Draft

Ca^{++} Flux Analysis via FACS

protocol by Susannah D. Barbee

1. Prepare single-cell suspensions of primary thymocytes or lymphocytes. Resuspend cells at 10^6/mL in RT or 37°C Cell-Loading Buffer (CLB: HBSS + 1% FBS.

2. Add Indo-1 (2 mg/mL in DMSO, Molecular Probes I-1203) to a final concentration of 2 µg/mL.

3. Incubate 30 min 37°C.

4. For simultaneous staining of surface antigens: stain with azide-free FITC- and PE-conjugated antibodies for 20 min at RT†.

5. Resuspend in CLB at 5 x 10^5/mL. Keep at RT until analysis (within 2 hrs).

6. Incubate sample aliquots for 5 min at 37°C before running.

7. Collect a baseline ratio for 30 sec before adding stimulants, collect 8-10 min.

8. Use Ionomycin to measure maximal flux: dilute stock (500 µg/mL in DMSO) 1/5 with CLB to 100 µg/mL. Add 10 or 20 µL per 1 mL sample for 1 or 2 µg/mL final concentration.
** Always flush lines with DMSO and then CLB after stimulations to remove any excess ionomycin.

FACS Ca^{++} calibration

1. Prepare solutions of known Ca^{++} concentration with Calcium Calibration Buffer Kit #1 (Molecular Probes C-3008):

Buffer	Add	to volume of B	Total $[Ca^{++}]$ (mM)	$[Ca^{++}]_i$ (nM)
10 mM K_2EGTA (**A**)			0	0
2	4 mL of **A**	1 mL	2.00	27
3	3.6 mL of **2**	1 mL	3.74	65
4	3.2 mL of **3**	1 mL	5.23	120
5	2.8 mL of **4**	1.2 mL	6.67	218
6	2.4 mL of **5**	1.4 mL	7.89	410
7	2.0 mL of **6**	1.8 mL	8.89	876
10 mM CaEGTA (**B**)			10	~33 µM

2. Wash Indo-1-loaded cells 1x CLB, and resuspend aliquots of 5 x 10^5 cells in 1 mL each of the buffers.

3. Incubate 90 min 37°C to equilibrate, then collect steady-state indo-1 ratio.

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