

Calcium Flux on LSRII

Background

This method describes the loading of cells with indo-1 AM for the purpose of measuring cellular calcium levels. Indo-1 is excited by the UV laser, its emission depends on whether it is bound to calcium (~420nm) or free (~510nm). The ratio of these two wavelengths can indicate changes in intracellular calcium concentration.

Please note the concentration of indo-1 varies with cell type (ranging from 0.1 to 6 μ M) and therefore requires optimisation.

Materials

1. **DMSO**
2. **PBS**
3. **Indo-1 AM** 50 μ g vial (Invitrogen cat. No. I1223)
4. **Ionomycin** in DMSO at 1 mg/ml (Calbiochem cat: 407950)
5. **Calcium-free Media**
6. **Cell Loading Media (CLM)**
7. **EGTA** (to inhibit calcium flux, add it to a final concentration of 2mM)

Additional Considerations

Ionomycin is used as a positive control to establish a maximum Calcium flux ratio and EGTA inhibited cells are used to approximate minimal response.

Equipment

1. **Centrifuge**
2. **Pipettes.** You will need one in the range of 2-10 μ l, one in the range of 10-100 μ l, and another ranging from 100-1000 μ l.
3. **12x75 mm polystyrene/polypropylene tubes.** Depending on which machine you wish to use (LSR II prefers polystyrene while the CyAn prefers polypropylene).
4. **Ice bucket with cover.** Generally, cells are more stable and tolerate insult better when they're cold. The cover keeps light out, which could bleach the fluorochromes.
5. **Flow cytometer.** You need a flow cytometer fitted with a 350nm UV laser. Our four-laser-17-colour BD LSRII is convenient for this assay.

Reagent preparation

1. Dissolve 50 μ g Indo-1 in 25 μ l dry DMSO to have a final concentration of 2mg/ml (2mM). (Can be stored at -20°C).
2. Dissolve Ionomycin in DMSO to a final concentration of 1mg/ml (1.4mM). Aliquot in 10 μ l and store at -20°C. Add 5-10 μ l Ionomycin to 1ml Indo-1 loaded cells (Ionomycin final concentration: 1-10 μ g /ml (or 1-1.4 μ M)).

3. CLM is PBS or any cell medium containing 1mM calcium, 1mM magnesium, and 0.5% BSA. Store at 4°C.

Sample preparation

1. In a 15ml tube, resuspend the cells in 1ml **calcium-free** medium at a concentration of 10^7 cells per ml.
2. Add 2µl of 2mg/ml Indo-1 (4µM final concentration).
3. Incubate the cells with Indo-1 for 30 minutes at 37°C.
4. Pellet cells at 400g for 5 minutes; aspirate the supernatants being careful not to disturb the pellet.
5. Resuspend the cells in 1ml CLM.
6. Rest cells for 15 minutes before acquiring any data.
7. 100µl (1×10^6 cells total) can be taken off and used for further staining.

Cell Surface Staining (if required):

1. Add the appropriate mAb to 1×10^6 cells (100µl 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°C for several hours. At the time of analysis, cells should be warmed up to 37°C. Each tube should be warmed separately just before running.**

Instrument Set up:

1. Verify filter configuration: 530/30 PMT A (Indo-1 blue) and 405/20 PMT B (Indo-1 violet) on the UV trignon and close violet laser shutter (ask lab staff to do it for you) to prevent violet laser light interfering with Indo-1 violet emission.
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. Check Linear scale for Indo-1 (violet), Indo-1 (blue).
 - d. Under the ratio tab click add and choose Indo-1 (violet) for numerator and Indo-1 (blue) for the denominator.
 - e. 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 flow rate (see LSR II dashboard).
2. Install a sample stained with Indo-1.
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 (cells should form a 70 degree angle with the x-axis).
7. Adjust ratio scaling to set the baseline at 50,000 unit by selecting ratio tab and changing the % scaling until it reaches 50,000 units.

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. As positive control for calcium flux, install a 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 10 μ l Ionomycin to the tube and mix thoroughly. ***DO NOT PUT THE INSTRUMENT ON STANDBY.***
6. Re install the tube and stop recording when cells are no longer reacting to Ionomycin.
7. Install a sample of interest and repeat step 4, 5, and 6, using your own stimulus.