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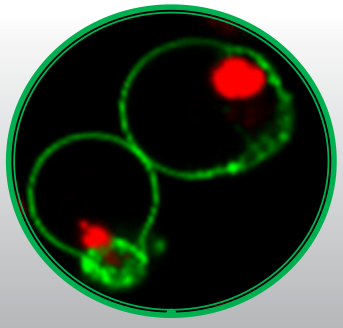
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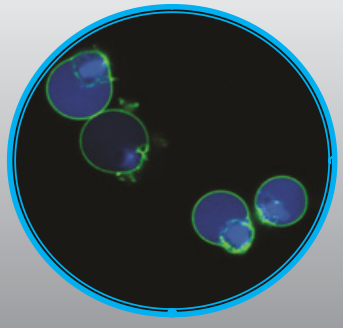
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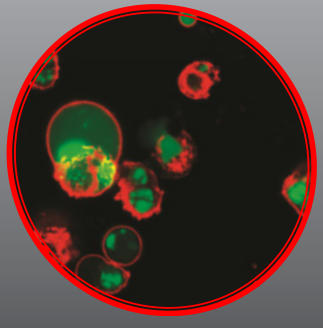
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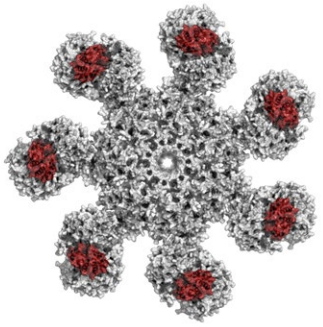
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Apoptosis: Two Roads to a Single Destination



"[caspases] play a central role in apoptosis and ultimately ensure that programmed cell death occurs in a controlled manner with minimal impact to surrounding cells"

Detecting apoptosis is important for many areas of biological research, as well as for the development of new drugs; the ability to monitor apoptosis is key in the assessment of anticancer drugs for example. It's brought about by two distinct pathways: the intrinsic pathway, and the extrinsic pathway.

The Long and Winding Road: Intrinsic Pathway

The intrinsic pathway is characterized by the perturbation of the mitochondrial membrane. Mitochondria are key organelles in the survival and proliferation of eukaryotic cells. Their primary role is to generate energy, ATP, via the electron transport chain (ETC). This occurs through a strong electrochemical proton motive force – a consequence of successive electron reduction – that allows protons to build up in the intermembrane space between the inner and outer mitochondrial membranes. This mitochondrial membrane potential (MMP) then helps the protons back into the mitochondria along with mitochondrial ATP synthase, which completes the ETC and produces ATP.

Loss of MMP indicates membrane perturbation, which causes cytochrome c to be released into the cytoplasm. This goes on to form a complex known as the apoptosome, along with Apoptotic protease activating factor-1 (Apaf-1). The apoptosome initiates activation of the caspase cascade via caspase 9.

Several events can contribute to loss of MMP. For example, it may be calcium-induced[1] or caused by pro-apoptotic Bcl-2 protein family members such as Bax-like proteins, or short-form Bcl-x.[2]

The Path that Leads to Trouble: Extrinsic Pathway

Apoptosis can also be initiated externally via death receptors on the cell surface such as TNF-R1 and Fas. Upon binding to their ligands (i.e. TNFα and FasL) the receptors recruit adaptor proteins and members of the Bcl-2 family of proteins, which form homodimers in the outer membrane of mitochondria. Proapoptotic homodimers make the mitochondrial membrane permeable, allowing caspase cascade activators to be released. Ultimately, via the extrinsic pathway, pro-caspase 8 is cleaved into caspase 8, which initiates the process of cell death.

The Common Link: Caspases

Initiator caspases 2, 8, 9, 10, 11, and 12, and effector (executioner) caspases 3, 6, and 7 all play a central role in apoptosis and ultimately ensure that programmed cell death occurs in a controlled manner with minimal impact to surrounding cells.[3]

When initiator caspases are activated by activator proteins, they go on to proteolytically cleave effector pro-caspases into their active form. Effector caspases then carry out further proteolytic degradation of a whole host of other intracellular proteins. This includes the cleavage and activation of enzymes involved in breaking down cell structures and DNA.

Real-time

Real-time analysis of apoptosis detection is vital for understanding the mechanisms of programmed cell death for many applications such as in drug discovery and toxicological profiling.

Novel, highly membrane permeable, and non-toxic fluorogenic caspase 3 substrates have been developed that are ideal for kinetic studies of caspase 3 activation and real-time apoptosis detection by flow cytometry, microscopy or live imaging. They consist of a caspase 3 recognition sequence DEVD, linked to a DNA-binding dye which is initially rendered non-fluorescent. Upon cleavage by active caspase 3, the dye is released and becomes highly fluorescent on binding to DNA.[4]

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VISUALIZING APOPTOSIS AS IT HAPPENS

IT'S JUST A PHASE

THE BIOCHEMICAL & MORPHOLOGICAL STAGES OF APOPTOSIS

INTRINSIC PATHWAY
Mitochondria-mediated apoptosis

EXTRINSIC PATHWAY
Death receptor-mediated apoptosis



INTRINSIC SIGNAL

The intrinsic pathway may be activated by an internal signal such as cell stress, viruses, or severe DNA damage.



BCL-2 FAMILY PROTEINS

Bcl-2 family sensor proteins induce Bcl-2 family effectors (such as Bax and Bak) to act on the mitochondria, inducing the formation of the mitochondrial permeability transition pore.



RELEASE OF CYTOCHROME C

Cytochrome c is released from the mitochondria through the pore induced by Bax / Bak.



FORMATION OF THE APOPTOSOME

Cytochrome c then binds with Apoptotic protease activating factor-1 (Apaf-1) to create an apoptosome, converting pro-caspase 9 to its active form, initiator caspase 9.



BREAKDOWN OF THE CELL

The cell shrinks and becomes rounded due to the breakdown of the cytoskeleton, organelles become tightly packed, chromatin condenses against the nuclear envelope, and DNA fragmentation is brought about by a nuclease known as caspase-activated DNase (CAD).



PHAGOCYTOSIS

Dying cells display signals to mark the cell for phagocytosis by other cells such as macrophages. A common signal is the translocation of phosphatidylserine from the inner leaflet to the outer leaflet with the help of the enzyme scramblase.



EXTRINSIC SIGNAL

A ligand such as TNFα or FasL interacts with its death receptor on the outside of the cell surface.



RECRUITMENT OF ADAPTOR PROTEINS

Adaptor proteins bind to the death receptor on the inner cell surface, setting off the chain of events leading to the start of the caspase cascade.



RECRUITMENT OF INITIATOR CASPASES

Initiator caspases include caspases 2, 8, 9, and 10. In the extrinsic pathway, adaptor proteins recruit and activate pro-caspase 8 into its active form. Initiator caspases then activate effector caspases via proteolytic cleavage.



EFFECTOR (EXECUTIONER) CASPASES

Once activated by initiator caspases, effector caspases 3, 6, and 7 degrade various intracellular proteins by proteolysis in order to carry out the last steps in programmed cell death.



BLEBBING AND APOPTOTIC BODY FORMATION

Blebbing and formation of apoptotic bodies is brought about in a process of disassembly.

Please Eat Me: Apoptosis Signs and Signals

"Externalized, and with its usual asymmetry now disturbed, [phospatidylserine] is exposed and acts as a signal to macrophages, which in turn sets off the chain of events leading to phagocytosis."

Several biochemical and morphological changes take place during the process of apoptosis that can be measured or monitored in programmed cell death research. The 'eat me' signals in mammalian systems are diverse and often consist of either an alteration to sugars, lipids, and proteins on a cell's plasma membrane, or a detectable change to internal cell components such as the mitochondria.

All Shook Up: Mitochondrial Membrane Perturbation

Loss of MMP is thought to occur due to the opening of the mitochondrial permeability transition pore, which in turn lets in a flow of ions and small molecules. This eventually results in the decoupling of the respiratory chain due to an altered ion equilibrium state, leading to the subsequent release of cytochrome c and pro-apoptotic proteins.

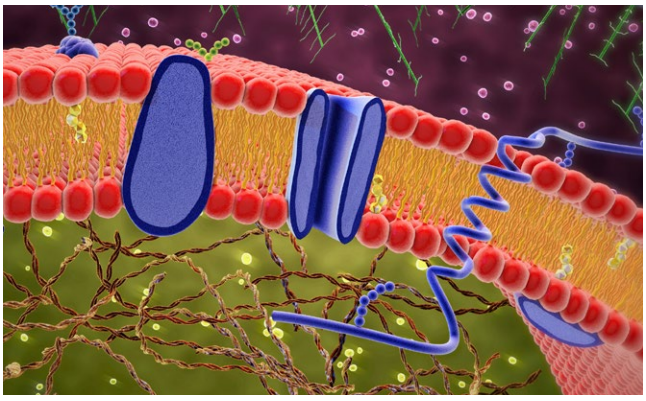
Most techniques to track MMP rely on using fluorescent microscopy or imaging methods with positively-charged fluorescence dyes, which can accrue in the electronegative interior of the mitochondrion. These dyes allow for the visualization of intact MMP; the fluorescent signal is then lost when the membrane is perturbed and the potential is lost. Other dye protocols specifically target live cells.

The mitochondrial permeability transition pore may also be specifically targeted by probing the release of mitochondria-entrapped fluorescent dyes such as calcein.[1]

Fluorescent dyes can target several aspects of mitochondria to monitor a variety of properties; fluorescence increase with increasing calcium concentration, fluorescence decrease upon opening of the transition pore, or fluorescence increase with increasing superoxide concentration.

Phospatidylserine: The Cell Membrane's White Flag

One of the best-known membrane alterations is the translocation of the phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the membrane via the enzyme scramblase. Externalized, and with its usual asymmetry disturbed, PS acts as a signal to macrophages, which in turn sets off the chain of events leading to phagocytosis. Several methods utilizing fluorescent dyes exist to track this exquisite event.



A conundrum? PS translocation to the cell surface is not exclusive to apoptosis; it also occurs in necrosis. These processes can be differentiated by using membrane-impermeable dead cell stains, which are able to enter into necrotic cells.

In Vogue: Annexin V

Annexin V is a Ca^{2+} dependent phospholipid-binding protein with a high affinity for PS and the most commonly used PS probe. Annexin V may be conjugated to fluorescent molecules and detected via several methods, including flow cytometry and fluorescent imaging. Fluorescent semiconductor nanoparticles may also be used for Annexin V labeling.

New in Town: Low-Molecular-Weight Chelators

Relatively new in the world of PS detection, low-molecular-weight high-affinity PS chelators can mimic the binding mechanism of annexin and act as cell death molecular imaging agents. Synthetic zinc(II)-bis(dipicolylamine) (Zn2BDPA) coordination complexes are an example of one of these agents. However, there is a need to improve their *in vivo* imaging performance.[2]

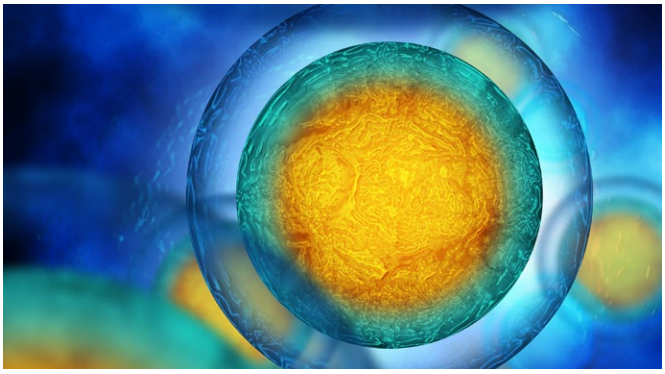
Surface Deep

When negatively charged PS translocates to the outer leaflet, it substitutes for the neutral lipids and generates a change in surface charge. Since this charge becomes more negative, cationic dyes can be readily adsorbed on the cell surface. Several dyes are in development including a poly(*p*-phenylene vinylene) derivative[3] and dyes from the 4′-(dialkylamino)-3-hydroxyflavone family.[4] In terms of ease of use, charge-based methods are an attractive alternative to annexin-based assays.

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The Nucleus: At the Center of it All



"[Caspase-activated DNase] is activated by the caspase cascade, which in turn leads to specific cleavage of DNA at internucleosomal linker sites. The leftovers? Double-stranded DNA breaks that appear as DNA ladders after separation by gel electrophoresis."

The final stages of apoptosis induction take place in the nucleus, with the condensation of chromatin, disassembly of nuclear scaffold proteins, and DNA fragmentation. These three hallmark signaling events of apoptosis occur during the execution phase[1] and involve the carefully controlled movement of certain nuclear proteins to other compartments. These movements thus make excellent options for probing in monitoring apoptosis.

DNA fragmentation is brought about by a nuclease known as caspase-activated DNase (CAD). CAD is activated by the caspase cascade, which in turn leads to specific cleavage of DNA at internucleosomal linker sites. The leftovers? Double-stranded DNA breaks that appear as DNA ladders after separation by gel electrophoresis. This is a distinctive feature of DNA degraded by CAD, which is a key event during apoptosis. Fragmented DNA from necrotic cells shows no clear pattern of DNA laddering.

Classical methods using agarose gel electrophoresis can detect DNA ladders. However, Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assays allow DNA fragmentation to be analyzed by flow cytometry or microscopy.

There's Light at the End of the TUNEL

TUNEL assays involve the application of a nuclear stain to identify cells, followed by a TUNEL label which detects DNA double-strand breaks. The method works thanks to the ability of the TdT enzyme to label blunt ends of double-stranded DNA, allowing the unique nuclear events of apoptosis to be directly visualized.

Going Nuclear

These nuclear events can also be visualized using fluorogenic nuclear dyes, which target caspase activity. For example, novel cell-membrane permeable fluorogenic caspase 3 substrates are available for detecting caspase 3 activity within live cells in real time. Initially non-fluorescent,[2] the substrate is cleaved in the cytosol by caspase 3 released during the apoptosis caspase cascade, allowing the dye to bind DNA in the nucleus and fluoresce. Apoptotic cells can then be visualized and quantified through imaging techniques and flow cytometry.

Taking a Cell-fie

Labeling using fluorescent dyes allows for the application of various imaging methods. Advantages to imaging include the ability to directly visualize cell health and to monitor the progress of cell death, its high reproducibility, and the specific data that can be obtained, such as the number and percentage of apoptotic cells.

Sensitive Soul

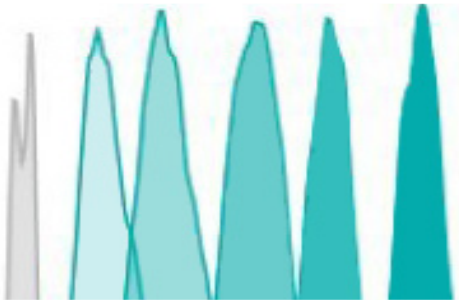
Flow cytometry offers highly sensitive assays – useful in detecting early or late events – as well as a wide range of assays for the detection of different parameters. The wide selection of laser excitation sources also means cytometry methods are compatible with various fluorescent dyes. Advantages to using flow cytometry are its ability to analyze vast numbers of cells and collect huge amounts of quantitative data.

Why not both?

Imaging cytometers are another option. The advantages to combining imaging and flow cytometry come from the simplified workflow, increased data parameters, high sensitivity, and the ability to normalize plate reader assay data to the number of cells in each well. The right analytical choice ultimately boils down to the experimental data required.

References

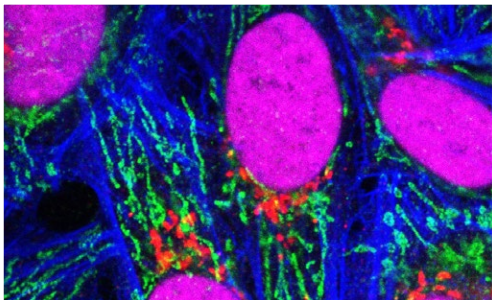
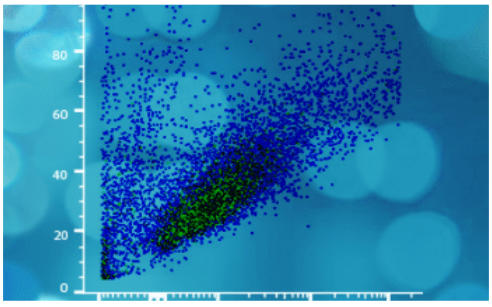
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