



Rational design of genetically encoded reporter genes for optical imaging of apoptosis

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Published online: 4 July 2020
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Abstract

Apoptosis is a process in which cells are genetically regulated to cause a series of changes in morphology and metabolic activity, which ultimately lead to cell death. Apoptosis plays a vital role in the entire life cycle of an organism. Too much or too little apoptosis can cause a variety of diseases. Therefore, efficient and convenient methods for detecting apoptosis are necessary for clinical treatment and drug development. Traditional methods for detecting apoptosis may cause damage to the body during sample collection, such as for flow cytometry analysis. So it is necessary to monitor apoptosis without invasion *in vivo*. Optical imaging technique provides a more sensitive and economical way for apoptosis visualization. A subset of engineered reporter genes based on fluorescent proteins or luciferases are currently developed to monitor the dynamic changes in apoptotic markers, such as activation of caspases and exposure of phosphatidylserine on the surface of dying cells. These reporters detect apoptosis when cells have not undergone significant morphological changes, providing conditions for early diagnosis of tumors. In addition, these reporters show considerable value in high-throughput screening of apoptosis-related drugs and evaluation of their efficacy in treating tumors. In this review, we will discuss the recent research progress in the optical imaging of apoptosis based on the genetically encoded reporter genes.

Keywords Reporter genes · Optical imaging · Apoptosis · Tumor treatment

Introduction

Apoptosis, also known as programmed cell death, is a process in which a cell is genetically regulated to cause a series of changes in morphology and metabolic activity that ultimately lead to cell death. This process plays a vital role in the entire life of the organism, and dysregulation of apoptosis can cause a variety of diseases [1–3]. Too little apoptosis leads to diseases such as tumors and autoimmune diseases, whereas too much apoptosis leads to neurodegenerative diseases [4, 5]. During apoptosis, it is essential that caspase cleaves downstream substrates to deliver apoptotic signal. The caspase family is a class of proteolytic enzymes that contains a cysteine active site and

specifically cleave the peptide bond after the aspartic acid residue. Caspases are commonly used as target molecules because of their vital role in the execution of cell death [6]. There are intrinsic and extrinsic pathways that can trigger apoptosis (Fig. 1). The former is stimulated by a proapoptotic protein Bcl-2 family such as Bax that changes mitochondrial permeability after receiving an apoptotic signal, causing Smac and Cytochrome c to be released from the mitochondria, activating initiator caspase, caspase-9 [7]. The latter is triggered by binding of death receptors to ligands, such as Fas–Fas ligand and TNF–TNF ligand. Ligand-bound receptors will recruit and activate caspase-8 or caspase-10 (both initiator caspase) [8]. No matter which apoptotic pathway activates caspase-3 (executioner caspase), it will continue to transmit and amplify apoptotic signals, triggering two landmark events, phosphatidylserine (PS) exposure and DNA fragmentation. PS is normally located on the inner leaflet of the cell membrane. Caspase-3 cleaves and inactivates Flippase (PS-dependent ATPase) so that it cannot flip or actively translocate PS from the outer leaflet to the inner leaflet of the plasma membrane. At the same time, caspase-3 cleavage activates scramblase (Xkr8-BSG

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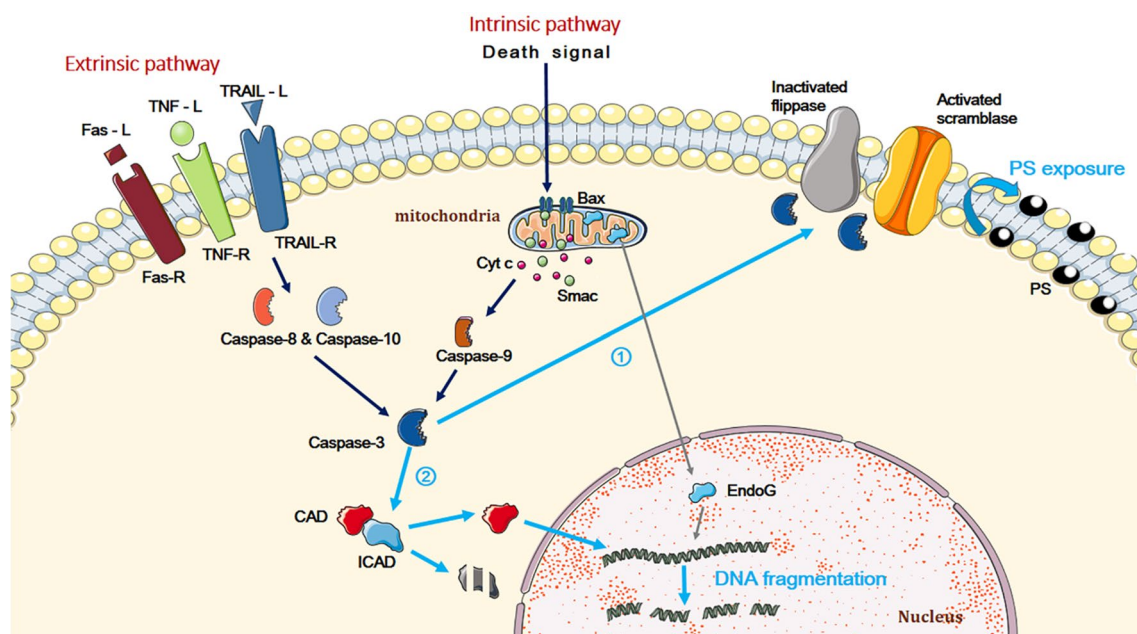


Fig. 1 Intrinsic and extrinsic pathways of apoptosis. The intrinsic pathway is stimulated by a proapoptotic protein Bcl-2 family such as Bax that changes mitochondrial permeability after receiving an apoptotic signal, causing Smac and Cytochrome c to be released from the mitochondria, activating initiator caspase, caspase-9. The extrinsic

pathway is triggered by binding of death receptors to ligands, such as Fas–Fas ligand and TNF–TNF ligand. Ligand-bound receptors will recruit and activate caspase-8 or caspase-10. Both pathways activate caspase-3, resulting in DNA fragmentation and PS exposure

heterodimer), resulting in irreversible exposure of PS to the cell surface [9]. DNA fragmentation mainly depends on two nucleases, DNA fragmentation factor (DFF) and endonuclease G (EndoG). DFF is also known as caspase-activated DNase (CAD). In non-apoptotic cells, CAD forms a complex with inhibitor of CAD (ICAD) present in the cytoplasm, existing as an inactive enzyme. In apoptotic cells, activated caspase-3 cleaves ICAD at two specific sites and inactivates its activity. CAD released from the inhibited state enters the nucleus to degrade chromosomal DNA [10–12]. EndoG transfers from mitochondria to the inside of the nucleus to cut DNA into short fragments [13].

Traditional methods for detecting apoptosis include morphological methods such as electron microscope and biochemical methods such as DNA electrophoresis and flow cytometry by AV/PI [14]. However, owing to the complexity of different forms of programmed cell death, morphologically similar cell deaths have a great degree of diversity in function, biochemistry, and immunity. Similarly, a single biochemical readout is not sufficient to be a clear indicator of the precise death modality [15]. Therefore, traditional methods cannot accurately distinguish different forms of programmed cell death. In addition, these methods have their own limitations. For instance, morphological observation by electron microscope is the most classic and reliable method for judging apoptosis by far. It is considered as the gold standard for determining apoptosis, but has the

following disadvantages: (1) it can only be qualitative but not quantitative; (2) The specimen processing process is complicated and the equipment is relatively expensive, which are not suitable for the inspection of a large number of specimens; (3) When observed by electron microscopy on tissue sections, it is sometimes difficult to distinguish apoptotic cells from mitotic division of normal cells, because chromatin condensation can occur in both cases. When agarose gel electrophoresis was performed on the DNA of apoptotic cells by DNA electrophoresis, the DNA fragments showed a step-like electrophoresis band spaced 180–200 bp apart. This typical change is considered to be a reliable indicator of apoptosis. However, this detection method also has some problems that need to be solved, such as low sensitivity and specificity, and inability to accurately quantify. Flow cytometry is an important means to detect morphological changes and cascades of apoptotic cells [16]. The Annexin V/propidium iodide (PI) method is the most commonly used method for detecting apoptosis by flow cytometry. In addition to the activation of caspase-3, the hallmark of apoptosis is the flipping of PS. Annexin V is a protein with a strong affinity for PS. Under normal conditions, PS is located in the inter leaflet of cell membrane and cannot bind to Annexin V. When the cell is apoptotic, PS is flipped out of the membrane and binding to Annexin V [4]. Annexin V can detect this change and specifically binds to fluorescein isothiocyanate. Therefore, Annexin

V can be used as a detection medium for PS turnover. PI is a fluorescent dye that cannot stain living cells. Hence, normal live cells are not stained, apoptotic cells are stained with Annexin V, and necrotic cells are stained with PI [17]. However, the drawback of flow cytometry is that it cannot be applied to in vivo detection because cells need to be suspended in a single state.

In the past two decades, the development of modern molecular imaging technologies has made it possible to image apoptosis noninvasively in living cells and mammals. Molecular imaging mainly includes the following modes: optical imaging, nuclear medicine imaging, and magnetic resonance imaging (MRI) [18, 19]. Among them, optical imaging has an important role in the imaging field because of its strong sensitivity and economic [20]. Meanwhile, various kinds of optical probes are also developed [21], bringing a bright future in visualizing protein–protein interactions [22], tumor cells [23], and gene therapy effects [24], etc. In this review, we mainly summarize the recent research progress on optical imaging of apoptosis based on the genetically encoded reporter genes. These reporter genes are designed to visualize the dynamic process of apoptosis in cells or mammals by modifying the fluorescent protein or luciferase enzyme to reflect the dynamic changes of apoptotic markers such as caspase or PS. Besides, reporter genes that combining optical imaging with other imaging modality are also discussed.

Optical imaging of apoptosis

Optical imaging is a convenient and highly sensitive imaging method. Optical imaging mainly includes fluorescence imaging (FI) and bioluminescence imaging (BLI) (Table 1). The recently developed optical reporter genes for apoptosis imaging in vivo will be discussed here.

FI of apoptosis based on fluorescent protein

Fluorescence imaging is an in vivo imaging method in which a fluorescent protein gene is introduced into a cell to produce a fluorescent protein through transcription and translation. After being excited by excitation light, the fluorescent protein emits fluorescence that can be detected by charge coupled device (CCD) cameras. Fluorescent proteins are mainly divided into blue fluorescent proteins (BFP), cyan fluorescent proteins (CFP), green fluorescent proteins (GFP), yellow fluorescent proteins (YFP), and red fluorescent proteins (RFP) [25, 26]. GFP was isolated from jellyfish *Aequorea Victoria* and was the first fluorescent protein discovered [27]. Its fluorescence is almost absent in other cell types, and no additional substrate is needed. Therefore, GFP is widely used in cell imaging. The key to fluorescent imaging of apoptosis is enabling to observe significant changes in fluorescence signals before and after apoptosis, such as fluorescence from dark to bright, changes in the spatial position of fluorescence, and changes in fluorescence color (Table 2). Two feasible ways to quench and restore fluorescence are as follows: (1) A fluorescent quencher is attached to the fluorescent protein to make the fluorescence signal temporarily disappear; (2) The self-assembling of split fluorescent proteins achieves fluorescence from dark to bright. Specific examples of reporter genes based on the above principles will be described in detail as below.

Self-assembly based fluorescent reporter

Green fluorescent protein is a special barrel-shaped structure composed of eleven β strands. Chromophore is located in the middle. The eleventh β strand catalyzes the maturation of the chromophore [52]. Therefore, GFP can be divided into two parts: one part is ten β strands of GFP (β 1–10), and the other is the eleventh β strand of GFP (β 11). β 1–10 and β 11 has the high binding affinity so that GFP emits green fluorescence only when the two structures are combined for several tens of minutes. If the two

Table 1 Strengths and weaknesses of optical imaging

Imaging modality	Reporter types	Strengths	Weaknesses
Optical imaging	Self-assembly	High sensitivity	Delay caused by fluorescence maturation
	Translocation	Able to quantify apoptosis	Higher background signal
	Color	Able to monitor initiator and executioner caspase simultaneously	Low depth penetration
	Bioluminescence	Low background signal; Able to monitor initiator and executioner caspase simultaneously No phototoxicity	Need for injecting substrate or cofactor

Table 2 Types of fluorescence imaging of apoptosis

Fluorescence change	Method	Apoptosis marker	Fluorescent protein used	References	
From dark to bright	Split and self-assembly of fluorescent protein	caspase-3,7	Venus	[28]	
		caspase-3	GFP and IFP	[29]	
		caspase-3	GFP	[30, 31]	
		Bax and Cyt c	YFP	[32]	
		Smac	GFP	[33]	
Translocation	Adding quencher peptide	caspase-7	GFP	[34]	
		caspase-3	GFP	[35]	
		caspases Drice and Dcp1	mRFP and EGFP	[36]	
Color	FRET	PS	YFP	[37]	
		caspase-3	CFP-YFP	[38–40]	
		caspase-3,9	ECFP-Venus	[41–44]	
		Caspase-3	mNeonGreen-mRuby2	[45]	
		caspase-3,8/9	CFP-DsRed and YFP-DsRed	[46]	
		caspase-2,3	ECFP-DsRed	[47]	
	BRET	BRET	caspase-3,8	seCFP-Venus -(mRFP1)	[48]
			caspase-3	LSS-mOrange-mKate2	[49]
			caspase-3,7	CBGt-dTomato	[50]
		PS	Rluc-QDs	[51]	

GFP green fluorescent protein, *EGFP* enhanced green fluorescent protein, *YFP* yellow fluorescent protein, *Venus* a variant of YFP, *CFP* cyan fluorescent proteins, *ECFP* enhanced cyan fluorescent protein, *seCFP* super-enhanced CFP, *mRFP* monomeric red fluorescent protein, *IFP* infrared fluorogenic protease, *CBG* click beetle green luciferase, *Rluc Renilla* luciferase, *QDs* quantum dots

parts are distributed in the same spatial position, caspase cuts the connecting peptide, and then GFP quickly fluoresces after self-assembly. Xiaokun Shu et al. designed a GFP-based fluorogenic reporter, dubbed ZipGFP, for apoptosis imaging based on this principle. DEVD is a sequence that can be specifically recognized and cleaved by caspase-3. In order to improve the sensitivity of the probe, a coiled coil composed of $\beta 11$ and $\beta 1-10$, is linked with $\beta 1-10$ and $\beta 11$ through DEVD, respectively. In the cells undergo apoptosis, caspase-3 cleaves the DEVD sequence, both $\beta 11$ and $\beta 1-10$ are pulled apart, which makes self-assembly possible and leads to enhanced fluorescence of ZipGFP reporter [30]. Similarly, GFP can also be divided into three parts, $\beta 1-9$, $\beta 10$, and $\beta 11$. $\beta 10$ and $\beta 11$ strand can form an antiparallel structure fitting well to $\beta 1-9$. Therefore, Xiaokun Shu et al. also designed a FlipGFP reporter by connecting $\beta 10$, E5, $\beta 11$ and K5 in turn and inserting a DEVD sequence between $\beta 11$ and K5 (Fig. 2). Thus, the heterodimer formed by K5 and E5 parallels $\beta 10$ and $\beta 11$ to prevent self-assembly of GFP. When caspase-3 cleaves DEVD, $\beta 11$ flips back and forms an antiparallel structure with $\beta 10$, allowing self-assembly with $\beta 1-9$ and resulting in enhanced fluorescence of FlipGFP [31]. They applied FlipGFP to image apoptosis of HeLa cells and zebrafish embryos. The results showed that

the fluorescence of HeLa cells was significantly enhanced after treatment with staurosporine. In addition, during zebrafish embryo development, apoptosis occurs in the forebrain and retina. Other fluorescent proteins such as Venus [28] and infrared fluorescent proteins [29] using similar self-assembly principles have also been reported for apoptosis imaging.

If the spatial positions of the two parts of GFP are placed on the cytoplasm and the mitochondrial membrane respectively, the mitochondrial permeability changes in apoptotic cells so that the two parts are distributed in the same space to complement spontaneously, releasing a fluorescent signal. For instance, Smac, a protein found in the mitochondrial membrane space, is used as a target for apoptotic imaging in vivo (Fig. 3). The Smac was connected to $\beta 11$, while the rest of GFP was located in the cytoplasm, so the self-assembly of GFP was hindered by the difference in spatial location of the two fragments. Under the stimulation of apoptotic signal, Smac- $\beta 11$ is released from the mitochondria into the cytoplasm, enabling GFP to complement and to recover fluorescence [33]. Another study developed a split yellow fluorescent protein (YFP) based reporter, which are fused to Bax and cytochrome c, to monitor mitochondrial-related apoptotic events [32]. The change in mitochondrial permeability is the initiation stage of apoptosis [14], and caspases are

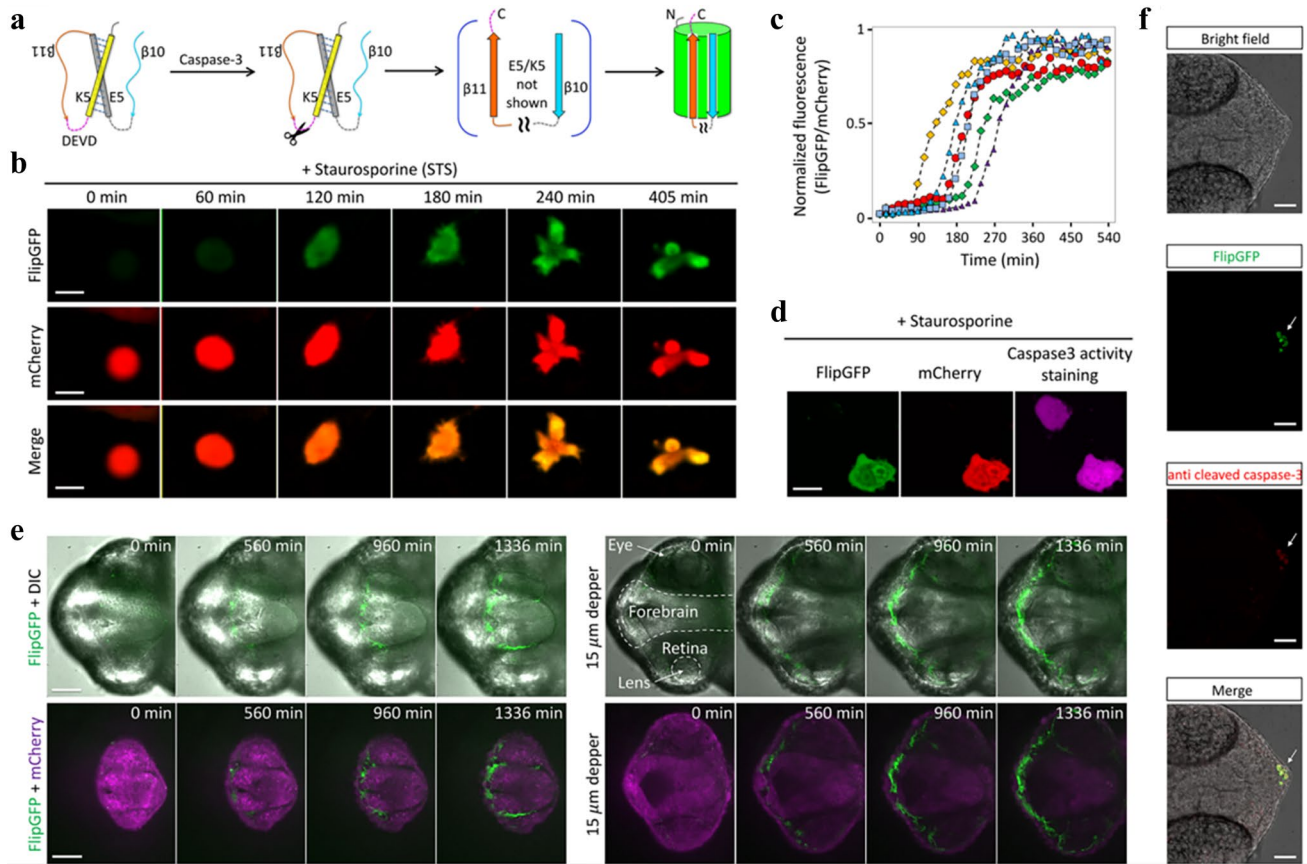


Fig. 2 FlipGFP reporter in imaging apoptosis in mammalian cells and in zebrafish. **a** Schematic of FlipGFP. **b** Fluorescence images of HeLa cells after injection of staurosporine. **c** Representative cells. **d** FLICA staining. **e** Time-lapse images of zebrafish embryo (FlipGFP: green, mCherry: purple). Imaging started at 24 h postfertilization. **f** Whole

mount immunostaining of zebrafish embryo expressing FlipGFP. Arrow points to apoptotic embryo cells. Scale bars: 20 μ m (**b**, **d**); 70 μ m (**e**); 50 μ m (**f**) (Reprinted with permission from [31]. Copyright 2019 American Chemical Society.)

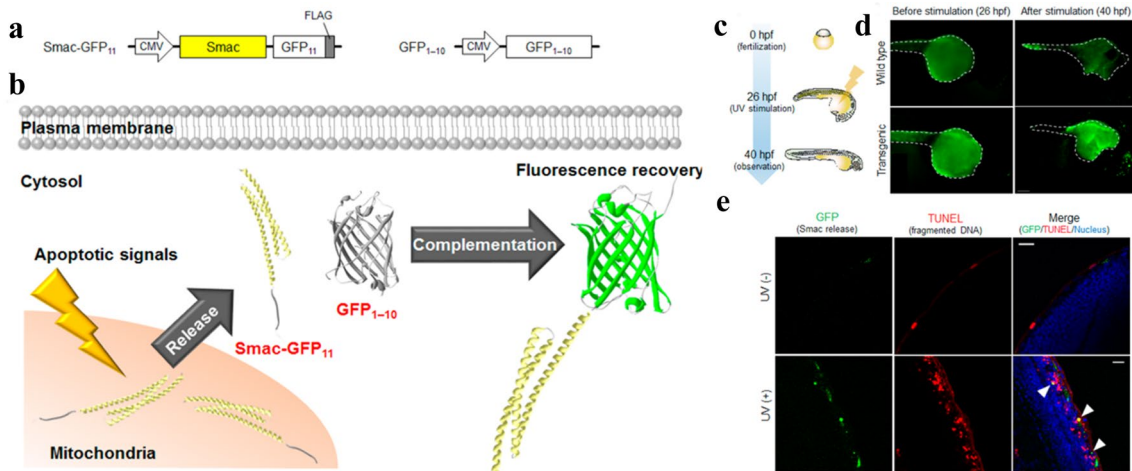


Fig. 3 Smac-GFP11/GFP1-10 reporter and images of apoptosis in a zebrafish embryo. **a** Schematic of the reporter. **b** Upon apoptotic stimulation, Smac-GFP11 is released from mitochondria into cytoplasm. It is spontaneously complemented with cytosolic GFP1-10, resulting in the recovery of the fluorescence. **c** Procedure for detec-

tion of apoptosis in a zebrafish embryo. **d** Fluorescence images of zebrafish embryos. **e** TUNEL assay in transgenic zebrafish. Scale bars: 20 μ m (Reprinted with permission from [33]. Copyright 2019 American Chemical Society.)

subsequently activated to induce apoptosis. Therefore, targeting mitochondrial apoptosis markers is more conducive to detect the occurrence of apoptosis at an early stage. But the shift of the spatial position may cause the reporter sensitivity to decrease.

Quencher-based fluorescent reporter

Caspase activatable-GFP (CA-GFP), reported by Samantha B. Nicholls et al., is a strong and effective reporter gene designed to temporarily curb the fluorescence of GFP by quenching peptides [34]. The quencher peptide is a 27-amino acid peptide from the tetrameric proton channel domain of M2 influenza protein, which is connected to the carbon terminus of GFP through a caspase recognition sequence. Due to the presence of the quenching peptide, CA-GFP is in a tetrameric state. The environment of the chromophore in the tetramerized CA-GFP changes slightly, causing GFP to almost completely lose fluorescence. However, when CA-GFP is expressed in the presence of active caspases, GFP is released from CA-GFP and undergoes the necessary structural rearrangements, which eventually mature the chromophore and produce fluorescence. Studies have shown that there is a similarity between the circular dichroism spectrum and thermal stability between CA-GFP and GFP_{β1-10} [53]. This may be the basis for the structure of CA-GFP as a dark-to-bright reporter. CA-GFP is neither dependent on mitochondria nor on the nucleus, never affected by other biological processes in monitoring the activity of caspase. In mammals, fluorescence increases threefold after activation. The model of CA-GFP can be used to develop multiple reporter genes by replacing the cleavage site and fluorescent protein. Peng Wu et al. distinguished caspase-6 and caspase-7 by changing the sequence of the cutting site. In addition, they also constructed CA-Cerulean, CA-Citrine and CA-mNeptune reporters with CFP, YFP and RFP to monitor the activity of caspase alone or in multiple channels [54]. These reporter genes further expand the application of CA-GFP, such as fluorescence activated cell sorting or multiplex microscopy.

Spatial translocation based fluorescent reporter

Changing the spatial position of fluorescence is also an ingenious idea. It is usually used in combination with other methods, for example, the splitting of fluorescence and fluorescence resonance energy transfer (FRET) technology. pCasFswitch is designed to use the location change of GFP signal to reflect the occurrence of apoptosis. This reporter contains a nuclear localization sequence (NLS), a plasma membrane localization sequence (PLS), a GFP sequence and a caspase-3 specific recognition fragments (DEVDG). The NLS is attached to the N-terminus of GFP,

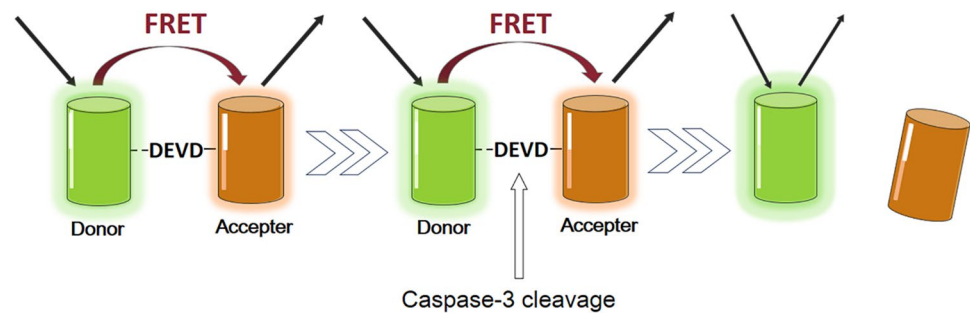
and the PLS is attached to the C-terminus of GFP through DEVDG. If PLS is stronger than NLS, the green fluorescent signal will be located on the plasma membrane of the cell. When active caspase-3 cleaves DEVDG, the remainder of pCasFswitch, GFP-NLS, will be located in the nucleus [35]. Another Spatial translocation based fluorescent reporter introduces two fluorescent proteins. Pierre-Luc Bardet et al. devised a fluorescent reporter of caspase activity named as Apoliner. This reporter includes mRFP connected to the transmembrane domain and eGFP connected to the NLS. A fragment of *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) links the carbon end of mRFP to the nitrogen end of NLS. The DIAP1 fragment contains the BIR1 domain, which can be recognized and cleaved faster by the effector caspases Drice and Dcp1. Therefore, in living cells, both fluorescent signals should be present on the cell membrane. When caspase is activated, mRFP remains on the cell membrane but NLS-eGFP should be released, accumulating in the nucleus. Unique caspase recognition sequence of Apoliner enhances efficiency and specificity [36]. In addition, the transfer of signals from intracellular to extracellular is also a form of transfer. For example, Annexin V linked to a secretory signal peptide is fused with YFP to form the secA5-YFP reporter gene. This fusion protein is secreted outside the cell and then binds to the cell membrane of apoptotic cells that undergo PS turnover. This secreted reporter protein can mark morphological changes of apoptotic cells during biological development [37]. However, this secreted protein may not have been secreted in the cell or it may be outside the cell but not bound to PS, resulting in relatively high background noise.

FRET based fluorescent reporter

The change in color is essentially a change in emission wavelength, usually caused by energy resonance transfer. Energy resonance transfer occurs between a donor and an acceptor with a distance of less than 10 nm (the absorption spectrum of the two have a certain overlap). Energy is transferred from the donor to the acceptor so that the acceptor energy increases and the donor energy decreases. There are two kinds of energy resonance transfer, one is fluorescence resonance energy transfer (FRET), and the other is bioluminescence resonance energy transfer (BRET). Both the donor and acceptor of FRET are fluorescent proteins. The FRET-based fluorescence reporters usually employ caspase-specific cleavage fragments to connect two fluorescent proteins [36, 38–44, 46–49] (Fig. 4).

CFP and YFP and their variants are very common FRET combinations, because in addition to the overlap of the donor emission spectrum and the acceptor excitation spectrum, their fluorescence ranges are far away from each other [55]. Kiwamu Takemoto et al. developed a

Fig. 4 Schematic of FRET based fluorescent reporter. The donor and acceptor are connected by DEVD, because the close distance between the donor and acceptor triggers FRET. When apoptosis occurs, the caspase-3 cleavage increases the distance between the two fluorescent proteins and the FRET disappears



FRET-based fluorescent probe SCAT3, which consists of a donor (a cyan fluorescent protein) and an acceptor (a mutant of yellow fluorescent protein), which are connected by DEVD peptide. When apoptosis occurs, the longer distance between these two fluorescent proteins causes FRET to disappear. At this time, only the fluorescence emitted by the donor can be detected [41]. SCAT3 was used to process salivary glands in *Drosophila* during apoptosis [43]. This probe can be also applied to monitor the tumor cell apoptosis induced by photodynamic therapy (PDT) [42, 44]. FRET between CFP and YFP can also be applied to understand T cell contraction and tumor damage caused by T cells. Béatrice Breart et al. used the CFP-DEVD-YFP reporter to image tumor cell apoptosis in vivo in studying the connection between T cell adoptive therapy and tumor cell elimination. When UVB irradiation induced apoptosis of EG7 tumor cells transfected with CFP-DEVD-YFP reporter, DEVD was cleaved after activation of caspase-3, causing FRET loss. In the control group, EG7 tumor cells were transfected with CFP-DEVD-YFP reporter. Since caspase-3 could not cleave DEVD, FRET did not change. For the mice bearing with EG7 tumor cells transfected with CFP-DEVD-YFP reporter, intravital two-photon imaging revealed that tumor cells in contact with cytotoxic T cells were more likely to undergo apoptosis than tumor cells without surrounding cytotoxic T cells [38]. This result provides a visual evidence that cytotoxic T cells directly mediate tumor cell elimination. Similarly, Kym R. Garrod et al. used the CFP-DEVD-YFP reporter to reflect the loss of motility and cell death of T cells at the peak of the response. Less than 10% of T cells with FRET loss remained motile, while T cells with no FRET change characterized high motility [39]. This evidence proves that changes in CFP-DEVD-YFP reporter represent T cells undergoing apoptosis. The application of FRET based fluorescent reporter for function and dynamic changes of T cells has opened up a perspective for us in investigating apoptosis imaging of immune cells. Kathy Q. Luo et al. constructed two sensors based on the FRET to compare the dynamic changes of caspase-8 and caspase-3 in HeLa cells [40]. Because the specific cleavage ability of caspase-8 is weaker than that of caspase-3, two caspase-8 specific

cleavage sites (IETD) are connected in series between CFP and YFP to enhance its cleavage ability. Simultaneously, CFP-DEVD-YFP was constructed to monitor the changes of caspase-3. The results showed that in the process of apoptosis induced by TNF- α , the activation process of caspase-8 was slower than that of caspase-3, and the activation time was much earlier than the activation of caspase-3. This study provides in vivo data on the interaction of caspase-8 and caspase-3 pathways.

Apart from CFP and YFP, some red fluorescent proteins are also often used as receptors in FRET, combined with other fluorescent proteins as reporter genes to detect apoptosis. In order to figure out the dynamic changes of B cell apoptosis in germinal centers, Christian T. Mayer et al. produced transgenic mice expressing apoptosis reporter (INDIA) [45]. INDIA contains a FRET pair consisting of mNeonGreen and mRuby2. The two fluorescent proteins are connected by peptide DEVDG. Apoptosis is induced by staurosporine, and activated caspase-3 cleaves the specific site DEVDG, causing FRET loss and mNeonGreen emission increased. Hiroshi Kawai et al. produced six versions of FRET-based reporter for caspase activity through different combinations of CFP, YFP, GFP and DsRed, namely GFP-peptide-CFP (GC) YFP-peptide-CFP (YC) YFP-peptide-GFP (YG) CFP-peptide-DsRed (CR) GFP-peptide-DsRed (GR) YFP-peptide-DsRed (YR) [46]. Peptide is a cleavage sequence of caspase. Through real-time monitoring of tumor necrosis factor (TNF)- α induced single cell apoptosis, it was found that CY, CR, GR, and YR performed well in detecting caspase activation. They also selected two reporters, YR and CR, to monitor caspase-8, 9 (initiator caspase) and caspase-3 (effector caspase) in the same cell. The result revealed that the initiator caspase and effector caspase were activated almost simultaneously. Juqiang Lin et al. used ECFP-DsRed to image caspase 2 and caspase 3 activity changes in HeLa cells [47]. In addition, a reporter based on dual-FRET system composed of three fluorescent proteins has been developed. Katsuya Kominami et al. established a fusion protein called CYR83, consisting of super-enhanced CFP, a variant of YFP (Venus) and monomeric red fluorescent protein 1 (mRFP1). The peptides connecting these three proteins are IETD and DEVD, respectively [48]. They

converted fluorescence in CYR83-expressing single cells into an image indicating of the emission ratio changes of FRET. The reduction of emission ratio represented the increase of caspase activity. Thus, the dual-FRET system is able to simultaneously monitor the dynamic changes of caspase-8 and caspase-3 activation in single cells in real time. However, FRET is affected by the depth of the tissue and the sensitivity of the probe is insufficient. Fluorescence lifetime imaging microscopy (FLIM) can image animal models in a three-dimensional environment without being limited by tissue depth. Fluorescence lifetime is the physical property of the fluorescent molecule itself, which means the time that the fluorophore is in the excited state before releasing the photons back to the ground state. In FRET, the energy transfer of donor fluorescence is a quenching process that cuts down the excited state of the donor, so the fluorescence lifetime of the donor fluorescent protein is shortened. FLIM displays the spatial distribution of reporter lifetime in living cells and accurately measures the shortened lifetimes of the donor [56]. Johanna M. Buschhaus et al. monitor the dynamic changes of LSS-mOrange-DEVD-mKate2 through FLIM. LSS-mOrange and mKate2 are donor and acceptor fluorescent proteins, respectively. In cells without caspase-3 activity, the fluorescence lifetime of LSS-mOrange is shortened, while FRET is reduced in cells undergoing apoptosis, and the lifetime of LSS-mOrange is extended [49, 57]. The fluorescence spectra of EYFP and EGFP are similar, so the combination of EYFP and EGFP is difficult to use as a FRET-based reporter gene. However, FLIM can be used to determine the fluorescence lifetime of the acceptor and the donor, and a FRET-based EYFP-DEVD-EGFP reporter gene was constructed to detect changes in caspase-3 activity during apoptosis in a single cell [58]. But this reporter gene has not yet been applied to in vivo apoptosis imaging. Yet FLIM still cannot overcome the defect that FRET requires excitation light to excite the donor protein to fluoresce, which may cause phototoxicity to cells. The donor of BRET is luciferase, so the donor does not require external excitation light. Fluorescent proteins [50] or quantum dots [51] can be selected as receptors for BRET.

BLI of apoptosis based on luciferase

There are some organisms in nature that emit light spontaneously. The luciferase present in these creatures catalyzes luciferin (the substrate of luciferase) to produce bioluminescence. Bioluminescence imaging (BLI) was developed based on this principle and has been widely used in novel light-producing transgenic animals and imaging the biodistribution of exosomes in vivo [59]. According to the source, luciferases used for BLI are divided into firefly luciferase (Fluc) from *Photinus pyralis*, click beetle

luciferase (Cluc) from *Pyrophorus plagiophthalmus*, *Renilla* luciferase (Rluc) from *Renilla reniformis*, and *Gaussia* luciferase (Gluc) from *Gaussia princeps*. In marine creatures, luciferase only needs the substrate, coelenterazine analogs, to emit light; while in coleoptera creatures, the substrate of luciferase is D-Luciferin, which requires oxygen, Mg^{2+} and consumes ATP to emit light [60]. Among them, Fluc is often modified for in vivo apoptotic imaging. There are two main methods of modification: split and cyclization.

Split luciferase based reporter

One probe design method for detecting apoptosis is to split luciferase into two parts, NLuc and CLuc, which first applies to imaging protein–protein interactions [22, 61]. The use of reporter genes designed by split-Fluc for monitoring early in vitro apoptosis events has been reported [62], but this invasive way to detect apoptosis can cause some damage to cells. Luciferase, unlike fluorescent protein, is unable to emit fluorescence by self-assembly, so it is necessary to connect peptide A and peptide B to both ends of NLuc and CLuc, respectively (Fig. 5). Peptide A (MNEAYVHDGVPVRS LN) and peptide B (MKARKEAELAAATAEQ) are two strongly interacting peptides that can minimize their impact on the structure of NLuc and CLuc because of the small size of two peptides [63]. When apoptosis occurs, caspase-3 will cleave DEVD linked between NLuc and CLuc so that the strong affinity between peptide A and peptide B allows the two parts of luciferase to be intact to restore luciferase activity [64]. Using this caspase-3 sensor as an effective tool, they also noninvasively visualized apoptosis for evaluation of

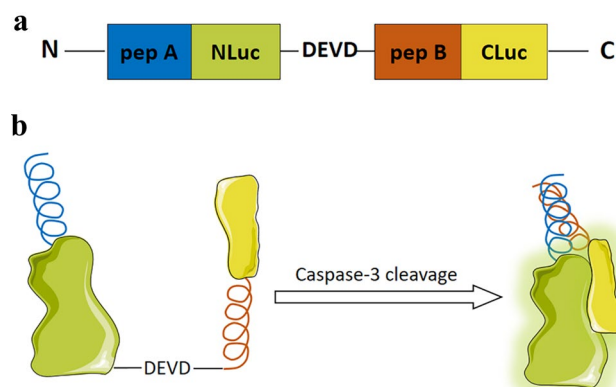


Fig. 5 Strategy for imaging of caspase-3 activity using Split luciferase based reporter. **a** Schematic of Split luciferase based reporter. This reporter includes an N-terminus of luciferase and a C-terminus of luciferase adjoining peptide A and peptide B, respectively. A connecting sequence, DEVD, is a caspase-3 recognition sequence **b** when apoptosis occurs, caspase-3 will cleave DEVD linked between NLuc and CLuc so that the strong affinity between peptide A and peptide B allows the two parts of luciferase to be intact to restore luciferase activity

therapeutic efficacy as well as for optimization of natural killer (NK) cell-based immunotherapy [65]. Qiuxia Fu et al. improved the probe based on this principle and used this probe to monitor changes of caspase-3 activity in the liver of mouse in real time [66]. The fragment of attB was inserted into the upstream of pepA-NLuc-DEVD-pepB-CLuc to construct the reporter plasmid. The reporter plasmid and Φ C31o integrase plasmid were delivered to the mouse liver by hydrodynamic injection procedure. Φ C31o integrase improves the recombination efficiency of in mice liver and could be an alternative approach to create genetically modified laboratory mice models. Using split luciferase method, the signal was reduced in vivo compared to that in vitro. To address this problem, Stefanie Galban et al. screened a thermal stable luciferase, a Fluc mutant, which increased the signal-to-noise ratio by enhancing the intrinsic stability of luciferase in the absence of tightly interacting peptides [67].

Cyclic luciferase based reporter

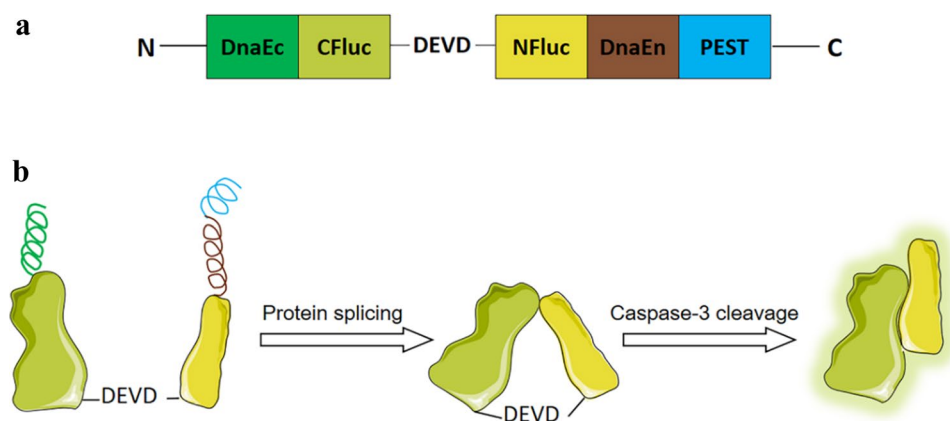
Another approach for detecting apoptosis is to construct a cyclic luciferase encoded by a gene as a probe called pcFluc-DEVD for in vivo imaging (Fig. 6) [68, 69]. The amino terminal end of Fluc (NFluc) and the carboxy terminal end of Fluc (CFluc) are connected by DEVD. The C- and N-terminal fragments of DnaE are connected with CLuc and NLuc. The DnaE intein makes CFluc-DEVD-NFluc form a cyclic protein by protein splicing. The luciferase activity disappears through distorting its structure and restores after DEVD is cleaved. In addition, the PEST sequence was attached to the C-terminal fragment of the fusion construct as a signal for protein degradation. Since the cyclic Fluc does not have a PEST sequence, the PEST sequence will only cause degradation of unspliced products. Takeaki Ozawa et al. used this pcFluc-DEVD probe in a hepatic ischemia/reperfusion (I/R) mouse model and in vivo monitoring of liver apoptosis by caspase-3 activity [70]. In

addition, Xiaoyuan Chen et al. employed pcFluc-DEVD as a reporter to successfully quantify doxorubicin-induced apoptosis in vivo [71]. Our group also successfully used pcFluc-DEVD to detect sanguinarine-induced apoptosis via activation of reactive oxygen species (ROS) in living cells and mice [72]. This method of separation and recombination of luciferase can significantly reduce the luminescence signal of the background, thereby significantly increasing the sensitivity of the probe. In addition, Luciferase and luciferase substrates are relatively cheap and available. Compared with positron emission tomography (PET) and MRI, BLI does not involve radioisotopes and the instrument of BLI is easy to operate. These advantages provide conditions for the wide application of pcFluc-DEVD. In contrast to the split luciferase based reporter, the split fragments are too large and may cause a decrease in transfection efficiency. Moreover, if the two peptides do not fully interact with each other, the bioluminescence signal will be weakened.

Fused luciferase based reporter

Apart from modifying the structure of luciferase, it is also possible to fuse luciferase with Annexin V to form reporters that can rapidly detect PS. Mahboobeh Nazari et al. reported a probe that connects RLuc and Annexin V through recombinant fusion protein technology [73]. They used PCR and cloning approaches to clone full-length cDNA of RLuc into a vector pPROEX HTa/Annexin V that expressing Annexin V. Then, the new construct vector was transformed into *E. coli* BL21 and expressed under the control of T7 promoter. After purification, the RLuc/Annexin V fusion protein was obtained and added to the apoptotic cells stimulated by actinomycin D. In cells with normal structure and function, PS is located in the leaflet of the cell membrane, and Annexin V cannot bind to it. In apoptotic cells, RLuc/Annexin V combines with PS flipping to the outer leaflet in the presence of Ca^{2+} . The fluorescent signal of the fusion protein can be captured by a

Fig. 6 Strategy for detection of caspase-3 using cyclic luciferase based reporter. **a** Schematic of Cyclic luciferase based reporter. DnaE helps luciferase cyclization and PEST sequence guides degradation of uncyclized reporter. **b** The reporter formed inactive cyclized luciferase after protein cleavage. If caspase activates and cleaves the reporter during apoptosis, luciferase resumes activity and emits a luminescence signal



luminometer after adding the substrate, coelenterazine. The chosen luciferase was Rluc because it does not need post-translational modifications and ATP to participate in the catalytic reaction. So the cell activities will not be disturbed. Trajen Head et al. employed a biosensor composed of the fusion of a stable mutant of Rluc (Rluc8) and Annexin V to detect apoptosis in vivo and in vitro, and named it ArFP [74]. Rluc8 improves the stability of the reporter by 200 times and the bioluminescent intensity by 4 times. They investigated the feasibility of ArFP in Jurkat (human T lymphocyte) cells cultured in vitro and in corneal and retinal tissues of mice. The results show that ArFP can specifically detect apoptosis in vitro and in the corneal tissue of mice. Better stability and stronger bioluminescent intensity enabled the probe to successfully achieve apoptosis imaging for the cornea and retina tissues in mice. Therefore, Annexin V/Rluc fused reporters may be more widely applied to other apoptosis-related diseases and drug screening. In the future, the feasibility of fusion of other luciferases and Annexin V to develop a series of reporters with different characteristics requires further investigation.

Secreted luciferase based reporter

In mammalian cells, under similar conditions, bioluminescent intensity of Gluc is more than 100 times higher than Fluc and Rluc [75]. The high bioluminescent intensity gives Gluc some unique advantages as a reporter gene. But in the natural state, Gluc has a secretion signal peptide composed of 16 amino acids at the N-terminus. When the *Gluc* gene is expressed, Gluc is secreted out of the cell. This outward secretion feature limits Gluc's application in monitoring cell internal activity [76]. Shuchi Gaur et al. used multiple repeated endoplasmic reticulum targeting peptides, KDEL, to increase the retention of Gluc in the cell without changing the luciferase activity. They obtained sGLUC-DEVD-(KDEL)₃ as a caspase sensor after many screenings [76]. In normal cells, sGluc remains in the cell under the action of endoplasmic reticulum targeting peptide, and when activated caspase-3 in apoptotic cells cleaves DEVD, sGluc is secreted outside the cell. The effects of this reporter were tested with doxorubicin and paclitaxel on breast cancer cells stably expressing sGLUC-DEVD-(KDEL)₃. The results showed that the release of sGluc was drug dose- and time-dependent. Further study of the effectiveness of the caspase sensor in animal models is needed. sGLUC-DEVD-(KDEL)₃ may reduce the difficulty and cost of monitoring tumor treatment in vivo by collecting blood or urine samples to detect secreted report signals.

Other reporters

Other researchers monitored apoptosis in vivo by using β -galactosidase (β -gal) or luciferase to interact with a chromogenic substrate to generate luminescence. It has been reported that the estrogen receptor regulatory domain (ER) is used to regulate the function of proteins with no catalytic ability such as myc, dhfr, and fos [77–79]. Due to the "protein inactivation" function of the ligand-binding domain of steroid receptors, ER can inhibit other activities on the same polypeptide chain in the absence of hormone [78]. Bharathi Laxman et al. confirmed that ER can also inhibit the activity of luciferase, a kind of catalytic enzyme [80]. After the ER-DEVD-Luc-DEVD-ER they designed was cleaved by caspase-3, luciferase is released, catalyzing luciferin to produce bioluminescence. The ER-LACZ-ER constructed by Divya Khanna et al. also used the function of ER to minimize the catalytic activity of β -gal [81]. ER-mediated steric hindrance prevents tetramerization of the β -gal. The β -gal released by ER-LACZ-ER after being cleaved by caspase-3 forms the β -gal tetramer. DDAOG is a chromogenic substrate formed by conjugating β -gal and 7-hydroxy-9H-{1,3-dichloro-9,9-dimethylacridin-2-one (DDAO). After β -gal catalysis, DDAOG becomes DDAO which can emit near infrared fluorescence signal. This strategy of constructing reporters can also be extended to PET or other imaging modality, that is, replacing Luc gene with herpes simplex virus thymidine kinase gene. Also, this reporter can be used to efficiently screen for protease inhibitors.

Multiple modality imaging

Each imaging method has its own advantages and disadvantages, and it is difficult to meet the requirements for sensitivity, specificity, and targeting at the same time. The molecular probes in multimodal molecular imaging can overcome the shortcomings of a single imaging method, achieve complementary advantages, and broaden the application scope of molecular imaging technology.

Dual fluorescence imaging is the first step from single-modality imaging to multi-modality imaging. In the process of apoptosis, condensation of chromatin and disassembly of nuclear scaffold proteins occur in the nucleus. Meng Yang et al. used two fluorescent proteins to visualize the dynamic changes in the size of the cytoplasm and nucleus [82]. Histone H2B-GFP and RFP are used to label the nucleus and cytoplasm of human prostate cancer cells, respectively. Standard fluorescence microscope imaged real-time changes in cytoplasmic and nuclear size and nuclear fragmentation to reflect the early apoptosis of cells. The results showed that after paclitaxel treatment, the cytoplasm emitting red fluorescence and the nuclei emitting green fluorescence

swelled, and the nuclei fragmented, forming ring-like structures of fragmented chromatin. After vinblastine treatment, swollen nuclei and cytoplasm but no ring-like structures were observed. Therefore, they believe that nuclear fragmentation caused by paclitaxel-induced apoptosis is different from vinblastine-induced apoptosis. Dual bioluminescence imaging has also been utilized to detect apoptosis *in vivo*. Khalid Shah et al. employed Fluc and Rluc to monitor the effect of gene delivery in human glioma Gli36 cells and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis *in vivo* in real time [83]. Fluc and Rluc constructed in replication-deficient HSV-derived amplicon vectors. Gli36 cells stably transfected with Fluc-HSV formed a Gli36fluc⁺ cell line with luciferase activity. Subsequently, Gli36fluc⁺ cells were implanted into nude mice, and solid tumors appeared after 7–10 days. After injection of luciferase substrate (luciferin), the tumor volume was observed by bioluminescence imaging. After injection of HSV amplification vector with Rluc gene in the same tumor, coelenterazine was injected to image the distribution of gene delivery 36 h later. They also used HSV amplification vectors to deliver TRAIL to tumor cells for inducing apoptosis. It was found that the TRAIL-amplicon vector reduced the volume of glioma by real-time bioluminescence imaging. Therefore, dual bioluminescence imaging of Fluc and Rluc has the potential to monitor the delivery of therapeutic genes and antitumor effects. However, many types of tumors are not sensitive to TRAIL. In order to solve this problem, they developed a dual fluorescence reporter gene system to identify molecules, MS-275, that assist TRAIL to improve the efficiency of inducing tumor apoptosis. This approach may advance the application of TRAIL in Phase I/II trials [84]. Another approach to design apoptosis reporter is to create a caspase-3-dependent reporter gene—pepA-NLuc-DEVD-CLuc-pepB and Rluc to assess the targeted therapy effectiveness of adenovirus-mediated apoptotic drugs (TRAIL/FasL) [85]. The limitation of bioluminescence imaging is that the bioluminescent intensity is not enough, which is unfavorable to imaging in deep tissues. Nevertheless, after the luciferase is folded by the endoplasmic reticulum, the bioluminescent intensity increases to two thousand times. Therefore, the reporter gene of GFP-DEVD-ssGluc was designed to transfer to the endoplasmic reticulum via carrying a signal sequence on Gluc. The DEVD in apoptotic cells is cut off so that Gluc is released into the blood after passing through the endoplasmic reticulum and Golgi apparatus [86]. The advantage of this reporter gene is that not only the location of tumor cells is displayed by GFP, but also circulating tumor cells in the blood can be detected for real-time dynamic analysis of caspase.

As mentioned above, there are two main types of luciferase substrates, D-Luciferin and coelenterazine.

Bioluminescence imaging seems to only for the reporter gene that utilizing these two substrates. However, the newly discovered Vluc and its corresponding substrate vargulin can also be used to bioluminescence imaging. Casey A Maguire et al. developed a reporter system consisting of Fluc, Rluc, and Vluc for sequential monitoring three different events that occur in the same biological system after TRAIL is delivered via an adeno-associated virus (AAV) [87]. The secreted soluble variant of TRAIL was cloned into AAV to construct AAV-sTRAIL. Similarly, Vluc was cloned into AAV to construct AAV-Vluc. U87 glioma cells stably express Fluc after transfection with lentivirus vector (U87-Fluc). Gluc and nuclear factor- κ B (NF- κ B) responsive elements were constructed into a lentiviral vector and then the vector transfected the U87-Fluc cells, resulting in U87-Fluc/NF-Gluc cells. U87-Fluc/NF-Gluc cells were implanted into nude mice. AAV-Vluc and AAV-sTRAIL were injected at the site of tumor formation 19 days later. 10 days after injection of the AAV vector, the Vluc signal obtained after intravenous injection of the vargulin substrate represents gene delivery. Fluc imaging and Gluc imaging were performed 22 days and 23 days after injection of AAV vector, respectively. Fluc signal represents tumor volume, Gluc signal represents TRAIL binding to glioma cells triggering NF- κ B pathway activation. This reporter system containing three luciferases could be expanded to other areas where multiple parameters need to be monitored simultaneously. Optical imaging can also be combined with other modalities to perform apoptosis imaging *in vivo*. For instance, Pritha Ray et al. described a multi-modality reporter named as MTF to monitor the activation of caspases in living animals with melanoma cells by different imaging techniques. This reporter contains red fluorescent protein (mRFP1), firefly luciferase (FL), and HSV1-sr39 truncated thymidine kinase (tTK), which are connected by DEVD (Fig. 7a) [88]. To normalize the decline in cell viability during imaging, they established a dual sensor cell line, *B16F10-mtf-hrl* cell line, stably expressing humanized *Renilla* luciferase (RL) and fusion protein (MTF). TK and FL signals reflected caspase activation, while RL signal reflected the cell viability. *B16F10-mtf-hrl* cells were implanted into the shoulder of nude mice. When the tumor reached 4–6 mm in diameter, the changes of RL signal, TK signal and FL signal were imaged *in vivo* before and 24 h after injection of staurosporine (Fig. 7b). After 24 h of treatment with staurosporine, the RL signal decreased significantly while the FL and TK signals increased 1.2-fold and 1.4-fold, respectively (Fig. 7c). After normalization using RL signal, the FL signal and TK signal increased by 2.4 times and 2.2 times, respectively (Fig. 7d). Therefore, this normalization method does describe the increase in signal more accurately. Also, the application of MRI to multimodal imaging of apoptosis has been reported. Fan

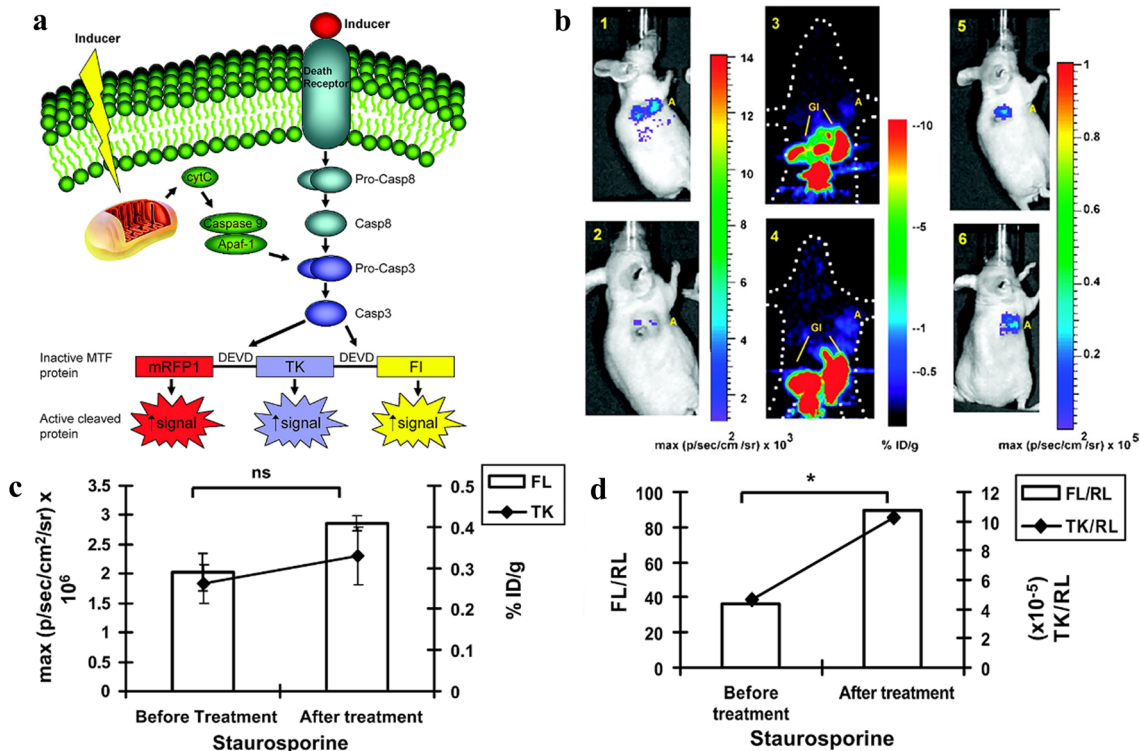


Fig. 7 Schematic diagram of activation of multimodal fusion proteins and imaging of fusion proteins in living animals. **a** Caspase-3 activated by the endogenous and exogenous pathways cleaves MTF fusion protein into three reporter proteins. The activity of each reporter protein was significantly increased. **b** Bioluminescence and microPET imaging of *B16F10-mtf-hrl* tumor-implanted living mouse before staurosporin injection and 24 h after staurosporin injection, respectively. (1) RI activity after mouse were injected with coelenterazine (50 µg/mouse). (5) FI activity of the mouse injected with coelenterazine 30 min. (3) The same mouse was then injected with ~200 µCi of [¹⁸F] FHBG and scanned for 10 min in a micro-

PET after 1 h. RL (2), FL (6), and TK (4) imaging of tumor-bearing mouse 24 h after intratumoral injection of staurosporine (50 µmol/L). **c** Without RL signal normalization, FL (photons/s/cm²/sr) signal increased 1.4 times and TK (%ID/g) signal increased 1.2 times before and after staurosporine injection in *B16F10-mtf-hrl* tumors of nude mice (n=4). **d** With RL signal normalization, FL/RL and TK/RL signals show a significant increase after injection of staurosporine in *B16F10-mtf-hrl* tumors of nude mice (n=4). The FL signal activity increases by 2.4 times and the TK signal activity increases by 2.2 times (*, P<0.05) (Reprinted with permission from [88]. Copyright 2019 American Chemical Society.)

Zhang et al. employed circular luciferase to detect apoptosis without significant changes in tumor morphology under the action of doxil, and diffusion-weighted MRI showed the morphological changes of tumor cells at the late stage of apoptosis. At the same time, PET assisted monitoring of tumor metabolism and proliferation. This approach presents apoptotic kinetics from multiple perspectives [89].

Conclusion

Apoptosis is an extremely complicated physiological process, which is closely related to the occurrence of many diseases. The early diagnosis of cancer associated with apoptosis and the evaluation of the effect of drug treatment are necessary for the control of related diseases. Traditional detection methods will cause more or less damage to the body, which promotes the rapid growth of noninvasive

imaging in vivo. This article reviews the reporters of several endogenous fluorescent or bioluminescent reporter genes fused to proteins or peptides associated with apoptosis markers. In addition to imaging apoptosis using optical imaging, the reporter gene also plays an important role in other physiological processes and gene therapy through other imaging methods such as magnetic resonance or nuclear medical imaging [20, 90].

Among the two pathways of apoptosis, the reporter gene is mainly designed by detecting the caspase family, pro-apoptotic protein and PS. Furthermore, other key proteins in the apoptotic process can also be used as detection targets to design reporter genes to increase the diversity of reporters. The researches of apoptotic reporter in mammals provide many valuable preclinical data for clinical application, but the following three aspects need to be conquer to translate into clinical application. First of all, the depth of detection of apoptotic reporter genes. Although optical imaging has

high resolution, it often has limited penetrating power to tissues due to light scattering when imaging *in vivo*. The combination of multiple imaging modalities can overcome the shortcomings of optical imaging. For example, photoacoustic imaging has the dual characteristics of high resolution of optics and tissue penetration of ultrasound [91], which is conducive to improving the detection depth of apoptosis reporter genes. Recently, apoptotic probes based on photoacoustic imaging have been reported [92, 93]. It is an inevitable trend that more multi-modal reporter genes are being developed. Second, the efficiency of vector transfection and targeting. The vector needs a certain degree of targeting to accurately reach the site to be detected. In addition, the success rate of transfection is the key to whether the apoptosis reporter gene can reach the required amount for imaging. Therefore, it is necessary to develop a more efficient, low-toxic or even non-toxic vector system that can deliver reporter genes to specific organs or tissues. Finally, the instrument should be continuously improved to meet the needs of the apoptotic reporter gene. With the successive development of multimodal reporters, the corresponding imaging equipment has a lot of room for improvement. The development of PET/MRI simultaneously offer information on morphology and function, improving the contrast of imaging in soft tissue. Imaging instruments that combine optical imaging with nuclear imaging or MRI are also being developed. The multiple advantages of these multimodal instruments provide a more convenient and fast means for imaging of apoptotic reporter genes.

Despite the extensive application of the aforementioned techniques for optical imaging, the reporter probes for apoptosis imaging are still in their infancy and have a long way to go. The reporter gene has a broad prospect for imaging apoptosis and the utilization of these reporter genes *in vivo* will definitely lead to progress in drug development and clinical gene therapy in the future, as well as in early detection, diagnosis and treatment tailored for individual patients.

Acknowledgements This work was supported by National Natural Science Foundation of China (No. 81772010) and The National Key Research and Development Program of China (973 Program) (Grant No. 2017YFA0205202).

Compliance with ethical standards

Conflict of interest No potential conflicts of interest were disclosed.

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