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Review

Shining light on cell death processes – a novel biosensor for necroptosis, a newly described cell death program

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Cell death contributes to the maintenance of homeostasis, but mounting evidence has confirmed the involvement of programmed cell death in some diseases. The concept of programmed cell death, which was coined several decades ago to refer to apoptosis, now also encompasses necroptosis, a newly characterized cell death program. Research on programmed cell death has become essential for the development of some new therapies. To study cell death signaling and its molecular mechanisms, new biochemical and fluorogenic approaches have been devised. Here, we first provide an overview of programmed cell death modes and the importance of dynamic cell death studies. Next, we focus on both apoptotic and necroptotic signaling and their mechanisms by providing a systematic review of all the methods and approaches that have been used. We emphasize the contribution of advanced approaches based on fluorescent probes, reporters, and Förster resonance energy transfer (FRET)-based biosensors for studying programmed cell death. Because apoptosis and necroptosis signaling pathways share some effectors molecules, we discuss how these new tools could be used to discriminate between apoptosis and necroptosis. We also describe how we developed specific FRET-based biosensors for detecting necroptosis. Finally, we touch on how dynamic measurement of biomolecules in living models will play a role in personalized prognosis and therapy.

Keywords: Biosensors · Caspase · Cell death · Cytochrome C · Receptor interacting protein kinase

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Abbreviations: **AIF**, apoptosis-inducing factor; **Bak**, Bcl-2 homologous antagonist/killer; **Bax**, Bcl-2-associated X protein; **Bcl-2**, B cell lymphoma-2; **BH**, Bcl-2 homology; **Bid**, BH3 interacting domain death agonist; **CFP**, cyan fluorescent protein; **Cyt c**, cytochrome c; **FADD**, Fas-associated protein with death domain; **FRET**, Förster resonance energy transfer; **GFP**, green fluorescent protein; **IMS-RP**, intermembrane space reporter protein; **MOMP**, mitochondrial outer membrane permeabilization; **PARP**, poly(ADP)-ribose polymerase; **PCD**, programmed cell death; **PI**, propidium iodide; **PS**, phosphatidylserine; **RIPK1/3**, receptor interacting protein kinase 1/3; **ROS**, reactive oxygen species; **TNF**, tumor necrosis factor; **TNFR1**, tumor necrosis factor receptor 1; **TRAIL**, TNF-α-related apoptosis-inducing ligand; **YFP**, yellow fluorescent protein

1 Introduction

In adult humans, per minute, several million cells die to balance the number of newly formed cells. Every cell in an organism is programmed to respond specifically to extracellular signals. The delivery of this information to subcellular levels, which requires the mobilization of intracellular signaling pathways, modulates the functions of subcellular compartments such as mitochondria, endoplasmic reticulum, and nucleus to generate the appropriate response. Pre- and post-translational modifications of molecules regulate cellular processes quickly and reversibly. At the organism level, cellular homeostasis is essential and relies on three processes: proliferation, differentiation and cell death. In the past, much attention

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was given to the study of cell proliferation and differentiation, while cell death was relatively neglected.

The concept of programmed cell death (PCD) was first introduced, in 1923, to describe the response of a plant cell infected by an incompatible fungus [1]. In plants, PCD is involved in xylogenesis, loss of foliage in autumn, cellular responses to counter pathogens and the preservation of the organism under environmental stress [2–5]. While plant scientists allowed physiologists to generalize this concept to other contexts in animal cells, it was not until the 1970s that attention was given to cell death. The term "programmed cell death" was then used to define particular cellular events that occur during embryonic development [6–8]. In animals, PCD has been characterized in the cells of interdigital spaces, disappearance of certain embryonic structures, remodeling of early neural structures to establish functional synaptic connections, and death of certain cells of the immune system to prevent autoimmune diseases (for review see [8]).

PCD is fundamental in both plant and animal physiology, playing a prominent role in the regulation and maintenance of cellular homeostasis. PCD is genetically regulated, relies on specific signaling pathways, and has been strongly conserved throughout evolution [9–15]. So it has become essential to gain a deep understanding of this process and to elucidate its mechanisms at the molecular level.

Mutations in cell death genes contribute to the development of many diseases such as cancer [16, 17] and immunological [18–20], neurodegenerative [21, 22] and cardiovascular diseases [23–25]. The involvement of PCD malfunction in pathogenesis has raised interest and contributed to the emergence of a new biomedical research field.

Indeed, cardiovascular diseases, including heart failure, myocardial infarction and ischemia/reperfusion, are the leading cause of death worldwide. Cell death mechanisms are involved in the development of these disorders, and pharmacological inhibition of PCD decreases the severity and progression of cardiac injuries (for review see [26]).

Alzheimer's disease is characterized by progressive neuronal cell death, mainly in the neocortex and limbic system structures. Accumulation and aggregation of amyloid β peptides promote cell cytotoxicity and alter the activation state of several kinases; this provokes subsequent activation of the cell death signaling pathways [27, 28].

Some viruses can hijack the cell's defense mechanisms, to the detriment of the cell. Viruses can counteract the defense mechanisms of infected cells by taking control of both extrinsic and intrinsic apoptotic machinery that lead to cell suicide. However, depending on the cellular context, certain cells can escape viral attacks by triggering necrotic cell death [19, 20, 29].

In cancer, depending on the severity and accumulation of mutations, cells either repair the defects or, to preserve genetic integrity, they activate signaling pathways leading to cell death. However, evasion of cell death is a hallmark of cancer (for reviews see [30, 31]), as some cells can escape PCD and engage in tumorigenesis [31, 32].

Based on morphological and biochemical criteria, three distinct major types of cell death have been identified: apoptotic cell death (type I), autophagic cell death (type II) and necrotic cell death (type III) [33]. While autophagy has been considered more as a cell survival mechanism, it has also been shown to promote cell death in a cell context-specific manner [34]. Autophagic cell death is a type of cell death that lacks chromatin condensation but involves massive autophagic vacuolization of the cytoplasm. Unlike apoptotic cells, which are efficiently cleared by macrophages, autophagic cells are not engulfed by phagocytes [33].

Recently, two new types of cell death have been described. The first, charontosis, is a type of PCD occurring in mouse embryonic stem cells (mESCs) upon etoposide (ETO) treatment [35]. Another type of cell death, ferroptosis, is a unique iron-dependent form of non-apoptotic cell death that is induced by erastin [36].

In this review, we focus on apoptotic and necroptotic cell death. We review the biochemical and live-cell imaging approaches developed to track specific markers and discriminate between these two types of PCD. Because cellular processes are dynamic, we emphasize approaches that capture this dynamic feature in living cells.

2 Importance of dynamic studies of signaling pathways

Recently, many papers have shown that the biological response is dictated by the duration, magnitude and subcellular compartmentalization of enzymatic activity [37–39]. Specifically, the response of the cell is based on the modulation of the spatiotemporal dynamics of kinase activity. One of the most striking examples is the kinase mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK). A central question about the MAPK/ERK signal transduction cascade is how the same protein kinase can elicit different cellular responses [38, 40, 41].

In cell death processes, cell fate is the result of a dynamic balance between extracellular signals promoting survival or cell death. In the absence of trophic factors, cells initiate PCD. Depending on cell type and stimulus, ERK dynamics can mediate different PCD modes, such as apoptosis, autophagy or senescence in various cellular models (for review see [42]). The involvement of ERK in various cell death forms requires its sustained and sequestered activity. These two characteristics are recognized as hallmarks of ERK-mediated cell death. Sustained cytoplasmic ERK activity triggers senescence or autophagy by activating pro-death proteins in the cyto-

plasm [43, 44]. By contrast, sustained nuclear compartmentalization of ERK activity can promote apoptosis [45]. It has been suggested that signals provoking sustained activation of ERK in different subcellular compartments trigger cell death.

It is essential to determine the spatiotemporal patterns of initiator and executioner molecules that lead progressively and irreversibly to a particular type of cell death. The specific enzyme activity profiles required for cell death initiation and execution should be identified in living cells. Biosensors and reporters are suitable for such approaches [46]. It is important, however, to distinguish between biosensors and bioprobes. A bioprobe provides only a snapshot of the cellular state at a given time, and is often discarded after its use. A biosensor can follow modifications of biomolecules in living cells with improved spatiotemporal resolution, while a reporter will indicate the occurrence of a specific event.

There are two main types of genetically encoded biosensors: environment-sensitive fluorophores and biosensors based on Förster resonance energy transfer (FRET). Environment-sensitive fluorophores are the result of fluorescent protein engineering. In this situation, biomolecules binding to specifically engineered fluorophores modulate the fluorescence intensity of the fluorophore, which provides information about the concentration changes of the biomolecule inside the cell. Environment-sensitive biosensors include those reporting on the concentration of several ionic and reactive oxygen species (for reviews see [47–50]).

By contrast, FRET biosensors consist of two parts: the "bioreceptor" part allows specific recognition of the biomolecule of interest, whereas the "transducer" part converts biochemical information into a photo-physical signal that is then recorded by a microscope (for review see [51]).

It is important to distinguish intermolecular from intramolecular FRET biosensors [41]. Intermolecular FRET biosensors consist of two distinct proteins of interest, each of which is fused to a fluorescent protein [52, 53], and are well suited for protein–protein interaction studies. Intramolecular FRET biosensors useful for the study of PCD include cleavage reporters and conformational change biosensors, which sense post-translational modifications such as proteolysis and phosphorylation in living cells (for reviews see [54, 55]). FRET biosensors are based on the arrangement of several components, and in particular two fluorescent proteins flanking a molecular recognition element (MRE). Many types of FRET biosensors are now available, in particular kinase activity reporters (KARs) (for review see [46]). While KARs show an increase in FRET between the two fluorophores upon phosphorylation, protease biosensors, such as caspase (cysteine-dependent aspartate-directed protease) reporters, are based on FRET decrease after protease cleavage at a specific site between two FRET compatible fluorophores.

Many caspase reporters have been developed in attempts to identify the type of PCD, and to monitor the activity of initiator and executioner caspases in cells undergoing cell death [56–58]. They are reviewed below.

3 Apoptosis

Apoptosis is essential for cell homeostasis and during embryonic development [59, 60]. An excellent example of apoptosis playing a central role in morphogenesis is the development of the vertebrate limb [60].

Morphologically, apoptosis is characterized by membrane blebbing, cell shrinkage, chromatin aggregation, and the appearance of apoptotic bodies [59]. Depending on the initial signal, two major signaling pathways can be distinguished: the intrinsic and the extrinsic signaling pathway. Intrinsic signaling involves the mitochondrial pathway and is usually induced by internal stimuli, such as DNA damage, inhibition of cell survival factors, defective cell cycle, hypoxia, or other forms of cellular stress. The signaling cascade induced in the mitochondrial pathway depends on the release of pro-apoptotic proteins from the mitochondrial intermembrane space (IMS), such as cytochrome c (Cyt c). This protein binds apoptotic protease-activating factor 1 (Apaf-1), inducing a conformational change and oligomerization; this leads to the formation of a caspase activation platform called the apoptosome. The formed complex recruits, dimerizes and activates initiator caspase-9, which in turn cleaves and activates caspase-3 [61]. On the other hand, extrinsic signaling is induced by activation of transmembrane receptors of the death receptor family, including Fas/CD95, tumor necrosis factor (TNF)-α receptor 1 (TNFR1), and two death receptors (DR4 and DR5), which bind to TNF-α-related apoptosis-inducing ligand (TRAIL) [62]. As the signaling of the extrinsic pathway is shared by apoptosis and necroptosis (the regulated form of necrosis) we will emphasize its molecular mechanism.

3.1 Molecular pathways of apoptosis

3.1.1 The intrinsic pathway of apoptosis

This pathway is engaged at the mitochondrial level by mitochondrial outer membrane permeabilization (MOMP), which is tightly regulated by members of the B cell lymphoma-2 (Bcl-2) family. The coordinated function of Bcl-2 family proteins is critical during apoptosis. The Bcl-2 family members have some homologous regions known as Bcl-2 homology (BH) domains. These molecules can be divided into two groups: anti-apoptotic proteins such as Bcl-2, Bcl-X_L (Bcl-extra large), Bcl-w and Mcl-1 (induced myeloid leukemia cell differentiation protein), which contain BH domains 1–4; and pro-apoptotic proteins, including the multi-domain effectors (comprising BH domains 1–3) Bax (Bcl-2-associated X protein), Bak (Bcl-2 homolo-

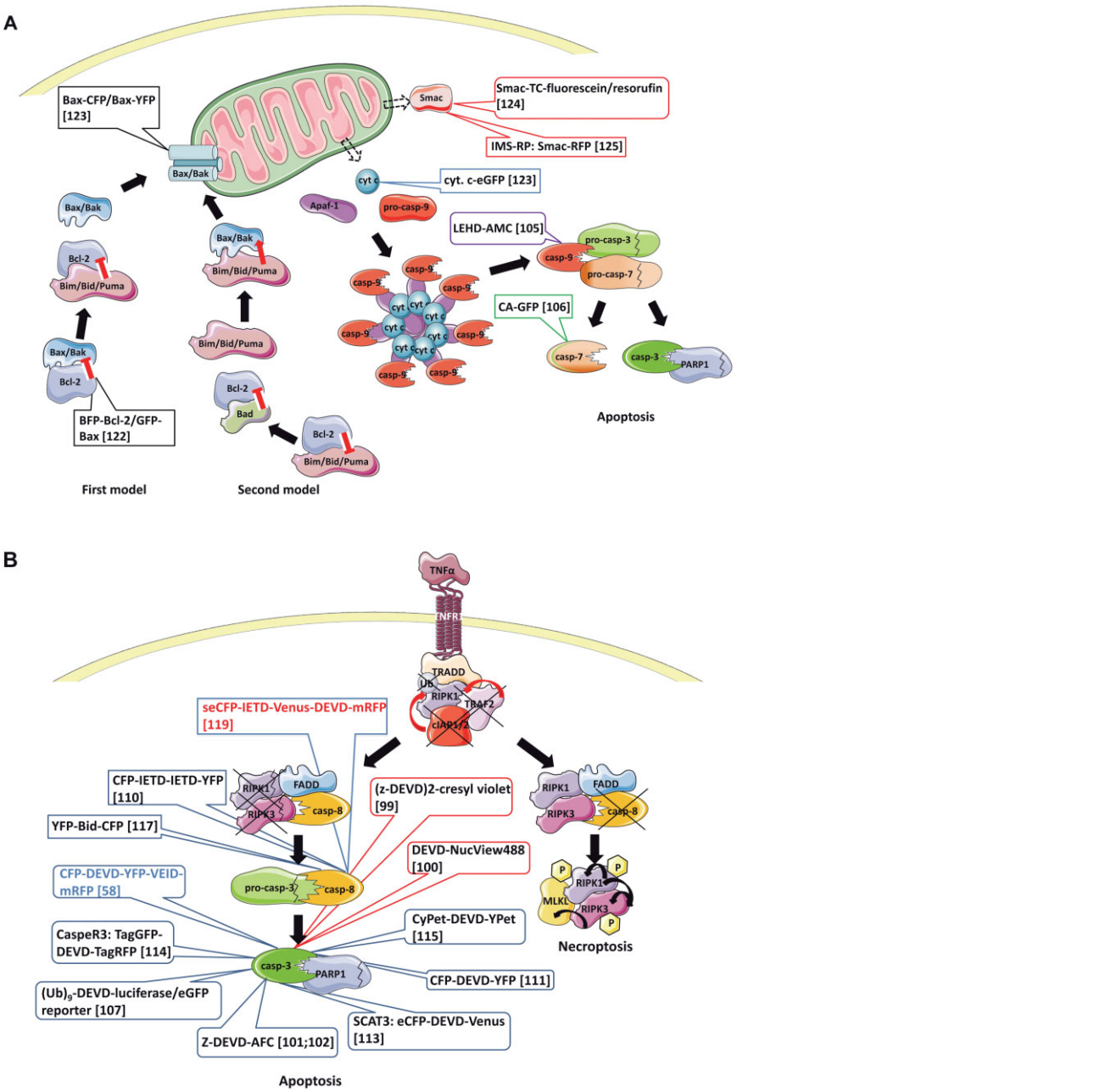

Figure 1. Programmed cell death signaling pathways and dedicated fluorescent probes and FRET-based biosensors. (**A**) The intrinsic pathway. Signaling pathways are indicated by solid-line arrows. Release of Cyt c and Smac from mitochondria is indicated by a dashed-line arrow. Red T signs indicate protein inhibition. Red arrows indicate protein activation. Cleavage of the protein is denoted by zigzag lines. Fluorescent probes and biosensors are indicated by callouts (rectangles: biosensors; rounded rectangles: bioprobes; black: conditional biosensors; blue: for Cyt c; red: for Smac, green: for caspase-7; purple for caspase-9). Names and domain compositions of fluorescent indicators are indicated and are followed by the references in parentheses. Domain names are linked with hyphens from the N to the C terminus. (**B**) The extrinsic pathway. Signaling pathways are indicated by solid-line arrows. Curved arrows indicate post-translational modifications (red: ubiquitination; black: phosphorylation). Crossovers denote the absence of the functional protein or post-translational modification. Zigzag lines designate the cleavage of the protein. Fluorescent probes and biosensors are indicated by callouts (rectangular: for caspase-8; rounded rectangular: for caspase-3; oval: for caspase-9; red callout: fluorescent probe; blue callout: biosensor). Names and domain compositions of fluorescent indicators are designated and are followed by the references in parentheses (black font: single caspase indicators; red font: biosensor designed for caspase-8 and -3; blue font: biosensor designed for caspase-3 and -6). Domain names are linked with hyphens from the N to the C terminus. casp, caspase; cIAP1/2, cellular inhibitor of apoptosis protein 1/2; eGFP, enhanced GFP; MLKL, mixed lineage kinase-like domain protein; P, phosphate; RFP, red fluorescent protein; TRADD, TNF-α receptor associated death domain; TRAF2/5, TNF-α receptor associated factor 2/5; Ub, ubiquitin. (Figure designed using references [58, 99–102, 105–107, 110, 111, 113–115, 117, 119, 122–125].)

gous antagonist/killer), Bok (Bcl-2 related ovarian killer) and BH3-only proteins such as Bid (BH3 interacting domain death agonist), Bim and Bad (Bcl-2-associated death promoter) (for review see [63]). The induction of MOMP is promoted by pro-apoptotic proteins Bax and Bak, which oligomerize to form pores in the outer mitochondrial membrane (OMM) [64]. BH-3 only proteins function as either direct activators of Bax and Bak, or as de-repressors of anti-apoptotic Bcl-2 and Bcl-X_L [65, 66]. Currently, there are two opposite models describing the activity of BH-3 only proteins (see review [67]). According to one model, Bax and Bak are constantly repressed by the anti-apoptotic activity of Bcl-2-like proteins. Activated BH3-only proteins associate with Bcl-2-like proteins, thereby releasing Bax and Bak to promote MOMP. The other model indicates that some BH3-only proteins, such as Bid, Bim and Puma (p53 upregulated modulator of apoptosis), directly bind to and activate Bax and Bak. However, under normal conditions, their exposed BH3-domains interact with and are sequestered by anti-apoptotic Bcl-2 proteins. Binding of other BH3-only proteins, such as Bad, to the anti-apoptotic proteins results in the release of Bid, Bim and Puma, allowing them to activate Bax (for review see [63]). Figure 1A shows a representation of the mitochondrial pathway.

3.1.2 *The extrinsic pathway of apoptosis*

As mentioned above, several stimuli, such as Fas ligand and TRAIL, can lead to apoptosis. However, the most extensively studied mechanism is the one induced by TNF. Binding of TNF to the extracellular domain of TNFR1 [68] results in the formation of complex I, which contains the TNF-α receptor-associated death domain (TRADD), the E3-ubiquitin ligases TNF-α receptor associated factor 2/5 (TRAF2/5), and the cellular inhibitor of apoptosis proteins (cIAP1/2), which promote K63-linked ubiquitination of receptor interacting protein kinase 1 (RIPK1) [69, 70].

RIPK1 is a serine/threonine kinase of the RIPK family and is considered as a molecular switch between RIPK1 kinase-dependent apoptosis and necroptosis [71]. The RIPK1 ubiquitination state is crucial because it determines whether RIPK1 functions as a molecular platform for the recruitment of molecular adapters that can elicit a pro-survival response, or as a kinase that promotes cell death.

In apoptosis, K63-linked ubiquitination of RIPK1 finally leads to the induction of the nuclear factor-kappa B signaling pathway, which generates a pro-survival response. When the ubiquitination of RIPK1 is inhibited [72–74], it dissociates from complex I and forms, together with Fas-associated protein with death domain (FADD) and initiator caspase-8, the cytoplasmic death-inducing signaling complex (DISC) [75]. Recruitment and oligomerization of caspase-8 monomers in the DISC result in its autocatalytic activation [76]. Functional caspase-8 subsequently activates executioner caspases-3 and -7 by limited proteolysis within their interdomain linker. In contrast to caspase-8, the executioner caspase zymogens exist in the cytosol as inactive dimers [77]. The activated executioner caspases finally mediate apoptosis by proteolytically processing their substrates, such as poly(ADP)-ribose polymerase (PARP) [78, 79]. Figure 1B shows a representation of the apoptosis and necroptosis signaling pathways and key molecular effectors.

3.2 Approaches for apoptosis assessment

Apoptosis plays an essential role in cell homeostasis. To be able to modulate this therapeutically, apoptosis has to be fully characterized to define specific molecular targets. Multiple analytical procedures have been developed to determine the presence of apoptosis and its extent. These approaches can be divided into two groups: biochemical and fluorogenic assays.

3.2.1 *Biochemical assays*

Apoptosis is characterized by the cleavage of DNA at internucleosomal (linker) sections by an activated endonuclease [80], and by membrane blebbing [81]. The fragmented DNA shows a typical ladder pattern in electrophoretic gels [80]. The morphological changes in the plasma membrane can be detected by atomic force microscopy (AFM), which has been successfully applied in studies on K562, HeLa and Ishikawa cell lines [81, 82].

An alternative approach for identifying apoptotic cells is based on the detection of DNA strand breaks by labeling their 3'OH termini using an exogenous terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay [83]. The labeling substrates include biotin- or digoxigenin-conjugated nucleotides [84, 85] and 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) [86]. The TUNEL assay has been successfully used, at earlier stages of apoptosis, to detect the activation of a serine protease that hydrolyzes protein(s) associated with the internucleosomal linker DNA. In this way, the serine protease increases the accessibility of DNA to the apoptosis-associated endonuclease [84]. Moreover, by performing TUNEL staining on organ sections, cell death can be precisely localized in situ [87].

Another method widely used to detect apoptosis takes advantage of another characteristic, i.e. the external exposure of phosphatidylserine (PS), which is normally located on the inner surface of the lipid bilayer [88]. The anticoagulant protein annexin V binds PS with high affinity. Upon induction of apoptosis, cell surface-exposed PS is available to annexin V. During the early stages of apoptosis, cells can still exclude cationic dyes such as propidium iodide (PI). Therefore, during the early stages of apoptosis cells bind annexin V and exclude PI, becoming PS^+/PI^-. At later stages of apoptosis, cells can no longer exclude PI and become double fluorescent, PS^+/PI^+. The use of fluorochrome-conjugated annexin V in flow cytom-

Table 1. Biochemical approaches for assessing apoptosis

Cellular structure/biomolecule	Approach/technique	Reference
DNA cleavage at internucleosomal sections	Gel electrophoresis	[80]
Membrane blebbing	Atomic force microscopy	[81, 82]
DNA strand break	TUNEL	[83–87]
External exposure of PS	Flow cytometry	[89–91]
	Electrochemiluminescence cytosensing	[92]
Cytoplasmic Cyt c release	Fractionation techniques followed by western blotting	[93, 94]
Caspase activation	Western blotting against caspase substrates (PARP1) or activated caspases	[78, 91]
	Flow cytometry against caspase substrates (PARP1) or activated caspases	[91, 95]
	Immunohistochemistry against activated caspases	[87]
	Positron emission tomography	[96–98]

etry is now widespread [89, 90]. In 2000, Belloc et al. [91] used flow cytometric analysis with annexin V and anti-active caspase-3 antibodies to show that a partial caspase-3 activation precedes external exposure of PS. Recently, a new technique called electrochemiluminescence (ECL) cytosensing has been introduced. This technique uses annexin V immobilized on L-cysteine-capped cadmium sulfide quantum dots (CdS-QDs)/polyaniline nanofibers. Interaction between PS on the surface of apoptotic cells and annexin V can be probed by ECL. This method is highly sensitive, selective, reproducible and simple [92].

An alternative approach frequently used to detect apoptosis is the measurement of Cyt c release. This analysis can be performed using various fractionation techniques in which cells are either permeabilized with digitonin or mechanically ruptured followed by separation of the cytosolic fraction from the heavy membrane fraction containing mitochondria. Both fractions are analyzed by western blotting to determine Cyt c distribution and amount [93]. This method has been successfully applied to demonstrate that cell death stimuli such as Bax- or Bax/Bak-dependent pro-apoptotic drugs induce hierarchical release of the mitochondrial factors involved in the induction of cell death [94].

As mentioned above, the relatively early caspase activation is a major event and a hallmark of apoptosis [78]. One way to determine caspase activation is by detection of the cleavage products of caspase substrates such as PARP1 [78, 79] by western blotting [78] or flow cytometry [95]. The latter technique has been used to show the difference in kinetics between apoptosis induced by camptothecin and that caused by TNF-α together with cycloheximide [95]. Another way to assess activation of caspases is by detecting the epitope of the activated (cleaved) caspases with specific antibodies using western blotting analysis or flow cytometry [91]. Like TUNEL staining, immunohistochemistry can be used to detect apoptotic cell death, and an antibody directed against activated caspase-3 can be used on organ sections to identify the localization of cell death in situ [87]. The use of specific substrates for caspase-3 has been successfully applied in vivo. By performing positron emission tomography in mice, apoptosis could be traced using caspase-3 substrates labeled with CP18 or ICMT-11 [^{18}F] [96–98]. Table 1 summarizes the biochemical approaches used to assess apoptosis.

3.2.2 *Fluorogenic assays*

The techniques mentioned above are useful for detecting cells undergoing apoptosis. However, most of these techniques have one major disadvantage: the analysis requires lysis or fixation of the cells, which hampers spatiotemporal resolution. Besides, these methods cannot characterize the dynamics of key biomolecules involved in apoptosis. Therefore, approaches that can monitor apoptosis in living cells have been developed. One example of such a system is the use of fluorogenic caspase substrates [99–103]. These are peptides that are not fluorescent but emit strong fluorescence upon caspase-induced cleavage. As they are highly permeant and not cytotoxic, these reporters can be used effectively in living cells even during long-term incubations [100]. A high content screening with the cell death small interfering RNA (siRNA) pool and using NucView488^{TM} as a caspase-3 fluorogenic substrate demonstrated the reliability of fluorescent signal production [104]. Kasili et al. [105] described the use of a fluorogenic probe containing a cleavage site for caspase-9 linked to 7-amino-4-methylcoumarin (AMC). This probe was used to reveal caspase-9 involvement in apoptotic MCF-7 cells after photodynamic therapy (PDT) with d-aminolevulinic acid (ALA).

Recently, several caspase activity reporters have been described that use green fluorescent protein (GFP) as a fluorescent label. One of them, caspase-activatable-GFP (CA-GFP), is specific for caspase-7 and contains a version of GFP that does not show any fluorescence due to the presence of a hydrophobic quenching peptide that tetramerizes GFP, thus preventing its maturation. The fluorescence can be completely restored by catalytic removal of the quenching peptide following caspase activation. The reporter appears to be more sensitive than

other apoptosis reporters: in mammalian cells it provides a threefold increase in fluorescence upon induction of apoptosis and activation of caspases. The system is effective for time-resolved observation of apoptosis [106]. Another recently described reporter contains an enhanced GFP (eGFP) and a luciferase encompassing a caspase cleavage sequence flanked by a proteasome recognition site. In the absence of active caspases, the proteasome recognition site gets ubiquitinated and is degraded by the proteasome. However, when caspases are activated, the caspase recognition sequence is cleaved, releasing luciferase and GFP. The presence of both luciferase and GFP makes this versatile reporter suitable for both in situ and in vivo use. Using this reporter, Huang et al. [107] demonstrated caspase-3 activation in solid tumors during radiotherapy.

Another approach that is gaining much attention is the use of FRET-based biosensors and probes to assess the initiation and execution of apoptosis. This method makes use of two GFP derivatives encompassing a peptide substrate specific for a certain caspase [108]. In the absence of active caspase, the two proteins are brought together and non-radiative energy is transferred from the donor to the acceptor fluorophore. But when caspase activity is present, the peptide is cleaved, separating the two fluorophores and eliminating FRET [108]. The two most commonly used fluorophores are cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) [109–112] or its improved version Venus [113]. Another combination is TagGFP (an enhanced bright mutant of the GFP-like protein) and TagRFP (a monomeric red fluorescent protein) generated from the wild-type RFP [114]. This biosensor (TagGFP-DEVD-TagRFP sensor) has a wide dynamic range, bright fluorescence, and enhanced pH and photo-stability [114]. Nguyen and Daugherty [115] described an improved FRET pair, CyPet-YPet, that has a 20-fold ratiometric FRET signal change, which is much greater than the 3-fold change of the parental pair FP-YFP.

Currently used apoptotic biosensors are specific for the activation of caspase-3 [111, 113, 114, 116] or caspase-8 [110, 117]. They enable visualization of the dynamics of caspase activity with high spatial resolution [110, 113]. For instance, the use of a caspase-8 biosensor that contains the Bid protein, a known caspase-8 substrate [118], fused to YFP and CFP at the N and C terminus, respectively, showed a difference in caspase-8 activation in SK-N-SH cells by anti-Fas antibody and Aβ or tunicamycin. Moreover, this biosensor has been used for real-time detection of cytoplasmic caspase-8-mediated cleavage of Bid, which is followed by translocation of truncated Bid (tBid)-CFP [118] to mitochondria [117]. In 2006, Wu et al. [58] developed a dual FRET biosensor for caspase-3 and caspase-6. The study highlighted a difference in activity kinetics between caspase-3 and -6. The analysis revealed that following apoptosis induction, caspase-3 activation preceded caspase-6 by ~30 min [58]. Another apoptotic biosensor recently developed by Kominami et al. [119] monitors the activities of both caspase-8 and caspase-3. This biosensors detected distinct activation patterns of caspase-8 and -3 in response to different apoptotic stimuli in mammalian cells. This study emphasizes the necessity for positive feedback amplification of caspase-8 activation.

Cell death can be characterized by monitoring not only caspase activity but also the activation of the intrinsic apoptotic pathway, which includes the assessment of mitochondrial transmembrane potential (MTP) modulation. MTP modulation can be recorded by tetramethylrhodamine-based fluorescent probes, such as tetramethylrhodamine ethyl ester (TMRE) and tetramethylrhodamine methyl ester (TMRM). These probes are cell permeant, positively charged dyes that readily accumulate in active mitochondria due to their relative negative charge. Depolarized or inactive mitochondria fail to sequester the probes due to their decreased membrane potential. Using TMRE in HeLa cells stimulated with staurosporine, actinomycin D or TNF together with cycloheximide, Goldstein et al. [120] showed that a decrease of MTP is an early event in apoptosis. By contrast, Vanden Berghe et al. [121] showed that mitochondria membrane is hyperpolarized in L929sAhFas cells (L929 cells stably expressing human Fas receptor). This study demonstrated that the kinetics of hyperpolarization is stimulus dependent. Upon TNF stimulation, mitochondria were hyperpolarized with biphasic kinetics, whereas H_2O_2 stimulation caused immediate hyperpolarization that rapidly returned to baseline.

Activation of the intrinsic apoptotic pathway can be monitored using FRET-based approaches that can be used for biosensing as well as for protein–protein interaction studies. By performing FRET measurements on NIH3T3, baby hamster kidney cells (BHK-21) and porcine aortic endothelial cells co-expressing blue fluorescent protein (BFP)-Bcl-2 and GFP-Bax, Mahajan et al. [122] not only showed their cellular localization but also demonstrated interaction between the two proteins during apoptosis. A similar approach showed multimerization of Bax at the mitochondria following mitochondrial permeability transition pore (PTP) opening in COS-7 cells expressing either CFP-Bax or YFP-Bax. In the same study, use of Cyt c coupled to eGFP also indicated that the release of Cyt c following PTP opening takes place within minutes, but occurs only several hours after the PTP has been locked in the open conformation [123].

Researchers also investigated the kinetics of cytosolic release of other mitochondrial IMS proteins during apoptosis, such as Smac (second mitochondria-derived activator of caspases), Omi and adenylate kinase-2. Recombinant proteins were fused to a short tag of 10–15 amino acids containing a tetracysteine motif (TC). This motif is specifically recognized by a cell-permeant fluorescein- or

resorufin-based fluorescent dye that binds covalently to the tag. A confocal microscopy-based, real-time approach using fluorescently labeled proteins revealed that Cyt c, adenylate kinase-2, Smac, and Omi are released rapidly and simultaneously, and that their release does not depend on caspase activity [124]. More recently, Albeck et al. [125] described a fluorescent reporter for MOMP based on a fusion of mitochondrial import sequence of Smac and RFP. The IMS reporter protein (IMS-RP) differs from the Smac fusion protein described above through the absence of an IAP-binding motif, and is consequently biochemically inactive. Using IMS-RP in combination with specific initiator caspase reporters revealed that MOMP occurs at a later stage during apoptosis in HeLa cells, and is preceded by the activity of initiator caspases.

4 Necrosis and necroptosis

For a long time necrosis was considered as an accidental cell death. In 1988, it was discovered that TNF stimulation triggers distinct responses in different cell lines. It was then observed that some cell lines show 'classical' features of apoptosis in response to TNF, while others exhibit a rounded up morphology without nuclear disintegration [126]. Two decades later, in 2005, a new term 'necroptosis' was introduced to describe a new regulated form of necrotic cell death [127]. Necroptosis is an important mechanism of virus-induced inflammation and innate immune control of viral infections [128, 129]. In animal models deficient in caspase-8 or FADD, necroptosis appears to play an important role during development [130, 131]. In addition, necroptosis is involved in pathologies in animal models of acute pancreatitis [132], hypoxic/ischemic injury [127], and septic shock [133]. Inhibition of necroptosis reduces tissue damage in models of cardiac infarction [134] and ischemic brain injury [127]. These findings demonstrate the biological importance of necroptosis, but the molecular components regulating this newly reported cell death program are not well defined.

4.1 Molecular pathway of necroptosis

As mentioned above, the molecular pathway triggered by TNF is shared by apoptosis and necroptosis. Functional caspase-8 proteolytically inactivates RIPK1 and RIPK3, which are important in necroptosis, thus promoting the initiation of apoptosis [135]. However, when the proteolytic activity of caspase-8 is pharmacologically or genetically inhibited, the cell undergoes a shift from apoptosis to necroptosis. In such a cellular context, RIPK1 and RIPK3 are not proteolytically inactivated, and they form a complex called the necrosome, which regulates various downstream effectors of signaling cascades [129, 135, 136]. A new component of the necrosome was recently identified: the mixed lineage kinase-like domain protein (MLKL). This protein is phosphorylated by RIPK3 and then recruited to the necrosome, triggering necroptosis [137, 138]. Figure 1B shows a representation of the signaling pathway of necroptosis and highlights the cross-talk with the apoptosis pathway.

The serine/threonine kinases, RIPK1 and RIPK3, of the RIPK family have similar modular structures consisting of an N-terminal kinase domain (KD) and an intermediary domain (ID). Both domains play a significant role in necrosome formation. The ID is important for RIPK1-RIPK3 interaction because it contains the RIP homology interaction motif (RHIM). Mutation of RHIM disturbs the RIPK1–RIPK3 interaction and, therefore, necrosome formation. The kinase activity of the kinase domains is necessary for the phosphorylation of RIPK1 and RIPK3, because it promotes necrosome formation and complex stabilization [129, 135]. Unlike RIPK3, RIPK1 contains an additional C-terminal death domain (DD). The DD belongs to the 'death-fold' superfamily of homotypic interaction motifs [139], and is necessary for the interaction of RIPK1 with other DD proteins, such as TNFR1 and FADD [140].

Although the molecular mechanisms of necroptosis initiation are well characterized, less is known about the downstream signaling cascades responsible for the disruption of specific cellular functions. Cellular functions associated with necroptotic cell death include increased mitochondrial production of reactive oxygen species (ROS) [141], reduction of the cellular energy by unregulated ATP-consuming processes and disturbed ATP synthesis [142], lysosomal membrane permeabilization [121], and plasma membrane permeabilization [121] at the final stage of necroptosis. Some of these perturbations have been linked to the formation of the necrosome complex. The kinase activity of RIPK3 is central in necroptosis, as it plays a role in the overactivation of various metabolic cascades. The resulting increased concentration of breakdown products enhances the activity of Krebs cycle, which results in increased ROS production by mitochondria [136]. A sudden decrease in cellular ATP during necroptosis can be partially explained by the RIPK1-mediated inhibition of the adenine nucleotide translocase (ANT). This ATP/ADP antiporter is located at the inner mitochondrial membrane and is necessary for keeping the mitochondrial ADP concentration in balance for efficient ATP production and transport to the cytoplasm. Temkin et al. [142] showed that RIPK1 inhibits ANT following TNFR1 stimulation. This leads to inhibition of ADP/ATP exchange and a decrease of cellular ATP, and subsequently to necrotic cell death.

4.2 Approaches for necroptosis assessment

The methods for detecting necroptosis can be divided into two groups: biochemical and fluorogenic assays.

Table 2. Biochemical approaches for assessing necroptosis[a)]

Cellular structure/biomolecule	Approach/technique	Reference
Cellular swelling and formation of balloon-like structure	Time-lapse microscopy or electron microscopy	[87]
RIPK1-dependent RIPK1/RIPK3 phosphorylation	In vitro kinase assay	[129, 135, 136]
Formation of the necrosome complex	Inhibition by RIPK-1-specific inhibitor Necrostatin-1, knockdown of RIPK3 or MLKL followed by western blotting	[127, 137, 144]
AlphaII-spectrin breakdown by calpains	Western blotting or immunocytochemistry against breakdown products of alphaII-spectrin	[136]

a) MLKL, mixed lineage kinase-like domain protein

4.2.1 *Biochemical assays*

Necroptotic cell can be analyzed morphologically by time-lapse microscopy or electron microscopy [87]. During necroptosis cellular morphology is characterized by cellular swelling and formation of a balloon-like structure, known as oncosis [87]. In the absence of caspase activity, which is a characteristic of necroptosis, the involvement of necroptosis can be assessed using a novel necroptotic marker, RIPK1-dependent RIPK1/RIPK3 phosphorylation [129, 135, 136]. Normally, RIPK1 and RIPK3 are phosphorylated in a biphasic way: an early wave (5–15 min after TNF stimulation) and a late wave (90–120 min after TNF stimulation). Only the late phosphorylation peak of RIPK1/3, which is dependent on RIPK1, is linked to necroptosis induction [143]. Therefore, the second RIPK1/3 phosphorylation has to be blocked by adding the RIPK1 kinase inhibitor, Necrostatin-1 [87].

Necroptosis can be assessed using the RIPK1-specific inhibitor Necrostatin-1 [127], or by knocking down RIPK3 [144] or MLKL [137] expression, and then determining the phosphorylation profiles of necroptotic kinases by western blot analysis.

Calpains, ubiquitous cysteine proteases activated by calcium signaling, have important roles in cell death programs. Although this molecular effector is not described here, the interconnection with caspases is crucial and approaches to determine caspases and calpain activities simultaneously have been considered in neurosciences [145]. AlphaII-spectrin, a substrate of both caspases and calpain, was used in a biochemical approach to determine necroptosis and to distinguish it from apoptosis. The study performed by Zhang et al. [145] in pheochromocytoma-12 (PC-12) cells after various treatments triggering either apoptosis or necroptosis aimed at assessing caspase versus calpain activity relied on determining multiple alphaII-spectrin breakdown products using specific antibodies. Table 2 summarizes the biochemical approaches to assess necroptosis.

4.2.2 *Fluorogenic assays*

To distinguish necroptotic cells from apoptotic cells undergoing secondary necrosis, the nucleus is stained with PI. As secondary necrotic cells have already passed through an apoptotic stage, their nuclei are condensed and/or fragmented. The internucleosomal fragmentation of the DNA in apoptotic cells produces a homogenous DNA staining with PI. As necrotic cells retain their chromatin structure, their nucleoli show prominent staining [87].

A calpain FRET biosensor designed to follow synaptic activity in dendritic spines [146] was characterized in cell lines and dissociated hippocampal neurons. This biosensor is composed of the micro-calpain cleavage site from alpha-spectrin flanked by eCFP and eYFP fluorescence proteins and should be used in combination with caspases activity reporters in a different cell death context.

To date, only caspase FRET biosensors can be used negatively. Because caspase biosensors function on the basis of FRET decrease upon caspase activation, and caspase activity is inhibited during necroptosis, it might be possible to monitor non-FRET change in living cells. However, if the cells are in secondary necrosis, this approach is not valid because caspases are activated during apoptosis and the protease biosensor is intrinsically irreversible.

Although RIPK1 and RIPK3 kinases are involved in initiating necroptosis, no FRET biosensors have so far been developed for these molecules. Thus, development and optimization of FRET biosensors for "positive" detection of this particular PCD should be considered. In line with this idea, we are using a genetically encoded FRET biosensor of the sandwich design to develop RIPK kinase activity reporters (RIP-KARs). Specific RIPK1 and RIPK3 substrates were identified by proteomic approaches in cells undergoing necroptosis. RIP-KARs are currently being tested and optimized (personal communication).

Finally, cathepsins, which are proteases involved in final protein degradation by lysosomes, have been used to monitor apoptosis. Lysosomes are tightly linked with cell death. Several studies reported that release of cathepsins mediated by lysosome rupture leads to mitochondrial

membrane permeabilization and a subsequent Cyt c leakage into the cytoplasm, resulting in further caspase-9 activation (for review see [147, 148]). A relationship between the amount of lysosomal proteins released following lysosomal rupture and the type of cell death has been established. It was reported that a small leakage of lysosomal proteins into the cytoplasm triggers apoptosis, whereas extensive lysosomal rupture leads to rapid necrosis [149]. Thus, cathepsin activity should be monitored during necrosis. Cathepsin activity has been assessed mainly with chemical activity-based probes both in vitro and in vivo (for review see [150]). To investigate cathepsin B activity, Yhee et al. [151] developed a cathepsin B nanoprobe (CB-NP) consisting of a near-infrared fluorescence (NIRF) dye, a dark quencher, and highly specific cathepsin-sensitive substrate. CB-NP was delivered in mouse tumor tissues to monitor the fluorescent signal in the cytosol in response to cathepsin B activity [152]. Cathepsin probes are often used to monitor the effects of various pharmacological inhibitors of several cathepsins at different stages of tumor progression [153].

5 Perspectives

In this review, we also focus on the recent contribution of fluorogenic approaches using biosensors and reporters for cancer therapy design and treatment.

5.1 Necroptosis: The new player in cancer therapy

Most anti-cancer therapeutic strategies cause the death of tumor cells by inducing damage that is recognized as an apoptosis signal. However, cancer cells undergo mutations of key effector proteins of the apoptotic machinery, such as p53, and thereby acquire resistance to therapeutic agents [154]. Before 2004, apoptosis was the only cell death program considered in attempts to block tumor growth. In most solid tumors, cancer cells metabolism is completely reorganized, undergoing a complex metabolic reprogramming [155]. Generally, cancer cells present oxidative phosphorylation (OXPHOX) defects and an increase in the aerobic glycolysis pathway ("Warburg phenomenon"). Although OXPHOX is more effective than glycolysis in terms of energetic yield, cancer cells prefer using this metabolic shunting to generate intermediates for anabolic reactions [156]. Cancer-specific metabolic molecules production may, therefore, favor cell death evasion, angiogenesis, tissue invasion, metastasis and immunosuppressive effects. Interestingly, mutations of oncogenes and/or tumor suppressor genes contribute to the control of cancer cells metabolic reprogramming [157].

In 2004, alkylating agents were shown for the first time to provoke necroptosis in tumor cells [158, 159]. Indeed, alkylating agents activate the DNA repair PARP, which catalyzes the synthesis of poly(ADP-ribose) chains on specific nuclear proteins. Under these experimental conditions, cells using aerobic glycolysis undergo rapid depletion of ATP and cell death, while cells maintaining OXPHOS are resistant to ATP depletion and cell death. Based on the fact that most cancer cells rely on aerobic glycolysis for ATP production, this mechanism is proposed for the induction of tumor cell death following treatment with alkylating agents.

The mitochondrial IMS protein, apoptosis-inducing factor (AIF), also mediates necroptosis. Indeed, in response to DNA alkylation and subsequent PARP activation, AIF is released in the cytosol and rapidly relocalizes to the nucleus, where it promotes DNA degradation, leading to apoptosis or necroptosis [160, 161]. In this AIF-dependent necroptosis, RIPK1 participates in AIF release from mitochondria [162]. Emerging anticancer therapeutic strategies targeting AIF-mediated PCD have been developed for patients with metastatic breast and pancreatic cancers. These compounds can induce high levels of ROS, leading to AIF release from mitochondria and necroptosis of cancer cells without affecting neighboring normal cells (for review see [163]).

In contrast to apoptosis, necroptotic cancer cells drive a local inflammatory reaction by releasing pro-inflammatory molecules into the extracellular space, such as the nuclear factor high-mobility group protein B1 (HMGB1) [164], which promotes tumor cell death. Cancer cells can become resistant to current chemotherapeutic agents, and targeting necroptosis has become a potential alternative approach for fighting cancer (for review see [165]). Recent studies have confirmed the sensitivity of cancer cells to necroptosis [166, 167].

The main objective of therapeutic strategies used to be the targeting of caspases in cancer cells to trigger apoptosis. Nowadays, induction of necroptosis is recognized as an effective means for killing cancer cells, especially when they are apoptosis resistant. It is important to keep in mind that apoptosis and necroptosis are not mutually exclusive processes that are entirely separated [168–170]. Apoptosis and necroptosis share many common molecular effectors. Depending on cell context, and specifically on the caspase activation state, a cell can die by apoptosis or necroptosis [136, 171, 172]. In line with this view, detailed analysis of the enzyme activation state of a tumor cell line is thought to be crucial for establishing a prognosis before treatment (Fig. 2). Applying this approach might help predict effective treatment for killing tumor cells. Genetically encoded FRET biosensors are particularly relevant for this purpose because several parameters could be measured dynamically, including activation state and concentration of specific biomolecules. The monitoring of biochemical events in real time in living cells can yield qualitative and quantitative information on signal integration mechanisms and the kinetics of signal transduction. Very recently, Earley et al. [173] used MOMP probes [125] to visualize drug-induced

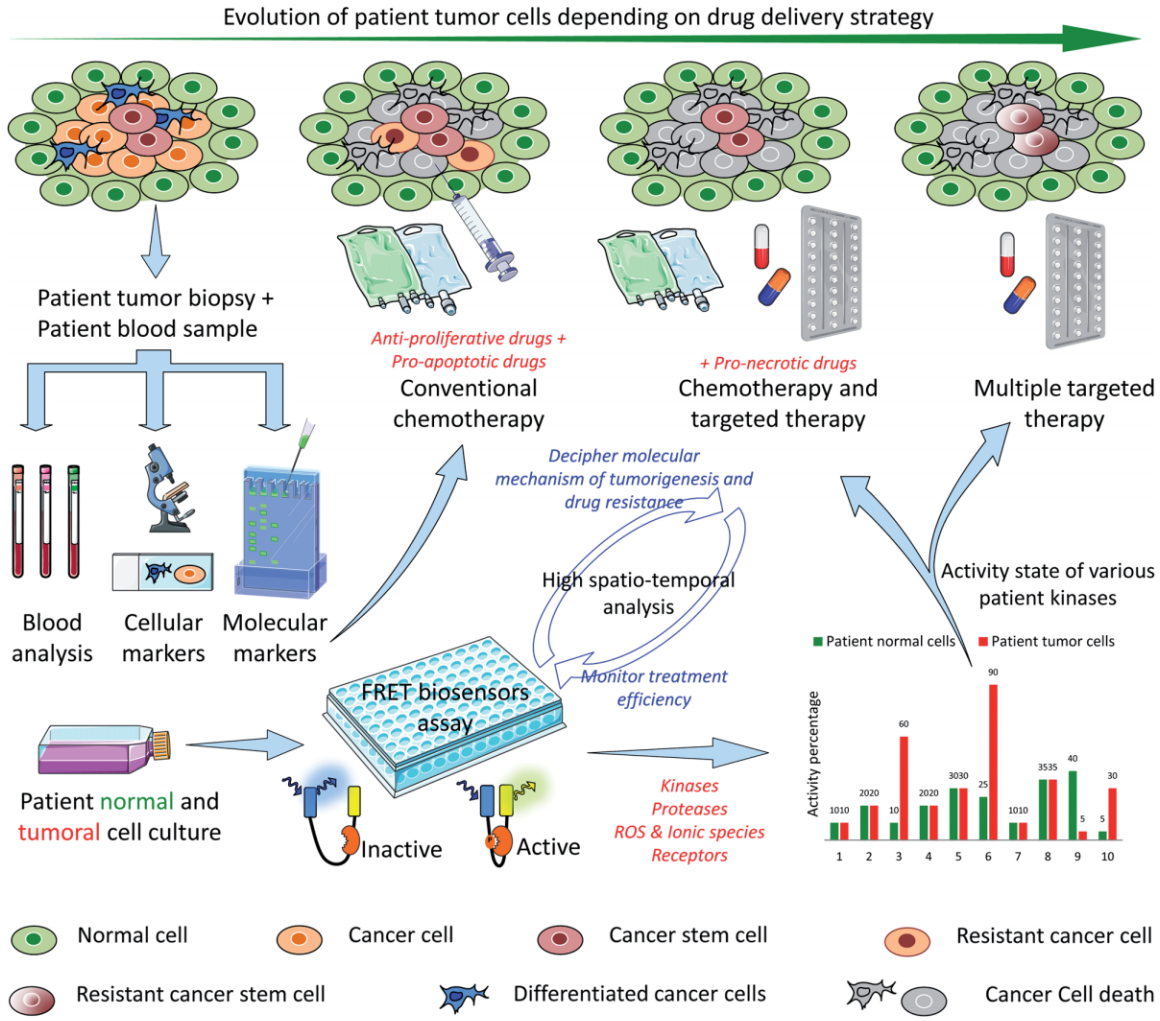

Figure 2. The evolution of therapeutic strategies and biotechnologies for cancer therapy and the use of FRET biosensor strategies for evaluating cancer drug combinations. A few years ago, treatments were mainly based on cellular and molecular markers and limited to chemotherapy with both anti-proliferative and pro-apoptotic drugs. These treatments remained ineffective against various resistances developed by cancer cells. Nowadays, new strategies tend to analyze the activation state of the enzymes within patient tumor cells to tailor treatment, including both chemotherapeutics and agents that target specific enzymes responsible for the aggressiveness of the tumor. FRET biosensors provide powerful tools for accurate determination of the activation state profiles of key regulators in a patient's tumor cells. Live-cell, biosensor-based dynamic approaches can be used to refine therapy design and to monitor treatment efficacy. Biosensors and reporters can also be used for in cellulo screening of new drug candidates. In the near future, therapeutic strategies relying on biosensors will surely contribute to personalized medicine.

apoptosis of single cells in live mice. Breast and pancreatic cancer cell lines stably expressing an IMS-RP were established to detect IMS-RP release into the cytoplasm upon induction of apoptosis. Comparison of apoptotic rates between in cellulo and in vivo models showed that cancer cells outside the tumor are more sensitive to drugs. This study highlights not only the importance of improving high-resolution intra-vital microscopy [174] for in vivo biosensing, but also the necessity of taking into account the pathophysiological context of tumors for the development of adapted therapy. Recently, Bagci-Onder et al. [175] elegantly used both bioluminescence and fluorogenic reporters to overcome TRAIL resistance in glioblastoma multiforme (GBM) cells in cellulo and in vivo. Using dual bioluminescence imaging of the death receptors DR4/DR5, it was shown that chemical modulators of DR4/DR5 expression sensitize GBM cells to apoptosis [176]. Live-cell imaging of GBM cells expressing FRET caspase biosensors allowed the monitoring of the effects of TRAIL stimulation on GBM cells. This study also evaluated GBM cell responses to engineered neural stem cells delivering secreted TRAIL [176] in cellulo and in vivo. This innovative and original approach provides an effective method using live-cell imaging technology, relevant biosensors, and pertinent use of stem cells properties.

5.2 FRET biosensors for prognosis and therapy design

A combination of several FRET biosensors and/or ionic species probes such as ROS can be used for multiparameter assays in living cells with high spatiotemporal resolution ([56, 177]; for review see [178]). This "lab-on-chip" approach based on biosensors and reporters might increase our understanding of the molecular mechanisms of tumorigenesis and tumor resistance to therapeutic agents. This approach could detect cellular abnormalities and identify kinetic changes in enzyme activation and/or aberrant enzymatic activities in order to develop more effective strategies for cancer therapy. For example, if tumor cells are resistant to apoptosis, the treatment can be aimed at inducing necroptosis. In addition, biosensors can also be used to monitor cellular responses to therapeutic agents [179].

A very recent study has identified a specific targeted molecule that was able to bypass apoptosis resistance of $p53^{-/-}$ colon carcinomas cells. Analysis of carcinoma cells by tissue microarray revealed overactivation of glycogen synthase kinase 3b (GSK3b) compared to normal cells from the same patient. Downregulation of the kinase in combination with chemotherapy promoted AIF-mediated necroptosis of drug-resistant colon carcinoma cells [180]. A "lab-on-chip" approach using a specific GSK3b FRET biosensor may help in identifying GSK3b as overactivated in the cellular context, so that treatment could be adapted to the phenotype of tumor cells, and, finally, in monitoring the necroptotic process. At present there is only an atypical half-life biosensor for measuring the activity of GSK3 kinase [181].

The concept of personalized prognosis and therapy design relying on FRET-based biosensors was illustrated in a study by Mizutani et al. [182]. They developed a reliable genetically encoded FRET-based biosensor to measure breakpoint cluster region-abelson (BCR-ABL) activity directly in living chronic myeloid leukemia (CML) cells obtained from a patient. Since chimeric BCR-ABL kinase exists only in tumor cells, imatinib (US) and Gleevec (EU) work specifically in tumor cells without affecting neighboring normal cells. This biosensor approach is radically different from conventional techniques trying to capture a snapshot of BCR-ABL activity by assessing the phosphorylation level of its substrate v-Crk avian sarcoma virus CT10 oncogene homolog-like (CrkL) in vitro. This novel dynamic approach has enabled the identification of a small population of CML cells resistant to Gleevec. The biosensor approach was then applied to optimize therapy design for targeting both drug-sensitive and drug-resistant CML cells according to the patient's tumor stage [183, 184]. Interestingly, this biosensor approach was also used to evaluate and compare BCR-ABL mutation effects on cell sensitivity to several inhibitors in order to predict the most appropriate treatment, thus providing a new method for prognosis.

In conclusion, to overcome the drawback of biochemical approaches, biosensors and reporters with greater spatiotemporal resolution have been developed by molecular engineering to cope with new challenges in biology, namely, the measurement of activity and interaction of biomolecules in living cells in real time. These new powerful and optimized tools are slowly but surely joining the biologist's toolbox to determine the dynamics of biomolecules in their physiological context [185–187]. These dynamic approaches will surely help us deepen our understanding of the molecular mechanisms of PCD, and will allow us to develop new therapeutic strategies to improve the efficacy of personalized treatment.

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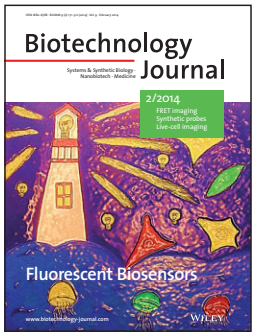

The Fluorescent Biosensor special issue of *Biotechnology Journal* is edited by Dr. May Morris and Prof. Marc Blondel. The cover image is an artistic interpretation of how fluorescent biosensors function as molecular beacons for scientists navigating a sea of molecules. Image courtesy of L. Divita and R. Wintergerst.

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