

Application Note

Simultaneous Analysis of Four Stage-Specific Markers of Apoptosis with the Accuri C6 Flow Cytometer[®] and CFlow[®] Plus Software

Introduction

The study of apoptosis (programmed cell death) has broad application in the fields of cell biology, cancer research and developmental biology. A wide variety of products designed to monitor the progressive stages of apoptosis by flow cytometry have been developed over the past ten years. Often, the limiting factors for investigators wishing to develop apoptosis studies are an understanding of reagent compatibility and access to flow cytometry for analysis.

The Accuri C6 Flow Cytometer System with CFlow Plus Software provides many advantages for apoptosis studies:

Absolute counts

Cell concentration can be calculated directly from CFlow software statistics tables without the addition of counting beads.

Six detection parameters

The dual laser (blue and red) excitation, 4-color detection system allows flexibility in reagent choice.

Post collection data analysis

The unique 7-log dynamic range and pre-optimized detector performance minimizes the potential for data loss due to incorrect instrument set up.

Digital signal processing

Data signal processing allows post-data collection, fluorescence compensation and gating decision adjustments.

Results

Apoptosis Detection

The stage of apoptosis was assessed immediately following induction with either staurosporin or anti-Fas antibody using the following reagents:

FLICA-Caspases 3 & 7 (C6 FL1 detector): Carboxyfluorescein-labeled caspase inhibitor peptide FAM-DEVD-FMK, binds to caspases 3 & 7 (Immunochemistry Technologies).

TMRM (C6 FL2 detector): Tetramethylrhodamine, indicates mitochondrial membrane potential ($\Delta\Psi_m$) (Immunochemistry Technologies).

7-AAD (C6 FL3 detector): 7-aminoactinomycin D is excluded by cells with maintained membrane integrity (Accuri Cytometers, Inc.).

Annexin-APC (C6 FL4 detector): Phosphatidyl serine detection (eBiosciences).

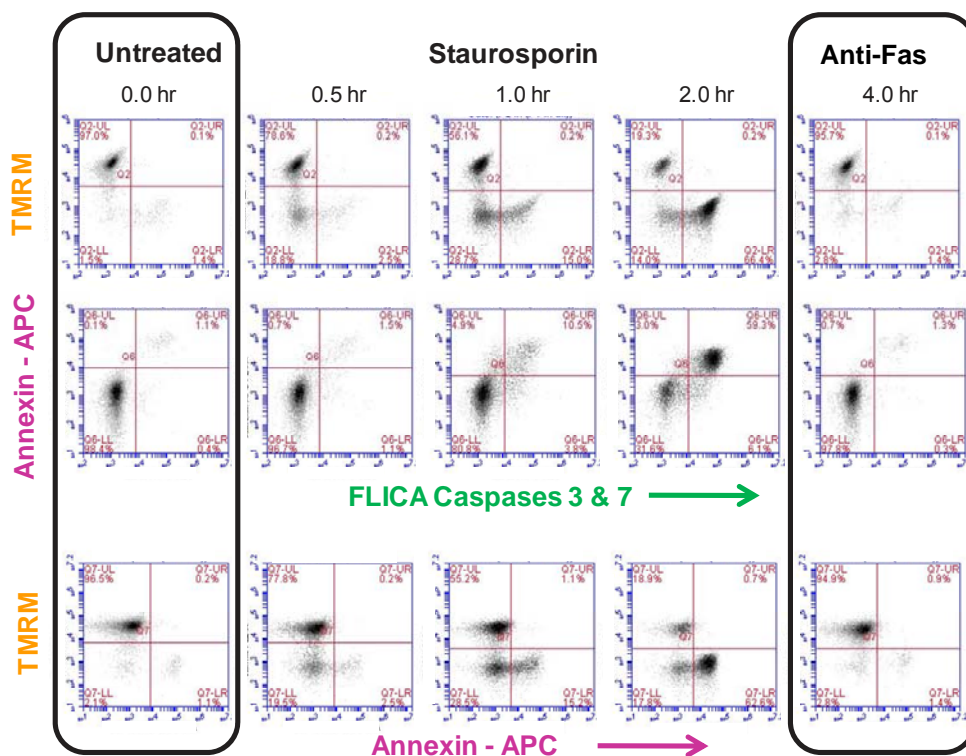


Figure 1. All plots have been gated to exclude 7-AAD positive (late stage) cells. Progression of apoptosis in Jurkat cells treated short term with either staurosporin, or anti-Fas antibody, as indicated by:

1. Decreased TMRM signal
2. Increased expression of caspases 3 & 7 and
3. Binding of annexin to phosphatidyl serine on the outer cell membrane.

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Results, cont.

Staurosporin treated Jurkat cells

The plots in rows 1 and 2 of Figure 1 show that a loss of $\Delta\Psi_m$ (mitochondrial membrane potential), indicated by decreasing TMRM fluorescence, precedes expression of caspases 3 and 7 and the appearance of phosphatidyl serine (PS) on the outer membrane (annexin positivity). The plots in row 2 suggest that the activation of caspases 3 and 7, and expression of PS occur nearly simultaneously.

Anti-Fas treated Jurkat cells

Cells treated with anti-Fas antibody for 4 hours (column 5) do not show signs of $\Delta\Psi_m$ loss, or increased expression of caspases 3 & 7 or PS on the outer membrane, as compared to untreated control cells (column 1).

Viable cell number determinations

7-AAD exclusion (cells in gate P2 of Figure 2b) was used as an indication of late stage apoptosis and probable cell death. The number of cells falling in both gates P1 (whole cell FSC vs.SSC) and P2 (intact outer membrane) of Figure 2a, and the volume collected by the C6 for each sample, was copied from the CFlow Plus statistics tables, pasted into a spreadsheet program and the number of viable cells per mL of sample determined (Table 1).

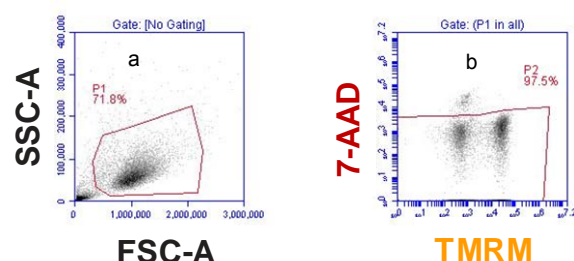


Figure 2. Light scatter gate P1 (a) and 7-AAD negative gate P2 (b) for Jurkat cells apoptosis analysis. 7-AAD is excluded by cells with intact outer membrane integrity and its uptake occurs in the latest stages of apoptosis. All plots in Figure 2 were gated on 7-AAD negative cells

Sample	7-AAD Neg Cell #	Volume (μL)	Percent Viable	Viable cells $10^3 / \text{mL}$
Untreated	13,313	41.2	97.50%	323
Stauro 0.5 hr	10,069	40.1	96.10%	251
Stauro 1.0 hr	10,133	28.4	97.50%	357
Stauro 2.0 hr	9,805	19.4	98.70%	505
Stauro 3.0 hr	10,405	19.1	97.70%	545
Anti-FAS 4.0 hr	10,229	25.1	97.90%	408

Table 1. Viable cell number per mL of sample. The data in columns 2-4 were copied directly from the CFlow Plus statistics tables and pasted into a spreadsheet program. The viable cells per mL values (last column) were calculated from the data in columns 2 and 3.

Materials and Methods

Cell Treatment and Staining

Jurkat cells were cultured for 0.5, 1.0, 2.0, or 4.0 hour with either 1 μM staurosporin or 100 ng/mL anti-Fas antibody to induce apoptosis. After culture, cells were transferred to a 96-well plate for staining. After pelleting, cells were resuspended in 100 μL of 1x Annexin Binding Buffer pre-warmed to 37°C containing FAM-DEVD-FMK, 100 nM TMRM, and/or 2 μL APC-conjugated Annexin-V and incubated at 37°C for 20 minutes in the dark. After incubation, cells were washed twice with 1x Annexin Binding Buffer before resuspension in 200 μL of the same buffer with or without 7-AAD at a 1:100 dilution.

Flow Cytometric Analysis

Samples were kept at RT and analyzed immediately on an Accuri C6 Flow Cytometer with CFlow Plus Software.

References

- 1) Jayaraman S. JIM 306:68-79, 2005
- 2) Rasola A. et al. Cytometry 45: 151-157, 2001.
- 3) Degeterev et al. Nature Reviews 9:378-390, 2008.