Flow Cytometry Core Facility

Barts and The London
School of Medicine and Dentistry

The Blizard Institute of Cell and Molecular Science Flow Cytometric Functional Assays

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Cytoplasmic Calcium Flux

Activated cells have a high demand for calcium resulting in a flux of calcium into the cytoplasm of the cells which can be detected flow cytometrically in real-time by use of calcium indicator dyes, such as Fluo-4 and Indo-1. Indo-1 is the preferred dye for flow cytometric measurements as the dye is ratio-metric and when bound or not bound to calcium emits at 390nm (violet) and at 490nm (green) respectively after excitation by a UV laser. Modern bench-top instruments can then ratio the signals from the bound and unbound signals giving a processed ratio measurement. When calcium is in the external environment of the cells the signal detected is a combination of calcium moving into the cell and the calcium released from internal stores. The amount of flux from the external environment can thus be determined by comparing the calcium signal from cells in a calcium free environment, see Figure 1.

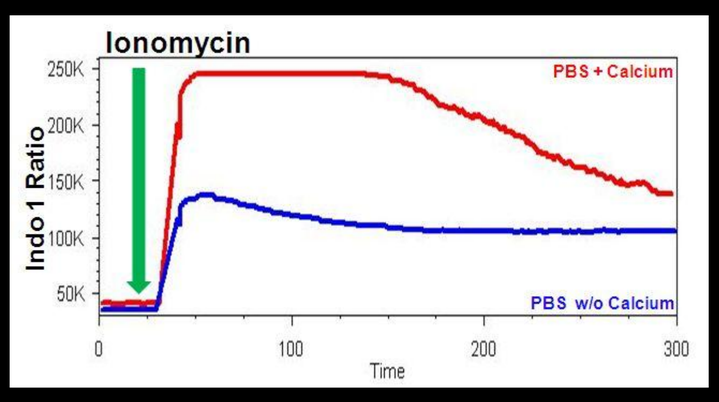

Figure 1. Jurkat T-cells were loaded with 1μM Indo-1 and in the absence or presence of external calcium activated by 10μg/ml Ionomycin. The profile without calcium shows the release of calcium from internal stores of calcium which are released into the cytoplasm. While the profile with calcium shows the amount a calcium flooding into the cytoplasm from the external environment and the internal stores.

Cell Organelle Calcium Flux

Rhod-2 is a mitochondrial specific calcium binding dye which is sub-optimally excited at 488nm and emits at 575nm. Use of the general cell activator ionomycin can detect the amount of calcium flux to mitochondria when cells are in a calcium-free solution. The use of endoplasmic reticulum (ER) calcium ATPase pump inhibitor, thapsigargin (Tg) which releases calcium from the ER to the mitochondria and is then detected by Rhod-2 giving a read-out of the ER internal calcium stores, Figure 2.

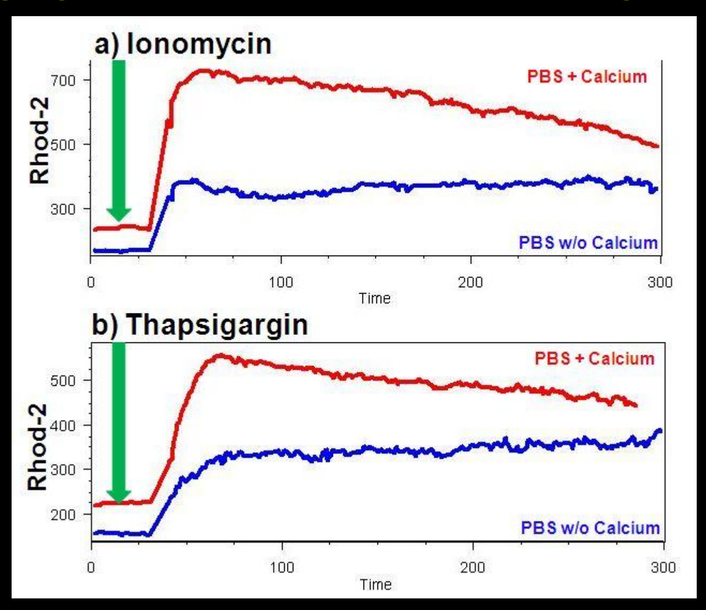

Figure 2. Jurkat T-cells were loaded with 0.5μg/ml Rhod-2 and in the absence or presence of external calcium activated by 10μg/ml Ionomycin a). The profile without calcium shows the release of calcium from internal stores of calcium which are released to the mitochondria. While the profile with calcium shows the amount of calcium flooding to the mitochondria from the external environment and the internal stores. Use of Tg allows the measurement of the movement of calcium from the ER to the mitochondria when the cells are in calcium free media b). The use of Tg with cells in calcium – rich media shows the movement of calcium from the ER and the external media to the mitochondria b).

Reactive Oxygen Species

ROS (Reactive Oxygen Species) is often produced during apoptosis, this includes hydrogen peroxide, super oxide and nitric oxide which can all be detected flow cytometrically. Fluorescein analogue, H_2DCFDA detects preferentially hydrogen peroxide, this dye when esterified by cytoplasmic esterase's increases in fluorescence when binding hydrogen peroxide. Superoxide is detected preferentially by Dihydroethidium which upon contact with superoxide is oxidised to ethidium which then fluoresces upon binding to DNA and RNA. Figure 3 shows an example of H_2DCFDA detection of hydrogen peroxide.

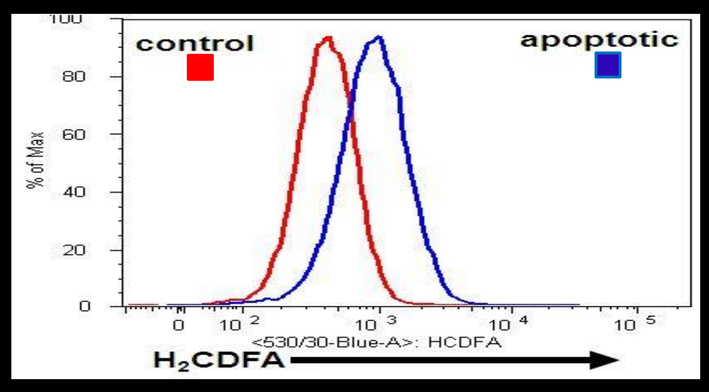

Figure 3. Jurkat T-cell line were treated with 1 μM staurosporine (STS) for 2h at 37°C. Cells were then incubated with 1 μM H_2CDFA for 30 min at 37°C and washed. Cells (30,000 events) were then analysed on a BD LSRII and data collected in 530/30nm channel to determine H_2CDFA fluorescence. Control cells (untreated) had a median H_2CDFA fluorescence of 397, while the STS apoptotic cells had a median H_2CDFA fluorescence of 872 or a 2.2 fold increase in hydrogen peroxide compared to controls.

Mitochondrial Dysfunction

Production of ROS is often associated with mitochondrial dysfunction. Dihydroethidium detection of superoxide can be combined with $DiOC_6(3)$ measurement of the potential difference of the inner mitochondrial membrane; the fluorescence of which decreases as the potential difference decreases during apoptosis.

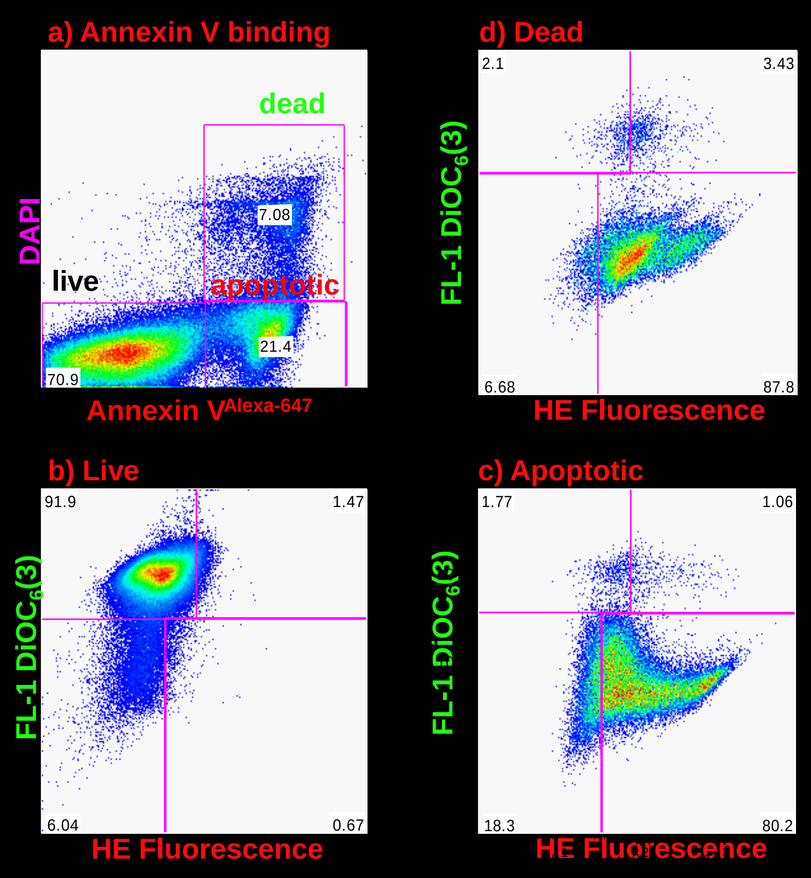

Figure 4. Jurkat T cells were UV-irradiated and incubated with 4 nM $DiOC_6(3)$ and Dihydroethidium (HE) at 5μM at 37°C for 30 mins. Cells were then labelled with Annexin V alexa-647 and DAPI (200ng/ml) for 15 min at RT. a) Annexin V binding assay cells were then gated on b) live c) apoptotic d) dead to show superoxide production and mitochondrial membrane potential as detected by HE and $DiOC_6(3)$.

Cell Proliferation - CFSE

CFSE can be used to determine the proliferation rate of stimulated cells in terms of percentage cells in each generation at different time points throughout the stimulation. The proliferation rate is determined by computer software such as FlowJo which calculates the deconvolution under the proliferation curve, see Figure 5 below. The cell cycle phase of such cells can be determined by loading aliquots of cells at each time point test with Hoechst 33342. The degree of cells synthesising DNA can be determined in live cells at each time point analysed giving the investigator more information on the proliferating cells.

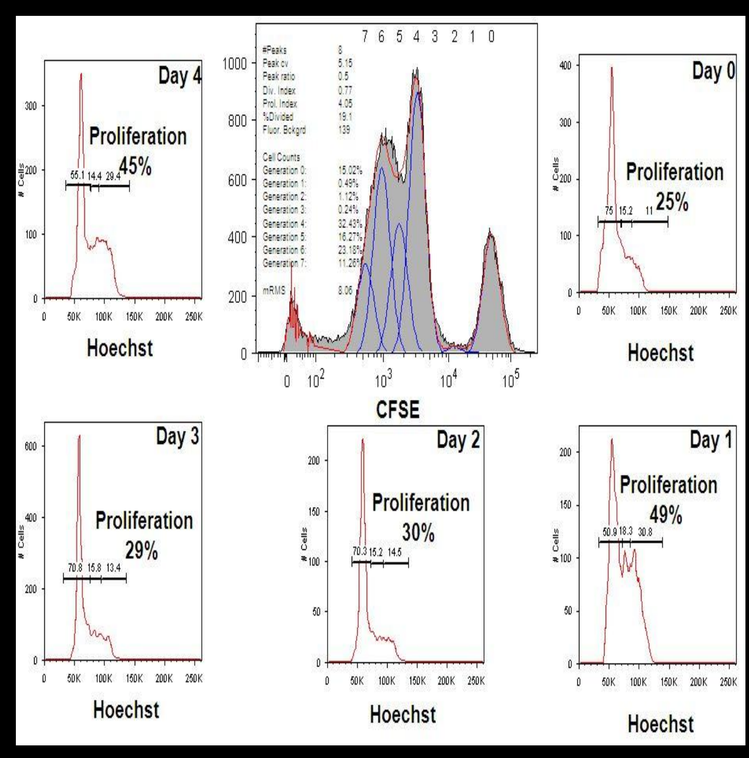

Figure 5. Jurkat cells were incubated with 2 μM CFSE for 15min at 37°C and washed before returning to culture. At various time points, cells were washed and 10μlg/ml Hoechst added for 45min at 37°C. PI was added just prior to FACS analysis to detect viable cells only.

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