The apoptosis analysis guide

to help you progress faster

Contents

Cell death

Cell death happens when a cell fails to maintain essential life functions and can be non-programmed, in the case of injury or trauma, or programmed, as in processes like apoptosis and autophagy.

Cell death can be classified according to its morphological appearance (such as apoptotic or necrotic), enzymological criteria (with or without the involvement of distinct proteases), functional aspects (programmed or non-programmed), or immunological characteristics (immunogenic or non-immunogenic)[1].

Before studying cell death mechanisms, researchers should ensure cell death has happened. The Nomenclature Committee on Cell Death (NCCD) has proposed that researchers should define a cell as dead when the following features are observed[1]:

1. The cell has lost the plasma membrane integrity

2. The cell has undergone complete disintegration

3. Whatever is left of the cell has been phagocytosed by the neighboring cells *in vivo*

This guide aims to provide you with an overview of apoptosis, the most studied and well-known type of cell death, the most common parameters used to assess apoptosis, and tools you can use to study cell death in your research.

Apoptosis

Apoptosis is a type of programmed cell death that is critical for numerous normal physiological processes. Historically, apoptosis has been defined by its morphological features, mostly described in the 1970s by John Kerr[2]. Apoptotic morphology includes cell shrinkage, membrane blebbing, chromosome condensation (pyknosis) and nuclear fragmentation (karyorrhexis), DNA laddering, and the eventual engulfment of the cell by phagosomes. In contrast to necrosis, the apoptotic cell does not provoke an inflammatory response, and only individual cells are affected by apoptosis *in vivo*.

Mechanisms of apoptosis

Apoptosis is characterized by the activation of a family of cysteine-aspartate proteases known as caspases, involved in the restricted proteolysis of over 400 proteins. The two main pathways through which apoptosis is initiated are the intrinsic and extrinsic cell death pathways, resulting in caspase activation.

The intrinsic cell death pathway is governed by the Bcl-2 family of proteins, which regulate commitment to cell death through mitochondrial permeabilization. Many intracellular death signals are communicated through the intrinsic cell death pathway, such as DNA damage, oncogene activation, growth factor deprivation, ER stress, and microtubule disruption. The key step in the intrinsic cell death pathway is permeabilization of the mitochondrial outer membrane, which has been identified as a 'point of no return', after which cells are committed to cell death.

Following permeabilization, the release of various proteins from the mitochondrial intermembrane space promotes caspase activation and apoptosis. Cytochrome c binds to apoptosis protease-activating factor-1 (APAF1), inducing its oligomerization and forming a structure called the apoptosome that recruits and activates caspase-9. Caspase-9 is an initiator caspase that cleaves and activates the executioner caspases, caspase-3 and -7, leading to apoptosis.

Activation of the extrinsic cell death pathway occurs following the binding on the cell surface of "death receptors" to their corresponding ligands such as Fas, TNFR1, or TRAIL. These death receptors have two distinct signaling motifs: death domains (DD) and death effector domains (DED) that allow them to interact and recruit other adaptor molecules, such as FAS-associated death domain protein (FADD) and caspase-8, which can then directly cleave and activate caspase-3 and caspase-7, leading to apoptosis[3].

Hallmarks of apoptosis

Apoptosis occurs via a complex signaling cascade that is tightly regulated at multiple points, providing many opportunities to evaluate the proteins involved (Figure 1).
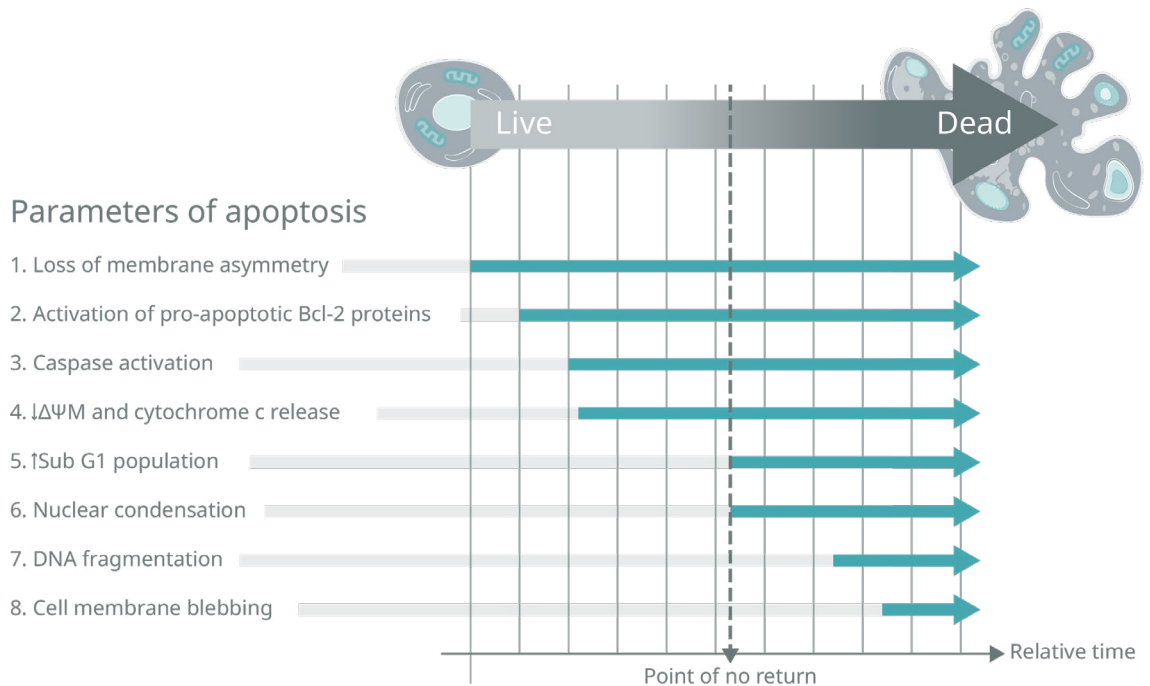
Figure 1. Hallmarks of apoptosis. These events do not happen in a sequential order, and many of them will overlap and occur simultaneously. Loss of membrane asymmetry or caspase cascade initiation are biochemical features of apoptosis that do not necessarily lead to cell death. However, other downstream features such as a decrease of the mitochondrial membrane potential (ΔΨm) and simultaneous release of cytochrome c into the cytosol, are generally considered points of no return, after which it is doubtful the cell will survive.

In the following chapters, we will describe the different tools you can use to detect apoptosis in your particular samples and help you identify the most appropriate method for your experimental settings.

Detecting apoptosis

Activation of pro-apoptotic members of the Bcl-2 family

The Bcl-2 family consists of several evolutionarily conserved proteins that share one or more of the Bcl-2 homology (BH) domains. The members of the Bcl-2 family can either promote or inhibit apoptosis, depending on the BH domains they contain. The anti-apoptotic members, such as Bcl-2 and Bcl-X(L), conserve all four BH domains; the pro-apoptotic members, such as Bax, Bak, or Bad, always contain the BH3 domain and may have lost other domains.

There is a dynamic balance between anti- and pro-apoptotic proteins. When the intrinsic pathway is activated, some of the pro-apoptotic members will dimerize and form pores on the outer mitochondrial membrane, leading to the release of APAF1 and other mitochondrial proteins for subsequent activation of downstream proteins like caspases[4].

One of the simplest methods to look at activation of pro-apoptotic Bcl-2 proteins is by looking at the change in protein levels by western blot using a specific antibody. For example, Figure 2 shows how expression levels of Bax change in the spleen and liver from a mouse after aspirin treatment.
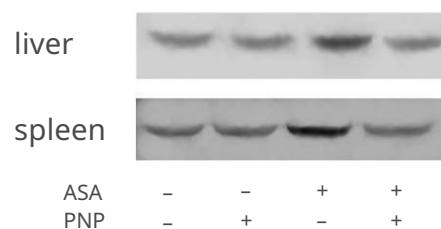

liver			
spleen			
ASA	– –	+	+
PNP	– +	–	+

Figure 2. Levels of pro-apoptotic Bax were monitored in rat liver and spleen homogenates treated with aspirin (ASA) and with or without phyllanthus niruri protein (PNP), a novel antioxidant protein. Bax was detected using anti-Bax antibody [E63] (ab32503). Image adapted from Bhattacharyya S. et al.[5].

However, protein expression levels might not always change in a particular cell type or injury. In these instances, several other techniques can be used to detect the activation of Bcl-2 pro-apoptotic members:

— Assess the oligomerization with other pro-apoptotic proteins:
This can be done by immunoprecipitating one of the proteins and detecting its oligomerization partner by western blot.

— Assess the mitochondrial localization of the activated proteins:
This can be done by performing western blot on cell fractions or by looking at the localization of the activated proteins in the mitochondria together with mitochondrial dyes by immunofluorescence.

Loss of membrane asymmetry

The loss of cellular membrane asymmetry is an early sign of apoptosis where embedded phosphatidylserine (PS) residues in the inner plasma membrane become externalized and signal phagocytosis.

Annexin V, a human placental protein that specifically binds to PS in the presence of calcium and fluorochrome-conjugated annexin V, is a commonly used tool to detect and quantify the PS exposure characteristic of membrane asymmetry[6].

The binding of fluorochrome-conjugated annexin V to exposed PS can be detected by flow cytometry or fluorescence microscopy (Figure 3). While fluorescence microscopy allows visualization of the event, flow cytometry is the most useful method as it provides quick and accurate quantification of cells with exposed PS.
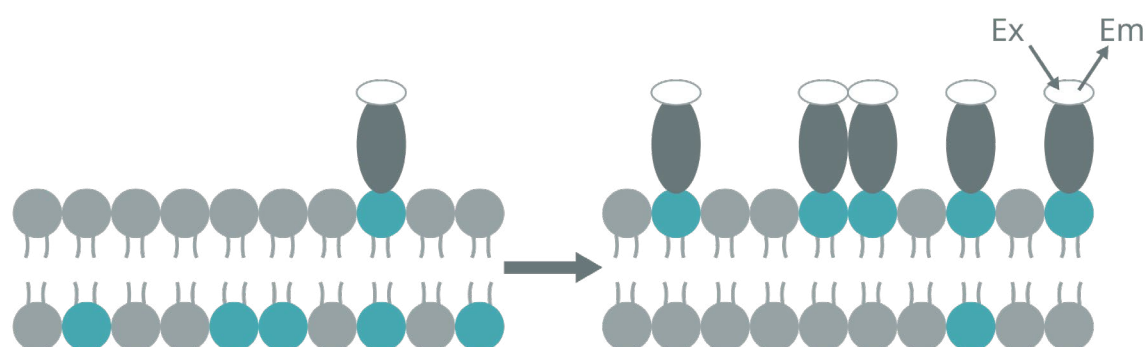

Figure 3. In a normal healthy cell (left), PS residues are mainly located in the inner plasma membrane. After initiation of apoptosis (right), PS residues relocate to the outer plasma membrane and provide an anchorage point for annexin V binding. Upon excitation, fluorochrome-conjugated annexin V will emit a fluorescent signal.

Annexin V detection should be paired with the use of cell viability reagents such as propidium iodide (PI) or 7-AAD, which are not able to penetrate the plasma membrane and can be used to differentiate between apoptotic and necrotic cells. Table 1 shows a quick representation of how to interpret data obtained from annexin V and viability dye staining.

Table 1. Stage of cell depending on the annexin V and viability dye staining.

	Annexin V	**Annexin V**
viability dye (PI/7-AAD)	Viable cells	Apoptotic cells
viability dye (PI/7-AAD)	Dead cells	Late apoptotic/necrotic cells

It has recently been described that PS exposure is not exclusive to apoptosis and also happens in other types of cell death such as necroptosis[7]. Therefore, loss of membrane asymmetry can be a more confirmatory assay for apoptosis than a defining assay and should be run alongside other apoptosis assays.

Annexin V protocol to detect apoptosis

A. Incubate cells with annexin V-FITC

1. Induce apoptosis by the desired method.

2. Collect 1–5 x 10^5 cells by centrifugation.

3. Resuspend cells in 500 µL of 1X binding buffer.

4. Add 5 µL of annexin V-FITC (ab14085) and 5 µL of propidium iodide (PI) (ab14083).

5. Incubate at room temperature for 5 min in the dark.

6. Proceed to B or C below depending on the method of analysis.

B. Quantify by flow cytometry

1. Analyze annexin V-FITC binding by flow cytometry (Ex = 488 nm; Em = 350 nm) using FITC signal detector (usually FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2).
    – For adherent cells, gently trypsinize and wash cells once with serum-containing media before incubation with annexin V-FITC (Steps A3–5).

C. Detect via fluorescence microscopy

1. Place the cell suspension from step A.5 on a glass slide. Cover the cells with a glass coverslip. For analyzing adherent cells, grow cells directly on a coverslip.

2. Following incubation (Step A5), invert coverslip on a glass slide and visualize cells. The cells can also be washed and fixed in 2% formaldehyde before visualization.
    – Cells must be incubated with annexin V-FITC before fixation since any cell membrane disruption can cause non-specific binding of annexin V to PS on the inner surface of the cell membrane.

3. Observe the cells under a fluorescence microscope using a dual filter set for FITC and rhodamine.
    – Cells that have bound annexin V-FITC will show green staining in the plasma membrane.
    – Cells that have lost membrane integrity will show red staining (PI) throughout the nucleus and a halo of green staining (FITC) on the cell surface (plasma membrane).

Caspases

The caspase family is made up of highly conserved cysteine proteases that play an essential role in apoptosis. Mammalian caspases can be subdivided into three functional groups: initiator caspases (caspase-2, -8, -9 and -10), executioner caspases (caspase-3, -6 and -7), and inflammatory caspases (caspase-1, -4, -5, -11 and -12). Initiator caspases initiate the apoptosis signal, while the executioner caspases carry out the mass proteolysis that leads to apoptosis. Inflammatory caspases do not function in apoptosis but are instead involved in inflammatory cytokine signaling and other types of cell death, such as pyroptosis[8].

Initially synthesized as inactive pro-caspases, caspases become rapidly cleaved and activated in response to granzyme B, death receptors, and apoptosome stimuli. Caspases will then cleave a range of substrates, including downstream caspases, nuclear proteins, plasma membrane proteins, and mitochondrial proteins, ultimately leading to cell death.

Activation of caspases can be detected using multiple methods (Table 2). The answer provided by different experimental methods will confirm whether one or more specific caspases are active or inactive. It is always best practice to use more than one method to confirm specific caspase activation.

Table 2. Caspase activation detection.

Sample type	Detection method	Best to use when you want to
Live cells (suspension or adherent)	Flow cytometry	Quickly detect and quantify how many cells have active caspases using a specific substrate.
Fixed cells (suspension or adherent)	Fluorescence microscopy	Visualize which cells have active caspases. Commonly used when you want to visualize other proteins at the same time.
	Flow cytometry	Quickly detect and quantify how many cells have active caspases using a specific antibody.
Cell or tissue lysates (fresh or frozen)	Western blot	Detect a caspase in its cleaved form as well as in its pro-caspase form using specific antibodies.
	Absorbance/ fluorescence assay	Quickly detect caspase activation in a cell population using a specific substrate. Easily adaptable for HTP analysis.
	Absorbance/ fluorescence assay	Quickly detect caspase activation in a cell population using a specific antibody against the active form.
Tissue sections (frozen or paraffin)	IHC	Visualize caspase activation with a specific antibody in discrete cells in a heterogeneous tissue (patient sample, mouse or rat tissue).

Antibody-based methods

The ability to detect active caspase relies on the specificity of the antibody and where the epitope is located. Therefore, choosing the right antibody for chosen method is essential.
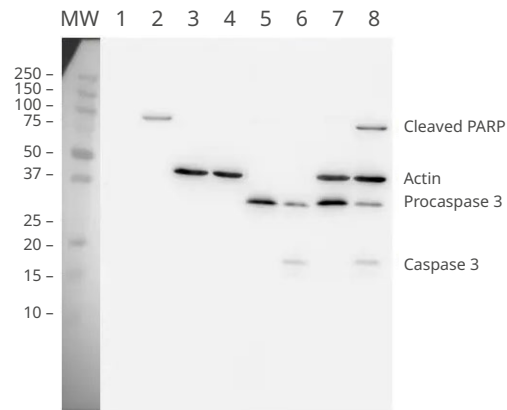

Figure 4. Detection of caspase-3 by western blot. HeLa cells were left untreated (lanes 1, 3, 5, 7) or treated with 1 μM staurosporine for 4 hours (lanes 2, 4, 6, 8). Apoptotic proteins (PARP and caspase-3) and loading control (actin) were detected with Apoptosis western blot cocktail (ab136812).

The antibody against caspase-3 used in Figure 4 recognizes an epitope found in both uncleaved (proenzyme) and cleaved (active form) caspase-3. Western blot analysis is the recommended assay for these antibodies as it allows differentiation of both forms and bands to be used as transfection controls (i.e. there has been no issue in the transfer process).

To determine caspase-3 activation by immunostaining (Figure 5), it is more appropriate to use an antibody that only detects the cleaved active form so that only apoptotic cells are stained.
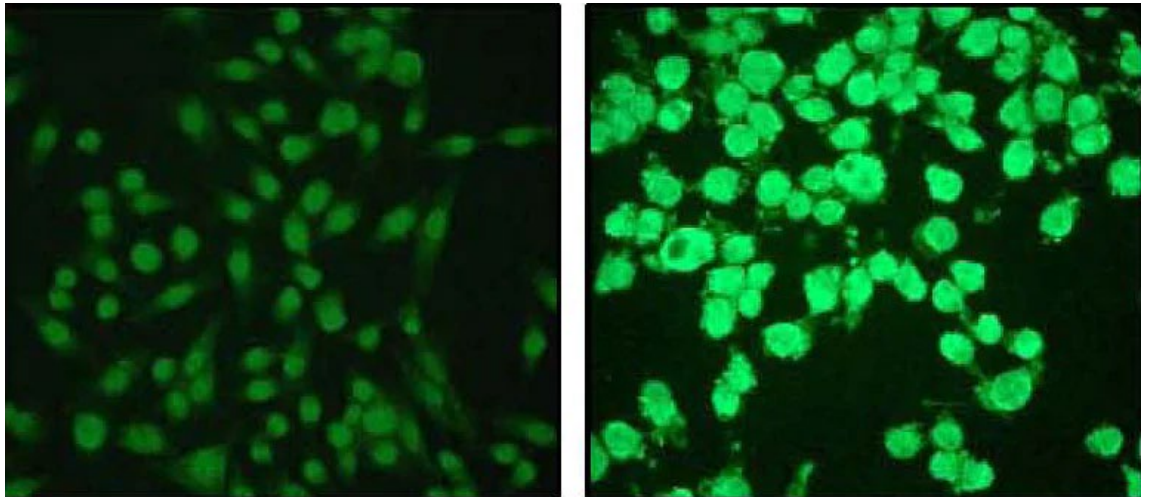

Figure 5. Detection of active caspase-3 by immunofluorescence. HeLa cells were left untreated (left) or treated with 1 μM staurosporine for 4 hours (right) and stained with Active Caspase 3 antibody [E83-77] (ab32042) at 1:100 dilution.

<u>Substrate-based methods</u>

Biochemical substrates consist of short peptides containing specific cleavage sequences that are recognized by the caspase and covalently attached to a colorimetric or fluorogenic detection probe. Upon cleavage of the substrates by the cognate caspase, the colorimetric or fluorogenic compound is liberated, producing an increase in absorbance (colorimetric substrate) or fluorescence light (fluorogenic substrate). The resulting signal is proportional to the amount of caspase activity present in the sample; however, many cleavage sites are similar and can be cleaved by other caspases. For example, caspase-3 and -7 have very similar cleavage sites. In these situations, using specific activators and/or inhibitors of caspase activity is essential to determine the correct activity.

Another important consideration when looking at caspase activation is ensuring the specific caspase is activated in the chosen sample. For example, the breast cancer cell line MCF-7 lacks a functional caspase-3. Therefore, determination of caspase-7 activity would be preferable.

Calpain and cathepsins

Caspases are the main proteases involved in apoptosis. However, other proteases, such as calpains and cathepsins, also contribute to the regulation of apoptosis in some specific cellular systems.

Calpains are non-lysosomal calcium-dependent cysteine proteases composed of one or two subunits. Calpain cleavage sites are not sequence-specific, and tertiary structure elements, rather than primary amino acid sequences, appear responsible for directing cleavage to a specific site. In endothelial cells, activated calpain-1 cleaves Bid, leading to cytochrome c release. In cardiomyocytes, calpain-1 has been shown to activate caspase-3 and PARP following TNF induction, although the mechanism by how they regulate apoptosis remains unclear[9].

Calpain activity can be easily detected in many cell types using a specific calpain substrate linked to a colorimetric or fluorogenic detection molecule that will be released upon cleavage of the substrate. There are, however, a few considerations to keep in mind when investigating apoptosis-related calpain activity:

— Ensure that there is no contamination from protease-rich lysosomes or other organelles that would give a false positive.
  – Consider using inhibitors to lysosomal proteases (such as cathepsins) to limit nonspecific activity.

— Use caspase inhibitors such as z-FA-FMK in your study to differentiate between the activities of the different cysteine proteases.

— Use cells treated with calpain-specific inhibitors as negative controls to ensure the activity you are measuring is from calpain.

— Calpain is likely to be active at some level in the cell, so use the appropriate background controls (untreated cells) to provide a baseline for calpain activation.

— Calpain activity itself is not an indication of apoptosis, as calpain has other functions and is activated during necrosis.
  – Ensure that you are using appropriate controls.

Cathepsins are proteases found in all animals. While most cathepsins are cysteine proteases (cathepsins B, F, K, L, and S), cathepsin D is an aspartic protease, and cathepsin G is a serine protease. Cathepsins are generally found in lysosomes. They become activated at the low pH found in this organelle and have been historically associated with necrosis. Some cathepsins remain active at neutral pH (in the cytosol)[10] and are associated with apoptosis signals such as caspase-8 activation through TNF alpha[11]. However, cathepsins can cause cell death without the involvement of the mitochondria or in a caspase-independent manner[12].

Like caspase and calpain activity, cathepsin activity can be easily detected in many cell types using a specific cathepsin substrate linked to a colorimetric or fluorogenic detection molecule that will be released upon cleavage of the substrate.

Here are a few things to keep in mind when investigating apoptosis-related cathepsin activity:

— Use specific cathepsin inhibitors as negative controls to ensure the activity you are measuring is cathepsin-specific.

— Use caspase inhibitors in your study to differentiate between the activities of the different cysteine proteases.
   – Unfortunately, some general caspase inhibitors (such as z-FA-FMK) can also inhibit cathepsin activity so be sure you include cathepsin inhibitors as controls.

— Cathepsins are also activated during necrosis, and cathepsin activation is not a unique indication of apoptosis, so make sure that you are using appropriate controls and appropriate cell lines to give context to your study.

Mitochondrial transmembrane potential

A distinctive feature of apoptosis is the disruption of normal mitochondrial function, especially changes that affect the mitochondrial (trans)membrane potential ($\Delta\Psi$m). $\Delta\Psi$m is critical for maintaining the physiological function of the respiratory chain to generate ATP. Opening the mitochondrial permeability transition pore (MPTP) leads to the collapse of the $\Delta\Psi$m and subsequent release of cytochrome c into the cytosol.

Mitochondrial membrane potential is commonly detected using cationic (positively-charged) fluorescent dyes that accumulate in the negatively-charged mitochondrial matrix. The dye accumulates in inverse proportion to $\Delta\Psi$m: the more negative the $\Delta\Psi$m, the more dye accumulates. This means that a healthy cell will contain more dye while an apoptotic cell will contain less.

These dyes can be used qualitatively with fluorescence microscopy or quantitatively in flow cytometry or microplate spectrophotometry[13]. Table 3 highlights some of the mitochondrial membrane potential probes available and the applications they are most suitable for.

Table 3. Mitochondrial membrane potential probes.

Probe	Description	Visualization	Ex/Em (nm)	Best to use for
TMRE/TMRM (tetramethylrhodamine, ethyl ester/tetramethylrhodamine, methyl ester)	Cationic red-orange dye that readily accumulates in active mitochondria Depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE.	Healthy cells: bright orange fluorescence. Apoptotic cells: weak orange fluorescence.	Ex = 549 Em = 575	Time-lapse fluorescence microscopy and immunofluorescence staining.
	Cationic green-red dye that exhibits potential-dependent accumulation in mitochondria. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.	Healthy cells: red fluorescence. Apoptotic cells: green fluorescence.	Ex = 530 Em = 530–570	Flow cytometry or microplate spectrophotometry.
	JC-10, a derivative of JC-1, has improved solubility in aqueous media and the ability to detect subtle changes in mitochondrial membrane potential loss.	Healthy cells: orange fluorescence. Apoptotic cells: green.	Ex = 490 Em = 520–570	Ideal for comparative measurements.
MitoOrange dye	Cationic dye. In normal cells, the red fluorescence intensity increases when dye accumulates. In apoptotic cells, the fluorescence intensity of the dye decreases following the collapse of the ΔΨm.	Healthy cells: red fluorescence. Apoptotic cells: weak red fluorescence.	Ex = 540 Em = 590	Flow cytometry or microplate spectrophotometry.
MitoNIR dye		Healthy cells: nearinfrared (NIR) fluorescence. Apoptotic cells: weak NIR fluorescence.	Ex = 635 Em = 660	Multiparametric study of apoptosis.

FCCP (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) is an ionophore uncoupler of oxidative phosphorylation. Treating cells with FCCP depolarizes mitochondrial membrane potential, making FCCP a very good positive control for these types of studies.

Cytochrome c release

The collapse of the $\Delta\Psi$m is a reasonably catastrophic event that leads to the opening of the MPTP and the subsequent release of cytochrome c into the cytosol. Some studies suggest that cytochrome c can be released independently of the MPTP[13–15], although the importance and mechanism of action of this event is unclear. Once the mitochondrial pores are opened and cytochrome c is released, the apoptotic cascade reaches a "point of no return" from which it is very unlikely that the cell can recover, meaning death is the most likely outcome.

The most common technique for detecting cytochrome c release is western blot on protein extracted from different subcellular compartments. It is crucial to ensure that the different subcellular fractions are not contaminated with other fractions. This can be easily checked with specific and reliable subcellular markers:

— Cytoplasmic markers: GAPDH, actin

— Mitochondrial markers: VDAC1, PDH-E1

Douglas Green's laboratory created a GFP-cytochrome c protein that enables time-lapse fluorescence microscopy of live cells to follow cytochrome c release from the mitochondria in different cell types during apoptosis[16]. However, a similar result can be obtained by using immunofluorescence staining of fixed cells with a cytochrome c antibody to visualize cytochrome c release from the mitochondria into the cytoplasm.

There are few things to consider when studying cytochrome c release from the mitochondria:

— Timing: perform pilot studies to determine which time points work best for your cell type.

— Control: if you want to show that cytochrome c release is due to specific apoptotic stimuli and not a secondary event related to mitochondrial dysfunction, make sure you use a proper positive control (e.g., a cell line where this process has been shown) and a negative control (e.g., a biochemical that inhibits this process).

Chromatin condensation

During apoptosis, chromatin undergoes a phase change from a heterogeneous, genetically active network to an inert, highly condensed form. When stained with DNA-binding nuclear dyes, the compacted chromatin will be brighter than the chromatin from non-apoptotic cells, and the condensed nuclei can be easily identified by fluorescence microscopy (qualitative detection) or flow cytometry (quantitative detection).

Genomic DNA fragmentation

Condensed chromatin can be fragmented by a specific nuclease called caspase-activated DNase (CAD). Activation of CAD by the caspase cascade leads to cleavage of the DNA at the internucleosomal linker sites between the nucleosomes, generating fragments of ~ 200 base pairs known as DNA ladders.

Apoptosis DNA fragmentation analysis protocol

A. Harvest cells

1. Pellet cells.

2. Lyse cells in 0.5 mL detergent buffer: 10 mm Tris (pH 7.4), 5 mm EDTA, 0.2% Triton.

3. Vortex.

4. Incubate on ice for 30 minutes.

5. Centrifuge at 27,000 xg for 30 minutes.

6. Divide supernatants into two 250 µL aliquots.

7. Add 50 µL ice-cold 5 M NaCl to each followed by vortexing.

B. Precipitate DNA

8. Add 0.6 mL 100% ethanol and 150 µL 3 M Na acetate, pH 5.2, and mix by pipetting up and down.

9. Place at -80°C freezer for 1 hr.

10. Centrifuge 20,000 xg for 20 min.

11. Pool DNA extracts by re-dissolving by adding 400 µL (total) of Extraction buffer (10 mM Tris and 5 mm EDTA).

12. Add 2 µL (10 mg/mL) DNase free RNase. Incubate for 5 hr at 37°C.

13. Add 25 µL Proteinase K at 20 mg/mL and 40 µL of buffer (100 mm Tris, pH 8.0, 100 mM EDTA, 250 mM NaCl. Incubate overnight at 65°C.

14. Extract DNA with phenol: chloroform: isoamyl alcohol (25:24:1) and precipitate with ethanol.

C. Load DNA in agarose gel

15. Air-dry pellet and resuspend in 20 µL Tris-acetate EDTA buffer supplemented with 2 µL of sample buffer (0.25% bromophenol blue, 30% glyceric acid).

16. Separate DNA electrophoretically on a 2% agarose gel containing 1 µg/mL ethidium bromide and visualized under ultraviolet transillumination.

Tips on how to assay

— The DNA in the sample will make it very viscous and sticky. Use the DNA sample loading buffer at a higher concentration than you normally would to ensure the sample doesn't float away from the well. For example, use 10 µL 10X loading buffer in 50 µL to compensate for the higher viscosity.

— Prepare an agarose gel with 1.8–2% agarose. The high agarose concentration provides the necessary resolution to see the steps in the ladder.

— Run the gel at lower voltage for a longer time than you normally would to avoid overheating of the gel and subsequent deformation of the DNA bands.

The classic method to detect DNA ladders is to examine fragmented genomic DNA on an agarose gel[17]. This semi-quantitative method is falling out of use, but it is a simple technique that provides a robust answer.

An alternative method for detecting DNA fragmentation involves indentifying nicks (or strand breaks) using a The TdT dUTP nick end labeling (TUNEL) assay. The TUNEL technique is based on the ability of terminal deoxynucleotidyl transferase (TdT) to end-label nicks in the DNA brought about by caspase-activated deoxyribonuclease. TdT can label 3' blunt ends of double-stranded DNA with deoxyuridine, independently of a template[18].

The most common detection methods for TUNEL assay are flow cytometry and fluorescence microscopy.

Increase of sub G1 population

An increase in the percentage of cells stalled at G1 is another consequence of DNA fragmentation and can be readily detected with a flow cytometer. Once cells are permeabilized with, for example, a 70% ethanol solution, the DNA fragments will leak out and create a population of cells with reduced DNA content.

When cells are stained with a DNA dye such as propidium iodide (PI), a DNA profile representing cells in the different stages of the cell cycle (G1, S-phase, and G2M) can be observed by flow cytometry. The apoptotic cells are easily identified as the subG1 population seen to the left of the G1 peak. As the subG1 fraction will include all the dead cells in the population regardless of the type of cell, this parameter on its own is not a good indicator of apoptosis in a population that includes cells dying of other causes.

Flow cytometry analysis of cell cycle with propidium iodide

Method

1. Harvest the cells in the appropriate manner and wash in PBS.

2. Fix in cold 70% ethanol.
   – Add drop-wise to the pellet while vortexing as this should ensure fixation of all cells and minimize clumping.

3. Fix for 30 min at 4°C.

4. Wash 2 X in PBS.
   – Spin at 850 xg in a centrifuge and be careful to avoid cell loss when discarding the supernatant especially after spinning out of ethanol.

5. Treat the cells with ribonuclease.
   – Add 50 µL of a 100 µg/mL sock of RNase to ensure only DNA, not RNA, is stained.

6. Add 200 µL PI (from 50 µg/mL stock solution).

Analysis of results

— Measure the forward scatter (FS) and side scatter (SS) to identify single cells.

— Pulse processing is used to exclude cell doublets from the analysis. This can be achieved either by using pulse area vs. pulse width or pulse area vs pulse height depending on the type of cytometer.

— PI has a maximum emission of 605 nm so can be measured with a suitable bandpass filter.

Cell membrane blebbing

Together with DNA fragmentation, the final execution phase of apoptosis is characterized by dynamic membrane blebbing and cell contraction[19]. During apoptosis, the cell cytoskeleton breaks up, causing some parts of the cell membrane to bulge outwards. The bulges eventually separate from the cell, taking a portion of the cytoplasm with them and forming apoptotic bodies.

Membrane blebbing can be observed in live cells using phase-contrast microscopy. If you cannot use live cells or would like to use cells that you have prepared for studying other parameters (for example, cells harvested for DNA fragmentation quantification or chromatin condensation), you can detect caspase substrates associated with apoptotic membrane blebbing[20]. However, note this is an indirect method and it may give you false positive/negative results.

Caspase substrates associated with apoptotic membrane blebbing:

— Gelsolin

— ROCK-1 kinase

— P21-activated kinase (PAK)

Tips for apoptosis assays

There are several factors that can affect how successful your assays will be for detecting apoptosis.

Using apoptosis inducers and inhibitors

The use of activators or inhibitors for specific steps of the apoptosis cascade is essential for providing positive and negative controls. These ensure that the assay worked and confirm that apoptosis is occurring.

The table below highlights some known apoptotic inducers and inhibitors for functions linked to cell death.

Table 4. Highlighted apoptosis inducer and inhibitor compounds.

Apoptosis inducers	Biological description
Apoptosis Activator 2 (AAII) (ab141227)	Cytochrome c, caspase activator, and apoptosis inducer
Cycloheximide (ab120093)	Protein synthesis inhibitor
Mitomycin C (MMC) (ab120797)	Anticancer and antibiotic agent
Oxaliplatin (ab141054)	Platinum anticancer agent. Induces DNA cross-linking
CPT 11 (Irinotecan) (ab141107)	Potent DNA topoisomerase I inhibitor
Camptothecin (ab120115)	DNA topoisomerase inhibitor
Staurosporine (ab120056)	Protein kinase inhibitor
Apoptosis inhibitors	
z-VAD-(OMe)-FMK (ab120487)	Cell permeable, irreversible general caspase inhibitor
z-D(OMe)E(OMe)VD(OMe)-FMK (ab120488)	Cell permeable caspase-3 inhibitor
z-IETD-FMK (ab141382)	Caspase-8 inhibitor
Pifithrin-μ (ab120886)	Inhibitor of p53-mediated apoptosis

Analyze single cells

The time required to detect different apoptotic events or activities after apoptosis induction is often variable. As shown in Figure 6, Jurkat cells treated with anti-Fas antibody showed activation of caspase-8 after four hours, whereas caspase-3 activation was detected after 20 hours.
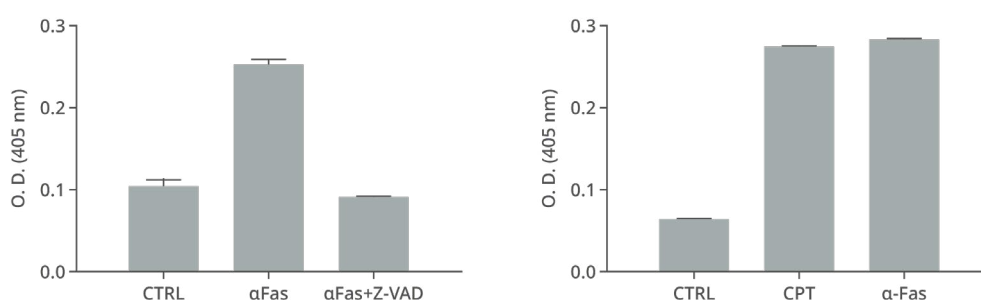

Figure 6. Initiator and executioner caspases have different activation times in Jurkat cells. Activation of caspase-8 was detected 4 hours after anti-Fas antibody treatment using Caspase 8 Assay Kit (Colorimetric) (ab39700) (left). Caspase-3 activation was detected 20 hours after anti-Fas treatment using Caspase-3 assay kit (Colorimetric) (ab39401) (right).

It is worth mentioning that activating caspases *in vitro* generally occurs at earlier time points than *in vivo*, and immortalized cells in culture will probably activate apoptosis at earlier time points than primary cells. Cells within a population will be in different phases of the cell cycle. This means individual cells within the same population will probably undergo apoptotic events at different times. For example, cells that have finished division will show G2/M arrest later than cells that have finished DNA replication (S phase). Therefore, it is important to analyze single cells whenever possible to track the activation of an apoptotic event.

Monitoring apoptotic events before or after the optimal time period will result in little or no signal, leading to the erroneous conclusion that the treatment did not induce apoptosis. Time course experiments are therefore essential to determine the optimal time to detect a given event.

Be aware of cell line variation

Different cell lines may respond differently to identical conditions, and certain treatments may induce apoptosis in one cell line but not others. Some apoptotic events might be underestimated because the cell line does not express a specific protein. For example, the breast cancer cell line MCF-7 lacks a functional caspase-3. Therefore, the determination of caspase activity using caspase-3 substrates or caspase-3 inhibitors will not yield any results.

In general, cultured cells treated with apoptosis-inducing stimuli will exhibit signs of apoptosis sooner than cells within tissues (scale of hours versus days). Some late apoptotic events may not even appear as apoptotic cells present in tissues tend to be phagocytized before these events can be detected.

General considerations

As we have mentioned throughout this guide, it is recommended to analyze more than one hallmark to identify apoptosis as a cause of death in the studied cell population. This is because several of these events can be observed in other types of cell death, such as necrosis or necroptosis.

Things to keep in mind:

— When choosing an apoptosis parameter to study, ensure that it is relevant to your experimental conditions.

— Perform an initial dose-response curve to identify the appropriate concentration of a compound and timing of the treatment for best results in your cells of interest.

— Minimize the number of processing steps to limit the possibility of losing dying cells.

— Published literature and protocols offer invaluable guidance but do not rely on them exclusively to set your experimental condition.

— Subtle lab-to-lab differences may affect your results.

Event	Detection method	Sample type
Loss of membrane asymmetry/PS exposure	Flow cytometry analysis of Annexin V binding	Live cells
	Time-lapse microscopy analysis of Annexin XII binding	Live cells
Changes in levels of anti-apoptotic Bcl-2 family proteins	Western blot assessment	Cell extracts
		Tissue extracts
	Flow cytometry/microscopy analysis of expression levels with antibodies	Fixed cells
Dimerization of anti-/ pro-apoptotic Bcl-2 family proteins	Immunoprecipitation	Cell extracts
		Tissue extracts
Caspase activation	Colorimetric/fluorometric substrate-based assays in microtiter plates	Cell extracts
		Tissue extracts
	Detection of cleavage of fluorometric substrate in flow cytometry/microscopy or by microtiter plates analysis	Live cells
	Western blot analysis of pro- and active caspase	Cell extracts
		Tissue extracts

	Flow cytometry/microscopy analysis with antibodies specifically recognizing the active form of caspases	Fixed cells
	Microplate spectrophotometry analysis with antibodies specifically recognizing the active form of caspases	Cell extracts
		Tissue extracts
Caspase substrate (PARP) cleavage	Microplate spectrophotometry analysis with antibodies specific for cleaved PARP	Cells extracts
		Tissue extracts
		Live cells (In-cell ELISA)
	Western blot analysis of cleaved PARP	Cells extracts
		Tissue extracts
Non-caspase proteases (cathepsins and calpain) activation	Colorimetric/fluorometric substrate-based assays in microtiter plates	Cells extracts
		Tissue extracts
Mitochondrial transmembrane potential (ΔΨm) decrease	Flow cytometry/microscopy/ microplate spectrophotometry analysis with MMP sensitive probes	Live cells
	Oxygen consumption studies	Live cells
Cytochrome c release	Western blot analysis of presence of cytochrome c in the cytosol	Cell extracts
		Tissue extracts
	Antibody-based microscopy analysis of presence of cytochrome c in the cytosol	Fixed cells
Increase of sub G1 population	Flow cytometry analysis of subG1 peak	Fixed cells
Nuclear condensation	Flow cytometry analysis of chromatin condensation	Live cells
	Microscopy analysis of chromatin condensation	Live cells
DNA fragmentation	Analysis of DNA ladder in agarose gel	DNA
	Analysis of DNA fragmentation by TUNEL	Live cells
Membrane blebbing	Light microscopy analysis of membrane blebbing	Live cells
	Western blot analysis of cleaved substrate (gelsolin, ROCK1)	Cell extracts
		Tissue extracts

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