Calcium Flux Analysis

Reagent Preparation:

1. Dissolve Indo-1 (Molecular Probes catalog No. I1226) in DMSO to a final concentration of 2 mg/ml (25 ul of DMSO into 50 ug of Indo-1).
2. Mix quantities of probenecid (Sigma Catalog No. P8761) and water to yield a 100 mM solution. Weigh 285 mg of probenecid, add 8 ml of dH_2O, then add 1 M NaOH until dissolved. The pH should be between 9 and 10. Store for 1 month at room temperature.
3. Dissolve Ionomycin (Calbiochem Catalog No. 407950) in 100% ethanol for a final concentration of 1 mg/ml. Store at -20^oC. This is used as a control for calcium flux.
4. Cell Loading Media (CLM) is Hanks Balanced Salt Solution (HBSS) containing 1 mM calcium, 1 mM magnesium, 1% FBS or 0.5% BSA and can be stored up to 1 month at 4^oC.

Sample Preparation:

1. In a 15 ml tube, suspend the cells in CLM at a concentration of 10^7 cells per ml.
2. Add 40 ul of a 100 mM probenecid stock to 1 ml of cells for a final concentration of 4 mM.
3. Add 2 ul of a 2 mg/ml Indo-1 stock to 1 ml of cells for a final concentration of 2 ug/ml.
4. Incubate the cells with Indo-1 AM and probenecid for 30 minutes at 37^oC.
5. Wash the cells twice in CLM.
6. Resuspend the cells in CLM at a concentration of 10^7 cells per ml.
7. Rest cells for 15 minutes before acquiring any data.
8. 100 ul (1×10^6 cells total) of the Indo-1 loaded cells can be used for additional cell surface staining.

Cell Surface Staining (if required):

1. Add the appropriate mAb to 1×10^6 cells (100 ul of the Indo-1 loaded cells).
2. Incubate for 20 minutes at room temperature.
3. Wash once in CLM.
4. Cells can be held at 4^oC for several hours. At the time of analysis, cells should be warmed up to 37^oC. Each tube should be warmed separately just before running.

Instrument Set up:

1. Verify filter configuration: 530/30 PMT A (Indo-1 blue) and 440/40 PMT B (Indo-1 violet) on the UV trigon.
2. In the Parameters Tab in the Instrument Frame:
    a. Delete all parameters except: FSC, SSC, FITC, PE, Indo-1 (violet), Indo-1 (blue).
    b. Check the Log box for FITC and PE
    c. Under the ratio tab click add and choose Indo-1 (violet) for numerator and Indo-1 (blue) for the denominator.

d. Create the following plots on a Global Worksheet: FSC vs. SSC, FITC vs PE, Indo-1 (violet) vs Indo-1 (blue) and Time vs Ratio Indo-1 (violet)/Indo-1 (blue).

Optimizing the Instrument Settings:

1. Acquire the cells on LO setting.
2. Install the Indo only sample.
3. Adjust FSC and SSC voltages, and FSC threshold.
4. Draw a gate around the population of interest if needed and format the remaining plots to show this population.
5. Adjust the FITC and PE voltages to place the negative population in the lower left corner.
6. Adjust Indo-1 (violet: on x-axis) and Indo-1 (blue: on y-axis) to optimize signal (70^{o} angle).
7. Adjust ratio scaling to set the baseline at 50,000 by selecting ratio tab and changing the % scaling until it reaches 50,000.

Recording Data to Apply compensation:

1. Record events for the Indo only tube.
2. Record events for the FITC/PE sample tube.
3. Draw a gate around the FITC+, FITC-, and PE+ populations on the FITC vs PE plot.
4. Create a Statistics View to display the FITC and PE mean values.
5. Adjust the compensation manually until the PE median value for the FITC+ and FITC- match
6. Adjust the compensation manually until the FITC median value for the PE+ and PE- match.

Recording Experimental Data:

1. For the calcium flux experiment set events to record to 1,000,000 and events to display 50,000.
2. Click the Next button on the Acquisition Controls frame to create a new Tube and Label
3. Install the FITC/PE sample and adjust flow rate to 200 events /second.
4. Click Record.
5. When 10,000 events have been recorded remove the tube and add the stimulus to the tube and mix thoroughly. ***DO NOT PUT THE INSTRUMENT IN STANDBY.***
6. Re install the tube and stop recording when cells are no longer reacting to the stimulus.