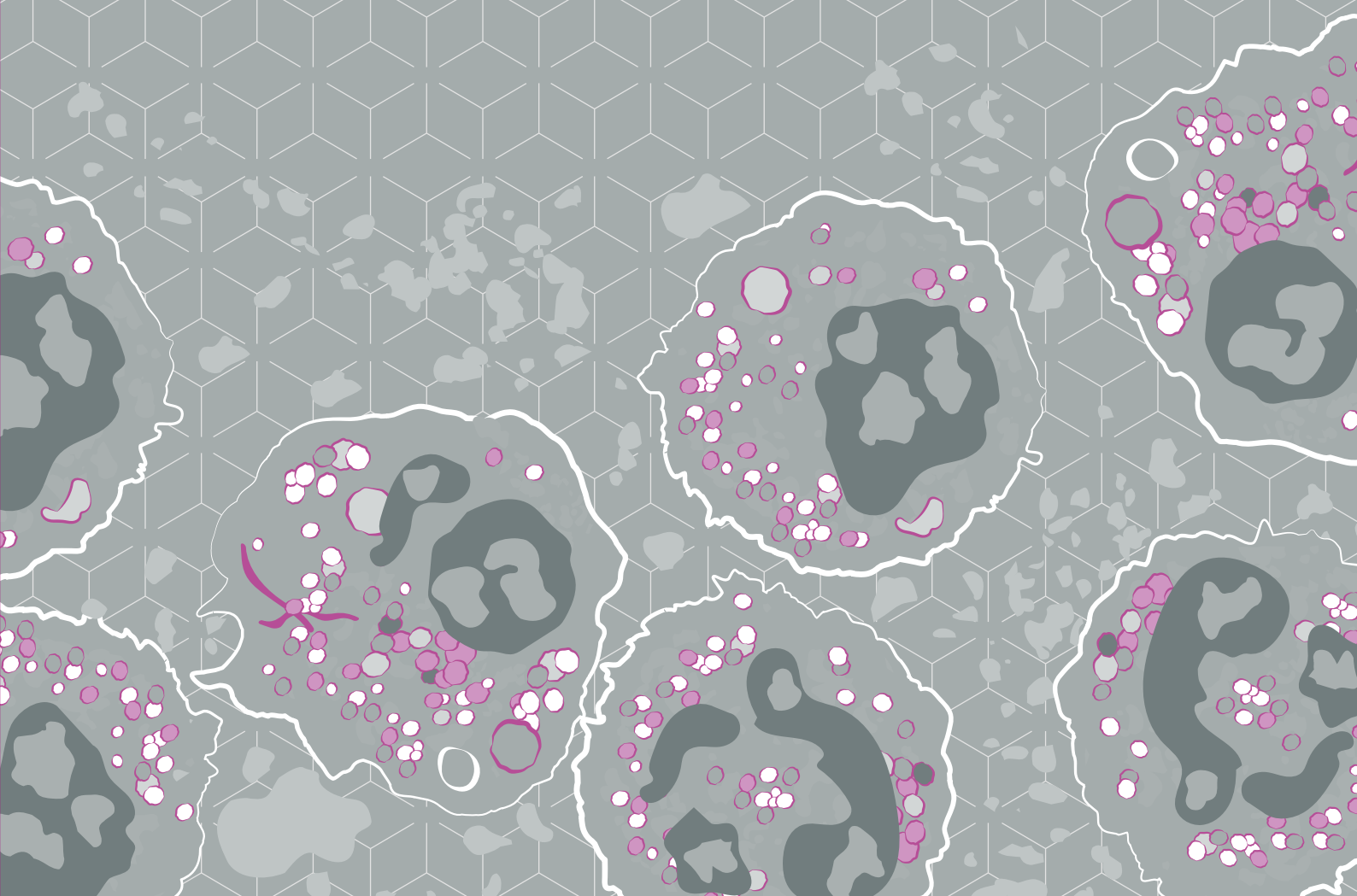
abcam

Necroptosis and other non-apoptotic cell death forms

Tools for cell death series 3



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.

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*

Classically, cell death has been divided into programmed apoptosis and unregulated necrosis. However, more recent research has defined a subset of non-apoptotic cell death modes that are controlled by distinct signaling pathways.

In this guide, we will examine the pathways of non-apoptotic forms of programmed cell death including necroptosis, pyroptosis and ferroptosis, and outline the different approaches that can be used to study them.

Necroptosis

Necroptosis is a programmed form of necrosis that is dependent on activation of receptor-interacting kinase 3 (RIPK3)[2] and the mixed lineage kinase domain-like (MLKL) psuedokinase[3]. This form of cell death is morphologically distinct from apoptosis, involving membrane rupture and release of cytoplasmic contents.

Necroptosis is activated in response to death receptor activation, although some death receptor-independent pathways are also a trigger. In most contexts, necroptosis is inhibited by proapoptotic caspase 8[4–6]; certain intracellular pathogens suppress apoptosis by inhibiting caspase 8, and necroptosis plays a role as a back-up to eliminate infected cells[7].

Necroptosis has been linked to inflammatory conditions such as multiple sclerosis[8], amyotrophic lateral sclerosis (ALS), ischemia-reperfusion injury and Crohn's disease, although it is unknown whether necroptosis is a driving factor for these diseases or a secondary consequence[9].

Death receptor dependent pathway

Necroptosis signaling: the intersection of prosurvival and apoptotic pathways

Necroptosis is initiated by ligand binding to death receptors including tumor necrosis factor receptor 1 (TNFR1), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors and Fas[10,11], although TNFR1-mediated necroptosis is the most studied. These death receptors are also involved in prosurvival signaling and apoptosis: the path the cell takes is dictated by which complex forms as a result of ligand binding (Figure 1).

Upon ligand binding, the cytosolic death domain of TNFR1 recruits prosurvival complex I, consisting of TNF-receptor-associated death domain (TRADD), RIPK1 and several ubiquitin E3 ligases. In complex I, RIPK1 is polyubiquitinated; however, subsequent RIPK1 deubiquitination causes RIPK1 dissociation and formation of one of two complexes: complex IIa mediates the activation of caspase 8 and initiates apoptosis, whereas complex IIb – otherwise known as the necrosome – assembles when caspase 8 is inhibited and triggers necroptosis[2].

The necrosome and downstream signaling

In complex IIb, RIPK1 recruits RIPK3 through rip homeotypic interaction motifs (RHIMs) contained within both proteins, resulting in their mutual phosphorylation. RIPK3 phosphorylation results in RIPK3 oligomerization, which is necessary for its activation. Once activated, RIPK3 phosphorylates MLKL at threonine 357 and serine 358[3].

Phosphorylated MLKL multimerizes and locates to the plasma membrane – a process that is crucial for necroptotic cell death[12]. It is currently uncertain exactly how MLKL triggers cell death[13]; however, there are some reports that it binds to phosphatidylinositol lipids and cardiolipin to directly permeabilize the membrane[14,15].

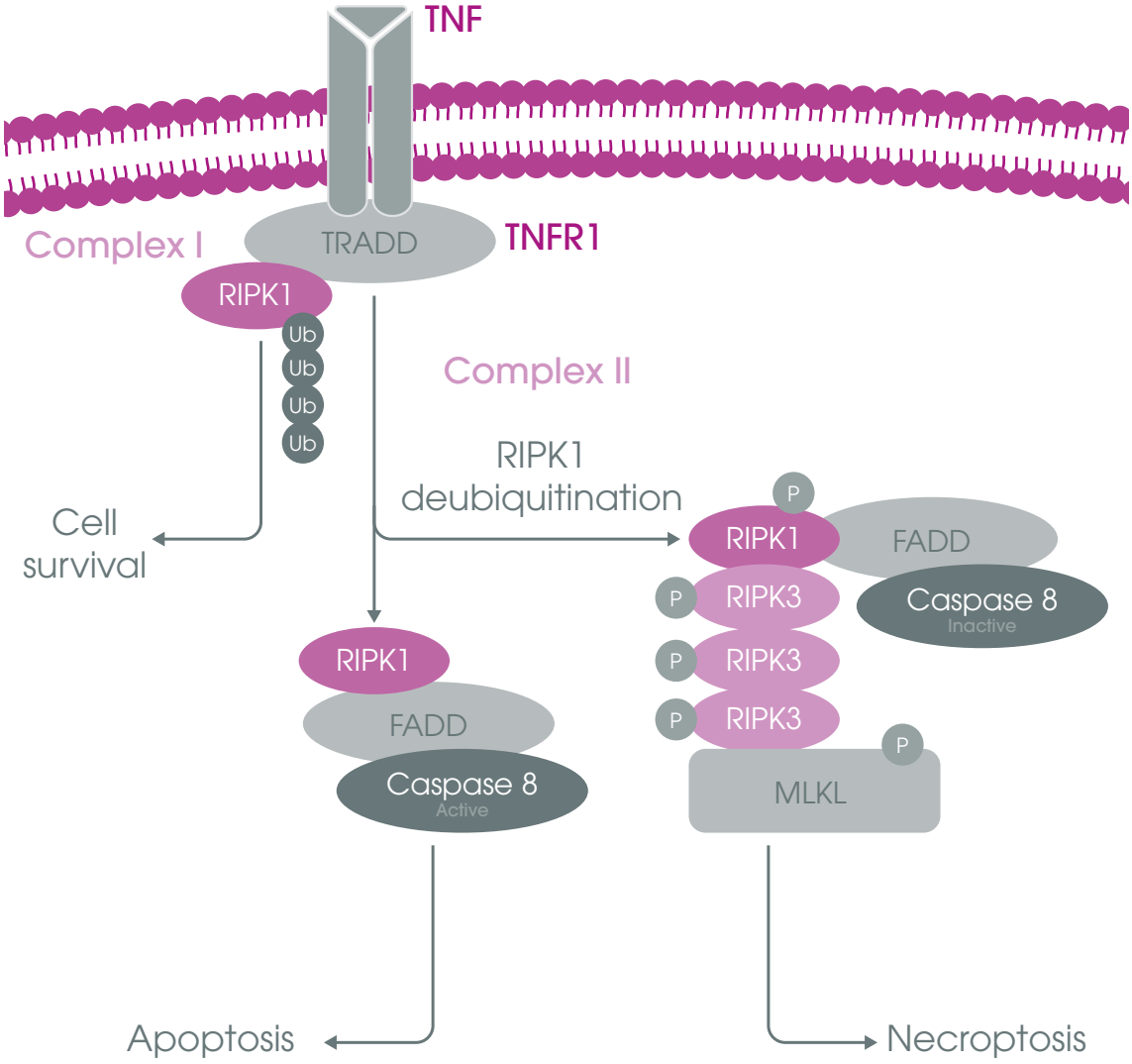


Figure 1. Necroptosis triggered by TNFR1.

Death receptor-independent pathway

Induction of necroptosis can also occur independently of death receptor pathways through engagement of Toll-like receptor TLR3 and TLR4[16], viral infection, type 1 and 2 interferons and viral expression of RHIM-containing proteins (Figure 2).

Toll-like receptors

These proteins are part of the innate immune system and sense cellular stress, damage and infection. Upon activation, the TIR-domain-containing adapter-inducing interferon-β (TRIF) adaptor protein forms a complex with RIPK3[16]. TLR activation-dependent necroptosis is dependent on RIPK3 and MLKL, but can proceed without RIPK1.

Viral DNA

RIPK3-dependent necroptosis is also stimulated by activation of DAI (DNA-dependent activator of IFN regulatory factors). DAI recognizes double-strand viral DNA, and contains a RHIM domain to recruit RIPK3 and form the necrosome.

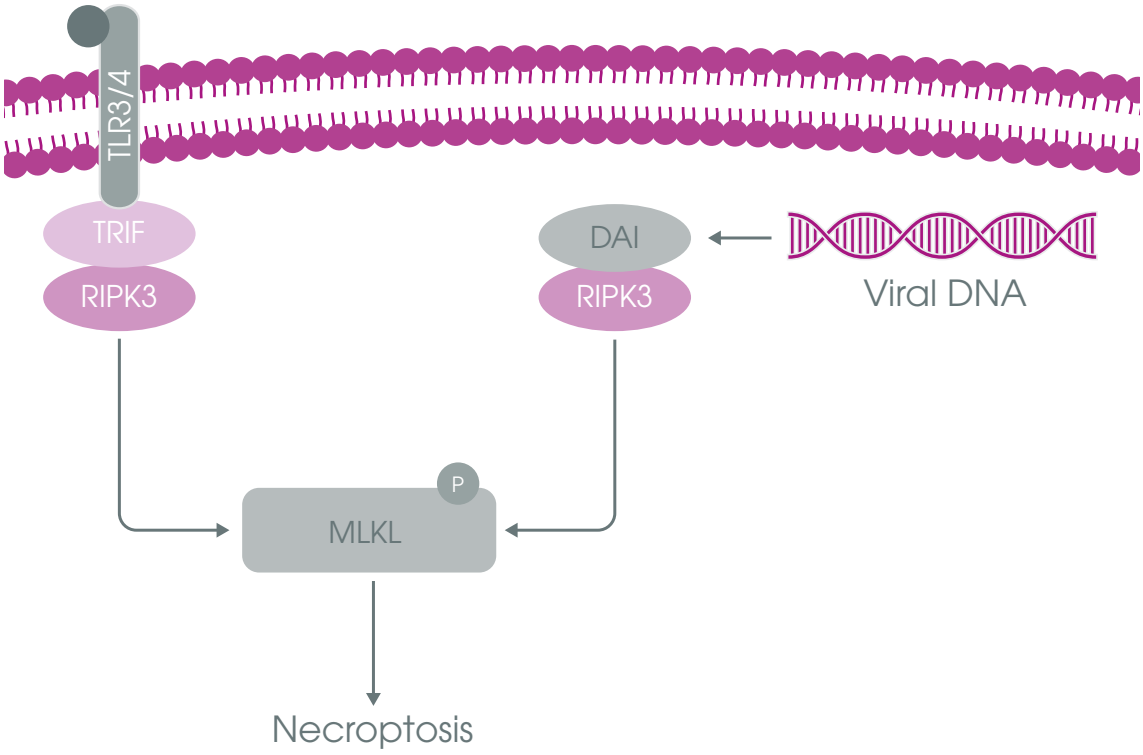


Figure 2. Death receptor-independent pathways of necroptosis.

Pyroptosis

Pyroptosis is an inflammatory caspase-dependent form of programmed necrosis that occurs in response to microbial infection. Morphologically, pyroptotic cells display cell swelling and rapid plasma membrane lysis. This form of cell death is driven by the inflammatory caspases: caspase 1, 4, 5 and 11.

Molecular pathway

Pyroptosis is initiated by the activation of canonical and non-canonical inflammasomes (Figure 3).

Non-canonical inflammasome pathway

In the non-canonical inflammasome pathway, lipopolysaccharides from gram-negative bacteria bind caspase 11 in mice, or caspases 4 and 5 in humans directly through their caspase activation and recruitment domains (CARDs). This induces caspase oligomerization and activation.

Canonical inflammasome pathway

Sensor proteins including Nod-like receptors (NLRs) detect pathogens and inflammatory agents. Pyroptosis triggered by activation of NLRC4 is the most extensively studied, although NLRC4 and NLRP1 inflammasomes can also trigger pyroptosis.

After activation, NLRC4 recruits the apoptosis-associated speck-like proteins containing CARD (ASC) adaptor protein. This complex dimerizes and activates caspase 1.

Gasdermin cleavage

Once caspases 1, 11, 4 or 5 have been activated by either the canonical or non-canonical inflammasome pathway, they trigger pyroptosis by cleaving gasdermin D between Asp276 and Gly277[17–19]. The resulting N terminal fragment is capable of inducing pyroptosis, whereas the C terminal fragment provides autoinhibition of gasdermin D.

Downstream events

Ultimately, pyroptosis initiation results in membrane pore formation, cell swelling followed by rupture. Events downstream of gasdermin D leads are currently unclear, although there is evidence that the N terminal fragment of gasdermin D oligomerizes in membranes to form pores[19].

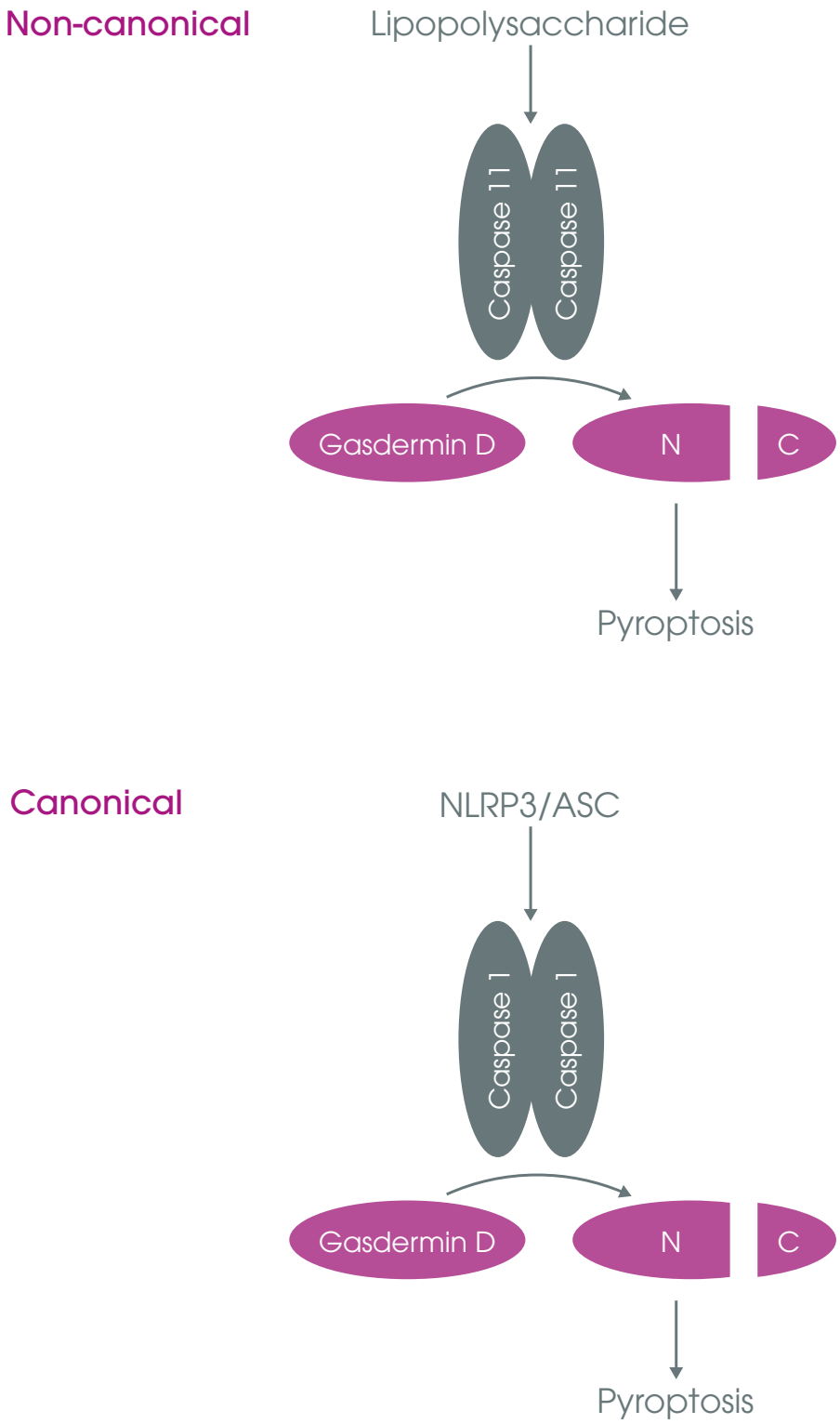


Figure 3. Initiation of pyroptosis by canonical and non-canonical inflammasome pathways.

Ferroptosis

Ferroptosis is another form of programmed non-apoptotic cell death, originally described in 2012[20]. The name "ferroptosis" derives from the dependence of this form of cell death on intracellular iron.

Morphologically, cells undergoing ferroptosis exhibit none of the features associated with apoptosis or necrotic forms of cell death, such as chromatin condensation, cytoplasmic swelling and membrane rupture. Necroptotic cells display subtler morphological features, including smaller than normal mitochondria with increased membrane density[20].

Molecular mechanisms

Ferroptosis occurs as a consequence of iron-dependent generation of lipid reactive oxygen species (ROS). Under non-ferroptotic conditions, build-up of lipid peroxides is tempered by glutathione (GSH) – an essential substrate for the activity of glutathione peroxidase 4 (GPX4), which is the only enzyme capable of reducing lipid hydroperoxides within biological membranes[21].

When GPX4 activity is reduced, antioxidant capacity is limited, leading to a build-up of lipid ROS and subsequent ferroptosis[22]. Ferroptosis can be triggered by reduced GPX4 activity by two distinct mechanisms (Figure 4):

1. Inhibition of the glutamate/cystine antiporter, x_c^-

Inhibition of the glutamate/cystine transporter reduces import of cysteine: a precursor for cysteine and glutathione synthesis. As a consequence, glutathione levels are reduced and GPX4 activity is inhibited. Experimentally, x_c^- can be inhibited with the small molecule erasatin[20].

2. Direct inhibition of GPX4 activity

Ferroptosis can also be triggered by direct inhibition of GPX4 activity. GPX4 can be inhibited by another small molecule, RSL3[23].

Unanswered questions

Mechanisms that trigger necroptosis have been identified by screening with small molecule libraries. What triggers ferroptosis *in vivo* has not yet been established, and remains an open question. Another aspect that remains unknown is how lipid peroxidation ultimately results in cell death.

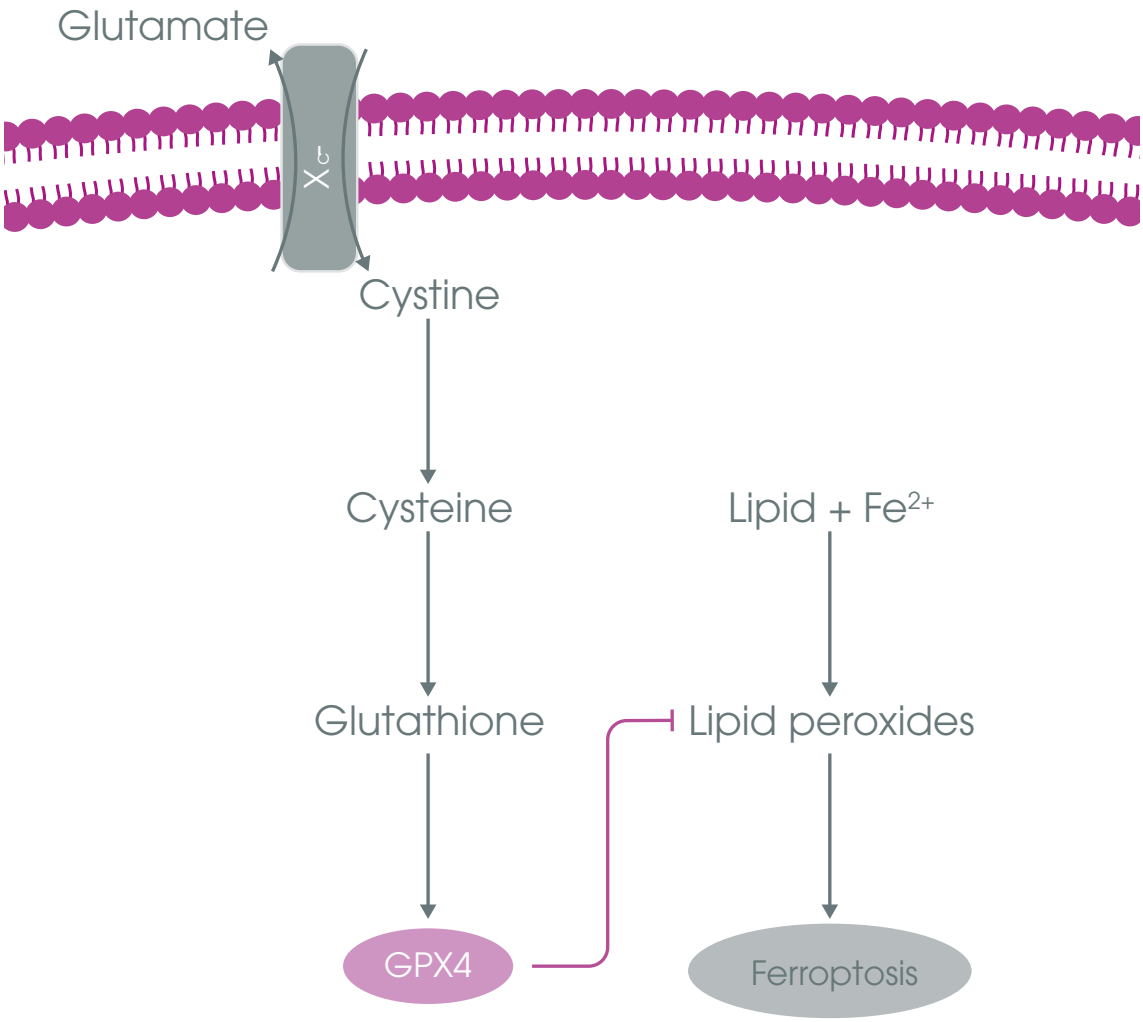


Figure 4. Ferroptosis activation pathway.

Other forms of cell death

Parthanatos

Parthanatos is a non-apoptotic form of cell death that has been implicated in a range of diseases, including Parkinson's disease, diabetes and stroke. Parthanatos initiation involves over-activation of the nuclear protein PARP1 (poly(ADP-ribose) polymerase 1)[24], which under physiological conditions, is involved in a variety of cellular process including DNA repair and transcription[25]. Ultimately parthanatos results in loss of cell membrane integrity.

Over activation of PARP1 is stimulated by DNA damage and ROS. When excessively activated, PARP1 uses NAD^+ to generate polymers of PAR. The generation of PAR polymers results in transferal of apoptosis inducing factor (AIF) from mitochondria to the nucleus[24]. The pathway for parthanatos is incompletely understood; parthanatos may cause cell death through depletion of cellular NAD^+ or AIF release, but at present the executioner mechanism has not been confirmed.

NETosis

NETosis is a pathogen-induced cell death mechanism in neutrophils, characterized by the release of neutrophil extracellular traps (NETs); chromatin structures that contain proteins capable of trapping exogenous bacteria and other pathogens.

NETosis occurs following PMA activation of protein kinase C and the raf-mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway.

Anoikis

Anoikis is a form of programmed cell death related to apoptosis. It is induced by cell detachment from the extracellular matrix. Under conditions where cells are attached to the extracellular matrix, anoikis is prevented by pro-survival signaling initiated by integrins – mediators of cell-ECM interactions.

Upon detachment, proapoptotic proteins Bid and Bim accumulate and drive apoptosis via the intrinsic pathway[26]. Alternatively, ECM detachment can release the mitochondrial Bit1 protein into the cytoplasm, which triggers apoptosis via a caspase-independent mechanism[27].

Loss of anoikis ability is a key step in cancer development. Research is currently underway to understand how cancer cells are able to evade anoikis.

For more information on apoptosis, see our apoptosis ebook.

Entosis

Cell death by entosis involves one cell invading into another. Like anoikis, this form of cell death occurs in response to cell detachment from the ECM. Entosis is triggered by adherens junction formation in the absence of integrin-ECM attachment. During entosis, cell invasion involves Rho GTPase and its downstream effector ROCK[28].

Studying cell death

Distinguishing between different forms of non-apoptotic cell death can be challenging, especially as many share similar morphological features (Table 1). However, the distinct regulatory pathways involved with each provide distinct protein markers that can be used for detection.

Table 1. Summary of main cell death pathways

	Apoptosis	Necroptosis	Pyroptosis	Ferroptosis
Trigger	Death receptor activation	Death receptor activation when caspase-8 is inhibited	Activation of inflammasomes by pathogens	Experimental small molecules such as erastin
Inflammatory response	No	Yes	Yes	Yes
Key proteins	– Caspase family	– RIPK1 – RIPK3 – MLKL	– Caspases 1, 4, 5 and 11 – Gasdermin D	– GPX4
Morphology	– Cell rounding – Blebbing – Formation of apoptotic bodies	– Organ swelling – Membrane rupture – Release of cell contents	– Plasma membrane rupture – Release of cell contents – Maintained mitochondrial integrity	– Small mitochondria, with increased membrane density

Whatever mode of cell death you are studying, a combination of different approaches should be used. Study of non-apoptotic cell death should use both specific positive indicators of the cell death mode of interest coupled with cell viability assays and techniques to rule out apoptosis[28].

Cell viability

Cell viability can be assessed using parameters such as cytolysis, metabolic activity or senescence. Your choice of assay will depend on what you want to detect, what instrumentation is available and the type of samples you have. Table 2 outlines the most common methods to look at viability and what results you should expect.

Table 2. Methods to study cell viability

	Cytolysis or membrane leakage assay		Metabolic activity assay	Cell cycle assay	Senescence assay
Mechanism of action	Dyes enter the cell upon cell membrane disruption	Cellular enzymes are released into media upon membrane disruption	Oxidation of a specific dye due to normal mitochondrial metabolism	Fluorescent dye intercalates with DNA in intact cells	Dye specifically stains cellular senescence markers
Markers or dyes	– PI – 7-AAD – DRAQ7™ – Trypan blue	– LDH	– LDH cell lysates – MTT/XTT (resarzurin) – WST-1 – Fluorescent dyes	– PI – DRAQ5™ – Nuclear Green CCS1 – Nuclear Red CCS1	Senescence-associated-β-gal
What to expect?	Increased signal: dying cells No signal: live cells	Increased signal: dying cells No signal: live cells	Increased signal: live cells Decreased signal: dead cells	Visualization of cell cycle phases	Blue staining: senescent No staining: quiescent or immortal
Detection method	– Flow Cyt – Microscopy – High content screening	– Microplate reader	– Microplate reader	– Flow Cyt	– IHC
Highlighted products	Live and Dead Cell Assay (ab115347) DRAQ7™ (ab109202)	LDH cytotoxicity assay kit II (ab65393) LDH Cytotoxicity Assay Kit (Fluorometric) (ab197004)	Mitochondrial Viability Stain (ab129732) WST-1 Cell Proliferation Reagent (ab155902)	Propidium Iodide Flow Cytometry Kit (ab139418) Cell Cycle Assay Kit (Fluorometric - Green) (ab112116)	Senescence Detection Kit (ab65351)

Abbreviations: LDH = lactate, PI = propidium iodide, 7-AAD = 7-aminoactinomycin D, WST1 = water-soluble tetrazolium salt, Flow Cyt = flow cytometry, IHC = immunohistochemistry, β-gal = β-galactosidase.

Ruling out apoptosis

Different approaches can be used to rule out the presence of apoptosis. These are outlined briefly below, but for more detailed information on detecting apoptosis, see our apoptosis ebook.

Caspase 3 activity

Caspase-3 is the primary executioner caspase in apoptosis, required for the mass proteolysis that leads to apoptosis. Caspases are initially synthesized as inactive pro-caspases, and they are activated by cleavage at specific sites. The method used to detect caspase-3 will depend on the available instrumentation and how the samples have been prepared (Table 3).

Table 3. Detection of caspase 3 activity

Sample type	Detection method	Best used when you want to…	Highlighted products
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	Western blot	Detect a caspase in its cleaved form as well as in its pro-caspase form using specific antibodies	ab32042 ab136812
	Absorbance/ fluorescence assay	Quickly detect caspase activation in a cell population using a specific substrate. Easily adaptable of HTP analysis	ab39401 ab39383
	Absorbance/ fluorescence assay	Quickly detect caspase activation in a cell population using a specific antibody against the active form	ab181418 ab168541
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)	ab32042

Morphological changes

The morphology of dying cells can give clues to the type of cell death occurring. This can be assessed using imaging techniques (Table 4).

Table 4. Morphological differences between apoptotic and necrotic cell death.

Apoptosis	Necrosis
– Cell rounding – Blebbing – Formation of apoptotic bodies	– Cell swelling – Membrane rupture – Release of cell contents

Secondary necrosis (associated with apoptosis) can be distinguished from primary necrosis by propidium iodide (PI) staining; homogenous PI staining indicates chromatin fragmentation indicative of apoptosis, whereas necrotic cells display staining primarily in the nucleoli.

Chromatin condensation and DNA fragmentation

During apoptosis, chromatin becomes highly condensed. 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).

Condensed chromatin is then fragmented by a specific nuclease – caspase-activated DNase (CAD), generating fragments of around 200 base pairs. These can be detected by examining DNA on an agarose gel. Although this semi-quantitative method is falling out of use, it is a simple technique that provides robust answers.

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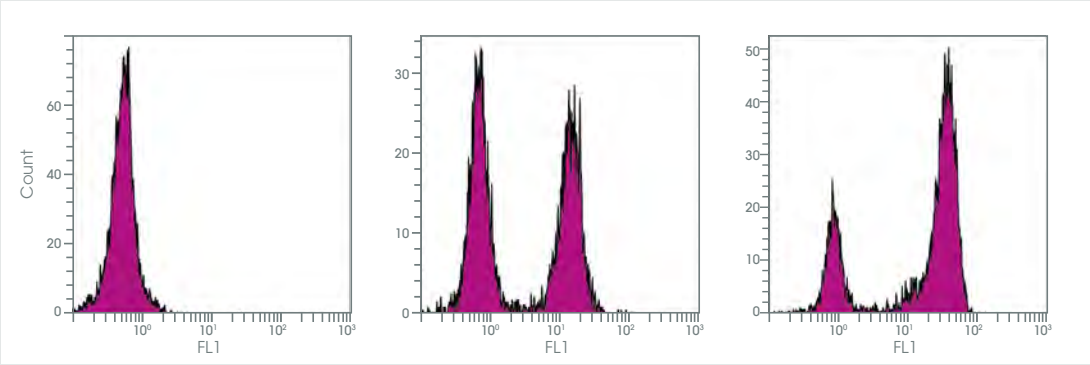

In situ direct DNA fragmentation (TUNEL) assay kit

TUNEL assay analysis. RAW 264.7 cells were left untreated (A) or treated with increasing concentrations of camptothecin (B, 2 µM; C, 10 µM) during 24 hours prior staining with In situ direct DNA fragmentation (TUNEL) assay kit (ab66108). This assay uses a deoxyuridine nucleotide labeled with FITC, which can be measured in the FL1 channel.

Apoptosis DNA fragmentation analysis protocol

Detecting necroptosis

Although many proteins are involved in the necroptotic pathway (Table 5), the most reliable method to detect necroptosis is measuring MLKL phosphorylation status and by specific inhibition of the pathway.

Table 5. Key proteins involved in necroptosis

Protein	Function	Role in necroptosis
RIPK1	Protein kinase involved in inflammation and cell death pathways	Recruits RIPK3 to the necrosome
RIPK3	Protein kinase essential for necroptosis	Oligomerizes and recruits MLKL to the necrosome
MLKL	Kinase-domain like protein, phosphorylated by RIPK3	Translocates to the membrane to mediate cell death
CYLD	Deubiquitinase	Deubiquitinylates RIPK1, allowing formation of the necrosome.
cIAP1/2	Ubiquitin E3 ligases	Ubiquitinylate RIPK1 preventing formation of the necrosome
Caspase-8	Proapoptotic caspase	Inhibits necroptosis

MLKL phosphorylation

MLKL is activated by RIPK3-mediated phosphorylation. The activation state of MLKL can be determined by assessing phosphorylation status of Thr357 and Ser358. Phospho-MLKL is detected by antibody-based methods, including western blot, IHC and flow cytometry.

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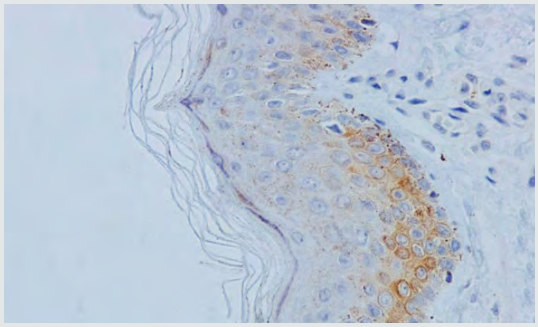

Anti-MLKL (phospho S358) antibody (EPR9514)

Detection of phosphorylated MLKL (pS358) by immunohistochemistry. Formalin/PFA-fixed paraffin-embedded sections (FFPE) of human skin tissue were stained with the rabbit monoclonal anti-MLKL (phospho S358) antibody (EPR9514) (ab187091) at 1:250 dilution.

Necroptosis inhibition

Targeting components of the necroptosis pathway – either by chemical inhibition or with transgenic models – can be used to tell whether cell death is dependent on them.

Table 6. Chemical inhibitors of necroptosis

Compound	Target
Necrostatin-1 (Nec1)	RIPK1
7-Cl-O-Nec-1 (Nec1s)	RIPK1
GSK'872	RIPK3
Necrosulfonamide	MLKL

Considerations for using inhibitors:

- Nec1 has some off-target activity: Nec1s is more specific[29].
- RIPK1 can contribute to apoptosis[30]. Be aware that RIPK1 inhibitors may also block apoptosis under some circumstances.
- Using transgenic models is the best method for confirming presence of necroptosis.

Detecting pyroptosis

Pyroptosis can be studied by looking at caspase activation, gasdermin D cleavage, or by inhibiting or ablating key components of the pyroptotic pathway (Table 6).

Table 7. Key proteins involved in pyroptosis

Protein	Function	Role in pyroptosis
Caspase 1	Inflammatory caspase, activated by sensor proteins and inflammatory agents	Cleaves gasdermin D
Caspase 11 (mouse) Caspase 4 and 5 (human)	Inflammatory caspase, activated by bacterial polysaccharides	Cleaves gasdermin D
Gasdermin-D	Cleaved by caspases	Executes pyroptosis

Caspase activity

Active caspases are cleaved from their inactive pre-caspase forms during pyroptosis. Caspase cleavage can be assessed by western blot and a specific caspase antibody.

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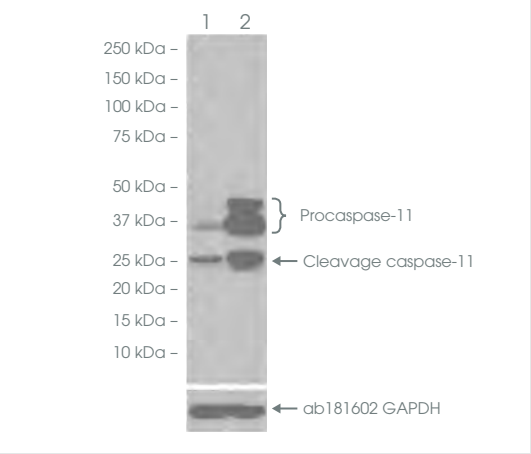

Anti-Caspase 11 antibody (EPR18628)

Detection of caspase 11 in RAW 164.7 (mouse macrophage) cell lysate with rabbit monoclonal anti-caspase 11 antibody (EPR18628) (ab180673) (1:1000 dilution). Lane 1: untreated, lane 2: cells treated with 10 mg/mL lipopolysaccharide for 8 hours. GAPDH was used as loading control.

Although active caspases are cleaved, observing caspase cleavage alone is not proof of caspase activation and other methods should also be used to confirm pyroptosis. Caspase activation can be detected directly using caspase activation assays.

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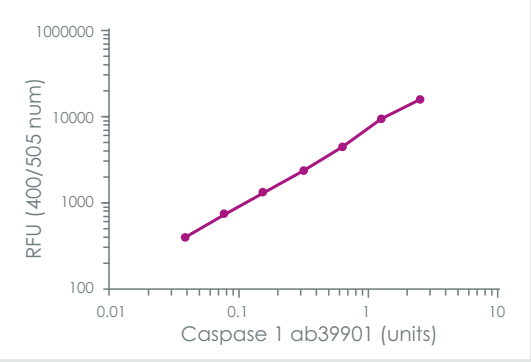

Caspase 1 Assay Kit (Fluorometric)

Activity of known concentrations of active caspase 1 (background subtracted) was detected using Caspase 1 Assay Kit (Fluorometric) (ab39412) in only 2 hours.

Gasdermin D

Pyroptosis involves cleavage of gasdermin D (53 kDa), resulting in a 30 kDa N-terminal fragment. Cleaved gasdermin D can be detected by western blot by the presence of a band at 30 kDa.

We recommend using our anti-gasdermin D rabbit polyclonal (ab155233), which detects the N-terminal region of gasdermin D.

Pyroptosis inhibition

Dependence on caspase 1, 11, 4 or 5 is essential to distinguish pyroptotic cell death from other forms of necroptosis and apoptosis. Determine if cell death still occurs after ablation of activity of relevant caspases, either by chemical inhibition or using transgenic models.

Caspase 1 activity can be ablated by chemical inhibition with z-YVAD-fmk (ab141388).

Detecting ferroptosis

Ferroptosis can be identified by looking at whether cell death is prevented by inhibitors, and by measuring lipid peroxides.

Table 8. Key proteins involved in ferroptosis.

Protein	Function	Role in ferroptosis
GPX4	Reduces lipid hydroperoxides within lipid membranes	Activity reduced in ferroptosis
Glutathione	Substrate for GPX4	Protein is depleted if ferroptosis mechanism though X_{c^-} inhibition

Inhibiting ferroptosis

The presence of ferroptosis can be confirmed using chemical inhibitors known to prevent ferroptosis. As ferroptosis is caused by reduction of GPX4 activity, knockdown is not an effective method.

Table 9. Ferroptosis inhibitors and their modes of action

Inhibitor	Mode of action
Ferrostatin-1	Lipid ROS scavenger[20]
Liproxstatin-1	Unknown. Possibly reduction of free radicals[21]

Accumulation of lipid peroxides

Ferroptosis is dependent on lipid ROS accumulation. Several methods are available to detect the presence of lipid ROS.

Table 10. Methods to detect the presence of lipid ROS

Assay	Mechanism	How to measure
C11- BODIPY	Detects free radical-induced oxidation	Quantification by flow cytometry
Malondialdehyde quantification	Bi-product of lipid peroxidation	Lipid peroxidation (MDA) assay kit
4-HNE quantification	Bi-product of lipid peroxidation	Antibody-based quantification

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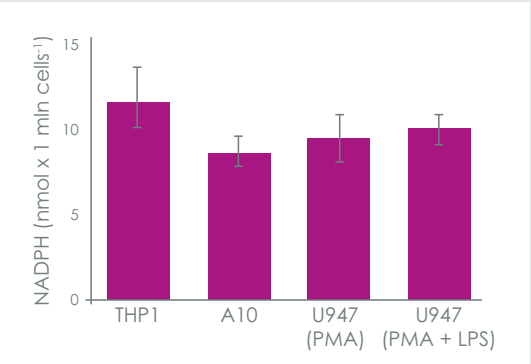

Glutathione Peroxidase Assay Kit

Glutathione peroxidase (GPX) activity was measured in cell lysates using Glutathione Peroxidase Assay Kit (ab102530). Lysates were prepared from THP1 (human monocytic leukemia), A10 (rat thoracic aorta) and U947 (human monocytic).

General considerations

As we have mentioned throughout this guide, it is recommended to analyze more than one parameter to identify which type of cell death we see in the studied cell population. This is because multiple parameters can be observed in different types of cell death such as necrosis or necroptosis.

Things to keep in mind:

- When choosing 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 condition – Subtle lab-to-lab differences may affect your results

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