Analysis of apoptosis by propidium iodide staining and flow cytometry

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Since its introduction, the propidium iodide (PI) flow cytometric assay has been widely used for the evaluation of apoptosis in different experimental models. It is based on the principle that apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Use of a fluorochrome, such as PI, that is capable of binding and labeling DNA makes it possible to obtain a rapid (the protocol can be completed in about 2 h) and precise evaluation of cellular DNA content by flow cytometric analysis, and subsequent identification of hypodiploid cells. The original protocol enhanced the capacity for a rapid, quantitative measure of cell apoptosis. For this reason, since its publication, the PI assay has been widely used, as demonstrated by the large number of citations of the original paper and/or the continuous use of the method in many laboratories.

INTRODUCTION

Apoptosis is a common form of cell death in eukaryotes, playing a fundamental role during embryogenesis, in the homeostatic control of tissue integrity, tumor regression and immune response development[1]. On receiving specific signals, a number of distinctive biochemical and morphological changes occur in the cell. A family of proteins known as caspases, and perhaps other proteases, are activated in the early stages of apoptosis. These proteins cleave key cellular substrates that are necessary for normal cellular function, including structural proteins in the cytoskeleton and nuclear proteins. The caspases can also activate other degradation enzymes such as DNases, which begin to cleave the DNA at the linker regions between oligonucleosomes[2]. The result of these biochemical events is the appearance of morphological changes in the cell and extensive DNA cleavage. The products of DNA degradation are nucleosomal and oligonucleosomal DNA fragments (180 bp and multiples of 180 bp), which generate a characteristic "ladder" pattern during agarose gel electrophoresis[3]. Because the DNA in apoptotic cells is partially degraded, the fraction of low-molecular-weight DNA can be extracted, whereas the non-degraded DNA remains in the cell nucleus[4]. Because DNA fragments are lost from apoptotic nuclei and nuclear DNA content can be easily measured by flow cytometry, after nucleic acid staining with specific fluorochromes[5], methods have been developed for a quantitative evaluation of apoptotic nuclei.

In 1991, we published a rapid and simple flow cytometry method for measuring apoptosis in propidium iodide (PI)-stained mouse thymocytes[6]. With slight changes to the original procedure, the method has been applied to many other kinds of cells, including adherent tumor cell lines. Since then, other similar methods have been reported, using different fluorochromes and procedures (see refs. 7–9 for review). In this protocol, we discuss the steps of our method, as well as its advantages and pitfalls.

Basically, the method we describe uses PI for nuclear staining. PI is a fluorogenic compound that binds stoichiometrically to nucleic acids[10–12] so that fluorescence emission is proportional to the DNA (and RNA, which has to be removed if DNA is to be measured) content of a cell. When apoptotic cells are stained with PI and analyzed with a flow cytometer, they display a broad hypodiploid (sub-G_1) peak, which can be easily discriminated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channels.

This method appears to offer a number of advantages. It allows (i) a rapid, reliable and reproducible estimate of apoptosis, (ii) simultaneous analysis of cell-cycle parameters of surviving cells and (iii) when necessary, simultaneous analysis of cell surface antigens recognized by fluorescein isothiocyanate- or Alexa 488-conjugated monoclonal antibodies and the extent of apoptosis[13].

It should be stressed, however, that many types of apoptosis exist, and the extensive DNA fragmentation and loss of DNA fragments is not a universal finding in apoptotic death. Also, necrotic cells sometimes display some degrees of DNA degradation that may result in hypodiploid nuclei. Furthermore, the 'sub-G^1' peak can represent, in addition to apoptotic cells, nuclear fragments, clumps of chromosomes, micronuclei or nuclei with normal DNA content but different chromatin structure and diminished accessibility of fluorochrome to DNA (i.e., cells undergoing differentiation)[8]. Therefore, the presence of a hypodiploid DNA peak is not a *bona fide* proof of apoptotic death. Morphological (microscopic observation of apoptotic bodies)[14], biochemical (DNA ladder in agarose gel)[3] or specific demonstration of DNA breaks (terminal deoxynucleotidyl transferase assay[15]) should be used to confirm apoptosis before quantitative analysis by flow cytometry.

Another important concern in quantitative evaluation of apoptotic cells by flow cytometry is the discrimination of true apoptotic nuclei from nuclear debris. A proper setting of acquisition parameters (volume of particles, usually measured as forward scatter (FSC)) and of diploid DNA peak by using a calibration standard (DNA check beads) and negative and positive cell controls is essential before using the method with a cell line that has never been analyzed before.

It should also be remembered that apoptosis is a dynamic process and that there is a short "time window" during which apoptotic cells display their characteristic features. For this reason, different methods can produce different results depending on the time of the apoptosis process[16]. For example, in early phases of apoptosis,

terminal deoxynucleotidyl transferase can be positive for DNA breaks, and the cell membrane can expose phosphatidylserine which is Annexin-V positive. However, morphological observation can be negative for apoptotic bodies and flow cytometric analysis can be negative for the sub-G_1 peak, as DNA fragments are still maintained in the nucleus. Accordingly, the DNA ladder cannot be observed with agarose gel electrophoresis[8].

However, when used appropriately, the PI flow cytometric assay is a rapid and easily reproducible method that can be adapted for apoptosis evaluation in different kinds of cells.

MATERIALS

REAGENTS

- PBS
- Triton X-100
- Sodium citrate
- PI
- Deionized/distilled H_2O
- 70% (v/v) ethanol
- RNase (DNase free; boil RNase for 10 min if it is not DNase free)
- 0.2 M Na_2HPO_4

EQUIPMENT

- Tissue culture equipment
- 12×75 polypropylene tubes
- 4 °C and −20 °C refrigerators
- Incubator (37 °C, 5% CO_2; v/v)
- Centrifuge
- Flow cytometer (488-nm laser line, for excitation)
- pH meter

REAGENT SETUP

Fluorochrome solution 0.1% sodium citrate (wt/v), 0.1% Triton X-100 (v/v), 50 mg l^{-1} PI in deionized/distilled water). Fluorochrome solution can be kept in the dark at 4 °C for months. ! CAUTION Use gloves while preparing and using fluorochrome solution as PI is mutagenic.

DNA extraction buffer Mix 192 ml of 0.2 M Na_2HPO_4 with 8 ml of 0.1% Triton X-100 (v/v). Adjust the pH to 7.8.

DNA staining solution Dissolve 200 µg of PI in 10 ml of PBS. Add 2 mg of DNase free RNase. ▲ CRITICAL Prepare fresh staining solution just before use. ! CAUTION Use gloves while preparing and using DNA staining solution as PI is mutagenic.

PROCEDURE

1| Suspend cells at 1–2×10^6 cells ml^{-1} in 1 ml of PBS in 12×75 tubes.

2| Centrifuge at 200*g* for 5 min at room temperature.

3| Aspirate off the PBS.

4| Follow the quick method for thymocytes and non-adherent mononuclear cells (option A) and the standard method for multinuclear cells growing in suspension and for adherent cells (option B).

(A) Quick method (direct DNA staining in PI hypotonic solution)

(i) Gently resuspend the cell pellet in 1 ml of fluorochrome solution.
▲ CRITICAL STEP Resuspend cells with caution to avoid nuclei fragmentation and separation of apoptotic bodies from apoptotic nuclei. Since the hypotonic shock removes the majority of the RNA, RNase treatment is not required.

(ii) Place the tubes in the dark at 4 °C, before flow cytometry analysis, for at least 1 h and no longer than 24 h.
▲ CRITICAL STEP One hour is necessary for appropriate staining of the nuclei; cells can be maintained for 24 h, in the dark at 4 °C without any substantial change in DNA profile.

(B) Standard method (PI staining after alcoholic fixation)

(i) Resuspend cell pellet in 500 µl of PBS.

(ii) Fix cells by adding 4.5 ml of 70% (v/v) cold ethanol to the cell suspension keeping the tubes on ice.
■ PAUSE POINT Cells can be stored in ethanol solution at −20 °C for several weeks.

(iii) Centrifuge at 400*g* for 5 min and remove the supernatant (ethanol solution).

(iv) Wash cells in 5 ml of PBS and centrifuge at 400*g* for 5 min.
▲ CRITICAL STEP Cells with extensive DNA degradation can be directly resuspended in DNA staining solution without any further treatment.

(v) If DNA is not extensively degraded, resuspend cells in 0.5 ml of PBS and add 0.5 ml of DNA extraction buffer. Incubate at room temperature for 5 min and centrifuge at 400*g* for 5 min.

(vi) Remove the supernatant and resuspend cells in 1 ml of DNA staining solution.

(vii) Incubate resuspended cells for at least 30 min at room temperature in the dark.

5| Analyze cells by flow cytometry. Use 488-nm laser line for excitation. Measure red fluorescence (>600 nm) and side scatter. Collect at least 20,000 events. Gate-out residual debris (**Fig. 1a**). Measure hypodiploid and diploid DNA peaks (**Fig. 1b**).
▲ CRITICAL STEP A precise calibration of the flow cytometer is necessary to properly gate out debris. This procedure depends on the type and producer of the flow cytometer, but can be basically performed as follows: (i) use a suspension of multilabeled 10- and 2.5-µm microspheres (Microbeads Molecular Probes); (ii) adjust the FSC channel around the 600 channels of a 1,024-channel linear scale for the 10-µm beads and annotate the channel corresponding to the 2.5-µm microsphere; (iii) adjust the DNA peak corresponding to diploid thymocytes (or to the G_0/G_1 cell-cycle phase of tumor cells) around the

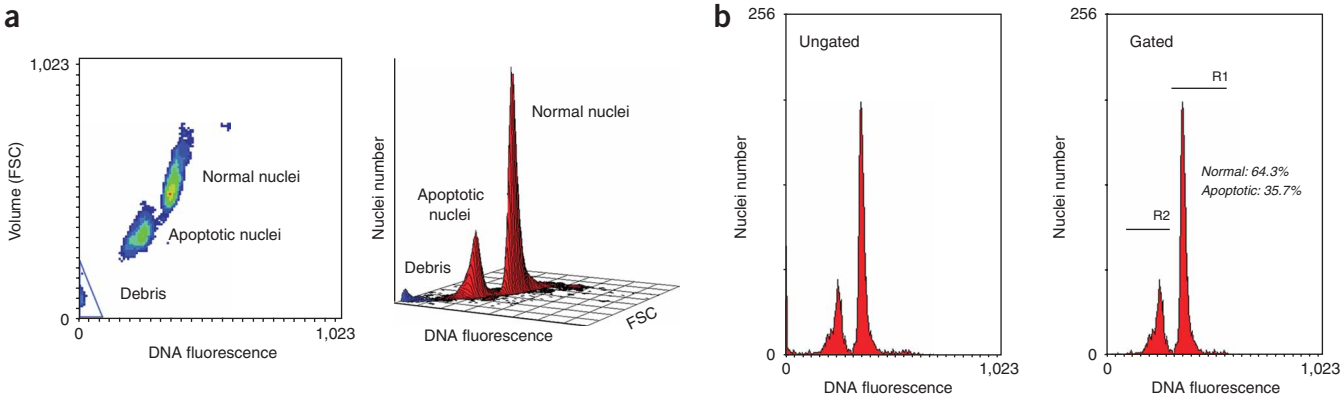

Figure 1 | Measure of apoptotic thymocytes. (**a**) Dot-plot analysis of thymocytes undergoing apoptosis. Debris and residuals of necrotic cells (the bulk of which has been eliminated during acquisition with the flow cytometer) can be recognized (and gated-off by eliminating the corresponding region) by the lower diameter (FSC) and reduced DNA fluorescence compared to apoptotic thymocytes. The right panel shows a 3D representation of the peaks corresponding to normal nuclei, apoptotic nuclei and debris. (**b**) A precise and reproducible estimate of the percentage of normal and apoptotic nuclei can be obtained by analysis of the DNA histogram after elimination of residual debris.

350 channel; (iv) running diploid thymocytes, exclude the bulk of debris from the analysis, increasing progressively the FSC threshold until the unwanted signals in the 1–100 channels of the red fluorescence are $<1\%$; (v) and record flow cytometer parameters and collect samples maintaining these settings. Residual debris, if any, will be gated-out during analysis (see ANTICIPATED RESULTS).

? TROUBLESHOOTING

● TIMING

The procedure can be completed in less than 3 h including analysis. Specifically, the steps require the following time:
Sample preparation with the "Quick method": less than 30 min
Sample preparation with the "Standard method": less than 1 h
Sample incubation for staining: at least 30 min
Flow cytometer calibration and analysis: about 30 min

? TROUBLESHOOTING

Lysis of mitotic cells, micronuclei and chromosome aggregates can be erroneously identified as apoptotic cells, particularly when PI hypotonic solution (quick method) is used. The use of a linear rather than a logarithmic scale in the PI emission histogram provides better exclusion of objects/events with minimal DNA content.

If cell debris still strongly affects the percentage of hypodiploid nuclei, check the samples by fluorescence microscopy, and if cell lysis is extensive, use an alternative method.

Flow cytometric analysis that fails to demonstrate a hypodiploid peak despite the presence of apoptosis as revealed by other methods (morphological observation and/or Annexin-V positivity) can be due to an absence or very low DNA loss from apoptotic nuclei due to the presence of large DNA fragments. In this case, a specific extraction procedure should be used as indicated above (point 4(B)v of PROCEDURE).

ANTICIPATED RESULTS

Flow cytometric analysis of PI-stained apoptotic nuclei results in a broad peak of hypodiploid particles, clearly separated from the sharp diploid DNA peak of normal cells. Residual debris can still be present, particularly when adherent cell lines are examined. However, debris and residuals of necrotic cells can be easily gated-off (**Fig. 1a**) during the analysis of data, as they

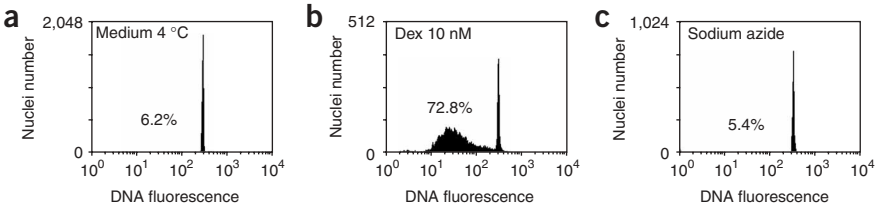

Figure 2 | The quick method can be safely used with mouse thymocytes. In this case, logarithmic amplification of the DNA fluorescence histogram allows a more precise separation of normal and apoptotic nuclei. (**a**) DNA histogram of normal thymocytes. (**b**) Apoptosis induced by dexamethasone treatment. (**c**) The persistence of normal peak characteristics of diploid nuclei when necrotic death of thymocytes was produced by sodium azide.

have minimal DNA fluorescence and reduced diameter (FSC) when compared to apoptotic nuclei. After gating, hypodiploid and diploid nuclei can be easily, precisely and reproducibly measured in the DNA fluorescence histogram (**Fig. 1b**).
The quick method is very useful for thymocyte analysis, as it is rapid, cheap and does not require alcohol fixation and RNase treatment. This procedure can also be used in other cell lines growing in suspension, such as Jurkat, U-937, 3DO T-cell hybridoma and AKR1. In these cells, a better resolution between the two peaks can be obtained with a logarithmic amplification of DNA fluorescence. The results are totally superimposable on those obtained with the classic procedure (**Fig. 2**). The quick procedure and logarithmic amplification are not suitable for analysis of all cells, particularly adherent cell lines, because in these cases a good separation of nuclear fragments from apoptotic nuclei is problematic when nuclei are exposed to hypotonic solution before fixation. The standard method has been successfully used in a number of adherent cell lines (MCF-7, NIH-3T3, L-929, 293, PC-12, LNCaP and Cos-1).

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