Reactive Oxygen Staining – MitoSOX
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Supplies:
M36008 MitoSOX Red Mitochondrial superoxide indicator (Invitrogen)
DMSO
Hanks Balanced Salt Solution w/ Ca and Mg
Labeled microcentrifuge tubes
Labeled flow cytometry tubes.
Paraformaldeyde
PBS

Sample Preparation:
NOTE: At time of seeding 3 sets (9 wells) should be just TCPS. One will be one the unstained sample, TCPS, and one LPS. The LPS should be added with seeding.

1. The unstained samples should be cells that are fixed with no fluorphores added.
2. LPS at 1 microM/mL should be introduced to one of the TCPS culture sets. This should be at the time of seeding.

Procedure:
Unstained sample

1. Add 0.25% trypsin to each plate and place in incubator for 5 minutes.
2. Stop trypsin with RPMI + 10% FBS. Place in labeled microcentrifuge tube and spin down at 3100 RPM for 10 minutes.
3. Resuspend with 3.7% paraformaldeyde.
4. Let stand at room temperature for 20 minutes.
5. Centrifuge at 3100 RPM for 10 minutes
6. Aspirate and resuspend in 400 microliters of PBS
7. Place in labeled FACs tube and store in refrigerator

Procedure:
Stained sample

1. Dissolve one vial of MitoSOX (50 micrograms) into 13 microliters of DMSO. This makes a 5mM MitoSOX solution.
2. Dilute in HBSS
    a. Add 5mL of HBSS to entire tube.
3. Wash cells with warm HBSS
4. Add staining solution to cells
    a. 0.25mL per 24-well plate.
    b. 0.5 per 12-well plate.
5. Incubate at 37 degrees Celsius protected from light for 10 minutes.
6. Wash cells 3 times with HBSS.
7. Transfer surfaces to fresh 24-well plate, except unstained, TCPS, and LPS surfaces.
8. Detach cells with 0.25% trypsin. 5 minutes or so in incubator. Shake plates gently if needed. Check under scope for detachment.
9. Stop trypsin with RPMI + 10% FBS. Place supernatant in labeled microcentrifuge tube and spin down at 3100 RPM for 10 minutes.
10. Aspirate off supernatant and resuspend in 3.7% paraformaldeyde. Let set at room temperature for 20 minutes.
11. Centrifuge at 3100 RPM for 10 minutes. Aspirate supernatant and resuspend in 400 microliters of PBS.
12. Place in labeled and capped FACS tube and store in refrigerator until time of flow cytometry.