



## APOPTOSIS: Quantitative Analysis Techniques

### Introduction

The study of programmed cell death, or **apoptosis**, has emerged as a topic of intense research activity at UIUC. Many investigators are interested in rapid assays to detect and quantify apoptosis in cell populations. Recently, the staff of the BTC Flow Cytometry Facility tested several kits and methodologies designed to identify apoptotic cells. This brief summary of **flow cytometric techniques** for the investigation of apoptosis was prepared to update UIUC faculty regarding the technologies available in this area.

### Characteristics of Apoptosis

Apoptosis is a form of cell death in which an individual cell undergoes an internally controlled or "programmed" transition from an intact metabolically active state into a number of shrunken remnants that retain their membrane bound integrity (1,2).

Flow cytometry and fluorescence microscopy can be used to detect several of the morphological changes characteristic of cells undergoing apoptosis. In particular, flow cytometry can be used to follow the percentage of apoptotic cells present in a cell population over the course of an experiment. Below is a short list of detectable characteristics.

#### Detectable Characteristics

1. DNA Fragmentation
2. Changes in cell size and granularity
3. Changes in plasma membrane permeability
4. Cell surface modification (externalization of phosphatidylserine)
5. Formation of Apoptotic bodies

### Methods and Results

Each of the listed characteristics can be discriminated using either image or flow cytometry. The following is a brief summary of each method, along with representative data.

Apoptosis was induced in U937 cells (human monocyte cell line) by treatment with TNF- $\alpha$  (tumor necrosis factor). Apoptosis was induced in CHO cells by exposure to UV radiation ( $\lambda=254\text{nm}$ ).

#### 1. DNA fragmentation

These tests were conducted using the *APO-DIRECT* and *APO-BRDU* kits sold by Phoenix Flow Systems, Inc. DNA fragmentation due to internucleosomal cleavage exposes many 3'-hydroxyl termini of DNA. Apoptotic cells can be identified by labeling the DNA strand breaks with fluorescently tagged deoxyuridine triphosphate nucleotides (F-dUTP) or with bromodeoxyuridine triphosphate (Br-dUTP) and FITC labeled antiBrdU antibody. Fluorescence intensity measured by flow cytometry will then be proportional to the number of strand breaks. This technique is often referred to as a **TUNEL** assay. Figure 1 shows typical data for a sample containing 10% apoptotic cells. These methods require cell fixation and the entire cell preparation requires approximately 4 hours.

#### 2. Cell size and granularity

Morphologically, rapid cell shrinkage and increased cell granularity are the most obvious changes often associated with apoptosis (1). These changes can allow populations of apoptotic and viable cells to be distinguished based on light scatter properties measured by flow cytometry. Figure 2 shows a comparison of the forward and side scatter histograms of normal and apoptotic CHO cells.

#### APO-DIRECT Labeling

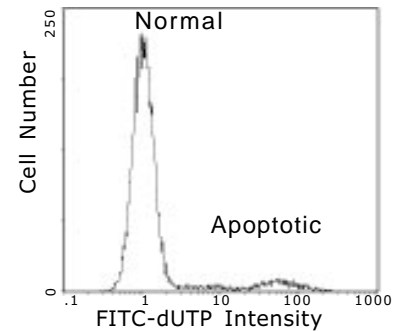
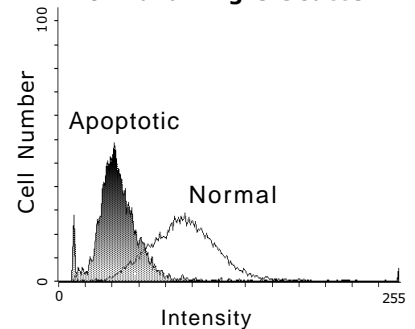


Figure 1

Example of F-dUTP labeling of apoptotic cells using the *APO-DIRECT* kit. Approximately 10% of the cells are identified as apoptotic.

#### Forward Angle Scatter



#### Side Scatter

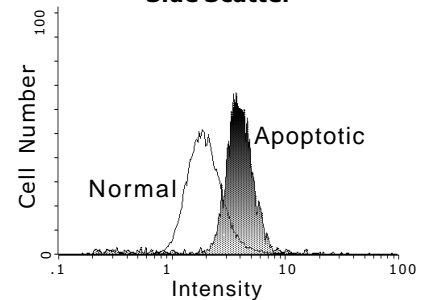


Figure 2

Variations in light scatter between apoptotic and non-apoptotic cells. Apoptotic cells tend to shrink, as indicated by the forward angle light scatter intensity. They also increase in granularity, as indicated by the side scatter intensity.



### 3. Changes in plasma membrane permeability

It has been reported that apoptotic cells exhibit increased plasma membrane permeability to certain fluorescent dyes(4). Short exposure of cells to a low concentration of Hoechst 33342 results in more intense labeling of apoptotic cells, as compared to control cells. By counterstaining with a viability stain, such as propidium iodide (PI), it is possible to distinguish among normal, apoptotic and necrotic cells(5). Figure 3 shows an example of a cell population that contains 30% apoptotic cells as determined by this method. The PI histogram is not shown, but the fraction of necrotic cells is easily measured. The time required for preparing and analyzing a sample by this method is about 15 minutes.

### 4. Quantitation of annexin V binding to apoptotic cells

These tests were conducted using the Annexin V Apoptosis Detection Kit sold by R&D Systems. This assay is based on the observation that phosphatidylserine (PS), a negatively charged phospholipid, is translocated from the inner to the outer leaflet of the plasma membrane during apoptosis (6,7). Annexin V conjugated to FITC has been demonstrated to preferentially bind to PS in a  $Ca^{+2}$  dependent manner. No fixation is required for this technique. It is a fast, and reasonably accurate method for identifying apoptotic cells. Since Annexin V can be conjugated to FITC or PE, the assay can be run on the bench-top flow cytometry analyzers. Figure 4 shows example data for this method for a sample with 40% apoptotic cells. Apoptotic cells bind Annexin V and appear as the higher fluorescence intensity population. The time required for preparing and measuring a sample is about 60 minutes.

### Fluorescence Microscopy

Acridine Orange (AO) and ethidium bromide (EB) are intercalating, nucleic acid specific, fluorochromes which emit a green and orange fluorescence, respectively, when they are bound to DNA. Of the two, only AO can cross the plasma membrane of viable and early apoptotic cells (8). Viewed by fluorescence microscopy, viable cells appear to have a bright green nucleus with intact structure while apoptotic cells exhibit a bright green nucleus showing condensation of chromatin as dense green areas. Late apoptotic cells and necrotic cells will stain with both AO and EB, however EB produces the highest intensity emission. Late apoptotic cell cells have an orange nucleus showing condensation of chromatin and necrotic cells display an orange nucleus with intact structure. This is a simple assay, that provides a very useful qualitative evaluation. The total time required for preparing and measuring a sample is 5 to 10 minutes

### References

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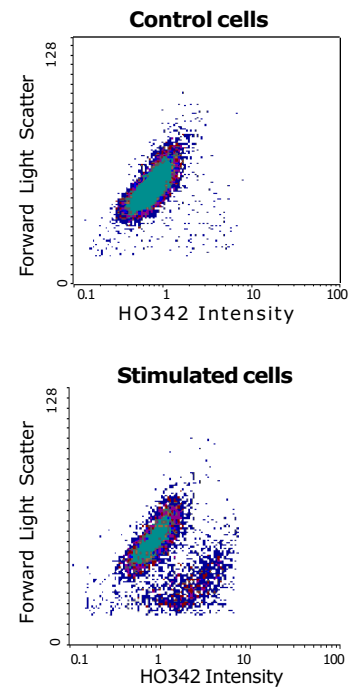


Figure 3  
The plasma membranes of apoptotic cells are more permeable to Hoechst. Apoptotic cells in the stimulated cells plot as having increased Hoechst fluorescence and decreased FALS.

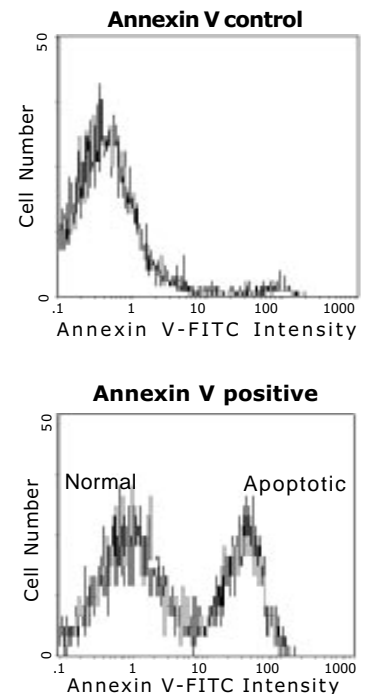


Figure 4  
Annexin V-FITC labels PS which flips to the outside of the plasma membrane during apoptosis.

