Flow Cytometry Core Facility

Barts and The London School of Medicine and Dentistry

The Blizard Institute of Cell and Molecular Science Novel Flow Cytometry Techniques

G. Warnes & S. Martins, Flow Cytometry Core Facility

FRET

FRET – Fluorescence Resonance Energy Transfer allows investigator to study protein interactions or biological activity of proteins. FRET is characterized by the non-radiative transfer of energy between donor and acceptor fluorophores. This occurs via a long range dipole-dipole coupling mechanism when molecules are in a parallel orientation and within 10 nm. CFP & YFP FRET can be used to determine the proximity of two proteins. FRET can also be used to measure the biological activity of proteins when CFP and YFP are attached to the same protein. Changes in biological activity can be determined by comparing the degree of FRET in live or fixed cells, see figure 1 below.

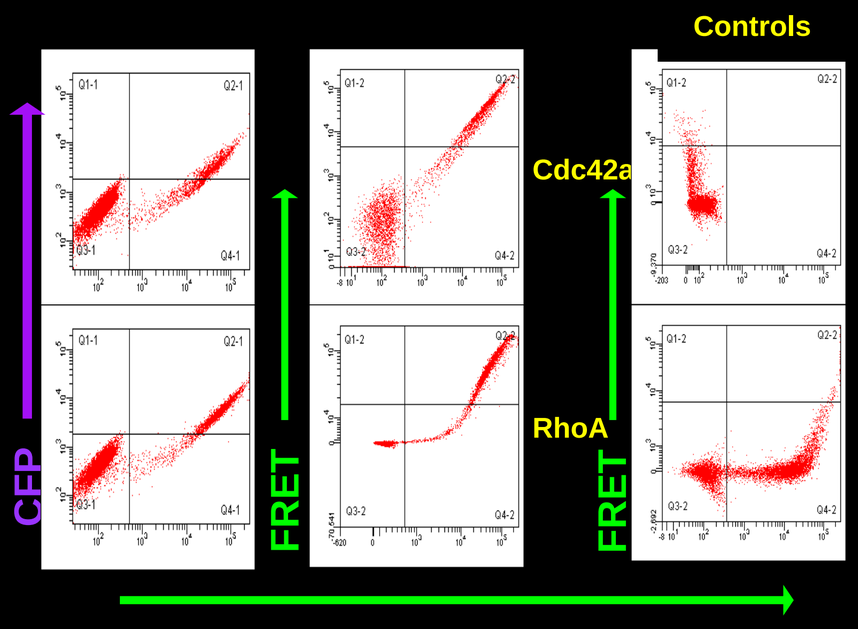

Figure 1. HeLa cells transfected with Cdc42a and RhoA doubled labelled with CFP and YFP were showed to display FRET at 530nm after 405nm violet excitation. CFP was detected at 450nm after 405nm violet excitation. YFP was detected at 530nm after 488nm blue excitation. Cdc42a and RhoA transfections showed a high degree of FRET at 18% and 28% respectively, when compared to and double single transfected controls.
M. Dittmar, Centre for Infectious Diseases, ICMS.

Protein Translocation

The translocation of proteins from one type of cell organelle to another is quantitated by Western Blot, a time consuming assay requiring a large number of cells. Quantitation of fluorescence by conventional image analysis is time consuming and inaccurate. Conventional flow cytometry can be used to quantitate protein translocation by the novel use of cell organelle fluorescent dyes.

Cell nuclei were labelled with the DNA stain, Hoechst 33342 (Ex350-Em461nm) and mitochondria with MitoTracker Orange (Ex488-575nm). After induction of apoptosis and cells were permeabilised and labelled with antibody against protein of interest. Cells were then lysed manually in a glass homogeniser and the resulting cell lysate analysed by flow cytometry.

Cell organelles were then identified by specific nuclear stain *e.g.* nuclei and mitochondria. The degree of protein translocation from mitochondria to nucleus can was then quantitated by the fluorescent antibody labelling of the apoptotic protein *e.g.* Apoptosis Inducing Factor, AIF.
S Leverrier, D Bergamaschi, L Ghali, A Ola, G Warnes, B Akgul, K Blight, R Garcia-Escudero, A Penna, A Eddaoudi, A Storey. Role of HPV E6 proteins in preventing UVB-induced release of pro-apoptotic factors from the mitochondria. *Apoptosis* 12 (3), 549-60, 2007. See figure 2 over.

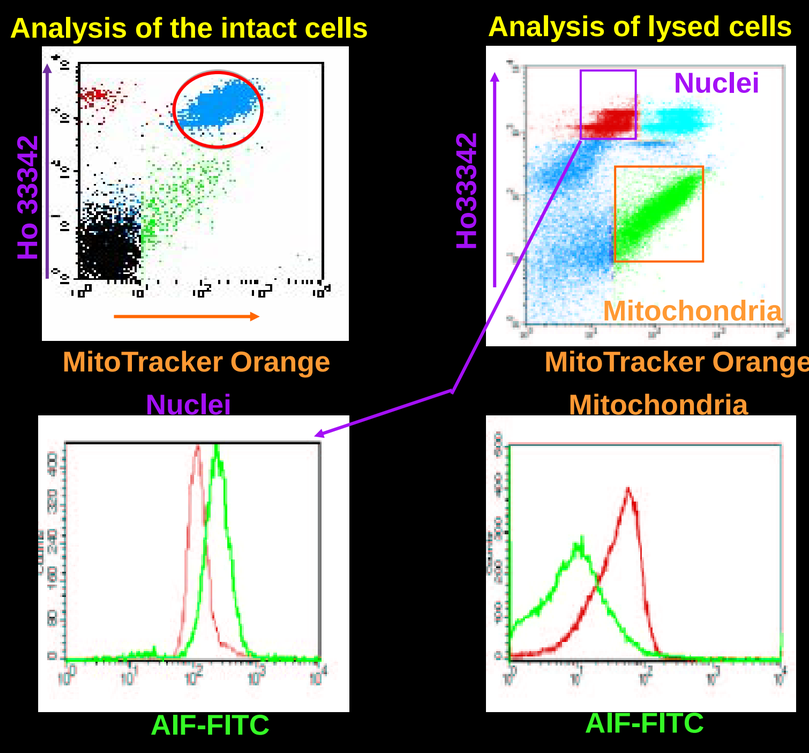

Figure 2. Fibrosarcoma cell line HT1080 were labelled with MitoTracker Orange. Apoptosis was induced by UV -irradiation. Cells were fixed and permeabilised and labelled with Hoechst 33342 and αAIF^{FITC} or isotype^{FITC}. Cells were analysed flow cytometrically then lysed. Cell lysates were then re-analysed and nuclei gated on Ho33342^{+ve}/MitoTO^{-ve} events with mitochondria gated on Ho33342^{low+ve}/MitoTO^{+ve} events. AIF^{FITC} in nuclei and mitochondria of treated and untreated cells were then compared. F. Ismail, Centre for Cutaneous Research, ICMS.

Multi-Functional Assays

The advent of commercial cytometers with multiple lasers has allowed researchers to multiplex assay's together in the same tube. This is especially useful when studying apoptosis as this multi-parametric process previously required several flow cytometric tests to be done separately to elucidate the processes involved. Multiplexing parameters such as annexin V binding, viability, mitochondrial membrane potential and reactive oxygen species shows the apoptotic processes involved from early to intermediate stages. These cells can then be fixed in 70% ethanol and sub G_1 analysis to show late stage apoptosis. See figure 3 below.

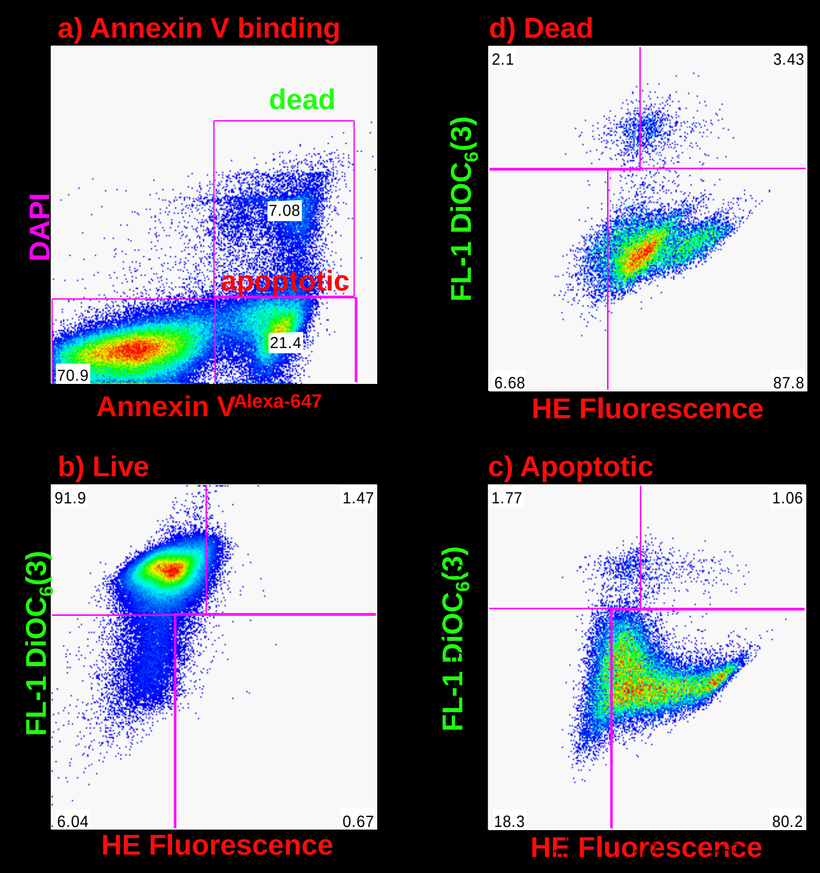

Figure 3. Jurkat T cells were UV-irradiated and incubated with 4 nM DiOC$_6$(3) and Dihydroethidium (HE) at 5μM at 37^{o}C for 30 mins. Cells were then labelled with Annexin V^{647} and DAPI (200ng/ml) for 15 min at RT. a) Annexin V binding assay cells were then gated on b) live c) apoptotic d) dead to show Superoxide production and mitochondrial membrane potential as detected by HE and DiOC$_6$(3).

Senescence Assay

Senescence can be studied flow cytometrically by the use of nucleic acid fluorescent dyes Hoechst 33342 and pyronin Y which bind to DNA and RNA in a linear manner respectively. Senescent cells such as stem cells have previously been studied with Hoechst 33342 which show a characteristic blue-red shift. Here senescent cells are characterized by being in the G_o phase of the cell cycle whilst cycling cells are in G_1, S phase and G_2m. Most fluorescent DNA dyes do not distinguish between G_o and G_1 cells. The use of Pyronin Y which binds solely to RNA when used in conjunction with Hoechst 33342 allows the investigator to sort living senescent and cycling cells, see figure 4 below.

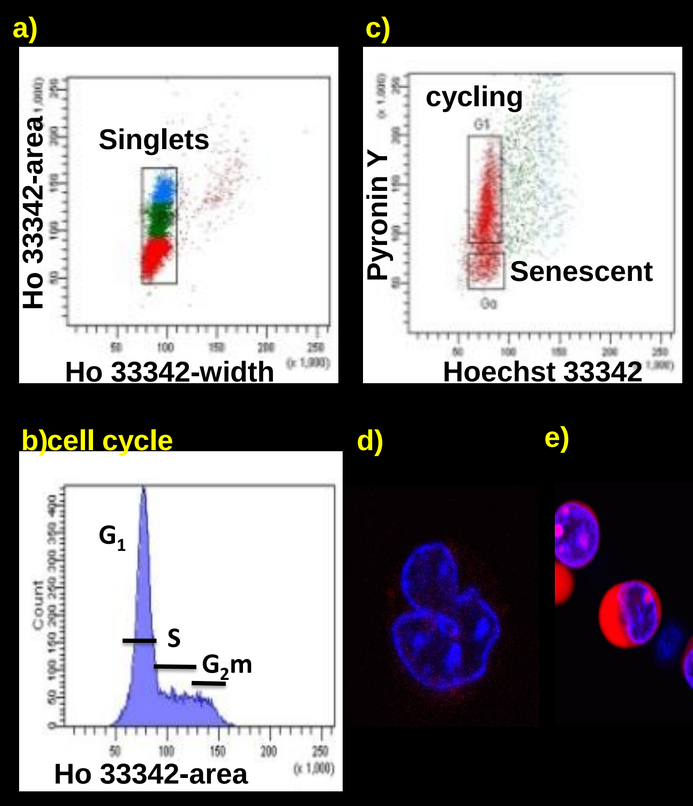

Figure 4. Jurkat T cells were incubated with Ho33342 (15 μg/ml) and Pyronin Y (0.5 μg/ml) for 45 minutes at 37^{o}C. Doublet discrimination was achieved by use of the width and area Ho33342 parameters a) normal cell cycle analysis of Ho33342 with G_1 70%, S phase 20% and G_2m 10% b); Senescence was determined in a Ho33342 v Pyronin Y dot-plot G_o cells (14%) having low amounts of RNA or Pyronin Y signal whilst cycling G_1 cells (48%) have higher amounts RNA or Pyronin Y signal c). Sorting cells in G_o & G_1 shows G_o have little RNA present d); G_1 cells have RNA present in the cytoplasm as indicated by the red Pyronin Y signal.

Please contact:- Dr Gary Warnes & Sandra Martins on g.warnes@qmul.ac.uk and s.martins@qmul.ac.uk
tel. no. 020-7882-2402

www.http://www.icms.qmul.ac.uk/flowcytometry/

Flow Cytometry Core Facility