

## Mitochondrial Transmembrane Potential ( $\Psi_m$ ) Assay Using TMRM

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**[Abstract]** During cellular respiration, nutrients are oxidized to generate energy through a mechanism called oxidative phosphorylation, which occurs in the mitochondria. During oxidative phosphorylation, the gradual degradation of molecules through the TCA cycle releases electrons from the covalent bonds that are broken. These electrons are captured by NAD<sup>+</sup> through its reduction into NADH. Finally, NADH transports the electrons to the complexes of the electron chain in the internal membrane of mitochondria. These complexes use the energy released by the electrons to pump protons into the intermembrane space, generating an electrochemical gradient across the internal membrane of mitochondria, which provides energy for the ATP-synthase complex, ultimately producing adenosine triphosphate (ATP). We assessed the mitochondrial membrane potential ( $\Psi_m$ ) using tetramethylrhodamine methyl ester (TMRM), a cell-permeant, cationic, red fluorescent dye. To measure specifically the mitochondrial membrane potential ( $\Psi_m$ ) we quantified the fluorescence intensity before and after applying FCCP, a mitochondrial electron chain uncoupler. The difference of intensity before and after applying FCCP corresponds specifically to the mitochondrial membrane potential. We analyzed mitochondrial membrane potential ( $\Psi_m$ ) by cytofluorimetry. The ratio between the total level of signal and the signal generated after uncoupling provided a normalized value for the difference in cell size. Furthermore, to normalize for the different size of cells that we were analyzing we have analyzed TMRM in live imaging using IN Cell Analyzer, so that the level of mitochondrial membrane potential could be detected per unit of mitochondrial membrane area measured. Thus, our protocol can also be used to compare the mitochondrial membrane potential of cells that are different in size.

### Materials and Reagents

1. Murine Embryonic Fibroblasts (MEF)
2. Phenol-red free HBSS (Gibco®, catalog number:14175-079)
3. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco®, catalog number: 15630-080)
4. Tetramethylrhodamine methyl ester (TMRM) (Life Technologies, catalog number: T668)
5. Cyclosporin H (Enzo Life Sciences, catalog number: ALX-380-286-M001)

6. Hoechst 33342
7. Carbonylcyanide-p-trifluoromethoxyphenyl hydrazone FCCP (Sigma-Aldrich, catalog number: C2920-10MG)
8. Trypsin (451) - Trypsin, 0.05% (1X) with 0.53 mM EDTA 4Na, liquid, 20 x 100 ml (Gibco®, catalog number: 25300096)

### **Equipment**

1. IN Cell Analyzer 1000 (General Electric Company)
2. 37 °C 5% CO<sub>2</sub> Cell culture incubator
3. 12 well plates (Corning, Costar®, catalog number: CLS 3513)
4. FACScan (BD)
5. Centrifuge spinning speed: 15682,186 x g (G-force) (13,000 rpm, 8.3 cm radius)

### **Software**

1. IN Cell Investigator Analysis software (General Electric Company)
2. BD CellQuest software
3. FlowJo software

### **Procedure**

#### **A. For quantitative real-time analysis**

1. Cells are plated at 50% cell confluence.
2. Cells are incubated for 30 min at 37 °C in phenol-red free HBSS with 10 mM HEPES, 20 nM TMRM, 2 µM cyclosporine-H, inhibitor of multidrug resistance pump activity but not of the permeability transition pore (multi drug resistance pump activity can affect the mitochondrial loading with TMRM) and 2 µg/ml Hoechst 33342, nuclear dye that can be used in living cells.
3. Sequential images were taken before (3 time points) and after 4 µM FCCP (3 time points) was injected in a motorized way for TMRM (535 nm excitation filter; 600 nm emission) and Hoechst 33342 (360 nm excitation filter; 460 nm emission) in different fields (4 fields acquired for each well and 6 wells for each experimental condition) every 3 min with an IN CELL Analyzer 1000.
4. Images are automatically analyzed with IN Cell Investigator Analysis software to measure the TMRM intensity. Fluorescence intensity is measured on the average of 10 points

randomly selected for each field at the first and the last time point, the background corresponding to an area without cell is removed for each field.

5. The fluorescence intensity measured at the last time point (after applying FCCP) is normalized by the fluorescence intensity measured at the first time point of the experiment.

#### B. For FACS analysis

1. Cells are plated at 50% confluence.
2. Cells are trypsinized (at least 2 millions of cells).
3. Cells are centrifuged for 5 min at G-force 13,62.
4. Cells are washed in PBS, resuspended in phenol-red free HBSS with 10 mM HEPES and counted.
5. Cells (300,000 cells per tube, each condition done in triplicate) are resuspended the in phenol-red free HBSS with 10 mM HEPES and 20 nM TMRM in the presence of 2  $\mu$ M of the multidrug resistance pump inhibitor cyclosporine-H and incubated for 30 min at 37 °C. In parallel, cells are incubated 5 min in the same conditions with an uncoupling agent, 4  $\mu$ M FCCP, to measure the specific mitochondria staining.
6. FACS analysis for TMRM was performed on BD FACScan with CellQuest software and analysed using FlowJo software. Fluorescence intensity of TMRM was calculated by gating on live cells.
7. The intensity of the fluorescence with FCCP was normalized by the intensity without FCCP in order to rule out the possibility that the difference of the intensity is only or mainly caused by the cell sizes.

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#### **References**

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