A GUIDE TO DRUG DISCOVERY

Cell death assays for drug discovery

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Abstract | Cell death has an important role in many human diseases, and strategies aimed at modulating the associated pathways have been successfully applied to treat various disorders. Indeed, several clinically promising cytotoxic and cytoprotective agents with potential applications in cancer, ischaemic and neurodegenerative diseases have recently been identified by high-throughput screening (HTS), based on appropriate cell death assays. Given that different cell death modalities may be dysregulated in different diseases, it is becoming increasingly clear that such assays need to not only quantify the extent of cell death, but they must also be able to distinguish between the various pathways. Here, we systematically describe approaches to accurately quantify distinct cell death pathways, discuss their advantages and pitfalls, and focus on those techniques that are amenable to HTS.

Dysregulated cell death is a common feature of many human diseases, including cancer, stroke and neurodegeneration, and modulation of this cellular response has proved to be an effective therapeutic strategy. For example, many cytotoxic agents are potent anticancer therapeutics[1], whereas cytoprotective compounds may be used to avoid unwanted cell death in the context of stroke, myocardial infarction or neurodegenerative disorders[2].

The complex mechanisms and pathways that control cell death are increasingly becoming understood and it is now clear that different cell death subroutines have a critical role in multiple diseases. Unwarranted apoptotic and necrotic death of postmitotic cells contributes to the aetiology of many acute and chronic diseases, including ischaemic, toxic, neurodegenerative and infectious syndromes[3,4]. Conversely, impaired apoptosis is frequently associated with hyperproliferative conditions such as autoimmune diseases and cancer. Defective autophagy has been associated with developmental disorders and muscular dystrophy[5,6]. In many instances, the modality by which cells die is critical for the outcome of cell death at the organismal level. Necrosis has long been recognized as a pro-inflammatory process, whereas apoptosis was considered as a non-immunogenic (if not tolerogenic) cell death subroutine. Recently, immunogenic instances of apoptosis have been unveiled[7], and it has clearly been shown that immunogenic cell death plays an important part in the response to anticancer therapy *in vivo*[8].

Cell-based tests that measure cell death-related phenomena are broadly used for drug development. In addition to quantifying cell death, it is critical that such tests accurately determine how potential drugs may be modulating cell death. That is, they should identify the cell death subroutine that is implicated, as well as allow the deconvolution of the biochemical cascades and the identification of the molecular targets that are being inhibited or enhanced by the investigated compounds. Thus, drug discovery assays must first address two critical questions: do the cells die and, if so, through which lethal pathway do they die? Following this, target deconvolution and identification must be undertaken to develop novel drugs from promising candidates.

Novel cell death subroutine-specific inducers or inhibitors may provide great advantages over nonspecific cytotoxic or cytoprotective agents for at least two reasons. First, highly selective agents (that is, agents with one target or a few targets) are less prone to provoke side effects than compounds that target whole classes of proteins. Second, there are several instances in which the activation or inhibition of one specific cell death modality over others might be clinically desirable. One example is the induction of necrosis in apoptosis-resistant cancer cells[9].

During the past decade, owing to major technological advances in the field of combinatorial chemistry in addition to the sequencing of an ever-increasing number of genomes, high-content chemical and genetic libraries have become available, raising the need for high-throughput

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Apoptotic bodies
Membrane-surrounded vesicles that are shed from dying cells during the late stages of apoptosis, and that may include portions of the nucleus and/or seemingly normal organelles.

Autophagic cell death
For a long time, this term has erroneously been used to indicate cell death that manifests with autophagic vacuolization; thus implying that cell death is mediated by rather than accompanied by autophagy.

Caspases
Cysteine proteases that cleave their substrate after an aspartic acid residue. Caspases have a critical role in both the initiation (caspase 2, caspase 8, caspase 9 and caspase10) and execution (caspase 3, caspase 6 and caspase 7) of apoptosis.

Necroptosis
A regulated form of necrosis that requires the catalytic activity of receptor-interacting protein kinase 1 (RIPK1) and RIPK3.

Anoikis
A particular form of apoptosis that is triggered by the detachment of cells from the extracellular matrix.

screening (HTS) approaches (BOX 1). In response to this demand, multiple conventional cell death detection methods have been adapted to HTS and many novel HTS-amenable techniques have been developed[10].

Several articles have recently been published with general guidelines for the use and interpretation of tests that measure specific cell death modalities[10–12] (TABLE 1). Here, we provide a systematic overview of the methodologies that can accurately quantify cell death and precisely identify lethal biochemical cascades from a drug discovery-oriented perspective. We review the design and use of biosensors of apoptosis, autophagy and necrosis, as well as the implementation of cell death assays on HTS platforms.

Mechanisms of cellular suicide

The existence of different cell death modalities was first suspected in the 1960s, when John Kerr reported that "after ischemia, some (few) hepatocytes looked as small masses of cytoplasm containing condensed nuclear chromatin"[13], a phenotype that he dubbed "shrinkage necrosis"[13]. A few years later, this expression was abandoned in favour of the Greek neologism 'apoptosis' (meaning 'falling off', like leaves from a tree), indicating a cell death subroutine that is morphologically distinct from 'necrosis', which, at that time, was considered to be a purely accidental route to cellular demise[14].

Apoptosis usually manifests with pseudopodia retraction, detachment from the substrate, shrinkage (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis) and shedding of apoptotic bodies, which are cleared by neighbouring phagocytes *in vivo*[3]. By contrast, cells undergoing necrosis display none of these morphological features nor cytoplasmic vacuolization (which was considered as a prelude to autophagic cell death). However, necrotic cells do exhibit common traits, including an increasingly translucent cytoplasm, swelling of cytoplasmic organelles, condensation of chromatin into circumscribed and irregular patches, and an increase in cell volume (oncosis) that culminates in mechanical rupture of the plasma membrane (FIG. 1). Unlike their apoptotic counterparts, necrotic cells do not fragment into discrete bodies, nor do their nuclei, which have indeed been reported to accumulate in necrotic tissues[4].

Since the introduction of the apoptosis–necrosis dichotomy, the field of cell death research has witnessed a tremendous expansion, with two major consequences: first, the progressive characterization of the biochemical mechanisms underlying apoptosis and, to a lesser extent, necrosis; and second, the description of several putative novel cell death mechanisms[3].

Prominent biochemical events that have been linked to apoptotic cell death include activation of caspases, mitochondrial membrane permeabilization (MMP) (accompanied by the release of proteins that are normally sequestered within the mitochondrial transmembrane space), internucleosomal DNA fragmentation and the surface exposure of the phospholipid phosphatidylserine[3]. Although necrotic cell death has been regarded as a purely accidental event, the recent discovery of multiple instances of regulated necrosis, such as tumour necrosis factor receptor-elicited necroptosis[2], has initiated the exploration of necrosis-related biochemical processes. These include the activation of receptor-interacting protein kinase 1 (RIPK1) and RIPK3, a metabolic burst associated with overproduction of reactive oxygen species (ROS), as well as MMP and lysosomal membrane permeabilization (LMP)[4].

During the past two decades, dozens of neologisms have been introduced to define new putative cell death modalities, but these expressions should be used cautiously, as only a few of them truly reflect the discovery of cell death modalities that are biochemically and morphologically distinct from apoptosis and necrosis. Thus, although terms including mitotic catastrophe[15], anoikis[16], entosis[17], pyroptosis[18], parthanatos[19] or autoschizis[20] are being used by the scientific community, most often they refer to cell death modes that are executed (at least in part) by the molecular machinery of apoptosis or necrosis and that, as a consequence, manifest some of the corresponding biochemical and morphological traits[3].

The role of autophagy in the execution of cell death remains controversial. Although in 1973 Schweichel and Merker[21] identified autophagy as a *bona fide* executioner of cell death, recent results suggest that this is only true in a limited number of pathophysiological settings[22]. Thus, with some exceptions, such as the involution of salivary glands during the development of the fruitfly *Drosophila melanogaster*[23], autophagy constitutes an epiphenomenon of cell death that can be interpreted as an ultimate but unsuccessful attempt by cells to cope with stress and re-establish homeostasis. The notion that autophagy usually is a cytoprotective mechanism is corroborated by the fact that its pharmacological or genetic inhibition accelerates rather than prevents cell death in most cases[22].

Box 1 | Cell death-related high-throughput screening in drug discovery

The basic requirement for a primary high-throughput screening (HTS) application is its ability to test a robust parameter in a reproducible and preferably inexpensive fashion, under a very high number of conditions. Several factors are critical to the success of the primary screen. These include: in-depth knowledge of the underlying biology, a well-defined and simple question as well as numerous pilot experiments (to select and optimize the cell model, inducers, durations and read-outs). Robust statistical approaches are also necessary to avoid hit under- or overestimation. Once a number of primary hits (possibly predefined) are identified, secondary screens should be performed to further characterize and select potential drug candidates[127]. Finally, such secondary hits must be validated in hypothesis-driven, low-throughput experiments. This final phase of target deconvolution and identification, which relies mostly on chemical inhibitors and RNA interference-based approaches, is critical for the development of lead compounds with a confirmed mechanism of action.

In the context of cell death research, the design of HTS approaches must consider the highly dynamic and multifaceted nature of cell death. Thus, whenever feasible, multiple markers should be monitored at the primary screening stage, as this may allow researchers to answer many questions at the same time. Indeed, the most successful HTS approaches to date were designed to address multiple questions. For instance, live cell screening by fluorescence microscopy or impedance-based tests could provide an elegant way to enhance the temporal resolution in primary and secondary screens[34]. However, in many cases, technical or biological constraints limit an excessively complex and multiparametric HTS approach.

Cell death assays

Cells should be considered as dead when they fulfil at least one of the following criteria: the plasma membrane has lost its integrity, the cell has fragmented into apoptotic bodies or the corpse or its fragments have been taken up by neighbouring cells[3]. Additionally, as dying cells cease all functions, a decrease in metabolism can be observed at the level of the cell population. Thus, cell death assays fall into two major groups: assays that measure *bona fide* cell death and tests that quantify biochemical processes that are viewed as surrogate viability markers[3]. Dozens — if not hundreds — of techniques are currently available to assess cell death, either directly or indirectly through surrogate markers. The detailed description of these methods, their advantages and their drawbacks have recently been reviewed in two comprehensive sets of guidelines[11,12].

Vital dyes. The most common means of assessing the death of cultured cells is provided by vital dyes, which are fluorescent or coloured molecules that discriminate between living and dead cells. The vital dyes most frequently used in cytofluorometry (owing to its intrinsic statistical power) include so-called exclusion dyes, which cannot cross intact plasma membranes (for example, propidium iodide or 4′,6-diamidino-2-phenylindole (DAPI)) and hence only label dead cells. By contrast, fluorogenic esterase substrates (for example, calcein acetoxymethylester (calcein-AM)) can be used to selectively label living cells. This is possible because these lipophilic, non-fluorescent compounds, which readily penetrate into cells, are hydrolysed by intracellular esterases to generate fluorescent and plasma membrane-impermeant products, which are then retained exclusively by living cells[24]. However, although exclusion dyes provide robust, artefact-free information, the enzymatic activity of intracellular esterases may be affected by cell death-unrelated phenomena.

Intracellular proteins. Plasma membrane rupture can also be biochemically quantified by measuring the spillage of intracellular proteins (most often enzymes) into cell culture supernatants. For example, kits for the fluorometric or colorimetric detection of glucose-6-phosphate dehydrogenase (G6PD) and lactate dehydrogenase (LDH) are commercially available. However, a major drawback of these techniques is that the activity of these enzymes may be affected by physicochemical parameters (for example, changes in the pH of the culture medium or side effects of pharmacological inhibitors) and enzyme activity may decay with time in the extracellular milieu[10]. Nevertheless, the quantification of G6PD or LDH release is suitable for HTS protocols, as it is relatively inexpensive and can easily be implemented in 96- or 384-well plates.

Cellular metabolism. The most widely used surrogate biochemical marker for viability is ATP, based on the assumptions that living cells produce ATP and it is indispensable for cellular life. Luciferase-based assays allow for the sensitive quantification of intracellular ATP and are amenable to HTS studies. However, decreased intracellular ATP concentrations may result from non-lethal perturbations, including cessation of proliferation (for example, owing to senescence, starvation or contact inhibition) and inhibited mitochondrial respiration. Thus, measuring ATP does not always directly correlate with cell viability[10].

Several other assays that measure specific facets of cellular metabolism have been extensively used to monitor viability, including the well-known 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. MTT and other tetrazolium derivatives are colourless salts that are readily taken up by living cells and converted by mitochondrial reductases into coloured compounds that can be easily quantified by measuring their absorbance using a spectrophotometer[25]. Unfortunately, similarly to the ATP-based assays, a number of other factors may inhibit mitochondrial reductases, thus implying that the conversion of tetrazolium derivatives *per se* cannot provide unequivocal information on cell viability.

ATP-based and MTT-based assays are highly susceptible to metabolic interference; consequently, they may generate false positive results. Thus, although metabolism-oriented tests are very useful to obtain preliminary information, their results must be validated in secondary screens based on *bona fide* cell death markers. Irrespective of these issues, cell metabolism has recently been used to successfully screen chemical and genetic libraries (see Supplementary information S1 (table)). In 2008, we performed the first genome-wide study of regulated necrosis based on ATP levels, which led to the identification of more than 400 *bona fide* regulators of necroptosis[26]. Arora and colleagues[27] monitored ATP levels in Ewing's sarcoma cells to identify kinase-specific small interfering RNAs (siRNAs) that kill tumour cells in a limited, yet successful screening effort. Similarly, MTT conversion has been exploited to screen 5,600 compounds from several commercially available libraries, leading to the identification of camptothecin as an agent that is highly active against alveolar rhabdomyosarcoma[28].

Cell attachment. The degree of attachment of cells to the culture substrate has also been proposed as an *in vitro* marker of viability. Most non-haematopoietic cells firmly adhere to an adequate culture substrate, whereas injured cells progressively round up and eventually detach, which is coincident with cell death. On the basis of these observations, multiwell plates with microelectrodes that measure the impedance of the surface occupied by cells have been proposed as an indirect indicator of viability[29–31]. There are two main advantages to this technique. First, it allows for non-invasive, label-free determinations[32], thereby avoiding probe-related interferences (which are common and often disregarded)[33]. Second, it permits real-time monitoring and hence allows resolution (at least to some extent; that is, until control cells reach full confluence) of the temporal dimension in the medium-throughout applications or in follow-up assays[34]. However, it should be kept in mind that impedance might be influenced by factors that are not related to cell death; for example, changes in cellular morphology and changes in the polarization of the plasma membrane.

Entosis
A recently discovered and debated cell death mode in which one cell engulfs one of its live neighbours, which may then die within the phagosome.

Pyroptosis
A pyrogenic cell death modality that is accompanied by the activation of caspase 1 and the secretion of the pro-inflammatory cytokine interleukin-1β. Pyroptosis can manifest with features of apoptosis or necrosis.

Parthanatos
Caspase-independent cell death induced by poly(ADP-ribose) polymerase 1 and mediated by apoptosis-inducing factor.

Autoschizis
A cell death modality that is initiated in certain cancer cells by combinatorial treatment with vitamins C and K3, and leads to the self-excision of cytoplasmic portions.

Table 1 | **Comparison of methods used to measure cellular viability**

Method	Technological platform	Main advantages	Main disadvantages	Recommended for HTS*
ATP consumption	Luminometer	Rapid and highly sensitive	• Highly sensitive to metabolism-related ATP fluctuations • Cannot identify cell death modes	1
Autophagic flux	Luminometer	Rapid	• Expensive	2
	Fluorescence microscopy	Allows real-time monitoring	• Different inducers might influence the pH of the lysosomes	2
	Immunoblotting	Assessed by routine methods	• Time-consuming	N/A
Autophagosome quantification	Electron microscopy	Provides precise ultrastructural information	• Expensive and time-consuming	N/A
	Fluorescence microscopy	Rapid and inexpensive	• Cannot distinguish between increased autophagy and decreased degradation	1
Caspase activation (fluorescent substrates or FRET constructs)	Cytofluorometry	Allows automated analyses on a per-cell basis	• Caspase activation may occur in cell death-unrelated settings • Fluorescent substrates may emit upon unspecific degradation	2
	Fluorescence reader	Rapid		1
	Fluorescence microscopy	Can be coupled with other cell-death markers		1
Cell detachment	Impedance reader	Allows real-time monitoring	• Unable to discriminate between different cell death modes	2
$\Delta\psi_m$ dissipation	Cytofluorometry	Rapid, inexpensive and no need for permeabilization	• Temporary $\Delta\psi_m$ dissipation may occur in cell death-unrelated settings • Fixable probes are suitable for end-point determinations only	2
	Fluorescence microscopy	Non-fixable probes allow for real-time $\Delta\psi_m$ monitoring		1
Extracellular release of proteins	Absorbance reader	Inexpensive	• Unable to discriminate between different cell death modes	1
	Fluorescence microscopy	Allows the study of late events and is applicable to real-time monitoring	• Detection might be aggravated by morphological changes of dying cells	1
Immunological methods	Cytofluorometry	Allows automated analyses on a per-cell basis	• Rely heavily on the performance of primary antibodies • Poorly expressed antigens may be under-detected or undetected • Antigens may get denatured during sample processing	2
	Electron microscopy	Irreplaceable for precise colocalization studies		N/A
	Fluorescence microscopy	Compatible with sample fixation and storage		1
	Immunoblotting	Allows the study of early biochemical parameters		N/A
	Light microscopy	Allows the detection of early cell death-related events		N/A
IMS protein release	Fluorescence microscopy	Release of fusion proteins can be followed in real-time	• Release of more than one IMS protein must be assessed to avoid false positive results	N/A
	Immunoblotting	Subcellular fractionation allows the detection of multiple proteins		1
LC3 lipidation	Immunoblotting	Assessed by routine methods	• Expensive and time-consuming	N/A
Mitochondrial swelling	Absorbance reader	Allows the study of mitochondria in the absence of metabolic interference	• Laborious and time-consuming	N/A
Morphological determinations	Electron microscopy	Provides precise ultrastructural information	• Expensive	N/A
	Fluorescence microscopy	Inexpensive and fixable dyes available	• Morphological alterations can be caused by cell death-independent mechanisms	N/A
	Light microscopy	Rapid and inexpensive, can be used for monitoring cell cultures	• Prone to underestimation, unsuitable for quantitative studies	1
PS exposure	Cytofluorometry	Rapid, specific for an early event of apoptosis execution	• PS exposure can occur independently of apoptosis	2

Table 1 (cont.) | **Comparison of methods used to measure cellular viability**

Method	Technological platform	Main advantages	Main disadvantages	Recommended for HTS*
ROS overgeneration	Cytofluorometry	Inexpensive	• ROS generation can occur independently of cell death	N/A
	Fluorescence microscopy			N/A
Tetrazolium salt conversion	Absorbance reader	Rapid and inexpensive	• Cannot discriminate between cytotoxic and antiproliferative effects	1
Translocation of cell death mediators	Fluorescence microscopy	Applicable for live cell monitoring	• Detection might be complicated by morphological changes of dying cells	1
Detection of DNA-strand breaks	Cytofluorometry	Useful in co-staining protocols; compatible with long term sample storage	• TUNEL positivity can derive from sample processing • Expensive	N/A
	Fluorescence microscopy			N/A
	Light microscopy	Seen as the gold standard for detecting cell death *in situ* (in tissues slides) by IHC		N/A
Vital dyes	Cytofluorometry	Routinely employed in co-staining protocols	• Unable to discriminate between different cell death modes	1
	Fluorescence microscopy	Facilitates the identification of dead cells by visual inspection		1

FRET, Förster resonance energy transfer, IHC, immunohistochemistry; IMS, intermembrane space; LC3, microtubule-associated protein 1 light chain 3 (also known as MAP1LC3); $\Delta\psi_m$, mitochondrial transmembrane potential; PS, phosphatidylserine; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling. These methods are further discussed in REFS 10, 11.* 1, suitable for primary screening; 2, suitable for secondary screening and validation; N/A, not applicable. Examples of recent successful high-throughput cell-based assays for the detection of cell death and/or cell survival-related variables can be seen in Supplementary information S1 (table).

Fluorescence microscopy. With the advent of robotized high-throughput microscopes and automated image analysis tools, fluorescence microscopy can now be exploited for HTS[10]. This has allowed the use of nuclear numbers as a read-out for cell viability in image-based HTS. These nuclear numbers can be observed from fluorescent chromatin dyes, such as Hoechst 33342 or DAPI, or from cell lines stably expressing nuclear markers, such as histone 2B fused to red fluorescent protein (H2B–RFP). Although this technique presents the same problems as other cell viability assays in that it does not distinguish between cell death and proliferation arrest, it does provide additional information — for example, on nuclear morphology (see below) — to help make this distinction. In addition, other fluorescent probes may be used to simultaneously acquire data on cell morphology or other parameters, such as the activation of specific signalling pathways[35].

Time-lapse fluorescence microscopy can also be used to document cell fate, including cell death. Recently, the H2B–GFP (green fluorescent protein) biosensor has been used to screen a genome-wide siRNA library[36]. Following two-day live imaging, cell phenotypes were quantitatively assessed by computational image processing, which enabled the identification of hundreds of human genes involved in biological functions, including cell division, migration and survival. The success of this study and similar systems biology studies underscores the importance of high-content, HTS-amenable fluorescent markers in the discovery of novel small molecules and candidate targets for drug development.

Model organisms. Organisms other than immortalized mammalian cells can be used for the identification of cell death modulators. Yeast are simple and genetically tractable organisms that share most autophagy-relevant proteins[11] and some (but probably not all) constituents of the apoptotic and necrotic cell death machineries with mammalian cells[37]. Dying yeast cells exhibit a number of biochemical processes that are reminiscent of mammalian apoptosis (such as MMP) and necrosis (such as nuclear release of high mobility group protein B1 (HMGB1), see below).

Thus, yeast may be used to determine the cytotoxic profile of compounds and to discriminate between specific cell death subroutines. The viability of yeast cells can be determined by means of exclusion dyes or, preferentially, by clonogenic assays[38]. Other non-mammalian model organisms — including the fruitfly *D. melanogaster* and zebrafish (*Danio rerio*) — have been successfully used in cell death-oriented screens[39–41]. However, the use of these models is limited to institutions with suitable animal facilities.

Apoptosis assays

Although a number of specific assays have now been developed, apoptosis cannot be accurately determined by monitoring only one single parameter (BOX 2). This is because certain events that have long been associated with apoptosis have recently been shown to also occur during non-apoptotic cell death[4,42]. Moreover, several apoptotic processes manifest in cell death-unrelated settings and mediate non-lethal, physiological effects[43]. Consequently, as some commonly used apoptotic markers are shared with other cell death modes (although they manifest with different kinetics), it is challenging to accurately monitor apoptosis based on single end-point determinations. Thus, real-time approaches or the analysis of multiple time-points should always be

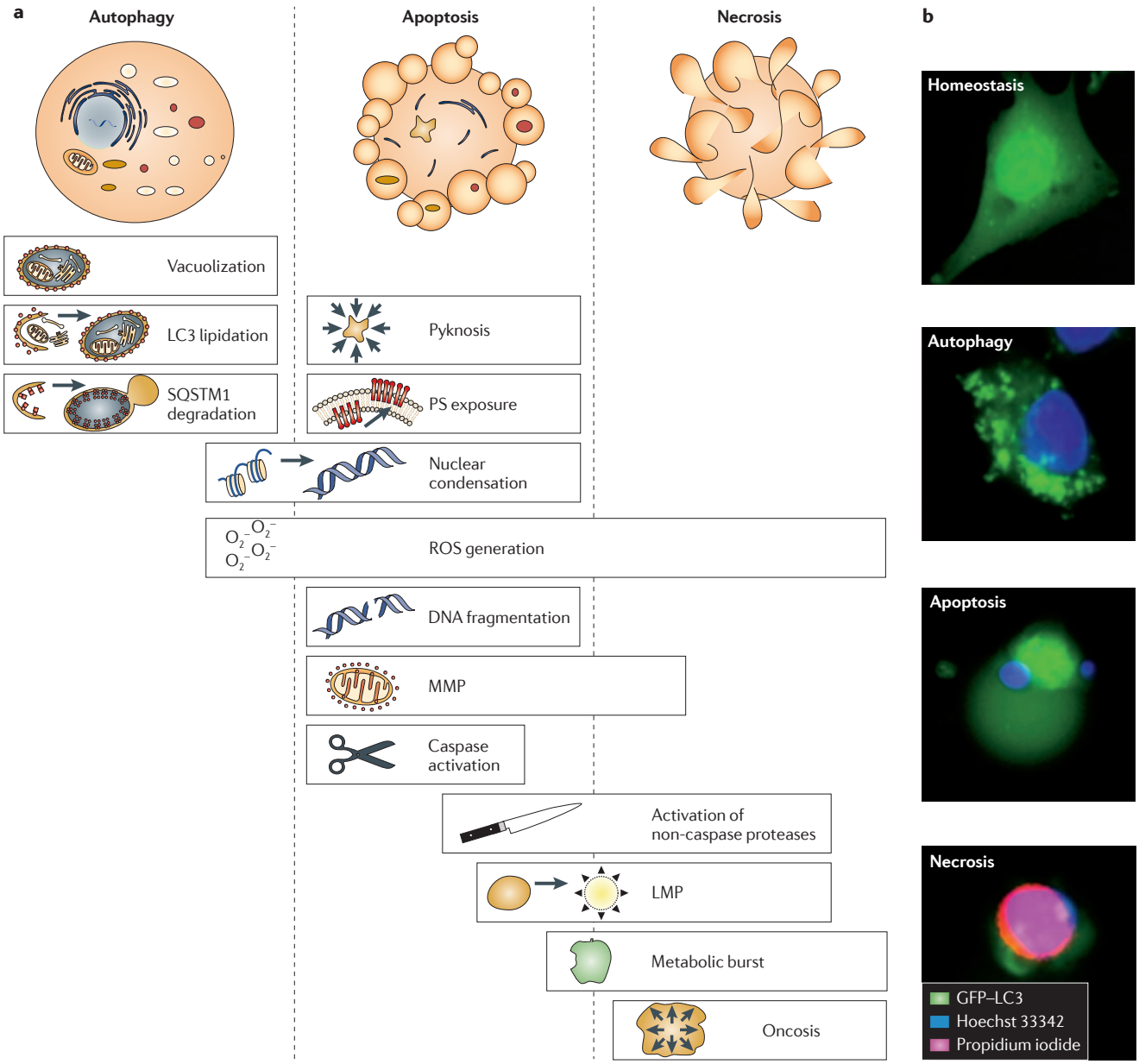


Figure 1 | **Molecular characteristics of autophagy, apoptosis and necrosis. a |** Macroautophagy, which is phenotypically detectable by massive vacuolization, manifests with the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3; also known as LC3) as it incorporates into the nascent membrane of the autophagosome. Lysosomal fusion initiates the breakdown of the autophagosomal content, including LC3-bound sequestosome 1 (SQSTM1). Apoptosis typically shows signs of mitochondrial membrane permeabilization (MMP), followed by a self-amplifying caspase cascade. In specific cases of apoptosis, non-caspase proteases can be activated alternatively or the caspase cascade is activated in response to cell death receptor signalling without the primary involvement of mitochondria. The cleavage of several substrates by apoptotic proteases and the activation of nucleases leads to the characteristic phenotype of apoptotic cells, including nuclear condensation and pyknosis followed by DNA fragmentation. In many cases, reactive oxygen species (ROS) are generated and lysosomal membrane permeabilization (LMP) occurs. The exposure of phosphatidylserine (PS) on the membrane surface serves as an uptake signal and facilitates removal of apoptotic corpses by macrophages. Necrosis typically manifests with oncosis and shares some features with apoptosis, such as MMP, LMP, activation of non-caspase proteases and ROS generation. **b** | Simultaneous detection of multiple cell death-related parameters: human osteosarcoma U2OS cells stably expressing a green fluorescent protein (GFP)–LC3 chimaera were kept in control conditions (homeostasis) or treated for 12 hours with 1 μM of rapamycin (an autophagic stimulus) or 1 μM of staurosporine, either alone (triggering apoptosis) or together with 20 μM of Z-VAD-fmk (leading to necrosis). Cells were then co-stained with the nuclear dye Hoechst 33342 and the exclusion dye propidium iodide. Autophagic, apoptotic and necrotic cells manifest GFP–LC3 aggregation, nuclear condensation and propidium iodide uptake, respectively.

Box 2 | **Common problems of cell death assays**

Single morphological or biochemical features have long been used to monitor specific cell death modalities, based on oversimplified ideas. Some of these include the idea that 'caspase activation is equal to apoptosis' and that 'autophagic vacuolization is equal to autophagic cell death'. However, most phenomena that are associated with cell demise can also occur in cell death-unrelated scenarios[10]. As well as caspase activation — which reportedly mediates several physiological processes, including the maturation of haematopoietic cell precursors — these phenomena also include the dissipation of the mitochondrial transmembrane potential, which can occur in a rapid and reversible fashion under selected circumstances; the limited release of mitochondrial intermembrane space proteins; the exposure of phosphatidylserine on the outer leaflet of the plasma membrane; the transient increase in intracellular levels of reactive oxygen species; and the accumulation of autophagic vacuoles, which, in most cases, constitutes a cytoprotective response to stress[10].

Another common problem with cell death measurements derives from the diversity of cellular systems to which these techniques are applied. In particular, cancer cell lines, which are often relatively resistant to cell death induction, are highly heterogeneous, owing to the genetic and epigenetic alterations affecting cell death-regulatory molecules. This diversity implies that some of the biochemical processes that are routinely monitored to quantify cell death may not occur, or they may occur with very different kinetics in various experimental settings. For instance, autophagy-deficient cells fail to expose phosphatidylserine during apoptosis[53] and mouse embryonic fibroblasts lacking the pro-apoptotic BCL-2 family members BAX and BAK1 die in response to chemotherapy without manifesting the morphological and biochemical signs of apoptosis[128].

To overcome these (and other) sources of bias, multiple cell death-relevant parameters must be measured for the precise quantification of the extent of death and its precise subroutine[10].

preferred for precisely discriminating between apoptosis and non-apoptotic cell death[10].

Apoptosis assays can, for the most part, be subdivided into two categories: assays that detect events occurring in most (if not all) instances of apoptotic cell death and assays that measure pathway-specific processes, the occurrence of which depends on the apoptosis-initiating stimulus and the precise cellular context of lethal signalling.

Universal assays. Techniques that are suitable for the detection of apoptosis in most instances include morphological methods that quantify pyknosis, nuclear condensation and the generation of apoptotic bodies, as well as assays that detect DNA fragmentation, such as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)[10]. These parameters are most readily assessed visually, and following the recent advances in high-throughput microscopy and image analysis, they can now be used in HTS[10]. Although the TUNEL technique has several drawbacks[10] and should never be used as a stand-alone method for apoptosis detection, it is flexible with regard to its read-out methodology (fluorescence or light microscopy; or fluorescence-activated cell sorting (FACS)) and it can be combined with other staining methods to visualize cell morphology or detect activation of cell death signalling pathways.

Caspase activity. Caspase activation was regarded as a universal marker of apoptosis before the discovery of instances of caspase-independent cell death that manifest with apoptotic morphology[3]. Moreover, it has recently been found that initiator and executioner caspases — such as caspase 8 and caspase 3, respectively — exert non-lethal functions as intracellular signalling molecules (for example, in inflammation or in the differentiation of haematopoietic precursors)[44]. Nevertheless, assessment of caspase activity remains an important part of apoptosis detection.

Caspase activation is often quantified by immunological methods using antibodies against active (cleaved) forms of these enzymes or against their cleaved substrates. Alternatively, caspase activation can be assessed by other reagents that are compatible with HTS applications, such as exogenous pro-luminescent or fluorogenic caspase substrates[10]. Some of these substrates are bifunctional compounds that allow the real-time assessment of caspase activity and changes in nuclear morphology[45]. In addition, fluorescent biosensors can be introduced into cells[10] (FIG. 2). Typically, these are Förster resonance energy transfer (FRET) fluorescent protein partners that are linked by a short stretch of amino acids containing a caspase cleavage site. Upon proteolytic cleavage, the FRET efficiency is decreased, thus quenching the emission of the FRET acceptor[46].

Alternatively, caspase activation can be monitored by bimolecular fluorescence complementation. In this case, non-fluorescent fragments of the fluorescent protein Venus are fused to one particular caspase and the activation-associated oligomerization yields a fluorescent signal[47]. This technique is intrinsically unsuitable for measuring the activity of caspases that do not oligomerize (for example, caspase 3). It has not yet been shown if this technology is sufficiently robust for HTS.

Irrespective of the detection method used, it is important to remember that caspase activation during apoptosis is transient, and the timing of the assay needs to be carefully optimized before HTS application[48].

The caspase-mediated cleavage of several intracellular proteins accounts for characteristic apoptosis-associated cellular changes. Thus, the cleavage and consequent activation of RHO-associated, coiled-coil containing protein kinase 1 (ROCK1) leads to plasma membrane 'blebbing'[49]. In addition, cleavage of pannexin 1 increases pannexin 1 channel activity, in turn augmenting plasma membrane permeability and hence the influx of small exclusion dyes such as the cyanines YOPRO1 and TOPRO3, but not the influx of larger molecules such as propidium iodide[50]. Thus, changes in plasma membrane morphology and a selective and partial increase in its permeability constitute a hallmark of caspase-dependent apoptosis.

Phosphatidylserine exposure. The exposure of phosphatidylserine on the outer leaflet of the plasma membrane constitutes an early event in apoptosis. Ectophosphatidylserine can be readily detected by fluorescent or streptavidin-labelled Annexin V, allowing cytofluorometric, immunofluorescence and immunohistochemical determinations[51]. When the plasma membrane is permeabilized, Annexin V binds intracellular phosphatidylserine, implying that this staining is incompatible with sample permeabilization, and that necrotic cells, irrespective of their origin, will stain positively for Annexin V[10].

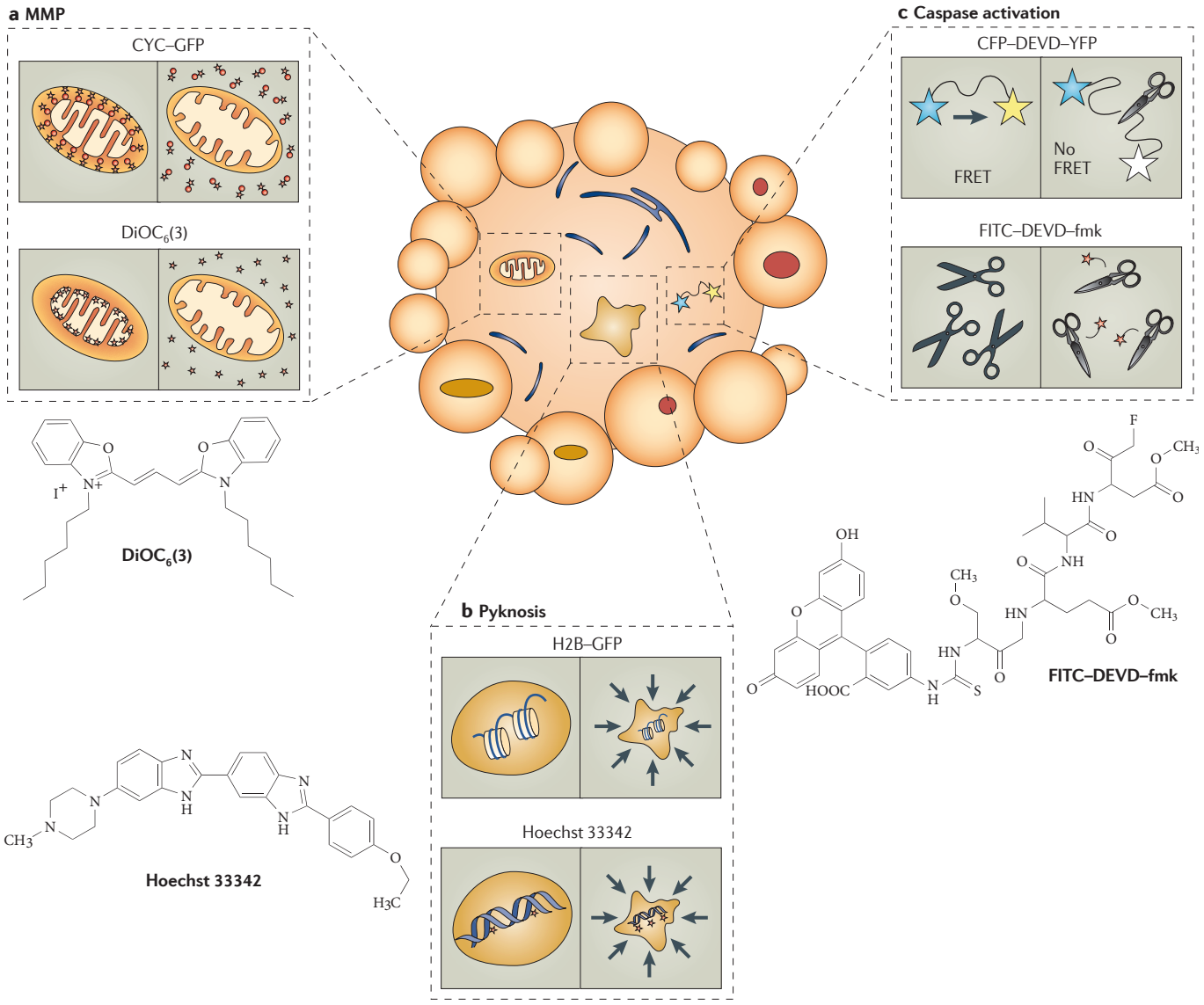


Figure 2 | **Apoptosis assays amenable for high-throughput screening. a** | Mitochondrial membrane permeabilization (MMP) is one of the principal characteristics of many instances of apoptosis. Fluorescent biosensor cell lines that are engineered to express the intermembrane space proteins cytochrome *c* (CYC) or second mitochondria-derived activator of caspase (SMAC, also known as DIABLO) fused to a fluorescent protein (for example, green fluorescent protein (GFP)) allow for real-time determinations in a high-throughput screening (HTS)-compatible format. Alternatively, cells can be stained with fluorescent membrane potential-sensitive dyes such as $DiOC_6(3)$ or tetramethylrhodamine ethyl ester (TMRE), which should be used in combination with exclusion dyes such as propidium iodide or 4′,6-diamidino-2-phenylindole (DAPI), respectively. **b** | Pyknosis, which is characteristic of apoptosis, can be visualized by monitoring histone 2B (H2B)–GFP constructs or by chromatin stains such as Hoechst 33342. **c** | The activation of caspases can be monitored with adequate microscopy hardware by measuring changes in Förster resonance energy transfer (FRET) in suitable recombinant caspase substrates (for example, cyan fluorescent protein (CFP)–DEVD–yellow fluorescent protein (YFP)). For end-point assays, membrane-permeable fluorescent caspase substrates (for example, FITC–DEVD–fmk) can be utilized.

To distinguish apoptotic cells (which are Annexin V-positive but with intact plasma membranes) from non-apoptotic cells, Annexin V staining is usually combined with cell-impermeable dyes, such as propidium iodide. Some cell types do not expose phosphatidylserine during apoptosis, and this is an aberration that has been linked to defects in the phosphatidylserine-exposing enzyme phospholipid scramblase 1 (REF. 52) or in the autophagic machinery[53]. Finally, phosphatidylserine exposure can occur in cell death-unrelated conditions, for instance when T lymphocytes are activated[54].

MMP. MMP can be initiated by pro-apoptotic members of the apoptosis regulator BCL-2 protein family on the outer mitochondrial membrane, or by the so-called mitochondrial permeability transition pore at the inner

mitochondrial membrane[55]. MMP is widely considered as the point of no return in the biochemical cascade that leads to intrinsic apoptosis and often also contributes to extrinsic apoptosis. The most prominent consequences of MMP include dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$), arrest of mitochondrial ATP synthesis, overproduction of ROS and cytosolic release of cytotoxic proteins that are normally retained within the mitochondrial intermembrane space[55].

Any of these events can be detected to assess the involvement of mitochondria in cell death. For example, $\Delta\psi_m$-sensitive probes allow for $\Delta\psi_m$ quantification by fluorescence microscopy and cytofluorometry[56], with the caveat that $\Delta\psi_m$ dissipation can also occur in a temporary and reversible fashion that does not lead to cell death[43]. Dihydroethidium (also known as hydroethidine) exemplifies a cell-permeant probe that changes its emission properties upon oxidation by ROS[57]. Alternatively, fluorescence microscopy can be used to determine ROS-mediated DNA damage by monitoring the nuclear relocalization of the DNA repairing enzyme 8-oxoguanine DNA glycosylase fused to GFP[58].

ROS production, however, not only derives from apoptotic MMP but it also can constitute an early signalling event in some instances of necrosis[55] and autophagy[59], and can contribute to the execution of necroptosis[4]. The cytoplasmic translocation of intermembrane space proteins such as cytochrome *c* (CYC) and apoptosis-inducing factor (AIF) can be monitored by immunofluorescence microscopy, which is laborious and not suitable for HTS.

Alternatively, several transgenic cell lines have been engineered to express intermembrane space proteins fused to fluorescent proteins (for example, CYC–GFP[60]), and they allow real-time determinations in an HTS-compatible format. However, the limited release of mitochondrial intermembrane space proteins has also been shown to occur in cell death-unrelated settings[61]. Mitochondrial dysfunction is often paralleled by changes in mitochondrial morphology[62], which can be assessed by the expression of mitochondrial matrix-targeted fluorescent proteins or by transmembrane-insensitive mitochondriophylic dyes[63]. Importantly, it should be kept in mind that changes in mitochondrial morphology can also derive from cell death-unrelated interferences in the mitochondrial fission and fusion machineries.

Cell-free systems. The fact that mitochondria have a major role in apoptosis regulation has been exploited in the development of cell-free assays for HTS. In these systems, isolated mitochondria are exposed to an array of cytotoxic agents and the decrease in light scattering at 540 nm is measured, which reflects mitochondrial swelling[64]. For example, a collection of 1,040 US Food and Drug Administration-approved drugs were tested on isolated rat liver mitochondria, and 28 compounds that are able to prevent mitochondrial swelling were identified[65]. These compounds may exert neuroprotective effects *in vivo*[65].

Alternatively, isolated mitochondria can be incubated in the presence of $\Delta\psi_m$-sensitive fluorochromes that can continuously monitor their $\Delta\psi_m$[55]. Moreover, the $\Delta\psi_m$ of isolated mitochondria may be quantified using a microfluidic on-chip microelectrode device[66]. Recently, Deniaud and colleagues[67] have developed a cell-free system in which purified mitochondria and endoplasmic reticulum vesicles were used to measure endoplasmic reticulum-dependent mitochondrial swelling and $\Delta\psi_m$ dissipation. Similar multiorganelle cell-free systems may allow the identification of agents that modulate the pro-apoptotic crosstalk between distinct subcellular compartments.

In the intrinsic apoptotic pathway, mitochondrial factors released from the intermembrane space lead to the activation of caspases within a multimolecular complex: the apoptosome. Such mitochondrial caspase activators include CYC, which is one of the necessary components of the minimal apoptosome that also contains deoxyATP (dATP), apoptotic protease-activating factor 1 (APAF1) and caspase 9 (REFS 68,69). Under suitable conditions (in particular when exogenous dATP is added), addition of CYC to cytosolic extracts can lead to caspase activation, which can be easily monitored by fluorogenic reactions[70]. Using this *in vitro* set-up, it is possible to identify endogenous proteins and exogenous pharmacological agents that prevent caspase activation by apoptosome inhibition[71,72]. The potential nephroprotective effects of chemical apoptosome inhibitors are now being investigated in preclinical studies[73].

Although the quantification of pathway-specific biochemical features is not recommended as a primary screening method — as these features *per se* are not an accurate measure of apoptosis — the precise identification of the molecular mechanisms that underlie the cytotoxic or cytoprotective action of lead compounds is a critical step in drug development. For this reason, hundreds of assays have been developed to monitor post-translational changes in signalling proteins that exert pro-apoptotic and anti-apoptotic functions, mostly based on modification-specific antibody staining. Recently, some of these methods have been adapted to HTS applications, including high-throughput FACS and the in-cell-western assay[74].

Other HTS-compatible techniques that detect signalling-associated changes in protein–protein interactions include bimolecular fluorescence complementation[75] and fluorescent reporter proteins or protein fragments that specifically bind to signalling intermediates such as phosphatidylinositol (an intracellular second messenger) and its derivatives[35]. Additionally, the translocation of vital and lethal factors between subcellular compartments can be measured using antibody or fluorescent reporter-based assays. For example, cells expressing the pro-apoptotic BCL-2-like protein BAX fused to GFP can be used to identify inducers or inhibitors of BAX-mediated MMP[76]. Automated fluorescence microscopy coupled to software-assisted image analysis allows the implementation of these assays in HTS-compatible formats.

Owing to their ease and low cost of implementation (see above), most HTS approaches used so far have relied on the detection of 'universal' cell death

Intrinsic apoptosis
Intracellular stress is sensed by mitochondria, which undergo mitochondrial membrane permeabilization and hence activate caspase-dependent and caspase-independent cell death executioner mechanisms.

Extrinsic apoptosis
Apoptosis can be initiated by extracellular molecules that bind to 'death' receptors (for example, FAS), in turn activating the caspase 8–caspase 3 cascade.

Box 3 | **Immunogenic cell death**

Cancer cells can succumb to immunogenic cell death when they are treated with anthracyclins or ionizing irradiation. This cell death variant is characterized by the early, pre-apoptotic plasma membrane exposure of calreticulin (CRT) and the exposure or release of other chaperone proteins, including members of the heat shock protein family[8]. The exposure of these 'eat me' signals facilitates the uptake of parts of the dying cancer cells by dendritic cells; the uptake receptor that mediates this is still elusive. At later stages, dying cancer cells release the damage-associated molecular pattern high-mobility group protein B1 (HMGB1) as well as ATP. HMGB1 and ATP are recognized by two receptors expressed on the surface of dendritic cells: toll-like receptor 4 (TLR4) and purinergic receptor 7 (P2RX7), respectively. Binding of HMGB1 to TLR4 and subsequent activation of MYD88-dependent signalling stimulates antigen processing by dendritic cells, as it inhibits the fusion of lysosomes to the antigen-containing phagophore[129]. Binding of ATP to P2RX7 purinoreceptors initiates the assembly of the inflammasome that is in turn responsible for a caspase-mediated interleukin-1β release[130]. These immunogenic events eventually enable dendritic cells to prime T cells that are specific for tumour antigens. Many of the immunogenic parameters of cell death can be screened via fluorescent biosensor cell lines[79].These include fusion proteins comprising CRT–green fluorescent protein (GFP)–endoplasmic reticulum retention sequence (KDEL) or CRT–HaloTag to assess CRT relocalization from the endoplasmic reticulum to the periphery of the cells and the plasma membrane surface, and HMGB1–GFP fusion proteins to assess HMGB1 liberation from the nucleus. ATP release is measured via the fluorescent ATP-binding probe quinacrine (which labels intracellular ATP-containing vesicles) or by luciferase-based techniques that allow the detection of extracellular or intracellular ATP.

markers. However, some apoptosis-specific indicators have also been successfully applied to HTS (see Supplementary information S1 (table)). For example, in 2008, 2-chloro-*N*-(4-methoxyphenyl)-*N*-methylquinazolin-4-amine (EP128265) was identified as a potent inhibitor of breast and prostate cancer cell proliferation using a cell-based high-throughput caspase 3 activation assay[77].

One year later, Irshad *et al.*[78] used an enzyme-linked immunosorbent assay (ELISA)-based method to screen a library of 150,000 human cDNA clones for their ability to induce internucleosomal DNA fragmentation in tumour cells. This led to the discovery that the solute carrier family 22 member 13 (SLC22A13; also known as ORCTL3) has synthetic lethality with mutations in the oncogene *HRAS*, and is therefore a viable candidate for the development of tumour-specific cytotoxic therapies. These studies suggest that, at least in some instances, apoptosis-specific markers can be successfully applied to both chemical and genetic HTS studies.

It should be noted that apoptosis is a non-uniform process with respect to its precise biochemical features and physiological consequences. Although apoptotic cell death is mostly 'silent' — in the sense that the cells are removed without leaving a trace or a consequence, especially when it is part of normal development or tissue homeostasis — apoptosis can be immunogenic under specific circumstances[8]. Inducing immunogenic cell death with the goal of stimulating an anticancer immune response to control residual tumour cells could be highly desirable for anticancer therapy (BOX 3). Thus, it may be useful to develop HTS assays to monitor biochemical alterations associated with immunogenic cell death, such as exposure of calreticulin on the plasma membrane surface[79].

Autophagy assays

In spite of the fact that autophagy (BOX 4) is rarely — if ever — directly responsible for cell killing[22], autophagy inducers or inhibitors constitute attractive drug candidates. Autophagy-stimulating chemicals exert consistent cytoprotective and anti-ageing effects and hence may be useful — alone or in combination with cell death inhibitors — for the therapy of ischaemia, myocardial infarction or neurodegeneration[80]. Autophagy inhibitors are considered as potent chemosensitizers and radiosensitizers, and might therefore be introduced into combination regimens for optimal anticancer therapies[81].

Steady-state methods to measure autophagy include electron microscopy for quantifying autophagosomes and autophagolysosomes, immunoblotting for detecting the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3; also known as LC3), or immunofluorescence microscopy for monitoring the redistribution of LC3 from a diffuse pattern of distribution to a punctate pattern of distribution (FIG. 3). The main limitation of these techniques is their inability to distinguish between accumulation of LC3-positive autophagosomes, owing to increased initiation of autophagy, and decreased autophagic degradation, caused, for example, by defects in lysosomal fusion or degradation. Moreover, the autophagosomal accumulation of LC3 can be influenced by lysosomal acidification[82], indicating that results from LC3 aggregation assays must be confirmed by complementary techniques[11].

In contrast to steady-state techniques, flux measurements enable the determination of whether autophagic degradation proceeds to completion. The discrimination between efficient and arrested autophagic processing is critical because the former generally exerts cytoprotective functions, whereas the latter frequently represents a pathological event[83]. The autophagic flux can be assessed in a number of ways: by pulse-chase, immunoblotting or luciferase-based methods for monitoring the degradation of autophagic substrates, including sequestosome 1 (SQSTM1), mutant α-synuclein, or proteins with expanded polyglutamine repeats[84]; by assessing the turnover of LC3II (the mature, autophagosome-associated form of LC3) in the presence and absence of lysosomal inhibitors (for example, E64D or pepstatin) by immunoblotting or by immunofluorescence microscopy; by monitoring the turnover of autophagosomes in the presence of inhibitors of the initiation of autophagy (for example, 3-methyladenine or wortmannin) by electron microscopy or GFP–LC3 fluorescent imaging; or by using fluorescence microscopy to monitor the autophagosome–lysosome colocalization, for example, with tandem monomeric RFP–GFP-tagged LC3 (REF. 11).

HTS. Most of the techniques mentioned above — with some notable exceptions — are incompatible with primary HTS, yet they are well adapted for secondary screens or subsequent validation. Cell lines stably expressing GFP–LC3 in combination with automated fluorescence microscopy and computer-based image analysis have successfully been used in HTS studies[85,86]. *Renilla reniformis* luciferase coupled to LC3 has recently been used to develop a real-time HTS-compatible assay

Box 4 | **Autophagic flux**

The term autophagy, which derives from Greek and means self (auto) eating (phagy), refers to any catabolic pathway that leads to the lysosomal degradation of cytoplasmic structures. Macroautophagy is a specific form of autophagy that involves a membranous organelle other than the lysosome: the autophagosome[42]. Autophagosomes are double-membraned vesicles that progressively engulf cytoplasmic constituents (including protein aggregates as well as old, damaged and supernumerary organelles) and deliver them to lysosomes for degradation. The origin of the precursors of autophagosomes — phagophores, also known as isolation membranes — has been the subject of extensive debate, and it is possible that membranes from the endoplasmic reticulum, the Golgi apparatus and mitochondria contribute to the formation of autophagosomes[131]. Once they are sealed, autophagosomes fuse with lysosomes to generate the so-called auto(phago)lysosomes, and this is coincident with the acidification of the luminal microenvironment and the activation of lysosomal hydrolases. Shortly after the autophagosomal–lysosomal fusion, auto(phago)lysosomes appear as single-membraned and electron-dense vesicles, owing to the degradation, by lysosomal enzymes, of both the autophagosomal inner membrane and its cargo. Eventually, newly generated macromolecules are exported into the cytosol and reused by bioenergetic or biosynthetic metabolic circuitries[132].

Although baseline autophagy contributes to the maintenance of cellular homeostasis, autophagic flow is upregulated in response to many adverse conditions, including nutrient or growth factor deprivation, accumulation of unfolded proteins and intracellular infection. Thus, autophagy frequently exerts cytoprotective functions by acting as a stress response mechanism[22]. However, in some settings, autophagy may contribute to the execution of cell death[23].

The intracellular incidence of autophagosomes is one of the parameters currently being used to estimate autophagic flow. However, autophagosomes do not only accumulate when autophagy is upregulated (increased on-rate), they also accumulate when the autophagosomal–lysosomal fusion is inhibited (decreased off-rate). These two possibilities can be distinguished by monitoring the autophagic flux, either by comparing autophagosome formation in the presence or the absence of lysosomal inhibitors (which block the last step of autophagy)[11] or by assessing the abundance of typical autophagic substrates such as the protein sequestosome 1 (REF. 11).

for monitoring the autophagic flux[87]. Additionally, firefly luciferase fused to a long amino-terminal polyglutamine stretch has been shown to constitute a preferential autophagic substrate and is hence a *bona fide* marker of autophagic degradation that may be applicable to HTS studies[88]. Finally, a FACS-based method to quantify GFP–LC3 turnover has recently been developed[89].

To date, only automatic fluorescence microscopy, which is used to monitor the autophagosomal translocation of GFP–LC3, has been successfully used in large-scale autophagy HTS. In 2009, He and colleagues[90] used this method to screen a library of 1,050 human cDNA clones, which led to the discovery of three genes (*TM9SF1*, *TMEM166* and *TMEM74*) whose overexpression induces high levels of autophagosome formation. Similarly, Roberge and co-workers[91] screened a library of 3,500 chemical compounds and identified perhexiline, niclosamide, amiodarone and rottlerin as autophagy-stimulatory drugs that act via the inhibition of mammalian target of rapamycin (mTOR) complex 1. More recently, a genome-wide siRNA screen identified 236 autophagy-relevant genes and characterized the molecular pathways regulating autophagy under normal nutritional conditions[35]. These examples underscore the importance of cell-based HTS methods for monitoring autophagy. It is not yet known whether these techniques or autophagic flux assays will enable the discovery of clinically useful autophagic modulators.

Non-mammalian systems. Autophagy is conserved from yeast to humans, enabling the extrapolation of information obtained in this simple model organism to mammalian systems. In fact, the molecular mechanisms of autophagy were initially characterized in yeast, and HTS studies in non-mammalian systems have yielded important insights.

For example, Rubinsztein and colleagues[92] conducted a primary small-molecule screen in yeast, analysing 50,729 compounds for their capacity to modify rapamycin-induced growth arrest. These findings were validated in human cells, and three small-molecule enhancers that induce autophagy independently or downstream of mTOR were identified. More recently, a yeast screen monitoring the translocation of a GFP-tagged mitochondrial protein to the vacuole led to the identification of 32 genes specifically involved in mitophagy[93]. Additionally, genetic screens in *Caenorhabditis elegans* and in flies have successfully uncovered novel autophagy-relevant genes[94,95]. It remains to be determined whether these genes will be pertinent to mammalian autophagy and whether they are *bona fide* targets for drug development.

Upstream regulatory pathways. In addition to monitoring the extent of autophagy, recent progress in our understanding of the underlying molecular mechanisms has led to the development of assays for assessing the activity of upstream regulatory pathways. These assays include immunoblotting for determining the phosphorylation status of substrates of critical autophagy-regulator kinases, such as mTORC1 and UNC51-like kinase 1 (ULK1, also known as ATG1); enzymatic assays for quantifying such kinase activities[11]; and assays to measure the activity of the phosphoinositide 3-kinases (PI3Ks) and the cellular levels of their products. Several of these assays, including in-cell-western assays to monitor mTORC1 activity have been recently adapted for HTS[35,74]. Similarly, the FYVE (zinc finger domain)–RFP reporter has been successfully used to detect the autophagy-associated accumulation of the type 3 PI3K product phosphatidylinositol-3-phosphate[35,85]. As for apoptosis pathway-specific biochemical assays, these

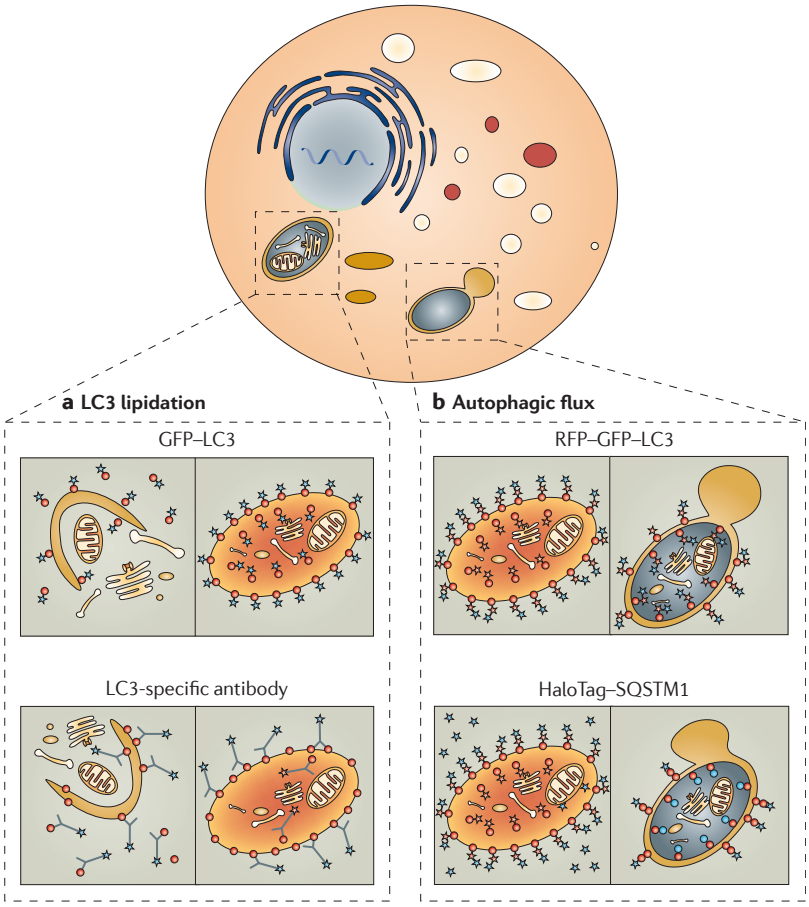


Figure 3 | **Autophagy assays. a** | Autophagy is accompanied by the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3; also known as LC3), which is incorporated into the nascent autophagosome. Fluorescent biosensor cell lines stably expressing green fluorescent protein (GFP)–LC3 can be used to monitor the formation of autophagic vacuoles. Alternatively, antibodies that are specific for LC3 can be utilized to detect autophagic vacuoles in fixed and permeabilized cells. **b** | Autophagic flux, which comprises vacuole formation and subsequent destruction of their content by lysosomal proteases, can be assessed by cells expressing a red fluorescent protein (RFP)–GFP–LC3 tandem protein, as the pH-sensitive GFP signal is quenched following lysosomal fusion. Cell lines expressing HaloTag–sequestosome 1 (SQSTM1) fusion protein — which readily degrades after lysosomal fusion — are an alternative way to detect autophagic vacuoles and they offer the possibility of using a fluorescent ligand that can be selected to meet hardware specifications.

methods should not be used as a primary means of quantifying the levels of autophagy; they should instead be used to identify the underlying molecular mechanisms.

Cell-free systems. The development of reliable cell-free systems to measure autophagy that can be applied to HTS is eagerly awaited. Recently, the autophagosomal–lysosomal fusion during macroautophagy was characterized independently of vesicular trafficking, using isolated autophagosomes and lysosomes. This led to the identification of the basic requirements for heterotypical fusion processes[96,97]. Similarly, attempts to reconstitute autophagy-relevant conjugation systems — such as those causing the lipidation of LC3 — are in progress[98], suggesting that autophagic sequestration could soon be recapitulated *in vitro*.

Necrosis-oriented assays

In the absence of a phagocytic system, secondary necrosis represents the end point of all cell death modalities (for example, in cultured cells). At the end stage of the process, the plasma membrane ruptures and the intracellular content is released in a disorganized manner. On the contrary, primary necrosis — be it accidental or programmed (necroptosis) — constitutes a cell death subroutine in its own right (see above)[3]. The recent discovery that necrosis is not a purely accidental process and that necroptosis has an active role in several human diseases[4], has prompted much interest in the development of necrosis modulators. Theoretically, inducers of necrosis might be useful for the treatment of apoptosis-resistant tumours. Necrosis inhibitors may limit necroptosis-mediated cell death in diseases that have been linked to pathological cell loss, such as stroke.

In practical terms, there are only a few specific markers of necrosis. Most of the biochemical processes that characterize necroptosis can also occur during apoptotic cell death. Some examples include LMP and MMP, abrupt ATP consumption, ROS overproduction by mitochondria and non-mitochondrial sources, cytosolic calcium overload and activation of non-caspase proteases such as cathepsins and calpains[99]. In this context, the temporal dimension has a critical role. Necrotic cell death can indeed be differentiated from apoptosis by monitoring the kinetics of appearance of such common markers.

For example, whereas during apoptosis the integrity of the plasma membrane is retained until secondary necrosis intervenes or apoptotic bodies are cleared, primary necrosis is characterized by an early plasma membrane collapse[3]. Accordingly, unlike apoptotic cells, necrotic cells do not become Annexin V-positive (see above) before taking up exclusion dyes. Thus, the development of necrosis-oriented assays should take the timing of this process into careful consideration. A combination of microscopic and impedance-based technologies — which is currently under development — would allow the detection of necrotic markers (see below) in a time-resolved fashion.

Necrosis-specific markers. One commonly used assay for necrosis detection measures the extracellular release of HMGB1, a non-histone chromatin-binding protein that normally regulates transcription[100]. Commercial kits are available for the ELISA-based end-point quantification of HMGB1 in cell culture supernatants as well as in bodily fluids. Moreover, cell lines that have been engineered for the stable expression of HMGB1 fused to a fluorescent protein (for example, GFP or RFP) can be utilized to monitor necrosis in real-time by time-lapse microscopy, even in HTS settings[101] (FIG. 4). The major caveat of this detection system is that HMGB1 release has also been shown to occur in some cell death-unrelated settings (for example, following monocyte and/or macrophage activation)[102] and in some cases of apoptosis[103].

An additional biomarker for necrotic cells has recently been described. Peptidylprolyl isomerase A (PPIA; also

known as CYPA) is specifically released from necroptotic cells[104]. Release of CYPA appears to be dependent on cell membrane permeabilization, which occurs in the early stages of necrotic cell death. Cyclosporin A and other drugs that bind and inhibit CYPA have been identified using CYPA binding and enzymatic assays[105–107]. These inhibitors might be used to develop CYPA-oriented assays. ELISA kits for the quantitative measurement of CYPA are commercially available.

An enzymatic assay measuring PPI activity has been used to measure CYPA in human serum and tissue culture samples[108]. This protocol has been optimized for HTS and used to identify inhibitors of CYPA[109]. However, the PPI activity in biological samples may not be solely attributable to CYPA, owing to many other proteins that also function as PPIs. Similarly to HMGB1, CYPA release is not entirely specific for necrosis: macrophages and other cells can also liberate CYPA in response to inflammatory stimuli or oxidative stress[110]. Thus, CYPA-oriented or HMGB1-oriented assays may be adapted to HTS assays for detecting necrotic cell death.

Because of the limited availability of specific readouts, successful HTS for necrosis relied on cells that preferentially undergo non-apoptotic cell death. For example, Degterev and colleagues[111] induced necroptosis by stimulating cells with tumour necrosis factor in the presence of the pan-caspase inhibitor Z-VAD-fmk. This system was used to screen a compound library, and resulted in the discovery of necroptosis-specific small-molecule inhibitors termed necrostatins.

The discovery of necrostatins and the characterization of necrostatin 1 (REFS 2,111) as an enzymatic inhibitor of RIPK1 that is able to protect against non-apoptotic cell death *in vitro* and *in vivo*[2], initiated the molecular characterization of the mechanisms that mediate programmed necrosis. Currently, necrostatin 1 and several of its derivatives are under clinical investigation as cytoprotective drugs, and they also constitute potent research tools for the study of necroptosis[112]. It should be noted that some — but not all — necrostatins inhibit RIPK1, suggesting that anti-necrotic drugs can act on pharmacological targets other than RIPK1.

At present, our knowledge of the molecular mechanisms of necroptosis is relatively limited, and no specific cell-free systems have been developed to assess particular facets of this cell death mode. However, it can be speculated that cell-free systems that are designed to assess particular necroptosis-associated phenomena such as LMP[113] may help in the future development of cytoprotective agents.

Detection of mitotic catastrophe

Mitotic catastrophe has been defined as a cell death modality that occurs either during or shortly after failed mitosis[3]. Often, mitotic catastrophe manifests with micronucleation and multinucleation[15]. However, cells that are dying following failed mitosis eventually activate the molecular machinery for apoptosis or necrosis and develop morphological traits of either of these two processes[114], suggesting that mitotic catastrophe might represent a prelude to death rather than a cell death

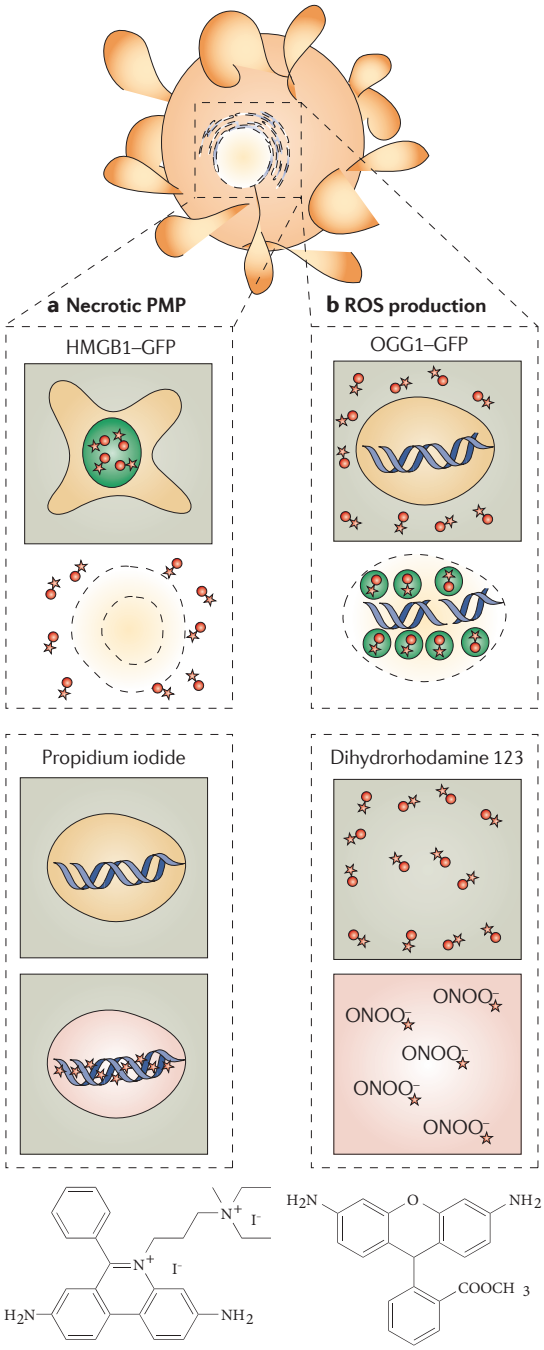


Figure 4 | **Imaging necrosis. a** | Necrotic plasma membrane permeabilization (PMP) can be assessed by fluorescent biosensor cell lines that express the non-chromatin-binding high mobility group protein B1 (HMGB1) fused to a fluorescent protein (for example, green fluorescent protein (GFP)). The liberation of HMGB1 during cellular disintegration can be determined in real-time assays. Alternatively, cells can be stained with exclusion dyes (for example, propidium iodide) that only label cells that have lost the integrity of their plasma membrane. **b** | The recruitment of a GFP–8-oxoguanine DNA glycosylase (OGG1) fusion protein to the nucleus (and in particular, to reactive oxygen species (ROS)-induced DNA-strand breaks) can be used as an indicator of ROS-mediated DNA damage. Chemical ROS probes such as dihydrorhodamine 123 offer an alternative means of determining ROS production.

Necrostatin 1
The tryptophan-based molecule 5-(1*H*-indol-3-ylmethyl)-3-methyl-2-thioxo-4-imidazolidinone that was first identified as a specific and potent inhibitor of necroptosis.

Micronucleation and multinucleation
The presence of multiple nuclei often derives from mitotic problems — for instance, from chromosomes or chromosomal fragments that have not been evenly distributed at anaphase — or from deficient cytokinesis.

subroutine on its own. This hypothesis is further supported by the fact that in addition to cell death, mitotic catastrophe can lead to senescence or the generation of viable, polyploid cells[115,116].

At present, there are no biochemical markers that are specific for mitotic catastrophe. Activation of caspase 2 has been correlated with some cases of mitotic catastrophe[15]. However, as caspase 2 can be activated in response to mitosis-unrelated stimuli — including DNA damage and heat shock[117,118], and some instances of mitotic catastrophe can proceed in a caspase 2-independent fashion[114,119] — caspase 2 activation cannot be used as a reliable indicator for mitotic catastrophe. Moreover, the most widely used markers of apoptosis and necrosis (see above) may become apparent in cell death occurring before, during or after mitosis, thus implying that standard end-point procedures for the detection of apoptosis or necrosis cannot discriminate between pre-mitotic, mitotic and postmitotic cell death. Thus, until recently, the only reliable method of detecting mitotic catastrophe relied on the continuous observation of cells by conventional microscopy, which is not applicable to HTS.

In 2010, we proposed the first fully automated, fluorescence video microscopy assay for the detection of mitotic catastrophe[120]. This technique is based on isogenic colon carcinoma cell lines that differ from each other in ploidy and tumour protein p53 status, yet they express similar amounts of fluorescent biosensors for the simultaneous visualization of chromatin (H2B–GFP) and centrosomes (centrin fused to *Discosoma striata* RFP (Ds-Red)). By coupling fluorescence video microscopy and automated image analysis, this method allows the monitoring of several indicators of the nuclear status, including size, mean intensity and granularity and the establishment of cell fate profiles (FIG. 5). Combined with appropriate algorithms, these parameters can be used to automatically identify cells displaying abnormal nuclear morphology that is indicative of mitotic catastrophe[120].

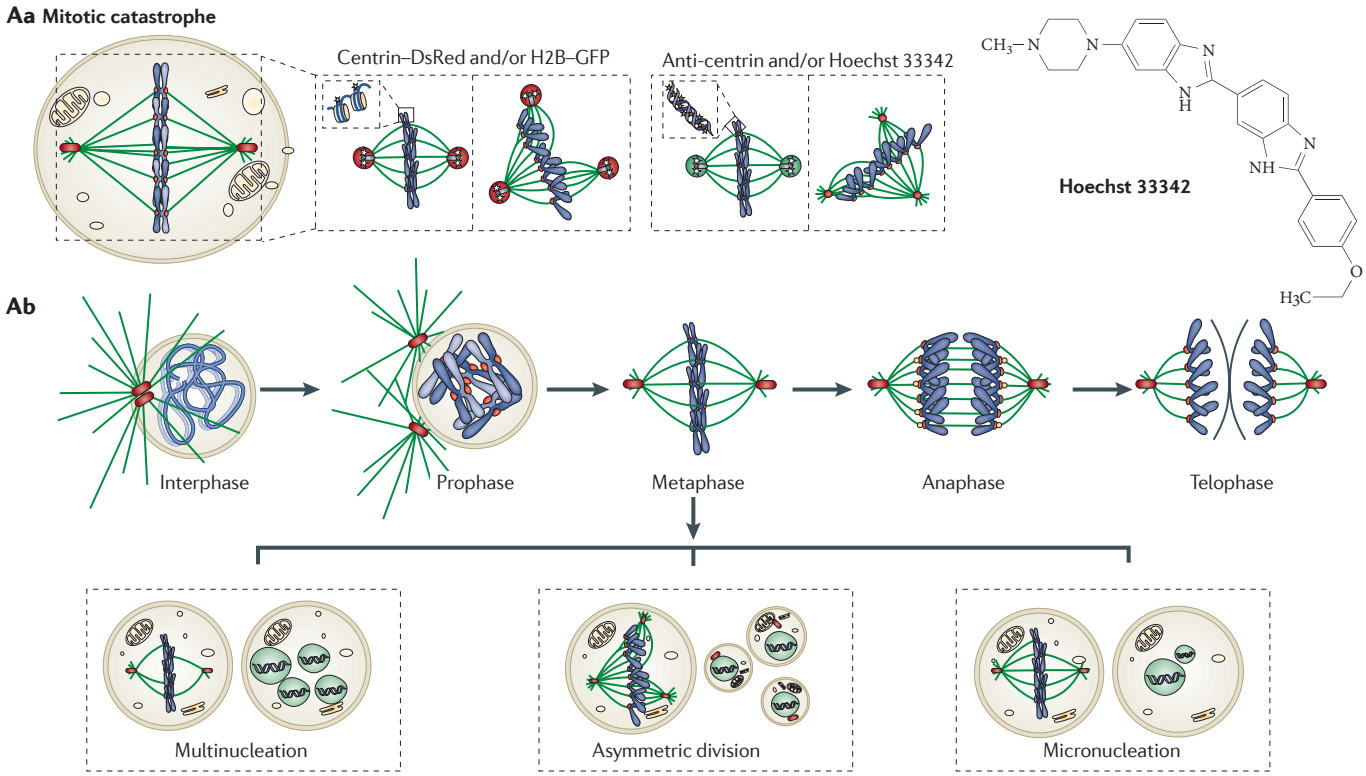
Figure 5 | **Cell fate profiling. Aa** | Fluorescent biosensor cell lines stably expressing the histone subunit 2B fused to green fluorescent protein (H2B–GFP) along with centrin fused to DsRed allow the assessment of mitotic events in living cells. Alternatively, centrin-specific antibodies together with nuclear dyes (such as Hoechst 33342) can be used to visualize mitotic events in dividing cells in end-point assays. **Ab** | Cells can undergo normal mitotic divisions (upper panel) or they can fail to establish a linear metaphase plane and undergo mitotic catastrophe, which manifests in multinucleation, micronucleation or asymmetric division (sometimes followed by apoptosis). **B** | This can be documented in cell fate profiles, which allow the visualization of the behaviour of multiple individual cells. Graphs represent the cell fate profiles of control cells (left) versus cells treated with a mitotic spindle poison, like paclitaxel (right). Each line represents the fate of one cell and its daughter cells. The different colours depict different cell fates. Graphs are reproduced, with permission, from REF. 120© (2010) Macmillan Publishers Ltd. All rights reserved.

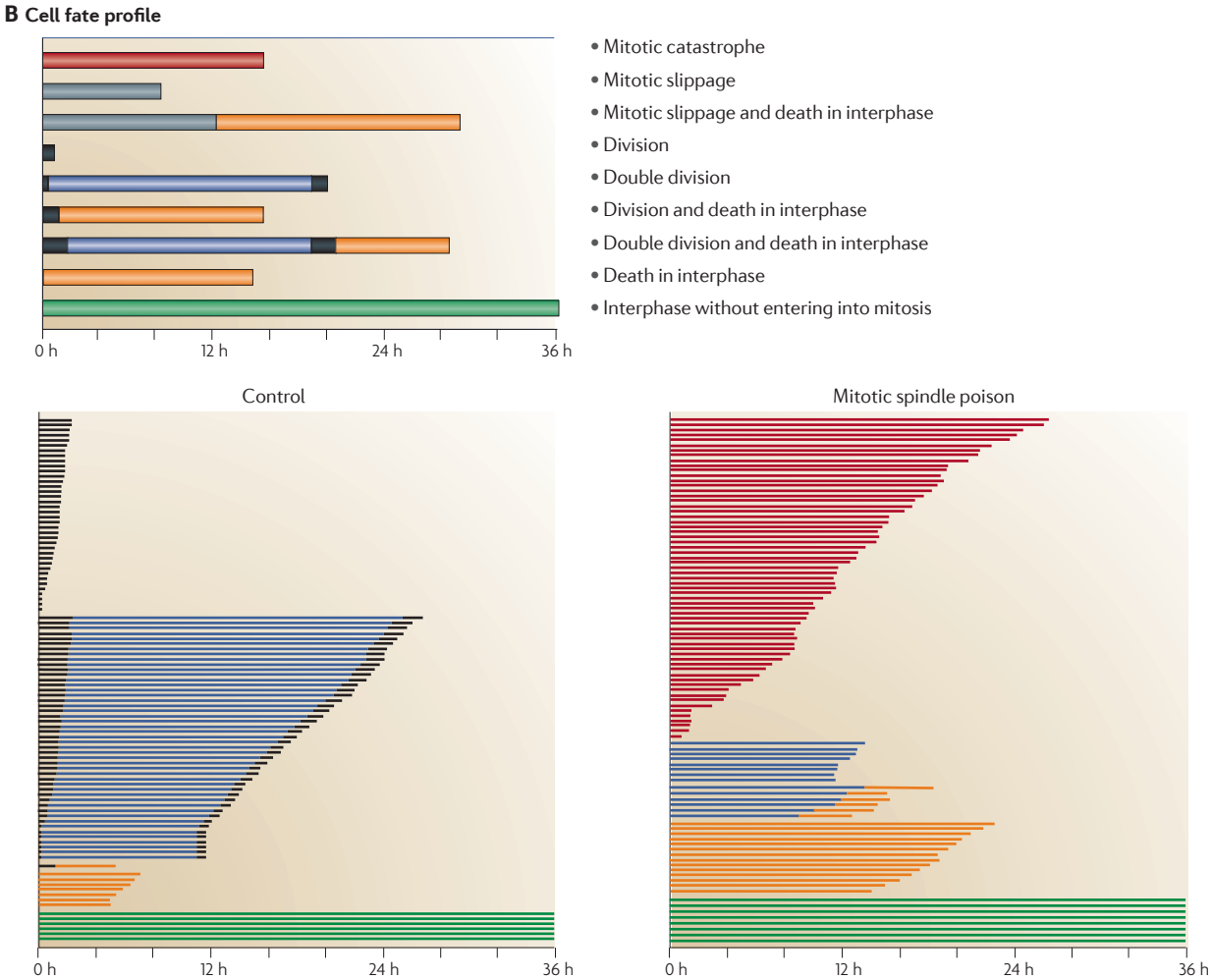
Problems and perspectives

Compared to specific target-oriented HTS methods that are usually performed by monitoring the activity of purified enzymes, cell-based assays have a number of advantages. In addition to the fact that cell-based assays filter chemical libraries for the identification of cell-permeable agents, they also lead to the exclusion of nonspecific toxic agents, especially when cytoprotection is the goal of the assay. Cytotoxic or cytoprotective agents that are identified in cell-based assays have no predefined mode of action, meaning that the identification of the target may constitute a challenge that has to be undertaken by chemical and/or biological analyses. This may be accelerated, at least in some cases, by using cell-free systems and/or a battery of organelle and pathway-specific tests[75] that evaluate the action of a compound on specific subcellular compartments or processes.

The main drawback of apoptosis-specific and necrosis-specific assays is that neither of these assays can provide unequivocal information on the occurrence of one specific cell death modality compared to the other. Similarly, reliable stand-alone methods for the detection of autophagy have not yet been developed. It is therefore recommended that at least two complementary techniques are used to avoid a consistent proportion of false positive or false negative results. Except for high-content microscopy-based assays, this is rarely — if ever — feasible during primary HTS, which often involves more than 500,000 separate assays. Therefore, apoptosis-specific or necrosis-specific assays will usually be used at the validation step, following a primary screen based on more robust universal cell death markers such as exclusion dyes.

During the past decade, progress in combinatorial chemistry has led to the generation of impressively large chemical libraries. Moreover, the information generated by the sequencing of an ever-growing number of animal genomes has resulted in the construction of more complete, genome-wide libraries that allow the overexpression or knockdown of most (if not all) transcripts[121]. The availability of large libraries of pharmacological and genetic tools coupled to the need to dissect and understand the molecular cascades that regulate cellular demise, has driven the optimization of apoptosis, necrosis and autophagy assays that are amenable to HTS. The advent of fully automated fluorescence microscopy and imaging stations is contributing to the optimization of these assays, by allowing the use of fluorescence-based living biosensors in miniaturized HTS procedures. To date, clinically promising compounds have been identified by cell death-based HTS approaches, including necrostatin 1 (see above)[2], NSC23925 (which is an inhibitor of multidrug resistance in cancer cells)[122] and carnosic acid (which is an interesting lead compound for the development of neuroprotective agents)[123]. It can be anticipated that the combination of increasingly sophisticated high-content and high-throughput microscopic methods[124,125] with microfluidic devices or on-chip technologies[126] will accelerate the cell-based identification of cell death-modulatory drugs.





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Competing interests statement

The authors declare no competing financial interests.

FURTHER INFORMATION

Author's homepage: http://kroemerlab.com/index.htm

SUPPLEMENTARY INFORMATION

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