**TITLE:**

Use of Electron Paramagnetic Resonance in Biological Samples at Ambient Temperature and 77 K

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**SUMMARY:**

Electron paramagnetic resonance (EPR) spectroscopy is an unambiguous method to measure free radicals. The use of selective spin probes allows for detection of free radicals in different cellular compartments. We present a practical, efficient method to collect biological samples that facilitate treating, storing, and transferring samples for EPR measurements.

**ABSTRACT:**

The accurate and specific detection of reactive oxygen species (ROS) in different cellular and tissue compartments is essential to the study of redox-regulated signaling in biologicalsettings.Electron paramagnetic resonance spectroscopy (EPR) is the only direct method to assess free radicals unambiguously. Its advantage is that it detects physiologic levels of specific species with a high specificity, but it does require specialized technology, careful sample preparation, and appropriate controls to ensure accurate interpretation of the data. Cyclic hydroxylamine spin probes react selectively with superoxide or other radicals to generate a nitroxide signal that can be quantified by EPR spectroscopy. Cell-permeable spin probes and spin probes designed to accumulate rapidly in the mitochondria allow for the determination of superoxide concentration in different cellular compartments.

In cultured cells, the use of cell permeable 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) measures total nitroxide signal. This probe is highly selective for superoxide and does not react with hydrogen peroxide. The treatment of samples with and without cell-impermeable superoxide dismutase (SOD) pretreatment, or use of cell-permeable PEG-SOD, then allows for the differentiation of extracellular from cytosolic superoxide. The mitochondrial 1-hydroxy-4-[2-triphenylphosphonio)-acetamido]-2,2,6,6-tetramethyl-piperidine,1-hydroxy-2,2,6,6-tetramethyl-4-[2-(triphenylphosphonio)acetamido] piperidinium dichloride (mito-TEMPO-H) allows for measurement of mitochondrial ROS (predominantly superoxide).

Spin probes and EPR spectroscopy can also be applied to *in vivo* models. Superoxide can be detected in extracellular fluids such as blood and alveolar fluid, as well as tissues such as lung tissue. Several methods are presented to process and store tissue for EPR measurements and deliver intravenous 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH) spin probe *in vivo*. While measurements can be performed at room temperature, samples obtained from *in vitro* and *in vivo* models can also be stored at -80 °C and analyzed by EPR at 77 K. The samples can be stored in specialized tubing stable at -80 °C and run at 77 K to enable a practical, efficient, and reproducible method that facilitates storing and transferring samples.

**INTRODUCTION:**

While measures of oxidative stress and reactive oxygen species are important to the study of diverse diseases across all organ systems, the detection of reactive oxygen species (ROS) is challenging due to a short half-life and high reactivity. An electron paramagnetic resonance (EPR) technique is the most unambiguous method for detecting free radicals. Spin probes have advantages over the more commonly used fluorescent probes. Though fluorescent probes are relatively inexpensive and easy to use and provide rapid, sensitive detection of ROS, they do have serious limitations due to artifactual signals, an inability to calculate ROS concentrations, and a general lack of specificity1.

To facilitate the use of EPR for biologicalstudies, a variety of spin probes have been synthesized that can measure a range of biologically relevant free radical species as well as pO2, pH, and redox states2-7. Spin traps have also been developed to capture short-lived radicals and form long-living adducts, which facilitates detection by EPR8. Both classes (spin probes and spin traps) have advantages and limitations. One commonly used class of spin probes are cyclic hydroxylamines, which are EPR-silent and react with short-lived radicals to form a stable nitroxide. Cyclic hydroxylamines react with superoxide 100 times faster than spin traps, enabling them to compete with cellular antioxidants, but they lack specificity and require the use of appropriate controls and inhibitors to identify the radical species or source responsible for the nitroxide signal. While spin traps exhibit specificity, with distinct spectral patterns depending on the trapped species, they have slow kinetics for superoxide spin trapping and are prone to biodegradation of the radical adducts. Applications for spin trapping have been well-documented in biomedical research9-13.

The goal of this project is to demonstrate practical EPR methods for designing experiments and preparing samples to detect superoxide using spin probes in different cellular compartments *in vitro* and in different tissue compartments *in vivo*. Several manuscripts have published protocols relevant to these goals, using cell-permeable, cell-impermeable, and mitochondrial targeted spin probes to target different cellular compartments *in vitro* and process tissue for analysis in mouse models14,15. We build upon this body of literature by validating an approach to measure superoxide using a 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) spin probe in different cellular compartments *in vitro* to ensure accurate measurements, highlighting potential technical problems that may skew results. We also provide methods to perform EPR measurements in blood, bronchoalveolar lavage fluid, and lung tissue using the CMH spin probe. These studies compare different methods to process the tissues as well as present a method to inject another spin probe, CPH, into mice prior to harvesting tissue. Finally, we develop a practical method to store samples in polytetrafluoroethylene (PTFE) tubing to allow for the storage and transfer of samples before EPR measurements at 77 K.

**PROTOCOL:**

All animal studies were approved by the University of Colorado Denver Institutional Animal Care and Use Committee.

1. **Preparation of Reagents**
   1. **Diethylenetriaminepentaacetic acid (DTPA) stock (150 mM)**
      1. Add 2.95 g of DTPA (393.35 g/mol) to 10 mL of deionized water.
      2. To dissolve DTPA, add 1 M NaOH dropwise and bring to a pH of 7.0.
      3. Bring the volume to 50 mL with water for a final DTPA concentration of 150 mM, and store at 4 °C.
   2. **Phosphate buffer saline (PBS) (50 mM, pH 7.4)**
      1. Prepare 5 M of sodium chloride (NaCl) (58.44 g/mol; 29.22 g/100 mL).
      2. Prepare 1 M of potassium phosphate dibasic HK2PO4 (174.18 g/mol; 17.42 g/100 mL)
      3. Prepare 1 M of potassium phosphate monobasic KH2PO4 (136.1 g/mol; 13.61 g/100 mL). Mix 3 mL of 5 M NaCl with 4.24 mL of 1 M potassium phosphate dibasic and 0.760 mL of 1 M potassium phosphate monobasic. Check the pH.
      4. Bring the volume to 100 mL with deionized water.
      5. Store at room temperature (RT) for short-term (days) and at 4 °C for long-term (weeks) storage.
   3. **Krebs-Henseleit buffer (KHB) containing 100 µM DTPA** 
      1. In 50 mL conical centrifuge tube, add 33.3 µL of 150 mM DTPA stock solution.
      2. Bring to a 50 mL volume with Krebs-Henseleit buffer (KHB).
      3. Prepare fresh buffer with DTPA each day and keep it at RT.
   4. **Tris-EDTA buffer containing sucrose**
      1. Prepare 0.5 M Tris stock: dissolve 15.14 g of Tris base (121.14 g/mol) in 150 mL of deionized water. Using HCl, adjust the pH to 7.8 and bring to a final volume of 250 mL.
      2. Dissolve 21.4 g of sucrose (342.29 g/mol; final concentration = 0.25 mM) in 150 mL of deionized water.
      3. Add 5 mL of Tris stock to sucrose to achieve a 10 mM final Tris concentration.
      4. Add 0.5 mL of 0.5 M EDTA stock to Tris-sucrose to achieve a 1 mM final concentration.
      5. Check the pH and adjust it to 7.4.
      6. Bring to a final volume of 250 mL with deionized water and store at 4 °C.
   5. **Bovine erythrocyte Cu/Zn superoxide dismutase (SOD) stock (30,000 U/mL)** 
      1. Reconstitute 30,000 U of SOD in 1 mL of PBS (approximately 5.7 mg, depending on activity of SOD lot).
      2. Mix well, aliquot, and store at -20° C for short-term (6-12 months) and at -80 °C for long-term storage.
   6. **SOD working solution (1000 U/mL)**
      1. Transfer a 30 µL aliquot of 30,000 U/mL SOD stock into a 1.5 mL tube.
      2. Dilute to 1000 U/mL by adding 870 µL of sterile PBS.
      3. Keep the solution on ice, and use it fresh.
   7. **Phorbol 12-myristate 13-acetate (PMA) stock (5 mM)**
      1. Dissolve 1 mg of PMA (616.83 g/mol) in 325 µL of DMSO (final concentration = 5 mM).
      2. Aliquot a 5 mM PMA solution and store it at -20 °C.
   8. **PMA working solution (125 µM)**
      1. Dilute a 10 µL aliquot of 5 mM PMA stock into 390 µL of sterile PBS.
      2. Keep the solution on ice and use it fresh.
      3. For a vehicle control for PMA, use 10 µL of DMSO in 390 µL of PBS.
   9. **Diphenyliodonium chloride (DIP) (2.5 mM)**
      1. Dissolve 3.2 mg of DIP (316.57 g/mol) in 4 mL to obtain a 2.5 mM stock.
      2. Prepare the solution and use it fresh.
   10. **Deferoxamine mesylate salt (DFO) (20 mM)**
       1. Dissolve 4.5 mg of DFO (656.79 g/ mol) in 340 µL to obtain a 20 mM stock.
       2. Prepare the solution and use it fresh.
   11. **Preparation of antimycin A (AA) stock (5 mM)**
       1. Dissolve 5.4 mg of AA (532 g/mol) in 2 mL of ethanol (final concentration = 5 mM).
       2. Aliquot the stock in glass vials and store at -20 °C.
   12. **Preparation of spin probes** 
       1. Bubble 50 mM phosphate buffer containing 100 µM DTPA with nitrogen for 30 min to remove dissolved oxygen from the buffer.
       2. Remove the spin probe from the -20 °C freezer and allow the container to come to RT (10-15 min).
       3. Weigh out 2.4 mg of 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine·HCl (CMH) (237.8 g/mol)
       4. Dissolve CMH into 1 mL of the deoxygenated phosphate buffer for a final concentration of 10 mM.
       5. Weigh out 5 mg of 1-hydroxy-4-[2-triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine,1-hydroxy-2,2,6,6-tetramethyl-4-[2-(triphenylphosphonio)acetamido]piperidinium dichloride (mito-TEMPO-H) (529.1 g/mol).
       6. Dissolve mito-TEMPO-H into 1 mL of the deoxygenated phosphate buffer for a final concentration of 9.5 mM.
       7. Weigh out 4.9 mg of 1-hydroxy-3-carboxy-2, 2, 5, 5-tetramethylpyrrolidine·HCl (CPH) (223.7 g/mol).
       8. Dissolve CPH into 1 mL of the deoxygenated phosphate buffer for a final concentration of 22 mM.
       9. Aliquot and store at -80 °C (freeze-thaw is not recommended).
2. **Detection of Superoxide *in vitro***
   1. **Detection of total, extracellular, and intracellular superoxide in PMA-stimulated RAW 264.7 cells at RT**
      1. Following proper aseptic technique, thaw RAW 264.7 cellsand passage them in DMEM media supplemented with 10% FBS (low endotoxin-free) and 1% antimycotic/ampicillin at 37 °C in CO2 incubator.
      2. Seed RAW 264.7 cells at 1 x 106 cells/well into 6-well plates one day prior to treatment.
      3. Gently remove media and wash the cells once with 1 mL of KHB buffer.
      4. Add KHB containing 100 µM DTPA to each well, and treat in a total volume of 500 µL with the following:
      5. For wells pretreated with SOD, add 15 µL/well of SOD working solution (1000 U/mL; final concentration of SOD = 30 U/mL) and incubate for 10 min at 37 °C prior to addition of CMH and PMA.
      6. Add 12.5 µL/well of 10 mM CMH stock (final concentration = 0.25 mM).
      7. Add 40 µL/well of 125 µM PMA working solution (final concentration = 10 µM) or 40 µL vehicle (stock 10 µL of DMSO in 390 µL of PBS).
      8. Incubate for 50 min at 37 °C in a CO2 incubator.
      9. Remove the plates from the incubator and place them immediately on ice.
      10. Collect buffer from each well in separate, 1.5 mL, labeled tubes. Keep on ice throughout.
      11. Add 100 µL of fresh KHB buffer containing 100 µM DTPA, gently scrape the cells, and resuspend by pipetting up and down several times. Keep on ice throughout cell resuspension.
      12. Load the sample collected in steps 2.1.10 and 2.1.11 (50 µL) in each of the capillary tubes. Seal both ends and run the EPR.

NOTE: Always test a tube or well (without cells) containing the probe in buffer (same concentration = 0.25 mM), treated under the same conditions as the cells (same incubation time and temperature) as a control, since background intensity of the probe is temperature- and time-dependent.

* + 1. Set the EPR acquisition parameters to the following: microwave frequency = 9.65 GHz; center field = 3432 G; modulation amplitude = 2.0 G; sweep width = 80 G; microwave power = 19.9 mW; total number of scans = 10; sweep time = 12.11 s; and time constant = 20.48 ms.
  1. **Detection of mitochondrial superoxide in RAW 264.7 cells**
     1. Follow steps 2.1.1 and 2.1.2 to seed RAW 264.7 cells one day prior to the experiment.
     2. Remove media and wash the cells once with 1 mL of KHB buffer.

* + 1. Add 200 µL of KHB containing 100 µM DTPA to each well.
    2. Add 5.3 µL/well of 9.5 mM mito-TEMPO-H stock (final concentration = 0.25 mM)
    3. Incubate for 10 min at RT.
    4. Add 1 µL/well of antimycin A (AA), 5 mM stock solution in ethanol (final concentration = 25 µM).
    5. Incubate for 50 min at 37 °C in a CO2 incubator.
    6. Remove the plates from the incubator and place them immediately on ice.
    7. Gently scrape the cells and resuspend by pipetting up and down. Keep on ice.
    8. Load the sample in a capillary tube. Seal both ends.
    9. See the previous section for EPR setting.
  1. **Detection of superoxide in RAW 264.7 cells at 77 K**
     1. Place the buffer collected in step 1.1.10 in pre-prepared PTFE tubing 1-2 inches in length (3/16" OD x 1/8" ID). Make sure the PTFE tubing is straight so it can be easily inserted and removed from the finger dewar. Use a rubber stopper to close one end of the PTFE tubing, pipette the buffer or cell suspension (100 to 150 µL) into the PTFE tubing, and seal the tubing with a second stopper.
     2. Flash freeze the sample in liquid nitrogen. The sample can be transferred to a labeled cryopreservation tube for storage at -80 °C or run immediately.
     3. Fill the finger dewar with liquid nitrogen and insert the PTFE tubing containing the sample into the finger dewar. Make sure the sample is centered in the active space of the resonator and run EPR at 77 K.

NOTE: Start the nitrogen gas flow to your spectrometer 15-30 min before the measurements, and continue this flow throughout the measurements to prevent water condensation in the resonator.

* + 1. Set EPR acquisition parameters to the following: microwave frequency = 9.65 GHz; center field = 3438 G; modulation amplitude = 4.0 G; sweep width = 150 G; microwave power = 0.316 mW; total number of scans = 10; sweep time = 60 s; and time constant = 1.28 ms.

1. **EPR Measurements in Fluids**
   1. **Whole blood** 
      1. Treat mice (8-12 weeks old) with a single dose of intratracheal bleomycin (Bleo; 100 µL at 1 U/mL) dissolved in PBS or PBS alone as previously described16-17.
      2. Euthanize mice by administering inhaled isoflurane (1.5-4%) followed by exsanguination and cervical dislocation. Aspirate blood through the right ventricle into a syringe coated with heparin (1000 USP/mL) containing 100 µM DTPA and transfer to a 1.5 mL tube.
      3. In a separate 1.5 mL tube, add 15 µL of PBS containing 100 µM DTPA and 3 µL of CMH (10 mM) to 132 µL of blood for a total volume of 15 µL and final CMH concentration of 0.2 mM.
      4. Incubate blood for 10 min at 37 °C in a water bath.
      5. Remove the tubes from water bath.
      6. Take an aliquot by loading blood in a capillary tube and run EPR at RT with the following EPR acquisition parameters: microwave frequency = 9.65 GHz; center field = 3432 G; modulation amplitude = 1.0 G; sweep width = 80 G; microwave power = 19.9 mW; total number of scans = 3; sweep time = 12.11 s; and time constant = 20.48 ms. Alternatively, samples can be flash frozen as described in step 2.3 for measurements at 77 K. EPR acquisition parameters are the following: microwave frequency = 9.65 GHz; center field = 3438 G; modulation amplitude = 4.0 G; sweep width = 150 G; microwave power = 0.316 mW; total number of scans = 2; sweep time = 60 s; and time constant = 1.28 ms.
   2. **Bronchoalveolar lavage fluid (BALF)**
      1. After euthanasia (see step 3.1.2), collect BALF by slowly instilling and withdrawing 1 mL of PBS containing 100 µM DTPA three times in a syringe *via* a cannula placed in the trachea.
      2. In a 1.5 mL tube, treat 200 µL of BALF with 4 µL of CMH (10 mM) to obtain a final concentration of 0.2 mM.
      3. Incubate BALF for 50 min at 37 °C in a water bath.
      4. Take tubes out of the water bath and place them on ice.
      5. Load BALF in a capillary tube and run EPR at RT with the same EPR settings as used in step 1.1.13, or flash freeze in liquid nitrogen as described in step 2.3.
   3. **EPR measurements in blood and BALF at 77 K**
      1. Follow the protocol above to collect blood (steps 3.1.1. to 3.1.4) and BALF (steps 3.2.1 to 3.2.4).
      2. Place 150 µL of the treated blood or BALF in PTFE tubing (1-2 in). Use a rubber stopper to close one end of the PTFE tubing prior to adding the sample and another stopper to seal the tubing.
      3. Flash freeze the sample in liquid nitrogen.
      4. See section 2.3 for details on running EPR in frozen samples in PTFE tubing using the finger dewar at 77 K.
2. **EPR Measurements on Lung Tissue** 
   1. **Flash frozen lung tissue** 
      1. After collecting the BALF in step 3.2.1, the chest is opened and lungs flushed with 10 mL of cold PBS *via* the right ventricle to remove blood. Flash freeze the lung tissue in liquid nitrogen. Frozen lung tissue can be stored at -80 °C until use for EPR measurements.
      2. Stabilize the lung tissue on dry ice with tweezers and cut multiple small pieces (5-15 mg) of lung tissue using a single-edge blade.
      3. Weigh the tissue in a 1.5 mL tube, place the tube on the scale and tare the scale, then add the tissue pieces and record the weight.
      4. To the tissue in the 1.5 mL tube, add 196 µL of KHB containing DTPA and 4 µL of CMH (0.2 mM) to achieve a 200 µL total volume.
      5. Incubate for 1 h at 37 °C in a water bath.
      6. Spin down (for a few seconds) in a microcentrifuge at 3,884 x g.
      7. Place on ice and pipette 150 µL of the supernatant into the PTFE tubing and freeze for the 77 K measurements as described in section 2.3.

NOTE: For this method, the heterogeneity of the injury needs to be considered. For a bleomycin-induced lung injury, given that it is a highly heterogeneous injury, it is recommended to cut several tissue pieces from different parts of the lung from each mouse. Alternatively, a larger piece of tissue can be homogenized in KHB buffer containing 100 µM DTPA at a 1:6 weight-to-volume ratio (mg/µL) as described below.

* 1. **Fresh lung tissue preserved in sucrose buffer** 
     1. Flush the lavaged lungs with cold PBS to remove blood as done in step 3.1.2.
     2. Homogenize the fresh lung tissue in Tris-EDTA buffer containing 0.25 M sucrose with a 1:6 lung/buffer (mg/µL) ratio using Dounce tissue grinder with a glass or PTFE pestle.
     3. Add 50 µL of the lung homogenate to 450 µL of KHB containing 100 µM DTPA.
     4. In a 1.5 mL tube (in a total volume of 100 µL), to 98 µL of lung homogenate in KHB, add 2 µL of CMH of 10 mM stock to obtain a final concentration of 0.2 mM.
     5. Incubate for 20 min 37 °C in a water bath.
     6. Place the samples on ice and load them in a capillary tube. Run EPR at RT (settings used in step 2.1.13).
     7. To test the contribution of specific species and sources using different inhibitors, pre-treat 88 µL of lung homogenate +/- inhibitor, adjusting with KHB to achieve a final volume of 98 µL. In this experiment, the inhibitors included 10 µL of SOD (100 U/mL), 4 µL of deferoxamine (DFO; final concentration = 800 µM), or 4 µL of diphenyliodonium chloride (DIP; final concentration = 100 µM). Incubate for 20 min at 37 °C in a water bath.
     8. Add 2 µL of CMH and incubate for another 20 min at 37 °C, followed by EPR measurements as described above. Include a one-time matched blank sample with CMH KHB containing sucrose buffer. Alternatively, store aliquots of the remaining lung homogenates (step 3.1.2) at -80 °C for future measurements.

NOTE: The total volume can be scaled as needed.

* 1. **EPR measurements on lung tissue from mice injected with spin probes *in vivo* (at RT using tissue cell)**
     1. Prepare CPH stock solution by dissolving 4.9 mg of CPH in 1 mL of filtered and deoxygenated 50 mM phosphate buffer.
     2. Anesthetize mice with inhaled isoflurane (1.5-4%) for 20-30 seconds until unresponsive to toe pinch. Inject mice *via* retroorbital route with 100 µL of CPH spin probe for a 25 g mouse body weight (final dose = 20 mg/kg), and allow the probe to circulate for 1 h. Immediately after retroorbital injection, add one drop of 0.5% proparacaine HCl on the eye area to prevent eye pain and dryness. Monitor mice for 1 h and proceed to tissue harvesting.
     3. Harvest the lung tissue as described above and flash freeze the lungs.
     4. Cut 20-30 mg of frozen tissue on dry ice and record the exact weight.
     5. Gently wipe the tissue with cleaning wipes to absorb any surface water.
     6. Place the tissue within the window of the tissue cell (an accessory allows EPR measurements for tissue samples) and run EPR to determine total spins. The data can be expressed as total spins per mg of tissue.

1. **Data Analysis** 
   1. Simulate the EPR spectra using SpinFit module incorporated in the Xenon software of the bench-top EMXnano EPR spectrometer. Determine the nitroxide concentration by the SpinCount module. Alternatively, a calibration curve of a stable nitroxide such as 4-hydroxy-TEMPO or TEMPOL can be made, and the concentration can be obtained by comparing the intensity of the signal with the sample and standard.
   2. For the data collected at 77 K, use double integration followed by SpinCount.

**REPRESENTATIVE RESULTS:**

Superoxide detection using CMH was validated using the X/XO superoxide generating system to demonstrate that the nitroxide (CM.) signal was fully inhibited by SOD, while catalase had no effect. (**Figure 1A**) The total, extracellular superoxide was then evaluated in RAW 264.7 cells by incubating cells with the cell-permeable CMH spin probe +/- SOD pretreatment. The nitroxide concentration was measured in both the cell suspension and buffer, which demonstrated that the values in the two sample types were similar due to the permeable nature and rapid equilibration of the spin probe. (**Figure 1B**) The nitroxide radical signal increased in RAW 264.7 cells stimulated with PMA compared to control cells. This signal was significantly attenuated in cells pretreated with cell-impermeable SOD. Each color represents wells tested on different days, demonstrating the consistency of data collected on specific days and reproducibility of the results across time. The concentration of extracellular superoxide was determined by subtracting the signal in PMA cells pretreated with SOD from the signal after PMA in the absence of SOD (T). The remaining signal was attributed to intracellular superoxide(**Figure 1C**). **Figure 1D** illustrates the calculation of total and extracellular superoxide. (E) The intracellular signal was confirmed in PMA-treated cells after removal of the media and by the effect of PEG-SOD on the signal. In this graph, in contrast to (C), the CMH blank was not subtracted from the measurements, and the raw data is shown.

Mitochondrial superoxide in RAW 264.7 cells was detected using the EPR spin probe mito-TEMPO-H, which accumulates in mitochondia. (A) Representative EPR spectra for the baseline mito-TEMPO-H signal in buffer, the increased mito-TEMPO-H signal in control cells (Con), and the further enhanced signal in cells stimulated with the mitochondrial inhibitor Antimycin A (AA). The increase in the signal was attributed to the mitochondrial superoxide based on our previous study showing that SOD2 overexpression significantly attenuated measurements with mito-TEMPO-H10. In **Figure 2B**, the mitochondrial nitroxide concentration was determined by subtracting the mito-TEMPO-H signal in time-matched buffer from the cell measurements. The CM. signal obtained at low temperatures in RAW 264.7 cells after stimulation with PMA in the presence and absence of SOD. (**Figure 3A**) The CM. signal was attenuated in the presence of SOD, consistent with the room temperature data (**Figure 1**). **Figure 3B** shows the photograph of PTFE tubing with the stoppers used to collect data at 77 K for cells and *in vivo* samples.Superoxide production was detected in blood and BALF using the CMH spin probe. Blood or BALF samples were collected from PBS- and Bleo-treated mice and incubated immediately with CMH. The samples were transferred to the PTFE tubing and flash frozen, and EPR data was collected at 77 K. The concentration of nitroxide (CM.) accumulated in blood incubated with CMH (0.2 mM) at 37 degrees for 10 min. (**Figure 4A**).Nitroxide (CM.) concentration from BALF incubated for 50 min. (C) Nitroxide concentration represents the concentration of (CM.) accumulated in volume of blood or BALF used in the experiment (**Figure 4B**).

Three methods have been tested to evaluate several published techniques for tissue preservation and administration of spin probes *ex vivo* *vs*. *in vivo.* To perform EPR measurements on lung tissue, we first used flash frozen lung tissue from control or injured mice. **Figure 5A** shows the total CM. signal in the supernatant of a small piece of lung tissue incubated at 37 °C with CMH in PBS- and Bleo-treated mice, respectively. Due to heterogeneity of the lung injury after Bleo treatment, it is recommended to cut pieces from different regions of the lung and average several measurements to provide a more representative value. Alternatively, one can homogenize the entire lung and use one sample of this homogenate. Data collected at 77 K using PTFE tubing and finger dewar. **Figure 5B** shows representative spectra of nitroxide (CM.) signals from PBS- and Bleo-treated mice, respectively.

One limitation to treating lung tissue *ex vivo* is that it is not possible to reliably distinguish extracellular from intracellular superoxide due to the processing of the tissue that disrupts cell membranes. If this information is important to the experimental question, it can be addressed by using the *in vivo* CPH instillation method described below. Frozen tissue cannot be used to assess mitochondrial superoxide; though, for this measurement, the protocol can be adapted to use mito-TEMPO-H in the tissue or freshly isolated mitochondria.

As a second method for EPR measurements in lung tissue, fresh tissue was homogenized in sucrose buffer. The lung homogenate was incubated with CMH probe in KHB buffer containing DTPA. EPR measurements were carried out at RT. **Figure 6A** demonstrates the increase in CM. with Bleo. We presented an additional testing using different inhibitors that can be used to determine the species that contribute to the CM. signal. To elucidate the origin of CM. signal generated from lung tissue, we pretreated the lung homogenates with several scavengers and enzymes inhibitors. Lung homogenates were incubated with CMH in the absence or the presence of SOD, deferoxamine (DFO), and diphenyliodonium chloride (DIP) to account (respectively) for the contributions from superoxide, iron, or superoxide generated from flavin-containing enzymes (**Figure 6B**). This approach can be adapted to assess the specific radical species generated in a system or elucidate the contribution of other enzymatic sources (*e.g*., NOX, eNOS, or xanthine oxidase).

Mice were injected with CPH spin probes (20 mg/kg) *via* the retroorbital route to perform EPR measurements *in vivo*. It is unknown whether CMH can be safely administered to animals, while the CPH probe has been reported to be non-toxic; thus, we selected CPH for the *in vivo* experiments. Lung tissues were harvested and flash frozen in liquid nitrogen 1 h after circulation of CPH probes. Mice can be simultaneously treated with specific antioxidants to differentiate the species responsible for the signal. **Figure 7A** shows the higher CP. signal in Bleo-treated mice compared to control mice. Representative spectra of lung tissue from control and Bleo-treated mice are shown in **Figure 7B**. A mixed EPR spectra of CP. and ascorbic acid radical was observed. The values reported in **Figure 7A** are the concentrations of CP. components. Data were collected at RT using the tissue cell.

**FIGURE LEGENDS:**

**Figure 1: Detection of superoxide in different cell compartments.** (A) EPR spectra generated by 0.25 mM CMH in 0.5 mM hypoxanthine/xanthine oxidase (8 mU/mL) with and without SOD (30 U/mL). (B) RAW 264.7 cells (1 x 106 cells/well) were stimulated with 10 µM PMA in the presence of CMH for 50 min at 37 °C and nitroxide concentration (µM) detected in cell suspension (cells + buffer) and buffer collected from treated cells. (C) RAW 264.7 cells were stimulated with PMA *vs*. vehicle control (Con). One set of cells were pretreated for 10 min with 30 U/mL cell-impermeable SOD (PMA + SOD). Each color represents data from different experimental days and each point represents cells from an individual well. The nitroxide signal in a time-matched blank with CMH in KHB was subtracted from each signal to obtain final values. (D) Calculation of total and extracellular superoxide in PMA stimulated cells; T = total superoxide, EC = extracellular superoxide (SOD inhibitable signal). (E)To evaluate the intracellular superoxide signal (IC), the signal in buffer after PMA + SOD was compared to PMA-treated cells after the removal of buffer. To confirm, wells were pretreated with 60 U/mL cell-permeable PEG-SOD for 1.5 hours to determine the intracellular SOD inhibitable. The time-matched CMH blank is shown, and data reflect absolute nitroxide signal. Data expressed as mean ± SEM.

**Figure 2: Detection of mitochondrial superoxide in RAW cells stimulated with antimycin A.** (A) Representative spectra of the mitochondrial-specific EPR spin probe, 0.25 mM mito-TEMPO-H in RAW 264.7 cells without (Con) or with 25 µM antimycin A (AA) for 50 min at 37 °C. (B) CM. concentration (µM) in cells treated with AA compared to control. The nitroxide signal in a time-matched mito-TEMPO-H blank was subtracted from total signal to obtain final values. Data expressed as mean ± SEM.

**Figure 3: Detection of superoxide in RAW 264.7 cells at 77K**. (A) RAW 264.7 cells stimulated with 10 µM PMA and EPR spin probe, CMH 0.25 mM (50 min at 37 °C) with (black) or without (red) pretreatment with 30 U/mL SOD. 100 µL of supernatant was loaded in a 1-inch in length piece of PTFE tubing, then flash frozen in liquid nitrogen. The stoppers were removed, and frozen PTFE tubing was placed in the finger dewar for data acquisition at 77 K. (B) A photo of PTFE tubing and stoppers.

**Figure 4: EPR measurements in blood and BALF from control and bleomycin-treated mice**. Mice were treated with a single dose of intratracheal bleomycin (IT Bleo) (100 µL at 1 U/mL) or PBS vehicle. At 7 days, mice were anesthetized and euthanized. Blood was collected *via* right ventricular puncture into a syringe coated with 1000 USP/mL heparin containing 100 µM DTPA. Bronchoalveolar lavage fluid (BALF) was collected by lavaging the lungs with 1 mL of 100 µM DTPA in PBS. Blood and BALF were incubated for 10 or 50 min, respectively, with 0.2 mM CMH at 37 °C. 150 µL of blood or BALF was loaded in PTFE tubing flash frozen in liquid nitrogen and EPR data collected at 77 K using a finger dewar. Data show nitroxide concentrations in (A) blood and (B) BALF from PBS- and Bleo-treated mice (n = 4-6). Data expressed as mean ± SEM. (C) Representative spectra of nitroxide in blood from PBS- and Bleo-treated mice.

**Figure 5: EPR measurements in flash frozen lung tissue.** Mice were treated with a single dose of intratracheal bleomycin (IT bleo) (100 µL at 1 U/mL) or PBS vehicle. At 7 days, the lungs were flushed with cold PBS to remove blood and flash frozen in liquid nitrogen. 5-15 mg of flash-frozen lung tissue was incubated with 0.2 mM CMH in KHB containing 100 µM in 200 µL of total volume for 1 h at 37° C. Supernatant was collected and placed in PTFE tubing and run at 77 K in the finger dewar. (A) Nitroxide concentration (µM of nitroxide normalized to 1 mg of tissue). Data represent the average of 2-3 measurements for each lung. Data expressed as mean ± SEM. (B) Representative spectra of nitroxide in lung tissue from PBS- and Bleo-treated mice.

**Figure 6: EPR measurements in lung tissue preserved in sucrose buffer.** Mice were treated with a single dose of intratracheal bleomycin (100 µL at 1 U/mL). At 7 days post-treatment, the lungs were flushed with cold PBS to remove blood, and fresh lung tissue was homogenized in Tris-EDTA buffer containing 0.25 mM sucrose at a 1:6 lung weight/buffer volume (mg/µL) ratio. 50 µL of lung homogenate was preincubated with KHB with or without the following inhibitors for 20 min at 37 °C: SOD (100 U/mL), deferoxamine (DFO; 800 µM), and diphenyliodonium chloride (DIP); followed by incubation with 0.2 mM CMH in KHB containing 100 µM DTPA for 20 min at 37 °C. Data was obtained at RT using EPR capillary tubes. (A) Nitroxide concentration in lungs from PBS- and Bleo-treated mice. (B) Nitroxide concentration in Bleo lungs in the absence or the presence of inhibitors (n=3). Data expressed as mean ± SEM.

**Figure 7: EPR measurements in lung tissue from mice injected with CPH spin probe**. 100 µL of CPH was administered *via* retroorbital injection for a final concentration of 20 mg of CPH per kg of body weight. After 1 h of circulation, mice were euthanized, lungs were flushed with 10 mL of cold PBS *via* the right ventricle, and lung tissue was flash frozen. 20 to 30 mg of lung tissue was placed in tissue cell and EPR measurements performed at RT. (A) Data expressed as spins/mg. (B) Representative spectra of nitroxide signal in PBS and Bleo lung tissues (\* indicates the overlap with ascorbic acid radical). Data expressed as mean ± SEM.

**Table 1. Common inhibitors used to distinguish species responsible for spin probe oxidation.**

**DISCUSSION:**

The assessment of free radical production in biologicalsettings is important in understanding redox regulated signaling in health and disease, but the measure of these species is highly challenging due to the short half-life of free radical species and technical limitations with commonly used methods. EPR is a valuable and powerful tool in redox biology, as it is the only unambiguous method for detecting free radicals. In this project, we demonstrate practical EPR methods for designing experiments and preparing samples to detect ROS using spin probes in different cellular compartments *in vitro* and different tissue compartments *in vivo.* We also provide practical methods to handle biologicalsamples and store samples to improve efficiency.

Spin probes react efficiently with ROS and produce a stable nitroxide radical that can be detected with EPR. Several derivatives of the spin probe (cyclic hydroxylamine) have been synthesized with different permeability characteristics, which make them suitable for detecting free radical production in different cellular compartments10. This protocol utilized the cell-permeable spin probe, CMH; though, the impermeable spin probe 1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride HCl (CAT1H) can be used to detect extracellular superoxide. Similar to our prior study in human lymphoblast cell lines18, we were able to validate use of the permeable CMH spin probe with impermeable SOD and cell permeable PEG-SOD in RAW264.7 cells (a mouse lung macrophage cell line) stimulated with PMA to differentiate between extracellular and intracellular superoxide.

We also validated the rapid equilibration of CMH between the intra- and extra-cellular compartments, and we also found that the superoxide signal in cells drops significantly after washing the cells only once with KHB (data not shown). We confirmed utility of the mitochondrial specific spin probe mito-TEMPO-H in RAW 264.7 cells to measure the increased mitochondrial superoxide generated upon stimulation with mitochondrial electron transport chain inhibitor antimycin A. The specific contribution of mitochondrial superoxide production to the mito-TEMPO-H has been previously demonstrated and can be validated in experiments using isolated fresh mitochondria or systems with mitochondrial superoxide dismutase MnSOD (SOD2) overexpression10.

The assessment of ROS production *in vivo* is particularly challenging, but the ability to detect production of specific ROS provides important information when interrogating the role of oxidative stress or redox regulated signaling in biological settings. The appropriate handling of tissue when using spin probes and EPR is essential to generate reproducible and meaningful results. The use of spin probes with tissue will not likely measure superoxide radicals present at the time of tissue harvesting due to a short half-life, but instead it detects superoxide produced by enzymes such as NAPDH oxidase, uncoupled endothelial nitric oxide synthase, or xanthine oxidase when lung tissue or homogenates are incubated with the spin probe at 37 °C. The use of frozen tissue will not include superoxide generated by mitochondria, since freezing damages mitochondrial electron transport chain activity. To test mitochondrial superoxide, investigators need to isolate fresh mitochondria or use mitochondrial specific probes *in vivo* or in fresh tissue.

Several different protocols to preserve tissue have been published in the literature14,15. We compared three published methods for EPR measurements in lung tissue: 1) flash freezing tissue in liquid nitrogen, 2) homogenizing tissue in sucrose buffer, and 3) treating mice *in vivo* with a spin probe 1 hour before tissue harvesting. We compared control mice to mice with severe lung inflammation and oxidative stress induced by bleomycin to test each method’s ability to show consistent differences in nitroxide signals in injured lungs. All three methods showed a similar relative increase in nitroxide signal in the lungs of bleomycin-treated mice. The use of flash frozen tissue would likely be the easiest approach to collect tissue for most labs, negating the need to process tissue in the sucrose buffer at the time of harvesting. The injection of CPH to capture free radicals *in vivo* is powerful, but to confirm the specific species, this requires a treatment group including the appropriate antioxidant.

One challenge of using spin probes is that the oxidation of spin probes to nitroxide generates a similar three-line EPR spectrum regardless of the species responsible for the oxidation; thus, it does not distinguish between different ROS species. Also, it has been reported that there are potential reactions of hydroxylamine probes with photosynthetic electron transport chain and cytochrome c oxidase19,20. These observations should be considered when interpreting results. In this protocol, the photosynthetic system is not present, and the inclusion of DTPA with the buffer inhibits potential contamination of free ferric and cuprous ions10 . We demonstrated how to use a series of specific enzymes or chelators in lung tissue to establish the contribution of particular ROS or enzyme inhibitors to determine the source of ROS. This approach has been previously used with EPR to determine the contribution of ROS due to uncoupled eNOS13,15. We provide a list of common inhibitors used to distinguish species responsible for spin probe oxidation (**Table 1**).

We also demonstrated the importance of optimizing the incubation time for each experimental condition. When comparing spin probes to spin traps, spin traps generate unique spectra depending on the reactant which allows for specificity of the free radical species; however, they also exhibit slow kinetics for superoxide spin trapping and are prone to biodegradation. The treatment of lung tissue with the EPR probe *ex vivo* is also limited by an inability to adequately distinguish extracellular from intracellular superoxide due to the disruption of cell membranes during processing of the tissue (freezing or homogenizing). Use of the injected spin probe *in vivo* in conjunction with SOD or cell-permeable PEG-SOD can address this problem.

One goal was to establish a protocol to efficiently collect samples and store them at -80 °C prior to EPR measurements. We therefore developed a practical method to use PTFE tubing for holding the samples. This tubing is placed directly into the finger dewar for EPR analysis at 77 K without the need to clean the dewar between samples. This is an alternative to the recently published method involving the freezing of samples in 1 mL syringes. The measurements in frozen samples stored in PTFE tubing can be repeated over several days to demonstrate stability of the signal. This approach allows for batching the EPR measurements and facilitates transferring of the samples between laboratories so a remote EPR facility can run samples.

Overall, these protocols provide a straightforward approach to preparing cells and tissues for EPR measurements in biological systems. The protocols can be adapted to other models associated with oxidative stress and with the use of other spin probes. The timing and concentration of the spin probe will need to be adjusted for each experimental condition. The ability of EPR to determine the presence and production of free radical species unambiguously provides rigor to experimental approaches in the field of redox biology.

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**DISCLOSURES:**

The authors have nothing to disclose.

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