Topic Introduction

Dead Cert: Measuring Cell Death

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Many cells in the body die at specific times to facilitate healthy development or because they have become old, damaged, or infected. Defects in cells that result in their inappropriate survival or untimely death can negatively impact development or contribute to a variety of human pathologies, including cancer, AIDS, autoimmune disorders, and chronic infection. Cell death may also occur following exposure to environmental toxins or cytotoxic chemicals. Although this is often harmful, it can be beneficial in some cases, such as in the treatment of cancer. The ability to objectively measure cell death in a laboratory setting is therefore essential to understanding and investigating the causes and treatments of many human diseases and disorders. Often, it is sufficient to know the extent of cell death in a sample; however, the mechanism of death may also have implications for disease progression, treatment, and the outcomes of experimental investigations. There are a myriad of assays available for measuring the known forms of cell death, including apoptosis, necrosis, autophagy, necroptosis, anoikis, and pyroptosis. Here, we introduce a range of assays for measuring cell death in cultured cells, and we outline basic techniques for distinguishing healthy cells from apoptotic or necrotic cells—the two most common forms of cell death. We also provide personal insight into where these assays may be useful and how they may or may not be used to distinguish apoptotic cell death from other death modalities.

ASSAYS TO MEASURE CELL DEATH AND CELL SURVIVAL

Most cytotoxic stimuli trigger cell death by apoptosis; however, others may trigger alternative cell death mechanisms such as necrosis, autophagy, anoikis, or necroptosis. It is therefore wise to use a well-characterized model to evaluate and validate any protocol when embarking on cell death assays for the first time. Well-known inducers of apoptosis include death receptors such as TNF-related apoptosis-inducing ligand (TRAIL) (Corazza et al. 2009) and Fas ligand (Huang et al. 1999), serum withdrawal and other cell stressors (Schamberger et al. 2005), chemotherapeutic drugs including actinomycin D, staurosporine, or etoposide (Finucane et al. 1999a, 1999b; Waterhouse et al. 2001; Crowley et al. 2011), and ultraviolet (UV)- and γ -irradiation (Goldstein et al. 2000; Kulms and Schwarz 2000; Waterhouse et al. 1996; Lee et al. 2007). Although these stimuli normally trigger death by apoptosis, they can also result in different types of cell death, depending on the strength of the stimulus. For example, low levels of UV-irradiation have been shown to trigger apoptosis, while

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higher levels can trigger necrosis. Of note, some cell types may be resistant to a variety of these stimuli because they contain specific defects in key signaling molecules. Thus, it is critical to select a cell type that is known to be sensitive to the planned stimulus. We currently use actinomycin D (1 μM) or low levels of UV-irradiation (80 mJ/m^2) to trigger apoptosis in U937 cells or HeLa cells, and higher levels of UV-irradiation (500 mJ/m^2) to trigger necrosis. Treatment of cells with these stimuli is described in our protocols for Protocol: **Triggering Apoptosis in Hematopoietic Cells with Cytotoxic Drugs** (Crowley et al. 2015a) and Protocol: **Triggering Death of Adherent Cells with Ultraviolet Radiation** (Crowley and Waterhouse 2015a). We also regularly use leucine zipper-TRAIL (230 ng/mL), staurosporine (1 μM), mitoxantrone (5 μM), doxorubicin (5 μM), and γ -irradiation (10 Gy) to trigger apoptosis in a variety of cell lines.

The above concentrations are provided as a guide only; a more extensive titration may be required in your model. Also, some stimuli may require training or clearance before use, depending on the occupational health and safety regulations of your country. Any restrictions should be discussed with your institution before proceeding.

Assays for Quantitating Cell Death

In many cases, it is sufficient to know the extent of cell death in a sample but not necessary to determine the mechanism of death. Cell death can be measured by staining a sample of cells with trypan blue, as we describe in Protocol: **Measuring Cell Death by Trypan Blue Uptake and Light Microscopy** (Crowley et al. 2015b). Because trypan blue is a colorimetric dye that only stains dead cells with ruptured plasma membranes but is excluded from live cells that have intact plasma membranes, dead cells (blue) can easily be distinguished from live cells (colorless) using a standard bright-field microscope. The extent of cell death can be determined by manually counting the number of colorless vs. blue cells in the sample. This assay is convenient, as trypan blue is inexpensive and loss of plasma membrane integrity is a terminal event in all cell death in culture. However, it cannot be used to quantitate the extent of death in fixed cells or in tissue sections where the plasma membrane of cells has been compromised by fixation or sectioning. Quantitating cell death using trypan blue can also be tedious when assaying large numbers of samples, because the cells must be counted manually.

Propidium iodide (PI), a small fluorescent dye that is similarly excluded from live cells but stains the nuclei of cells with ruptured plasma membranes, can also be used to measure the extent of death in a sample of cultured cells; see Protocol: **Measuring Cell Death by Propidium Iodide Uptake and Flow Cytometry** (Crowley et al. 2015c).

PI is maximally excited by light at 535 nm and emits at 617 nm. Dead cells can therefore be detected using a fluorescence microscope equipped with a green excitation filter (e.g., 495–570 nm) and a red emission filter (e.g., 610–750 nm). Counting can be performed manually or automated using various image analysis programs. Modern flow cytometers, which rapidly and accurately count large numbers of cells, are also equipped with lasers and filters that can detect PI fluorescence. Flow cytometry is therefore ideal for quantitating cell death in samples stained with PI (see Protocol: **Measuring Cell Death by Propidium Iodide Uptake and Flow Cytometry** [Crowley et al. 2015c]). PI is relatively inexpensive and flow cytometry can be automated. This assay is therefore ideal for screening cell death in large numbers of samples or for performing initial titrations for drug toxicity before proceeding to specific assays that are often more expensive.

Assays for Quantitating Cell Survival

Both trypan blue and PI will stain cells that have reached the terminal stage of death, but neither can be used to detect cells that are dying or marked for death but are not yet terminal. This is an important distinction, because the damage caused by treatment with some cytotoxic agents may not have an immediate measureable effect (Sedelies et al. 2008). If used in long-term assays, trypan blue staining and PI uptake may also underestimate the toxicity of some treatments. For example, in a culture where 50% of the cells died and 50% divided once, the percentage of cell death will only be measured as 33% using the trypan blue or PI assays. Distinguishing healthy from dying cells is particularly important in

applications such as cancer chemotherapy, where any surviving cells have the potential to cause relapse. One way to address this issue is by performing a colony-forming assay to define the number of cells in a population that are capable of proliferating and forming large groups of cells (Ludlow et al. 2008; Sedelies et al. 2008). Because each colony is generally derived from a single surviving cell, the results provide a measure of the number of healthy cells in a sample. Colony-forming assays are easily performed on adherent cells by adding a defined number of cells to a flat-bottomed dish and incubating the cells until visible colonies form, as we describe in Protocol: **Measuring Survival of Adherent Cells with the Colony-Forming Assay** (Crowley et al. 2015d). The colonies can be counted under a light microscope or fixed and stained with Crystal Violet for quantitation at a later stage. The percentage confluence can be determined using image analysis software. It is more difficult to perform colony-forming assays on suspension cells because the cells disperse in medium. However, it is possible to prevent dispersal by culturing the colonies in soft agar; see Protocol: **Measuring Survival of Hematopoietic Cancer Cells with the Colony-Forming Assay in Soft Agar** (Crowley and Waterhouse 2015b).

Using the above basic techniques, it is possible to begin investigating cell death in culture models. These assays are particularly useful when it is sufficient to simply determine whether a cell has died to evaluate whether a specific treatment triggers, enhances, or blocks cell death. They are also applicable to large screens or initial titrations for determining optimal treatment conditions (e.g., drug concentration or assay duration) for inducing cell death. More specific cell death assays are needed to quantitate a specific type of cell death or determine the mechanism of cell death (see below).

ASSAYS TO DISTINGUISH APOPTOSIS FROM NECROSIS AND OTHER DEATH MODALITIES

Most forms of cell death are characterized by distinct morphologies that can be visualized by light microscopy. For example, apoptosis is characterized by rounding, shrinking, plasma membrane blebbing, and formation of apoptotic bodies. This is in contrast to necrotic cells which swell and lyse, releasing their cellular contents. The usefulness of light microscopy in analysis of cell death is often overlooked and underestimated, because researchers have developed more robust definitions based on biochemical characteristics (Elmore 2007). However, looking down a microscope at your cells is one of the most convenient tools for distinguishing live cells from dead or dying cells, and for distinguishing apoptosis from necrosis or other death modalities. We strongly recommend visual inspection as the first step in any procedure for measuring cell death (see Box 1). This method is convenient, because cells can be easily observed at regular intervals to confirm that an experiment is proceeding as expected, or to predict the optimum timing for additional assays. Further, it is a useful tool for recording the health and quality of any cell culture before sampling for subsequent experimentation. It can be used in combination with other protocols including cytopinning, 4',6-diamidino-2-phenylindole (DAPI) staining, or measuring immunofluorescence, or used as a visual reference for quantitative assays measured by flow cytometry (see below).

It is not always possible, however, to distinguish live cells from dead or dying cells using light microscopy. Some hematopoietic cells (e.g., T cells, B cells, or NK cells) and mitotic cells can have a similar appearance to apoptotic cells. Further, some cells also clump together, making it difficult to objectively measure cell death in individual cells. Finally, some treatments can induce morphological changes that appear similar to apoptosis. These issues can be addressed by staining and observing cellular structures such as the nucleus. The following techniques can be used to distinguish healthy cells from apoptotic or necrotic cells. Further assays will be required to distinguish these two most common forms of cell death from other modalities.

Detecting Nuclear Condensation

The nucleus is generally round in healthy cells but fragmented in apoptotic cells. Dyes such as Giemsa or hematoxylin, which are purple in color and therefore easily viewed using light microscopy, are

BOX 1. ANALYZING CELL DEATH BY LIGHT MICROSCOPY

The first and easiest question to ask when assaying cell death should always be “What do the cells look like?” Apoptotic and necrotic cells can easily be distinguished using standard light microscopes available in most laboratories. Observing cells by bright-field microscopy does not require a high level of expertise and can be performed by most laboratory staff. Images of the cells can be taken using a microscope camera attachment for further analysis. Cells that grow in suspension should be allowed to settle on the bottom of the dish before observation or imaging.

- Healthy cells generally have a round, well-defined nucleus surrounded by a cytoplasmic region enclosed by a continuous plasma membrane. In adherent lines, these cells remain attached as a monolayer on the bottom of the flask/dish (Fig. 1A).
- Apoptosis is characterized by nuclear condensation and fragmentation, cell rounding (in the case of adherent cells), shrinking, and blebbing of the plasma membrane (Fig. 1B.i and ii) (Hengartner 2000). Some cells eventually fragment into small, plasma membrane-bound apoptotic bodies, but other cells remain intact until they swell and lyse by a process known as secondary necrosis (Fig. 1B.iii). Cells may not progress to secondary necrosis *in vivo* because apoptotic cells are removed by phagocytosis (Tanaka et al. 2010), but this event provides a convenient end point for apoptosis in culture.

FIGURE 1. Images of cell survival and cell death. (A–C) Bright-field images of HeLa cells. (A) Untreated cells. (B) Cells treated with 230 ng/mL TRAIL for 4 h undergoing apoptosis. Apoptotic cells are indicated by asterisk (*). Features include plasma membrane blebbing, which appears as small bubble-like projections from the cell surface (shown at higher magnification in B(ii)), and plasma membrane extrusions during secondary necrosis, which appear as large bubble-like projections of the plasma membrane (shown at higher magnification with arrows in B(iii)). (C) Cells treated with UV 500 mJ/m² for 4 h undergoing necrosis. Features include large plasma membrane extrusions (shown at higher magnification with arrows in C(ii)). (D(i), D(ii)) Mitotic cells from untreated cultures. Features include rounding and detachment, appearance of DNA midlines or chromatin segregation. (E) U937 cells treated with 1 µM actinomycin D for 16 h. Indicated are a live cell (white arrow), an apoptotic cell (black arrow), and a necrotic cell (white arrow with black outline). (F) Autophagic cells treated with 25 µM chloroquine for 24 h. Features include large vacuoles within the cytoplasmic region of the cells (white arrows). (G) Cells treated with 1 µM staurosporine for 6 h.

- Necrosis is characterized by overt swelling of the plasma membrane, which appears as large bubble-like projections that eventually rupture (Fig. 1C.i and ii, black arrows). Adherent cells do not generally detach during necrosis, making it easy to distinguish these cells from rounded apoptotic cells or cells that have progressed through apoptosis to secondary necrosis. The nuclei of necrotic cells do not fragment like apoptotic cells and this provides another distinguishing feature for this type of cell death.
- In some cases, it may be difficult to differentiate live cells from dead or dying cells. For example, healthy cells such as mitotic (Fig. 1D.i and ii) or some hematopoietic cells (Fig. 1E, white arrow) are round, and may therefore be difficult to distinguish from apoptotic (Fig. 1E, black arrow) or secondary necrotic (Fig. 1E, white arrow with black outline).
- Other forms of cell death such as autophagy can also be distinguished by morphology. Cell death by autophagy is less well-characterized, but one distinguishing morphological feature is the formation of large vacuole-like structures (Fig. 1F, white arrows). It is wise to confirm autophagy by assaying molecular events such as increased expression of microtubule-associated protein light chain 3 (LC3-II), a protein that is associated with formation of autophagosomes (Crowley et al. 2011).
- Some treatments can also induce morphological changes that appear similar to apoptosis. For example, staurosporine can induce changes in the plasma membrane that resemble the early stages of apoptosis (Fig. 1G). Apoptosis should therefore be verified by staining the cells for specific events associated with cell death.

commonly used to stain the nucleus. Other features of apoptosis and necrosis, such as plasma membrane blebbing or rupture, can be identified by staining the cytoplasm with eosin. Eosin is pinkish in color and can also be viewed using light microscopy. Hematoxylin and eosin are, therefore, commonly used together to stain cells. We recommend use of a rapid stain (e.g., Rapi-Diff or Diff Quick) that has been optimized for staining the nucleus and cytoplasm by light microscopy (Yang and Widmann 2002) as we describe in Protocol: **Morphological Analysis of Cell Death by Cytospinning Followed by Rapid Staining** (Crowley et al. 2015e).

Fixing and staining adherent cells poses few problems; however, apoptotic cells round up and may detach from the culture plate. These cells can therefore be washed away during the fixing and staining procedure. Similarly, it can prove difficult to fix and stain nonadherent cells for analysis by light microscopy. To overcome this, cells may be gently centrifuged onto glass slides by cytopinning and then fixed and stained, as described in the above protocol. Cytospinning is also ideal for use with other dyes that are commonly used to stain the nucleus. These include DAPI and Hoechst (33258 and 33342), which bind to the DNA of live or fixed cells and fluoresce at 461 nm when excited by UV light (Excitation maximum ~350 nm). Cells stained with these dyes can be visualized using standard fluorescence microscopy equipped with a UV light source and a blue/cyan filter; see Protocol: **Analyzing Cell Death by Nuclear Staining with Hoechst 33342** (Crowley et al. 2015f).

Detection of DNA Fragmentation

The use of Hoechst 33342 and DAPI to identify and characterize apoptotic cells relies on a researcher's ability to distinguish the nuclear morphology of apoptotic cells from healthy cells or cells that have died by other mechanisms. Although this is generally easy, mitotic cells also have condensed chromatin that may be confused with apoptotic cells by inexperienced users. However, the condensed chromatin of mitotic cells differs from that of apoptotic cells because the DNA of latter is fragmented but the DNA of the former is not. The fragmented DNA in apoptotic cells can be labeled with fluorescently tagged UTP by terminal deoxynucleotidyl transferase, and terminal dUTP nick end labeling (TUNEL) can be used to specifically label apoptotic cells. UTP can be tagged with a variety of fluorochromes, including fluorescein isothiocyanate (FITC), which is excited by light at 495 nm and emits at 520 nm. FITC-labeled UTP can be used in combination with Hoechst 33342 or DAPI to easily distinguish apoptotic cells from mitotic cells using fluorescence microscopy using the protocol for

Protocol: Detection of DNA Fragmentation in Apoptotic Cells by TUNEL (Crowley et al. 2015g). The number of apoptotic cells can be determined as a percentage of the total by counting both the TUNEL-positive and the Hoechst 33342- or DAPI-positive cells. Cell counting of TUNEL-stained cells can also be automated using flow cytometry, as only the apoptotic cells are fluorescently labeled (Trapani et al. 1998a, 1998b).

Apoptotic cells with fragmented DNA can also be identified and distinguished from live cells by staining with PI and measuring DNA content by flow cytometry. This assay, described in **Protocol: Measuring the DNA Content of Cells in Apoptosis and at Different Cell-Cycle Stages by Propidium Iodide Staining and Flow Cytometry** (Crowley et al. 2015h), is based on the fact that PI intercalates with DNA at a specific ratio. The level of PI staining is therefore directly proportional to the DNA content of the cell. PI staining is commonly used for cell cycle studies, as cells in G2/M have twice the DNA content as cells in G0/G1, and therefore bind twice the level of PI. Because apoptotic cells have fragmented DNA, and therefore show lower fluorescence than cells in G0/G1, PI staining is also ideal for quantitating the level of apoptosis in a sample. It should be noted that this assay, in which all cells are fixed and stained with PI, is distinct from the PI uptake assay in **Protocol: Measuring Cell Death by Propidium Iodide Uptake and Flow Cytometry** (Crowley et al. 2015c), in which live cells exclude the dye. It should also be noted that cells can die by apoptosis in the absence of nuclear fragmentation (e.g., because of defects in activation of caspase-activated DNase [Kagawa et al. 2001; Zhang et al. 2001]). Although this is rare, lack of nuclear fragmentation should not be used in isolation to determine that cells are healthy or have not died by apoptosis. This issue can be addressed by performing assays for other apoptotic events that occur in the absence of DNA fragmentation.

Detecting Phosphatidylserine Exposure

Apoptosis is also characterized by exposure of phosphatidylserine (PS) on the outside of apoptotic cells, which acts as a signal that triggers removal of the dying cell by phagocytosis (Komoriya et al. 2000). Annexin V, a 36 kDa calcium-dependent protein, has been found to selectively bind to PS, and can be used to label apoptotic cells in which PS is exposed. Purified annexin V can be conjugated to various fluorochromes, which can then be visualized by fluorescence microscopy or detected by flow cytometry. Binding of fluorescently tagged annexin V is therefore ideal for detecting and quantitating the number of apoptotic cells in a sample. However, it should be noted that annexin V can also enter cells with ruptured membranes and bind to PS in necrotic cells. These cells can easily be distinguished from apoptotic cells by assaying PI uptake in combination with annexin V binding as we describe in **Protocol: Quantitation of Apoptosis and Necrosis by Annexin V Binding, Propidium Iodide Uptake, and Flow Cytometry** (Crowley et al. 2015i). Using this method, apoptotic cells are stained with annexin V but not PI (annexin V+/PI−), necrotic cells are stained with annexin V and PI (annexin V+/PI+) and healthy cells are not stained with either dye (annexin V−/PI−). It is wise to select an early time point to detect large numbers of apoptotic cells in this assay, because the annexin V+/PI− population is transient, and all apoptotic cells will eventually progress to secondary necrosis (annexin V+/PI+) (D'Amours et al. 1998). In fact, the majority of cells in long-term assays are either annexin V−/PI− (healthy) or annexin V+/PI+ (dead), regardless of how they died. Using annexin V binding in such assays is therefore unlikely to yield extra information compared to measuring PI uptake alone.

Detecting Caspase Activity

DNA fragmentation and PS exposure are both orchestrated by caspases during apoptosis. Caspases are a family of cysteine proteases that cleave their substrates at the carboxyl terminus of specific aspartic acid residues. This generally results in substrate proteolysis at only one or two sites (Martin et al. 1995, 1996; Waterhouse et al. 1996; Sedelies et al. 2008). The cleaved fragments can therefore be detected as distinct bands by western blotting using substrate-specific antibodies. Initially, the number of caspase

substrates was believed to be limited, but the number of proteins known to be cleaved by caspases is now quite sizeable (Luthi and Martin 2007; Timmer and Salvesen 2007; Poreba et al. 2013). Caspases themselves are also activated by cleavage and undergo autolytic processing. Detection of cleaved caspases or proteins cleaved by caspases is therefore commonly used as a marker of apoptosis. Although western blotting is convenient, it can be difficult use this technique to quantitate the level of apoptosis in a sample. However, antibodies that specifically recognize the cleaved fragments of caspases and their substrates can be used to specifically detect caspase activity in apoptotic cells by immunocytochemistry. Flow cytometry (using primary antibodies conjugated to fluorescent molecules, or by counterstaining with fluorescently labeled antibodies against the primary antibody) can then be used to quantitate the number of apoptotic cells. This assay, described in Protocol: **Detecting Cleaved Caspase-3 in Apoptotic Cells by Flow Cytometry** (Crowley and Waterhouse 2015c), is particularly useful because the activation of specific caspases (e.g., caspase-3) is unique to apoptotic cells, and it is possible to detect cleaved caspases in cells that have died by apoptosis but progressed to secondary necrosis.

Detecting Mitochondrial Damage

Caspases are activated during apoptosis by proteolysis. This may occur via direct cleavage by granzyme B or by the caspases themselves (Martin et al. 1996; Sedelies et al. 2008). The latter can be facilitated by bringing several caspase zymogens together in sufficient proximity to allow autoactivation. This can be triggered by death-inducing signaling complexes formed following ligation of death receptors (the extrinsic pathway), or by apoptosome formation following the release of cytochrome *c* from mitochondria (the intrinsic pathway) (Waterhouse and Green 1999; Waterhouse et al. 2001). Cytochrome *c* release is a critical event in the intrinsic pathway to apoptosis and can be measured using several different methods, including western blotting, immunocytochemistry, and flow cytometry. The choice of assay will depend on a variety of criteria (Waterhouse and Trapani 2003). Protocol: **Analysis of Cytochrome *c* Release by Immunocytochemistry** (Crowley et al. 2015j) is an ideal starting point, because the difference between a cell with intact and permeabilized mitochondria is visually striking. Cytochrome *c* release can also be quantitated by flow cytometry using a modified immunocytochemistry protocol (Christensen et al. 2013). Alternatively, flow cytometry can be used to quantitate the number of cells that have reduced mitochondrial transmembrane potential, which is commonly associated with cytochrome *c* release during apoptosis; see Protocol: **Measuring Mitochondrial Transmembrane Potential by TMRE Staining** (Crowley et al. 2015k). Measuring loss of mitochondrial transmembrane potential by flow cytometry is easier than measuring cytochrome *c* release; however, it should be noted that there are cases where measurements of these events are not interchangeable. The relative benefits and pitfalls of measuring release of cytochrome *c* versus loss of mitochondrial transmembrane potential are discussed in the two protocols above and by Christensen et al. (2013).

CONCLUSION

Apoptosis was originally defined by distinct morphological features such as nuclear condensation and plasma membrane blebbing (Kerr et al. 1972; Wang et al. 2005). These features are still important in identifying and characterizing apoptosis, but new assays that measure key molecular events are more convenient for analyzing and quantitating apoptosis in a sample. Although the protocols introduced here comprise an effective overall strategy for measuring cell death, it is essential to understand the benefits and limitations of each assay. The importance of morphology should also not be underestimated for distinguishing apoptosis from other death modalities. Essentially, when it comes to assaying cell death: Keep it simple at the start, use “seeing is believing,” and do not rely on the outcomes of one assay alone. These protocols should be used in combination to develop a basic understanding of cell death in your model.

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