

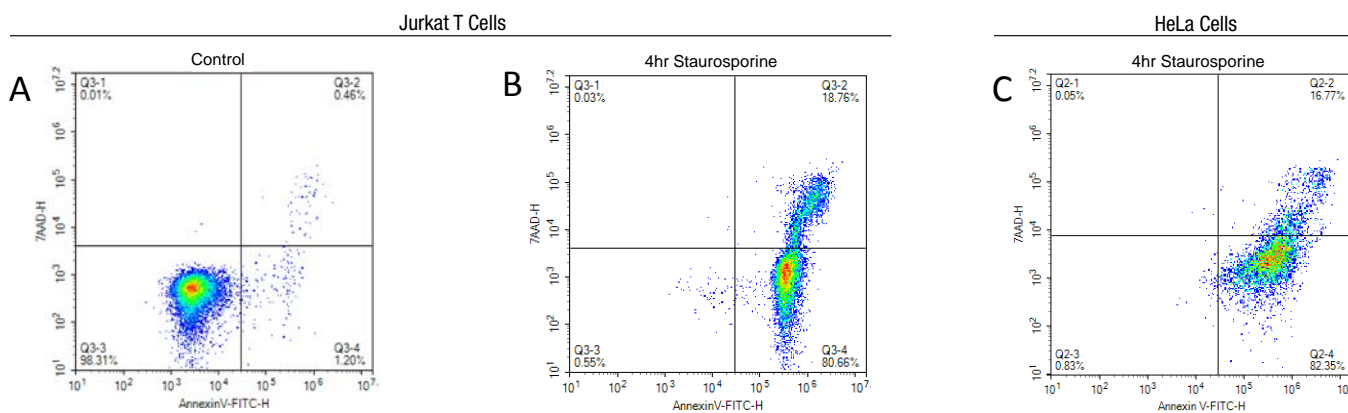
### Quantitative measurements of apoptosis using the NovoCyte Flow Cytometer

Apoptosis, or programmed cell death, is an active process by which cells regulate how they die by triggering specific pathways that cause the cell to shrink, condense, and eventually be cleared by phagocytosis. This is in contrast to necrotic cell death where cells die uncontrollably due to external factors and fall apart, leading to detrimental effects such as the activation of an immune response. Therefore, apoptotic cells that die in a very orderly fashion have no or limited disruption of nearby cells and tissue.

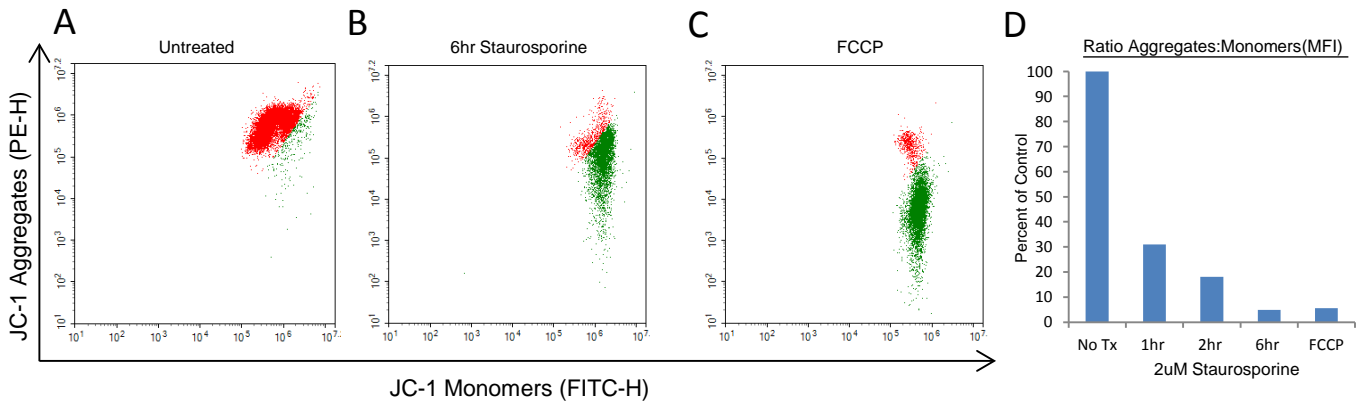
Apoptosis is a temporal and multistep process that can be interrogated at different stages depending on the context. At an early apoptotic stage, the mitochondria loses membrane potential and is unable to function properly leading to cytochrome c release and the activation of caspases. Another event at an early stage of apoptosis involves phosphatidylserine (PS) translocation from the inner cellular membrane to the outer leaflet, exposing it to the extracellular environment. During later stages of apoptosis, cellular membranes become permeable compromising their integrity and selective transport of materials.

In order to demonstrate the flexibility and versatility of the NovoCyte system for assessment of apoptotic events, we show three different assays that can measure apoptosis in response to staurosporine, a broad kinase inhibitor known to induce apoptosis in many cell types. The first assay uses Annexin V to bind exposed PS on cell membranes for labeling early apoptotic cells and the DNA binding dye, 7-AAD, for labeling late apoptotic cells. With this assay, we show that staurosporine induces apoptosis in both Jurkat T cells and HeLa cells in Figure 1, and can distinguish cells at early and late stages of apoptosis. The second assay makes use of the fluorescent dye JC-1, to measure the loss of mitochondrial membrane potential in early apoptotic cells. JC-1 is cell permeable and aggregates inside mitochondria when membrane potential is maintained, emitting fluorescence in the PE channel. Yet, when the membrane potential is lost, JC-1 will not localize to the mitochondria and will reside in the cytoplasm in its monomeric form, emitting fluorescence in the FITC channel. Thus, the ratio of PE to FITC fluorescence can represent changes in the mitochondria membrane potential. As shown in Figure 2, compared to untreated controls, Jurkat T cells treated with staurosporine have reduced mitochondrial membrane potential that continuously decreases over time up to 6 hours after treatment. Such a response is similar to that observed on cells treated with a known mitochondrial membrane depolarizing agent, FCCP. The third assay quantifies caspase activity. Probe molecules containing a cleavage site specific for caspase 3 & 7 are added to cells. When cleaved by these activated caspases in early apoptotic cells, the probe molecules enter the nucleus, bind to DNA, and fluoresce. This enables the direct readout of activated caspases in cells. As shown in Figure 3, staurosporine treated Jurkat T cells have more caspase activity than those for the untreated, control cells.

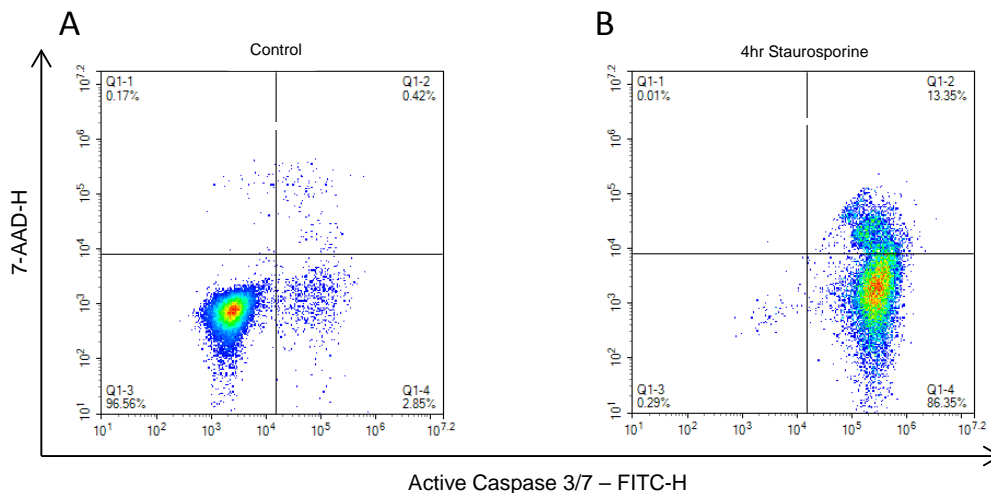
The three assays described here offer quantitative, robust and reproducible assays for measuring populations of cells which are undergoing apoptosis at different stages. These apoptotic assays are easy to perform with the NovoCyte flow cytometer due to its automatic compensation capability and a wide dynamic range of fluorescence detection, thereby eliminating the need for PMT voltage adjustments.



**Figure 1. Detection of early apoptosis using Annexin V/7-AAD in Staurosporine treated cells.** Jurkat T cells were subjected to A) vehicle control or B) 2uM Staurosporine and C) HeLa cells were subjected to 2uM Staurosporine for 4 hours. Following treatment, cells were analyzed for early and late apoptotic cells by staining for phosphatidylserine with Annexin V-FITC and with the DNA binding dye 7-AAD (BioLegend), followed by flow cytometric analysis. Cells were classified as early apoptotic with Annexin V only positive staining and late apoptotic (or dead) cells with Annexin-V and 7-AAD positive staining.



**Figure 2. Staurosporine compromises mitochondrial membrane potential in Jurkat T cells.** Jurkat T cells were treated with A) vehicle, B) 2uM Staurosporine for 6 hours, or with C) FCCP. JC-1 dye (Life Technologies) was added to the cells for flow cyometry measurement of cells' mitochondrial membrane potential. In healthy cells, the dye is localized to the mitochondria and forms aggregates, emitting fluorescence in the PE channel. In cells where the mitochondrial membrane potential has been compromised, the dye localizes to the cytoplasm as monomers, emitting fluorescence detectable in the FITC channel. The effect of compound treatment of the cells can be evaluated based on the ratio of PE to FITC fluorescence. D) A bar graph representing the ratio for Jurkat T cells at different time points following treatment with staurosporine. All treated cells (including FCCP positive control) show a lower ratio than the vehicle treated control.



**Figure 3. Detection of caspase 3/7 activity in Jurkat T cells treated with Staurosporine.** Jurkat T cells were treated with 2uM staurosporine for A) 0h or B) 4h and stained with the cell permeable DEVD-conjugated fluorescent nucleic acid binding dye (Life Technologies) and 7-AAD dye (BioLegend). Untreated control cells show minimal caspase activity. Upon 4 hr treatment with Staurosporine, cells exhibit an increase in caspase 3/7 activity (99.7%).

### References

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2. Susan Elmore. Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol.* 2007; 35(4): 495-516



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