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Measurement of Cell Death in Mammalian Cells

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Abstract

Described in this unit are methods used to assess cell death in mammalian tissue. The unit is divided into five sections: (1) a brief overview of cytotoxicity and pathways of cell death, (2) a method to measure cell death using lactate dehydrogenase (LDH) release as a marker of membrane integrity, (3) a flow cytometry method that simultaneously measures two types of cell death, necrosis and apoptosis, (4) use of fluorescent microscopy and nuclear morphology to assess apoptosis and necrosis, (5) the use of multi-well plates and high content analysis imaging systems to assess nuclear morphology, and (6) a discussion of the use of cytotoxicity assays to determine the mechanisms of cell death.

INTRODUCTION

Cytotoxicity: Necrosis versus Apoptosis

Cytotoxicity is caused by the adverse actions of chemicals and physical agents on cells. Methods used to assess cytotoxicity typically compare cellular function and/or integrity in control cells to those exposed to a toxicant, stress or disease. The function being measured depends on the cell type, the injurious agent used, and the type of cell death being studied. The identification and characterization of modes of cell death has implications for both pharmacology and toxicology. Each type of cell death is mediated by multiple signaling pathways, which serve as potential targets for cellular toxicity. Understanding how a compound can activate the various pathways of cell death is essential for determining the acute, subacute, and chronic effects of drugs and chemical toxicants. For example, pharmaceutical therapies designed to activate cytotoxicity pathways in tumor cells must be effective against multiple cancers, while maintaining some selectivity to minimize destruction of normal tissue. Systematic assessment of the activation cell death facilitates the design and testing of safer and more effective pharmaceuticals.

Cell death typically occurs by necrosis, apoptosis, or autophagy (Figure 12.8.1, Table 12.8.1). Necrosis, which is ATP-independent, is characterized by cell and organelle swelling, pyknosis, loss of ion gradients, increased permeability with loss of cell membrane integrity, and the release of intracellular contents (Cummings and Schnellmann, 2001; Tait, 2008). In contrast, apoptosis is ATP-dependent and is characterized by cell shrinkage, maintenance of plasma membrane integrity, chromatin condensation, nuclear fragmentation, and activation of a family of cysteine-containing, aspartate-directed proteases called caspases (Lemasters 1999). *In vivo*, necrotic cell death typically induces inflammation, while apoptotic cell death does not (Proskuryakov and Gabai, 2010). Apoptosis eventually

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leads to necrosis. The modifiers “apoptotic” and “necrotic” specify the predominant pathways responsible for the cell death (Majno and Joris, 1995; Levin et al., 1999).

Several features of apoptosis and necrosis may be shared, depending on the cell model and the chemical or physical agent responsible causing cell death (Figure 12.8.1, Table 12.8.1). For example, the mitochondrial membrane permeability transition (MPT), which is the result of the opening of a high-conductance pore in the mitochondrial inner membrane, can be altered by either apoptosis or necrosis (Lemasters et al., 1999; Lemasters et al. 2009). Thus, apoptosis ensues if cellular ATP levels are maintained during MPT. In contrast, necrosis predominates if ATP levels are significantly reduced. Other features of cell death present in both apoptotic and necrotic cells are DNA degradation and nuclear condensation. Protocols designed to differentiate between necrosis and apoptosis should assess at least two exclusive markers for each of these different modes of cell death (see Fig 12.8.1 and Table 12.8.1).

Historically, caspase activation and activity was thought to be necessary for apoptosis and were, therefore, used as a marker for this type of cell death. However, numerous investigators have observed caspase-independent apoptosis (Cummings and Schnellmann, 2002; Norberg et al., 2010). Caspase-independent apoptosis is similar to classical apoptosis – in terms of cellular and nuclear morphology and maintenance of membrane integrity. Caspase-independent apoptosis is characterized by cell and nuclear apoptotic morphology in the presence of either a broad-spectrum caspase inhibitor, such as ZVAD-fmk, or in cells devoid of caspase activity using antisense or knockout technologies. The absence of caspase activity must be confirmed by quantification of caspases using immunoblot analysis or by measuring caspase activity (see Table 12.8.2). However, it is difficult to prove that all caspase activity is completely inhibited or that inhibition of one caspase isoform does not result in the activation of another. Furthermore, it is difficult to account for unknown caspases. Despite these problems, several studies have provided evidence of caspase-independent apoptosis induced by anticancer agents (Cande et al., 2002; Cummings and Schnellmann 2002), serum deprivation, and oxidants (Cande et al., 2002). Some studies also suggest that caspase-independent apoptosis is actually autophagy.

Autophagy is a mechanism in which subcellular membranes enclose cytoplasm and organelles and transport them to lysosomes or vacuoles for degradation. The role of autophagy in causing cell death is still being elucidated; however it is clear that when apoptosis is inhibited prolonged autophagy can induce necrosis (Walsh and Edinger, 2010). Autophagic cell death is characterized by vacuolization of the cytoplasm without condensation of chromatin. Cells that die by autophagy may not attract phagocytes, unlike apoptotic cells which are cleared through lysosomal degredation (Orrenius et al, 2011).

The existence of multiple pathways for cell death necessitates the development of different protocols for measuring and identifying the type of cell death (Table 12.8.2). Cell death has been evaluated using measurement of mitochondrial dehydrogenase activities (Fanning et al., 1990), cellular respiration (Schnellmann, 1994), and mitochondrial membrane potential using the flourometric dyes 5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazocarbocyanine iodide (JC-1) and tetramethylrhodamine methylester, both available from Invitrogen (Reers et al., 1995; Lemasters et al., 1999). Cell toxicity has also been assessed by studying protein synthesis (Sorger and Germinario, 1983; Nony and Schnellmann, 2001), unscheduled DNA synthesis (Brambilla et al., 1979), DNA damage (Sorger and Germinario, 1983; Shen et al., 1991; Yan et al., 2000), and cytosolic free Ca^{2+} levels (Ogden et al., 1995). One of the best markers for cell death is membrane integrity. Plasma membrane permeability is assessed using propidium iodide or lactate dehydrogenase (see below), and lysosomal membrane integrity is determined using neutral red (Monks et al., 1988; Mertens et al., 1995). The activities of proteases such as calpains and caspases have also been utilized to assess cell

toxicity using zymography, immunoblot analysis, or analysis of cleavage of fluorometric-labeled peptide substrates (Cummings and Schnellmann, 2002; Liu et al., 2002).

Described in this unit are three protocols used, either separately or in tandem to measure cell viability, necrosis, and apoptosis. The first describes methods for assessing the release of the cytosolic enzyme lactate dehydrogenase (LDH; see Basic Protocol 1). Appearance of this protein in the extracellular milieu is a conventional marker for cell viability, loss of membrane integrity, and the presence of necrosis. The second and third protocols utilize flow cytometry (see Basic Protocol 2) and fluorescence microscopy (see Basic Protocol 3) for the simultaneous determination of apoptotic and necrotic cell death. Both assays employ propidium iodide (PI) as a marker for membrane integrity and necrosis. For apoptosis, the flow cytometry protocol involves the use of fluoresceinisothiocyanate (FITC)-conjugated annexin V as an indicator. Annexin V binds to phosphatidylserine, a phospholipid that is externalized relatively early during apoptosis. In contrast, the microscopy method uses nuclear morphology to identify apoptosis, which is visualized using the nucleic acid stain 4′, 6′-diamidino-2-phenylindole (DAPI). Any of these protocols can be employed with both freshly isolated cell suspensions and cells isolated from cultures. Each represents a reproducible and relatively straightforward method for the initial determination of the mechanisms of cell death.

NOTE: All protocols using cells isolated from live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to government regulations concerning the care and use of laboratory animals.

BASIC PROTOCOL 1

MEASUREMENT OF PLASMA MEMBRANE INTEGRITY AND VIABILITY USING LDH RELEASE

During necrosis, cell viability is lost through the breakdown of the cellular membrane. This results in the release of intracellular constituents, including enzymes such as lactate dehydrogenase (LDH). Traditionally, LDH release was measured using an NADH-linked UV-visible spectrophotometric method (Schnellmann and Mandel, 1986). However, this analytical approach has several limitations, including the fact that the samples are usually run serially, resulting in a time-intensive protocol. The method described below is an NADH-linked LDH assay using a fluorescence plate reader (Moran and Schnellmann, 1996). This results using this method correlate well ($r^2 = 0.95$) with the UV-visible spectrophotometric technique and allows for the parallel processing of multiple samples of smaller volumes, decreasing analysis time and costs.

The LDH assay can be used with cells isolated from fresh tissue or from adherent cultures. The results are normally expressed as the percentage LDH activity released from the cells into the media compared to the total LDH activity (LDH activity in cells plus that present in the media or cell-free buffer). If adherent cultures are used, LDH activity should be quantified in a cell-free sample of the media and compared to the total activity in media isolated from treated cells, detached cells, and in attached cells.

Materials

Cellular material (~1 mg protein/ml)

Toxicants

2:1 (v/v) *n*-butyl phthalate/dioctyl phthalate (store at room temperature for up to 1 month)

Cell medium or buffer

Liquid nitrogen

16 mM pyruvic acid in LDH-PO_4 buffer (prepare fresh daily)

LDH-PO_4 buffer: 50 mM K_2HPO_4 and 9 mM KH_2PO_4, pH 7.4 (prepare fresh weekly)

0.3 mM β-NADH in LDH-PO_4 buffer (prepare fresh daily)

1.5-ml microcentrifuge tubes

48-well transparent plate

Fluorometric plate reader with a 360-nm excitation filter (40 nm bandwidth) and a 460-nm emission filter (BMG Laboratories, Molecular Devises, and Bio-Tek)

1. Treat cells with desired concentrations of toxicants or solvent controls.
2. Prepare three 1.5-ml microcentrifuge tubes for sample collection for each treatment group. Place 0.4 ml of 2:1 *n*-butyl phthalate/dioctyl phthalate solution into the first microcentrifuge tube and 0.5 ml of medium, or buffer, into the second microcentrifuge tube.

   The ratio of n-butyl phthalate to dioctyl phthalate may be altered depending on the density of cell sample. The proper ratio is that which results in no cellular material being present in the supernatant after centrifugation. If cellular material is present, then the ratio should be increased. The presence of cellular material in the supernatant can be determined visually using microscopy. The purpose of the phthalate solution is to separate cells from the extracellular buffer or media.

   The cell-free medium will be transferred to the third microcentrifuge tube.
3. After incubation for a predetermined time, remove a 1-ml aliquot of cells, layer it on the top of the 2:1 *n*-butyl phthalate/dioctyl phthalate solution in the first microcentrifuge tube, and centrifuge for 2 min at 10,000 × *g* at room temperature. Transfer the supernatant, which contains the LDH activity released into the medium (i.e., the cell-free medium), into the third microcentrifuge tube.
4. Remove a 0.5-ml aliquot from the cell suspension and place it into the second microcentrifuge tube.

   This mixture represents the total LDH activity in the cells and medium.
5. If working with adherent cells grown in culture, scrape the cells in the plate with medium and transfer a 0.5-ml aliquot to the second microcentrifuge tube.

   This tube will be used to determine the "total" LDH activity.
6. To obtain cell-free medium when working with adherent cells grown in culture, transfer a portion of the medium to the first microcentrifuge tube, centrifuge 2 min at 10,000 × *g* at room temperature, and transfer the supernatant to the third microcentrifuge tube.

   At this point all samples may either be analyzed immediately or stored at −80°C for at least 2 weeks.
7. At the time of analysis, subject all samples to three freeze/thaw cycles using liquid nitrogen to lyse the cells.

*Previous studies reveal that the freeze-thaw process does not decrease LDH activity (*Moran and Schnellmann, 1996*). An alternate method for lysing the cells is to treat media and "total" samples with 1% (v/v) Triton X-100.*

8. Add 100 μl of the cell-free medium sample (supernatants from step 3 or 6) and 25 μl of the corresponding "total" sample (samples from step 4 or 5) to adjacent wells in a 48-well transparent plate. Leave 4 wells empty for blanks.
9. Add 50 μl of 16 mM pyruvic acid (the LDH substrate) to each sample and to the four blank wells.
10. Swirl the plate gently by hand to mix contents.
11. Adjust the volume in the wells to 450 μl using LDH-PO_4 buffer (25°C) and gently swirl the plate by hand to mix contents.
12. Immediately prior to scanning on the fluorometric plate reader, add 50 μl of the β-NADH solution to each sample and gently swirl the plate by hand to mix contents.
13. Scan the entire plate using 360 nm excitation (40-nm band width) and 460 nm emission for five cycles of 24 sec each. Calculate the change in fluorescence (or ΔF) by comparing the values obtained in the first to the fifth cycle.

    The ΔF value should decrease as NADH is consumed by LDH.
14. Subtract the average ΔF in the blank wells from the average ΔF in the cell-free medium and total samples. Determine percent LDH release (Equation 12.8.1) by dividing the corrected ΔF value of the cell-free medium sample by that of the total sample and adjust for the dilution factor (8 in the above example).

$$\frac{\Delta \mathrm{F}_{\text{medium sample}} - \Delta \mathrm{F}_{\text{average blank}}}{\Delta F_{\text{suspension/adherent/total sample}} - \Delta \mathrm{F}_{\text{average blank}}} \times \text{dilution factor} \times 100 = \% \text{ LDH release}$$

Calculation of the cellular LDH activity retained, as opposed to percent LDH release, is an alternative was to assess cell viability.

Cell viability can also be measured using a number of different fluorescent or spectrophotometric vital dyes such as trypan blue, neutral red, MTT exclusion, CellTiter-blue, or DNA staining using propidium iodide. These markers are often used in tandem in a single model to assess viability. As an example, the time-dependent toxicity of GSH-conjugated hydroquinones to a renal proximal tubular cell line as assessed using LDH activity and MTT and neutral red staining is shown in Figure 12.8.2 *(*Mertens et al., 1995*). Decreases in LDH activity correlate with the decreases in MTT and neutral red exclusion after both 2 and 3 hr of treatment.* Figure 12.8.2 *illustrates the use of multiple vital dyes and LDH to determine cell viability in a single model.*

BASIC PROTOCOL 2

MEASUREMENT OF NECROSIS AND APOPTOSIS USING FLOW CYTOMETRY

Flow cytometry is a valuable tool for the simultaneous assessment of necrosis and apoptosis in a single population of cells. Necrosis is detected by measuring the permeability of the plasma membrane to a normally impermeable fluorescent dye, such as the DNA-binding dye propidium iodide (PI). Apoptosis is detected by measuring the externalization of phosphatidylserine on the plasma membrane using fluorescent-tagged annexin V. Additionally, flow cytometry can be employed to determine alterations in cell size (Bortner

and Cidlowski, 2001; Warnes et al. 2011). Flow cytometers equipped with cell sorters have the added advantage of being able to recover cellular material for additional studies, such as immunoblot analysis.

While the cost of flow cytometers are significant, they offer numerous advantages over other analytical approaches, including the fact that relatively small numbers of cells are needed for analysis, decreasing both reagent cost and the time required to conduct the experiment. Furthermore, flow cytometers are equipped with software that allows determination of results, such as the percentage of cells present in an apoptotic region (see below). Flow cytometers also make it possible to quantify both necrosis and apoptosis in the same population of cells. The concentrations of fluorescent markers used to determine necrosis and apoptosis must be optimized for each cell type and marker. Care should be exercised when selecting multiple fluorescent markers to ensure that excitation and emission wavelengths do not overlap.

Materials

Cells

Toxicant (see Critical Parameters)

Phosphate buffered saline (PBS; Fisher or Sigma)

Annexin binding buffer (see recipe)

Annexin V–conjugated FITC (annexin V–FITC), typically at 25 μg/ml (i.e., Biovision or R&D Systems)

1 mg/ml propidium iodide (PI) in PBS

Orbital shaker

50-μm diameter nylon filter sieves5-ml snap-cap polypropylene tubes

Flow cytometer with FL-1 (FITC) and Fl-2 (PI) channels

NOTE: The amount of annexin V-FITC and PI needed for flow cytometry varies among cell types. In addition, the amount of FITC conjugated to annexin V can vary from source to source, and lot to lot. Thus, the amount of annexin V-FITC and PI used to obtain clear and discrete signals may change two- to five-fold for each cell type.

Prepare and treat cells

1. Treat cells with the desired toxicant (see Critical Parameters for suggested reference toxicants). Typically, use 1 million cells per treatment group (i.e., for each time or concentration point). Set up controls as follows:
    i. For the PI positive control, treat a separate aliquot or dish of cells with a toxicant at a concentration known to disrupt plasma membrane integrity.
    ii. For the annexin V positive control, treat a separate aliquot or dish of cells with a toxicant known to induce apoptosis.
    iii. For the no-stain negative control, do not expose the cells in this aliquot or dish to any kind of treatment.

    Be sure to include a separate set of controls for each experiment.
2. After incubation for a predetermined time, remove the toxicant and wash cells with PBS as follows:

a. If adherent cells are used, wash the monolayers twice with 1 ml of PBS.

b. If cellular suspensions are used or if cells have been released from the monolayers, centrifuge 5 min at 4000 × *g*, remove supernatant, and resuspend pellet in 1 ml PBS.

3. Add 600 μl of annexin binding buffer.

4. Based on the manufacturer's recommendation, add 2-5 μl of 25 μg/ml annexin V–FITC to each sample and to the annexin V positive control. Do not add annexin V–FITC to the no-stain negative control or PI positive control.

   Initially, use the amount of annexin V–FITC recommended by the manufacturer. The amount added may be altered depending on the positive controls (see below).

5. Add 25 μl of 1 mg/ml PI to each sample and to the PI positive control. Do not add PI to the no-stain negative control or annexin V positive control.

   The concentration of PI may be increased or decreased depending on the result of the PI positive control (see below).

6. Incubate the cells for 10 min at room temperature on an orbital shaker at 50 rpm in the absence of light.

7. Wash the cells to remove unbound annexin V–FITC and PI as follows:

   a. If adherent cells are used, aspirate buffer and add 1 ml of binding buffer without annexin V–FITC or PI. Incubate the cells for 10 min on an orbital shaker at 50 rpm. Shield cells from light at all times (i.e., cover with foil). Decant solution and repeat the wash and 10-min incubation in binding buffer two times. Add 500 μl annexin-binding buffer and gently remove the cells by scraping with a rubber policeman. Disperse the cells by pipeting, and filter the sample through a 50-μm nylon filter sieve into a labeled 5-ml snap-cap polypropylene tube.

      This step removes clumps and prevents clogging of the cytometer.

   b. If cell suspensions are used, centrifuge cells 5 min at 4000 × *g*, remove supernatant, and suspend the pellet in 1 ml of binding buffer. Repeat this process two times. After the final wash, add 0.5 to 1 ml of binding buffer to obtain 1×10^6 cells/ml.

Measure annexin V and PI staining

8 Analyze the cells using flow cytometry with the proper channels for FITC (typically FL-1) and PI (typically FL-2).

9 Read the no-stain negative control sample first. Adjust the forward and side scatter settings and the Fl-1 and Fl-2 compensations to produce plots similar to those shown in Figure 12.8.3A and B. Do not use gates to exclude cells (see Critical Parameters).

Because the no-stain negative control cells contain no fluorescent markers they should be mostly in the lower left hand corner (R1 and LL in Figure 12.8.3A and B, *respectively).*

There should only be one major population of control cells. These should contain at least 90% of the total events counted by the flow cytometer.

If adherent cells were detached using a rubber policeman, then the no-stain negative control will appear as shown in R1 in Figure 12.8.3A. *If cell*

suspensions were used, then the no-stain negative control will appear as shown in LL in Figure 12.8.3B.

10 Read the annexin V positive control.

An increase in fluorescence intensity on the Fl-1 channel will be observed. The pattern should be similar to that displayed in R4 and LR on Figure 12.8.3C and D*, respectively*.

A separate population may not be seen if adherent cells are used. Rather, a definite shift will be observed (see R4 Figure 12.8.3C*).*

If no shift in fluorescence intensity is seen along the Fl-1 channel, then the amount or concentration of annexin V–FITC added may be too low. Alternatively, the agent used to induce apoptosis was inactive.

11 Read the PI positive control.

An increase in fluorescence intensity on the Fl-2 channel should be observed. The pattern should be similar to that displayed in R2 and UL in Figures 12.8.3E and F*, respectively*.

The PI positive controls are typically similar for both cellular suspensions and adherent cells released using a rubber policeman.

If no shift in fluorescence intensity is seen along the Fl-2 channel, the amount or concentration of PI added may have been too low. Alternatively, the agent used to induce necrosis may be inactive

12 Adjust the Fl-1 (annexin V–FITC) and Fl-2 (PI) channel compensations. Determine the settings that result in the highest amount of signal for each marker with the least amount of fluorescent overlap (bleed-over). Read the controls and samples under these conditions.

If the compensation on the FL-2 (PI) channel is maximized, there is the possibility that the FL-1 (FITC) signal will be reduced resulting in a masking or loss of the annexin V–FITC signal. Similarly, if the compensation of the Fl-1 (FITC) channel is maximized, then the Fl-2 (PI) signal may be reduced.

Once the forward scatter, side scatter, and the Fl-1 and Fl-2 compensations are set for the no-stain negative control and the annexin V and PI positive controls, they should not be altered during the rest of the analysis. If a change is made in any of these parameters all controls must be re-verified and samples re-tested.

Analyze data

13 For all analyses, use the no-stain negative control to determine the absolute boundaries for both annexin V–FITC and PI positive cells (Fig 12.8.3A and B).

*Samples can be examined by quadrant, histogram, or region analysis. Quadrants work well for cell suspensions (*Fig. 12.8.3B*) while region analysis is better than quadrants for adherent cells removed from cultures by mechanical methods (*Fig. 12.8.3A*)*.

14 After determining the proper type of analysis (gates, histograms, or regions), perform statistics on every sample, including the no-stain negative control and annexin V and PI positive controls. Be sure to compare data generated with control cells to that of the no-stain negative control. If using region analysis for adherent cultures, subtract the no-stain negative control values from the actual samples. Failure to do so could result in artificially high values for cell death

due to the basal events present in the R2, R3, and R4 regions of the no-stain negative control (Fig. 12.8.3A).

These statistical analyses can be performed using the software supplied with the flow cytometer.

Control cells will remain in the same region or quadrant as the no-stain negative control (denoted R1 and LL in Fig. 12.8.3A and B, *respectively). Apoptotic cells are those with increased fluorescent intensity on the annexin-FITC axis (Fl-1 scale), denoted in R4 and LR on* Fig. 12.8.3C and D, *respectively. Necrotic cells are identified by their increased fluorescent intensity on the PI axis (Fl-2 scale), denoted in R2 and UL in* Figure 12.8.3E and F, *respectively*.

Cells may also be present in the R3 region or UR quadrant. This type of staining may indicate late apoptosis when membrane integrity is finally lost, or necrosis when annexin-V–FITC freely enters the cells and binds to intracellular phosphatidylserine (see Anticipated Results)

Ideally, the control levels of annexin V–FITC binding should be ≤10%, while PI staining should be <5%. These values can be cell-type dependent (see Critical Parameters and Troubleshooting).

BASIC PROTOCOL 3

DETERMINATION OF NUCLEAR MORPHOLOGY AND MEMBRANE INTEGRITY

Alterations in nuclear morphology occur in both necrotic and apoptotic cells, and can be determined by assessment of nuclear staining using fluorescent stains and fluorescent microscopy. Healthy cells display diffuse staining of the nucleus (Fig. 12.8.4A). The types of nuclear morphology observed in injured or dead cells depend on both the cell type and the toxicant used, but generally fall into three classes: (1) chromatin condensation (Fig. 12.8.4B): chromatin margination without nuclear condensation; (2) nuclear fragmentation (Fig. 12.8.4C): chromatin margination with nuclear condensation; and (3) nuclear condensation (Fig 12.8.4D): a decrease in nuclei size without chromatin margination.

Margination refers to the clustering of chromatin at the edge, or periphery, of the nuclear membrane resulting in a loss of staining in other parts of the nucleus. Nuclear condensation refers to a decrease in the size of the nucleus compared to control nuclei. Nuclear condensation can be present in both apoptotic and necrotic cells. Nuclear condensation that occurs during necrosis, termed pyknosis, coincides with the loss of membrane integrity. Nuclear fragmentation is commonly associated with apoptosis, but can also occur with necrosis. Chromatin condensation is also typical of apoptotic cells and often precedes nuclear fragmentation. Because nuclear fragmentation and condensation can occur during both necrosis and apoptosis, other markers of cell death (i.e., flow cytometry analysis of PI and annexin V–FITC staining) must be employed for making firm conclusions about the mechanisms of cell death.

Assessment of nuclear morphology by fluorescence microscopy can be performed using cell-permeable nucleic acid stains, such as 4′,6′-diamidino-2-phenylindole (DAPI) and Hoechst 33342. While PI can also be used to assess nuclear morphology, DAPI and Hoechst are preferred because of their cell permeability. The protocol detailed below employs DAPI to assess nuclear morphology in both cell suspensions and cultures using fluorescence microscopy. Using microscopy to assess nuclear morphology allows direct comparison of nuclear to cell morphology. However, practice is required to accurately identify the different

types of nuclear morphology. Alternate Protocol 1 describes the tandem use of PI and DAPI to distinguish apoptosis from necrosis.

High-throughput screening requires the adaptation of smaller scale methods for measuring cell death to a higher density format. Multi-well plates make it possible to measure the cytotoxic effects of multiple compounds and concentrations at the same time. Additionally, high-throughput scaffolds allow the use smaller volumes of reagents and the analysis of multiple cellular endpoints in a single plate thereby conserving both resources and time. Finally, high content imaging systems can be utilized to automate and quantify changes in nuclear morphology. This improves data quality and reduces variability. Described in Alternate Protocol 2 is the use of DAPI in 96-well plates to examine cell viability and nuclear morphology using a high content imaging system.

NOTE: If possible, use a double-blind protocol during both data acquisition and data analysis to minimize bias in the interpretation of the results.

Materials

Cultured cells on coverslips, in 35-mm cell culture dishes, or in 96-well tissue culture plates; or a suspension of cells at a concentration of ~1 mg protein/ml or 1-2 × 10^6 cells/ml

Phosphate buffered saline (PBS)

10% neutral buffered formalin solution

100 μM DAPI in PBS

Mounting media

Crystal mount35-mm culture dishes

Orbital shaker

Coverslips

Glass slides

Fluorometric microscope with 350-nm excitation and 486-nm emission filters

For adherent cells in 35-mm cell culture dishes

1a Wash the monolayers twice with 1 ml of PBS.

2a If cells are grown on coverslips, place them into 35-mm cell culture dishes.

3a If cells are to be fixed, add 10% neutral buffered formalin solution and incubate the samples for 10 min at room temperature on an orbital shaker at 50 rpm. Aspirate the formalin solution and, using an orbital shaker, wash the cells three times with PBS at room temperature for 10 min each.

The washing steps remove excess fixative.

The DAPI staining can be performed in either fixed or unfixed cells. If cells are counterstained with markers of cell viability, such as PI, then DAPI staining should be initially performed in unfixed cells (see below). Fixation of cells can be accomplished after the final wash step with PBS (step 7a). In general, it is advantageous to fix cells as they can be stored and analyzed later.

4a	Aspirate the final PBS wash and add 500 μl PBS and 100 μl of 100 μM DAPI to each dish.
5a	Incubate the samples for 10 min at room temperature under light-shielded conditions on an orbital shaker set at 50 rpm.
6a	Aspirate the DAPI solution and wash the tissue three times with 750 μl PBS for 10 min at room temperature under light-shielded conditions on an orbital shaker set at 50 rpm.
7a	After the final wash, remove the PBS, add two drops of mounting media, and apply coverslips.
8a	If the cells are grown on coverslips, remove the PBS, add 1 drop of mounting media to the cells, invert, and place them onto a glass slide.
	An adhesive, such as crystal mount, may be needed to hold the cover slip in place. Only use an adhesive if the cells are fixed.
	Samples may be stored under light-shielded conditions at 4°C for months. Samples can be stored longer if an anti-fade reagent is used.
9a	Analyze by fluorescence microscopy using 350-nm excitation and 486-nm emission wavelengths. Proceed to step 10.
	For cell suspensions
1b	After treatment, isolate 1 million cells from the suspension by centrifuging in a micorcentrifuge tube for 5 min at 4000 × *g*, at 4°C.
	If cells are to be fixed, suspend them in 10% neutral buffered formalin fixative and incubate them for 10 min at room temperature on an orbital shaker set at 50 rpm. Remove the fixative by centrifugation and suspend the cells in 500 μl PBS.
2b	Suspend cells in 500 μl of PBS and add 100 μl of 100 μM DAPI.
	DAPI will stain live cells. This analysis must be performed on the same day because a certain amount of cell death occurs normally over time in unfixed cells.
3b	Incubate the samples for 10 min at room temperature under light-shielded conditions on an orbital shaker set at 50 rpm.
	Alternatively, place the cells on a shaker rocker.
4b	Pellet the cells by centrifugation for 5 min at 4000 × *g* at 4°C.
5b	Gently suspend the cell pellet in 0.5 - 1.0 ml PBS to a final concentration of 1.0×10^6 cells/ml.
6b	Add an equal amount of cells to a microscope slide and apply a cover slip. If the cells are fixed, add 1 drop of mounting media prior to application of the cover slip, and seal the cover slip to the slide using an adhesive such as crystal mount.
7b	Analyze by fluorescence microscopy using 350-nm/486-nm excitation/emission filters. Proceed to step 10.
	DAPI staining will appear blue under these conditions.

Count nuclei

10 Count the number of nuclei in the field displaying nuclear fragmentation, chromatin condensation, or nuclear condensation. Refer to the definitions provided above and the examples shown on Figure 12.8.4.

- **i.** For nuclear condensation, compare the size of the nuclei in the experimental samples to control cell nuclei. Nuclei undergoing condensation should be at least 50% smaller than control nuclei (Fig. 12.8.4D).
- **ii.** Nuclear fragmentation is the most obvious of all of these nuclear morphologies since the cells have undergone both nuclear condensation and chromatin margination (Fig. 12.8.4C).
- **iii.** Chromatin condensation is characterized by an increase in DNA staining at the periphery, or edge, or the nuclei without a decrease in nuclei size compared to the controls (Fig. 12.8.4B).

If there is uncertainty about the type of nuclear morphology, contact an experienced pathologist.

11 Count the total number of nuclei, including the apoptotic ones. Count at least 200 nuclei per sample.

12a Divide the number of altered nuclei by the total number of nuclei and multiply by 100.

This value, which represents both the percent apoptosis if morphological changes that occurred in the absence of a loss of membrane integrity

12b Alternatively, determine the percent of each type of nuclear morphology separately. This is done by dividing the number of nuclei displaying the morphology in question by the total number of nuclei (including those displaying the other two types of morphology), and multiplying by 100. Thus, the percent chromatin condensation, nuclear condensation, and nuclear fragmentation can be directly compared.

ALTERNATE PROTOCOL 1

ASSESSMENT OF NUCLEAR MORPHOLOGY AND MEMBRANE INTEGRITY USING DAPI AND PI

An alternative way to assess nuclear morphology during cell injury and death is to stain cells with PI (25 to 50 μg/ml). Because live cells do not accumulate PI, this method can only be used with fixed cells. Also, since PI stain RNA, cells should be treated with RNase (100 Kunitz U/ml) prior to staining. Morphological alterations in PI-stained nuclei are visualized using fluorescence microscopy employing 350 nm excitation and 486 nm emission wavelengths. The PI staining appears red under these conditions. Nuclear morphologies induced during cell death are not dependent on the type of nucleic acid stain used. One advantage of PI is that its excitation and emission wavelengths are different than those of DAPI or Hoechst 33342, providing additional options for co-localization.

Assessment of nuclear morphology and membrane integrity using DAPI and PI is a valuable method for distinguishing between cell death mediated by apoptosis or necrosis. This approach can also be used for determining the stage of apoptotic cell death (early or late apoptosis). Basic Protocol 3 is performed as described except both PI (25 - 50 μg/ml) and DAPI are added to living cells. Staining must be performed on living cells, although the cells may be fixed after staining. Data analysis is exactly the same except that nuclei are also

analyzed in the same field at 568 nm excitation/590 nm emission. While this doubles the amount of time required for acquisition and analysis of data, this drawback is small compared to the substantial increase in information obtained on the mechanism and stage of cell death.

The reason for using PI and DAPI in tandem to measure apoptosis and necrosis is that DAPI enters all cells while PI only enters necrotic cells, or those undergoing late apoptosis (or secondary necrosis/necrosis) when membrane integrity is lost. Regardless of the type of cell death, loss of membrane integrity is correlated to nuclear morphology in the same cell. Cells displaying normal nuclear morphology in the absence of PI staining are healthy cells. Cells displaying nuclear fragmentation, chromatin condensation, or nuclear condensation in the absence of PI staining are apoptotic cells. Cells positive for nuclear fragmentation or chromatin condensation in the presence of PI are typically undergoing late apoptosis. In contrast, cells positive for PI and displaying normal nuclear morphology or nuclear condensation are undergoing necrosis.

ALTERNATE PROTOCOL 2

ASSESSMENT OF NUCLEAR MORPHOLOGY USING MULTI-WELL PLATES

For adherent cells in 96-well plates

1c Wash cell monolayers twice with 200 μl of PBS.

2c If cells are to be fixed, add 4% neutral buffered formalin solution and incubate the samples for 10 min at room temperature on an orbital shaker set at 50 rpm. Aspirate the formalin solution and the wash cells three times for 10 min each with PBS at room temperature.

3c Aspirate the final PBS wash. Add 200 μl PBS and 50 μl of 100 μM DAPI to each well.

4c Incubate 10 min at room temperature under light-shielded conditions on an orbital shaker set at 50 rpm.

5c Aspirate the DAPI solution and wash the samples three times with 200 μl PBS for 10 min at room temperature under light-shielded conditions on an orbital shaker set at 50 rpm.

6c After the final wash, add 200 μl PBS to each well

If the samples are fixed they can be stored in sealed 96-well plates under light-shielded conditions at 4°C for months. Samples can be stored longer if an anti-fade reagent is used.

7c Analyze the samples by fluorescence microscopy using 350-nm excitation and 486-nm emission wavelengths.

A High Content Analysis (HCA) imaging system can be utilized to measure and analyze morphological parameters automatically. The present method was developed employing an IN Cell Analyzer (GE Healthcare). Similar devices have been developed by Molecular Devices and BD Biosciences.

Count nuclei

8c Collect four images per well using the HCA imaging system.

9c Utilizing an HCA software package (e.g. Molecular Devices, BD Biosciences, GE Healthcare), set the parameters for normal nuclear morphology and size.

While for many cell types the nuclear area ranges between 100- 200 μm^2, it is important to determine the precise parameters for the particular cell type of interest.

10c Set the parameters for both micronuclei and binuclei compared to the control nuclei for a given cell type. Micronuclei should be at least 50% smaller than the control nuclei (Fig. 12.8.4D). *To optimize analysis consider using a positive control that causes the formation of micronuclei and/or binuclei (e.g. mitomycin C)*.

11c Count the number of normal, micro- and bi- nucleated cells in the four fields for each well. Refer to the definitions given above and the examples provided on Figure 12.8.4. *If there is uncertainty about the type of nuclear morphology, contact an experienced pathologist*.

12c To determine cell viability, subtract the number of altered nuclei from the number of total nuclei.

13c To determine the percent apoptosis, divide the number of altered nuclei by the total number of nuclei and multiply by 100.

This value represents the percent apoptosis if morphological changes occurred in the absence of a loss of membrane integrity. Membrane integrity can be determined using PI staining or LDH release.

This value also represents overall cell viability.

15c. Alternatively, determine the percent of each type of nuclear morphology separately. This is accomplished by dividing the number of nuclei displaying the morphology in question by the total number of nuclei (including those displaying the other two types of morphology), and multiplying by 100.

BASIC PROTOCOL 4

MEASUREMENT OF TIME-DEPENDENT TOXICITY USING CELL DEATH MARKERS

The aim of this assay is to conduct time-dependent experiments to relate the onset and progression of cell death to a given concentration of toxicant. For example, flow cytometric analysis of mouse leukemic L1210 cells exposed to 2 μM staurosporine for 24 hr results in 24% of the attached cells staining positive for annexin V only (a marker of phosphatidylserine externalization) compared to 0.2% of the cells at 12 hr (Fig. 12.8.5A and B, observe the percent staining in the lower right quadrant). Increasing the time of staurosporine exposure to 36 hr results in 30% of the cells staining annexin V positive (Fig. 12.8.5B and C). The appearance of cells staining positive for PI only reflects the number of L1210 cells undergoing necrosis while cells staining positive for both PI and annexin V reflects cells progressing to late apoptosis (Fig. 12.8.5C and D; Fig. 12.8.1).

While the data on Figure 12.8.5 illustrate time-dependent toxicity, it does not reveal how these time points were chosen. This is of concern with toxicants, because very early time points may result in false negatives (Fig. 12.8.5A) whereas late time points could lead to a false conclusion that staurosporine is inducing only necrosis in L1210 cells (Fig. 12.8.5D). For some toxicants and cell types, starting points for time-dependent studies can be gleaned from the literature. Alternatively, an initial approach is to monitor cell morphology for cell detachment, swelling, shrinkage, sloughing, rounding, flattening, or nuclear condensation using light microscopy. Always compare morphological changes in treated cells to those in control cells. Making observations before the first observable detection of cell death makes it possible to study events early in the genesis of cell death, while measurements made after

the first observable detection of cell death allows for one to study the progression of cell death. Once time points are chosen, proceed as detailed in the above protocols to measure the indicated marker (i.e., LDH release in Basic Protocol 1, flow cytometry in Basic Protocol 2, and nuclear morphology in Basic Protocol 3).

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**.*

Annexin binding buffer

10 mM HEPES

140 mM NaCl

5 mM KCl

1 mM $MgCl_2$

1.8 mM $CaCl_2$, pH 7.4 at 4°C

Prepare fresh

Ca^{2+} is essential for annexin V to bind to externalized phosphatidylserine.

COMMENTARY

Background Information

Typically, the goals of cell death assays are to determine the relative potency or safety of chemicals or other agents, or to determine the mechanism or pathway of cell death relayed to a disease state. The protocols described in this unit can be used to determine whether cell death is through apoptotic, necrotic, or caspase-independent mechanisms. Several markers used for this purpose are listed in Table 12.8.3 and are discussed below. It is important to note that the markers and methods listed on this table are useful for the assessment, but not the definitive determination, of the mechanism of cell death. For example, increases in chromatin condensation and annexin V binding in the absence of PI staining do not prove that caspases have been activated. Rather, chromatin condensation and annexin V binding only indicate that cells are dying by apoptosis. Caspase activity must be verified using different assays.

A second point concerning Table 12.8.3 is that it is assumed that multiple markers of cell death and time points are being tested. Time-dependent analysis is critical if the aim of the study is to assess the mechanisms of cell death. For example, a cell positive for caspase activity, chromatin condensation, and cell shrinkage, but negative for PI staining at 12 hr, is dying by apoptosis. However, the same cell analyzed at a latter stage of death (i.e., at 24 hr when membrane integrity is lost) could also be positive for PI and release LDH. If LDH and PI are the only markers used to assess cell death, and if 24 hr is the only time point analyzed, the incorrect conclusion could be made that the cell had died by necrosis. This example highlights both the pitfalls of selecting the wrong markers for assessing the mechanism of cell death and the importance of performing time-dependent studies.

Limitations

An important limitation of the assays listed on Table 12.8.3 is that apoptosis is the culmination of multiple events, with every potential alteration not being required for apoptosis to occur. Multiple assays are needed to include/exclude apoptosis as the method of cell death. An excellent approach for studying mechanism of cell death is to correlate

alterations in cytotoxicity markers to cell morphology over time. Cell morphology can be analyzed using a variety of methods, including phase-contrast light microscopy, confocal laser scanning microscopy, and flow cytometry. In general, alterations in cell morphology should correlate to alterations in cytotoxicity markers. For example, since cells swell during necrosis, an agent causing an increase in a necrotic cell marker, such as PI staining or LDH release, may also increase cell size. In contrast, cells shrink during apoptosis. As a result, increases in apoptotic cell markers, such as annexin V or chromatin condensation, should coincide with decreases in cell size. However, cell shrinkage is believed to be independent of caspase activity (Bortner and Cidlowski, 1999), and could indicate caspase-independent apoptosis. Regardless of the mechanism of cell death, analysis of both cell morphology and the markers of cytotoxicity listed on Table 12.8.3 is a sound approach for studying the mechanisms of cell death.

Many of the protocols detailed in this unit are also limited in that they can only be used to determine toxicity in single cells after separation from the tissue. During the process of cell isolation it is possible for artifacts of cell damage to be introduced. This could result in a mischaracterization of cytotoxicity. Additionally, many of the methods listed on Table 12.8.3 are not sensitive enough to determine the cytotoxic effects of compounds on tissues or in organs with differing volumes or cell types. To address these limitations, new protocols are constantly being developed to measure alterations in cell morphology, both in cultured tissues as well as *in vivo* utilizing intravital microscopy (Hasibur et al. 2011).

Critical Parameters and Troubleshooting

LDH release—The following list of critical parameters must be considered when measuring LDH release as a marker of cell membrane integrity and viability during necrosis: (1) The rate of NADH consumption must be linear; (2) The cellular material used to assess total LDH activity must be fully permeabilized; (3) In general, control values for percent LDH release should be <10%. If the levels of LDH activity in controls are high, it is necessary to ensure that the solvent used as a negative control is not toxic. Treatment of cells with the solvent alone should not result in an increase in LDH release compared to cells receiving no treatment. If control LDH activities are high it also important to make certain the sample processing did not damage the cells and, using microscopy, to be sure that cell-free media is totally cell-free;(4) The sensitivity of the fluorescence plate reader used must be adequate to detect changes in the rate of NADH consumption.

Flow cytometry—Selecting appropriate apoptosis and PI positive controls is critical for this assay. These controls do not have to be the toxicants under investigation. A search of the literature with regard to the cell type usually yields several possibilities. In general, 1-2 μM staurosporine is a good positive control for apoptosis, while oxidants, such as 500 – 1000 μM *t*-butylhydroperoxide, are positive controls for necrosis. Regardless of the compounds used, preliminary concentration- and time-dependent analyses should be performed to determine appropriate assay conditions. The concentrations and time points chosen for apoptosis should be those that yield the highest amount of annexin V staining in the absence of any PI staining. The concentrations chosen for the PI positive control should be those that induce a high level of PI staining with no corresponding staining of the annexin V–only region or gate. From these studies, choose a single time and concentration for subsequent studies.

Basic Protocol 2 assumes that the majority of cells grown in culture are still attached to the plate after treatment. Cells that have detached can be isolated from the media by centrifugation and prepared for measurement using the protocols for cellular suspensions. Detached cells are usually good positive controls.

If cells exhibit high levels of PI staining, then the FL-2 channel signal will extend into the FL-1 channel and mask the annexin V–FITC signal. This will be apparent by the fact that the PI positive control results in a signal on the FL-1 channel (i.e., the PI positive control resembles the annexin V positive control). Lower the concentration of PI, or increase the time and number of washes, to overcome this problem.

Care should be taken to verify that control cells are not undergoing significant amounts of necrosis or apoptosis due to solvent toxicity.

A high degree (>10%) of annexin V–FITC background staining may be seen in control cells. To reduce the background level of annexin V–FITC staining, dilute the annexin V–FITC prior to addition to the cells, or increase the time and number of cell sample washes.

One purpose of gates is to exclude cellular debris present as the result of isolation or preparation of cells for analysis. Avoid gates when studying cell death because it is impossible to determine whether cellular debris results from isolation or preparation of cells, or is the result of cell death. Note that this refers to the use of gates during sample acquisition only, and does not refer to the use of gates/regions to analyze data. Make sure that 100% of the cells are accounted for when using gates or regions to analyze data.

Nuclear morphology—If the level of DAPI staining is too low to identify changes in nuclear morphology, increase either the time of exposure or the concentration of DAPI.

If the level of DAPI staining is too intense, reduce the light power source, the exposure time, or the contrast of the microscope used. Alternatively, it may be best to decrease the concentration of DAPI.

If background levels of DAPI staining in the cell periphery (cytosol) are high, increase the length of the wash time (i.e., 10 to 20 min), or decrease the DAPI concentration.

Find a toxicant (e.g., from the literature), such as cisplatin or staurosporine, that induces apoptotic nuclear morphology for use as a positive control.

Make sure the magnification used is sufficient to accurately assess each nucleus (400× usually works well).

A higher concentration of DAPI may be needed for unfixed cells.

Anticipated Results

LDH release—The LDH release assay for cell membrane permeability and viability is straightforward. If a given injurious agent decreases cell viability, the percent LDH activity released from the cells should increase over time, or with higher concentrations of the test agent.

Flow cytometry—If a toxicant induces apoptosis a time- or concentration-dependent shift in cells from the control region to the annexin V region will be observed. If a toxicant causes necrosis, a shift in cells from the control region to the PI region will be observed. Late apoptosis (secondary necrosis) or necrosis will be indicated if cells are observed in the annexin V and PI positive region (R2 or UR in Fig.12.8.3). A time-course experiment may be needed to determine which possibility is correct (see Basic Protocol 4). If increases in annexin V staining occur prior to increases in PI staining, then the cells are dying by apoptosis. Necrosis is responsible if increases in PI staining precede increases in annexin V staining. Additionally, annexin V should bind to the extracellular face of the plasma

membrane during apoptosis and should not be present in the cytosol. Fluorescence microscopy can be used to verify the localization of annexin V–FITC in cells.

Time Considerations

The amount of time required for any of the protocols described in this unit depends on the number of samples analyzed.

LDH release—In general, a sample set of 50 should take no longer than 2 hr to analyze. However, samples can be frozen and analyzed at a later time if necessary.

Flow cytometry—The time required for flow cytometry varies depending on the number of samples and whether cell suspensions or adherent cell cultures are used. Excluding the time needed for treatment of cells with toxicants, 50 samples generally require 2 hr of preparation and 1 hr of analysis.

Nuclear morphology—Generally, Basic Protocol 3 requires 4 hr, including the time for acquisition of data. If fixed cells are used, the time needed increases by ~2 hr. Nuclear morphology need not be analyzed the same day if fixed cells are employed. If live cells are used, then the cells should be analyzed directly. Images can be saved directly onto a computer or disk and analyzed later to reduce the amount of time needed for acquisition. If images are stored, care should be taken to ensure the same microscope settings (i.e., brightness and contrast) are used for all samples.

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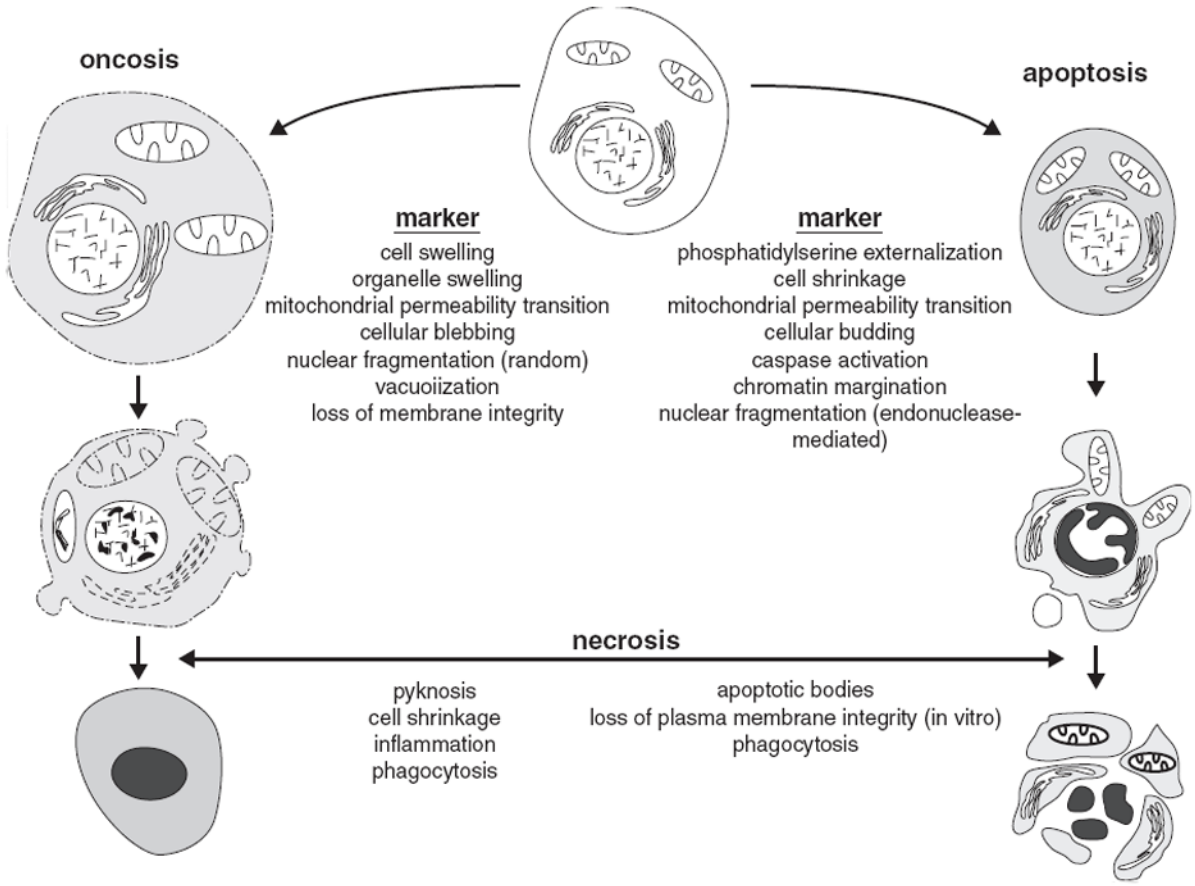

Figure 12.8.1.

Pathways and markers of cell death. Cells typically die by one of two pathways, necrosis or apoptosis, both of which proceed in distinct phases. Necrosis and apoptosis are cellular processes that ultimately lead to necrosis. Necrosis is the term used to describe dead cells or tissue, regardless of the pathway or process involved. Cells dying by necrosis have distinct morphology compared to those dying by apoptosis. Further, cells dying by necrosis and apoptosis can be identified by markers of each pathway. However, depending on the type of cell being studied and the toxicant used, some markers or events occur in both necrosis and apoptosis. Adapted from Majno and Joris (1995).

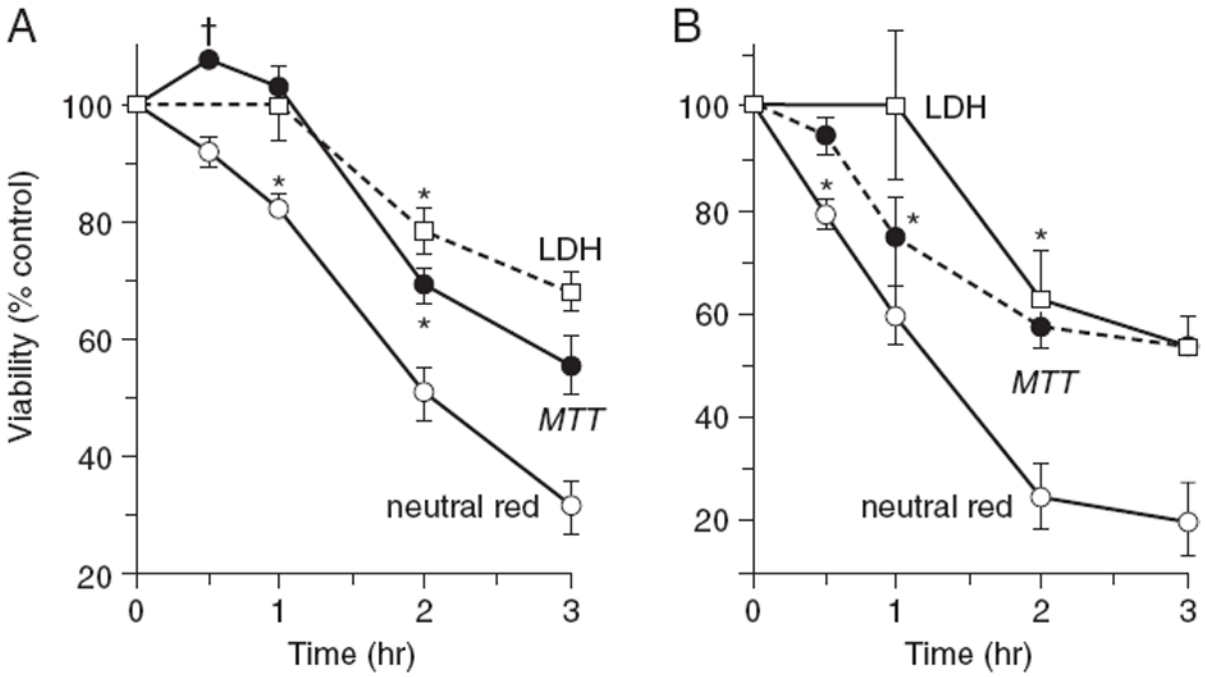

Figure 12.8.2.

Differences in cytotoxicity of **(A)** 2-Br-(diglutathione-S-yl)hydroquinone and **(B)** 2-Br-6-(glutathione-S-yl)hydroquinone to renal tubular epithelial cells (LLC-PK_1) as assessed by neutral red accumulation, MTT reduction, or intracellular LDH activity (as indicated in panels **A** and **B**). Cells were exposed to 0.5 mM 2-Br-(diglutathione-S-yl)hydroquinone and 2-Br-6-(glutathione-S-yl)hydroquinone for 2 hr. After removal of toxicants, cells were incubated for 1 hr in neutral red solution, MTT solution, or Earle's balanced salt solution, and LDH activity was determined.*, Earliest time at which a significant decrease in viability was observed with each assay. For 2-Br-(diglutathione-S-yl)hydroquinone, $p < 0.05$ for LDH at 2 hr. For 2-Br-6-(glutathione-S-yl)hydroquinone, $p < 0.002$ at 0.5 hr for neutral red, $p < 0.05$ at 1 hr for MTT, and $P < 0.06$ for LDH at 2 hr. Reprinted with permission from Mertens et al. (1995).

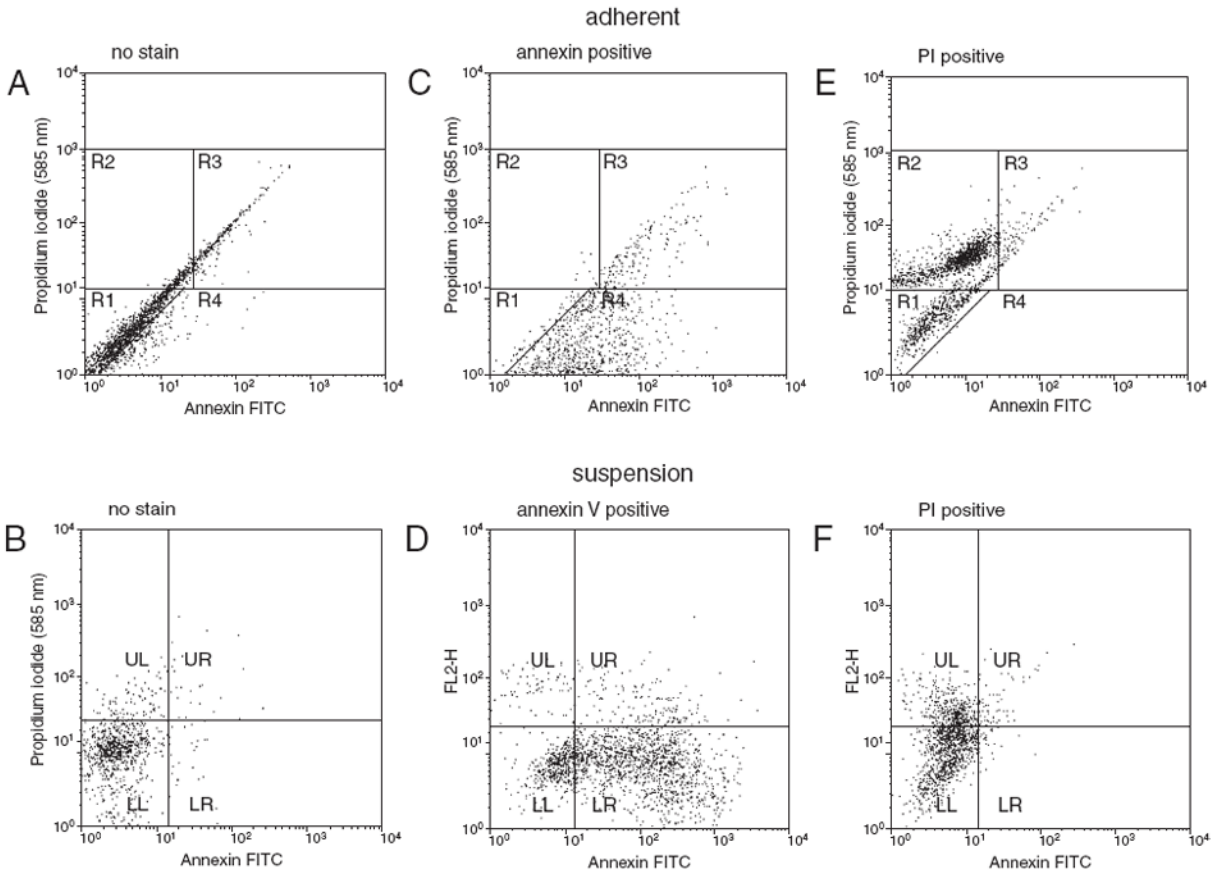

Figure 12.8.3.

Representative dot plot for negative and positive controls generated during a typical flow cytometry experiment. Panels **A, C**, and **E** represent data generated using adherent cells treated, stained in culture dishes with annexin V–FITC and PI, and then released by mechanical methods. Panels **B, D**, and **F** represent data generated using freshly isolated suspensions of cells, or those treated in culture dishes and released from the dish using enzymatic methods, prior to staining with annexin V–FITC and PI. R1, R2, R3, and R4 in panels **A, C**, and **E** denote region 1, 2, 3, and 4, respectively. UL, UR, LL, and LR in panels **B, D**, and **F** represent upper left, upper right, lower left, and lower right, respectively. The regions in panels **A, C**, and **E** and quadrants in panels B, D, and F are included as instructional guides.

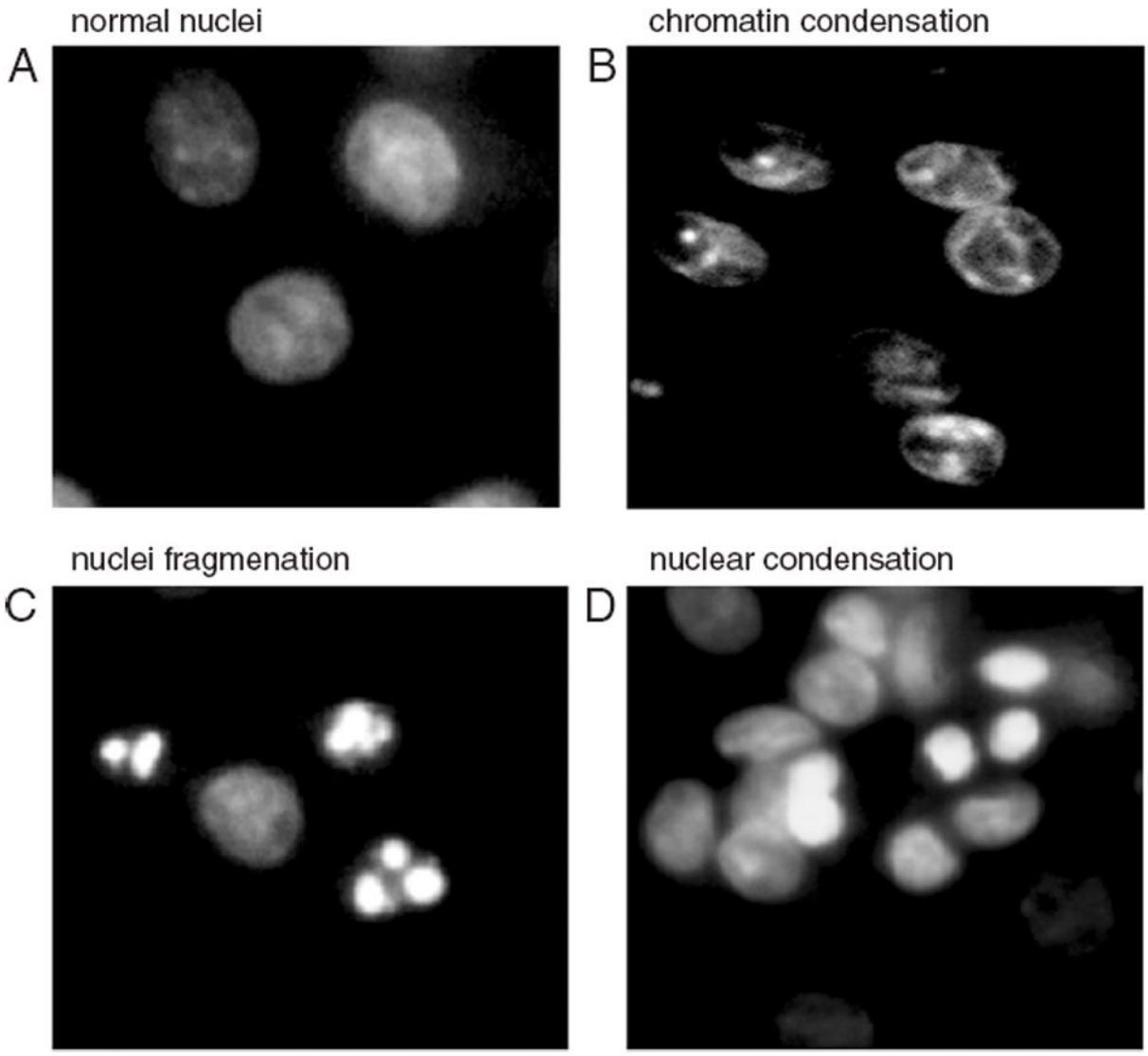

Figure 12.8.4.
Representative DAPI staining in primary cultures of renal proximal tubular cells. (**A**) Typical staining for nuclei in control cells. (**B**) Typical chromatin condensation induced by 50 μM cisplatin after 24 hr of exposure. (**C**) Typical nuclear fragmentation induced by 2 μM staurosporine after 24 hr of exposure. (**D**) Typical nuclear condensation induced by 2 μM vincristine after 24 hr of exposure.

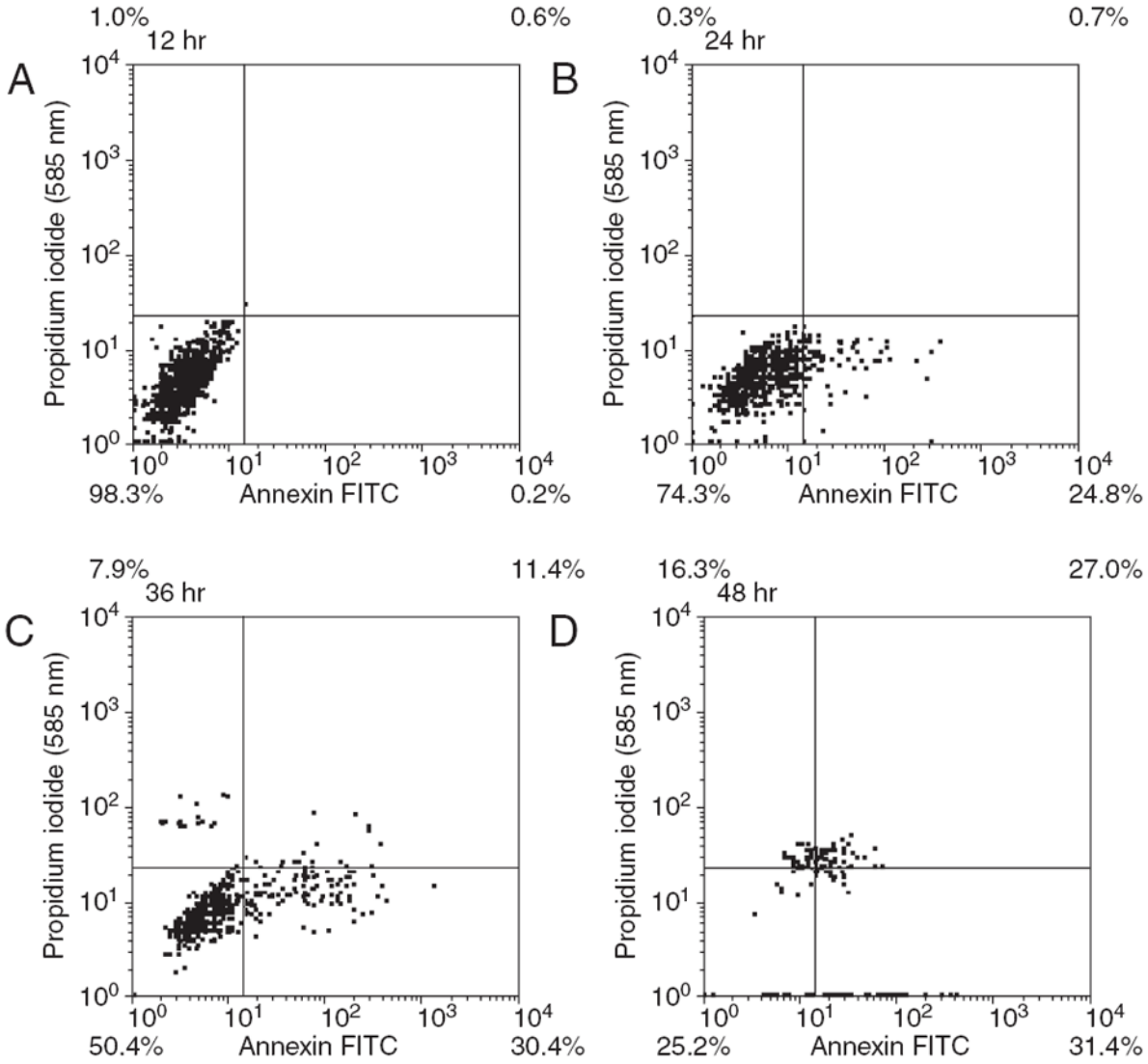

Figure 12.8.5.

Examples of time-dependent toxicity as measured by annexin V and propidium iodide (PI) staining using flow cytometry. Time-dependent increases in annexin V and/or PI staining in mouse leukemic L1210 cells exposed to 2 μM staurosporine for 12 hr (**A**), 24 hr (**B**), 36 hr (**C**), and 48 hr (**D**). Note the percent staining for each quadrant is listed in the corner of each quadrant.

Table 12.8.1

Morphological and Biochemical Features of Apoptosis and Necrosis

Apoptosis	**Necrosis**
Externalization of phosphatidylserine	Phosphatidylserine degraded, internalized, or released
Activation of caspases	Caspase are not activated
Maintenance of membrane integrity	Loss of membrane integrity
Decrease in cellular volume (cell shrinkage)	Increase in cellular volume (cell swelling)
No release of intracellular contents	Release of intracellular contents
No inflammation (*in vivo*)	Inflammation (*in vivo*)
Formation of cellular buds and fragments	Formation of cellular blebs
Chromatin condensation	No chromatin condensation
ATP concentrations are slightly decreased or maintained	Loss of ATP concentrations
Organelles retain integrity	Organelle swelling and loss of integrity
Plasma membrane Ca^{2+} gradients maintained	Loss of Ca^{2+} gradients
Shared features	
Membrane permeability transition	
DNA degradation	
Nuclear condensation	

Table 12.8.2

Markers and Methods Used to Assess Cytotoxicity

Morphological or biochemical event	Marker	Reference
Externalization of phosphatidylserine	Annexin V binding	Schutte et al., 1998; Cummings and Schnellmann, 2002
Activation of caspases	Fluorometric substrate cleavage	Liu et al, 1996; Thornberry et al., 1997; Saraste and Pulkki, 2000
	Expression of specific caspases	
	Cytochrome c translocation	
Maintenance of plasma and lysosomal membrane integrity	PI staining	Ferlini et al., 1996; Singh, 2000
	Neutral red	
Cellular volume	Cell size	Bortner and Cidlowski, 2001
Release of intracellular contents	LDH	Mertens et al., 1995; Moran and Schnellmann, 1996
Inflammation	Inflammatory cell infiltration or expression of markers of inflammation	Jaeschke et al., 1996; Licht et al., 1999
Formation of cellular buds, fragments, or blebs	Cell morphology	Lemasters et al., 1987; Zhang et al., 1999
Chromatin condensation	DAPI or Hoechst staining	Lieberthal et al.1996
ATP levels	HPLC analysis	Groves and Schnellmann, 1997
DNA fragmentation	DAPI or Hoechst staining Agarose gel electrophoresis DNA hypoploidy	Singh, 2000; Cummings and Schnellmann, 2002
Ca^{2+} gradients	FURA-2	Lemasters, 1999; Lemasters et al., 1999
Mitochondrial function and integrity	MTT	Reers et al., 1995; Lemasters, 1999; Lemasters et al., 1999; Cummings and Schnellmann, 2002
	JC-1 Tetramethylrhodamine	
Cellular and mitochondrial respiration	O_2 consumption	Schnellmann, 1994

Table 12.8.3

Cytotoxic Marker Distribution During Cell Death

Marker	Necrosis	Apoptosis	Caspase-independent apoptosis/autophagy	References
Annexin V binding[a]	−	+	+	Schutte et al., 1998; Licht et al., 1999
PI staining[b]	+	−	−	Ferlini et al., 1996; Hamel et al., 1996
Neutral red exclusion[c]	+	−	−	Modha et al., 1993; Mertens et al., 1995
Trypan blue staining	+	−	−	Lash and Tokarz, 1989; Modha et al., 1993
Caspase activation	−	+	−	Thornberry et al., 1997; Cummings and Schnellmann, 2002
LDH release[b]	+	−	−	Moran and Schnellmann, 1996
JC-1 transition[d]	+	+	*e*	Reers et al., 1995
Mitochondrial dehydrogenase activity	+	+	*e*	Fanning et al., 1990; Yang et al., 1998
Cell shrinkage	−	+	+	Trump et al., 1997
Cell swelling	+	−	−	Lieberthal et al., 1996; Trump et al., 1997
Loss of Ca^{2+} gradients	+	−	*e*	Lemasters et al., 1987
Decreases in ATP	+	−	*e*	Groves and Schnellmann, 1996
Mitochondrial swelling	+	−	−	Schnellmann et al., 1989
DNA degradation	+	+	+	Shen et al., 1991; Vanags et al., 1996; Saraste, 1999
Cell blebbing	+	−	−	Lemasters et al., 1987; Trump et al., 1997
Cell budding	−	+	+	Kerr, 2002; Otsuki et al., 2003
Nuclear fragmentation	+	+	+	Weaver et al., 1993; Takemura et al., 2001
Chromatin condensation	−	+	+	Barry et al., 2000; Otsuki et al., 2003
Nuclear condensation	+	+	+	Lipton and Nicotera, 1998; Saraste, 1999
Inflammation	+	−	−	Saraste, 1999
Phagocytosis	+	+	*e*	Licht et al., 1999; Saraste, 1999
Cytochrome c translocation	−	+	−	Liu et al., 1996
Translocation of proapoptotic proteins (eg. Bax and Bak)	−	+	−	Lindsten et al., 2000

[a] Must be binding to the external plasma membrane and not internalized.

[b] Present during late stages in all types of cell death.

[c] Dye is absent from dying or dead cells.

[d] Fluorescence absorbance is shifted from 525 nm in healthy cells to 590 nm in injured cells.

[e] ?, unknown.