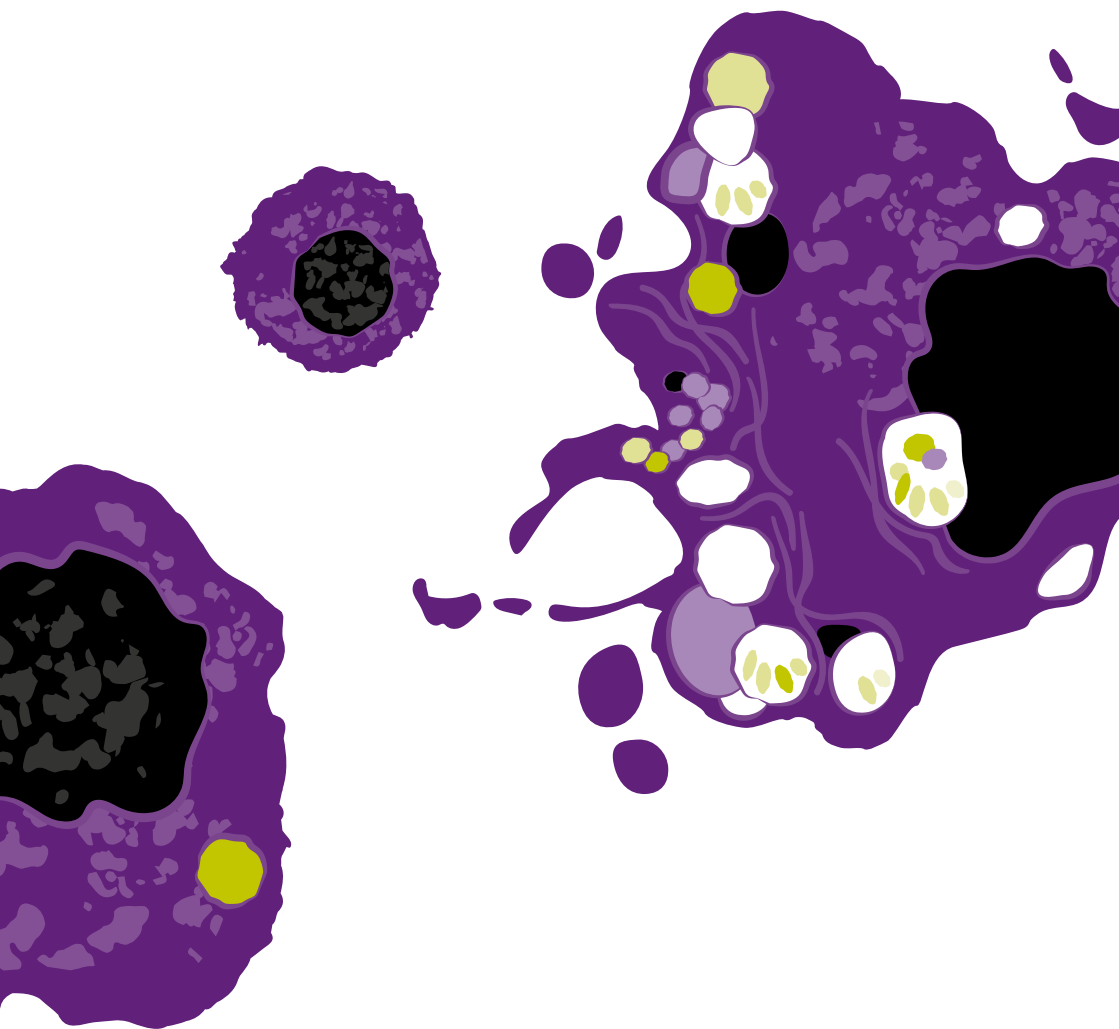


Apoptosis



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Cell Death

Cell death happens when a biological cell fails to maintain essential life functions. This may be the result of the natural process of development or protection against cellular defects, of normal cells replacement, or factors such as disease or localized injury.

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

It can be difficult sometimes to know which cellular and biochemical parameters should be measured when studying cell death. In this guide we aim to provide you with an overview of the most common parameters that researchers measure to assess apoptosis, a particular form of cell death, and tools you can use to detect apoptosis in your research.

Apoptosis

Apoptosis is a form of programmed cell death and is critical for numerous normal physiological processes including cellular homeostasis, embryogenesis and post-embryonic development. Apoptosis is characterized by several biochemical features including cell shrinkage, membrane blebbing, chromosome condensation (pyknosis), nuclear fragmentation (karyohexis), 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*.


Apoptosis and necrosis represent two extremes of cell death, with a full range of variations in between. There are other forms such as necroptosis or autophagy, which share some of the characteristics of either form. As cell death can occur by several different paths, both morphologically and biochemically, researchers need to examine multiple biochemical markers at carefully selected time points to determine the mechanism of cell death in their particular experimental system.

Apoptotic mechanism

During apoptosis, a family of cysteine-aspartate proteases known as caspases accelerates cell death through restricted proteolysis of over 400 proteins. The two main pathways through which cell death is initiated are the intrinsic and extrinsic cell death pathways, both of which converge on caspase activation.

The intrinsic cell death pathway is governed by the Bcl-2 family of proteins, which regulate commitment to cell death through the mitochondria. A myriad of 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, release of various proteins from the mitochondrial intermembrane space promotes caspase activation and apoptosis. Cytochrome C binds apoptosis protease-activating factor 1 (APAF-1), inducing its oligomerization and thereby forming a structure called the apoptosome that recruits and activates an initiator caspase, caspase 9. Caspase 9 cleaves and activates the executioner caspases, caspase 3 and 7, leading to apoptosis.

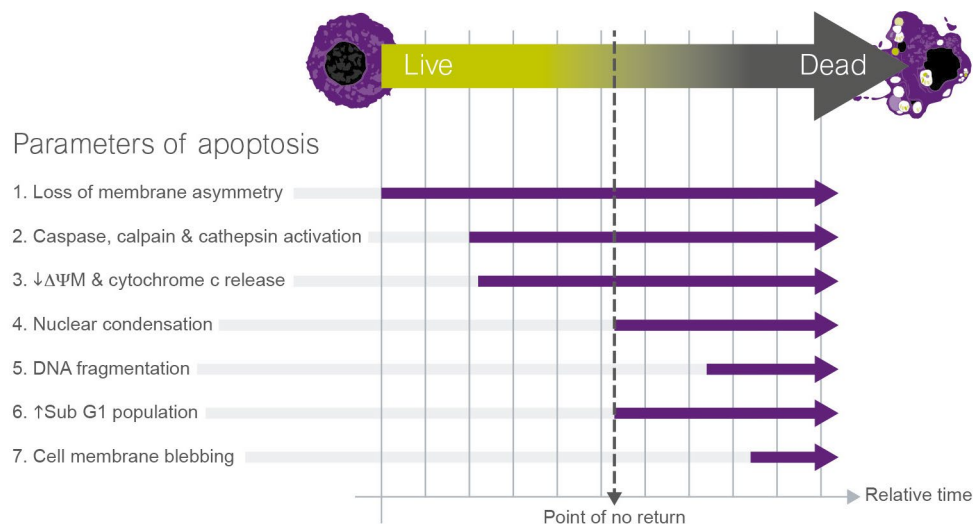
Activation of the extrinsic cell death pathway occurs following the binding on the cell surface of “death receptors” to their corresponding ligands such as Fas, TNFR1 or TRAIL. These death receptors have two distinct signaling motifs, death domains (DD) and death effector domains (DED), that allow them 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.

 [Click here to download your apoptosis pathway card](#)

Crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8 cleavage and activation of the BH3-only protein BH3-interacting domain death agonist (BID), the product of which (truncated BID, known as tBID) is required in some cell types for death receptor-induced apoptosis.

Parameters of Apoptosis

Apoptosis occurs via a complex signaling cascade that is tightly regulated at multiple points, providing many opportunities to evaluate the proteins involved. The image below shows the main parameters of apoptosis and the approximate relative time when they are likely to be detected:



These parameters do not happen in a sequential order, and many of them will overlap and occur at the same time. Loss of membrane asymmetry or initiation of caspase cascade are biochemical features of apoptosis which do not necessarily lead to cell death. However, other downstream features such as decrease of the mitochondrial membrane potential ($\Delta \Psi m$) and concomitant release of cytochrome C into the cytosol, are generally considered points of no return, after which it is very unlikely the cell will survive.

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

Methods and Technologies for Detecting Apoptosis

Loss of membrane asymmetry

The loss of cellular membrane asymmetry is an early sign of apoptosis, wherein embedded phosphatidylserine (PS) residues in the 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 in particular is a commonly used tool to detect and quantify the PS exposure characteristic loss of membrane asymmetry. Often, annexin V binding is paired with cell viability reagents such as propidium iodide (PI) that are normally not able to penetrate the plasma membrane to differentiate between apoptotic and necrotic cells.

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

Annexin V protocol to detect apoptosis:

A. Incubation of cells with annexin V-FITC

1. Induce apoptosis by desired method.
2. Collect $1-5 \times 10^6$ 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, optional).
5. Incubate at room temperature for 5 min in the dark.
6. Proceed to B or C below depending on method of analysis.

B. Quantification by flow cytometry

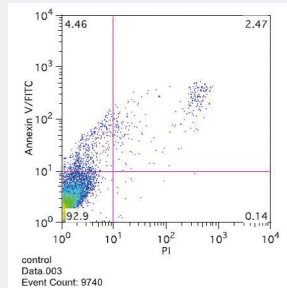
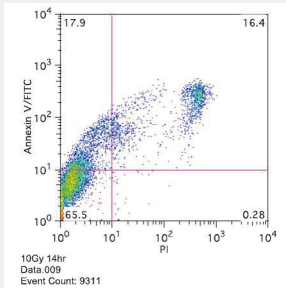
Analyze annexin V-FITC binding by flow cytometry (Ex = 488 nm; Em = 350 nm) using FITC signal detector (usually FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2). For adherent cells, gently trypsinize and wash cells once with serum-containing media before incubation with annexin V-FITC (Steps A.3–5).

C. Detection by 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. Following incubation (Step A.5), 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).
2. Observe the cells under a fluorescence microscope using a dual filter set for FITC & 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).

+ Product highlight:

Annexin V-FITC Apoptosis Detection Kit (ab14085)



Detect PS exposure in only 10 minutes.

+ Click here to search the right annexin assay for you

Apoptosis-related proteases: Caspases, calpain and cathepsins

Caspases

The caspase family of highly conserved cysteine proteases 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 rather involved in inflammatory cytokine signaling.

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 depending on the instrumentation available and how the sample has been prepared. This table outlines the most common methods to detect caspase activation:

Table 1: Caspase activation detection

Sample type	Detection method	Best to use when you want to ...	Highlighted Products
Live cells (suspension or adherent)	Flow Cytometry	Quickly detect and quantify how many cells have active caspase using a specific substrate	ab112130
Fixed cells (suspension or adherent)	Fluorescence Microscopy	Visualize which cells have active caspase. Commonly used when you want to visualize other proteins at the same time	ab65613
	Flow Cytometry	Quickly detect and quantify how many cells have active caspase using a specific antibody	ab65613
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	ab2324 ab32042 ab136812 ab131385
	Absorbance/ Fluorescence Assay	Quickly detect caspase activation in a cell population using a specific substrate. Easily adaptable for HTP analysis	ab39401 ab39383
	Absorbance/ Fluorescence Assay	Quickly detect caspase activation in a cell population using a specific antibody against the active form	ab181418 ab173187 ab168541 ab154469 ab119507 ab119508
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)	ab2302

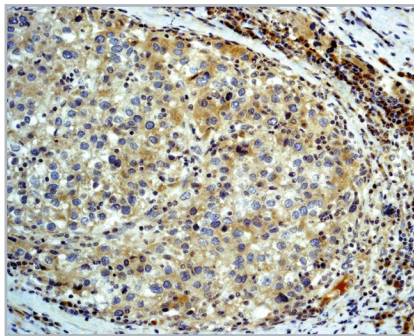
The answer provided by the different experimental methods will confirm whether one or more specific caspases are active or inactive. It is always best practice to use more than one method to confirm specific caspase activation.

Antibody-based methods [flow cytometry, microscopy, western blot and immunostaining]

The ability to detect active caspase relies on the specificity of the antibody and where the epitope is located. If the antibody recognizes an epitope found in both the pro- and active forms of the caspase, you will only be able to differentiate both forms by visualization in Western blot. Therefore, it is key to choose the right antibody for the right method.



The image above shows HeLa cells untreated (lanes 1, 3, 5, 7) or treated (lanes 2, 4, 6, 8) with 1 μ M staurosporine for 4 hours. Apoptotic proteins (PARP and caspase 3) and a loading control (actin) were detected with Apoptosis Western Blot Cocktail (ab136812). The antibody against caspase 3 can detect both uncleaved (proenzyme) and cleaved (active form) caspase 3.

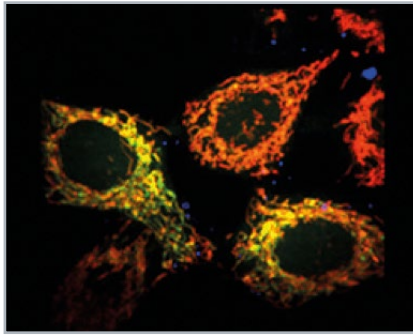


On the other hand, to determine caspase 3 activation by immunostaining (image above), it would be more appropriate to use an antibody that detects the cleaved active form only so that only apoptotic cells are stained.

Substrate-based methods [flow cytometry, microscopy, absorbance/fluorescence assay]

Biochemical substrates consist of short peptides that contain a specific cleavage sequences that are recognized by the caspase and are 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 of the 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, the use of specific activators and/or inhibitors of caspase activity is essential to ensure determination of the correct activity.

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



[+](#) Click here to view the Caspase activation webinar

In this webinar, Dr Baucher-Hayes presents a new method to look at caspase activation by FRET using caspase substrates linked to fluorescent proteins. This allows the detection of caspase activity in live cells.

Calpain and Cathepsins

Inhibition of caspases only results in a delay and not a complete inhibition of apoptosis, highlighting the essential role of non-caspase proteases such as calpains and cathepsins.

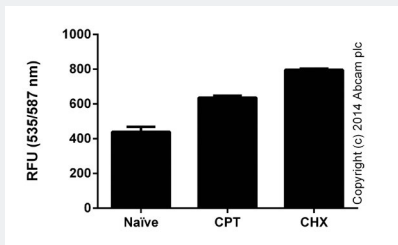
Calpains are cytosolic calcium-dependent cysteine proteases composed of one or two subunits. Calpain cleavage sites are not sequence-specific, and tertiary structure elements rather than primary amino acid sequences seem to be responsible for directing cleavage to a specific site. A broad range of proteins are calpain substrates, including cytoskeletal proteins such as α -fodrin, ion channels, growth factor receptors and adhesion molecules. Calpain activation has been implicated in neuronal apoptosis in ischemic brain injury and neurodegenerative diseases such as Alzheimer's.

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 trying to investigate 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.
- Similarly, it is a good idea to use caspase inhibitors (such as **z-FA-FMK**) in your study to be able 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. Make sure that you are using appropriate controls.

+ Product highlight:

Calpain activity assay (ab65308)



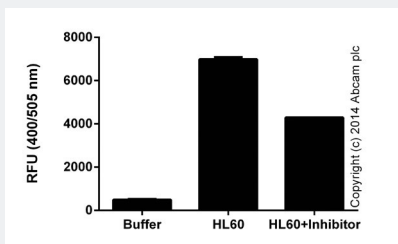
Calpain activity measured in Jurkat cells in the absence (naive) or presence of 10 μ M Camptothecin (CPT) or 10 μ g/mL Cyclohexamide (CHX) for 4 hours.

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 a serine protease. Cathepsins are generally found in lysosomes. They become activated at the low pH found in this organelle, and thus have been historically associated with necrosis. Some cathepsins remain active at neutral pH (in the cytosol), and are associated with apoptosis signals such as caspase 8 activation through TNF alpha.

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, which will be released upon cleavage of the substrate.

+ Product highlight:

Cathepsin B activity assay kit (fluorometric) (ab65300)



Sample: Cell extracts, tissue extracts

Assay time: 2 hours

Quantification of basal Cathepsin B activity in HL60 cell lysates in absence or presence of Cathepsin B inhibitor.

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. Make sure that you are using appropriate controls and appropriate cell line to give context to your study.

 [Click here to view our range of Cathepsin activity kits](#)

Mitochondrial transmembrane potential ($\Delta\Psi_m$)

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 in fluorescence microscopy or quantitatively in flow cytometry or microplate spectrophotometry. The table below highlights the mitochondrial membrane potential probes available from Abcam:

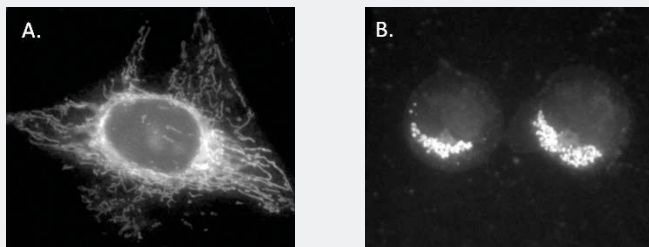
Table 2: Mitochondrial membrane potential probes

Probe	Healthy/apoptotic cells	Ex/Em	Best to use for:
TMRE (tetramethylrhodamine, ethyl 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 nm Em = 575 nm	Time-lapse fluorescence microscopy and immunofluorescence staining.
JC-1 = 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. Apoptotic cells: green.	Ex=530 nm Em=590 nm	Flow cytometry or microplate spectrophotometry. Ideal for comparative measurements.
JC-10 = a derivative of JC-1, has improved solubility in aqueous media and the ability to detect subtler changes in mitochondrial membrane potential loss.	Healthy cells: orange fluorescence. Apoptotic cells: green.	Ex=490 nm Em = 520 -570 nm	Flow cytometry or microplate spectrophotometry. Ideal for comparative measurements.
MitoOrange dye and MitoNIR dye = cationic dyes. In normal cells, the fluorescence intensity increases when dye accumulates in normal cells. In apoptotic cells, the fluorescence intensity of the dye decreases following the collapse of the $\Delta\Psi_m$.	Healthy cells: red or near infrared (NIR) fluorescence. Apoptotic cells: weak fluorescence.	MitoOrange: Ex=540 nm Em=590 nm MitoRed: Ex=635 nm Em = 660nm	Flow cytometry or microplate spectrophotometry. Multiparametric study of apoptosis.

FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) is a ionophore uncoupler of oxidative phosphorylation. Treating cells with FCCP eliminates mitochondrial membrane potential, which makes FCCP a very good positive control for these type of studies.

Product highlight:

TMRE – Mitochondrial Membrane Potential Assay Kit (ab113852)



HeLa (A) and Jurkat cells (B) were stained with 200 nM TMRE for 20 minutes and immediately imaged.

Cytochrome C release

The collapse of the $\Delta\Psi_m$ is a fairly catastrophic event as it leads to the opening of the mitochondrial permeability transition pores in the mitochondrial membrane and the subsequent release of cytochrome C in the cytosol, which in turn triggers other downstream events in the apoptotic cascade.

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 and death is the most likely outcome.

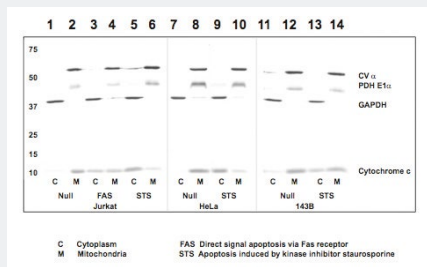
The most common technique to detect cytochrome C release is through western blot on protein extracted from different subcellular compartments. It is very important in this case to ensure that the different subcellular fractions are not contaminated with other fractions. This can be easily checked with specific and reliable subcellular markers:

- Cytoplasmic markers: [GAPDH](#), [actin](#)
- Mitochondrial markers: [VDAC1](#), [PDH-E1](#)

 [Click here to learn more about organelle controls](#)

+ Product highlight:

Apotrack™ Cytochrome C Apoptosis WB Antibody Cocktail (ab110415)

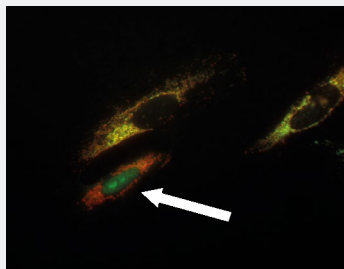


This is a western blot antibody cocktail that allows the detection of cytochrome C in cytoplasmic and mitochondrial fractions. This product contains a set of organelle control markers to help you monitor and optimize your fractionation protocol.

Douglas Green's laboratory has 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. This tool, however, might not be available for all researchers. A similar, if less elegant, result can be obtained by using immunofluorescence staining of fixed cells with a cytochrome C antibody at selected time points to visualize cytochrome C release from the mitochondria into the cytoplasm.

+ Product highlight:

Apotrack™ Cytochrome C Apoptosis ICC Antibody Cocktail (ab110417)



HeLa cells treated with staurosporine were stained with anti-cytochrome C antibody (green) and anti-ATP synthase subunit alpha antibody (red). The white arrow indicates cytochrome C release from mitochondria.

+ Click here to view our antibodies for cytochrome C detection

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 link cytochrome C release to apoptosis due to a specific stimulus, 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., use of a biochemical process).

Nuclear condensation, DNA fragmentation and increase of subG1 population

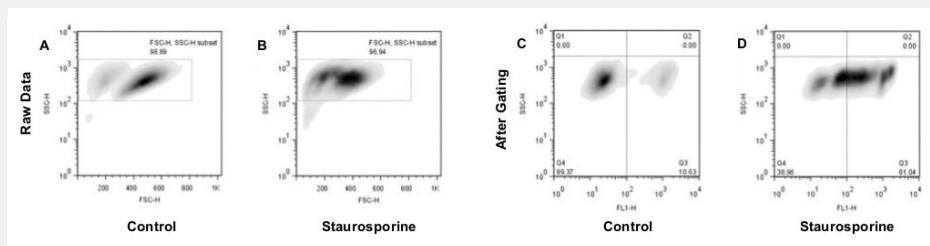
Chromatin condensation and genomic DNA fragmentation, together with cell membrane blebbing, are considered morphological hallmarks of the terminal stages of apoptosis.

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) and/or flow cytometry (quantitative detection).

+ Product highlight:

Nuclear Condensation Assay Kit (ab139479)



Flow cytometry analysis of untreated and staurosporine-treated Jurkat cells. The raw data shown in panels A and B (left) was used for gating cell populations (rectangles) for analysis. Panels C and D (right) represent the separation of healthy and apoptotic nuclei based on their characteristic fluorescence.

+ Click to select the right nuclear dye for your experimental setting

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 specific cleavage of the DNA at the internucleosomal linker sites between the nucleosomes, generating fragments of ~ 200 base pairs known as DNA ladders.

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

Apoptosis DNA fragmentation analysis protocol:

Procedure:

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 x g for 30 minutes
6. Divide supernatants into 2 – 250 μ L aliquots
7. Add 50 μ L ice cold 5 M NaCl to each followed by vortexing.

B) Precipitate DNA

8. Add 0.6 mL 100% EtOH and 150 μ L 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 x g for 20 min
11. Pool DNA extracts by re-dissolving by adding 400 μ L (total) of Extraction buffer (10 mM Tris and 5 mM EDTA).
12. Add 2 μ L (10 mg/ml) DNase free RNase. Incubate for 5 hr at 37°C .
13. Add 25 μ L Proteinase K at 20 mg/ml and 40 μ L 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 EtOH.

C) Load DNA in agarose gel

15. Air-dry pellet and resuspend in 20 μ L Tris-acetate EDTA buffer supplemented with 2 μ L of sample buffer (0.25% bromphenol blue, 30% glyceric acid).
16. Separate DNA electrophoretically on a 2% agarose gel containing 1 μ g/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 does not 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.

An alternative method for detecting DNA fragmentation involves the identification of nicks (or strand breaks) using a TUNEL assay. The TdT dUTP nick end labelling (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 has the ability to label 3' blunt ends of double stranded DNA with deoxyuridine, independently of a template.

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

Increase of subG1 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 staining dye such as 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

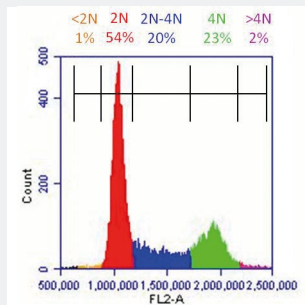
1. Harvest the cells in the appropriate manner and wash in PBS.
2. Fix in cold 70% ethanol. Add drop wise to the pellet while vortexing. This should ensure fixation of all cells and minimize clumping.
3. Fix for 30 min at 4°C.
4. Wash 2 X in PBS. Spin at 850 g in a centrifuge and be careful to avoid cell loss when discarding the supernatant especially after spinning out of ethanol.
5. Treat the cells with ribonuclease. Add 50 μl of a 100 $\mu\text{g}/\text{ml}$ stock of RNase. This will ensure only DNA, not RNA, is stained.
6. Add 200 μl PI (from 50 $\mu\text{g}/\text{ml}$ 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 it can be measured with a suitable bandpass filter.

+ Product highlight:

A healthy population of HeLa cells analyzed with Propidium Iodide Flow Cytometry Kit (ab139418)



Flow cytometer software was used to establish markers on a histogram plot to quantify the percentage of cells with <2N (dead cells), 2N (G1 phase), 2N-4N (S phase), 4N (G2 phase) and >4N (M phase) content.

Nuclear cell membrane blebbing

Together with DNA fragmentation, the final execution phase of apoptosis is characterized by dynamic membrane blebbing and cell contraction. 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 what are known as apoptotic bodies.

Membrane blebbing can be observed in live cells using phase-contrast microscopy. If you are not able to 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. Note, however, that 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)

General considerations

As mentioned elsewhere in this document, it is recommended to analyze more than one parameter to identify apoptosis as a cause of death in the studied cell population. This is because several of these parameters can be observed in other types of cell death such as necrosis or necroptosis.

Table 3: Summary biochemical and cellular parameters of apoptosis and detection method

Parameters	Detection methods	Sample type	Highlighted Products
Loss of membrane asymmetry/ PS exposure	Flow cytometry analysis of Annexin V binding	Live cells	ab14085
	Time-lapse microscopy analysis of Annexin XII binding	Live cells	ab129817
Cleavage of anti-apoptotic Bcl-2 family proteins	Western blot assessment of protein cleavage	Cell extracts	ab134001
		Tissue extracts	ab32370
Caspase activation	Colorimetric / fluorometric substrate-based assays in microtiter plates	Cell extracts	ab39383
		Tissue extracts	ab65607 ab39700
	Detection of cleavage of fluorometric substrate in flow cytometry / microscopy or by microtiter plates analysis	Live cells	ab112130 ab65614 ab65613
	Western blot analysis of pro- and active caspase	Cell extracts Tissue extracts	ab13847 ab138485 ab32539
	Flow cytometry / microscopy analysis with antibodies specifically recognizing the active form of caspases	Live cells (F) Fixed cells (M)	ab47537 ab2302
	Microplate spectrophotometry analysis with antibodies specifically recognizing the active form of caspases	Cell extracts Tissue extracts	ab157404 ab181418
Caspase substrate (PARP) cleavage	Microplate spectrophotometry analysis with antibodies specific for cleaved PARP	Cells extracts Tissue extracts Live cells (In cell ELISA)	ab174441 ab110215 ab140362
		Western blot analysis of cleaved PARP	Cell extracts Tissue extracts
Non-caspase proteases (cathepsins and calpain) activation	Colorimetric / fluorometric substrate-based assays in microtiter plates	Cell extracts Tissue extracts	ab65302 ab65300 ab65306 ab126780

Mitochondrial transmembrane potential ($\Delta\Psi_m$) decrease	Flow cytometry/microscopy /microplate spectrophotometry analysis with MMP sensitive probes	Live cells	ab113852 ab113850 ab112134
	Oxygen consumption studies	Live cells	ab197243
Cytochrome C release	Western blot analysis of presence of cytochrome C in the cytosol	Fixed cells	ab110415 ab65311 ab133504
	Antibody-based microscopy analysis of presence of cytochrome C in the cytosol	Fixed cells	ab110417 ab133594 ab13575
Increase of sub G1 population	Flow cytometry analysis of subG1 peak	Fixed cells	ab14083 ab139418
Nuclear condensation	Flow cytometry analysis of chromatin condensation	Live cells	ab139479 ab112151 ab115347
	Microscopy analysis of chromatin condensation	Live cells	ab14083 ab108410
DNA fragmentation	Analysis of DNA ladder in agarose gel	DNA	ab66090 ab65627 ab66093
	Analysis of DNA fragmentation by TUNEL	Live cells	ab66110 ab66108
Membrane blebbing	Light microscopy analysis of membrane blebbing	Live cells	
	Western blot analysis of cleaved substrate (gelsolin, ROCK1)	Cell extracts Tissue extracts	ab109014 ab13660

Tips for apoptosis assays

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

Use of apoptosis inducers and inhibitors

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

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

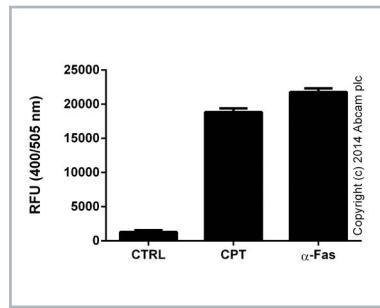
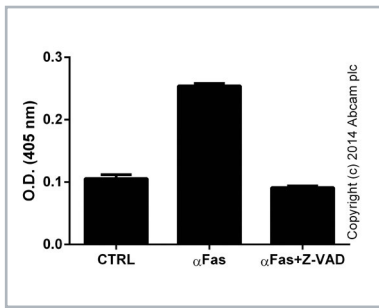
Table 4: Highlighted apoptosis inducer and inhibitor compounds

Product	Biological description
Apoptosis inducers	
Apoptosis Activator 2 (AAIL)	Cytochrome C, caspase activator and apoptosis inducer
Cycloheximide	Protein synthesis inhibitor
Mitomycin C (MMC)	Anticancer and antibiotic agent
Oxaliplatin	Platinum anticancer agent. Induces DNA cross-linking.
CPT 11 (Irinotecan)	Potent DNA topoisomerase I inhibitor
Camptothecin	DNA topoisomerase inhibitor
FCCP	Mitochondrial oxidative phosphorylation inhibitor
Apoptosis inhibitors	
z-VAD-(OMe)-FMK	Cell permeable, irreversible general caspase inhibitor
z-D(OMe)E(OMe)VD(OMe)-FMK	Cell permeable caspase 3 inhibitor
z-IETD-FMK	Caspase 8 inhibitor
Pifithrin-μ	Inhibitor of p53-mediated apoptosis

Timeline of events

Apoptosis typically progresses through the extrinsic or intrinsic death pathways, leading to activation of effector caspases, exposure of phosphatidylserine (PS) on the outer surface of the plasma membrane, cytochrome C release and genomic DNA fragmentation.

The specific events that occur will depend on the apoptotic pathways that are activated. Moreover, the time required to detect different events or activities after apoptosis induction is often variable. As shown in the image on the next page, Jurkat cells treated with anti-Fas antibody showed activation of caspase 8 after four hours (left) whereas caspase 3 activation was detected after 20 hours (right).



Cells within a population will be in different phases of the cell cycle. This means that the 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). Hence, it is important to analyze single cells whenever possible to track 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.

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, 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.

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.
- It is important to 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 conditions. Subtle lab-to-lab differences may affect your results.

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- Yeung MC. (2002) Accelerated apoptosis DNA laddering protocol. *Biotechniques.* 33(4):734, 736.

Other resources:

- www.abcam.com/tag/apoptosis
www.abcam.com/cancer
www.abcam.com/tag/webinars



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