Simultaneous Measurement of NF-kB Translocation and Apoptosis

Simultaneous Measurement of NF-κB Translocation and Apoptosis With the ImageStream® Multispectral Cell Imaging System

The ImageStream System

The ImageStream system combines the capabilities of microscopy and flow cytometry in a single platform for quantitative image-based cellular assays in large and heterogeneous cell populations. Here we demonstrate the simultaneous measurement of drug-induced apoptosis and NF-kB nuclear translocation using a combination of intensity-based and morphometric parameters. Apoptotic and necrotic cells are identified on the basis of intracellular caspase-3 staining and nuclear morphology, while the location of NF-kB relative to the nucleus is measured using a novel application of image correlation provided with the analysis software. These results demonstrate the power of analytical morphometry in the quantitation of apoptosis and nuclear translocation.

The ImageStream is operationally similar to a flow cytometer but it has the ability to simultaneously capture six images of each cell--at a rate of approximately 300 cells per second--with a resolution similar to that of a fluorescence microscope. Each cell is represented by a brightfield image, a darkfield image and up to four different fluorescence images. Using these data, the ImageStream provides quantitative information not just about the intensity of target molecules, but about their location within the cell as well. In addition, the system delivers statistically significant assessments of the cellular morphology of different populations.The combination of these flow cytometry and microscopy capabilities allows a new level of integrated quantitative analysis, both of the individual cell and the cell population.

Experimental Design and Results

Apoptosis is an organized, tightly regulated process by which a cell orchestrates its own destruction. Inappropriately low rates can result in cancer or autoimmunity while inappropriate high rates can result in neurodegenerative disorders or immunodeficiency. Development of agents that modulate the apoptotic process is an important goal of drug discovery research.

Factors that induce apoptosis initiate a cascade of signals that eventually result in the activation of intracellular effector proteases (caspases). These activated caspases in turn cleave cellular targets, resulting in the hallmark appearance of apoptosis, typified by nuclear condensation, chromosome fragmentation and cellular blebbing. In contrast, factors that inhibit apoptosis activate nuclear

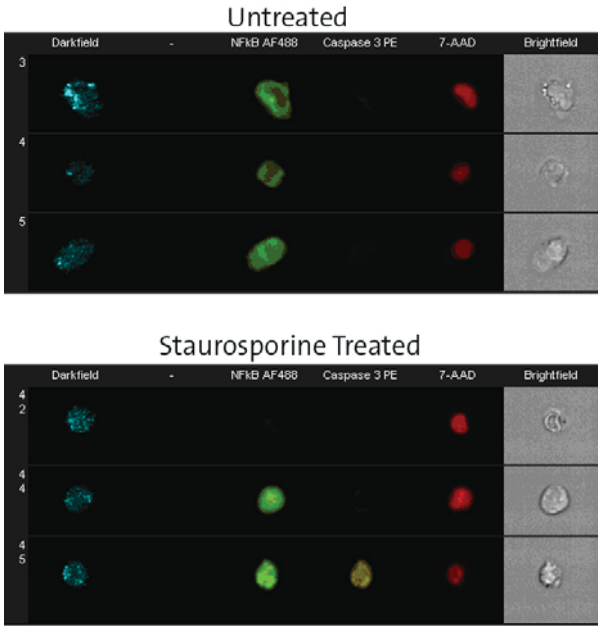

Figure 1. (Multi-spectral images of untreated (top) and staurosporine-treated (bottom) cells. Each cell is represented by the row of images that include Dark Field (488 nM side scatter, blue), NF-kB AF488 (green), activated caspase 3 PE (orange), 7-AAD (red), and Bright Field (white)

translocation of the transcription factor NF-kB. Once in the nucleus, NF-kB regulates the expression of genes that promote cell survival and proliferation, immune system function and inflammation. Agents that stimulate caspase activation but inhibit nuclear translocation of NF-kB may have great potential for the treatment of autoimmune disorders and cancer.

The extent of nuclear NF-kB translocation is usually assessed by microscope examination since the location of the factor within the cell is the key variable. Measurement of NF-kB location has historically been done on adherent cells which show large cytoplasmic area to nuclear area ratios. However, most cells of the immune system and many hematological tumors exist 'in suspension' (e.g., lymphocytes, monocytes, granulocytes) and adapting them to plates often proves difficult. In this study we introduce a method for measuring nuclear location of NF-kB in the THP-1 suspension cell line, which have very low cytoplasmic area to nuclear area ratios. We detail the simultaneous measurement of NF-kB translocation and cell death induced in a suspension-based cell line by the protein kinase inhibitor staurosporine. The data were acquired on the ImageStream imaging flow cytometer, which provides up to six channels of imagery for each cell. The ability to acquire multi-spectral images of large numbers of cells combined with morphology-based algorithms allowed for the multi-parameter assessment of cell death and NF-kB translocation in the same data file.

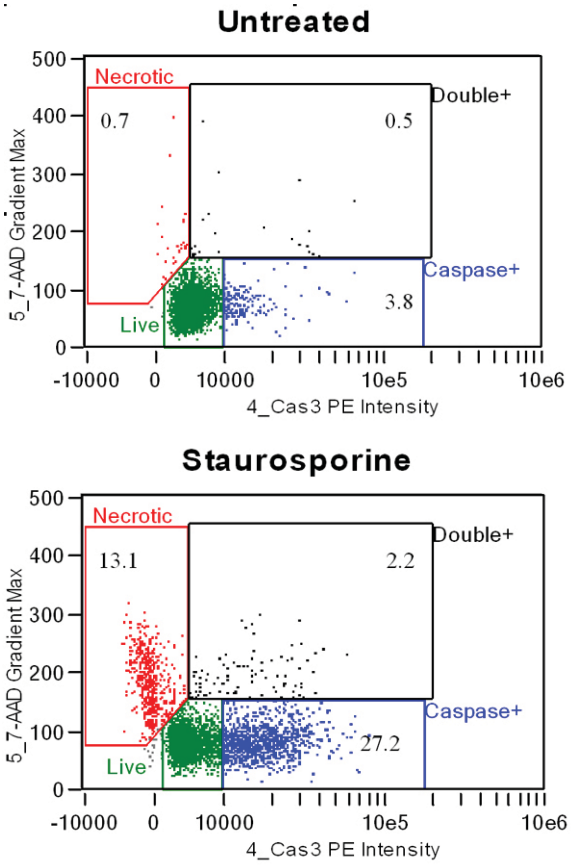

Figure 2A. Activated caspase 3 PE Intensity vs. 7-AAD gradient max bivariate plots and representative subpopulation imagery. These plots consist of single nucleated cells that were chosen by gating 7-AAD positive events with low nuclear area and high nuclear aspect ratios (measure of 'roundness'). The percentage of cells in each population is indicated by the number within the region.

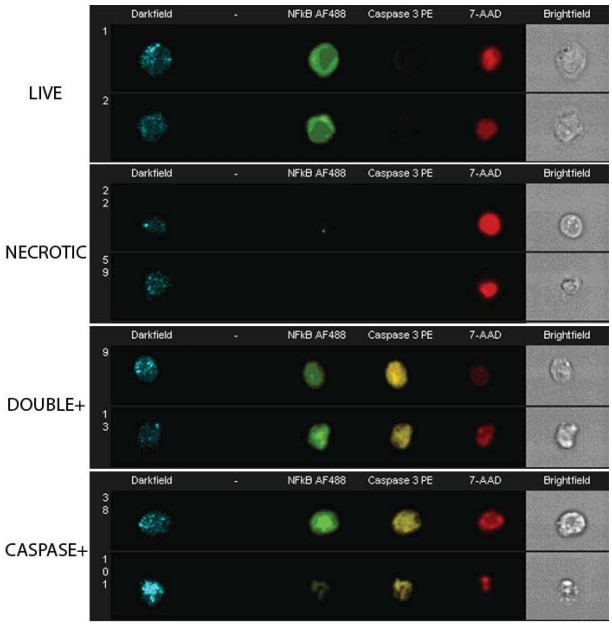

Figure 2B. . Representative six-channel images are shown for each of the populations defined by the gates shown in Figure 2A.

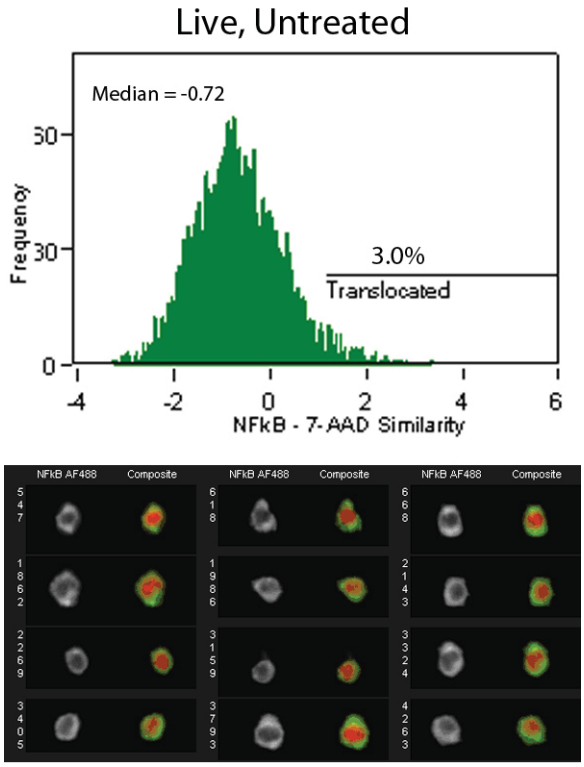

Figure 3A NF-KB – 7-AAD similarity score histogram for untreated THP-1 cells. In this analysis, a larger score indicates a greater degree of signal correlation between the NF-KB channel and nuclear channel and thus, translocation. The gate for the positive population threshold was determined by reference to the Dark Field – 7-AAD similarity score, a measure of uncorrelated images that is internally controlled (data not shown). The percentage of translocated cells is given above the 'Translocated' region bar, and the median of the entire 'Live' population is given in the upper right hand corner of the histograms. A sampling of cell images taken from the 'low similarity' regioin is shown below the histogram.

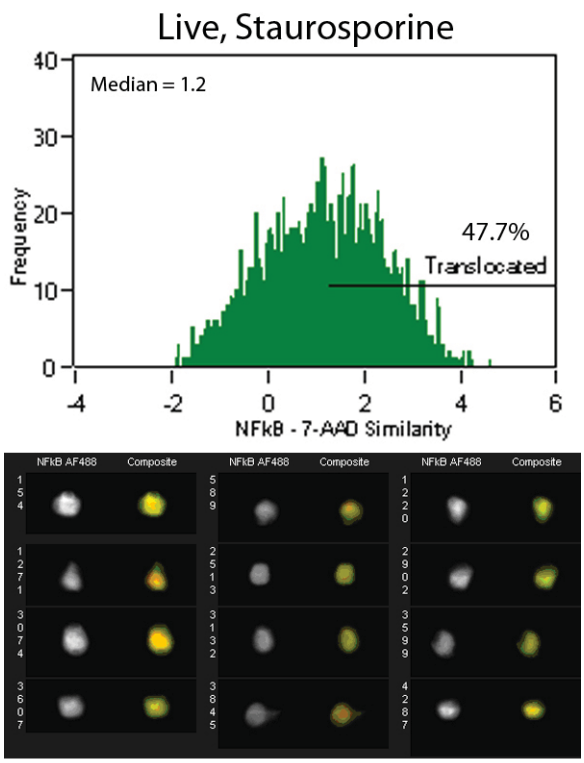

Figure 3B NF-KB – 7-AAD similarity score histogram for THP-1 cells treated with staurosporine. Values given in the histogram are as described in Figure 3A. A sampling of cell images from the 'high similarity' bin is shown below the histogram.

Apoptosis and NF-kB nuclear translocation were induced by treating THP-1 cells with 1 mM staurosporine for 4 hours. The cells were stained with fluorescent probes specific for NF-kB and caspase-3, followed by counterstaining the nucleus with 7-AAD. Multi-spectral images of 5000 events were collected on the ImageStream. Figure 1 shows representative images from the two experimental samples. Untreated cells generally had cytoplasmic NF-kB, low caspase3 staining and intact, uniformly staining nuclei. After staurosporine treatment, some cells expressed activated caspase 3 (e.g., cell #45), some had nuclear-localized NF-kB (e.g. cell #44), while others lacked detectable cytoplasmic staining (cell #42).

Measurements made on the target molecules were consistent with these images. Treatment induced caspase 3 activation in a large percentage of cells compared to the untreated control (Figure 2A). Drug treatment also induced a significant percentage of caspase 3 negative / high 7-AAD gradient max cells (the latter feature measures nuclear image contrast). Cells in this latter region were clas-

sified as necrotic because of their uniformly round nuclear staining patterns and potentially high plasma membrane permeabilities (indicated by a lack of intracellular NF-kB staining (Figure 2B)).

In addition to activating effector caspases, staurosporine is also known to induce nuclear translocation of NF-kB. We quantified the degree of nuclear translocation by calculating the 'similarity' of the NF-kB and 7-AAD images. The NF-kB – 7-AAD similarity score maps the pairs of images to a continuum ranging from uncorrelated to highly correlated. As shown in Figures 3A and 3B, staurosporine treatment induced significant NF-kB nuclear translocation in some, but not all, of these cells at the 4 hour time point: NF-kB images from selected low similarity histogram regions reveal a distinctly cytoplasmic distribution, while those from high similarity regions display a clearly nuclear distribution.

Conclusions

Cellular images provide a wealth of information that can be rapidly interpreted by the knowledgeable observer. However, objective and statistically rigorous interpretation requires quantitative analysis of large numbers of images. The ImageStream Imaging Flow Cytometer captures large numbers of multi-spectral digital images and provides powerful analytical algorithms, enabling robust quantitation of image-based assays.

In this study, we combined the use of intensity- and morphology-based features to monitor staurosporine-induced NF-kB translocation and cell death in one assay. Staurosporine induced two morphologically distinct forms of cells death: 1) apoptosis as measured by increased intensity of activated caspase 3 staining, indicating effector caspase activation; and 2) necrosis, as measured by the nuclear image contrast gradient. Staurosporine also induced significant nuclear translocation of NF-kB, as determined by the similarity algorithm that measures the correlation between the NF-kB and 7-AAD nuclear images. Thus, staurosporine-induced nuclear translocation of NF-kB is insufficient to protect THP-1 cells from cell death, although NF-kB is responsible for transcribing many genes involved in prevention of the apoptotic process, . This assay demonstrates the potential for monitoring the outcome of diverse signaling pathways simultaneously using the ImageStream imaging cytometer.