The many roads to cell death: discriminating between apoptosis, necrosis and autophagy

Cell death is crucial for the proper execution of normal and pathophysiological processes and is ubiquitous in biological systems. Programmed forms of cell death are responsible for producing morphological patterns during development, negative selection during immunity, and the molecular ‘arms race’ that occurs between viral and host genes during infection, as well as for tissue damage that occurs with environmental stressors such as genotoxins. Alterations within these cell death pathways can manifest as disease, including cancer, degenerative disorders and acquired immune deficiency syndrome (AIDS). The ability of tumour cells to elude programmed cell death is a hallmark of most types of cancer.

In recent years it has become clear that just measuring individual hallmarks of dying cells, such as nuclear fragmentation or membrane permeability, is not sufficient for discriminating between the different cell death pathways, which include apoptosis, autophagic cell death and necrosis. What is required is a multi-parameter assessment of cell death that reveals the mechanistic events occurring ‘behind the scenes’. According to recommendations of the Nomenclature Committee on Cell Death 2009, cell death-related processes (apoptosis, necrosis, autophagy) can be classified according to morphological appearance, enzymological criteria, functional aspects and immune characteristics.

Death in various forms
Apoptosis refers to a gene-directed cell suicide programme. A wide variety of cellular proteins, including cell surface receptors, proteases and mitochondrial components, regulate a delicate balance between cell survival and death by apoptosis. Mutations within these proteins may tip the balance, resulting in the uncontrolled survival and proliferation of tumour cells. Therefore, uncovering apoptosis mechanisms may result in new strategies to exploit this form of cell death for therapeutic purposes. Apoptosis is accompanied by a reduction of cell volume, nuclear condensation, DNA fragmentation, plasma membrane blebbing and engulfment by phagocytes. Additionally, apoptosis

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The progression of apoptosis can be studied by combining multiple assays into one analysis using multiparametric flow cytometry.
process. Certain imaging methods may have limitations on whether an equivalent number of cells are counted between sample to sample, or whether enough cells are counted, which could impact reproducibility and/or accuracy of the results. At the other end, methods such as multiparametric flow cytometry or imaging cytometry can be very powerful and provide content-rich data for cell death analyses, but these methods may also have limitations. For example, access to instrumentation may be limited, there may be a requirement for a great deal of expertise and training, and affordability may be a challenge.

There is a need in the industry for easy-to-use, affordable, accessible methodologies that can robustly, accurately and reproducibly provide answers to common, everyday questions that researchers are investigating in cell health and cell death. Established and emerging methods to effectively discriminate between apoptosis, necrosis and autophagy are described below.

**Imaging**

Cell death is ultimately a morphological process and the gold standard for analysing this process is to visualise the cells. When cell death is witnessed in culture, it becomes very obvious what is occurring. For example, during apoptosis, cells round up and appear to boil (‘blebbing’). There are multiple approaches available for imaging cell death, including simply visualising the cells real-time in culture, staining cells with acridine orange (a vital dye specific for apoptotic cells), histologically staining fixed tissue and electron microscopy. There are also new options available in image cytometry, as described further below.

**TUNEL**

The Terminal Deoxynucleotidal Transferase dUTP Nick End Labeling (TUNEL) procedure is a very common method for examining DNA fragmentation that results from apoptosis. TUNEL labels the terminal ends of DNA that are cleaved in the spacer region between nucleosomes in chromatin, and these DNA strand breaks are visualised by light microscopy. Although TUNEL is a very effective assay for apoptosis, one major limitation of this approach is that TUNEL detects apoptotic cells at the latest stage in the process.

**Caspase activation**

At the molecular level, a series of enzymes known as caspases are largely responsible for executing the apoptotic, but not necrotic, cell death pathway. In response to pro-apoptotic signals, caspases

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**Figure 2**

Kinetic and time course studies can provide more enriched information in cell death analysis. Jurkat cells were treated with staurosporine (top) and gambogic acid, an anti-tumour agent (bottom) for different periods of time, and analysed with the Muse® MitoPotential and Muse® Annexin V and Dead Cell Assay.
participate in a series of reactions that result in the cleavage of protein substrates, causing the disassembly of the cell. Caspases exist in every cell of the body as dormant proenzymes. During apoptosis, caspases form active enzymatic tetramer molecules that can activate or inactivate their substrates. These enzymes specifically recognise a four or five amino acid sequence on the target substrate, which includes an aspartic acid residue as the target for cleavage. Caspases function in other biological processes, such as immunity; in fact, many viral genomes encode for direct inhibitors of these caspases. Caspases can be detected via immunoprecipitation or immunoblotting techniques using caspase-specific antibodies, or by utilising fluorochrome substrates that become fluorescent upon cleavage by the caspase. Additionally, the involvement of caspases can be visualised by treating live cultures with Smac mimetic, a very potent inducer of apoptosis.

Mitochondrial detection
Mitochondria are key regulators of cell death processes such as apoptosis. Loss of the mitochondrial inner transmembrane potential is often, but not always, observed to be associated with the early stages of apoptosis and is believed to have a role in caspase-independent cell death. Fluorescence-based assays designed to evaluate the functional status of mitochondria are useful tools for examining the role of mitochondrial activity in the apoptosis cascade.

Phosphatidylserine and annexin V
Phosphatidylserine (PS), confined to the inner membrane leaflet of viable cells, translocates to the exposed membrane surface during the early stages of apoptosis. This event serves as a signal for phagocytic cells to attack. PS is exposed mainly at the surface blebs of apoptotic cells. Annexin V has a high affinity for membranes containing the negatively charged PS. These events can be visualised using flow cytometry.

Flow cytometry
Flow cytometry assays for apoptosis are now almost 25 years old. The earliest flow cytometry assays for apoptosis analysed changes in forward and side scatter and DNA fragmentation following ethanol treatment. Flow cytometry assays now target almost every stage of apoptosis, from the earliest mitochondrial changes to caspase activation, membrane changes and DNA damage. Unlike earlier assays, flow cytometry analyses apoptosis in individual cells.

Multiparametric flow cytometry is an ideal approach for combining multiple cell death assays. FLICA caspase detection, annexin V and a cell-impermanent DNA binding dye (e.g., propidium iodide) can be combined into a powerful, multi-stage assay for apoptosis (Figure 1). Combining these assays allows the progression of apoptosis to be analysed, and provides a much richer picture than any one assay alone can provide.

A bench-top flow cytometry platform can also provide quantitative cellular information instantly for many cell health, cell death, cell signalling, immunology and cell cycle analyses. One such instrument uses the principles of microcapillary cytometry, which allows for analysis of small sample sizes. The platform is closed, whereby the reagents and software are closely paired together for dedicated applications in order to simplify the process and to reduce the analytical complexity for performing and obtaining cytometry-based data. The technology allows for the analysis of two markers simultaneously and can be used to perform time course and dose response studies, thereby providing deeper insights into mechanisms at play as well as a more comprehensive picture of the cell death process (Figure 2).

To date, there is only one flow cytometry assay available to analyse autophagy, which involves measurement of light chain 3 (LC3). LC3 is a protein that segregates to autophagosomes that are phagocytosing defunct mitochondria and other intracellular organelles and eventually into lysosomes. Autophagy can be detected by flow cytometry using green fluorescent protein (GFP)-LC3 translocation (Figure 3). In this assay, a cell line is stably transfected with GFP-LC3. During induc-
tion, a lysozyme inhibitor is added to block the destruction of autophagosomes by lysosomes. If autophagy occurs, GFP-LC3 will accumulate in autophagosomes when the inhibitor is present and will not be released into the media. If autophagy does not occur, then GFP-LC3 will accumulate in the cytoplasm and will be released into the media. Autophagosome-associated GFP-LC3 can be detected in the intact cells by flow cytometry.

**Image cytometry**

Image cytometry allows for cytometric data and correlated cell images to be collected simultaneously, and many options now exist for these types of analyses. Because apoptosis is highly variable and pleiotropic, imaging can provide verification that apoptosis is occurring and can also characterise the process to some extent through the visualisation of chromatin condensation and cytoskeletal breakdown. Image cytometry also provides additional analysis options, including pixel-by-pixel analysis, that are useful for apoptotic analysis. Additionally, trypsin or Accutase® detachment, which can 'muddy' apoptotic labels, is not necessary.

Image cytometry also allows for the analysis of adherent cells without removal of the cells from their substrate. For this reason, many laboratories utilise image cytometry for the analysis of adherent apoptotic cells. Adherent cells that are ripped off their substrate, either through scraping or by trypsin, can disrupt the apoptotic phenotype, or even induce the apoptotic process itself. Image cytometry allows adherent cells to remain adherent throughout analysis by staining cells directly on the chamber slide. It is important to note that adherent cells will round up once they undergo apoptosis, so some of the cells that are in the later stages of apoptosis may be lost.

A stream-based image cytometry system also provides direct correlation between cytometry and imagery, and thereby combines cytometric and morphological analysis. In a stream-based imaging system the cells are not imaged on a slide; rather, they are imaged directly in the stream. When cytometry and imagery are correlated, viable, early-, intermediate- and late-stage apoptotic cells can be gated and analysed (Figure 4).

**Conclusion**

Cell death is a natural process essential for many normal physiological functions for both development and homeostasis. Additionally, alterations within cell death signalling pathways are associated with various diseases. The measurement of individual hallmarks of dying cells is not sufficient for
A stream-based scanning cytometry system is an excellent way to combine cytometric and morphological analysis. When cytometry and imagery are correlated, viable, early-, intermediate- and late-stage apoptotic cells can be gated and analysed discriminating between the different cell death pathways, which include apoptosis, necrosis and autophagy; instead, a multi-parameter assessment is needed that includes both descriptive and functional criteria. Recent advances in technology now enable deeper insights into the molecular markers and cellular changes that characterise each cell death pathway, and have also made it possible to conduct many of these analyses as routine bench-top experiments.

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