INDO-1 LOADING AND SAMPLE STAINING PROCEDURE FOR SIMULTANEOUS MEASUREMENT OF INTRACELLULAR CA^{2+} AND CELL SURFACE ANTIGEN EXPRESSION

MATERIALS:

1. 50 μg vial Indo-1 (e.g., from LifeTechnologies, Grand Island, NY)
2. DMSO (Sigma-Aldrich, St. Louis, MO)
3. RPMI 1640
4. Monoclonal antibodies (mAb), conjugated to suitable fluorochromes
5. Ionomycin (LifeTechnologies))
6. 37°C water bath, centrifuge, vortexer.
7. Agonists to test Ca^{2+} flux, e.g. anti-CD3, anti-IgG, ConA.
8. Serum (for RPMI with 2% serum, if cells require serum).

METHOD:

1. Incubate cells ($\leq 2x10^7$/mL) in RPMI with 1-5 μM Indo-1 (acetoxymethyl ester) at 37°C for 40 min for loading.

2. Incubate aliquots of Indo-1 loaded cells with saturating concentrations of fluorochrome-conjugated antibodies for 20 min. Incubate at 20° to 25°C unless the antigen is subject to capping, otherwise use 4° to 8°C. Note: mAbs must be azide free. Note: set-up single color stained cells for setting appropriate fluorescence compensation on the instrument.

3. Wash cells twice in RPMI and suspend them at the desired concentration (usually $2x10^6$/mL). Higher cell concentrations ($4x10^6$/mL) are required when the cells of interest represent less than 10% of the total population. Cells can be kept at 20° to 25°C unless the antigen is subject to capping, otherwise use 4° to 8°C.

4. Samples should be analyzed shortly after the cells were prepared.

5. Ionomycin (1-3μM final conc.) is used as a positive control for Indo-1 loading and maximum Ca^{2+} flux.

PREPARATION OF INDO-1:

1. Add 150 μL of DMSO to a 50 μg vial of Indo-1, cover with aluminum foil to protect from light.
2. Vortex well, then warm to 37°C for 5 min.
3. Transfer 150 μL of Indo-1 from vial to 4.85 mls of RPMI (=10μM). Wash out vial very well. If not the entire amount of Indo-1 dissolved in DMSO is used, the remainder can be stored dessicated at –20°C for a maximum of 6 months.
4. Cover the tube of 5 mL of 10 μM Indo-1 with foil.
5. Aliquot the appropriate amount of 10μM Indo-1 to the cell suspension (final conc.=1-5 μM). The optimal concentration is dependent on the cell type.
6. Store excess RPMI-diluted 10 μM Indo-1 at 4°C. In our laboratory, the 10 μM Indo-1 solution has been tested for stability up to 24 hrs.

PREPARATION OF IONOMYCIN:

1. Dissolve 1 mg of ionomycin in 1 mL DMSO.
2. Aliquot 13.5 μL of ionomycin solution into vials for later use and store at -20°C for less than one year.
3. Dilute one 13.5μL vial of ionomycin with RPMI to a volume of 3 mL (=6 μM).
4. Cover the 3 mL of 6 μM working stock with foil to protect from light.
5. 150 μL of working stock ionomycin is added to 300 μL of Indo-1 loaded cell suspension.

Special Note:

Indo-1 requires UV excitation and special filter sets for optimal detection, e.g., a 395-415 nm bandpass (BP) filter for Indo 'violet" and a 515-545 nm BP for Indo 'blue-green'.

Further Reading:

June CH, Rabinovitch PS. Intracellular ionized calcium. Methods Cell Biol. 1994;41:149-74.

June CH, Abe R, Rabinovitch PS. Measurement of intracellular calcium ions by flow cytometry. In: Current Protocols in Cytometry, Vol 2, Robinson JP, Darzynkiewicz Z, Hyun W, Orfao A, Rabinovitch P, eds., John Wiley & Sons, 1997, pp. 9.8.1 – 9.8.19.