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Current trends in luminescence-based assessment of apoptosis

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Apoptosis, the most extensively studied type of cell death, is known to play a crucial role in numerous processes such as elimination of unwanted cells or cellular debris, growth, control of the immune system, and prevention of malignancies. Defective regulation of apoptosis can trigger various diseases and disorders including cancer, neurological conditions, autoimmune diseases and developmental disorders. Knowing the nuances of the cell death type induced by a compound can help decipher which therapy is more effective for specific diseases. The detection of apoptotic cells using classic methods has brought significant contribution over the years, but innovative methods are quickly emerging and allow more in-depth understanding of the mechanisms, aside from a simple quantification. Due to increased sensitivity, time efficiency, pathway specificity and negligible cytotoxicity, these innovative approaches have great potential for both *in vitro* and *in vivo* studies. This review aims to shed light on the importance of developing and using novel nanoscale methods as an alternative to the classic apoptosis detection techniques.

Introduction

From the very beginning, cell death identification was and continues to be an important approach in the field of drug discovery and molecular biology.[1] Many of the classical techniques rely on fluorescent probes in order to point out regulated cell death (RCD) mechanisms by targeting specific cellular markers.[2] At the same time, the development of new dyes or probes for cell structures and organelles examination has allowed the indirect association of cell morphology and cell death. These modifications are mainly explored through microscopy imaging and usually only offer a qualitative analysis of cellular behaviour.

On the other hand, for the generation of statistically significant data regarding the incidence of RCD and/or abundancy of RCD specific markers, other approaches that allow quantitative analysis are used. In this regard, RCD is monitored using techniques such as: nuclear magnetic resonance,[3] flow cytometry,[4] imaging approaches,[5,6] photothermy[7] or ultrasound frequency evaluation.[8] Additionally, there are other analytical procedures for performing molecular biology assays that are suitable for cell death evaluation[9] like toxicology tests,[10] viability assays[11] and PCR assays.[12,13] These involve the analysis of specific genes, RNA or proteins, as valuable indicators of cell death. In this case, many of the techniques are engaging spectrophotometry. Moreover, mathematical models are offering new perspectives in cell death analysis especially regarding the most likely cell death pathway to be engaged in specific conditions.[14,15] Qualitative and quantitative examinations of cell death synergize to offer an accurate and valid result.

According to the recommendations of the Nomenclature Committee on Cell Death, in 2018, a novel classification for the RCD processes based on mechanistic and other important aspects was established.[16] Cell death categories are described depending on the signal molecules involved in and are named as follows: intrinsic apoptosis, extrinsic apoptosis, mitochondrial permeability transition driven necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic cell death, netotic cell death, lysosome-dependent cell death, autophagy-dependent cell death, non-lethal processes. This review aims to highlight the current and most innovative methods for the detection of different apoptosis pathways *in vitro* and *in vivo*, while briefly summarizing the most commonly used techniques, their drawbacks and the need of implementing new and more advanced approaches.

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Apoptosis is a physiological process described as an active mechanism involving cell shrinkage, membrane blebbing, vacuolated cytoplasm, chromatin fragmentation and the production of apoptotic bodies.[17] The main apoptosis signalling pathways are well described and can be seen in Fig. 1. This phenomenon happens relatively fast and its phases are difficult to capture. Beyond that, apoptotic bodies, if not cleared by phagocytes, can proceed to secondary necrosis[18] and may influence neighbouring cell populations by inducing inflammation and/or cellular stress due to the release of intracellular contents. Even though secondary necrosis is not a RCD mechanism itself, it shares similar morphological features to apoptotic[19] and necrotic cells and as a consequence, it can be challenging to decipher the exact cell death pathway. Moreover, other RCD mechanisms also resemble apoptosis when it comes to morphology and/or expression pattern of certain molecules

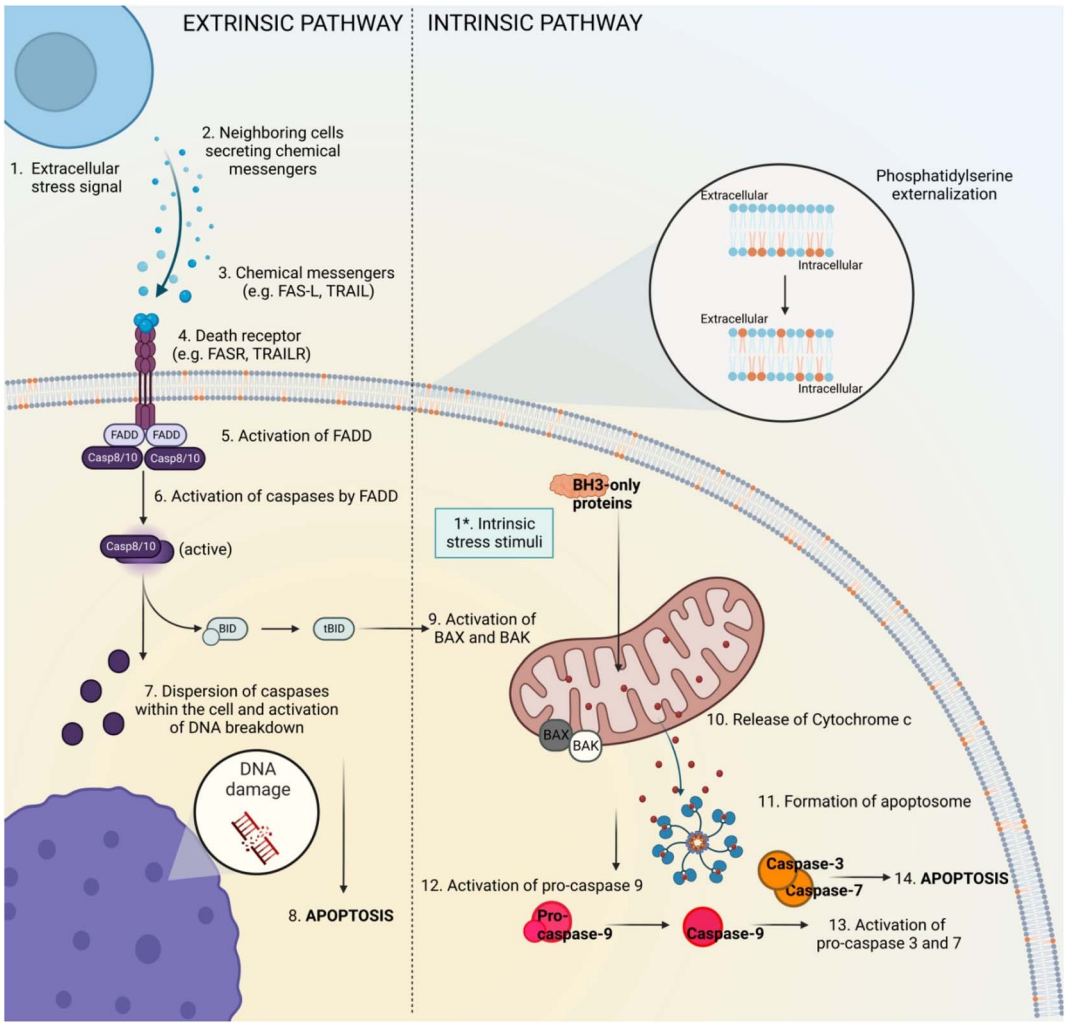

Fig. 1 Schematic representation of apoptosis. There are two main pathways that conduct to the installation of programmed cell death: extrinsic pathway and intrinsic pathway. The extrinsic one starts with a stress stimuli outside of cell (1) that determines the secretion and binding of specific ligands to death receptors. The chemical messengers can be secreted by neighbouring cells (2) and are often represented by FAS and TRAIL (3) that bind their specific receptors on the targeted cell (*e.g.* FASR, TRAILR) (4). The ligand–receptor interaction determines modification within the intracellular domain which activates FADD protein inside the cell (5). Activated FADD interacts with Pro-caspase-8 and pro-caspase-10, causing their activation through cleavage of proteins that keep them inactive (6). The active caspases (Caspase 8 and Caspase 10) are further dispersed within the cell cytoplasm and trigger the activation of specific molecules, including proteins involved in DNA breakdown (7), finally causing the installation of apoptosis (8). Activated Caspase 8 and Caspase 10 are also interacting with BID that is transformed in tBID (9). tBID moves into the mitochondria causing the activation of BAX and BAK (9) – this step is also the intersection point between the extrinsic pathway and the intrinsic one. The last ones are favouring the installation of mitochondrial outer membrane permeability (MOMP) that is allowing the release of cytochrome C from the mitochondria into the cytoplasm (10). The next steps involve the formation of complex of proteins termed "apoptosome" (11), complex that is managing the activation of Pro-caspase-9 (12). Activated Caspase-9 is triggering the activation of Caspase-3 and Caspase-7 (among other functions) (13), enzymes that begin the process of cellular materials breakdown that finally concludes to installation of apoptosis (14). The extrinsic pathway is triggered by an internal stress stimulus (*e.g.* DNA damage, hypoxia) (1*) which activates a class of proteins called BH3-only proteins (2*). Pro-apoptotic BH3-only proteins are managing the same activation of BAX and BAK, point which represents the intersection between the extrinsic and intrinsic pathway. This figure was created using https://www.biorender.com/.

involved in cell death establishment. This suggests the need of researchers to adapt their studies to the quickly developing field of cell death research by using novel and innovative techniques of RCD detection. Some of these methods, the targeted biomarkers correlated to the stages of apoptosis are presented in Table 1.

Classic methods for apoptosis detection and their main drawbacks

As previously mentioned, many techniques for the detection of apoptosis were developed, many of which showed impressive results *in vitro*. One of the most common approaches is the use of fluorescent dyes that specifically bind to target molecules associated to apoptosis pathways. Microscopy or spectroscopy techniques are used to visualize morphological changes and/or quantify labelled biomarkers. However, through intensive research, new distinct mechanisms of cell death have lately been identified and many of the classic dyes (PI, DAPI, annexin V) lack an acceptable specificity or selectivity for their pathways. As a result, they may enter/label dead or dying cells, but the researcher cannot accurately discriminate one cell death pathway from another. For instance, DAPI and PI cannot distinguish between early apoptotic and necrotic cells leading to potential false-positive results when used to detect apoptosis. Moreover, annexin V often uses expensive recombinant proteins which can affect cell activity or introduce artifacts. The lack of real-time monitoring given by the fact the most classic methods use endpoint measurements, makes it difficult to study cell dynamics over time, which is necessary for several research issues. These are just a few drawbacks, which can be overcome with the aid of other molecular techniques. Even though this strategy can be time-consuming, it continues to be a good method for validating the results or for providing more in-depth answers.

For the *in vitro* apoptosis detection, different methods are commonly used: microscopy, biochemical techniques, spectroscopy, electrochemistry and microfluidics.[95] Among the first procedures, time-lapse electron microscopy was applied for morphological hallmarks monitorization of the cells.[96]

On the other hand, *in vivo* detection of apoptosis has additional limitations which makes it more challenging when it comes to designing fluorescent probes. Most of these are related to the luminescent properties of the probe, interaction of the light with different tissues or biocompatibility. Notably, scattering of both the excitation or the emitted light by the biological tissues can drastically impact the resolution or intensity of the signal. Moreover, the tissue penetration depth is highly dependent to the wavelength of the light used. One reason for this is that tissue molecules interact distinctly with different wavelengths when it comes to the magnitude of scattering or absorption events. Hence, longer wavelength light has lower energy, which besides the fact that it is less likely to be scattered or absorbed, also has the advantage of a reduced photobleaching effect on the fluorescent probe used. Other difficulties that need to be overcome in order to design effective fluorescent probes for apoptosis detection *in vivo* include the low signal to noise ratio given by the autofluorescence of the complex biological environments; cytotoxicity or other events triggered by the interaction of the exogenous probe with the cells; biological barriers (such as blood–brain-barrier) which sometimes need to be worked through by the fluorescent probe to analyse specific tissues. Another aspect that needs to be considered is that the utilization of *in vivo* research for the detection of apoptosis may give rise to ethical considerations in certain instances, as well as provide technical obstacles in terms of sample acquisition and observation of events within living organisms.

From a biochemical point of view, some specific proteins and genes that are activated during apoptosis are recognized as key effectors[97] and can help investigate the cell death pathway with increased accuracy. Besides the simple quantification of these effectors, which remains a strong technique, the localization of particular molecules, such as the externalization of PS or cytochrome c release from the mitochondria, is also an appropriate method. Caspases are commonly studied key effectors of apoptosis. Their activity can be quantified by using fluorogenic caspase substrates *via* flow cytometry.[98] On the other hand, cytosolic cytochrome c is often assessed *via* fluorescence microscopy[52,99] or flow cytometry[47,100] for apoptosis detection and, other biomarkers, such as oligonucleosomal multimers of 180–200 bp, or the ratio of proapoptotic to anti-apoptotic proteins (ex. Bcl-2/Bax) can be quantified with the help of gel-based assays.[101–103]

Fluorescent dyes

Detection approaches are dependent on the fluorescence property of the probes used for apoptosis specific markers labelling engaging microscopy, flow cytometry or even genomic methods for more detailed investigations. In contrast, many widely used fluorescent dyes may exhibit limited specificity, since they have the potential to attach to other cellular structures or processes, hence resulting in potential instances of false positive results. The most used dye is annexin V conjugated to fluorescein isothiocyanate (FITC) which can detect middle to late stage apoptosis by binding to the exposed phosphatidylserine molecules on cell membrane.[104] However, integrating these dyes with complementary techniques can mitigate some of the limitations. In many protocols, annexin V-FITC is used in combination with propidium iodide (PI) which discriminates between live and necrotic cells by displaying red fluorescence.[105] In general, these two dyes are imaged using fluorescence microscopy, but for validation and numerical quantification, flow cytometry is usually required.[106] This method is an analytical technology that ensures a rapid examination of cell populations offering flexibility for different particularities of cell cycle events research.[107]

The combination of microscopy imaging and flow cytometry in one device makes the detection of apoptosis and necroptosis possible, as described by Pietkiewicz *et al.* 2015. They imaged the morphology of the nucleus at a single cell level using annexin V-FITC and propidium iodide staining protocol.[108]

Table 1 Methods for different stages of apoptosis detection and the main biomarkers

Apoptosis stage	Event	Biomarker	Methods used for the detection	Sources
Early	Loss of membrane asymmetry	Phosphatidylserine (PS) externalization	Light microscopy	20
			Carbon dots-annexin V probe & fluorescence spectroscopy	21
			AI & PI staining	22
			Flow cytometry	23 and 24
			Electrochemical probes	25
			Label free SERS sensing	26
			Fluorometric methods	27
			Iron oxide nanoparticles conjugated with *Pisum sativum* agglutinin & MR imaging & confocal microscopy	24
			Imaging flow cytometry & convolutional autoencoder	28
	Cell shrinkage and membrane damage	Decrease in cell size, presence of membrane blebs	Imaging techniques	29 and 30
			Flow cytometry	4 and 29
			Dielectrophoretic assay	31
	Chromatin changes	DNA condensation	Spectrofluorometric assay	32
			High resolution confocal imaging – single molecule localization microscopy	33
			Monoclonal antibodies against single-stranded DNA	34 and 35
			ELISA	36–38
			TUNEL assay	4,39
			DNA-specific fluorochromes & fluorescence microscopy	40–42
			Haematoxylin & eosin staining	43
	Mitochondrial changes	Depolarization of the mitochondrial membrane potential	Membrane permeable lipophilic and cationic fluorescent dyes & fluorescence microscopy	44 and 45
			Mitochondrial activity of streptolysin O permeabilised cells & luciferase luminescence assay	46
		Release of cytochrome c and other pro-apoptotic factors from the mitochondria to the cytosol	Flow cytometry – anti-cytochrome c monoclonal antibodies & fluorochromes	47
			Competitive immunoassay of ELISA, electrochemiluminescence ELISA	48 and 49
			Western blot	50
			Fluorometric assay based on aptamer/gold nanocluster probe	51
			Confocal laser scanning microscopy using carbon dots	52
		Activation of Bax proteins	Western blot, single molecule localization microscopy, confocal imaging, fluorescence cross correlation spectroscopy, protein-cross linking, and mass spectrometry	53
			Immunocytochemistry	54
	Activation of caspases and apoptosome formation	Caspase-8 activation by extracellular signals, like fas ligand or TNF	Colorimetric assay, Western blot	55
			ELISA	56 and 57
			Flow cytometry, fluorometric activity assay	58
			Immunofluorescence	59
			Membrane permeant fluorescent-labelled inhibitors of caspases & fluorescence microscopy	60
		Caspase-9 activation by cytosolic cytochrome c and the formation of the apoptosome together with Apaf-1	Colorimetric assay (*e.g.* using unlabelled DEVD containing peptide substrate and unmodified gold nanoparticles) & Western blot	55 and 61
			Mathematical simulation & computational methods	62 and 63
			Split luciferase complementation assay (*e.g.* Lumiptosome, lentivirus transfected split luciferase fragment)	64–66
			Calorimetry, NMR spectroscopy, site mutagenesis	63

Table 1 (*Contd.*)

Apoptosis stage	Event	Biomarker	Methods used for the detection	Sources
			Immunofluorescence	59
Mid	DNA damage	DNA fragmentation by caspase-activated DNases (CAD) into 180–200 bp long fragments (X)	SDS-PAGE	67
			TUNEL & single stranded DNA assay	68
			COMET assay	69
			Laser scanning cytometry, Halo assay, combination assay	22
			In situ ligation assay	70 and 71
			In situ end labelling	72 and 73
	Change in cell shape	Cell becomes spherical and detaches from neighbouring cells	Light microscopy, fluorescence microscopy TEM & SEM	30 and 74
	Apoptotic bodies formation	Activation of caspase-6 and caspase-7	Electrochemiluminescence ELISA assay using a neo-epitope antibody against cleaved Lamin A	75
			Coumarin based fluorescent substrates, irreversible inhibitors, selective aggregation induced emission luminogens combined with mass cytometry, immunofluorescence, confocal microscopy, and western blot	76
			Immuno blot, flow cytometry, luminescence & immunofluorescence	59 and 76–79
			SDS-PAGE, Coomassie staining, fluorography analysis, caspase activity assay by means of fluorimetry	79
		Membrane bound fragments called apoptotic bodies are formed in a controlled way, preventing the release of certain molecules in the extracellular matrix which could interfere with surrounding cells	Microscopy methods (phase contrast time lapse microscopy, confocal microscopy, TEM, SEM)	74 and 80–82
			Dynamic light scattering, liquid chromatography tandem mass spectrometry	74 and 80
			Fluorescence activated cell sorting, differential centrifugation & flow cytometry staining	81 and 83
			Morphometric area analysis & morphometric cell count	84
			Fluorescence labelling with Trp-BODIPY cyclic peptide	85
			In situ DNA ligation	86
Late	Phagocytosis	Apoptotic bodies are engulfed and degraded by other cells	Microscopy methods (light microscopy, phase contrast, fluorescence, confocal, TEM, SEM)	87–90
			Fluorescence activated cell sorting, differential centrifugation & flow cytometry staining	83, 88 and 89
			Fluorescence labelling with Trp-BODIPY cyclic peptide	85
	Degradation of cellular components	Organelles and other cellular structures are processed into component parts	Immunolabelling methods	91–93
			Microscopy methods (confocal microscopy, TEM &SEM)	91, 92 and 94

TUNEL assay

TUNEL assay is another representative apoptosis assay that can be performed *in vitro* on cell cultures, but also *in vivo* on animal models[109] and *ex vivo* on different tissues.[110,111] The technique name comes from terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick-end of DNA fragments labelling during chromatin condensation.[112] The analysis have involved flow cytometry[113] or microscopy.[41] Being one of the firsts methods used for apoptosis detection, TUNEL assay had a big disadvantage, its accuracy in differentiating between necrotic cells and late-apoptotic cells.[114] In the next years, an attempt to optimize these difficulties was made and the improvements consist in exploring other fluorophores[115] or antibodies[116] for labelling the DNA fragments or using different software platforms for the analysis.[84]

Considering these aspects, the significance of developing novel techniques for apoptosis detection lies in their potential to address the challenges associated with conventional methods. By doing so, researchers can obtain more precise, targeted, and comprehensive insights into apoptotic mechanisms within both research and clinical settings. This phenomenon contributes to an enhanced comprehension of cellular apoptosis mechanisms, pathological pathways, and the potential of establishing improved therapeutic approaches.

Advanced methods for the detection of apoptosis

Caspases

Caspases are cysteine proteases that act as key components in certain types of programmed cell death, such as apoptosis, pyroptosis, necroptosis, necrosis, autophagy and mitotic catastrophe.[117] Normally they are expressed as inactive zymogens but once stimulated, they become fully active subsequently autolytic cleavage.[118] Apoptosis involves the activation of caspases which degrade cell components. They are known to cleave a broad spectrum of structural proteins like actin, tubulin, vimentin, ROCK1, filamin-A and fodrin which might contribute to the specific apoptotic cell morphology.[119] This is followed by the formation of apoptotic bodies which are removed by surrounding cells *via* phagocytosis. Their active form and cleavage residue presence can be correlated to specific cell death types. Direct quantification methods for these cell death biomarkers that rely on antibody labelling are currently used, including ELISA, flow cytometry and Western blot. However, sample damage, long assay duration and the need of sophisticated instruments and expensive reagents, are some drawbacks that make them unsuitable for live cell assays, point-of-care applications and/or *in vivo* studies. Evaluation of apoptosis by monitoring the proteolytic activity of such caspases is a more reliable and less invasive approach that allows real-time monitoring of apoptosis both *in vitro* and *in vivo*. With hundreds of substrates identified so far,[119] proteomic studies were able to highlight the dominant motifs cleaved by specific members of the caspase family of proteins.[120] Since caspase 3 and 7 are the main proteases involved in cell death, many sensors for apoptosis rely on short peptides containing restriction sites for these two members. The optimal and most used sequence for caspase 3/7 activity sensor designing is a 4 amino acids peptide: DEVD (Asp-Glu-Val-Asp). Therefore, a wide variety of methods for apoptosis detection rely on linking a fluorophore to a quencher through a DEVD motif. The resulted construct will either switch from a fluorescent off state to an on state or significantly change its optic properties consequent to the specific proteolytic cleavage.

A wide variety of methods to quantify caspase activity for the indirect evaluation of apoptosis have been reported.

Caspase cleavage of bioengineered substrate molecules as a fluorescence on/off switch. Since these techniques only show a fluorescent signal as a response to activated caspases, it is a reliable approach to distinguish apoptosis from other types of cell death. Therefore, they can be used to highlight the efficacy of treatments that involve the use of apoptosis-inducing compounds.

Genetically modified cells to express switch-on fluorescence-base indicators that are specifically cleaved by caspase-3 like proteases have been used to detect cell death in real-time.[121] These indicators were designed by genetic engineering using Venus (a yellow fluorescent protein) as a template. The final genetically encoded construct is a cyclized protein, in a non-fluorescence state, that contains a caspase-3 cleavage site (DEVD). Fluorescence activity is only acquired after the caspase specific cleavage of DEVD. This method allows the evaluation of apoptosis in real-time in various conditions and is suitable both for 2D and 3D cell culture models.

One interesting and novel approach relies on aggregation induced emission (AIE) luminogens. AIE is a phenomenon that has gained significant interest in the domain of cellular imaging and theranostics. In contrast to other fluorescent molecules or nano-objects, AIE luminogens (AIEgens) present a strong emission when they form aggregates due to a restriction in intramolecular movements.[122] Since their discovery by Ben Zhong Tang's group,[123] they have become a key component in many applications, including the evaluation of cellular processes. Moreover, AIE based probes suitable for both apoptosis evaluation (through caspase-3/-7 activity quantification) and apoptosis inducing compounds screening have been described. One such probe consists of an AIE hydrophobic luminogen (TPE) in a fluorescence off-state due to the conjugation with a caspase-specific peptide (DEVD). When apoptosis is triggered following caspase-3/-7 activation, the two subunits are freed through caspase cleavage and the TPE residues aggregate followed by a turn-on in their fluorescence.[124]

Fluorescence resonance energy transfer (FRET) between two proteins (CFP and YFP) linked together by DEVD has been used for apoptosis detection in living zebra fish at single cell resolution. The gene for this fusion protein was inserted after a skin specific keratin 4 promoter leading to a green fluorescence signal (specific to YFP) under the excitation of the donor molecules (CFP) in skin cells. The cleavage of DEVD by caspase 3/7 would reduce the energy transfer, shifting the overall emission from green to blue (specific to CFP). Single apoptotic cells could be tracked in real time in a live vertebrate animal model.[125]

Caspase cleavage of luciferase prosubstrates and bioluminescence evaluation

Besides fluorescence, bioluminescence is another non-invasive optical imaging technology that has shown huge potential for the detection of biological processes *in vivo*. This method relies on the enzymatic conversion of a non-luminescent substrate into a light producing molecule. As a result, bioengineered cells that express luciferase can metabolise exogenous luciferin leading to a luminescent signal, which can further be quantified. Lately, numerous bioluminescent probes for apoptosis detection in animal models have been developed.

In one example, firefly luciferase positive cells were subcutaneously administered in SCID mice and tumours were allowed to grow. The authors used a modified substrate, Z-DEVD-amino luciferin, which could only be metabolised by luciferase after the initiation of RCD, the consequent activation of caspases and cleavage of the prosubstrate.[126] Therefore, they were able to provide a biocompatible, preclinical method for the detection of apoptotic cells.

Cytochrome c

Cytochrome c (Cyt c) is a hemeprotein that is connected to the inner mitochondrial membrane and functions as an electron transporter required for ATP production.[127]

The transfer of Cyt c from the intermembrane space of the mitochondria into the cytoplasm is an event unique to early apoptosis because, under normal circumstances, the outer mitochondrial membrane is only permeable to small metabolites up to ~5 kDa.[128]

This process, which is a crucial step in the intrinsic apoptosis pathway, was frequently referred to as the irreversible point of apoptosis,[99] even though more recent data suggest that cells might recover from late stages of programmed cell death through a mechanism called anastasis.[129] However, quantifying cytochrome c release from the mitochondria remains a powerful tool for the evaluation of apoptosis.

In dying cells, a Cyt c concentration between 1 and 10 μM (ref. 99) can be identified because a little portion of Cyt c released from the mitochondria can stimulate various amplification loops, which lead to the release of all of Cyt c from the mitochondria to the cytosol.[128,130] This will eventually lead to the formation of the apoptosome (Apaf-1/caspase-9/cytochrome c complex), which is in charge of cellular structure breakdown.

As a result, the quantification of Cyt c release is frequently employed to highlight mitochondria-dependent apoptosis.[100] However, the most popular techniques are either laborious or call for complicated processes including immunolabeling, subcellular separation, and western blotting.[47]

As a result, nanoparticle-based assays for Cyt c quantification have gained popularity lately. Many of these rely on carbon dots (CDs), some carbon-based nanoparticles, due to their unique optical properties, increased stability, biocompatibility, selectivity and sensitivity. Certain quenching mechanisms of Cyt c towards CDs have made them ideal candidates for the quantification of this molecule. Based on these properties, recent studies report the successful usage of CDs for apoptosis detection *in vitro*.[52,99] Moreover, Cyt c concentration was determined in human serum samples[131] and in living zebrafish.[99]

Phosphatidylserine externalization sensors

Phosphatidylserine externalization acts as an "eat me" signal of apoptotic cells to neighbouring phagocytes and is considered a hallmark of apoptosis.[132] However, recent studies suggest that the PS externalization process is inducible, reversible and not associated to other apoptosis specific events, such as mitochondrial Cyt c release, activation of caspases or DNA fragmentation.[132,133] Nevertheless, PS export to the outer plasma membrane leaflet lays the foundation for Fluorescein isothiocyanate-conjugated annexin V binding assay for apoptosis detection.[23] Notably, this process is regulated in a calcium dependent manner[133] and a complete inhibition of PS externalization was reported in some hematopoietic cell lines in the absence of extracellular calcium.[133] Moreover its externalization is also inhibited by calcium channel blockers,[132] making the annexin V assay through flow cytometry or fluorescence microscopy rely on the addition of external calcium ions. Similar to this technique, more recent methods that focus on labelling PS have been developed. The detection of single apoptotic cells using luminol electrochemiluminescence has been reported.[134] Other studies use nanomaterials such as carbon quantum dots to design annexin V conjugated apoptosis detection probes,[21] or silver nanoparticles followed by SERS analysis to highlight externalized PS.[26] Radiolabelled annexin V was also used to detect apoptotic cells *in vivo*.[135,136] Although, there are lots of commercially available FITC-annexin V kits to highlight and distinguish apoptosis from other RCD types, and a wide diversity of methods have been developed and used to assess cell death based on the same principle, their efficacy and specificity have lately been questioned due to recent studies which report PS exposure in non-apoptotic cells, such as cells that undergo necroptosis or pyroptosis.[137–140]

Computational tools

Label-free detection of apoptosis in live cells could help overcome the inconvenience of a potential interference between the fluorescent probe and the physiological functions of the cell. Although it can be difficult to achieve, deep learning- and activity recognition-based systems have allowed the detection and quantification of apoptotic events with high accuracy. The training model relies on large datasets of both apoptotic and nonapoptotic cells, multiple cell types and fluorescent labels and several imaging techniques. This computational tool can detect multiple apoptotic events in microscopy time-lapses and quantify cytotoxicity *in vitro* and *in vivo* based on live-cell imaging, such as intravital microscopy data.[141]

Other recent studies describe the use of deep learning to discriminate apoptosis from necroptosis using label-free digital holographic microscopy,[142] or to classify apoptosis and ferroptosis consequent to anti-transferrin receptor 1 antibody, DAPI and phalloidin-FITC staining.[143] The aforementioned methods are able to predict or identify these cellular processes with increased accuracy.

Clinical evaluation of apoptosis

The last years have witnessed an increasing amount of newly approved therapeutic schemes, especially in the oncological domain. These treatments aim to disrupt the carcinogenesis process by interfering with the components from the cell death pathway and induce the apoptosis.[144,145] While the main aspect behind these therapies is represented by efficient tumour clearance, a second goal is to achieve the installation of programmed cell death so that cancer cells can be eliminated by neighbouring phagocytic cells in a "quiet and quickly" manner.[146] Therefore, it is critical to implement in clinical practice imagistic techniques that can specifically evaluate the efficiency of apoptosis-inducing therapies for disease monitoring, especially cancer. The different approaches for apoptosis detection, their advantages, drawbacks and some applications are presented in Table 2.

Currently, in clinical setting, these methods primarily rely on magnetic resonance imaging (MRI) that can track the changes in the diffusive properties of a tissue or tumour mass (DW-MRI), where a tissue affected by cell death will have an increased water diffusion and diffusion coefficient respectively.[147,148] In addition to MRI techniques, high-frequency ultrasound (10 MHz or

Table 2 Comparison of different luminescence-based methods for apoptosis detection

Method	Source	Advantages	Drawbacks	Applications
Bioluminescence	Bioluminescence proteins (*e.g.*, firefly luciferase/Renilla luciferase)	High sensitivity	In some cases, the reporter gene must be introduced in the genome through genetic manipulation	*In vitro*, *ex vivo* and *in vivo* detection of apoptosis[165]
		Does not require external excitation light source	Lower resolution compared to other techniques	*In vitro*, *ex vivo* and *in vivo* drug screening of proapoptotic compounds[166]
	Bioluminescence resonance energy transfer (BRET) – based biosensor	Suitable for real-time detection	Transient signal	*In vitro* fluorescence-based detection and *in vivo* bioluminescence-based detection of apoptosis[167]
		Suitable for *in vivo* studies		BRET-induced NIR emission-based detection of apoptotic cells[168]
Fluorescence	Caspase activity reporters (fluorescent molecule (±quencher) bound to a peptidic substrate)	High sensitivity	The reporter gene must be introduced in the genome through genetic manipulation	Apoptosis monitoring and drug screening detection based on AIE[124]
		High specificity	Caspase kinetics alteration	Real-time monitoring of apoptosis in cultured cells[169,170]
		Suitable for real-time detection	Limited applicability for complex tissues and *in vivo* models	Caspase activity evaluation with potential applications in drug design and screening[171]
		Suitable for *in vivo* studies	Potential cytotoxicity Challenging data interpretation	
	Annexin V – conjugated probes	High sensitivity	In some cases, the reporter gene must be introduced in the genome through genetic manipulation	*In vivo* detection of cell death in murine cells and embryonic tissues[172]
		Suitable for real-time detection	May require exogenous calcium ions[133]	Vis/NIR-detection of cytotoxic compounds-induced apoptosis *in vitro* and *in vivo*[173]
		Suitable for *ex vivo* and *in vivo* studies	Specificity limitations (due to PS exposure in non-apoptotic cells)[137–140]	*In vitro* and *in vivo* real-time imaging of apoptosis[174]
	Nanomaterials (*e.g.*, carbon dots, hybrid nanoparticles)	High sensitivity	Potential cytotoxicity	Vis/NIR-detection of cytotoxic compounds-induced apoptosis *in vitro* and *in vivo*[173]
		High specificity	Challenging data interpretation	*In vitro* monitoring of apoptosis inducing compounds[52]
		Suitable for real-time detection	Limited use *in vivo*	Real-time detection of apoptosis[175]
	Fluorescent antibodies	High sensitivity	Endpoint measurement	Flow cytometry evaluation of apoptosis
		High specificity	Challenging data interpretation	Immunofluorescence assays[92]
		Suitable for *ex vivo* studies	High cost	
	Other fluorescence dyes (*e.g.*, DAPI/Hoechst/PI, FITC-annexin V, Mitotracker dyes)	Cost-effective	Limited use *in vivo*	*In vitro* detection of apoptosis[106]
		Some of them have high specificity for target biomarkers	Challenging data interpretation	
		Versatility	Often – endpoint measurement	
			May interfere with other cellular processes, leading to false positive results	
			Photobleaching	

Table 2 (*Contd.*)

Method	Source	Advantages	Drawbacks	Applications
Photoacoustic	Contrast agents (functionalized NPs, indocyanine green conjugates, apoptosis-specific nanobodies)	High sensitivity and spatial resolution Suitable for real-time detection Suitable for *in vivo* studies	Limited specificity May interfere with other cellular processes High costs of production Photostability limitations Challenging acquisition setup and data interpretation	*In vivo* real-time detection of tumour apoptosis[176,177] *In vivo* evaluation of stem cell location and apoptosis[178]
MRI	Contrast agents, paramagnetic/ultrasmall superparamagnetic iron oxide NPs	High sensitivity Suitable for real-time detection Suitable for *ex vivo* and *in vivo* studies	Potential cytotoxicity Limited specificity Long acquisition times High-cost equipment Challenging data interpretation	Detection of drug-induced apoptosis *in vitro*, *ex vivo* and *in vivo*[179] *In vivo* real-time detection of tumour apoptosis[177]
PET/SPECT	Specific radiotracers	Suitable for non-invasive, whole-body *in vivo* imaging High sensitivity Quantitative Versatility	Limited specificity and resolution High costs Radiation exposure Challenging data interpretation	*In vivo* evaluation of caspase activity[153,180] *In vivo* monitoring of externalized PS during chemotherapy induced apoptosis[181,182]
Ultrasound	Contrast agents	Non-invasive Suitable for real-time detection Low cost Versatility High spatial resolution	Limited specificity Limited contrast Limited depth penetration Limited to structural information Challenging acquisition setup and data interpretation	Detection of drug-induced apoptosis *in vitro*, *in situ* and *in vivo*[150] Drug screening, *in vitro* detection of different forms of cell death[183] Pre-clinical *in vitro* monitorization of radiotherapy[184] and photodynamic therapy effects[149]

greater) has shown promise in the detection of apoptotic cells, both *in vitro* and *in vivo*. This imaging modality utilizes the unique specular reflections produced by apoptotic cells, predominantly arising from the condensation and fragmentation of the cell nucleus during the later stages of apoptosis. Ultrasound transducers operating at frequencies of 10–60 MHz generate wavelengths of 25–150 μm, which closely match the size of individual cells and nuclei (10–20 μm), enabling sensitivity to change in cell size and nuclear morphology associated with apoptosis. Notably, the backscatter from apoptotic nuclei is significantly higher, up to six-fold, compared to non-apoptotic cellular nuclei. This distinctive signal can be observed in cultures treated with a variety of drugs and radiation. Consequently, the combination of high-frequency ultrasound with other imaging modalities, such as MRI, offers a comprehensive approach for evaluating apoptosis and provides valuable insights into the cellular and molecular changes occurring in pathological conditions.[149,150]

Magnetic resonance spectroscopy (MRS) is also used to measure lipid concentration and correlation of fat-water ratio for cell death quantification.[151,152] However, these techniques are not capable to timely and directly quantify the induction of apoptosis and fail to offer specific indicators. Other intensive tested methods involve the radiolabelling of Annexin-V or

aposense compounds and tracking of their uptake and biodistribution *via* positron emission tomography (PET) or single photon-emission tomography (SPECT). Although more specific, these methods are not yet efficient in specifically detecting and differentiating true cell death from growth arrest.[146] Experimental imaging techniques are now relying on the specific labelling of active caspases or substrates that are cleaved by these enzymes[153,154] and further quantification *via* PET imaging. Even so, there are issues related to the selectivity and specificity of the ligands or the uptake of the substrates.

Fluorescent imaging is one of the most commonly used technique for preclinical research involving early diagnosis, drug development, real-time therapeutic monitoring and prediction of prognosis. The reliability of this technique is supported by the sensibility of fluorescence, the stability of the fluorescent labels, advanced labelling techniques for specific molecular targets and ultimately, the reliable spatial resolution in comparison with the cellular dimensions.[155] Most of the techniques currently used in the clinic involve *ex vivo* analysis for quantification of specific molecules or events based on fluorescence assay. Such an example is the evaluation of apoptosis through terminal deoxynucleotidyl transferase (TdT) dUTP nick and labelling (TUNEL). This assay is measuring the fluorescent labelled dUTPs that are integrated *via* TdT into the damaged DNA of a biopsy sample, where the intensity of the fluorescence is corelated with the degree of apoptosis.[156,157] Similar principles are applied in the case of immunofluorescence techniques where specific tissue or circulatory molecules can be detected based on antibody – antigen affinity and use of fluorochromes to detect the location of the antibody. This technique can highlight the presence of specific molecules associated with the induction of apoptosis, or even multiple ones through simultaneous labelling with different fluorophores (*e.g.* FITC or rhodamine).[158] These assays are valuable to quantify the molecular changes related to cell death in tissue samples before and after treatment; however, there is a significant disadvantage consisting in the necessity of multiple biopsies and inability to monitor patients in real-time. While serial tumours sampling (or other tissue) is not necessarily a proper strategy in clinical trials, an advantageous technique could be represented by specific measurement of circulating molecules associated with cell apoptosis, considering the minimally invasive character of blood sampling. One group of such molecules are cytokeratins that represent up to 5% of the intracellular proteins.[159] Experimental evidence suggests that quantification of CK18 and/or CK19 in liquid biopsies could predict the cell death ratio in tumours, but are not able to differentiate apoptosis from necrosis: the release of soluble CKs takes place in both mechanisms.[160,161] However, combination techniques like the one between M30 apoptosense ELISA directed toward a caspase-cleaved neo-epitope on CK18 and M65 ELISA for detection of cleaved and intact CK18 from liquid samples could differentiate between different mechanisms of cell death in patients.[161] Currently used techniques for molecular diagnostics are based on ELISA assays that quantify the colorimetric reaction between an antibody directed toward a molecule of interest and linked to an enzyme, and a substrate that is processed by the specific enzyme.[158] However, considering the low amounts of circulatory apoptotic biomarkers in the liquid samples of the patients, a more advantageous approach could consist in fluorescence-linked immunosorbent assay (FLISA) or fluorescent ELISA (FELISA), techniques that are a more sensitive variation from the standard colorimetric ELISA and also more suitable for high throughput screening due tominimal amounts of required antibodies and less manipulation. However, at the current time, there are no extensive clinical studies based on this approach.

Nevertheless, an *ex vivo* approach for dynamically measuring cell apoptosis in patients during treatment is not necessarily feasible compared to the imagistic approaches. Fluorescent evaluation in the clinical setting is hampered by the unavailability of fluorescent dyes approved for patients use.[155] For the moment only two fluorophores, indocyanine green (ICG) and fluorescein, have been reported in the clinic for fluoresce based imaging of patients. ICG has been used to reconstruct in 3D an image of human breast cancer *via* fluorescence diffuse optical tomography (FDOT).[162] Fluorescein has been experimentally used in ophthalmology for diagnosis of cystoid macular edema (CME) *via* fundus fluorescein angiography (FFA). Even so, the standard optical coherence tomography (OCT) has been assessed as more superior in demonstrating axial distribution of fluid.[163] Although these studies are promising, ICG and fluorescein are non-specific exogenous fluorophores and thus are not suitable for targeted molecular imaging. However, these types of approaches are standing at the base of clinical trials based on targeted fluorophores for real life detection of specific biomarkers of apoptosis. Moreover, combination of fluorescence molecular tomography (FMT) with X-ray computed tomography (CT) or MRI will enhance the value of the imaging due to the possibility of specific mapping of molecular structures according to anatomical structures.[155]

Emerging data suggest that the dysregulation of apoptosis, through upregulation or downregulation, plays a significant role in the pathogenesis of various human diseases. However, the molecular mechanisms responsible for this dysregulation during disease initiation remain undefined. Consequently, future research should prioritize identifying the specific cellular and molecular entities that modulate apoptosis, with objectives centred on its inhibition or amplification based on therapeutic needs. It is crucial to emphasize functional assays to assess cellular viability, which provides a more precise evaluation of potential therapeutic agent efficacy compared to simply tracking cell retention or eradication.

A primary aim in biomedical research is harnessing insights into abnormal apoptosis to devise treatments enhancing patient outcomes in related diseases. Notably, in oncology, the observation that the majority of chemotherapeutic agents induce apoptosis in cancer cells has spurred investigations into treatments that target this dysregulated apoptosis. Furthermore, the integration of apoptosis imaging with imaging of other biological pathways, such as metabolism and angiogenesis, has potential to optimize clinical decision-making in diseases and interventions centred on apoptosis. Additionally, direct therapeutic interventions aiming at modulating apoptosis may exhibit reduced systemic toxicity compared to traditional chemotherapy. Such interventions could function as adjunctive treatments, augmenting the pro-apoptotic response

of tumour cells during chemotherapeutic exposure. In autoimmune disease contexts, inducing apoptosis in auto-reactive lymphocyte subsets by introducing specific autoantigens without co-stimulatory survival signals offers promise. The efficacy of this strategy has been demonstrated in murine models of experimental autoimmune encephalomyelitis.[164]

Conclusions

Apoptosis detection involves specific equipment and reagents that sometimes require high costs, laborious work, and prolonged time. Moreover, the lack of sensitivity and/or specificity are common in conventional apoptosis detection assays, making them inadequate to establish cell death conclusively. Consequently, a combination of complementary yet distinct techniques is highly recommended, such as fluorogenic assays with molecular quantification of apoptosis specific proteins. However, conventional methods have evolved to be used in more general assays, such as routine and preliminary experiments. Given these circumstances, it is essential to use comprehensive apoptosis detection techniques which add considerable value to both *in vitro* and *in vivo* research to ensure thorough understanding of the RCD events and factors influencing them.

The optical properties of various molecules or nanomaterials have been exploited to design improved probes with higher detection efficacy, increased signal to noise ratio and simplicity of approach, for example ON/OFF sensors and other probes which rely on event triggered changes in their optical properties. Furthermore, this allows to distinguish between RCD types that share similar characteristics and can investigate rapidly occurring cellular stages of such events. A novel trend is the use of computational tools such as deep learning in predicting apoptosis within a cell population, tissue or *in vivo*.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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