Mitochondrial membrane depolarization 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)

FCCP (CAS Number: 370-86-5, ref: Biosciences SIH-222)

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

100 nM TMRM 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 5-20 µM FCCP for 30 minutes

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

Aspirate medium

Stain

Add TMRM 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. TMRM: Emission detection range = 570 - 587 nm, Gain = 30

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

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.