however, there are a few minor issues to clarify.  
  
Minor Concerns:  
Query 1: In methods, additional information that should be included is about how the EPR signal calibration was done. Was a calibration curve performed with CM\* or CP\*? How should the preparation of these stable radicals be done? What concentrations should be used?

*EPR spectra were simulated using SpinFit, a program incorporated in the software of the spectrometer used here, and the concentration was obtained using SpinCount. Alternatively a calibration curve of a stable nitroxide such as 4-Hydroxy-TEMPO (TEMPOL) or 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl can be made and the concentration can be obtained by comparing the intensity of the signal from the sample and the standard.*

*CM and CP cannot be used for obtaining a calibration curve.*

Query 2:please confirm in the preparation of the spin probes the pH of the phosphate buffer. This solution is not included in the list of materials. PBS is also not included.

*The pH of the phosphate buffer and buffer preparation as well as the reagents were added to the methods and list of materials.*

Query 3:Please clarify the number of animals used in Figure 4. Panels A and B show data of 4-5, but C-F panels only show data of 2 animals per group. Samples were not obtained from the same animals? The legend does not show the n.

*In Figure 4, each point in the graph represents a different mouse. The same mice were used for both Fig 4A and Fig 4B, though in some mice, collection of the blood was unsuccessful, accounting for the variable n. The figure has been modified and now only includes the total nitroxide signal.*

Query 4:Discuss the choice of CPH for in vivo injection. CMH was used in all other in vitro and ex vivo experiments. Is there any advantage to using the CPH in this case?

*It is unknown if CMH can be safely administered to animals while injection of CPH probe has been reported to be non-toxic; therefore we selected CPH for in vivo experiments. We have clarified this in the methods.*