Flow Cytometry of Apoptosis

This unit describes the most common methods applicable to flow cytometry that make it possible to: (1) identify and quantify dead or dying cells, (2) reveal a mode of cell death (apoptosis or necrosis), and (3) study mechanisms involved in cell death. Gross changes in cell morphology and chromatin condensation, which occur during apoptosis, can be detected by analysis with laser light beam scattering. An early event of apoptosis, dissipation of the mitochondrial transmembrane potential, can be measured using a number of fluorochromes that are sensitive to the electrochemical potential within this organelle (see Basic Protocol 1). Another early event of apoptosis, caspase activation, can be measured either directly, by immunocytochemical detection of the epitope that characterizes activated caspase (see Basic Protocol 2), or indirectly by immunocyto-chemical detection of the caspase-3 cleavage product, the p85 fragment of poly(ADP-ri-bose) polymerase (see Basic Protocol 4). Exposure of phosphatidylserine on the exterior surface of the plasma membrane can be detected by the binding of fluoresceinated annexin V (annexin V–FITC); this assay is combined with analysis of the exclusion of the plasma membrane integrity probe propidium iodide (PI; see Basic Protocol 5). Also described are methods of analysis of DNA fragmentation based either on DNA content of cells with fractional (sub-G_1) DNA content (see Basic Protocol 6 and Alternate Protocol 1) or by DNA strand-break labeling (Terminal deoxynucleotidyltransferase–mediated dUTP Nick End Labeling, TUNEL; or In Situ End Labeling, ISEL; see Basic Protocol 7). Still another hallmark of apoptosis is the activation of tissue transglutaminase (TGase), the enzyme that cross-links protein and thereby makes them less immunogenic. Methods for analyzing TGase activation are presented in Basic Protocol 8 and Alternate Protocol 2.

STRATEGIC PLANNING

The choice of a particular method often depends on the cell type, the nature of the inducer of apoptosis, the desired information (e.g., specificity of apoptosis with respect to the cell cycle phase or DNA ploidy), and technical restrictions. For example, sample transportation or prolonged storage before the measurement requires prior cell fixation, thereby eliminating the use of "supravital" methods that rely on analysis of freshly collected live cells.

Positive identification of apoptotic cells is not always simple. Apoptosis was recently defined as a caspase-mediated cell death (Blagosklonny, 2000). Activation of caspases, therefore, appears to be the most specific marker of apoptosis (Shi, 2002). The detection of caspase activation, either directly (e.g., by antibody that is reactive with the activated enzyme; see Basic Protocol 2) or indirectly by the presence of poly(ADP-ribose) polym-erase (PARP) cleavage product (PARP p85; see Basic Protocol 4), provides the most definitive evidence of apoptosis. Extensive DNA fragmentation is also considered as a specific marker of apoptosis. The number of DNA strand breaks in apoptotic cells is so large that intensity of their labeling in the TUNEL reaction (see Basic Protocol 7) ensures their positive identification and discriminates them from cells that have undergone primary necrosis (Gorczyca et al., 1992). However, in the instances of apoptosis when internucleosomal DNA degradation does not occur (Collins et al., 1992; Catchpoole and Stewart, 1993; Ormerod et al., 1994; Knapp et al., 1999), the number of DNA strand breaks may be inadequate to distinguish apoptotic cells by the TUNEL method. Likewise, in some instances of apoptosis, DNA fragmentation stops after the initial DNA cleavage to fragments of 50 to 300 kb (Collins et al., 1992, Oberhammer et al., 1993). The frequency of DNA strand breaks in nuclei of these cells is low, and therefore, they may not be easily detected by the TUNEL method.

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The ability of cells to bind annexin V is still another marker considered to be specific to apoptosis. One should keep in mind, however, that use of the annexin V binding assay is hindered in some instances, e.g., when the plasma membrane is damaged during cell preparation or storage, leading to the loss of asymmetry in distribution of phosphatidyl-serine across the membrane. Furthermore, macrophages and other cells engulfing apoptotic bodies may also be positive in the annexin V assay (Marguet et al., 1999).

Apoptosis can be recognized with greater certainty when the cells are subjected to several assays probing different apoptotic attributes (Hotz et al., 1994). For example, the assay of plasma membrane integrity (exclusion of PI) and annexin V binding combined with analysis of PARP cleavage or DNA fragmentation may provide a more definitive assessment of the mode of cell death than can be determined by each of these methods used alone.

A plethora of kits designed to label DNA strand breaks and applicable to flow cytometry are available from different vendors. Most of these kits were designed by the authors (Gorczyca et al., 1992; Li and Darzynkiewicz, 1995). For example, Phoenix Flow Systems, BD PharMingen, and Alexis Biochemicals provide kits to identify apoptotic cells based on a single-step procedure utilizing either TdT and FITC-conjugated dUTP (APO-DIRECT; Li et al., 1995) or TdT and BrdUTP, as described in Basic Protocol 7 (APO-BRDU; Li and Darzynkiewicz, 1995). A description of the method, which is nearly identical to the one presented in this unit, is included with the kit. Another kit (ApopTag), based on a two-step DNA strand-break labeling with digoxygenin-16-dUTP by TdT, also designed by the authors (Gorczyca et al., 1992), was initially offered by ONCOR, later by Intergen, and most recently by Serologicals.

MITOCHONDRIAL TRANSMEMBRANE POTENTIAL ($\Delta\psi_m$) MEASURED BY RHODAMINE 123 OR DiOC$_6$(3) FLUORESCENCE

The critical role of mitochondria during apoptosis is associated with the release of two intermembrane proteins, cytochrome c and apoptosis-inducing factor (AIF), that are essential for sequential activation of pro-caspase 9 and pro-caspase 3 (Liu et al., 1996; Yang et al., 1997). AIF is also involved in proteolytic activation of apoptosis-associated endonuclease (Susin et al., 1997). Still another protein, Smac/Diablo, that interacts with the inhibitors of caspases, thereby promoting apoptosis, is released from mitochondria (Deng et al., 2002). Dissipation (collapse) of mitochondrial transmembrane potential ($\Delta\psi_m$), also called the permeability transition (PT), likewise occurs early during apoptosis (Cossarizza et al., 1994; Kroemer, 1998; Zamzani et al., 1998). However, a growing body of evidence suggests that this event may be transient when associated with the release of cytochrome c or AIF, and mitochondrial potential may be restored for some time in the cells with activated caspases (Finucane et al., 1999; Scorrano et al., 1999; Li et al., 2000).

The membrane-permeable lipophilic cationic fluorochromes such as rhodamine 123 (R123) or 3,3′-dihexyloxacarbocyanine iodide [DiOC$_6$(3)] can serve as probes of $\Delta\psi_m$ (Johnson et al., 1980; Darzynkiewicz et al., 1981, 1982). When live cells are incubated in their presence, the probes accumulate in mitochondria, and the extent of their uptake, as measured by intensity of cellular fluorescence, reflects $\Delta\psi_m$. A combination of R123 and PI discriminates among live cells that stain only with R123, early apoptotic cells that have lost the ability to accumulate R123, and late apoptotic/necrotic cells whose plasma membrane integrity is compromised and that stain only with PI (Darzynkiewicz et al., 1982; Darzynkiewicz and Gong, 1994). The specificity of R123 and DiOC$_6$(3) as $\Delta\psi_m$ probes is increased when they are used at low concentrations (<0.5 µg/ml). Still another probe of $\Delta\psi_m$ is the J-aggregate-forming lipophilic cationic fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Its uptake by

charged mitochondria driven by the transmembrane potential is detected by the shift in color of fluorescence from green, which is characteristic of its monomeric form, to orange, which reflects its aggregation in mitochondria (Cossarizza and Salvioli, 2001). In light of the recent evidence that the collapse of $\Delta\psi_m$ may not be a prerequisite for release of cytochrome c, AIF, and other apoptotic events (Finucane et al., 1999; Scorrano et al., 1999; Li et al., 2000), one should be cautious in interpreting the lack of collapse of $\Delta\psi_m$ as a marker of non-apoptotic cells.

Materials

Cells of interest in appropriate complete culture medium

10 µM rhodamine 123 (R123; see recipe) *or* 10 µM DiOC$_6$(3) (see recipe for 0.1 mM stock solution) *or* 0.2 mM JC-1 stock solution (see recipe)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark

12 × 75–mm tubes suitable for flow cytometer

Flow cytometer with 488-nm excitation and filters for collection of green, orange, and red fluorescence

Stain with R123 or DiOC$_6$(3) and PI

1a. Add either 20 µl of 10 µM R123 (200 nM final) or 5 µl of 10 µM DiOC$_6$(3) (50 nM final) to ~10^6 cells suspended in 1 ml complete tissue culture medium (with 10% serum), and incubate 20 min at 37°C in the dark.

2a. Centrifuge cells 5 min at $300 \times g$, room temperature. Resuspend cell pellet in 1 ml PBS.

3a. Add 10 µl PI solution and incubate 5 min at room temperature in the dark.

4a. Analyze cell fluorescence on the flow cytometer. Excite fluorescence with blue (488-nm) laser. Set the signal-triggering threshold on forward- and side-scatter signals. Collect green fluorescence [R123 or DiOC$_6$(3)] at 530 ± 20 nm and red fluorescence (PI) above 600 nm.

Stain with JC-1

1b. Suspend cell pellet (~10^6 cells) in 1 ml complete tissue culture medium with 10% serum.

2b. Add 10 µl of 0.2 mM JC-1 stock solution. Vortex cells intensely during addition and for the next 20 sec. Wash cells two times with PBS; centrifuge each time 5 min at $200 \times g$, room temperature.

   Addition of JC-1 to the cell suspension without vortexing may lead to formation of precipitate. Vortexing too vigorously, on the other hand, may cause cell damage.

3b. Incubate cells 15 min at room temperature in the dark.

4b. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation. Collect green fluorescence at 530 ± 20 nm and orange fluorescence at 570 ± 20 nm with a band-pass filter or above 570 nm with a long-pass filter.

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IMMUNOCYTOCHEMICAL DETECTION OF ACTIVATED CASPASES BY ZENON TECHNOLOGY

Caspases are activated by transcatalytic cleavage of their zymogen procaspase molecules into large and small subunits. The subunits then assemble to form the heterotetramer consisting of two small and two large subunits, which is the active caspase (Budihardjo et al., 1999; Earnshaw et al., 1999). Antibodies that are specific to activated caspase-3, caspase-8, and caspase-9 are now commercially available and one expects that antibodies reactive with other active caspases will soon be developed as well. It is possible, therefore, to detect caspase activation by immunocytochemical means. This protocol combines the use of activated caspase-specific antibody with staining of cellular DNA by propidium iodide (PI) to concurrently detect cells with activated caspases and relate caspase activation to the cell-cycle position.

The immunocytochemical detection of caspase-3 in this protocol makes use of Zenon technology (Haugland, 2002). Zenon technology consists of a labeling complex that is formed by a fluorochrome-labeled Fab fragment (Zenon Alexa Fluor 488) of an anti-IgG antibody that is directed against the Fc portion of a mouse (or rabbit) IgG_1 antibody. Mixing of the labeled Fab fragment with the primary antibody forms the labeling complex. Excess unbound labeled Fab fragments is removed by admixture of nonspecific mouse (or rabbit) IgG. The labeling complex is then used to stain cells in the same manner as a covalently labeled primary antibody (Haugland, 2002).

Materials

Cells of interest, both untreated (control) and induced to apoptosis (e.g., exponentially growing HL-60 cells incubated 2 to 4 hr with 0.15 µM camptothecin)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Fixatives:
  1% (v/v) methanol-free formaldehyde (Polysciences) in PBS, 0° to 5°C
  4% (v/v) methanol-free formaldehyde (Polysciences) in PBS, room temperature
  70% (v/v) ethanol, –20°C
Rinse solution (see recipe)
Primary antibody: cleaved (activated) caspase-3 antibody (Cell Signaling Technology, cat. no. 9661)
Zenon Alexa Fluor 488 rabbit IgG labeling kit (Molecular Probes, cat. no. Z-25302)
10% (v/v) Triton X-100 in PBS
DNA staining solution with PI (see recipe)

12 × 75–ml tubes suitable for use on flow cytometer
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend ~10^6 cells in 0.5 ml PBS.

2. Fix cells by transferring the above cell suspension into tubes containing 4.5 ml of 1% methanol-free formaldehyde in PBS at 0° to 5°C. Let stand 15 min at 0° to 5°C.

3. Centrifuge 5 min at $300 \times g$, room temperature. Decant supernatant.

4. Resuspend cell pellet in 3 ml of 70% ethanol at –20°C. Allow to sit at least 2 hr (cells can be stored several days in 70% ethanol at –20°C).

5. Bring the cell suspension in 70% ethanol to room temperature, add 2 ml PBS to this suspension, and let sit 5 min at room temperature.

6. Centrifuge 5 min at $300 \times g$, room temperature. Decant supernatant.

7. Resuspend cell pellet in 5 ml PBS and let sit 5 min at room temperature.

8. Centrifuge 5 min at $300 \times g$, room temperature. Decant supernatant.

9. Resuspend cell pellet in rinse solution. Let stand 30 min at room temperature.

10. Prepare the staining solution as follows.

   a. Mix 4 µl primary antibody (anti-caspase-3) with 16 µl rinse solution and with 5 µl solution A Zenon (from kit) in a 1.5-ml microcentrifuge tube.

   b. Keep 5 min in the dark at room temperature.

   c. Add 5 µl solution B Zenon (from kit).

   d. Keep 5 min in the dark at room temperature.

   e. Add 0.3 µl of 10% Triton X-100 in PBS.

11. Centrifuge the cell suspension (from step 9) 5 min at $300 \times g$, room temperature. Thoroughly drain the rinse solution by blotting on filter paper. Add 15 µl of the staining solution prepared in step 10, and 85 µl rinse solution, for a final volume of 100 µl. Resuspend the cell pellet.

12. Incubate cells with the staining solution 1 hr in the dark at room temperature.

13. Add 5 ml PBS, centrifuge 5 min at $300 \times g$, room temperature, and decant supernatant.

14. Resuspend cell pellet in 1 ml of 4% methanol-free formaldehyde in PBS and let stand 5 min at room temperature.

15. Centrifuge cells 5 min at $300 \times g$, room temperature. Decant supernatant.

16. Resuspend cell pellet in 1 ml DNA staining solution with PI.

17. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green Alexa 488 fluorescence at 530 ±20 nm and red PI fluorescence above 600 nm.

DETECTION OF APOPTOTIC CELLS USING FLUOROCHROME-LABELED INHIBITORS OF CASPASES (FLICAs)

BASIC PROTOCOL 3

Exposure of live cells to fluorochrome-labeled inhibitors of caspases (FLICAs) results in uptake of these reagents by apoptotic cells (Smolewski et al., 2001). Unbound FLICAs are removed from the nonapoptotic cells by rinsing the cells with wash buffer. The cells may also be fixed with formaldehyde; after fixation only apoptotic cells retain the label. Cells labeled with FLICAs can be examined by fluorescence microscopy, or their fluorescence can be measured by flow cytometry. FLICAs are convenient markers of apoptotic cells, and when used in combination with PI as described in the protocol below, they reveal three sequential stages of apoptosis. FAM-VAD-FMK, the inhibitor designed to react with all caspases, except perhaps caspase-2, is used in this protocol. It should be stressed, however, that FLICAs appear to react in apoptotic cells also with targets other than activated caspases. Cell labeling with FLICAs, therefore, although perhaps reflecting caspase activity and although reflecting caspase activation, cannot be interpreted as indicating reactivity with active enzyme centers of caspases only.

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Materials

Cells of interest

Medium supplemented with 10% (v/v) serum or 1% (w/v) serum albumin

FLICA kit (Immunochemistry Technologies) containing:

  FAM-VAD-FMK reagent (see recipe)

  Fixative

  Hoechst stain

Rinse solution: 1% (w/v) BSA in PBS (*APPENDIX 2A*)

1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark

12×75–ml tubes suitable for use on flow cytometer

Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend ~10^6 cells in 0.3 ml medium containing 10% serum or 1% serum albumin.

2. Add 10 µl FAM-VAD-FMK working solution to this cell suspension (final concentration 10 µM). Mix gently and incubate 1 hr at 37°C.

   Sulforhodamine-labeled FLICA (SR-VAD-FMK) may be used instead of FAM-VAD-FMK to make apoptotic cells fluorescence in the red.

3. Add 2 ml rinse solution, mix gently, and centrifuge 5 min at $200 \times g$, room temperature.

4. Resuspend cell pellet in 2 ml rinse solution and centrifuge as in step 3.

   Cells may be fixed 15 min in 1% formaldehyde in PBS, then suspended in 70% ethanol and stored for several days. A fluorochrome of a different color than FLICA may be used to counterstain other cellular components (e.g., DNA) or other markers of apoptosis (e.g., DNA strand breaks).

5. Resuspend cell pellet in 1 ml rinse solution. Add 1.0 µl of 1 mg/ml PI stock solution. Keep 5 min at room temperature.

   Protect samples from light at all times.

   Staining with PI is optional. It allows one to distinguish the cells that have compromised plasma-membrane integrity (e.g., necrotic and late apoptotic cells, cells with mechanically damaged membranes, or isolated cell nuclei) to the extent that they cannot exclude PI.

6. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FAM-VAD-FMK fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

DETERMINATION OF POLY(ADP-RIBOSE) POLYMERASE (PARP) CLEAVAGE

BASIC PROTOCOL 4

PARP is a nuclear enzyme that is involved in DNA repair and activated in response to DNA damage (de Murcia and Menissier-de Murcia, 1994). Early in apoptosis, PARP is cleaved by caspases, primarily by caspase-3 (Kaufmann et al., 1993; Lazebnik et al., 1994; Alnemri et al., 1996). The specific cleavage of this protein results in distinct 85-kDa and 24-kDa fragments, usually detected electrophoretically, and this is considered to be a hallmark of the apoptotic mode of cell death.

The development of antibodies that recognize the cleaved PARP products prompted their use as immunocytochemical markers of apoptotic cells. The antibody that recognizes the 85-kDa fragment (PARP p85) was initially used to score the frequency of apoptosis in tissue sections (Sallman et al., 1997; Kockx et al., 1998). Recently, this antibody has been adapted to label apoptotic cells for detection by flow cytometry and laser scanning

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cytometry (LSC; Li and Darzynkiewicz, 2000; Li et al., 2000). A good correlation was observed between the frequency of apoptosis detected cytometrically with PARP p85 Ab and that detected by the DNA strand-break (TUNEL) assay. However, at least in some cell systems, the cleavage of PARP occurs prior to the onset of DNA fragmentation (Li and Darzynkiewicz, 2000). In these instances, the correlation may not be apparent at early stages of apoptosis because the apoptotic index estimate based on PARP cleavage may exceed the estimate based on the TUNEL reaction. Cytometric analysis of cells differentially stained for PARP p85 and DNA, similar to the TUNEL assay, makes it possible not only to identify and score apoptotic cell populations but also to correlate apoptosis with the cell cycle position or DNA ploidy.

The classic immunocytochemical indirect (two-step) method to detect the 85-kDa PARP fragment is presented below. Alternatively, however, one can use the Zenon technology as described above (see Basic Protocol 2) for detection of activated caspases.

Materials

Cells of interest
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1% methanol-free formaldehyde (Polysciences) in PBS (*APPENDIX 2A*)
70% ethanol
0.25% (v/v) Triton X-100 (Sigma) in PBS (*APPENDIX 2A*); store at 4°C
PBS/BSA solution: 1% (w/v) bovine serum albumin (Sigma) in PBS; store at 4°C
Anti-PARP p85 antibody (Promega anti-PARP-85 fragment, rabbit polyclonal)
Fluorescein-conjugated anti-rabbit immunoglobulin antibody (Dako)
1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark
RNase stock solution (see recipe)

12×75–mm centrifuge tubes suitable for use on the flow cytometer
Pasteur pipets
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend ~10^6 cells in 0.5 ml PBS. Transfer this suspension to a 12×75–mm (5-ml) tube containing 4.5 ml of 1% methanol-free formaldehyde and fix cells 15 min on ice. Centrifuge the cells 5 min at $300 \times g$, 4°C, wash once with 5 ml PBS, centrifuge 5 min at $300 \times g$, and resuspend the cell pellet in 0.5 ml PBS. With a Pasteur pipet, transfer this cell suspension into a new 12×75–mm centrifuge tube containing 4.5 ml of ice-cold 70% ethanol.

   The cells may be stored several days in ethanol at −20°C.

2. Centrifuge cells 5 min at $200 \times g$, room temperature, and resuspend the cell pellet in 5 ml PBS; repeat centrifugation.

3. Resuspend cells in 5 ml 0.25% Triton X-100/PBS for 10 min.

4. Centrifuge cells 5 min at $300 \times g$, room temperature, and resuspend in 2 ml BSA/PBS solution for 10 min.

5. Centrifuge cells 5 min at $300 \times g$, room temperature, and resuspend in 100 μl BSA/PBS containing anti-PARP p85 Ab diluted 1:200. Incubate 2 hr at room temperature, or overnight at 4°C.

6. Add 5 ml BSA/PBS solution, let sit 5 min, and centrifuge 5 min at $300 \times g$, room temperature.

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7. Resuspend cell pellet in 100 μl PBS/BSA containing fluorescein-conjugated secondary Ab [F(ab′)$_2$ fragment, swine anti-rabbit immunoglobulin] diluted 1:30. Incubate 1 hr in the dark at room temperature.

8. Add 5 ml BSA/PBS, centrifuge 5 min at $200 \times g$, room temperature, and resuspend cell pellet in 1 ml PBS. Add 20 μl of 1 mg/ml PI and 50 μl RNase solution. Incubate 20 min in the dark at room temperature.

9. Analyze cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FITC-anti-PARP p85 fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

ANNEXIN V BINDING

Phospholipids of the plasma membrane are asymmetrically distributed between the inner and outer leaflets of the membrane. Phosphatidylcholine and sphingomyelin are exposed on the external leaflet of the lipid bilayer, while phosphatidylserine is located on the inner surface. During apoptosis, this asymmetry is disrupted and phosphatidylserine becomes exposed on the outside surface of the plasma membrane (Fadok et al., 1992; Koopman et al., 1994; van Engeland et al., 1998). Because the anticoagulant protein annexin V binds with high affinity to phosphatidylserine, fluorochrome-conjugated annexin V has found an application as a marker of apoptotic cells, in particular for their detection by flow cytometry (van Engeland et al., 1998). The cells become reactive with annexin V prior to their loss of plasma membrane ability to exclude cationic dyes such as PI. Therefore, by staining cells with a combination of annexin V–FITC and PI, it is possible to detect unaffected, non-apoptotic cells (annexin V–FITC negative/PI negative), early apoptotic cells (annexin V–FITC positive/PI negative), and late apoptotic ("necrotic stage" of apoptosis) as well as necrotic cells (PI positive).

Materials

Cells of interest
Fluorescein-conjugated annexin V (see recipe) in binding buffer (see recipe)
1 mg/ml propidium iodide (PI; Molecular Probes) in distilled water; store at 4°C in the dark
Flow cytometer with 488-nm excitation and filters for collection of green and red fluorescence

1. Suspend 10^5 to 10^6 cells in 1 ml fluorescein-conjugated annexin V in binding buffer for 5 min at room temperature in the dark.

2. Prior to analysis, add an appropriate volume of 1 mg/ml PI solution to the cell suspension to have a final PI concentration of 1.0 μg/ml. Incubate 5 min at room temperature in the dark.

3. Analyze cells on the flow cytometer, using 488-nm excitation. Set gates based on light scatter. Collect green annexin V fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

DNA FRAGMENTATION: DETECTION OF CELLS WITH FRACTIONAL (SUB-G₁) DNA CONTENT USING PI

Endonucleases activated during apoptosis target internucleosomal DNA sections and cause extensive DNA fragmentation (Kerr et al., 1972; Arends et al., 1990; Nagata, 2000). The fragmented, low-molecular-weight DNA can be extracted from the cells following their fixation in precipitating fixatives such as ethanol. Conversely, fixation with cross-linking fixatives such as formaldehyde results in the retention of low-molecular-weight DNA in the cell and therefore should be avoided. Generally, the extraction occurs during the process of cell staining in aqueous solutions after transfer from the fixative. As a result, apoptotic cells often end up with deficient DNA content, and when stained with a DNA-specific fluorochrome, they can be recognized by cytometry as cells having less DNA than G_1 cells. On the DNA content frequency histograms, they form a characteristic sub-G_1 peak (Umansky et al., 1981; Nicoletti et al., 1991; Gong et al., 1994). It should be noted that loss of DNA may also occur as a result of the shedding of apoptotic bodies containing fragments of nuclear chromatin.

The degree of DNA degradation varies depending on the stage of apoptosis, cell type, and often the nature of the apoptosis-inducing agent. Hence, the extractability of DNA during the staining procedure also varies. A high-molarity phosphate-citrate buffer enhances extraction of the fragmented DNA (Gong et al., 1994). With some limitations, this approach can be used to extract DNA from apoptotic cells to the desired level in order to achieve their optimal separation by flow cytometry.

Materials

Cells of interest
PBS (*APPENDIX 2A*)
70% ethanol
DNA extraction buffer (see recipe)
DNA staining solution with PI (see recipe)

12×75–mm tubes suitable for use on the flow cytometer
Flow cytometer with 488-nm excitation and filter for collection of red fluorescence

1. Suspend 1–2×10^6 cells in 0.5 ml PBS and fix cells by adding suspension to 4.5 ml of 70% ethanol in a 12×75–mm tube on ice.

   Cells may be stored several weeks in fixative at $-20°C$.

2. Centrifuge cells 5 min at $200 \times g$, decant ethanol, suspend the cell pellet in 5 ml PBS, and centrifuge 5 min at $300 \times g$, room temperature.

3. Suspend cell pellet in 0.25 ml PBS. To facilitate extraction of low-molecular-weight DNA, add 0.2 to 1.0 ml DNA extraction buffer.

4. Incubate 5 min at room temperature, then centrifuge 5 min at $300 \times g$, room temperature.

5. Suspend cell pellet in 1 ml DNA staining solution with PI. Incubate cells 30 min at room temperature in the dark.

6. Analyze cells on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect forward light scatter and red fluorescence above 600 nm.

DNA FRAGMENTATION: DETECTION OF CELLS WITH FRACTIONAL (SUB-G_1) DNA CONTENT USING DAPI

Cellular DNA may be stained with other fluorochromes instead of PI, and other cell constituents may be counterstained in addition to DNA. The following is the procedure used to stain DNA with DAPI. This protocol requires a UV laser.

Additional Materials *(also see Basic Protocol 6)*

    DNA staining solution with DAPI (see recipe)
    Flow cytometer equipped with UV excitation and filter for collection of blue
       fluorescence

1. Follow Basic Protocol 6, steps 1 to 4. Then, suspend the cell pellet in 1 ml DNA staining solution containing DAPI. Keep on ice 20 min.

2. Analyze cells on the flow cytometer, using UV excitation (e.g., 351-nm line from an argon-ion laser, or mercury lamp with a UG1 filter). Collect blue DAPI fluorescence in a band from 460 to 500 nm.

DNA FRAGMENTATION: DETECTION OF DNA STRAND BREAKS (TUNEL ASSAY)

DNA fragmentation during apoptosis, particularly when it progresses to internucleosomal regions (Arends et al., 1990; Oberhammer et al., 1993), generates a multitude of DNA strand breaks in the nucleus. The 3′-OH ends of the breaks can be detected by attaching a fluorochrome. This is generally done directly or indirectly (e.g., via biotin or digoxygenin) using fluorochrome-labeled deoxynucleotides in a reaction catalyzed preferably by exogenous terminal deoxynucleotidyltransferase (TdT; Gorczyca et al., 1992, 1993; Li and Darzynkiewicz, 1995; Li et al., 1995). The reaction is commonly known as TUNEL, from TdT-mediated dUTP-biotin nick-end labeling (Gavrieli et al., 1992). This acronym is actually a misnomer, since double-stranded DNA breaks are labeled, rather than single-stranded nicks. Of all the markers used to label DNA breaks, BrdUTP appears to be the most advantageous with respect to sensitivity, low cost, and simplicity of reaction (Li and Darzynkiewicz, 1995). When attached to DNA strand breaks in the form of poly-BrdU, this deoxynucleotide can be detected with a FITC-conjugated anti-BrdU Ab, the same Ab commonly used to detect BrdU incorporated during DNA replication. Poly-BrdU attached to DNA strand breaks by TdT, however, is accessible to the Ab without the need for DNA denaturation, which otherwise is required to detect the precursor incorporated during DNA replication.

It should be stressed that the detection of DNA strand breaks by this method requires pre-fixation of cells with a cross-linking agent such as formaldehyde. Unlike ethanol, formaldehyde prevents the extraction of small pieces of fragmented DNA. Thus, despite cell permeabilization and the subsequent cell washings required, the DNA content of early apoptotic cells (and the number of DNA strand breaks) is not markedly diminished through extraction. Labeling of DNA strand breaks in this procedure, which utilizes fluorescein-conjugated anti-BrdU Ab, can be combined with staining of DNA by a fluorochrome of another color (PI, red fluorescence). Cytometry of cells that are differentially stained for DNA strand breaks and for DNA allows one to distinguish apoptotic from non-apoptotic cell subpopulations and reveals the cell cycle distribution in these subpopulations (Gorczyca et al., 1992, 1993).

Materials

    Cells of interest
    1% (v/v) methanol-free formaldehyde (Polysciences) in PBS (*APPENDIX 2A*), pH 7.4
      (primary fixative)
    PBS (*APPENDIX 2A*)
    70% ethanol (secondary fixative)
    5× TdT reaction buffer (see recipe)
    2 mM BrdUTP (Sigma) in 50 mM Tris·Cl, pH 7.5
    TdT in storage buffer (both from Roche Diagnostics), 25 U in 1 µl
    10 mM cobalt chloride ($CoCl_2$; Roche Diagnostics)
    Rinsing buffer: PBS with 0.1% (v/v) Triton X-100 and 0.5% (w/v) BSA
    FITC-conjugated anti-BrdU MAb (see recipe)
    PI staining buffer: PBS with 5 µg/ml PI and 200 µg/ml DNase-free RNase
    Flow cytometer with 488-nm excitation and filters for collection of green and red
      fluorescence

Fix cells

1. Fix 1–5×10^6 cells in suspension 15 min in 1% methanol-free formaldehyde in PBS, pH 7.4, on ice.

2. Centrifuge 5 min at $200 \times g$, 4°C, resuspend cell pellet (~2×10^6 cells) in 5 ml PBS, centrifuge 5 min at $200 \times g$, 4°C, and resuspend cells in 0.5 ml PBS.

3. Add the 0.5-ml cell suspension from step 2 to 5 ml ice-cold 70% ethanol.

    The cells can be stored several weeks in ethanol at −20°C.

4. Centrifuge 5 min at $200 \times g$, 4°C, remove ethanol, and resuspend cells in 5 ml PBS. Repeat centrifugation.

Stain cells

5. Resuspend the pellet (not more than 10^6 cells) in 50 µl of a solution that contains:

      10 µl 5× TdT reaction buffer
      2.0 µl 2 mM BrdUTP stock solution
      0.5 µl TdT in storage buffer (12.5 U final)
      5 µl 10 mM $CoCl_2$ solution
      32.5 µl dH_2O.

6. Incubate cells in this solution 40 min at 37°C.

    Alternatively, incubation can be carried out overnight at 22° to 24°C.

7. Add 1.5 ml rinsing buffer and centrifuge 5 min at $200 \times g$, room temperature.

8. Resuspend cells in 100 µl FITC-conjugated anti-BrdU MAb solution.

9. Incubate 1 hr at room temperature or overnight at 4°C. Add 2 ml rinsing buffer and centrifuge 5 min at $200 \times g$, room temperature.

10. Resuspend cell pellet in 1 ml PI staining solution containing RNase. Incubate 30 min at room temperature in the dark.

11. Measure cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FITC-anti BrdU MAb fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

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18.8.11

DETECTION OF TISSUE TRANSGLUTAMINASE ACTIVATION BY CELL RESISTANCE TO DETERGENTS

Extensive protein cross-linking takes place during apoptosis. The ubiquitous transglutaminase TGase 2 (also called "tissue transglutaminase"; tTGase) was identified as the enzyme responsible for this reaction (Fesus et al., 1987; Melino and Piacentini, 1998). It is presumed that activation of TGase 2 during apoptosis prevents release of soluble and immunogenic proteins from dying cells because protein cross-linking makes these proteins less soluble and thereby decreases a possibility of induction of autoimmune reaction. Furthermore, protein packaging into apoptotic bodies may be facilitated when proteins remain in solid state rather than in solution. The additional role of TGase 2 as one of the "executor enzymes" during apoptosis is still being debated.

This protocol is a simple and rapid approach to identify apoptotic cells with activated TGase 2. The method is based on the propensity of cross-linked protein to withstand treatment with detergents. The authors have noticed that when live, nonapoptotic cells are subjected to treatment with solutions of nonionic detergents, lysis of their plasma membrane and release of the content of cytoplasm is complete, resulting in preparation of isolated nuclei. In contrast, apoptotic cells resist the detergent treatment; their cytoplasmic protein remains insoluble and attached to the nucleus in the form of a shell-like cover (Grabarek et al., 2002). It is possible, therefore, by flow or laser scanning cytometry to distinguish apoptotic cells from the nuclei isolated from nonapoptotic cells, by means of the abundance of protein in the former. In addition, bivariate gating analysis of cellular DNA and protein content makes it possible to reveal the cell cycle distribution separately for the population of cells with cross-linked protein (activated TGase 2) and for the population of cells that did not show protein cross-linking (Grabarek et al., 2002).

Alternate Protocol 2 combines the detection of TGase 2 activity by binding of fluoresceinated cadaverine (F-CDV) with analysis of the cell cycle.

Materials

Cells of interest
DAPI/sulforhodamine 101/detergent solution (see recipe)
Flow cytometer equipped with UV excitation and filters for collection of blue and red fluorescence

1. Collect ~10^6 cells from the culture and centrifuge 5 min at $300 \times g$, room temperature.

2. Suspend the cell pellet in 1 ml DAPI/sulforhodamine 101/detergent solution and vortex 20 sec.

3. Analyze cells on the flow cytometer, using UV excitation (e.g., 351-nm line from an argon-ion laser, or mercury lamp with a UG1 filter). Collect blue DAPI fluorescence in a band from 460 to 500 nm and red fluorescence of sulforhodamine 101 above 600 nm.

DETECTION OF TGase 2 ACTIVATION BY FLUORESCEINATED CADAVERINE (F-CDV) BINDING

This alternate protocol is based on the covalent attachment, by the activated TGase 2, of the fluorescein-tagged cadaverine to the respective protein substrates within the cell (Lajemi et al., 1998). This assay was adapted to flow cytometry and combined with concurrent analysis of cellular DNA content (Grabarek et al., 2002). Like the detergent-based assay, this method is simple and also offers good distinction between apoptotic cells with activated versus nonactivated TGase 2.

It should be noted that when the cost of the reagents for the procedure is taken into account, the detergent-based assay (see Basic Protocol 8) is less expensive by several orders of magnitude than this F-CDV assay.

Materials

    Fluoresceinated cadaverine solution (F-CDV; see recipe)
    Cells of interest
    100% methanol
    DNA staining solution with PI (see recipe)
    Flow cytometer with 488-nm excitation and filters for collection of green and red
        fluorescence

1. Add aliquots of F-CDV stock solution (1 part per 50) directly to cell cultures (10^6 to 10^7 cells) to obtain 50 μM final F-CDV concentration in the culture.

2. Incubate cells in the presence of F-CDV for the desired time interval during which activity of TGase 2 has to be detected (e.g., one to several hours).

    Because cross-linking is a cumulative process, intensity of cell labeling increases with time of incubation.

3. Harvest the culture by centrifuging 5 min at $200 \times g$, room temperature.

4. Suspend the cells in 0.5 ml PBS and fix in 5 ml of 100% methanol on ice. Keep in methanol ≥2 hr at 0° to 4°C; cells may be stored in the fixative for several days.

5. Centrifuge cells 5 min at $200 \times g$, room temperature, decant the fixative thoroughly, and suspend cell pellet in 2 ml DNA staining solution with PI.

6. Keep ≥30 min at room temperature.

7. Measure cell fluorescence on the flow cytometer, using 488-nm excitation (or a mercury arc lamp with a BG12 filter). Collect green FITC-CDV fluorescence at 530 ± 20 nm and red PI fluorescence above 600 nm.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX***.

Binding buffer

    10 mM HEPES-NaOH, pH 7.4
    140 mM NaCl
    5 mM $CaCl_2$
    Store several weeks at 4°C

DAPI/sulforhodamine 101/detergent solution

    Dissolve 100 μg DAPI, 2 mg sulforhodamine 101 (Molecular Probes), and 0.1 ml Triton X-100 in 100 ml PBS (or 0.1 ml Nonidet NP-40). Store several weeks at 0° to 4°C.

$DiOC_6$ (3) stock solution

    Prepare a 0.1 mM solution of $DiOC_6$ (3) (Molecular Probes) by dissolving 5.7 mg dye in 10 ml dimethyl sulfoxide (DMSO). Store for weeks in small (e.g., 0.5- or 1-ml) aliquots protected from light at −20°C. Prior to use, dilute ten-fold with PBS to obtain 10 μM concentration.

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DNA extraction buffer, pH 7.8

192 ml 0.2 M Na_2HPO_4
8 ml 0.1 M citric acid
Store for months at 4°C

DNA staining solution with DAPI

Dissolve 100 μg DAPI in 100 ml PBS. Store at 0° to 4°C for several weeks.

DNA staining solution with PI

200 μg propidium iodide (PI)
2 mg DNase-free RNase
10 ml PBS
Prepare fresh before use

FAM-VAD-FMK

Stock solution: following kit directions, dissolve FAM-VAD-FMK in dimethyl sulfoxide (DMSO) to obtain 150× solution. Store aliquots protected from light ≤3 months at −20°C.

Working solution: Just prior to use, prepare a 30× solution by diluting FAM-VAD-FMK stock solution 1:5 in PBS. Mix the vial until the solution becomes transparent and homogenous. Protect all FAM-VAD-FMK solutions from light. Discard unused portions. Do not store.

FITC-conjugated anti-BrdU MAb solution

100 μl PBS (*APPENDIX 2A*)
0.3 μg FITC-conjugated anti-BrdU MAb (Becton Dickinson)
0.3% (v/v) Triton X-100
1% (w/v) BSA
Prepare fresh before use

Fluoresceinated cadaverine solution

Dissolve 5-[(5-aminopentyl)thioureidyl]fluorescein dihydrobromide (F-CDV; Molecular Probes) in distilled water to obtain a 2.5 mM stock solution. Store aliquots (0.2 to 0.5 ml) of this solution several weeks at −20°C.

Fluorescein-conjugated annexin V

Dissolve FITC-conjugated annexin V (1:1 stoichiometric complex; e.g., from BRAND Applications) in binding buffer (see recipe) at a concentration of 1.0 μg/ml. This solution must be prepared fresh just prior to use.

JC-1 stock solution

Prepare a 0.2 mM solution of JC-1 (Molecular Probes) by dissolving 1.3 mg dye in 10 ml *N,N*-dimethylformamide (Sigma). Store for weeks in small (e.g., 0.5- or 1.0-ml) aliquots protected from light at −20°C. Use glass containers; *N,N*-dimethylformamide will dissolve plastics.

Rhodamine 123 (R123) stock solution

Prepare a 0.1 mM solution of R123 (Molecular Probes) by dissolving 0.38 mg dye in 10 ml methanol. Store for months in small aliquots protected from light at −20°C. Prior to use, dilute ten-fold with PBS to obtain 10 μM concentration.

Rinse solution

1 g BSA
0.1 ml Triton X-100
100 ml PBS (*APPENDIX 2A*)
Store up to 1 week at 4°C

RNase stock solution

Dissolve 2 mg RNase A (e.g., Sigma) in 1 ml distilled water. If the RNase is not DNase free, heat the solution 5 min at 100°C to inactivate any traces of DNase. Store ≤1 year at 4°C.

TdT reaction buffer, 5×

1 M potassium (or sodium) cacodylate
125 mM Tris·Cl, pH 6.6 (*APPENDIX 2A*)
0.125% bovine serum albumin (BSA)
Store for weeks at 4°C

COMMENTARY

Background Information

Applications of flow cytometry in cell necrobiology have two distinct goals (for reviews, see Darzynkiewicz et al., 1992, 1997; Ormerod, 1998; van Engeland et al., 1998; Vermes et al., 2000). One goal is to elucidate molecular and functional mechanisms associated with cell death. For this purpose, flow cytometry is used to measure cellular levels of components involved in the regulation and/or execution of apoptosis or cell necrosis. The most frequently studied are pro- and anti-apoptotic members of the Bcl-2 protein family, caspases, proto-oncogenes (e.g., *c-myc* or *ras)*, or tumor suppressor genes (e.g., p53 or pRB). Flow cytometry is also widely used to study functional attributes of the cell such as mitochondrial metabolism, oxidative stress, intracellular pH, or ionized calcium, all closely associated with mechanisms of apoptosis. The major advantage of flow cytometry in these applications is that it provides the possibility of multiparametric measurements of cell attributes. Multivariate analysis of such data reveals the correlation between the measured cell constituents. For example, when one of the measured attributes is cellular DNA content (the parameter that reports the cell cycle position or DNA ploidy), an expression of the other measured attribute can then be directly related to the cell cycle position (or cell ploidy) without a need for cell synchronization. Since individual cells are measured, intercellular variability can be assessed, cell subpopulations identified, and rare cells detected.

The second goal of cytometry applications is to identify and quantify dead cells and discriminate between apoptotic and necrotic modes of death. Dead-cell recognition is generally based on the presence of a particular biochemical or molecular marker that is characteristic for apoptosis, necrosis, or both. Numerous methods have been developed, especially for the identification of apoptotic cells. Apoptosis-associated changes in the gross physical attributes of cells, such as cell size and granularity, can be detected by analysis of laser light scattered by the cell in forward and side directions (Swat et al., 1981; Ormerod et al., 1995). Some methods rely on apoptosis-associated changes in the distribution of plasma membrane phospholipids (Fadok et al., 1992; Koopman et al., 1994). Others detect the loss of active transport function of the plasma membrane. Still other methods probe the mitochondrial transmembrane potential (Cossarizza et al., 1994; Kroemer, 1998; Zamzani et al., 1998). The detection of DNA fragmentation provides another convenient marker of apoptosis; apoptotic cells are then recognized either by their fractional (subdiploid, sub-G_1) DNA content due to extraction of low-molecular-weight DNA from the cell (Umansky et al., 1981; Nicoletti et al., 1991), or by the presence of DNA strand breaks, which can be detected by labeling their 3′-OH termini with fluorochrome-conjugated nucleotides in a reaction utilizing exogenous terminal deoxynucleotidyl transferase (TdT; Gorczyca et al., 1992, 1993; Li and Darzynkiewicz, 1995; Li et al., 1996). More recently, flow cytometric methods have been developed to detect activation of caspases and tissue transglutaminase (TGase 2; Grabarek et al., 2002). It should be noted, however, that the fluorochrome-labeled inhibitors of caspases (FLICAs), initially described as markers of caspase activation (Smolewski et al., 2001), or serine proteases, although convenient markers of apoptotic cells and most likely detecting activation of these proteases, do not have sufficient specificity to be used as specific probes of their active enzymatic centers (Pozarowski et al., 2003).

A variety of kits are commercially available to identify apoptotic cells using the methods presented in this unit. Since the reagents are

already prepackaged and the procedures are described in cookbook format, the kits offer the advantage of simplicity. Their cost, however, is many-fold higher than that of the individual reagents. Furthermore, the kits do not allow one the flexibility that is often required to optimize procedures for a particular cell system. In many situations, therefore, the preparation of samples for analysis by flow cytometry as described herein may be preferred.
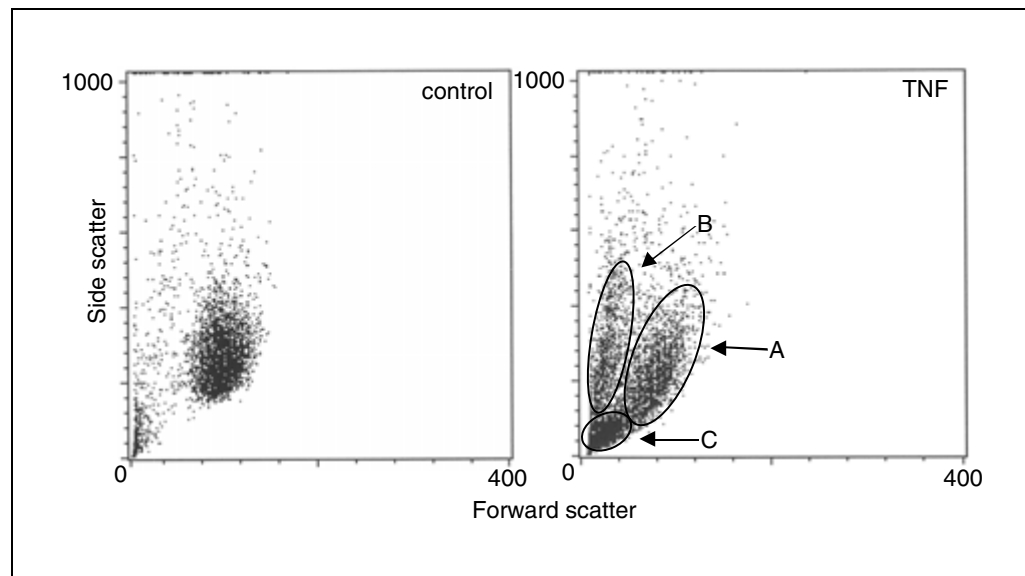
Light-scattering changes during apoptosis

Intersection of cells with the laser light beam in a flow cytometer results in light scattering. Analysis of light scattered in different directions reveals information about cell size and structure. The intensity of the forward light-scatter signal correlates with cell size. Side scatter, on the other hand, yields information on light-refractive and light-reflective properties of the cell and reveals optical inhomogeneity of the cell structure resulting from condensation of cytoplasm or nucleus, granularity, and so on.

As a consequence of cell shrinkage, a decrease in forward light scatter is observed at a relatively early stage of apoptosis (Swat et al., 1981; Ormerod et al., 1995). Initially, there is little change in side scatter during apoptosis. In fact, in some cell systems, an increase in intensity of side-scatter signal may be seen, reflect-

ing perhaps chromatin and cytoplasm condensation and nuclear fragmentation, the events that may lead to an increase in the light-refractive and light-reflective properties of the cell. When apoptosis is more advanced and the cells shrink in size, the intensity of side scatter also decreases (Fig. 18.8.1). Late apoptotic cells, therefore, are characterized by markedly diminished intensity of both forward- and side-scatter signals. In contrast to apoptosis, the early stages of cell necrosis are marked by cell swelling, which is detected by a transient increase in forward light scatter. Rupture of the plasma membrane and leakage of the cytosol during subsequent steps of necrosis correlate with a marked decrease in intensity of both forward- and side-scatter signals.

Analysis of light scatter is often combined with other assays, most frequently surface immunofluorescence (to identify the phenotype of the dying cell), or another marker of apoptosis. It should be stressed, however, that the change in light scatter alone is not a specific marker of apoptosis or necrosis. Mechanically broken cells, isolated nuclei, cell debris, and individual apoptotic bodies may also display diminished light-scatter properties. Therefore, the analysis of light scatter must be combined with measurements that can provide a more definite identification of apoptotic or necrotic cells.

Figure 18.8.1 Changes in light scattering properties of cells undergoing apoptosis. HL-60 cells were untreated (left panel) or treated 3 hr with TNF-α and cycloheximide (CHX) to induce apoptosis (right panel). Cell population **A** in the treated culture (right panel) represents cells that have light scattering properties similar to those of untreated cells. Early apoptotic cells (**B**) have diminished forward scatter and are very heterogenous with respect to side scatter. Late apoptotic cells (**C**) have both forward and side scatter diminished.

Mitochondrial potential

A point to consider in measuring $\Delta\psi_m$ is that the mitochondrial potential probes lack absolute specificity and also accumulate in the cytosol. Probe distribution in mitochondria versus cytosol follows the Nernst equation, according to which the ratio of mitochondrial to cytosolic free cation concentration should be 100:1 at 120 mV mitochondrial transmembrane potential (Waggoner, 1979). However, the specificity of particular mitochondrial probes towards mitochondria is higher at low probe concentrations. It is advisable, therefore, to use these probes at the lowest possible concentration. The limit for the minimal dye concentration that still provides an adequate signal-to-noise ratio during the measurement is dictated by sensitivity of the instrument (laser power, optics, photomultiplier sensitivity) and by the mitochondrial mass per cell; the latter varies depending on the cell type or upon mitogenic stimulation (Darzynkiewicz et al., 1981).

A series of MitoTracker dyes (chloromethyltetramethylrosamine analogs) of different colors was introduced by Molecular Probes as new mitochondrial $\Delta\psi_m$-sensitive probes. Some of these dyes remain attached to mitochondria following cell fixation using cross-linking agents (Poot et al., 1997; Haugland, 2002). It should be noted, however, that because these dyes bind to thiol moieties within mitochondria, their retention after fixation may not be a reliable marker of the transmembrane potential (Ferlini et al., 1998; Gilmore and Wilson, 1999). Furthermore, they are potent inhibitors of respiratory chain I and may themselves induce dissipation of $\Delta\psi_m$ (Scorrano et al., 1999). Because it is likely that other $\Delta\psi_m$ probes may predispose the cells to the permeability transition, one has to be cautious in interpreting the data on their use in analysis of apoptosis.

It has been reported that other mitochondrial probes, 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green, are markers of mitochondrial mass and are not sensitive to $\Delta\psi_m$ (Ratinaud et al., 1988; Poot et al., 1997). It was proposed, therefore, to measure both $\Delta\psi_m$ and mitochondrial mass by using a combination of $\Delta\psi_m$-sensitive and $\Delta\psi_m$-insensitive probes (Petit et al., 1995). Recent observations, however, indicate that 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green are quite sensitive to changes in $\Delta\psi_m$ and therefore, either alone or in combination with $\Delta\psi_m$-sensitive probes, cannot be used as markers of mitochondrial mass (Keiji et al., 2000).

Caspases

Caspases (cysteine-aspartic acid specific proteases) are activated in response to different inducers of apoptosis (Kaufmann et al., 1993; Lazebnik et al., 1994; Alnemri et al., 1996). The process of their activation is considered to be the key event of apoptosis, a marker of cell commitment to disassemble the machinery that supports cell life (for reviews, see Budihardjo et al., 1999; Earnshaw et al., 1999; Shi, 2002). Caspases recognize a four-amino-acid sequence on their substrate proteins; within this sequence, the carboxyl end of aspartic acid is the target for cleavage. Detection of caspase activation is of primary interest in oncology as well as in other disciplines of medicine and biology, and several methods have been developed to accomplish this.

One approach that is potentially useful for cytometry utilizes fluorogenic caspase substrates. The peptide substrates are colorless or nonfluorescent, but upon caspase-induced cleavage, they generate colored or fluorescing products (Gorman et al., 1999; Hug et al., 1999; Liu et al., 1999; Komoriya et al., 2000; Telford et al., 2002). Many kits designed to measure activity of caspases using fluorometric or colorimetric assays are commercially available (e.g., from Biomol Research Laboratories or Calbiochem). Some kits can be used to detect activation of multiple caspases, while other are based on the substrates that are specific for caspase-1, caspase-3, or caspase-8.

The second approach in studies of caspase activation applicable to cytometry is based on immunocytochemical detection of the epitope of these enzymes that is characteristic of their active form. This epitope appears as a result of conformational changes that occur during activation of caspases, such as those associated with the transcatalytic cleavage of the zymogen pro-caspases (for reviews, see Budihardjo et al., 1999; Earnshaw et al., 1999). Antibodies developed to react only with the activated caspases have recently become commercially available (e.g., from Promega). These antibodies can be used in standard immunocytochemical assays. Basic Protocol 3 describes the methodology of detection of caspase-3 activation based on this principle, combined with the convenient immunocytochemical Zenon technology (Haugland, 2002). Another approach that was proposed to probe caspase activation utilizes fluorochrome-labeled inhibitors of caspases (FLICAs; e.g., FAM-VAD-FMK), which are reagents designed as affinity labels to the active enzyme center of these enzymes (Smolewski et al.,

2001). It was recently found, however, that these reagents lack the desired specificity with respect to the active enzyme center of caspases (Pozarowski et al., 2003). However, they are convenient and specific markers of apoptotic cells and can be used as such, particularly when there is a need to distinguish between apoptotic and necrotic modes of cell death. Basic Protocol 3 describes application of FAM-VAD-FMK to identify three sequential stages of apoptosis. Finally, caspase activation can be detected indirectly, by identifying specific protein fragments which are the products of cleavage by particular caspases. For example, immunocytochemical detection of the poly(ADP-ribose) polymerase 85-kDa fragment (PARP p85 fragment), which results from cleavage of the PARP substrate by caspase-3, is described in Basic Protocol 4. Concurrent analysis of cellular DNA content allows one to correlate activation of caspases with the cell cycle position or DNA ploidy. Using immunocytochemical methods, however, one has to remember that, often, antibodies that are applicable to immunoblotting or immunoprecipitation may not always be useful in immunocytochemical assays, and vice versa.

Critical Parameters and Troubleshooting

Positive and negative controls

There are instances when cells die by a process of atypical apoptosis that lacks one or more characteristic apoptotic features. Obviously, apoptosis cannot be detected if the feature serves as a marker. It is also possible that the assay (kit) used to identify apoptotic cells malfunctions for technical reasons. For example, the enzyme TdT used in the TUNEL assay may be inactive due to improper storage. A mistake might be made during the staining procedure. It is essential, therefore, to distinguish between the genuine lack of apoptosis and the inability to detect it due to technical causes. The distinction can be made using a positive control consisting of cells known to be apoptotic (confirmed by a standard method and inspection of cell morphology). Such control cells have to be processed in parallel with the investigated sample through all the prescribed protocol steps. Some vendors provide positive and negative control cells with their kits (e.g., APO-BRDU from Phoenix Flow Systems).

The positive control cells can be prepared in large quantity and stored in aliquots to be used during each experiment. Such a convenient control may be, to give an example, exponentially growing HL-60 or U-937 leukemic cells treated 3 to 6 hr in culture with 0.2 µM camptothecin (CPT) to induce apoptosis. The cells so treated consist of subpopulations of apoptotic (S-phase) and non-apoptotic (G$_1$-phase) cells, present in the same sample. However, to induce apoptosis of S-phase cells with CPT, it is critical to have the cultures in the exponential phase of growth, at relatively low cell density (<800,000 cells/ml); subconfluent cultures are quite resistant to CPT. A large batch of cells treated in such a manner can be appropriately fixed in 70% ethanol and then stored in aliquots at −20°C to be used as a positive and negative control for each assay that utilizes fixed cells. For the assays that require live cells, controls should be freshly made and must not be fixed. Cells from healthy, untreated cultures may also serve as negative controls.

False-positive apoptosis

The exposure of phosphatidylserine on cell surfaces that occurs during apoptosis (Fadok et al., 1992; Koopman et al., 1994) makes apoptotic cells and apoptotic bodies attractive to neighboring cells, which phagocytize them. The ability to engulf apoptotic bodies is not solely the property of professional phagocytes, but is shared by cells from fibroblast or epithelial lineages. It is frequently observed, especially in solid tumors, that the cytoplasm of both nontumor and tumor cells located in the neighborhood of apoptotic cells contains inclusions typical of apoptotic bodies. The remains of apoptotic cells engulfed by neighboring cells contain altered plasma membrane, fragmented DNA, and other constituents with attributes characteristic of apoptosis. Thus, if the distinction is based on any of these attributes, the live, nonapoptotic cells that phagocytized apoptotic bodies cannot be distinguished from genuine apoptotic cells by flow cytometry. For example, nonapoptotic cells that engulf apoptotic bodies become reactive with annexin V (Marguet et al., 1999). Most likely this is due to the fact that during engulfment, the plasma membrane of apoptotic bodies fuses with the plasma membrane of the phagocytizing cell. It has also been shown that nonapoptotic cells (primarily monocytes and macrophages) in bone marrow of patients undergoing chemotherapy have large quantities of apoptotic bodies in their cytoplasm and are strongly positive in the TUNEL reaction (Bedner et al., 1999).

Even after relative mild treatment such as trypsinization, mechanical agitation, detach-

ment with rubber policeman, or electroporation, the plasma membrane of live nonapoptotic cells may have phosphatidylserine, reactive with annexin V, exposed on the surface. Such cells may also be erroneously identified as apoptotic cells.

Distinction between apoptosis, necrosis, and the "necrotic stage" of apoptosis

There are several differences between typical apoptotic and necrotic cells (Kerr et al. 1972; Arends et al., 1990; Majno and Joris, 1995) that provided a basis for development of numerous markers and methods that can discriminate between these two modes of cell death (Darzynkiewicz et al., 1992, 1997). The major difference stems from the early loss of integrity of the plasma membrane during necrosis. This event results in a loss of the cell's ability to exclude charged fluorochromes such as trypan blue or PI. In contrast, the plasma membrane and membrane transport functions remain, to a large extent, preserved during the *early stages* of apoptosis. A cell's permeability to PI or its ability to retain some fluorescent probes, such as products of enzyme activity (e.g., fluorescein diacetate hydrolyzed by esterases), is the most common marker distinguishing apoptosis from necrosis (Darzynkiewicz et al., 1994). A combination of fluorochrome-conjugated annexin V with PI distinguishes live cells (unstainable with both dyes) from apoptotic cells (stainable with annexin V but unstainable with PI) from necrotic cells (stainable with both dyes; Koopman et al., 1994). The same holds true for a combination of PI with FLICA (Smolewski et al., 2001). However, while this approach works well in many instances, it has limitations and possible pitfalls.

(1) Late-stage apoptotic cells resemble necrotic cells to such an extent that the term "apoptotic necrosis" was proposed to define them (Majno and Joris, 1995). This is a consequence of the fact that the integrity of the plasma membrane of late apoptotic cells is compromised, which makes the membrane leaky and permeable to charged cationic dyes such as PI. Since the ability of such cells to exclude these dyes is lost, the discrimination between late apoptosis and necrosis cannot be accomplished by methods based on the use of dye exclusion (PI, trypan blue) or annexin V binding.

(2) The permeability and asymmetry of plasma membrane phospholipids (accessibility of phosphatidylserine) may change, as a result of prolonged treatment with proteolytic enzymes (trypsinization), mechanical damage (e.g., cell removal from flasks by rubber policeman, cell isolation from solid tumors, or even repeated centrifugations), electroporation, or treatment with some drugs.

(3) Many flow cytometric methods designed to quantify the frequency of apoptotic or necrotic cells are based on the differences between live, apoptotic, and necrotic cells in the permeability of plasma membrane to different fluorochromes such as PI, 7-AAD, or Hoechst dyes. It should be stressed, however, that plasma membrane permeability probed by dye accumulation in the cell may vary depending on the cell type and other factors unrelated to apoptosis or necrosis (e.g., very active efflux mechanism that rapidly pumps dye out of the cell). The assumptions, therefore, that live cells maximally exclude a particular dye, while early apoptotic cells are somewhat leaky and late apoptotic or necrotic cells are fully permeable to the dye, and that these differences are large enough to identify these cells, are not universally applicable.

It is particularly difficult to discriminate between apoptotic and necrotic cells in suspensions from solid tumors. Necrotic areas form in tumors as a result of massive local cell death due to poor accessibility to oxygen and growth factors when tumors grow in size and their local vascularization becomes inadequate. Needle-aspirated samples or cell suspensions from the resected tumors may contain many cells from the necrotic areas. Such cells are indistinguishable from late apoptotic cells by many markers. Because the AI in solid tumors, representing spontaneous or treatment-induced apoptosis, should not include cells from the necrotic areas, one has to eliminate such cells from analysis. Because incubation of cells with trypsin and DNase selectively digests all cells whose plasma membrane integrity is compromised, i.e., primarily necrotic cells (Darzynkiewicz et al., 1994), such a procedure may be used to remove necrotic cells from suspensions. It should be noted, however, that late apoptotic cells have partially leaky plasma membrane and are also expected to be sensitive to this treatment.

In conclusion, examination of cells by microscopy may be the only way to distinguish between apoptosis and necrosis, based on their characteristic differences in morphology.

Preferential loss of apoptotic cells during sample preparation

Cell detachment in culture. Early during apoptosis, cells detach from the surface of culture flasks and float in the medium. The standard procedure of discarding the medium, trypsinizing the attached cells or treating them with EDTA, and collecting the detached cells results in selective loss of those apoptotic cells that are discarded with the medium. Such loss may vary from flask to flask depending on how the culture is handled, e.g., the degree of mixing or shaking, efficiency in discarding the old medium, and so on. Surprisingly, some authors still occasionally report discarding the medium and trypsinizing the cells. Needless to say, such an approach cannot be used for quantitative analysis of apoptosis. To estimate the frequency of apoptotic cells in adherent cultures, it is essential to collect floating cells, pool them with the trypsinized ones, and measure them as a single sample. It should be stressed that trypsinization, especially if prolonged, results in digestion of cells with a compromised plasma membrane. Thus, collection of cells from cultures by trypsinization is expected to cause selective loss of late apoptotic and necrotic cells.

Density-gradient centrifugation. Similarly, density-gradient separation of cells (e.g., using Ficoll-hypaque or percoll solutions) may result in selective loss of dying and dead cells, because early during apoptosis the cells become dehydrated and have condensed nuclei and cytoplasm, and therefore have a higher density than nonapoptotic cells. Knowledge of any selective loss of dead cells in cell populations purified by such an approach is essential when one is studying apoptosis.

Centrifugations, mechanical agitations. Repeated centrifugations lead to cell loss by at least two mechanisms. One involves electrostatic cell attachment to the tubes and may be selective for a particular cell type. For example, preferential loss of monocytes and granulocytes is observed during repeated centrifugation of white blood cells, while lymphocytes remain in suspension (Bedner et al., 1997). Cell loss is of particular concern when hypocellular samples ($<5 \times 10^4$) are processed. In such a situation, carrier cells in excess (e.g., chick erythrocytes) may be added to preclude disappearance of the cells of interest through centrifugation. The second mechanism of cell loss involves preferential disintegration of fragile cells. Because apoptotic cells are very fragile, especially at late stages of apoptosis, they may selectively be lost from samples that require centrifugation or are repeatedly vortexed or pipetted. Addition of serum or bovine serum albumin to cell suspensions, shortened centrifugation time, and decreased gravity force all may have a protective effect against cell breakage by mechanical factors. Apoptotic cells may also preferentially disintegrate in biomass cultures that require constant cell mixing.

It should be noted that sensitivity of apoptotic cells to mechanical factors depends on activation of TGase 2. Cells with activated TGase 2 have their cytoplasmic protein cross-linked and are resistant to mechanical stress. They also resist treatment with detergents. In contrast, apoptotic cells that do not activate TGase 2 are overly sensitive and easily undergo disintegration. It was observed that cells may often undergo apoptosis without evidence of TGase 2 activation (Grabarek et al., 2002).

Abundance of extractable (fragmented) DNA is not a quantitative measure of apoptosis

A common misconception in analysis of apoptosis is that the amount of fragmented (low-molecular-weight, "extractable") DNA detected in cultures, tissue or cell extracts, or other samples reflects incidence of apoptosis. Many methods have been developed to estimate the abundance of fragmented DNA and numerous reagent kits are being sold for that purpose. They include direct quantitative colorimetric analysis of soluble DNA, densitometry of DNA ladders on gels, and immunochemical assessment of nucleosomes. These approaches and the related kits are advertised as quantitative, in that they provide information regarding the incidence of apoptosis in cell populations. Such claims are grossly incorrect for the reason that the amount of fragmented (low-molecular-weight) DNA that can be extracted from a *single* apoptotic cell varies over a wide range depending on the *stage* of apoptosis. Although at an early stage of apoptosis only a small fraction of DNA is fragmented and extractable, nearly all DNA can be extracted from the cell that is more advanced in apoptosis. Thus, the total content of low-molecular-weight DNA extracted from the cell population, or the ratio of low- to high-molecular-weight fraction, does not provide information about the *frequency* of apoptotic cells (AI), even in relative terms, e.g., for comparison of cell populations. For this simple reason, biochemical methods based on analysis of fragmented DNA can be used to *quantitatively* estimate the frequency of apop-

tosis only when comparing cell populations that have identical distribution of cells across the stages of apoptosis.

DNA laddering observed during electrophoresis provides evidence of internucleosomal DNA cleavage, which is considered one of the hallmarks of apoptosis (Arends et al., 1990). Analysis of DNA fragmentation by gel electrophoresis to detect such laddering is thus a valuable method to demonstrate the *apoptotic mode* of cell death; however, it should not be used as a means to quantify the *frequency* of apoptosis.

It should be noted that in some cell types, particularly of fibroblast and epithelial lineages, apoptosis may occur without internucleosomal DNA cleavage. The products of DNA fragmentation in these cells are large (50- to 300-kb) DNA sections that cannot be extracted from the cell (Oberhammer et al., 1993). Obviously, in these cases, apoptosis cannot be revealed by the presence of DNA laddering on gels or by analysis of low-molecular-weight products. These large DNA fragments, however, can be identified by pulse-field gel electrophoresis.

Changes in cell morphology, the "gold standard" for identification of apoptosis

Apoptosis was originally defined as a specific mode of cell death based on very characteristic changes in cell morphology (Kerr et al., 1972; Fig. 18.8.2). These changes are still considered the "gold standard" for identification of apoptotic cells. Although particular markers may be used in conjunction with flow cytometry for detection and quantitative assessment of apoptosis in cell populations, the mode of cell death should always be confirmed by inspection of cells by light or electron microscopy. If there is any ambiguity regarding the mechanism of cell death, the morphological changes should be the deciding factor in resolving the uncertainty.

The characteristic morphological features of apoptosis and necrosis are listed in Table 18.8.1. The most specific (and generic to apoptosis) of these changes is chromatin condensation; the chromatin of apoptotic cells is very "smooth" (structureless) in appearance, and the structural framework that otherwise characterizes the cell nucleus is entirely lost. Because of the condensation, chromatin shows strong hyperchromicity with any of the DNA-specific
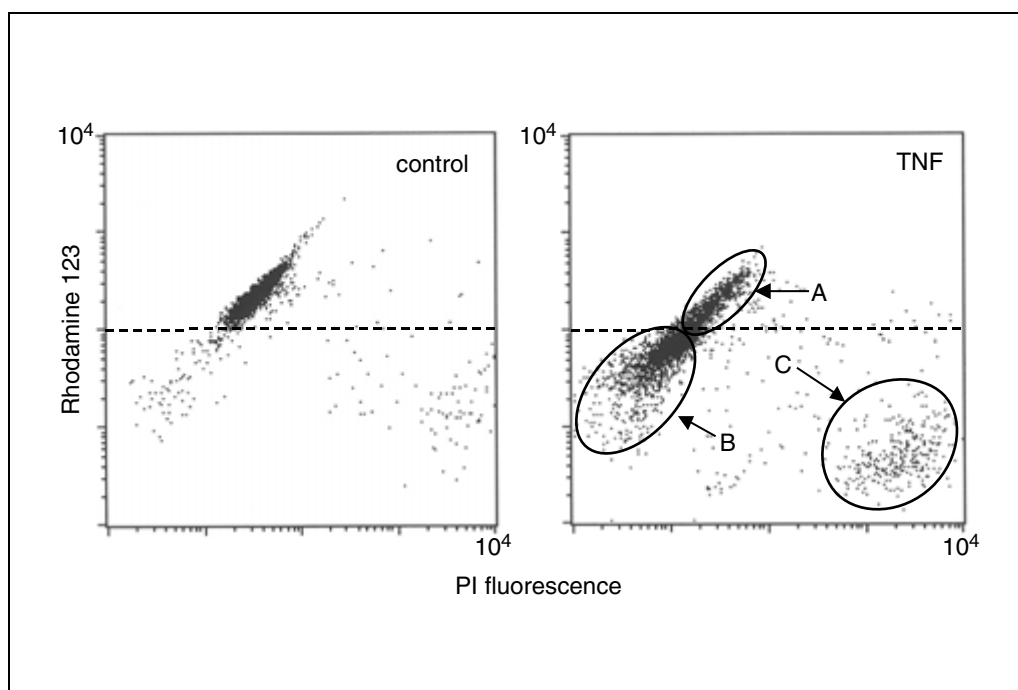
Figure 18.8.2 Detection of the collapse of mitochondrial electrochemical potential (ψ_m) by rhodamine 123 (R123). HL-60 cells were untreated (control; left panel) or treated 3 hr with TNF-α and CHX (right panel) to induce apoptosis. The cells were then incubated with R123 and PI according to Basic Protocol 1. The early apoptotic cells have diminished green fluorescence of R123 but exclude PI (cell population B). The late apoptotic (also necrotic) cells are stained strongly by PI (population C).

Table 18.8.1 Morphological Criteria for Identification of Apoptosis or Necrosis

Apoptosis	Necrosis
Reduced cell size, convoluted shape	Cell and nuclear swelling
Plasma membrane undulations ("blebbing," "budding")	Patchy chromatin condensation
Chromatin condensation (DNA hyperchromicity)	Swelling of mitochondria
Loss of the structural features of the nucleus (smooth, planate appearance)	Vacuolization in cytoplasm
Nuclear fragmentation (karyorrhexis)	Plasma membrane rupture ("ghost-like" appearance of lysed cells)
Presence of apoptotic bodies	Dissolution of nuclear chromatin (karyolysis)
Dilatation of the endoplasmic reticulum	Attraction of inflammatory cells
Relatively unchanged cell organelles	
Shedding of apoptotic bodies	
Phagocytosis of the cell remnants	
Cell detachment from tissue culture flasks	

dyes (Hotz et al., 1992). Apart from chromatin condensation, however, other changes are less generic to apoptosis and may not always be apparent. For example, nuclear fragmentation, although commonly observed during apoptosis of hematopoietic-lineage cells, may not occur during apoptosis of some epithelial- or fibroblast-lineage cells. Likewise, cell shrinkage, at least early during apoptosis, is not a universal marker of the apoptotic mode of cell death.

It should be stressed that optimal preparations for light microscopy require cytospinning of live cells followed by fixation and staining on slides. The cells become flat, facilitating assessment of their morphology. On the other hand, when cells are initially fixed and stained in suspension, transferred to slides, and analyzed under the microscope, their morphology is obscured by unfavorable geometry; the cells are spherical and thick, and require confocal microscopy to reveal details such as early signs of apoptotic chromatin condensation.

Differential staining of cellular DNA and protein of cells on slides with DAPI and sulforhodamine 101, respectively, is rapid and simple and provides very good morphological resolution of apoptosis and necrosis (Darzynkiewicz et al., 1997). A combined cell illumination with UV light (to excite the DAPI or other DNA fluorochrome) and light-transmission microscopy utilizing interference contrast (Nomarski illumination) is the authors' favorite method of cell visualization to identify apop-

totic cells. Other DNA fluorochromes, such as PI, 7-aminoactinomycin D, or acridine orange, can be used as well.

In conclusion, regardless of which cytometric assay has been used to identify apoptosis, the mode of cell death should be confirmed by inspection of cells by light or electron microscopy. Morphological changes during apoptosis have a very characteristic pattern (Kerr et al., 1972; Majno and Joris, 1995) and should be the deciding factor in situations where any ambiguity arises regarding the mode of cell death. It should be noted, however, that the laser scanning cytometer (LSC), offering many attributes of flow cytometry and simultaneously providing the means to examine morphology of the measured cells (Darzynkiewicz et al., 1999; Kamentsky, 2001), appears to be the instrument of choice in analysis of apoptosis (Bedner et al., 1999).

Membrane potential

It should be stressed that $\Delta\psi_m$, like other functional markers, is sensitive to minor changes in cell environment. The samples to be compared, therefore, should be incubated and measured under identical conditions, taking into an account temperature, pH, time elapsed between the onset of incubation and actual fluorescence measurement, and other potential variables.

Annexin V

Interpretation of the results may be complicated by the presence of non-apoptotic cells with damaged membranes. Such cells may have phosphatidylserine exposed on the plasma membrane and therefore, like apoptotic cells, bind annexin V. Mechanical disaggregation of tissues to isolate individual cells, extensive use of proteolytic enzymes to disrupt cell aggregates, to remove adherent cells from cultures, or to isolate cells from tissue, mechanical removal of the cells from tissue-culture flasks (e.g., with a rubber policeman), or cell electroporation all affect the binding of annexin V. Such treatments, therefore, may introduce experimental bias in the subsequent analysis of apoptosis by this method.

Even intact and live cells take up PI upon prolonged incubation. Therefore, fluorescence measurement should be performed rather shortly after addition of the dye.

DNA fragmentation

It should be stressed that the degree of extraction of low-molecular-weight DNA and consequently the content of DNA remaining in apoptotic cells for flow cytometric analysis varies markedly depending on the extent of DNA degradation (duration of apoptosis), the number of cell washings, and the pH and molarity of the washing and staining buffers. DNA fragmentation is often so extensive that most DNA is removed during the post-fixation rinse with PBS and in the staining solution, and a DNA extraction step is therefore unnecessary. Conversely, when DNA degradation does not proceed to internucleosomal regions but stops after generating 50- to 300-kb fragments (Oberhammer et al., 1993), little DNA can be extracted, and this method may fail to detect such apoptotic cells. It also should be noted that if G_2, M, or even late S-phase cells undergo apoptosis, the loss of DNA from these cells may not be adequate to place them at the sub-G_1 peak, as they may end up with DNA content equivalent of that of G_1 or early S-phase cells and therefore be indistinguishable from the latter.

It is a common practice to use detergents or hypotonic solutions instead of fixation in the process of DNA staining for flow cytometry (Nicoletti et al., 1991). Such treatments cause lysis of plasma membrane and release of the nucleus. Although this approach is simple and yields excellent resolution for DNA-content analysis, when used to quantify apoptotic cells it introduces bias owing to the fact that nuclei of apoptotic cells are often fragmented. Lysis of cells with fragmented nuclei releases nuclear fragments rather than individual nuclei, and consequently several fragments can be released from a single cell. Likewise, lysis of mitotic cells that happen to be in the specimen releases individual chromosomes or chromosome aggregates. In the case of micronucleation (e.g., after cell irradiation), the micronuclei are released upon cell lysis. Each nuclear fragment, chromosome, or micronucleus is then recorded by the flow cytometer as an individual object with a sub-G_1 DNA content. Such objects are then erroneously classified as individual apoptotic cells. This bias is increased if DNA content is displayed on a logarithmic scale. Such a scale allows one to record objects with DNA content as little as 1% or even 0.1% of that of G_1 cells, which certainly cannot be individual apoptotic cells.

Activation of TGase 2

Although apoptotic cells with activated TGase 2 can be detected using either Basic Protocol 8 or Alternate Protocol 2, the differences between these assays should be underscored. The most distinct is the difference in the length of the time window that may be measured by the respective assay. Namely, the detergent-based assay (see Basic Protocol 8) detects cumulative protein cross-linking, reflecting the integrated cross-linking, from its onset to the time of cell harvesting. In contrast, the assay based on incorporation of F-CDV (see Alternate Protocol 2) detects cross-linking that occurs only during the time interval when this reagent is present in the culture. Thus, if F-CDV is included at time zero, i.e., when the inducer of apoptosis is added, its incorporation is a reflection of the cumulative protein cross-linking and thus is comparable with the detergent-based assay. However, if it is added during the final hour or two, it will reflect the cross-linking that took place only during this 1- or 2-hr time window. This difference between the assays should be kept in mind when comparing frequency of TGase 2–positive cells, which may vary between the assays, depending on the length of the respective time window.

Situations (e.g., following treatment with particular drugs) may occur in which cell proteins may become less soluble and more detergent resistant, not necessarily because of TGase 2 activation, but because of alteration by the drug. Some treatments unrelated to TGase 2 activity may also result in attachment of F-CDV to cellular proteins. Some cell types may be

more resistant to detergents. In all these cases, the assay may detect the "false-positive" TGase 2–positive cells. As with other markers of apoptosis, one has to be careful and additionally identify these cells by microscopy based on the characteristic changes in their morphology.

Apoptosis can be induced and may progress in some cells with no apparent TGase 2 activation (Grabarek et al., 2002). In general, the activation of TGase 2 is seen to occur in cells that show a high degree of chromatin and cytoplasm condensation leading to pronounced nuclear and cell shrinkage, and that either lack or have very limited nuclear fragmentation. In contrast, the apoptotic cells that appear larger and whose nuclei are excessively fragmented do not show activation of TGase 2. Apoptosis without activation of TGase 2 appears to occur more frequently when induced with higher drug doses, i.e., when cells enter the apoptotic process more rapidly following treatment. Thus, a note of caution should be added, that since TGase 2 activation may not be detected in some instances of apoptosis, the absence of its activation should not be considered a marker of nonapoptotic cells.
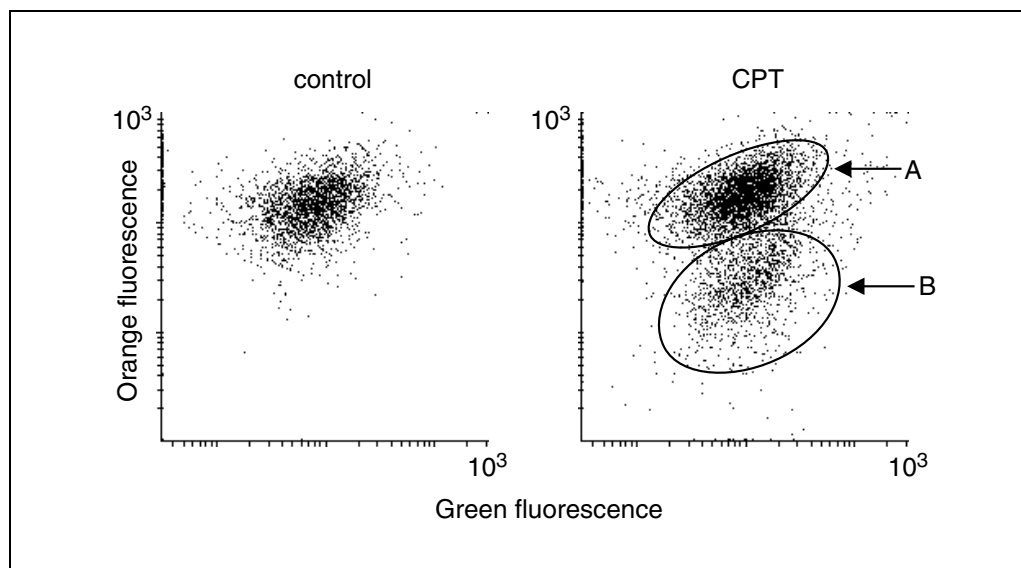
Anticipated Results

Light scatter. A decrease in forward light scatter characterizes early apoptotic cells (Fig. 18.8.1, cluster B). Late apoptotic cells and perhaps also larger apoptotic bodies show marked decrease in both forward and side light scatter (cluster C).

Mitochondrial potential. A combination of PI and R123 identifies nonapoptotic cells that stain only green, early apoptotic cells whose green fluorescence is diminished, and late apoptotic or necrotic cells that stain with PI and have red fluorescence only (Fig. 18.8.2). Likewise, a combination of $DiOC_6(3)$ and PI labels live nonapoptotic cells green, early apoptotic cells dim-green, and late apoptotic and necrotic cells red (not shown).
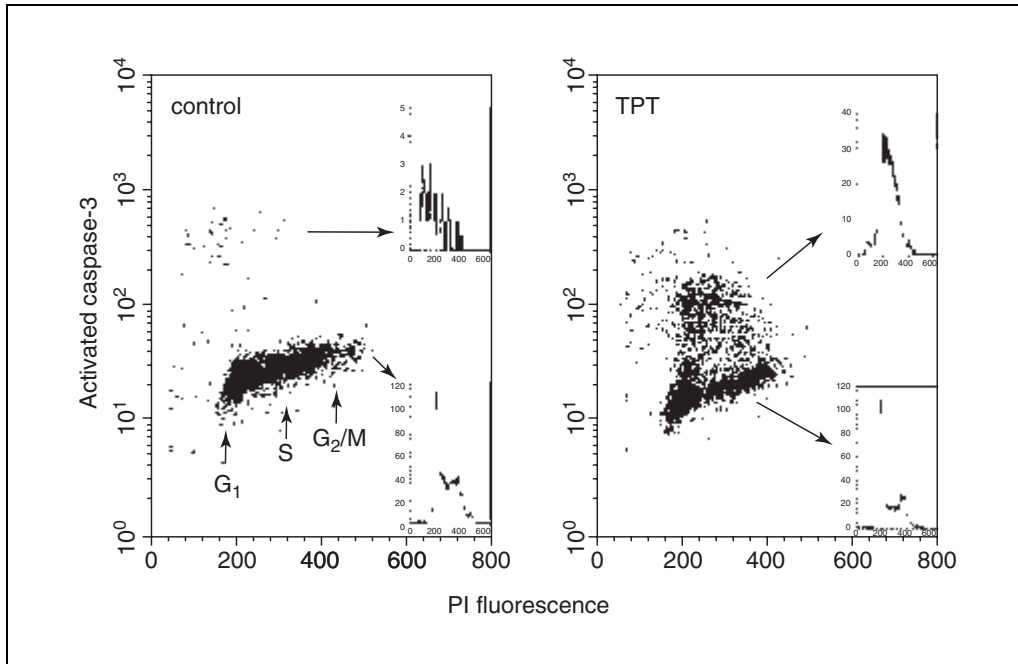
The change in binding of JC-1 is manifested by a loss of the orange fluorescence that represents the aggregate binding of this dye and that characterizes charged mitochondria (Fig. 18.8.3). JC-1 green fluorescence is expected to increase as a result of disaggregation of the complexed JC-1. However, either no change or a decrease in green fluorescence may be seen if JC-1 concentration within the cell is too high, which causes quenching of its fluorescence.

Caspases. Caspase-3 activation during apoptosis induced by topotecan (TPT), a camptothecin (CPT) analog, is reflected by the cells' ability to bind antibody that is reactive with the activated (cleaved) form of this enzyme (Fig. 18.8.4). Concurrent staining of cellular DNA with PI makes it possible to correlate caspase-3 activation with the cell cycle position. Note that activation of caspase-3 occurs preferentially in S-phase cells.
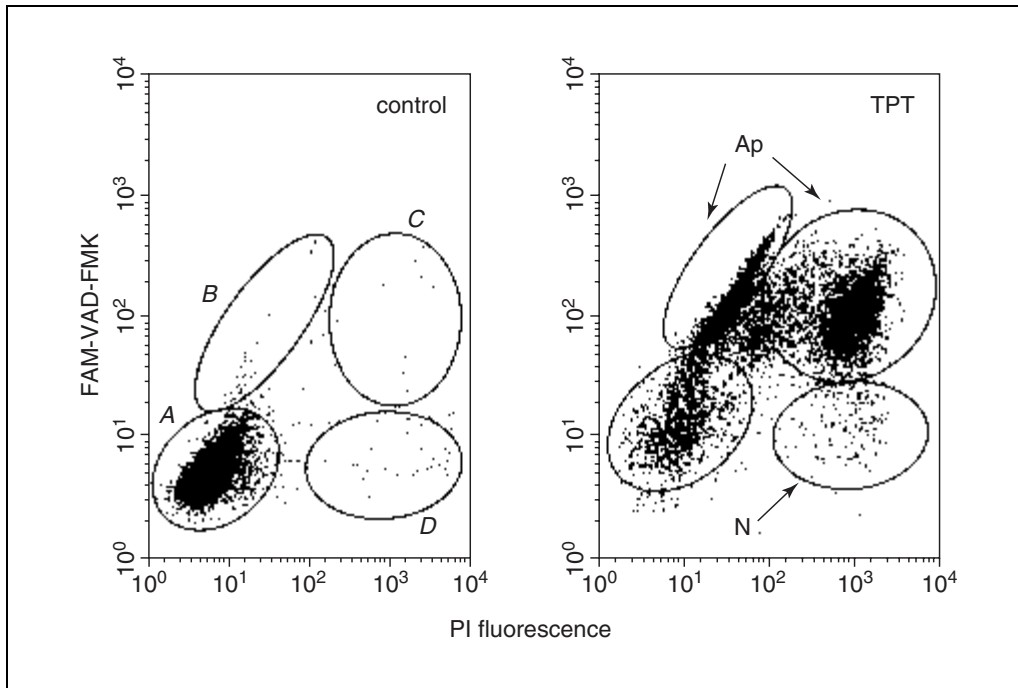

Figure 18.8.3 Detection of the collapse of mitochondrial electrochemical potential using the aggregate dye JC-1. HL-60 cells were untreated (control, left panel) or treated 3 hr with camptothecin (CPT, right panel) to induce apoptosis. Cells were then stained with JC-1 and their orange and green fluorescence was measured by cytometry, as described in Basic Protocol 2. Decreased intensity of orange fluorescence (subpopulation B) characterizes the cell with collapsed potential.

Figure 18.8.4 Immunocytochemical detection of caspase-3 activation using antibody reactive with the activated (cleaved) caspase-3. Apoptosis of HL-60 cells was induced by topotecan (TPT), an analog of CPT. Zenon technology (Haugland, 2002) was used to detect caspase-3 as described in Basic Protocol 2. Top and bottom insets in each panel show cellular DNA content frequency histograms of cells with activated and nonactivated caspase-3, respectively. Note that S-phase cells preferentially contain activated caspase-3 after induction of apoptosis by TPT.

Figure 18.8.5 Binding of fluorochrome-labeled inhibitor of caspases (FLICA; FAM-VAD-FMK) and PI during apoptosis. Apoptosis of HL-60 cells was induced by TPT. The cells were stained according to Basic Protocol 3. Green (FAM-VAD-FMK) and red (PI) cellular fluorescence was measured by flow cytometry. Four cell subpopulations (A to D) can be identified, differing in their capability to bind FAM-VAD-FMK and PI. They represent sequential stages of apoptosis, starting with binding of FAM-VAD-FMK (B), loss of plasma membrane integrity to exclude PI (C), and loss of reactivity with FAM-VAD-FMK (D).

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and Death**

18.8.25

The bivariate distributions (scatterplots) of green and red fluorescence intensity representing cells supravitally stained with FAM-VAD-FMK (FLICA) and PI reveal the presence of four distinct subpopulations (Fig. 18.8.5). Nonapoptotic cells show neither FLICA nor PI fluorescence (FLICA⁻/PI⁻; subpopulation A). Early apoptotic cells bind FAM-VAD-FMK and still exclude PI (FLICA⁺/PI⁻; subpopulation B). More advanced in apoptosis are the cells that bind FAM-VAD-FMK but lose the ability to exclude PI (FLICA⁺/PI⁺; subpopulation C). The cells most advanced in apoptosis are FAM-VAD-FMK negative and are stained with PI (FLICA⁻/PI⁺; subpopulation D). Because the late phase of apoptosis during which the plasma membrane becomes permeable to cationic dyes such as PI or trypan blue has been defined as the "necrotic stage" of apoptosis (Majno and Joris, 1995; Darzynkiewicz et al., 1997), the FLICA⁺/PI⁺ and FLICA⁻/PI⁺ cells thus represent two consecutive phases of the "necrotic stage." It should be noted that genuine necrotic cells, i.e., cells that die by the mode of necrosis ("accidental" cell death), not having activated caspases and unable to exclude PI (Darzynkiewicz et al., 1997), have the same properties (FLICA⁻/PI⁺) as very late apoptotic cells.

As mentioned earlier, because of lack of specificity, the labeling of apoptotic cells with FAM-VAD-FMK, while likely the marker of caspase activation, is not in and of itself evidence of its binding to the active enzymatic center of caspases (Pozarowski et al., 2003).
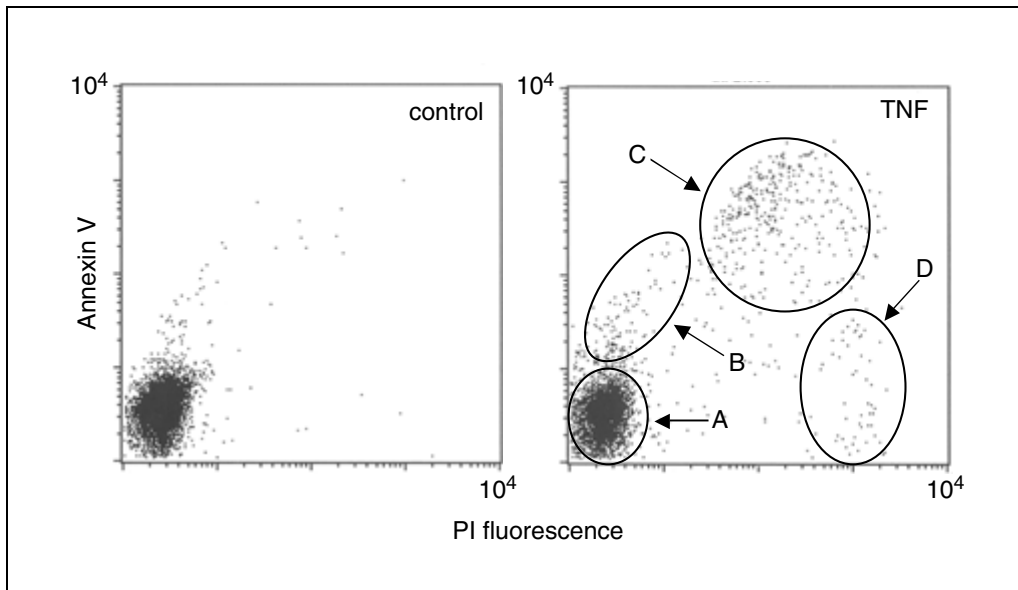
PARP cleavage. Differences in intensity of PARP p85 immunofluorescence versus PI fluorescence (cellular DNA content) allow one to identify apoptotic cells and reveal the cell cycle distribution of both apoptotic (PARP p85–positive) and nonapoptotic (PARP p85–negative) cells (Fig. 18.8.6). It is quite evident that predominantly S-phase cells were undergoing apoptosis upon CPT treatment (Li and Darzynkiewicz, 2000).

Annexin V. Live nonapoptotic cells stained according to Basic Protocol 5 have minimal green (annexin V-FITC) fluorescence and also minimal red (PI) fluorescence (Fig. 18.8.7; subpopulation A). At early stages of apoptosis, cells stain green but still exclude PI and therefore continue to have no significant red fluorescence (subpopulation B). At late stages of apoptosis, cells show intense green and red fluorescence (subpopulation C). It should be noted that isolated nuclei, cells with severely damaged membranes, and very late apoptotic cells stain rapidly and strongly with PI and may not bind annexin V (subpopulation D).
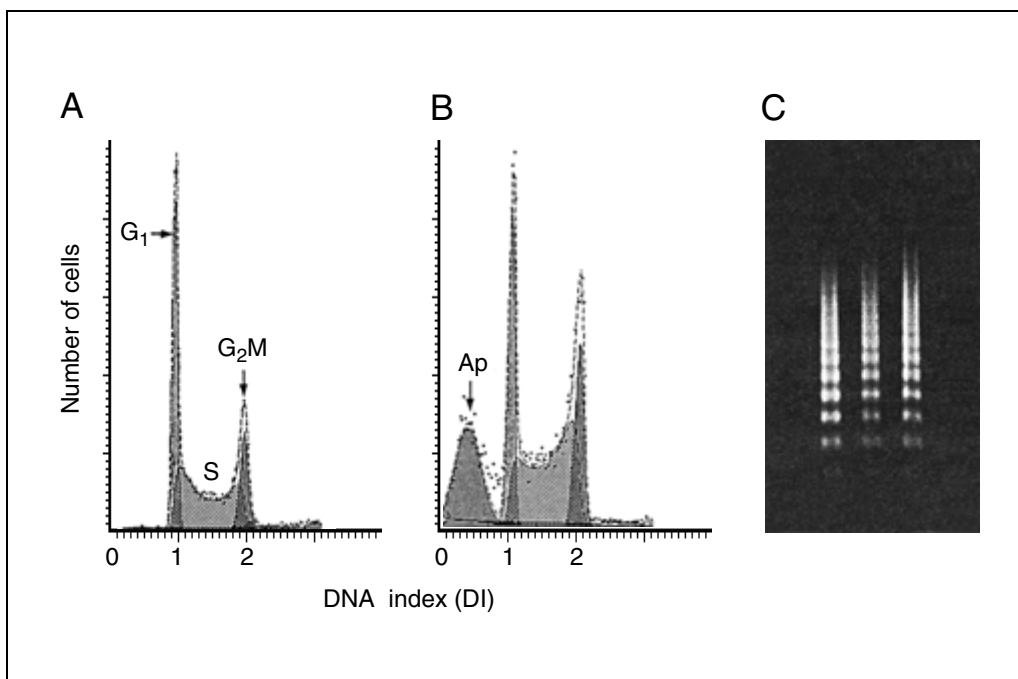
DNA fragmentation. Apoptotic cells have decreased PI (or DAPI) fluorescence and diminished forward light scatter relative to cells in the main peak (G_1; Fig. 18.8.8). Optimally, the sub-G_1 peak representing apoptotic cells should be separated from the G_1 peak of the
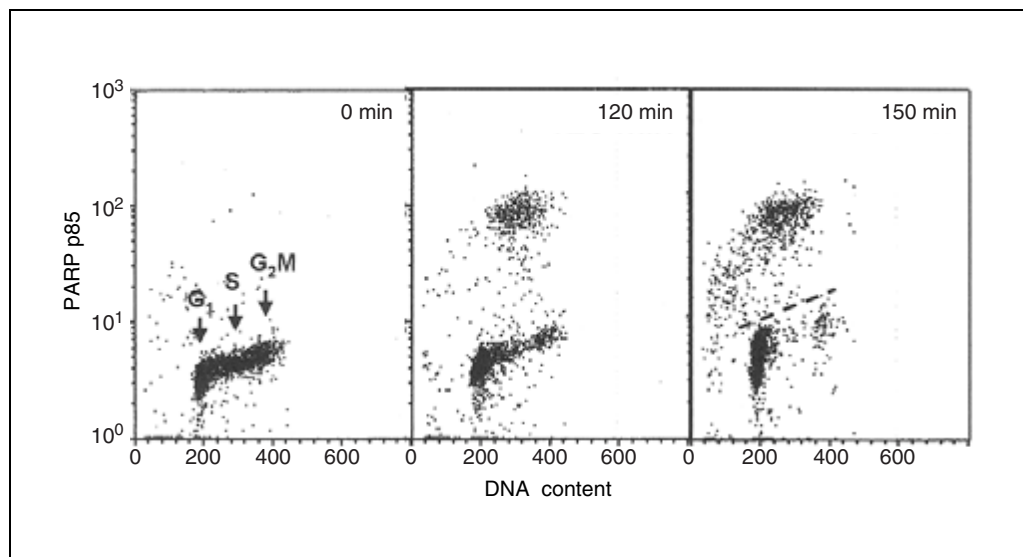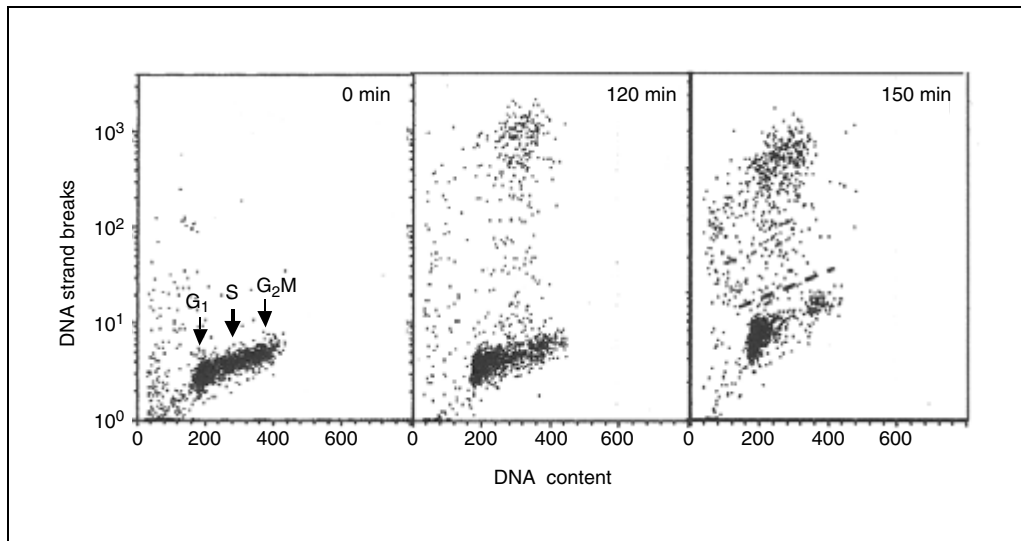

Figure 18.8.6 Identification of apoptotic cells by flow cytometry based on the immunocytochemical detection of the 85-kDa PARP cleavage fragment. To induce apoptosis, HL-60 cells were treated 60 min with TNF-α in the presence of CHX (Li and Darzynkiewicz, 2000). PARPp85 was detected immunocytochemically and DNA was counterstained with PI, as described in Basic Protocol 3.
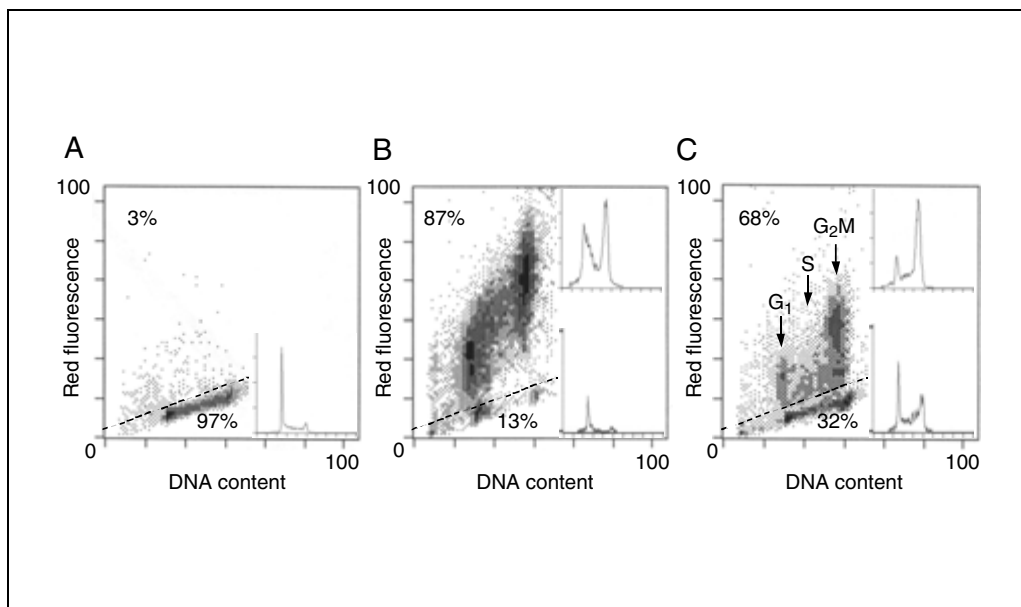
Flow Cytometry of Apoptosis

18.8.26

Figure 18.8.7 Detection of early and late apoptotic cells after staining with annexin V–FITC and PI. To induce apoptosis, HL-60 cells were treated 2 hr with TNF-α and CHX. Untreated (control; left panel) and TNF-α-treated (right panel) cells were then stained with annexin V-FITC and PI.
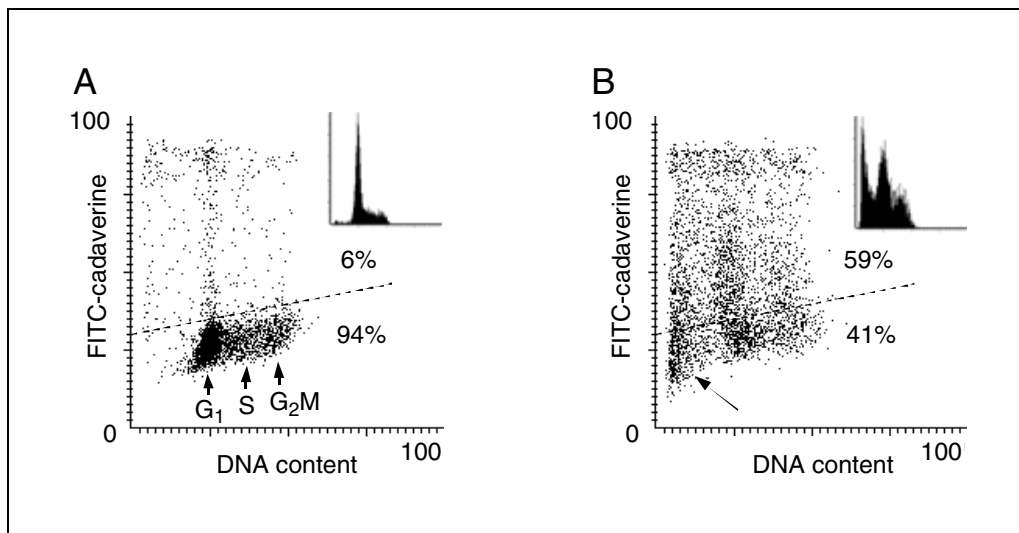

Figure 18.8.8 Detection of apoptotic cells by flow cytometry based on cellular DNA content analysis. (**A**) Normal cell plot. (**B**) To induce apoptosis, HL-60 cells were treated with the DNA topoisomerase II inhibitor fostriecin (Hotz et al., 1994). Cells were fixed in 70% ethanol, suspended in high-molarity phosphate buffer to extract fragmented DNA, and then stained with PI. A subpopulation of apoptotic cells (Ap) with fractional (sub-diploid) DNA content, i.e., with DNA index (DI) <1.0 (sub-G_1 cells), is apparent. Note also the increase in the proportion of S-phase cells in the nonapoptotic population. (**C**) The fragmented DNA extracted from the apoptotic cells by the buffer was subjected to gel elecrophoresis (Gong et al., 1994). Note "laddering" that reflects preferential DNA cleavage at internucleosomal sections, the characteristic feature of apoptosis (Arends et al., 1990).

Figure 18.8.9 Detection of apoptotic cells by flow cytometry based on the presence of DNA strand breaks. To induce apoptosis, HL-60 cells were treated 120 or 150 min with CPT (Li and Dar-zynkiewicz, 2000). DNA strand breaks were labeled with BrdUTP using exogenous terminal deoxynucleotidyl transferase. The cell cycle distribution of both apoptotic and nonapoptotic cell subpopulations can be estimated based on the DNA content of individual cells. Note that in analogy to PARP cleavage (Fig. 18.8.6), preferentially S-phase cells undergo apoptosis following CPT treatment.

Figure 18.8.10 Detection of tissue transglutaminase (TGase 2) activation during apoptosis by the acquired resistance of the cytoplasmic proteins to detergent. Bivariate distributions illustrating red fluorescence of sulforhodamine 101 (protein content) versus blue fluorescence of DAPI (DNA content) of HL-60 cells, untreated (**A**) or exposed to hyperthermia (72 hr at 41.5°C) in the absence (**B**) and presence (**C**) of the cytotoxic RNase onconase (1.67 μM; Grabarek et al., 2002). Following cell lysis by Triton X-100 and staining with DAPI and sulforhodamine 101, the isolated nuclei of nonapoptotic cells from control culture (**A**) show low and uniform intensity of red fluorescence, reflecting low protein content. Subpopulations of apoptotic cells in **B** and **C** have their cytoplasmic protein crosslinked and therefore are resistant to detergent. They stain intensely with sulforho-damine 101. Note differences in DNA content (cell cycle) distribution of the cells with activated (top insets; cells gated above the dashed line) versus nonactivated TGase 2 (bottom insets; cells gated below the dashed line) in **B** and **C**. Percentage of cells with activated and non-activated TGase 2 in the respective cultures is indicated in each panel.

Figure 18.8.11 Detection of TGase 2 activity in HL-60 cells using FITC-conjugated cadaverine (F-CDV) as the enzyme substrate. Cultures of untreated (**A**) and hyperthermia (5 hr at 41.5°C)-treated (**B**) HL-60 cells were incubated 5 hr with 100 μM F-CDV. Cells were then fixed and their DNA was counterstained with PI in the presence of RNase. Note the presence in the untreated culture (**A**) of few cells that incorporated F-CDV (spontaneous apoptosis), and large numbers of F-CDV-labeled cells in the treated culture (**B**). Note also that some apoptotic cells with fractional DNA content (sub-G_1 subpopulation) in the treated culture do not show incorporation of F-CDV (arrow). Percentage of cells with activated and nonactivated TGase 2 in the respective cultures is indicated. The inset shows the cellular DNA content distribution histogram of all cells (Grabarek et al., 2002).

nonapoptotic cell population with little or no overlap between these two.

TUNEL. DNA strand breaks in apoptotic cells are strongly labeled with fluoresceinated anti-BrdU Ab that distinguishes them from the nonapoptotic cells (Fig. 18.8.9). Because of the high intensity of their green fluorescence, an exponential scale (logarithmic PMTs) often must be used for data acquisition and display. Simultaneous measurement of DNA content makes it possible to identify the cell cycle position of cells in apoptotic and nonapoptotic populations. It should be noted, however, that late apoptotic cells may have diminished DNA content because of prior shedding of apoptotic bodies (which may contain nuclear fragments), or due to such massive DNA fragmentation that small DNA fragments cannot be retained in the cell even after fixation with formaldehyde. Such late apoptotic cells may have sub-G_1 DNA content as shown in Figure 18.8.8.

Tissue transglutaminase (TGase 2). Detergent (Triton X-100) treatment of nonapoptotic cells as well as apoptotic cells without activated TGase 2 results in cell lysis and release of isolated nuclei or nuclear fragments that have minimal protein content. Such isolated nuclei or nuclear fragments have very low red fluorescence after staining with sulforhodamine 101

(Fig. 18.8.10, panel A). Apoptotic cells with activated TGase 2, on the other hand, have cross-linked proteins and resist lysis under these conditions. Intensity of their red fluorescence is several times higher than that of the isolated nuclei (subpopulations represented by the scatter plots above the dashed lines in panels B and C). Note the high heterogeneity among individual TGase 2–positive cells in intensity of their red fluorescence.

Because cellular DNA content (PI fluorescence) is measured concurrently with protein content, induction of protein cross-linking can be correlated with the cell cycle position. It is evident that the effects are cell cycle phase specific. Both hyperthermia and onconase lead to preferential protein cross-linking in G_2/M-phase cells.

Activation of TGase 2 in HL-60 cells, detected by cell labeling with F-CDV combined with cellular DNA content analysis, is shown in Fig. 18.8.11. As in the case of protein content (Fig. 18.8.10), populations of cells with activated TGase 2 either undergoing spontaneous apoptosis in control culture (Fig. 18.8.11A) or subjected to hyperthermia (Fig. 18.8.11B) are heterogeneous in terms of their TGase 2–related fluorescence. In the hyperthermia-treated culture, a large number of cells have fractional

DNA content, forming a characteristic sub-G_1 population typical of apoptotic cells. This population is heterogeneous in terms of intensity of F-CDV fluorescence, with many F-CDV-negative sub-G_1 cells (arrow). Thus, the degree of activation of TGase 2 is uneven, and many cells with apoptotic features (sub-G_1 DNA content) have undetectable level of TGase activity.

Time Considerations

Basic Protocol 1 takes ~15 min to prepare cells for incubation with mitochondrial probes followed by an additional 15- to 30-min incubation.

Basic Protocol 2 (activated caspase detection by Zenon techology) requires ~2 hr to complete (cell fixation not included).

In Basic Protocol 3, FLICA (FAM-VAD-FMK) is added directly to cultures at least 30 min before cell centrifugation. Optimal labeling is achieved after a 1- to 2-hr incubation with FLICA. Cell rinses and staining with PI require an additional ~15 min before cells are measured by flow cytometry.

Basic Protocol 4 requires ~4 hr to process cells from fixation through primary and secondary Ab incubations followed by staining with PI before they are analyzed by flow cytometry.

Basic Protocol 5 is a rapid procedure that can be completed in 15 min.

Basic Protocol 6 requires ~40 min to carry out cell rinsing and staining with PI following removal from fixative.

In Alternate Protocol 1, cell rinsing and staining can be completed in 25 to 30 min.

Basic Protocol 7 takes ~3 hr to carry out all steps of cell rinsing and incubations with the respective reagents after removal of cells from fixative.

Basic Protocol 8 is a rapid procedure. Cells can be analyzed by flow cytometry ~5 min after their removal from cultures.

In Alternate Protocol 2, the reagent F-CDV is added directly to cultures at different time intervals. It takes ~40 min following cell fixation to carry out cell rinsing and labeling with PI.

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Figure 18.8.1 Changes in light scattering properties of cells undergoing apoptosis. HL-60 cells were untreated (left panel) or treated 3 hr with TNF-α and cycloheximide (CHX) to induce apoptosis (right panel). Cell population **A** in the treated culture (right panel) represents cells that have light scattering properties similar to those of untreated cells. Early apoptotic cells (**B**) have diminished forward scatter and are very heterogenous with respect to side scatter. Late apoptotic cells (**C**) have both forward and side scatter diminished.

Figure 18.8.2 Detection of the collapse of mitochondrial electrochemical potential (ψ_m) by rhodamine 123 (R123). HL-60 cells were untreated (control; left panel) or treated 3 hr with TNF-α and CHX (right panel) to induce apoptosis. The cells were then incubated with R123 and PI according to Basic Protocol 1. The early apoptotic cells have diminished green fluorescence of R123 but exclude PI (cell population B). The late apoptotic (also necrotic) cells are stained strongly by PI (population C).

Figure 18.8.3 Detection of the collapse of mitochondrial electrochemical potential using the aggregate dye JC-1. HL-60 cells were untreated (control, left panel) or treated 3 hr with camptothecin (CPT, right panel) to indice apoptosis. Cells were then stained with JC-1 and their orange and green fluorescence was measured by cytometry, as described in Basic Protocol 2. Decreased intensity of orange fluorescence (subpopulation B) characterizes the cell with collapsed potential.

Figure 18.8.4 Immunocytochemical detection of caspase-3 activation using antibody reactive with the activated (cleaved) caspase-3. Apoptosis of HL-60 cells was induced by topotecan (TPT), an analog of CPT. Zenon technology (Haugland, 2002) was used to detect caspase-3 as described in Basic Protocol 2. Top and bottom insets in each panel show cellular DNA content frequency histograms of cells with activated and nonactivated caspase-3, respectively. Note that S-phase cells preferentially contain activated caspase-3 after induction of apoptosis by TPT.

Figure 18.8.5 Binding of fluorochrome-labeled inhibitor of caspases (FLICA; FAM-VAD-FMK) and PI during apoptosis. Apoptosis of HL-60 cells was induced by TPT. The cells were stained according to Basic Protocol 3. Green (FAM-VAD-FMK) and red (PI) cellular fluorescence was measured by flow cytometry. Four cell subpopulations (A to D) can be identified, differing in their capability to bind FAM-VAD-FMK and PI. They represent sequential stages of apoptosis, starting with binding of FAM-VAD-FMK (B), loss of plasma membrane integrity to exclude PI (C), and loss of reactivity with FAM-VAD-FMK (D).

Figure 18.8.6 Identification of apoptotic cells by flow cytometry based on the immunocytochemical detection of the 85-kDa PARP cleavage fragment. To induce apoptosis, HL-60 cells were treated 60 min with TNF-α in the presence of CHX (Li and Darzynkiewicz, 2000). PARPp85 was detected immunocytochemically and DNA was counterstained with PI.

Figure 18.8.7 Detection of early and late apoptotic cells after staining with annexin V–FITC and PI. To induce apoptosis, HL-60 cells were treated 2 hr with TNF-α and CHX. Untreated (control; left panel) and TNF-α-treated (right panel) cells were then stained with annexin V-FITC and PI.

Figure 18.8.8 Detection of apoptotic cells by flow cytometry based on cellular DNA content analysis. (**A**) Normal cell plot. (**B**) To induce apoptosis, HL-60 cells were treated with the DNA topoisomerase II inhibitor fostriecin (Hotz et al., 1994). Cells were fixed in 70% ethanol, suspended in high-molarity phosphate buffer to extract fragmented DNA, and then stained with PI. A subpopulation of apoptotic cells (Ap) with fractional (sub-diploid) DNA content, i.e., with DNA index (DI) <1.0 (sub-G_1 cells), is apparent. Note also the increase in the proportion of S-phase cells in the nonapoptotic population. (**C**) The fragmented DNA extracted from the apoptotic cells by the buffer was subjected to gel electrophoresis (Gong et al., 1994). Note laddering that reflects preferential DNA cleavage at internucleosomal sections, the characteristic feature of apoptosis (Arends et al., 1990).

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Figure 18.8.9 Detection of apoptotic cells by flow cytometry based on the presence of DNA strand breaks. To induce apoptosis, HL-60 cells were treated 120 or 150 min with CPT (Li and Dar-zynkiewicz, 2000). DNA strand breaks were labeled with BrdUTP using exogenous terminal deoxynucleotidyl transferase. The cell cycle distribution of both apoptotic and nonapoptotic cell subpopulations can be estimated based on the DNA content of individual cells. Note that in analogy to PARP cleavage (Fig. 18.8.6), preferentially S-phase cells undergo apoptosis following CPT treatment.

Figure 18.8.10 Detection of tissue transglutaminase (TGase 2) activation during apoptosis by the acquired resistance of the cytoplasmic proteins to detergent. Bivariate distributions illustrating red fluorescence of sulforhodamine 101 (protein content) versus blue fluorescence of DAPI (DNA content) of HL-60 cells, untreated (**A**) or exposed to hyperthermia (72 hr at 41.5°C) in the absence (**B**) and presence (**C**) of the cytotoxic RNase onconase (1.67 µM; Grabarek et al., 2002). Following cell lysis by Triton X-100 and staining with DAPI and sulforhodamine 101, the isolated nuclei of nonapoptotic cells from control culture (**A**) show low and uniform intensity of red fluorescence, reflecting low protein content. Subpopulations of apoptotic cells in **B** and **C** have their cytoplasmic protein cross-linked and therefore are resistant to detergent. They stain intensely with sulforho-damine 101. Note differences in DNA content (cell cycle) distribution of the cells with activated (top insets; cells gated above the dashed line) versus nonactivated TGase 2 (bottom insets; cells gated below the dashed line) in **B** and **C**. Percentage of cells with activated and non-activated TGase 2 in the respective cultures is indicated in each panel.

Figure 18.8.11 Detection of TGase 2 activity in HL-60 cells using FITC-conjugated cadaverine (F-CDV) as the enzyme substrate. Cultures of untreated (**A**) and hyperthermia (5 hr at 41.5°C)-treated (**B**) HL-60 cells were incubated 5 hr with 100 µM F-CDV. Cells were then fixed and their DNA was counterstained with PI in the presence of RNase. Note the presence in the untreated culture (**A**) of few cells that incorporated F-CDV (spontaneous apoptosis), and large numbers of F-CDV-labeled cells in the treated culture (**B**). Note also that some apoptotic cells with fractional DNA content (sub-G_1 subpopulation) in the treated culture do not show incorporation of F-CDV (arrow). Percentage of cells with activated and nonactivated TGase 2 in the respective cultures is indicated. The inset shows the cellular DNA content distribution histogram of all cells (Grabarek et al., 2002).

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