Mitochondrial Superoxide production in Dermal Sheets

Materials

Reconstructed dermal tissue (referred to as Dermal sheets)

24-well plate, tissue culture treated (ref: Falcon 353047)

HBSS, with Ca and Mg (ref: Thermo Fisher 14025092)

PBS without Ca and Mg (ref: Eurobio CS1PBS01)

Lacquer (KIKO Milano Smart fast dry nail lacquer)

Equipment

Multiphoton Leica SP8 DIVE microscope

Microscope slide with spacer

Coverslips # 1.5 thickness

Biological safety cabinet, class 2 (SafeFAST Top, FASTER)

CO_2 Incubator (Binder world), adapted into a radiofrequency reverberation chamber (See ref. [1] for a detailed procedure)

Procedure

Prepare stain solutions

4 µM Hoechst in HBSS with Ca and Mg

2 µM mitoSOX red in HBSS with Ca and Mg

Keep them protected from light

Counter-stain

Aspirate growth medium

Add 4 µM Hoechst (1 ml)

Incubate for 30 min at 37 °C, 5% CO_2

Aspirate staining solution

Treatment

Negative control: sham RF exposure

Positive control: Incubate with 10-50 µM Antimycin A for 1h

RF exposure: 10, 50, 100 W/m^2 for 1h

Aspirate medium

Stain

Add mitoSOX red solution (1 ml per well)

Incubate 30 min at 37 °C, 5% CO_2

Wash with PBS

Microscope sample prepation

Place dermal sheet on a microscope slide with a spacer, using tweezers

Add some drops of HBSS (200-400 µl)

Place a coverslip on top of the spacer.

Make sure there are no air bubbles between the coverslip and the slide

Add some lacquer to keep the coverslip in place

Wait 1-2 minutes for the lacquer to dry

Microscopy

Mount the slide on the platform of the multiphoton microscope

Acquisition settings:

Resolution	1024 x 1024 px	**Zoom**	1 (25X)
Scan speed	400 Hz	**Pixel dwell time**	1.4 µs
Frame avg	1	**Frame accum**	2
Laser wavelength	800 nm	**Laser intensity**	1.5 %

With two hybrid detectors:

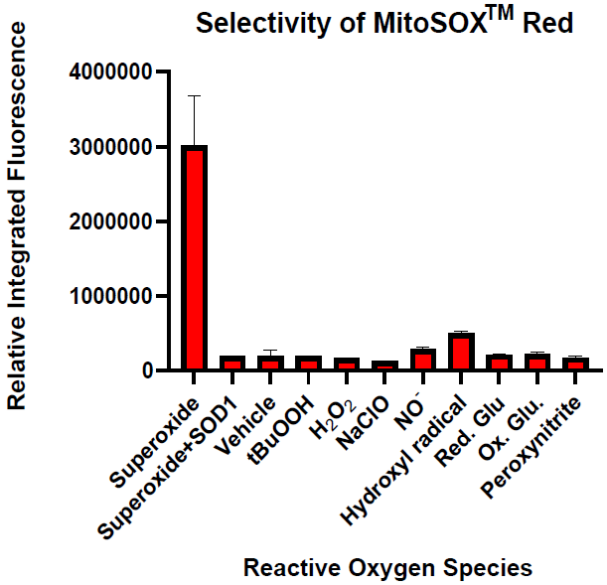
1. Hoechst (counter-stain): Emission detection range = 440 - 470 nm, Gain = 10
2. MitoSOX red: Emission detection range = 565 - 600 nm, Gain = 30

Acquire Z-stacks with a step of 1.5 µm in n ≥ 3 regions of the dermal sheet.

Notes

mitoSOX red is highly specific to superoxide [2]. High concentrations (over 2-5 µM) are toxic to cells, but signal is very low for lower concentrations (0.5 to 1 µM).

MitoSOX red is electrophoretically attracted to healthy mitochondria. The oxidated product of mitoSOX red is often located in the cell nucleus, where it intercalates in the DNA [3]. It escapes mitochondria due to membrane depolarization and/or mitochondria burst due to high ROS.



References

[1] R. Orlacchio *et al.*, "A Novel Reverberation Chamber for In Vitro Bioelectromagnetic Experiments at 3.5 GHz," *IEEE Trans. Electromagn. Compat.*, vol. 65, no. 1, pp. 39–50, Feb. 2023, doi: 10.1109/TEMC.2022.3216045.

[2] Invitrogen, "MitoSOX Green and MitoSOX Red Mitochondrial Superoxide Indicators." Accessed: Apr. 28, 2025. [Online]. Available: https://www.thermofisher.com/order/catalog/product/fr/en/M36008

[3] G. V. Raghuram *et al.*, "Cell-free chromatin particles released from dying cells inflict mitochondrial damage and ROS production in living cells," *Cell Death Discov.*, vol. 10, no. 1, pp. 1–9, Jan. 2024, doi: 10.1038/s41420-023-01728-z.