Mitochondrial staining of NK cells by flow cytometry

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We use this protocol and it's working

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DISCLAIMER

This protocol details how we follow the manufacturer's instructions for staining of mitochondrial features by flow cytometry.

ABSTRACT

Mitotracker Green (Thermo Fisher Scientific, cat. M7514) stains mitochondrial membranes, so gives a quick measure of mitochondrial mass per cell. However, it is not always as sensitive as quantifying mitochondrial mass by microscopy and staining intensity can be affected by mitochondrial swelling or reactive oxygen species.

Mitochondrial membrane potential assay kit (Cell Signaling Technology cat. 13296S) gives a fluorescent indication of the charge across mitochondrial membranes and thus the health of the mitochondria. A membrane charge uncoupler is included in the kit that depolarizes mitochondrial membranes and is thus a useful negative control.

MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific cat. M36008) detects reactive oxygen species within the cell. To see how much reactive oxygen species are generated in the face of oxidative stress, the cells can be pre-incubated with hydrogen peroxide and then stained with MitSOX indicator.

TO-PRO-3 (Thermo Fisher Scientific, cat. T3605) is a non-membrane permeable DNA-dye to discriminate live cells. Staining for CD56+ CD3- cells allows analysis of NK cells, not any contaminating cells.

These stains are performed on live cells and analyzed without fixation.

MATERIALS

Mitotracker Green (Thermo Fisher Scientific, cat. M7514)
Mitochondrial membrane potential assay kit (Cell Signaling Technology cat. 13296S) - contains TMRE and CCCP
MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific cat. M36008)
TO-PRO-3 (Thermo Fisher Scientific, cat. T3605)
anti-CD3-Bv785 (clone OKT3, Biolegend, cat. 317330, RRID:AB_2563507)
anti-CD56-PE-Cy7 (clone HCD56 cat. 318318, Biolegend, RRID:AB_604107)
RPMI (Gibco, cat. 2240-089)
Hanks' balanced salt solution (Gibco, 14025-092)
Human AB serum (Valley biomedical, cat. HP-1022HI)
UltraPure 0.5M EDTA, pH8 (Life Technologies, cat. 15575020)
PBS (Corning, 21-040-CV)
DMSO (Cat. BP231-100, Fisher Bioreagents)
Hydrogen peroxide solution (Sigma-Aldrich, cat. 216763-100ML)

Mitotracker and TMRM combined staining *(keep on ice)*

1 Resuspend 1x10^6 cells in 850 µL RPMI medium (no serum) for all tubes. Keep tubes on ice

2 Before diluting reagents, set up the CCCP negative controls:
- Add 1 µL CCCP to tubes containing 1x10^6 cells in 850 µL RPMI
- Incubate at 37°C (no CO_2) for 20 min.

3 Prepare staining reagents and keep on ice:
TMRM:
- Stock (can be freeze-thawed): prepare by adding 55 µL DMSO to lyophilized TMRM
- Working dilution: 2 µL stock into 1 mL RPMI

Mitotracker Green:
- Stock (can be freeze-thawed): Add 74.48 µL DMSO to tube contents to obtain 1 mM Mitotracker Green.
- Working dilution 100 nM in RPMI *(i.e. 1:100 to get 10 µM stock; then 1:100 to get 100 nM solution)*

4 After CCCP incubation, add TMRM and Mitotracker Green to ALL tubes (including CCCP controls):
- 100 µL TMRM

- 50 µL Mitotracker Green (from 100nM working solution)

Incubate at 37°C (no CO_2) for 20 min.

5 Wash with **flow buffer (1% human AB serum, 0.5 mM EDTA in PBS)**.
- If staining in a 96 well plate, wash 3 times with 200 µl flow buffer.
- If staining in a tube, wash once with 3 mL flow buffer.

Keep on ice until surface staining

(Optional) Stress mitochondria with hydrogen peroxide

6 Prior to harvesting cells for mitoSOX staining, they can be stressed with hydrogen peroxide:
- Resuspend 500,000 cells/condition in fresh media.
- Add a titration of H_2O_2 *e.g. 0 µM, 10 µM, 50 µM, 100 µM.*
- Incubate for 1h at 37ºC.

Proceed to staining.

MitoSOX staining *(keep on ice)*

7 Spin down 500,000 cells per tube and remove supernatant (400*g* for 5 min)

8 Dilute reagents:
- Stock (can be freeze-thawed if necessary): Dissolve MitoSOX reagent in 13 µL DMSO to make 5 mM solution
- Dilute 5mM solution 1:1000 in Hanks' balanced salt solution for 5 µM solution
- For DMSO control, dilute DMSO 1:1000 in Hanks' balanced salt solution.

9 Add 200 µL working solution to each tube

10 Incubate cells at 37°C for 15 min.

11 Wash cells with flow buffer
- If staining in a 96 well plate, wash 3 times with 200 µl flow buffer.
- If staining in a tube, wash once with 3mL flow buffer.

Stain for live NK cells

12 Prepare stain mix with anti-CD3 and anti-CD56
- anti-CD3 BV785 – 5 µL/sample
- anti-CD56 PE-Cy7 – 5 µL/sample
- Flow buffer – 90 µL/sample

13 After spinning down cells and completing washes (see above), add 100 µL stain/tube.

14 Incubate at 4°C for 15 min.

15 Wash twice with flow buffer

16 Dilute TO-PRO-3 dye (1:10,000) in flow buffer.

17 Add 300 µL flow buffer per tube

18 Add 200 µL diluted TO-PRO-3 per tube

19 Run immediately on flow cytometer (LSRII, BD Biosciences)

20 Live (TO-PRO-3-negative) single NK cells (CD56+ CD3-) cells can then be quantified for the amounts of mitochondrial stains relative to negative controls (DMSO and CCCP).