Chapter 4

The Multiplexing of Assays for the Measurement of Early Stages of Apoptosis by Polychromatic Flow Cytometry

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60549

Abstract

The detection of apoptosis has been a stalwart application for flow cytometric analysis for decades and this review of flow cytometric methods to detect early stages of apoptosis includes the use of the pivotal assay to detect early and late apoptosis, the Annexin V assay which when multiplexed with biologically functional fluorescent dyes to measure mitochondrial function and Reactive Oxygen Species (ROS) generation allows further identification of functionally different subsets within apoptotic populations. Here we show how this polychromatic approach can be used to demonstrate which subset of cells show changes in mitochondrial function and when ROS is generated in a time dependent manner. This polychromatic approach to flow cytometry leads to the identification of over ten sub-populations of cells during classic apoptosis or programmed cell death (PCD).

Keywords: DNA dyes, Annexin V, MitoTracker, ROS, Polychromatic

1. Introduction

Apoptosis can be defined as 'gene-directed cellular self-destruction'; or 'programmed cell death' (Type I PCD) [1-4], although this is really a phenomenon where cells are programmed to die at a particular point during the normal function of those cells e.g. during embryonic development [5]. The term 'cell necrobiology' is an encompassing term for all modes of cell death and can be defined as "various modes of cell death: the biological changes, which predispose, precede and accompany cell death; as well as the consequences and tissue response

to cell death" [2, 3]. The term necrobiology thus includes all the many forms of apoptosis besides 'classic' PCD, including Caspase independent apoptosis-like PCD, autophagy or Type II PCD, mitotic catastrophe, necrosis-like PCD, necrosis or oncosis defined as accidental cell death and cell senescence [2, 3].

Apoptosis can be activated by two pathways, the first the 'extrinsic pathway' which is mediated via the Cell Death Receptor pathway. This pathway is typically activated by TNF binding to the Death Receptor, FAS Associated Death Domain or FADD [6]. This results in cleavage of Caspase 8 which then cleaves pro-Caspase 3 to release active Caspase 3 resulting in the final stages of apoptosis with DNA fragmentation and cell shrinkage by nuclear and cytoplasmic condensation and fragmentation, membrane blebbing and the formation of apoptotic bodies [1-3]. The second pathway, the 'Intrinsic pathway' can be induced by a variety of drugs, including Staurosporine (STS) and Etoposide (ETOP), UV-B irradiation, γ-irradiation, and reactive oxygen and is mediated via mitochondrial responses to such cellular insults with resulting mitochondrial membrane depolarization. This depolarization causes the release of cytochrome *c* from the inner mitochondrial membrane into the cytosol where it binds to Apaf-1 recruiting pro-caspase-9 in an ATP dependent manner, this complex activates Caspase 9 which in turn activate effector Caspases [7].

There are a plethora of flow cytometry assays for the study of apoptosis several hours after induction, by the use of dual fluorescent DNA binding dyes, Annexin V binding, activation of Caspases, depolarization of the mitochondrial membrane potential, cytochrome *c* release from the inner mitochondrial membrane through to cell death as measured by permeability of the plasma membrane to fluorescent DNA binding dyes and DNA fragmentation in the form of estimations of Sub G_1 [8]. Early apoptosis can be measured by the use of fluorescent DNA dyes such as Hoechst 33342 (Ho33342) or YO-PRO-1 and propidium iodide (PI) which identifies live (non-stained), early apoptosis (single positive), late apoptosis and dead cells (double positive). This assay is based upon the fact that cells undergoing apoptosis have a varying permeability to Hoechst 33342, while only late apoptotic and dead cells are permeable to PI [9-11].

The now classic Annexin V assay, includes the use of this protein, a vascular anti-coagulant which binds in a calcium dependent manner to externalised phospholipid, phosphatidylserine (PS) located on the outer leaflet of the plasma membrane. This when combined with a fluorescent viability dye, show the presence of live, apoptotic and necrotic cells in a similar manner to that observed by dual DNA dye assays [9-11]. The Annexin V binding assay can be used as a pivot point to differentiate between early and late apoptotic events. A polychromatic approach can then be applied by the multiplexing of various live cell functional fluorescent dyes to the Annexin V assay. These include the measurement of mitochondrial membrane potential and ROS dyes allowing the identification of further subsets of the live and early apoptotic cell populations [12-20]. There are a range of such fluorescent dyes, such as TMRE, JC-1/9, a range of carbocyanine and rhodamine chemical based dyes, $DiOC_6$, $DiIC_1(5)$ and rhodamine 123. All these dyes record the level of mitochondrial membrane potential, with the higher the fluorescence the more polarized the inner mitochondrial membrane potential (except JC-1/9 were a colour change occurs) [16]. While little fluorescence indicates depolari-

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zation of the inner mitochondrial membrane and the presence of dysfunctional mitochondria within the cell [12-16].

Further multiplexing with Annexin V binding and mitochondrial membrane potential measurements can also be studied with measurement of ROS by the use of dihydroethidium (HE) which preferentially measures superoxide produced by the cell during apoptosis [17-20]. This allows the identification of multiple subsets within live and early apoptotic populations in terms of mitochondrial function and generation of ROS. The mode of action of a variety of chemicals and treatments to induce apoptosis, including UV-irradiation, Staurosporine (STS), Etoposide (ETOP), Curcumin [20] and Chloroquine (CQ) [21] can then be compared in a more complex fashion. To this end a time course study of STS induction of apoptosis from 1-30 h employing the multiplexing of Annexin V-FITC, DAPI for cell viability and mitotracker $DiIC_1(5)$ showed not only the changes in apoptotic culture dynamics but also that mitochondrial dysfunction occurs in live cells (Annexin V^{-ve}/$DAPI^{-ve}$) within an hour of treatment with STS and sequentially reduced over the time course [13-20].

More recently a non-calcium dependent plasma membrane dye F2N12S violet ratiometric asymmetry probe or 4'-N, N-diethylamino-6-(N, N, N-dodecyl-methylamino-sulfopropyl)-methyl-3-hydoxyflavone has been employed to measure the change in the outer surface charge of the plasma membrane which occurs when PS is flipped to the outer leaflet of the plasma membrane [22]. F2N12S does so by, after violet excitation (405 nm) emitting two fluorescence's at 530 and 585 nm (green and orange) were the ratio of orange/green fluorescence is high when the cells are alive and low when apoptotic.

Thus a polychromatic flow cytometric approach employing a range of assays can be achieved by the multiplexing of numerous assays allows the simultaneous analysis of the changes in functionality of cells as the apoptotic cascade is initiated, progresses to early apoptosis and eventually to cell death via late apoptosis.

2. Materials and methods

2.1. Cell lines

Jurkat T-cells were grown in RMPI-1640 with L-Glutamine (Life Technologies) supplemented with 10% Foetal Bovine Serum (FBS, Life Technologies) and penicillin and streptomycin (Life Technologies) in the presence of 5% CO_2 at 37°C.

2.2. Induction of apoptosis

Jurkat cells were treated with UV-irradiation, 1 μM Staurosporine (STS), 10 μM Curcumin, 75 μM Chloroquine (CQ), or 10 μM Etoposide (ETOP) for up to 30h to induce apoptosis (Sigma Chemicals). Time points analysed were 0, 1, 2, 3, 4, 5, 20, 24, 30h, (n=3) see cell labelling section below.

2.3. Labelling with DNA dyes

After the induction of apoptosis cells were labelled with by incubation of Hoechst 33342 (25 μg/ml) and PI (1 μg/ml) for 30 minutes on ice. Ho33342 was excited by UV or 405 nm lasers and emission collected at 440/40 nm and PI was excited at 488 nm and emission collected at 610/20 nm with 30, 000 events collected.

2.4. Annexin V labelling

After the induction of apoptosis cells resuspended in 100 μl calcium-rich buffer with Annexin-AF-647 or FITC (2.5 μl) (Life Technologies, Becton Dickinson). Cells were then incubated at Room Temperature (RT) for 15 min. DNA viability dyes, DAPI (200 ng/ml) (Sigma Chemicals) was added just before flow cytometric analysis. DRAQ7 (Biostatus, UK) was incubated at 5 μM for 10 min at 37^0C. Annexin V-FITC was excited at 488 nm and emission collected at 530/30 nm; Annexin V-AF-647 was excited at 633 nm and emission collected at 660/20 nm; DAPI was excited by UV or 405 nm lasers and emission collected at 440/40 nm; DRAQ7was excited at 633 nm and emission collected at 780/60 nm with 30, 000 events collected.

2.5. Dihydroethidium and Mitotracker dye loading

After the induction of apoptosis cells were loaded with 5 μM dihydroethidium (HE, Invitrogen) for 30 min at 37^0C and either carbocyanine dyes $DiOC_6(3)$ or $DiIC_1(5)$ at 40nM, (Invitrogen) by incubating cells with dyes for 15 minutes at 37^0C. Cells were then washed in PBS and resuspended in 100 μl calcium-rich buffer with Annexin-AF-647 or FITC (2.5 μl). Cells were then incubated at Room Temperature (RT) for 15 min. DNA viability dyes, DAPI (200 ng/ml) was added just before flow cytometric analysis. Annexin V-FITC or $DiOC_6(3)$ was excited at 488 nm and emission collected at 530/30 nm; Annexin V-AF-647 or $DiIC_1(5)$ was excited at 633 nm and emission collected at 660/20 nm; HE was excited at 488 nm and emission collected at 610/20 nm; DAPI was excited by UV or 405 nm lasers and emission collected at 440/40 nm with 30, 000 events collected. After gating on Annexin V and viability dye to define, live, early apoptotic, late apoptotic and dead cells, analysis of mitochondrial function +ve or -ve cells and HE +ve or -ve cells was determined by gating on the live cell population. The position of the vertical and horizontal axis can be placed to show low levels of ROS with the vertical axis and little mitochondrial dysfunction with the horizontal axis.

2.6. Violet Ratiometric Membrane Asymmetry Probe

After the induction of apoptosis cells resuspended in 100 μl calcium-rich buffer with Annexin-FITC (2.5 μl). Cells were then incubated at Room Temperature (RT) for 15 min. Violet Ratiometric Membrane Asymmetry Probe F2N12S (100 nM, Invitrogen) was then incubated for 10 mins at RT with DNA viability dye, PI (5 μg/ml). Annexin V-FITC was excited at 488 nm and emission collected at 530/30 nm; PI was excited at 488 nm and emission collected at 610/20 nm; F2N12S was excited by 405 nm laser and emission collected at 530/30 nm (Green) and 610/20 nm (Orange) with a Ratio set with Green as the denominator and Orange as the numerator with 30, 000 events collected. Live cells have a high orange signal with low green

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channel signal and thus have a high ratio of orange/green fluorescence. While dead cells have a high green and orange signal and thus have a low ratio of orange/green fluorescence, which are confirmed by back-gating on viability dye positive cells.

3. Results

3.1. Use of DNA binding dyes to detect apoptosis

Prior to the introduction of the Annexin V binding assay, fluorescent DNA binding dyes were used as a means to identify apoptotic cells in live cultures. With late apoptotic cells which have undergone DNA fragmentation being detected in fixed cells by measurement of cells 'Sub G_1' to the G_1 cells when analysing cells for their cell cycle [8]. Hoechst 33342 and propidium iodide (PI) was originally used to determine the presence of apoptotic cells by the nature of the selective permeability of live, apoptotic and dead cells to the fluorescent DNA binding dye, Hoechst 33342; while PI is only fully permeable to dead cells. This use of the dual DNA binding dye assay was the assay of choice to detect early apoptotic cells, with sub G_1 analysis of the cell cycle for the detection of late apoptotic cells commonly used in fixed cell preparations [8-11].

See the example confirming that Ho33342-PI labels the same cell populations as the Annexin V assay (Figure 1). Resting live cells are double negative for Ho33342^{-ve}/PI^{-ve}, while early apoptotic cells are Ho33342^{+ve}/PI^{-ve} and dead cells double positive Ho33342^{+ve}/PI^{+ve} see Figure 1A. The same resting cells were also labelled with Annexin V-FITC (Figure 1B) and were the same percentages were shown by Ho33342/PI are shown as double negative live cells in the Annexin V assay as AnnexinV^{-ve}/PE^{-ve}, early apoptotic are AnnexinV^{+ve}/PE^{-ve} and dead cells as double positive AnnexinV^{-ve}/PE^{+ve}. Likewise STS treated cells undergoing apoptosis as shown by Ho33342/PI labelling showed a greater level of cell death than resting cells (Figure 1C). Gated Ho33342^{+ve}/PI^{-ve} cells (Figure 1C) are shown to be AnnexinV^{+ve}/PE^{-ve} in Figure 1D.

3.2. Use of Annexin V assay to detect apoptosis

The advent of the Annexin V binding assay largely replaced the methodology above and identifies apoptotic cells by virtue that phosphatidylserine normally present in the internal leaflet of the plasma membrane which is externalised in apoptotic cells to which Annexin V binds (as well as phosphatidylethanolamine), [12]. After Annexin V labelling (with a fluorescent tag of choice), a range of fluorescent DNA dyes can be employed to determined viability, these commonly include PI, DAPI, 7-ADD and DRAQ7. Quadrants show live cells as not labelled or Annexin-V^{-ve}/viability dye^{-ve}, early apoptotic cells as Annexin-V^{+ve}/viability dye^{-ve}, late apoptotic and dead cells as Annexin-V^{+ve}/viability dye^{+ve} or Annexin-V^{-ve}/viability dye^{+ve}.

Figures 2 A-C shows the employment of Annexin V-AF-647 with DAPI after UV-irradiation and STS treatment for 24 and 3h respectively. This demonstrates that different inducers of apoptosis have very different times at which early apoptosis is detected, with both samples displaying a similar degree of cell death. Highlighted in Figures A is an intermediate area

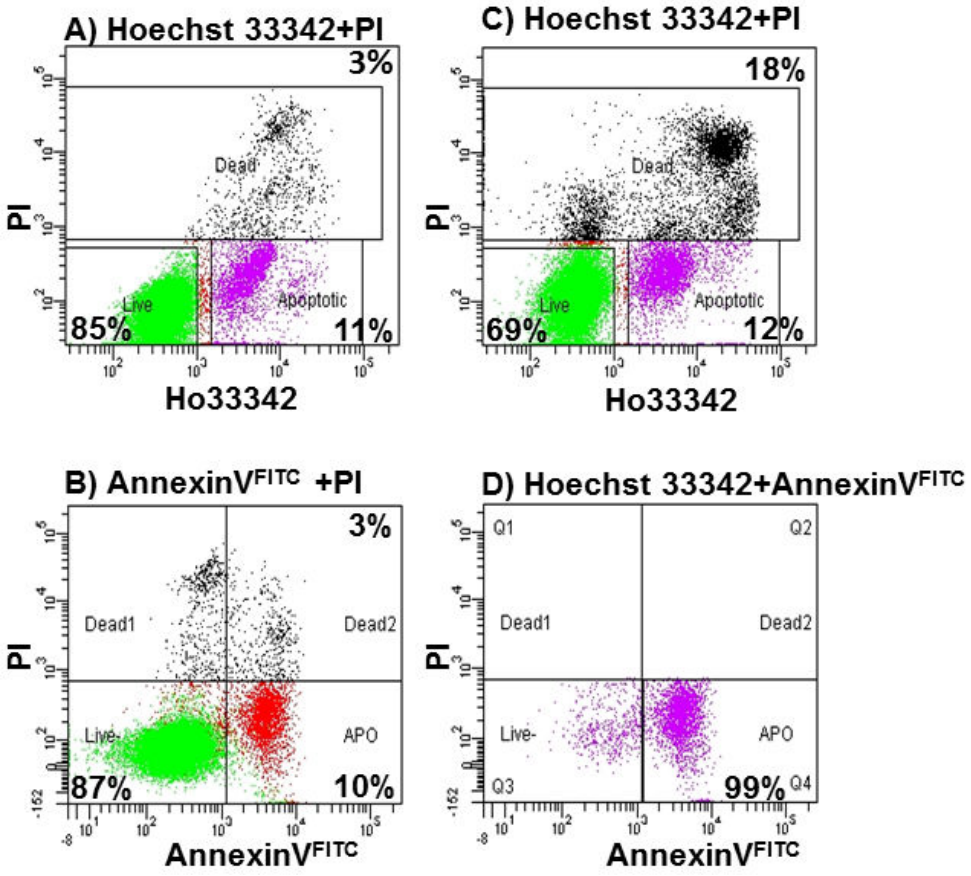

Figure 1. Jurkat T-cells (untreated) were incubated with B) Annexin V^{FITC} and PI with apoptosis at 10%. These cells were then incubated with 25 μg/ml Ho33342 for 30 min on ice to show apoptotic cells as $Ho33342^{+ve}PI^{-ve}$ at 11% A). STS treated cells showed a similar level of apoptotic cells (12%) when labelled with Ho33342 and PI C) but with more dead cells 18%. Back gating the apoptotic cells from C) shown in D) are apoptotic cells and are labelled with annexin V. Live, apoptotic (APO) and dead cells are shown in the quadrants, $AnnexinV^{-ve}/PI^{-ve}$, $AnnexinV^{+ve}/PI^{-ve}$, $AnnexinV^{+ve}/PI^{+ve}$ $AnnexinV^{-ve}/PI^{+ve}$ respectively.

between live and early apoptotic cells in which cells are starting the process of binding annexin V, moving minutes/hours later to the main Annexin-V^{+ve}/viability dye^{-ve} population. Later these cells start to become permeable to the viability dye and show a low degree of DAPI positivity. This population is classed as late apoptotic, with the fully DAPI labelled cells been necrotic or dead by the process of apoptosis.

Likewise Annexin V-FITC with DAPI can be used in a similar manner to that discussed above were Curcumin and ETOP has been employed for 24 h before labelling with Annexin V (Figure 2 D, E, F). A higher degree of apoptosis and cell death are shown in Figure 2 E, F than that produced by UV-irradiation and several hours of treatment with STS (Fig 2B, C).

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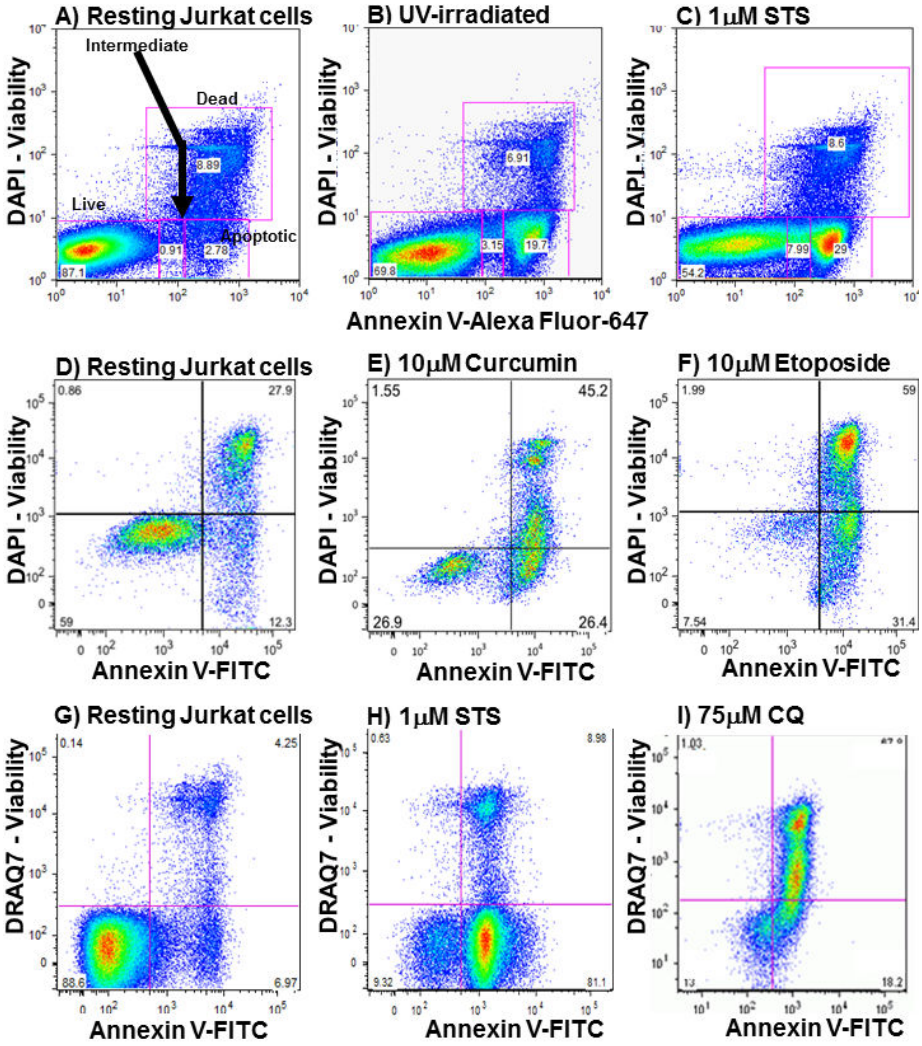

Figure 2. After induction of apoptosis cells were labelled with annexin V and a viability dye, A), and D) show resting cells labelled with Annexin V-647 or FITC and DAPI. Cells were UV-irradiated and labelled after 24 h B), while C) show cells treated with STS for 3 h; E) cells treated with Curcumin (Turmeric) for 24 h, and F) cells treated with ETOP for 24 h. Resting cells were also labelled with Annexin V-FITC and DRAQ7 G), and labelled likewise after H) STS treatment 6 h, and I) CQ treatment after 24 h. Live, apoptotic (APO) and dead cells are shown in the quadrants, AnnexinV^{-ve}/DAPI or DRAQ7^{-ve}, AnnexinV^{+ve}/DAPI or DRAQ7^{-ve}, AnnexinV^{+ve}/DAPI or DRAQ7^{+ve} AnnexinV^{-ve}/DAPI or DRAQ7^{+ve} respectively.

Also shown is the use of the new viability dye DRAQ-7 (Biostatus) with annexin V-FITC after STS and CQ treatment after 6 and 24 h respectively (Figure 2 G, H, I). Here a higher degree of cell death is shown in response to CQ and STS compared to after 3 h (Figure 2 C) [23].

3.3. Time course dynamics of apoptosis

The Annexin V assay gives the researcher a snap-shot of what is happening to a cell population for a particular time point (Figures 1, 2). The researcher should therefore use the Annexin V assay at multiple time points to get a fuller picture of the time maximal apoptosis is reached in any given experimental design. Figure 3 shows a 30 h time course of Jurkat STS induced apoptosis. At each time point (n=3) after the induction of apoptosis has been initiated the incidence of the live cell population showed a clear linear decline over time falling to 20% after 5 h. While the incidence of dead cells from 0-5 h remained constant at approximately 10% and only rose after the live population fell to 20% after 5 h of STS treatment. The dynamic population was the Annexin-V^{+ve}/viability dye^{-ve} cells which rapidly rose after 1 h <10% to over 60% at the 5 h time point which then declined as the incidence of the dead cell population rose and both live and apoptotic population fell from 20-30 h [13-20].

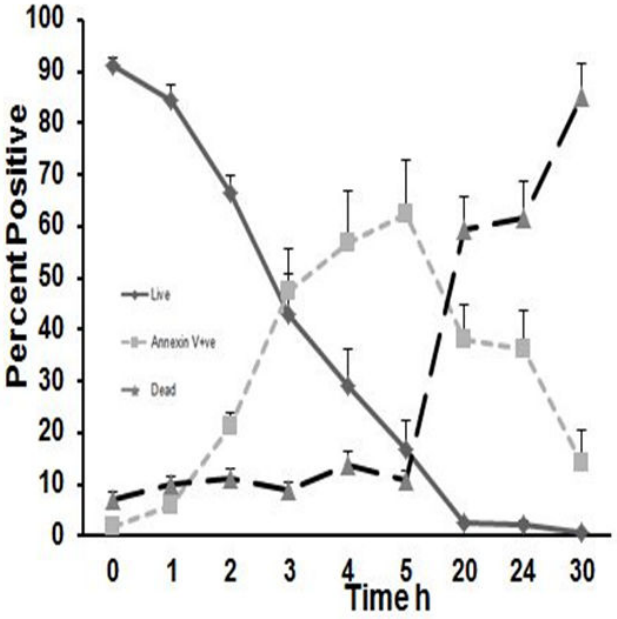

Figure 3. After induction of apoptosis cells with STS were labelled with annexin V-FITC and a DAPI at time points 0-30 h, with live, early apoptotic and dead cell populations plotted over this time, n=3, error bars, SEM. The population dynamics of apoptosis was shown to peak after 5 h and start to decline by 20 h at the same time the dead cell population significantly increased after 5 h. The live cell population systematically decreased over time.

3.4. Multiplexing other cell functions with the Annexin V assay

The multiplexing of fluorescent probes that measure functional or end products of biological processes with the standard Annexin V assay has allowed the researcher to further define the early events of apoptosis. The use of mitotracker carbocyanine dyes, $DiOC_6(3)$ or $DiIC_1(5)$,

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which measure the functionality or the polarization state of the inner mitochondrial membrane in cell cultures undergoing apoptosis, leads to the identification of live and early apoptotic cell populations with or without mitochondrial function. This can then be multiplexed with for example dihydroethidium (HE) to detect ROS allowing the further identification of more subsets within live, apoptotic and dead cell populations when used together with a mitotracker dye and the Annexin V assay [13-20].

UV-irradiated Jurkat cells after 3 h showed a high degree of early apoptosis (21%), a small amount of cell death (7%) and 71% of cells shown to be alive or Annexin-V^{-ve}/viability dye^{-ve} (Figure 4A). These live cell (or double negative events) can be divided into four sub-populations (Figure 4B), which include those with functional mitochondria $DiOC_6(3)^{+ve}/HE^{-ve}$ with no ROS production (86%). Those live cells with functional mitochondria $DiOC_6(3)^{+ve}/HE^{+ve}$ and ROS production (1.6%). Also live cells with no mitochondrial function or ROS production $DiOC_6(3)^{-ve}/HE^{-ve}$ (12%). Lastly live cells with no mitochondrial function with ROS production or $DiOC_6(3)^{-ve}/HE^{+ve}$ (0.1%) (Figure 4 B).

Likewise early apoptotic cells can be divided into four sub-populations (Figure 4C) which include those with functional mitochondria $DiOC_6(3)^{+ve}/HE^{-ve}$ with no ROS production (1.4%); those early apoptotic cells with functional mitochondria $DiOC_6(3)^{+ve}/HE^{+ve}$ and ROS production (1%). As well as early apoptotic cells with no mitochondrial function or ROS production $DiOC_6(3)^{-ve}/HE^{-ve}$ (55%), and lastly early apoptotic cells with no mitochondrial function with ROS production or $DiOC_6(3)^{-ve}/HE^{+ve}$ (42.6%) (Figure 4C).

Lastly late apoptotic and dead cells can be divided into four sub-populations (Figure 4D) which included those with functional mitochondria $DiOC_6(3)^{+ve}/HE^{-ve}$ with no ROS production (3%). Those late apoptotic and dead cells with functional mitochondria $DiOC_6(3)^{+ve}/HE^{+ve}$ and ROS production (4%), and those with no mitochondrial function or ROS production $DiOC_6(3)^{-ve}/HE^{-ve}$ (45%). Lastly late apoptotic and dead cells with no mitochondrial function with ROS production or $DiOC_6(3)^{-ve}/HE^{+ve}$ (48%) (Figure 4D).

In a further study employing STS and ETOP to induce apoptosis AnnexinV-FITC and DAPI was used in combination with the red mitotracker dye $DiIC_1(5)$ and HE (ROS) which showed a significantly different outcome in the distribution of cell subsets within live, early, late and dead cell populations compared to the previous example (Figure 5). Untreated cells showed a relatively high degree of apoptosis and cell death (20%, Figure 5A). However the live control cell population had a high degree of functional mitochondria (80%) and little ROS production (6%, Figure 5B). The STS and ETOP treated live cells had varying degrees of $AnnexinV^{-ve}/DAPI^{-ve}$ events (80 and 8% respectively, Figure 5E, I). However these live cell populations had similar incidence of cells with functional mitochondria 48 and 56% (without ROS production) respectively (Figure 5F, 5J). The different drugs also showed a very different degree of ROS production in that the functioning mitochondria in live cells treated with ETOP produced ROS to a high degree (17%, Figure 5J) compared to low levels in STS treated cells (3%, Figure 5F).

Early apoptotic cell populations after STS and ETOP treatment had a very different incidence of cells with functional mitochondria 18 and 1% (without ROS production) respective-

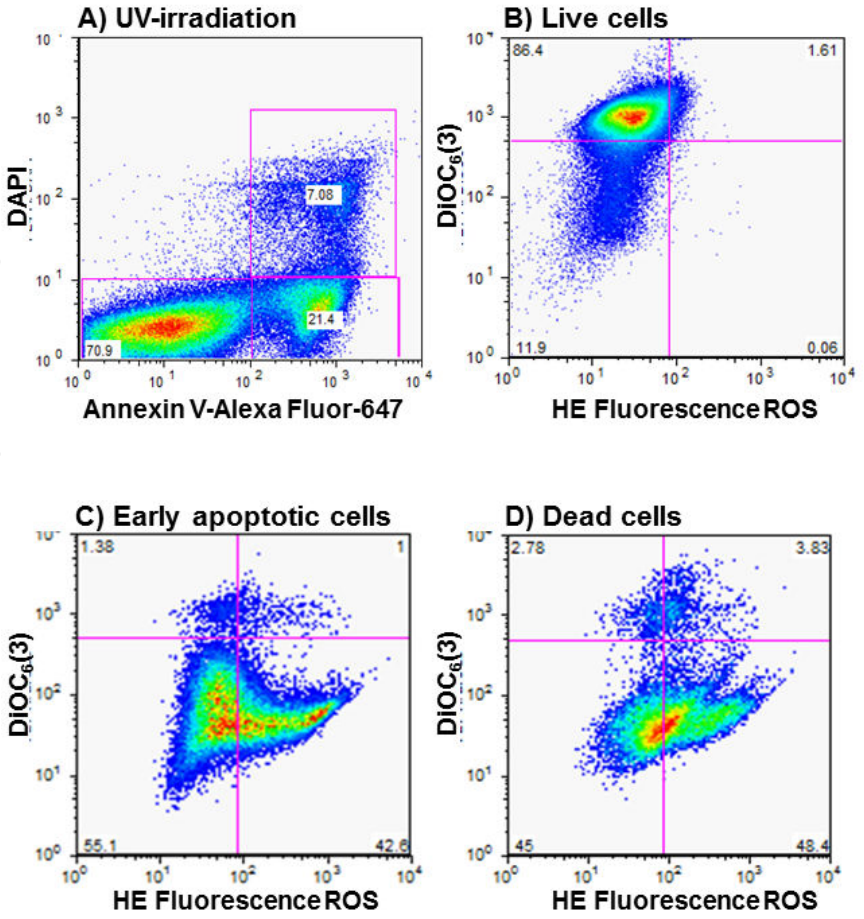

Figure 4. UV-irradiated Jurkat cells were labelled with HE, $DiOC_6(3)$ and Annexin V-FITC and DAPI after 3 h. The Annexin V and viability marker allows the live, apoptotic and dead cell populations to be determined A). Live B), apoptotic C) and dead D) populations were then analysed for mitochondrial function and ROS levels by plotting $DiOC_6(3)$ v HE parameters. $DiOC_6(3)^{+ve}/HE^{-ve}$ populations have fully functioning mitochondria, while $DiOC_6(3)^{+ve}/HE^{+ve}$ are functioning mitochondria with ROS generation. While $DiOC_6(3)^{-ve}/HE^{-ve}$ are cells with dysfunctional mitochondria and $DiOC_6(3)^{+ve}/HE^{+ve}$ are cells with dysfunctional mitochondria that have produced ROS.

ly (Figure 5G, 5K). The two drugs again also showed a very different degree of ROS production in that the cells with dysfunctional mitochondria treated with STS or ETOP produced ROS with an incidence of 17 and 54% respectively (Figure 5G, 5K). While late apoptotic and dead cells from both treatments show little or no mitochondrial function with most ROS+ve (86 and 79%, Figure 5H, 5L).

Following on from the observation that different drugs show varying affects upon the functionality of live and early apoptotic cell populations a time course study can reveal

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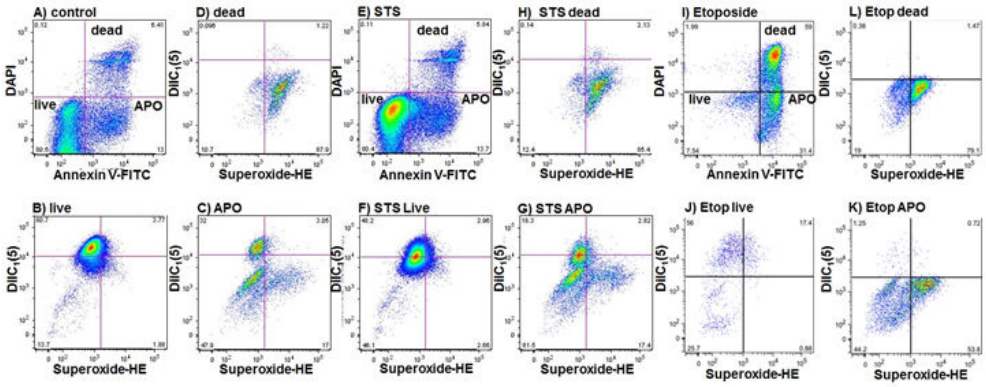

Figure 5. Untreated, STS (2 h) or ETOP (24 h) treated Jurkat cells were labelled with HE, $DiIC_1(5)$, Annexin V-FITC and DAPI. The Annexin V and viability dyes allow live, apoptotic and dead cell populations to be determined for untreated cells, STS or ETOP treatments A), E), I). Live B), F), J), apoptotic C), G), K), and dead D), H), L), populations were then analysed for mitochondrial function and ROS levels by plotting $DiIC_1(5)$ v HE parameters. $DiIC_1(5)^{+ve}/HE^{-ve}$ populations have fully functioning mitochondria, while $DiIC_1(5)^{+ve}/HE^{+ve}$ are functioning mitochondria with ROS production. While $DiIC_1(5)^{-ve}/HE^{-ve}$ are cells with dysfunctional mitochondria and $DiIC_1(5)^{+ve}/HE^{+ve}$ are cells with dysfunctional mitochondria that have produced ROS.

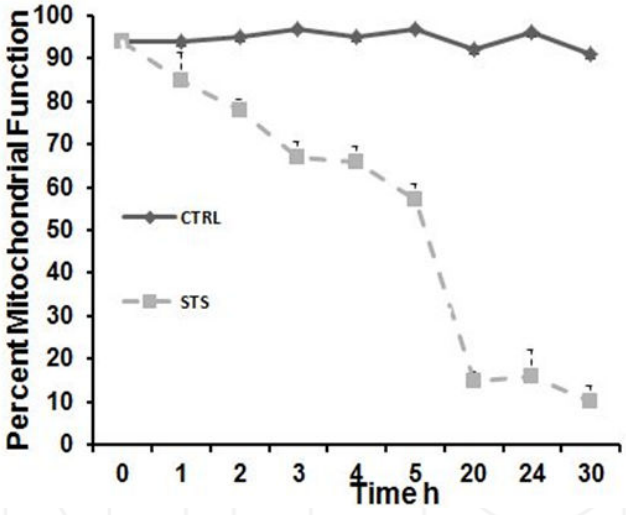

Figure 6. Jurkat cells were untreated or incubated with STS (1 μM) for 0, 1, 2, 3, 4, 5, 20, 24 and 30 h. Cells were loaded with $DiIC_1(5)$ to determine mitochondrial function and Annexin V-FITC and DAPI. Mitochondrial function was maintained throughout the assay in untreated cells but not in STS, n=3, error bar denote SEM.

interesting changes in these dynamics cell functions (*e.g.* mitochondrial function). A time course of live cell mitochondrial function can be plotted over 30 h after incubation with STS over this time course. After labelling with $DiIC_1(5)$ and Annexin V-FITC and DAPI the live cell

mitochondrial function can be plotted over time with the percentage of live cells with functioning mitochondria systematically decreasing over the 30 h with 40% of cells showing no function after 5 h (Figure 6) [13-17].

3.5. Use of Violet Ratiometric Membrane Asymmetry Probe

F2N12S, the violet ratiometric membrane asymmetry probe can also be used to measure apoptosis without the need for high calcium and is also not reversible unlike Annexin V binding to PS [22]. The use of the violet parameters, allows the blue and red laser parameters to be used for immunophenotyping without the need for separate labelling conditions as required for annexin V binding thus making a more flexible approach for labelling of samples. Here we compare F2N12S probe to Annexin V–PI labelling in untreated and STS treated cells, see Figure 7. Control cells labelled with Annexin V-FITC and PI are shown in Figure 7A with

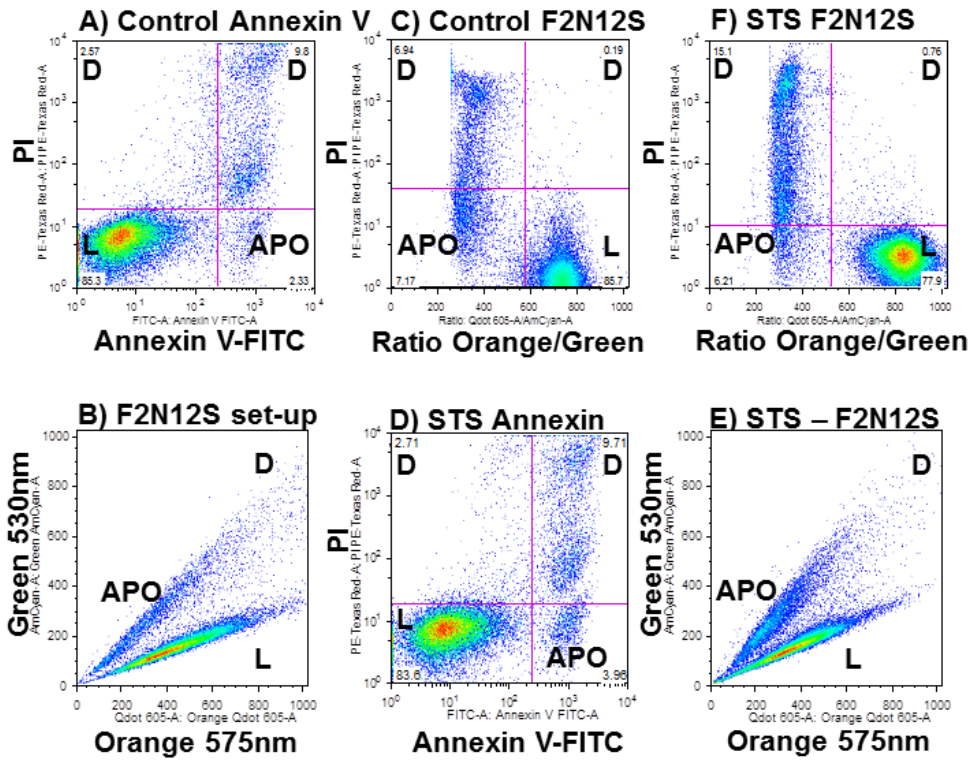

Figure 7. Jurkat cells were untreated or treated with 1 μM STS for 4 h and labelled with annexin V-FITC, the violet ratiometric membrane asymmetry probe, F2N12S and PI. A), D) show control and apoptotic cell cultures labelled with Annexin V-FITC and PI, L denotes live cells, APO apoptotic cells, and D denotes dead cells. B) and E) show the green and orange signals from F2N12S with live (L), apoptotic (APO) and (D) dead cells (confirmed by back-gating on PI positive events. Finally the F2N12S ratio signal of Orange/Green v PI indicates position of the live, apoptotic and dead cells for control and STS treated cells, C) and F).

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live cells indicated by (L), early apoptotic (APO) and dead cells (D). Jurkat cells loaded with F2N12S give orange (575nm) signals in live cells and green (530nm) signals in early, late and dead cell populations (Figure 7B). The electronic ratioing of the Orange/Green signals with the BD FACSDiva software allows the researcher to plot the Ratio v PI to give a dot-plot showing the presence of live cells (Ratio orange/green high), early apoptotic cells (APO, Ratio orange/green low), with dead cells being PI^{+ve} and Ratio orange/green low (Figure 7C). Likewise STS treated cells are shown labelled with Annexin V-FITC-PI (Figure 7D) and F2N12S with orange and green signals displayed in a dot-plot (Figure 7E) with the corresponding Ratio orange/green parameter vs PI in Figure 7D).

Acknowledgements

I would like to thank Dr Barry Wilbourn, GSK, UK for the Etoposide work included in this chapter.

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References

[1] Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972 Aug;26(4):239-257.

[2] Darzynkiewicz Z, Juan G, Li X, Gorcyzya W, Murakami T, Traganos F. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). Cytometry 1997;27:1–20.

[3] Wlodkowic D, Skommer J, Darzynkiewicz Z. Cytometry in cell necrobiology revisited. Recent advances and new vistas. Cytometry A 2010;77A:591–606.

[4] Fink SL, Cookson BT. Apoptosis, pyrotosis and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun 2005;73:1907–1916.

[5] Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell 1997 Feb 7;88(3):347-354.

[6] Yonehara S, Ishii A, Yonehara M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-down regulated with the receptor of tumor necrosis factor. J Exp Med 1989 May 1;169(5):1747-1756.

[7] Martinou JC, Youle RJ. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell 2011 Jul 19;21(1):92-101.

[8] Umansky SR, Korl BA Nelipovich PA. In vivo DNA degradation in thymocytes of gamma-irradiated or hydrocortisone treated rats. Biochem Biophys Acta 1981;655(1): 9-17.

[9] Pollack A, Ciancio G. Cell cycle phase-specific analysis of cell viability using Hoechst 33342 and propidium iodide after ethanol preservation. Methods Cell Biol 1990;33:19–24.

[10] Ormerod MG, Sun XM, Snowden RT, Davies R, Fearnhead H, Cohen GM. Increased membrane permeability of apoptotic thymocytes: a flow cytometric study. Cytometry 1993;14:595–602.

[11] Belloc F, Dumain P, Boisseau MR, Jalloustre C, Reiffers J, Bernard P, Lacombe F. A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. Cytometry 1994;17:59–6.

[12] Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis: Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled annexin V. J Immunol Methods 1995;184:39–51.

[13] Rasola A, Geuna M. A flow cytometric assay simultaneously detects independent apoptotic parameters. Cytometry A 2001;45A:151–157.

[14] Lugli E, Troaino L, Ferraresi R, Roat E, Prada N, Nasi M, Pinti M, Cooper EL, Cossaizza A. Characterization of cells with different mitochondrial membrane potential during apoptosis. Cytometry A 2005;68A:28–35.

[15] Poot M, Pierce RH. Detection of changes in mitochondrial function during apoptosis by simultaneous staining with multiple fluorescent dyes and correlated multiparameter flow cytometry. Cytometry 1999;35:311–317.

[16] Troiano L, Ferraresi R, Lugli E, Nemes E, Roat E, Nasi M, Pinti M, Cossarizza A. Multiparametric analysis of cells with different mitochondrial membrane potential during apoptosis by polychromatic flow cytometry. Nat Protoc 2007;2;2719-27.

[17] Lizard G, Fournel S, Genestier L, Dhedin N, Chaput C, Flacher M, Mutin M, Panaye G, Revillard JP. Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. Cytometry 1995;21:275–283.

[18] Yaffe, PB, Doucette CD, Walsh M, Hoskin DW. Piperine impairs cell cycle progression and causes reactive oxygen species-dependent apoptosis in rectal cells. Exp Mol Pathol 2013;94:109-114.

[19] Al-Mehdi AB, Shuman H, Fisher AB. Intracellular generation of reactive oxygen species during nonhypoxic lung ischemia. Am J Physiol 1997;272:L294-300.

[20] Thayyullathil F, Chathoth S, Hago A, Patel M, Galadari S. Rapid reactive oxygen species (ROS) generation induced by curcumin leads to caspase-dependent and –independent apoptosis in L929 cells. Free Radic Biol Med 2008;45:1403-12.

[21] Zhan L, Yang Y, Ma TT, Huang C, Meng XM, Zhang L, Li J. Mol Cell Biochem 2015; Epub DOI 10.1007/s11010-014-2298-6.

[22] Shynkar VV, Klymchenko AS, Kunzelmann C, Duportail G, Muller CD, Demchenko AP, Freyssinet JM, Mely Y. Fluorescent biomembrane probe for ratiometric detection of apoptosis. J Am Chem Soc 2007;129(7):2187-93.

[23] Vossenkamper A, Marches O, Fairclough PD, Warnes G, Stagg AJ, Lindsay JO, Evans PC, Luong LA, Croft NM, Naik S, Frankel G, McDonald TT. Inhibition of NF-kB signalling in human dendritic cells the enterpathogenic *Escherichia coli* effector protein NIeE .*J Immunol 2010*185: 4118-4127.

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