This protocol describes the loading of cells with indo1-AM for the purpose of measuring cellular calcium levels. This acetyloxymethyl ester form of the dye is cell permeant, passively diffusing across the cell membrane. Intracellular esterases cleave the acetyl groups, rendering the indo-1 impermeant.

Indo-1 is excited in the UV, and fluoresces at different wavelengths depending on whether it is bound to calcium (~420nm) or free (~510nm). The ratio of these two wavelengths can indicate changes in intracellular calcium concentration. Indo-1, because of the ratiometric quality, is the dye of choice for most flow applications. There are other dyes, most of which shift in fluorescence intensity rather than wavelength in reaction to changes in calcium levels. Alternatives to indo-1 may be useful, depending on your ultimate goal.

The optimal concentration of indo-1 varies with cell type, ranging from 1 to 10 mM. This typically requires empirical definition, but note that concentrations at the low end of the range tend to yield more sensitive measurements. Cells loaded with indo-1 are analyzed relative to a time parameter, and the change in fluorescence ratio over time can be related to changes in activation or stimulation by some agonist that will illicit a calcium flux. Ionophores, usually ionomycin, are used as positive controls. Calcium chelators such as EGTA serve as negative (Ca++ low) controls.

**Reagents:**

- DMEM
- PBS
- DMSO
- Cell Loading Medium (CLM; RPMI, 2% FCS, 25mM HEPES (pH 7.4))
- Indo-1 AM (Molecular Probes I-1203) (stock: 1mM in DMSO)
- Ionomycin (1mg/ml in DMSO)
- EGTA

**Procedure:**

1. Suspend 10-20 x 10^6 cells in 1ml CLM in 15 ml tube.
2. Load cells with indo-1, final concentration 1.5µM (1.5µl of 1mM stock in DMSO).
3. Incubate cells 45 minutes at 37°C in the dark.
4. Wash cells 2X with DMEM with 2% FCS.
5. Resuspend cells gently (DO NOT VORTEX) in CLM at 2.5 x 10^6 cells / ml. Store in the dark at room temperature until about 1 hour flow cytometric analysis.
6. Dilute cells to 1 x 10^6 / ml with CLM. Allow to equilibrate at 37°C in the dark for 30-60 minutes.
7. Analyze by flow cytometry. Run untreated cells to establish baseline; run “positive” control to establish maximum Ca++ flux ratio; run EGTA inhibited cells to approximate low (minimal) response.
Positive control: Ionomycin, 1ug/ml final concentration (stock 1mg/ml in DMSO)
Negative control: EGTA 8mM final concentration; add to indo1-loaded cells (step 6) approximately 1 hour prior to flow cytometric analysis.