Chapter 5

Measurement of Mitochondrial (Dys)Function in Cellular Systems Using Electron Paramagnetic Resonance (EPR): Oxygen Consumption Rate and Superoxide Production

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Abstract

The oxygen consumption rate (OCR) and superoxide production are crucial when assessing mitochondrial function and/or dysfunction. EPR spectroscopy allows the measurement of both components either independently or simultaneously in a same cellular or mitochondrial preparation. OCR determination using EPR oximetry is based on the change in EPR linewidth of a paramagnetic oxygen sensing probe (a perdeuterated nitroxide) in the presence of oxygen consuming cells in a closed system. Superoxide production can be monitored by the oxidation of cyclic hydroxylamines into nitroxides. The contribution of superoxide to the nitroxide formation is deduced from experiments in the presence and in the absence of SOD and PEG-SOD as appropriate controls.

Key words EPR, ETC, Mitochondrial function, Oximetry, Oxygen consumption rate (OCR), Superoxide, Nitroxide, Hydroxylamine

1 Introduction

Mitochondria have a pivotal role in cellular energetics including oxidative phosphorylation to produce cellular ATP, but they also have important roles in apoptosis and programmed cell death, and in ROS production. Mitochondrial dysfunction may lead to pathological processes such as diabetes, cardiovascular diseases, and neurodegenerative disorders. Over the years, several mitochondrial modulators have emerged, notably in the field of anticancer therapies targeting cellular oxygenation and mitochondrial metabolism. Their impact on the oxygen consumption rate (OCR) and bioenergetics of this organelle is nowadays well studied but little has been investigated on their potential underlying incidence on reactive oxygen species (ROS) production and oxidative damage occurring during those treatments. When the balance between antioxidant systems and cellular ROS is disrupted, overpowering oxidative

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stress may lead to the oxidation of macromolecules like DNA, proteins and lipids, consequently inducing cellular damage and defects in cell signaling [1]. More specifically, superoxide may trigger cell death when produced in large excess but it was also suggested to be a key driver promoting cancer cell migration and metastasis when produced at more moderate levels [2]. Therefore, the dual dose-dependent effects on oxygen consumption rate (OCR) and superoxide production are crucial to consider when assessing mitochondrial function and/or dysfunction.

Electron paramagnetic resonance (EPR) is a method of choice for assessing the oxygen consumption and the superoxide production. The OCR determination is based on the change in EPR linewidth of a paramagnetic oxygen sensing probe (here, a nitroxide) in the presence of oxygen consuming cells in a closed system [3–5]. This method has been successfully applied to characterize the effect of inhibitors of the electron transport chain in cancer cells [3, 6–11]. In addition, EPR allows the detection of superoxide produced by the oxidation of cyclic hydroxylamines into nitroxides [12–15]. Because their oxidation into paramagnetic nitroxide can be due to several redox reactions, the contribution of superoxide to the nitroxide formation has to be deduced from experiments carried out in the presence and in the absence of SOD or PEG-SOD (pegylated-superoxide dismutase) as appropriate controls [14]. As described in this paper, both parameters may be measured independently.

Recently, our lab also developed an integrated toolbox enabling the simultaneous analysis of the OCR and superoxide production on a single mitochondrial preparation [16]. For the purpose, there is a need to measure the variation of the EPR linewidth of one nitroxide, on the one hand, and the formation of another nitroxide, on the other hand. The separation of the EPR signals coming from both nitroxides can be done by using different isotopically-labeled probes: ^{15}N-PDT (4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-^{15}N-1-oxyl) is the nitroxide used for OCR measurement and ^{14}N-CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) is the cyclic hydroxylamine that will be transformed into a nitroxide after reaction with the superoxide. The EPR signal coming from both nitroxides are not superimposable as ^{15}N-PDT gives a doublet while ^{14}N-CMH gives a triplet (Fig. 1). Therefore, the variation of the linewidth of ^{15}N-PDT (linked to OCR) and the formation of ^{14}N-CM• (linked to superoxide production) can be recorded simultaneously on a single cellular or mitochondrial preparation [16].

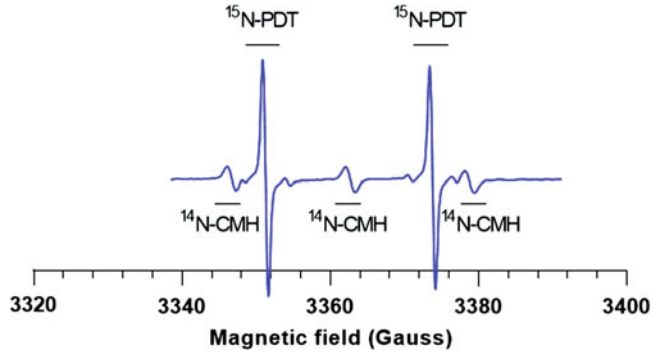

Fig. 1 Representative global EPR spectrum of ^{15}N-PDT and ^{14}N-CMH in PBS buffer injected into two separate capillaries, and gathered into a same quartz tube. Final concentrations of the probes are 100 μM and 0.5 mM, respectively

2 Materials

2.1 EPR Spectrometer

All EPR experiments were performed using a Bruker EMX-Plus spectrometer operating in X band (9.85 GHz) and equipped with a PremiumX ultra low noise microwave bridge and a SHQ high sensitivity resonator (*see* **Note 1**). The EPR cavity was heated at 310 K with air during all experiments. OCR and superoxide measurements can be performed on whole cells as well as on isolated mitochondria without changing material nor method. Specific concentrations for both models are informed for each assay. Skip the mitochondrial isolation part if using a whole cell model.

2.2 EPR Oximetry

1. Disposable microhematocrit capillaries in soda-lime glass (75 mm/75 μL).
2. Sealing gum for capillaries (*see* **Note 2**).
3. Phosphate buffered saline (PBS) from Gibco, pH 7.4, without calcium chloride nor magnesium chloride.
4. 20% Dextran from leuconostoc mesenteroides (average MW 60000–76000) solution diluted in PBS (*see* **Note 3**). Stock solution can be stored at 4 °C during a month.
5. ^{15}N-PDT (4-oxo-2,2,6,6-tetramethylpiperidined$_{16}$-^{15}N-1-oxyl) used as oxygen sensor (*see* **Note 4**). The required stock solution is 2 mM diluted into PBS. Store at 4 °C for short term use. Other aliquots should be stored at −80 °C until needed.
6. Biological system: depending on the type of experiment wanted: Stock solution of whole cells (around 5.10^6 cells/mL culture media) (*see* **Note 5**) or suspension of isolated mitochondria.

2.3 Superoxide Measurements

1. Gas-permeable polytetrafluoroethylene (PTFE) tubing (ZEUS) with inside diameter 0.025 in. and wall thickness 0.002 in., cut into 12 cm sections.
2. 1 mL syringes with 23G needles.
3. PBS (Gibco), pH 7.4, without calcium chloride nor magnesium chloride.
4. Parafilm.
5. Diethylenetriaminepentaacetic acid (DTPA): 100 mM stock solution in PBS. Store at room temperature.
6. Polyethylene glycol-superoxide dismutase (PEG-SOD): 4000 U/mL stock solution in PBS. Store at −20 °C.
7. Diamagnetic hydroxylamine for oxidation measurements: 1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine. HCl (CMH hydrochloride) used on mitochondria or (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride (MitoTempoH) for whole cell experiments. Stock solution are 10 mM and 1 mM, respectively, in PBS. Both probes are to be stored at −20 °C and flushed with argon at every use to avoid probe oxidation.
8. Biological system: depending on the type of experiment wanted: Stock solution of whole cells (around 20.10^6cells/mL culture media) or suspension of isolated mitochondria.

2.4 Mitochondrial Isolation

1. Cell line of interest, cultured in its recommended medium: about 40.10^6cells required resuspended in 1 mL of PBS (*see* **Note 6**).
2. Motor-driven Glass/Teflon potter homogenizer.
3. 0.1 M Tris/MOPS: Dissolve 1.21 g Tris in 40 mL distillated water, then adjust pH to 7.4 with MOPS powder. Bring to 50 mL and store at 4 °C.
4. 1 M EGTA/Tris: Dissolve 3.81 g EGTA in 40 mL distillated water, then adjust pH to 7.4 with Tris powder. Bring to 50 mL and store at 4 °C.
5. 1 M sucrose: Dissolve 34.2 g Sucrose in 100 mL distilled water and separate in 5 aliquots. Store at −20 °C.
6. Mitochondrial isolation buffer (100 mL to aliquot): Mix 10 mL of Tris/MOPS (0.1 M) and 1 mL of EGTA/Tris (1 M) to 20 mL of sucrose (1 M). Bring the mixture to 100 mL with distilled water and adjust pH to 7.4. Store at −20 °C.

2.5 Mitotoolbox

The Mitotoolbox combines OCR and superoxide measurements at the same time, meaning that all material listed for OCR and superoxide are also required for this experiment. Solutions and buffers

are kept on ice during the mitochondrial isolation procedure. Listed substrate supply includes NADH- and $FADH_2$-linked substrates to support state 3 respiration.

1. Isolated mitochondria (*see* "mitochondrial isolation" in **Methods**).
2. 100 mM EGTA: Dissolve 76 mg in 20 mL of water.
3. Mitochondrial Assay buffer (MAS (×2): This buffer is two times concentrated: Combine 12 g Sucrose, 20 g Mannitol, 68 mg potassium phosphate monobasic (KH_2PO_4), 0.5 g $MgCl_2$ hexahydrate, 23.8 mg HEPES, 1 g FA-free BSA and 5 mL of EGTA stock solution (100 mM) in 250 mL of water. Adjust pH to 7.4 and aliquot. Store at −20 °C.
4. 100 mM Pyruvate: dilute in PBS. Store at −80 °C.
5. 500 mM Succinate: dilute in PBS. Store at −80 °C.
6. 500 mM Malate: dilute in PBS. Store at −80 °C.
7. 100 mM ADP: Mix 425 mg ADP with 1.2 mL of PBS (ADP does not dissolve at this point). Neutralize with 5 M KOH (approx. 450 μL). ADP will dissolve after addition of KOH. Adjust pH to 7.4 and bring to 10 mL with PBS. Store at −80 °C.
8. Substrate Mix: Combine 300 μL of MAS (×2) with 216 μL PBS, 60 μL pyruvate (100 mM), 6 μL succinate (500 mM), 6 μL Malate (500 mM), and 12 μL ADP (100 mM). This final volume is enough for six combined measurements of OCR and Superoxide.

3 Methods

3.1 EPR Oximetry on Whole Cell Model

Keep the cell stock solution (lid open) in a 37 °C water bath during the experiments.

1. Turn on the X band EPR, heat the cavity at 310 K using continuous nitrogen flow (400 L/h) (*see* **Note 7**), and open Bruker Xenon Spin fit program.
2. Tune the EPR cavity at 19 dB attenuation (2.518 mW) with a sealed hematocrit capillary filled with PBS transferred in a quartz tube (*see* **Note 8**).
3. Set experimental parameters for acquisition: microwave power: 2.518 mW; modulation frequency: 100 kHz; modulation amplitude: 0.005 mT; Center field: 335mT; Sweep width: 1.5 mT; Sweep time:15 s.
4. Combine 60 μL of cell suspension to 40 μL of Dextran 20% solution.

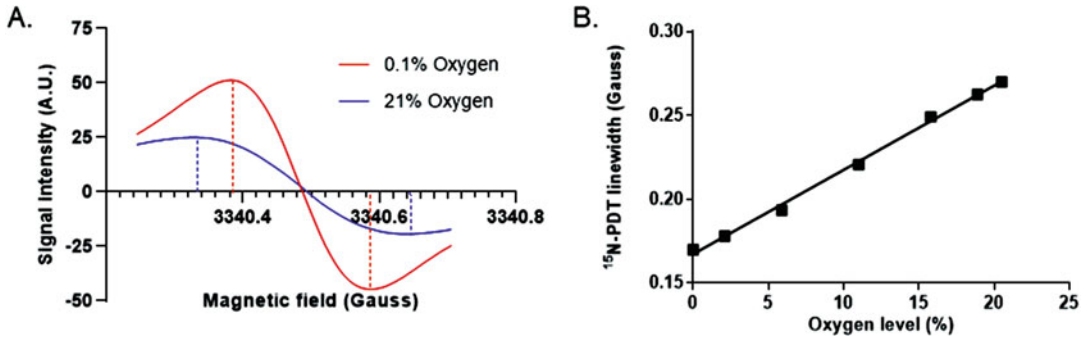

Fig. 2 (**a**): ^{15}N-PDT spectra at 0.1% (red) and 21% (blue) of oxygen mixed with nitrogen. (**b**): ^{15}N-PDT calibration curve linking the probe's linewidth (Gauss) to oxygen level (%) present into the sealed hematocrit capillary. Both spectra correspond to the high-field line

5. Add 4 μL of ^{15}N-PDT stock solution to the previous mixture and mix well.
6. Transfer mix into hematocrit capillary and seal with gum. Slip capillary carefully into a quartz tube and transfer it to the EPR cavity.
7. Tune the EPR cavity and wait 3 min after the probe was mixed before launching an automated "2D-field-Delay" measurement counting 15 points with a time delay of 60,000 ms.
8. Save file and proceed to data analysis after all experiments are done: switch to processing mode and load your file. Select "Peak picking" and define the region containing the peak on all slices with "region qualifier." Select pick > Peak and through > Dist Algorithm > All slices > Pick > Report distances. The final file can be saved as ASCII file to extract linewidth data at each point.
9. Correlate ^{15}N-PDT linewidth with the % of oxygen with a calibration curve (*see* **Note 9** and Fig. 2).
10. OCR corresponds to the slope of oxygen level during time.

3.2 Superoxide Measurement on Whole Cell Model

Keep the cell stock solution (lid open) in a 37 °C water bath during the experiments.

1. Turn on the X band EPR, heat the cavity at 310 K using continuous air flow (400 L/h) and open Bruker Xenon Spin fit program.
2. Tune the EPR cavity at 10 dB attenuation (20 mW) with a 12 cm long PTFE tube folded in 4 filled with PBS, transferred in an open quartz tube (*see* **Note 10**). Seal the end of the quartz tube with a patch of parafilm to avoid a potential leak into the EPR cavity. Set experimental parameters for acquisition:

microwave power: 20 mW; modulation frequency: 100 kHz; modulation amplitude: 0.1 mT; Center field: 336.5mT; Sweep width: 1.5 mT; Sweep time: 30.48 s.

3. For control condition, combine 37 μL of cell suspension to 0.5 μL od DTPA (100 mM), 5 μL of PBS and end by mixing 7.5 μL of MitoTempoH (1 mM). To measure superoxide contribution, make another measurement using the same conditions but replacing 2.5 μL PBS by adding 2.5 μL of PEG-SOD (4000 U/mL). Let incubate 10 min before adding the MitoTempoH. It is mandatory to flush MitoTempoH stock solution with argon before and during pipetting to avoid probe oxidation.
4. Transfer mix into PTFA tube cutting using the needle. Fold in 4 and insert into the open quartz tube. Seal quartz tube with a patch of parafilm and transfer it to the EPR cavity.
5. Tune the EPR cavity and wait 3 min after the probe was mixed before launching an automated "2D-field-Delay" measurement counting 11 points with a time delay of 40,000 ms.
6. Save file and proceed to data analysis after all experiments are done: switch to processing mode and load your file (Fig. 3). Select "Integration & derivative" and define the region containing the peak in all slices. Select "Double Integration" and copy results to primary. Then, switch to time domain > Store & return > Structure > Projection > Roof. The final file can be saved as ASCII file to extract double integration (DI) values for each timepoint (Fig. 4).
7. Subtract point 1 DI from point 11 DI for each condition. Superoxide contribution is measured by subtracting mean PEG-SOD DI to mean control DI.

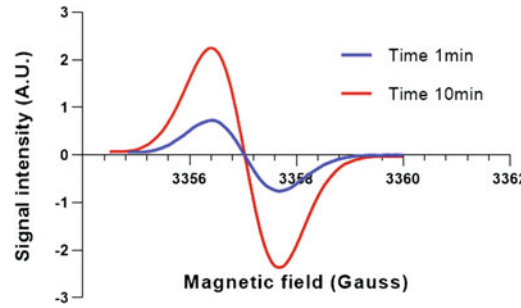

Fig. 3 Representative example of ^{14}N-CMH oxidation spectra after 1 and 10 min on whole cell model. These spectra are similar to those obtained with MitoTempoH probe. Both spectra correspond to the mid-field line

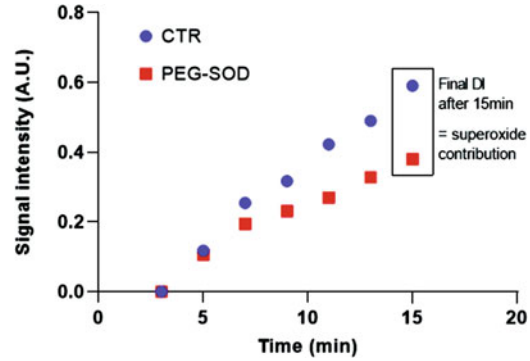

Fig. 4 Representative example of MitoTempoH DI spectra during time on isolated mitochondria. Same kind of spectra are obtained when using a whole cell model as well. Blue dots represent control condition and red dots represent PEG-SOD condition. Superoxide contribution is measured by subtracting the PEG-SOD DI to the CTR DI after 15 min

3.3 Mitochondrial Isolation

Mitochondrial isolation buffer (MIB) should be toughed in advance and stored on ice. All steps of mitochondrial isolation are performed on ice or at 4 °C to avoid damage by proteases and phospholipases. The potter homogenizer is precooled on ice before use. This protocol has been adapted from [17].

1. Harvest cultured cell line of interest to get around 40.10^6 cells (or more if needed, *see* **Notes 6** and **11**).
2. Resuspend cells in 1 mL PBS and centrifuge cells at 600 × *g* at 4 °C during 10 min.
3. Remove the supernatant and resuspend the cells in 3 mL of ice-cold MIB.
4. Transfer the cell mixture into the precooled potter and blend with the motor-driven pestle at 1600 rpm making 35 strokes. To maintain cooling temperature during homogenization, the entire device is placed in a box or beaker filled with ice (*see* **Note 12**).
5. Transfer the homogenate to a 15 mL falcon tube and centrifuge at 600 × *g* at 4 °C during 10 min.
6. Collect the supernatant and centrifuge at 7000 × *g* at 4 °C during 10 min.
7. Remove the supernatant and wash the pellet with 200 μL of MIB before transferring the homogenate to a 1.5 mL Eppendorf.
8. Centrifuge the suspension at 7000 × *g* at 4 °C during 10 min.
9. Discard the supernatant and gently homogenize the mitochondria pellet with the little remaining supernatant left, avoiding bubble formation.
10. Store on ice (*see* **Note 13**).

3.4 Mitotoolbox: OCR and Superoxide Simultaneous Measurement

The Mitotoolbox combines oximetry and superoxide measurements at the same time on the same mitochondrial isolate, meaning that all material listed for EPR oximetry and superoxide measurements are also required to perform the experiment. Of note, this simultaneous measurement of mitochondrial function can also be measured on a whole cell model using the same EPR device as well as EPR parameters with the cell stock solutions given in the **Material** section "EPR oximetry" and "superoxide measurements."

1. Store mitochondrial isolate and substrate mix on ice.
2. Respiration mix (for six independent measurements): combine 275 μL substrates, 137.5 μL of MAS (×2), 132 μL dextran 20%, and 5.5 μL DTPA. Mix well.
3. Superoxide mix (for six independent measurements): combine 125 μL substrates, 62.5 μL MAS (×2), 60 μL PBS, and 2.5 μL DTPA. Mix well.
4. Turn on the X band EPR, heat the cavity at 310 K using continuous air flow (400 L/h) and open Bruker Xenon Spin fit program.
5. Tune the EPR cavity at 19 dB attenuation (2.518 mW) with a 12 cm long PTFE tube folded in 4 filled with PBS and a sealed hematocrit capillary filled with PBS (*see* **Note 14** and Fig. 5).
6. Create and save a separate OCR parameters file on Xenon: microwave power: 2.518 mW; modulation frequency: 100 kHz; modulation amplitude: 0.005 mT; Receiver gain: 45 dB; Center field:334 mT; Sweep width: 1.5 mT; Sweeptime: 25 s.
7. Create and save a separate superoxide parameters file on Xenon: microwave power: 2.518 mW; modulation frequency: 100 kHz; modulation amplitude: 0.1 mT; Receiver gain: 60 dB; Center field: 334.4 mT; Sweep width: 1.5 mT; Sweeptime: 25 s.
8. Final OCR solution for EPR: combine 85 μL of respiration mix with 7 μL PBS, 3 μL of isolated mitochondria and add 5 μL of ^{15}N-PDT (2 mM) at the last minute.
9. Final superoxide solution for EPR: combine 32 μL superoxide mix with 3 μL PBS, 3 μL isolated mitochondria and add 2 μL of ^{14}N-CMH (10 mM) at the last minute.
10. ^{15}N-PDT and ^{14}N-CMH should be inserted to their mix at the same time. Hematocrit capillary and PTFA tubing are filled with their corresponding final solution, and put together into the open quartz tube. Transfer the device into the EPR cavity.
11. 3 min after probes were added to their final mix, launch first superoxide acquisition with the corresponding parameters and

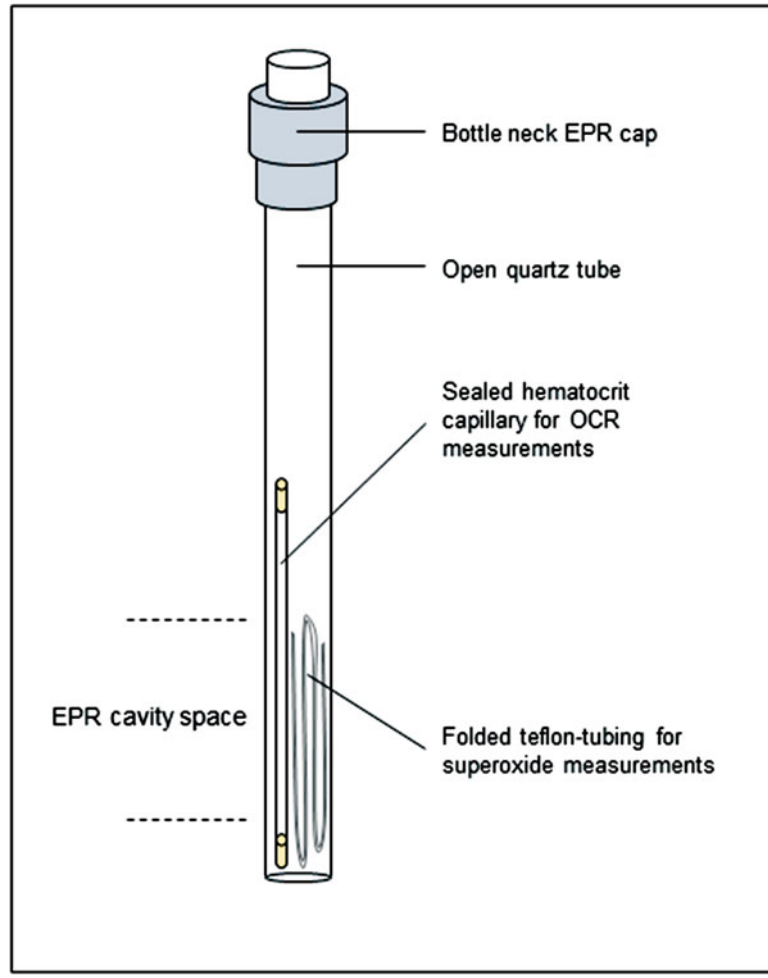

Fig. 5 Schematic representation of the double capillary device used to measure OCR and superoxide simultaneously

save file. Launch OCR acquisition every even number of minutes and superoxide on odd number of minutes after probe addition. Save files on Xenon for data analysis.

12. To measure superoxide contribution, add 5 μL of PEG-SOD to final OCR solution and 2 μL PEG-SOD to the final superoxide solution (adding less PBS in consequence).
13. Data analysis are performed the same way as stated in section "EPR oximetry" and section "superoxide measurement" on whole cell model.
14. Normalization by mitochondrial protein quantification is recommended (*see* **Note 15**).

4 Notes

1. Any EPR spectrometer can be used as long as it is capable of being equilibrated at 310 K with continuous gas flow.
2. Gum can contain paramagnetic manganese visible with EPR when inserted into the cavity's resonator. To avoid nonspecific spectra appearing during analysis, make sure the sealed part of the hematocrit capillary remains outside the resonator.
3. Dextran solution is used to keep cells or isolated mitochondria in suspension in the capillary during the whole measurement. Do not use dextran with a lower MW than stated, otherwise cells tend to sedimentate and EPR measurements will not be accurate.
4. ^{15}N-PDT (perdeuterated nitroxide) is a very sensitive probe to measure oxygen levels in a medium using EPR technology. Its linewidth is proportional to the oxygen level present into the capillary. A calibration linking the probe linewidth broadening to oxygen level should therefore first be performed in order to measure OCR during an experiment with cells or mitochondrial suspension.
5. For whole cells experiments, a stock solution of 5.10^6 cells/mL should be sufficient to measure a suitable oxygen consumption. However, depending on the cell type, this cell concentration can be fine-tuned beforehand by performing dose-response tests to get the best oxygen consumption curve (some cells are more rapidly consuming oxygen than others). This new stock concentration should therefore be used for all other oximetry experiments using this cell line.
6. Depending on the cell line, this initial cell number could be increased to have a better yield and perform more measurements from the same isolated batch of mitochondria. Typically, at the end of the process, enough mitochondria are collected (40 μL with a mean of 25 mg of protein/mL) to make four conditions with simultaneous measurements of OCR and superoxide, using 3T3 fibroblasts.
7. In this case, EPR cavity can also be heated with air but nitrogen is less expensive. The capillary being sealed off, the type of gas used to heat the cavity does not matter because it will not affect the sample.
8. The tuning of the EPR cavity is performed at 19 dB attenuation (2.518 mW) with the same device used for the actual oximetry experiments, namely a sealed hematocrit capillary filled with PBS and ^{15}N-PDT (oxygen sensor), inserted into a quartz tube that is then transferred to the EPR cavity. Mixing the oxygen sensor at this stage is not mandatory but recommended in

order to find the exact magnetic field value where the ^{15}N-PDT spectrum is appearing.

9. The calibration to link ^{15}N-PDT linewidth with the level of oxygen in a capillary is performed using an EPR spectrometer connected to an Aalborg gas mixer and an oxygen analyzer (Servomex OA540). Calibration mixture contains PBS and Dextran (20%) at a final ratio 1:1 mixed with 4 μL of ^{15}N-PDT (2 mM). ^{15}N-PDT linewidth is measured at different % of oxygen (7pts between 0 and 21%) mixed with nitrogen. Wait 40 s before each measurement for the oximeter to equilibrate. The obtained curve equation is then used to measure % of oxygen during experiments on biological systems.
10. To fill the PTFA tubing, insert needle into a tube segment and suck up the PBS or final cell solution until the segment is entirely filled. Fold in 4 and insert the tube at the open end of the quartz tube.
11. Do not treat cells with any agents before harvesting. Mitochondrial isolation is performed on control/non-treated cells and treatment of any kind should be added in final OCR or final superoxide solutions as a new condition to analyze its impact on mitochondrial function.
12. Homogenization by a motor-driven Glass/Teflon potter tends to heat up the cell mixture. To avoid damaging activation of proteases and phosphatases and maintain mitochondrial integrity, keeping the potter on ice is essential.
13. Isolated mitochondria should be used within 1–3 h for better responses.
14. The two tubes are inserted next to each other in the open quartz tube.
15. Protein quantification can be performed using BCA protein Assay. Mitochondrial isolates are diluted 1:20 and protein determination can be assessed following manufacturer's instructions.

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