

A low-cost flow cytometric assay for the detection and quantification of apoptosis using an anionic halogenated fluorescein dye

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We describe here a technical improvement of an established colorimetric method used to detect and measure the occurrence of apoptosis in mammalian cells during in vitro cell culture. This assay uses an anionic halogenated fluorescein dye that is taken up by apoptotic cells at the stage of phosphatidylserine externalization. We demonstrate that apoptotic cells stained with this dye can be detected by flow cytometric analysis. Furthermore, we show that the modified method compares well with the standard annexin-V-based apoptosis assay and that it is significantly more cost-effective than the annexin-V assay.

In viable cells, the distribution of phospholipids between the inner and outer leaflet of the plasma membrane is asymmetric. In this asymmetric distribution, phosphatidylcholine and sphingomyelin are predominantly located in the outer leaflet, while phosphatidylethanolamine and phosphatidylserine (PS) are found in the inner plasma membrane leaflet (1). However, during apoptosis PS translocates to the outer plasma membrane leaflet and can thus be used as a biomarker to identify apoptotic cells. It was demonstrated that annexin-V has a strong affinity for PS (2), and this facilitated the development of flow cytometry-based bioassays that use fluorescein isothiocyanate (FITC)-labeled or Phycoerythrin-labeled annexin-V to detect and quantify apoptosis (3).

Although the annexin-V assay is a well-established standard flow cytometric assay used to detect and quantify apoptosis based on PS externalization, it is relatively expensive, costing on average U.S. \$6 per sample. By contrast, the APOPercentage assay (Biocolor Ltd., Newtonabbey, Northern Ireland, UK), which also detects apoptosis at the stage when PS is externalized, costs on average about U.S. \$2 per sample.

The APOPercentage assay makes use of an anionic halogenated

fluorescein dye, the disodium salt of 3, 4, 5, 6,-tetrachloro-2', 4', 5', 7'-tetraiodo-fluorescein (TCTF), which is taken up by apoptotic cells, which gives such cells a purple-red appearance. Dye uptake can be visualized by bright-field microscopy and quantified by spectrophotometric measurement at 550 nm. The assay has been used with several adherent cell lines including V79, PC-3, DU145, LNCaP, MCF-7, MDA MB231, Huvec and Hek293 (4–6).

A limitation of the APOPercentage assay is that it cannot be used to detect apoptosis in suspension cell cultures. Furthermore, the quantification of apoptosis using the standard colorimetric method is quite laborious. One method involves the colorimetric or fluorometric measurement of dye released from apoptotic cells following alkaline lysis. The other method entails using analytical digital microscopy in which the intensity of dye staining helps assess the extent of apoptosis. A reference dye standard can be applied to determine absolute cell numbers labeled with TCTF. However, this type of analysis is quite ambiguous and requires that a reference standard be established for different cell types.

We modified the standard assay to facilitate the use of TCTF to perform absolute counts of apoptotic cells by flow cytometry. This not only made

the assay more robust but also made it possible to apply the assay to assess apoptosis in suspension cells. We also demonstrate that the modified assay compares well with the annexin-V assay in terms of accuracy and sensitivity. The flow cytometry-based assay using TCTF will cost about U.S. \$0.5 per sample. This is a ~90% reduction in cost in comparison to the annexin-V assay.

In our method we decreased the concentration of TCTF to a 1:160 dilution (~40 µg/mL) from the recommended 1:20 dilution (~325 µg/mL). Chinese hamster ovary (CHO) cells were cultured in 24-well cell culture plates and treated for 24 h with camptothecin (6 µM), ceramide (140 µM), and cisplatin (0.3 µM), which are well known as inducers of apoptosis. The cells were stained with TCTF (~40 µg/mL) for 30 min at 37°C, washed with PBS to remove the excess TCTF, and analyzed by flow cytometry. Cell staining was measured at 670 nm using filter 3 (FL3) on a Becton Dickinson FACScan instrument (BD Biosciences Pharmingen, San Diego, CA, USA) fitted with a 488 nm argon laser. The instrument settings were as follows: voltage set at 470 and AmpGain set at 1. Acquisition was done in Log mode. A minimum of 10,000 cells per sample were acquired and analyzed using CELLQuest PRO software (BD Biosciences).

To validate the use of the flow cytometry-based method for the detection and quantification of apoptosis using TCTF, the method was compared with the annexin-V assay. Cells were treated as described above and stained with annexin-V-fluorescein isothiocyanate (FITC) (BD Biosciences). The annexin-V assay was performed as described by the manufacturer, however the cells were not co-stained with propidium iodide or 7-amino-actinomycin D. Figure 1A shows a comparison of the number of apoptotic cells detected by the two assays. Illustrated in Figure 1B are overlaid histograms of the flow cytometry data for TCTF- and annexin-V-stained cells. Flow cytometric analysis showed that cells stained with annexin-V and TCTF essentially gave identical results for the quantification of apoptosis. To

Benchmarks

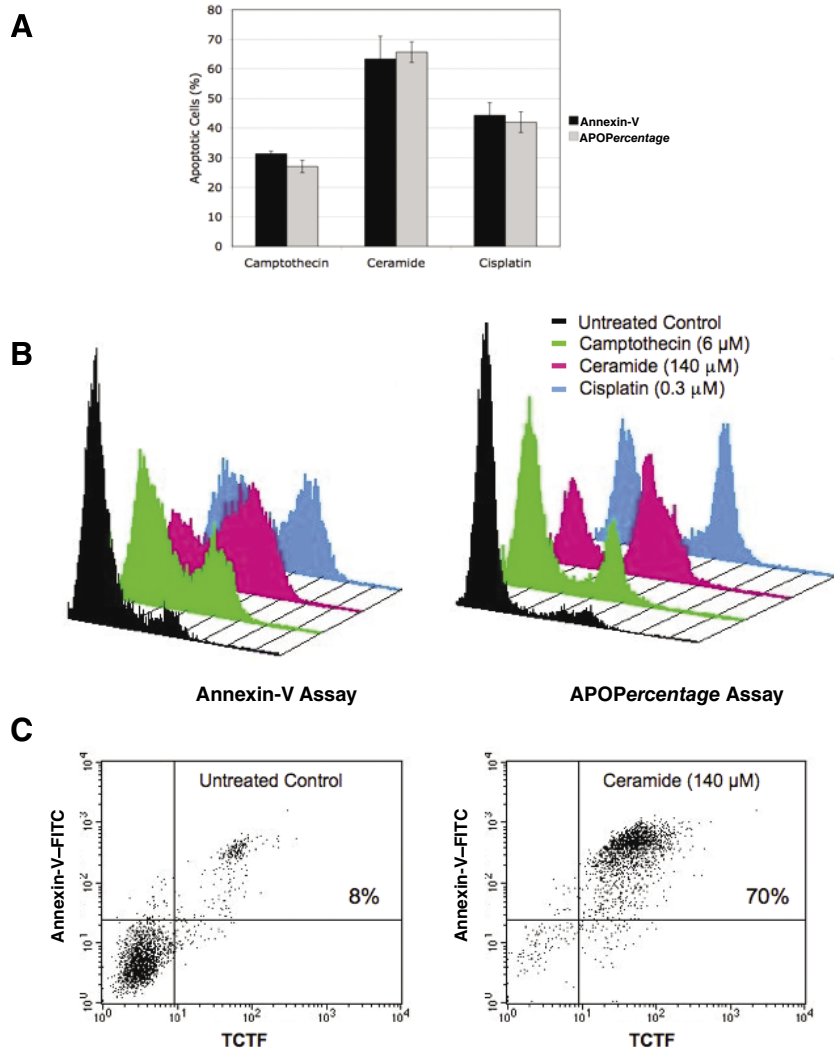


Figure 1. Quantification of apoptosis in Chinese hamster ovary (CHO) cells by flow cytometry. CHO cells were treated for 24 h with camptothecin, ceramide, and cisplatin. The cells were stained with either 3, 4, 5, 6-tetrachloro-2', 4', 5', 7'-tetraiodofluorescein (TCTF) or annexin-V-fluorescein isothiocyanate (FITC) and analyzed by flow cytometry. All treatments were performed in triplicate, and the number of apoptotic cells identified by the two assays is plotted on the bargraph (A). Also shown are overlaid histograms (B) of the relative fluorescence of the untreated control, camptothecin-, ceramide-, and cisplatin-treated cells. Cells were also co-stained with both annexin-V-FITC and TCTF. The dot plots (C) show the cell fluorescence measured by flow cytometry in FL1 (annexin-V-FITC) and FL3 (TCTF). The numbers in the upper-right quadrant of the plots indicate the number of cells staining positive for both annexin-V-FITC and TCTF.

further validate the modified assay we treated CHO cells with ceramide (140 μM) and co-stained the cells with annexin-V-FITC and TCTF. Since both assays detect apoptotic cells during PS externalization, it is expected that apoptotic cells should be positive for both annexin-V-FITC and TCTF. Cell staining for annexin-V-FITC was measured at 530/30 nm (FL1), while staining for TCTF was measured at 670 nm (FL3). Figure 1C illustrates that the apoptotic cells are positive for both annexin-V-FITC and TCTF.

To demonstrate that the flow cytometry-based assay can be used to quantify apoptosis in a variety of cell lines including suspension cultures, various cell lines including Jurkat T, HeLa, and MG63 cells were treated with ceramide (140 μM), and apoptosis was quantified using the flow cytometry-based assay. Figure 2 shows overlaid histograms of treated and untreated cells. The number of apoptotic cells detected by the flow cytometry-based assay for Jurkat T, HeLa, and MG63 cells were 75%, 74%

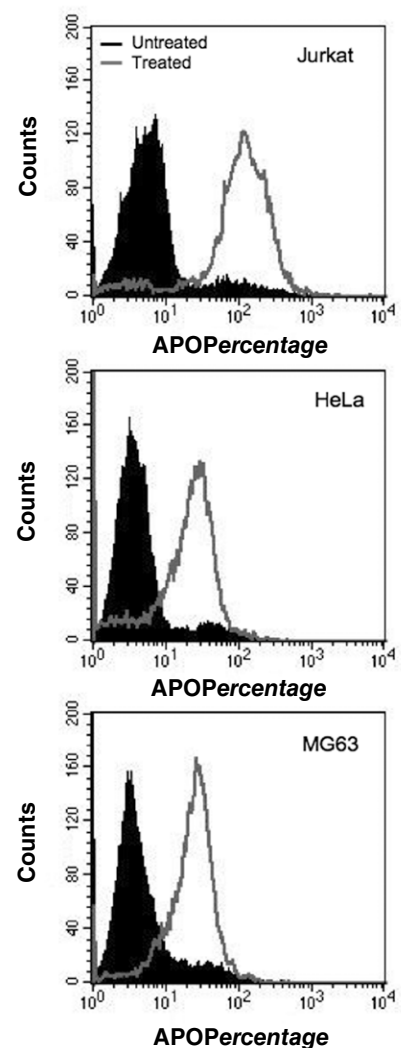


Figure 2. The quantification of apoptosis in Jurkat T, HeLa, and MG63 cells by flow cytometry. Jurkat T, HeLa, and MG63 cells were treated for 24 h with ceramide (140 μM). The cells were stained with 3, 4, 5, 6-tetrachloro-2', 4', 5', 7'-tetraiodofluorescein (TCTF) and analyzed by flow cytometry. Shown here are overlaid histograms of the relative fluorescence of the treated and untreated cells after staining.

and 83%, respectively. This shows that the flow cytometry-based assay is versatile and the use of this bioassay is not restricted to anchorage-dependent cells, which is a limitation of the standard colorimetric method.

A useful application of the flow cytometry-based assay can be as a high-throughput bioassay to determine LD₅₀ values based on the induction of apoptosis. Using an assay that measures apoptosis will be particularly valuable for anti-cancer drug screening since it is known that apoptosis plays

a role in the cytotoxic effects of anti-cancer drugs.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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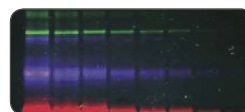
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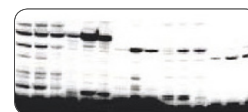
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