Reactive Oxygen Staining – Image-IT
From Ainslie Lab @ UNC ainslielab.web.unc.edu

Supplies:
I36007 Image-IT LIVE Green ROS Detection Kit (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 4 sets (12 wells) should be just TCPS. One will be unstained, TCPS, LPS and TBHP. 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.
3. TBHP should be used as a positive control 60-90 minutes prior to staining.
    a. Add 1.0 microliter of TBHP (Component C) to 77 microliters of DI water. Mix well.
    b. Dilute solution from (a) at a concentration of 1:1000 in RMPI with 10% FBS and 1% antibiotic. This produces a 100 microM solution.
    c. Replace the media of one set of TCPS culture sets with 100 microM TBHP solution.

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. Add 50 microliters of DMSO to one vial of carboxy-H_2DCFDA (Component A). This yields a stock solution of 10mM. Vortex vial until completely dissolved.
2. Add 10mM solution to HBSS to prepare a working solution of 25 microM
    a. 5.0 microliters of A to 2.0 mL of 37 degree Celsius HBSS
3. Wash cells with warm HBSS
4. Add staining solution to cells
    a. 0.25mL per 24-well plate.
5. 0.5 per 12-well plate.
6. Incubate at 37 degrees Celsius protected from light for 30 minutes.
7. Wash cells with HBSS

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8. Transfer surfaces to fresh 24-well plate, except TCPS, TBHP, and LPS surfaces.
9. Detach cells with 0.25% trypsin. 5 minutes or so in incubator. Shake plates gently if needed. Check under scope for detachment.

10. Stop trypsin with RPMI + 10% FBS. Place supernatant in labeled microcentrifuge tube and spin down at 3100 RPM for 10 minutes.
11. Aspirate off supernatant and resuspend in 3.7% paraformaldeyde. Let set at room temperature for 20 minutes.
12. Centrifuge at 3100 RPM for 10 minutes. Aspirate supernatant and resuspend in 400 microliters of PBS.
13. Place in labeled and capped FACS tube and store in refrigerator until time of flow cytometry.