Production of Superoxide Ion in the Mitochondria

Materials and Reagents:

- **MitoSOX Red Mitochondrial Superoxide Indicator** (Molecular Probes M36008) 10x50 µg
- **Storage:** -20°C in darkness, aliquot required.
- **Shelf life:** 6 months.
- **HBSS with Calcium and Magnesium (HBSS+)**
- **Sytox Blue** (Storage: -20°C, stable for 1 year, can be aliquoted)
- **APC Annexin V** (25 tests, 5 µL/test, store at 4°C in darkness, calcium-dependent)
- **Culture Medium:** DMEM/high glucose 10% FBS 1% penicillin/streptomycin
- **PBS without calcium and magnesium (1X)**
- **Trypsin-EDTA 0.05%**
- **35 mm culture dishes**
- **Flow cytometer** (excitation/emission settings detailed below)

MitoSOX Characteristics:

- **Excited by:** 488 nm blue laser
- **Ex:** 510 nm
- **Em:** 580 nm (similar to PE)
- **MitoSOX is oxidized by superoxide ions but not by other ROS or reactive nitrogen species.**
- **Oxidation is prevented by superoxide dismutase and becomes highly fluorescent upon binding to nucleic acids.**
- **The positively charged probe accumulates in the mitochondria.**

Annexin V Characteristics:

- **Excited by:** 650 nm red laser
- **Emission Peak:** 660 nm (similar to APC)

Sytox Blue Characteristics:

- **Excited by:** 405 nm violet laser
- **Ex:** 444 nm
- **Em:** 480 nm (similar to Pacific Blue)

Experimental Protocols

1. Cell Preparation

- **Day -3:** Seed **1.5 million SH-SY5Y cells** per **35 mm Petri dish** in **2 mL of culture medium**.
- **Day 0 (Experimentation)**
  - Ensure cells are **80% confluent**.
  - Replace the culture medium with **2 mL of pre-warmed PBS 1X** and wash **once**.

2. Solution Preparation

- **MitoSOX Reagent Solution (2.5 mM)**
  - Dissolve **50 µg MitoSOX** in **26.4 µL DMSO**.
  - Aliquot and store at -20°C.
  - Prepare fresh (< 15 min before use).
  - Can be reused once.
- **Culture Medium and HBSS:** Pre-warmed to **37°C**.

3. Cell Staining with MitoSOX Red:

1. Replace PBS with **1 mL of MitoSOX Red solution at 2.5 µM per dish**.
2. Incubate for **30 minutes at 37°C, protected from light**.
3. Wash cells with **1 mL PBS**.

4. Trypsinization and Collection:

1. Trypsinize cells with **500 µL trypsin per dish**, ensuring a single-cell suspension.
2. Add **1 mL of culture medium**, transfer to flow cytometry tubes.
3. Wash wells with **1 mL PBS** to recover all cells.
4. Wash cells **twice with HBSS+** (centrifuge **5 min, 1200 rpm, RT**).

5. Annexin V and Sytox Blue Staining

1. Discard the supernatant and gently mix the cell pellet.
2. Resuspend the cells in **100 µL of 1X Binding Buffer per 500,000 cells**.
3. Add **5 µL of Annexin V** and incubate for **20 minutes at RT in the dark**.
4. Add **400 µL of 1X Binding Buffer per tube**.
5. Add **3 µL Sytox Blue**.
6. Incubate **5 minutes at RT, protected from light**.

6. Flow Cytometry Analysis

1. Keep tubes **on ice** and analyze within **30 minutes**.
2. Exclude dead cells and analyze only the main cell population.

Flow Cytometry Parameters

- **Excitation:** 488 nm (MitoSOX), 405 nm (Sytox Blue), 650 nm (Annexin V)
- **Emission:** 580 nm (MitoSOX), 480 nm (Sytox Blue), 660 nm (Annexin V)
- **Compensation:** Include a tube with unstained cells to define baseline fluorescence.

Data Analysis

1. Compare **MitoSOX fluorescence intensity** between treated and untreated populations.
2. Verify **cell viability** using **Sytox Blue staining**.
3. Calculate the **Stain Index:**

   (Median Pop MitoSOX+ - Median Pop MitoSOX-) / SD pop-negative