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CYTOMETRY OF APOPTOSIS. HISTORICAL PERSPECTIVE AND NEW ADVANCES

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Characteristic changes in cell morphology paralleled by the appearance of a multitude of molecular and biochemical markers occur during apoptosis. These changes vary depending on the cell type, mechanism of induction of apoptosis, and the time-window at which the process of apoptosis is analyzed. By virtue of the capability of rapid measurement of individual cells the flow- and imaging-cytometry become preferred technologies to detect, identify and record incidence of apoptosis in large cell populations. It also provided a valuable tool to investigate molecular mechanisms in field of necrobiology. This review outlines the progress in development of the most commonly used cytometric methods probing cells death based on analysis of fragmentation of DNA, activation of caspases, analysis of mitochondrial potential, alterations in plasma membrane structure and other features that characterize programmed cell death. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".

***Key Words*: programmed cell death, cell necrobiology, fluorescent probes, cytometry, TUNEL, FLICA, SYTO probes, imaging cytometry.**

INTRODUCTION

This review is focused on the progress in development of cytometric methods probing apoptotic cell death. The term apoptosis is used here to define the "classical" apoptotic process, initially also called programmed cell death, which is inclusive of such hallmarks as: (I) mitochondrial changes manifesting by collapse of the transmembrane electrochemical potential and release of cytochrome c to cytosol, (II) activation of caspases, (III) chromatin condensation (pycnosis), (IV) activation of endonucleas(s) followed by internucleosomal DNA cleavage, (V) segregation of nucleoli, (VI) fragmentation of nucleus, and (VII) plasma membrane blebbing associated with formation of apoptotic bodies [1, 2].

It should be noted, however, that with passing time it became apparent that in addition to cell death that demonstrates "classical" features of apoptosis there are other ways of cell demise that also involve active participation of the cell [3–7]. The most recently recognized modes of cell death include: (I) extrinsic apoptosis, intrinsic apoptosis that is either (II) caspase-dependent or (III) -independent, (IV) regulated necrosis, (V) autophagic cell death and (VI) mitotic catastrophe [6]. Different mechanisms operate in each of these death modes, and this is reflected by somewhat different character of changes in cell morphology, expression of different molecular markers and some specific features. It is of importance, therefore, when describing cell death in general or apoptosis in particular, to define with as much detail as possible, the biomarkers that served to classify the mode of death.

The term "cell necrobiology" (biology of cell death) was proposed to encompass different modes of cell death, the range of stress signaling cascades leading to cell death, regulatory mechanisms, initiation, and execution steps of cell death, as well as cell remnants disposal mechanisms, all associated with cell demise [8–10; see *Cell Necrobiology* in Wikipedia].

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Abbreviations used: $\Delta\Psi_m$ – mitochondrial inner membrane electrochemical potential; AIF – apoptosis-inducing factor; BFP – blue fluorescent protein; CFP – cyan fluorescent protein; $DiOC_6$ (3) – 3,3'-dihexiloxa-dicarbocyanine; FLICA – fluorochrome-labeled inhibitors of caspases; FRET – fluorescence resonance energy transfer; GFP – green fluorescent protein; JC-1 – 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide; JC-9 – 3,3'-dimethyl-α-nafthoxacarboxycyaninine iodide; MOMP – mitochondrial outer membrane permeabilization; PARP – poly(ADP-ribose) polymerase; PI – propidium iodide; PT – permeability transition; TUNEL – TdT dUTP nick end labeling assay; YFP – yellow-fluorescent protein.

THE FIRST MARKERS ANALYZED BY CYTOMETRY — LIGHT SCATTER CHANGES, DNA FRAGMENTATION AND CHROMATIN CONDENSATION

The highly characteristic morphological changes that occur during apoptosis provided specific markers for identification of this mode of cell death prior to use of flow cytometry [1, 2]. Among the predominant features of apoptotic cells were their diminished size (as a result of dehydration), condensation of chromatin (often starting from nuclear periphery and forming "crescent moon" appearance), segregation of nucleoli, nuclear fragmentation and formation of apoptotic bodies [1, 2]. For several years these changes, considered the "gold standard" for recognition of apoptosis, served as markers for identification of apoptotic cells and used for assessment of frequency of apoptosis. Another hallmark of apoptosis was activation of endo-

nuclease that led to internucleosomal DNA cleavage and extensive fragmentation of nuclear DNA [2]. In these early studies, apoptosis-related fragmentation of DNA was recognized by a characteristic pattern of DNA "laddering" that revealed the presence of mono- and multi-nucleosomal size DNA fragments during gel electrophoresis [2].

The possibility of rapid measurement of individual cells in large cell populations offered by flow cytometry made it the methodology of choice to identify and quantify apoptotic cells as well as to study mechanisms associated with this mode of cell death [7–10]. The change in light scattering properties of cells dying by apoptosis was one of the earliest observations and served as a marker identifying apoptotic cells. Specifically, it was observed that at early stage of apoptosis the cell is characterized by distinctly reduced ability to scatter light in forward direction and by an increase in intensity in side (90° angle) scatter signal [11]. While forward light scatter reports cell size, the intensity of side light scatter relates to reflective and refractive properties of the cell. These early changes in forward light scatter are a result of cell shrinkage, whereas changes in side scatter reflect chromatin condensation, nuclear fragmentation and crosslinking of cytoplasmic proteins by activated transglutaminase, making the cell more refractive and reflective. At later stages of apoptosis, when cells shed-off apoptotic bodies and further reduce their size, the intensity of both, the forward and right angle light scatter signal become markedly decreased. The virtue of the assay based on light scatter measurement is its simplicity and the possibility of its combination with analysis of the cells surface immunophenotype [11] and/or with functional assays such as the mitochondria1 potential, calcium signaling or exclusion of charged fluorochromes by plasma membrane. It should be stressed, however, that the decreased forward light scatter signal is not a specific marker of apoptosis since mechanically broken and fragmented cells, isolated cell nuclei, and necrotic cells also have reduced light scatter properties. This approach, therefore, should be accompanied by another, more specific assay of apoptosis.

Activation of endonuclease that cleaves DNA at internucleosomal (linker) sections, one of the most characteristic events of apoptosis [2, 12], was also among the first markers of apoptosis recognized by flow cytometry. The low molecular weight (MW) fragments of DNA (mono- and oligo-nucleosomal sections) leak out from the ethanol-fixed or detergent-permeabilized apoptotic cells during rinse and staining procedures. The events with fractional DNA content recorded by flow cytometry, represented by the "sub-G_1" peaks on the DNA content histograms, were considered to be apoptotic cells [13–16]. It should be noted, however, that the detergent treatment of the unfixed cells as proposed in some studies [16] in addition to the intact nuclei, releases also individual nuclear/chromatin fragments. Therefore the number of the sub-G_1 events on DNA content frequency histograms from the assays utilizing detergent-permeabilized cells may severely overestimate incidence of apoptosis [17]. This is particularly pronounced when the DNA content histograms are plotted on exponential scale which allows one to detect and quantify objects having even less that 1% of DNA of the intact G_1 cells, which certainly cannot be individual apoptotic cells. The approach to identify apoptotic cells based on fractional DNA content was later advanced by selective extraction of low MW DNA (mono- and oligo-nucleosomal) from the ethanol-fixed cells using buffer of high molarity. A combination of analysis of fragmentation of so extracted DNA by gel electrophoresis ("DNA laddering") with the flow-cytometric detection of apoptotic cells based on their fractional DNA content after DNA extraction offered a complementary biochemical (DNA "laddering") and cytometric (sub-G_1) methodology of the same samples [17].

Furtner approach to identify apoptotic cells was based on the detection of DNA strand breaks in the nuclei by labeling their 3'OH termini with biotin- or digoxigenin-conjugated nucleotides in the enzymatic reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT) [18, 19]. This methodology is also known as the **T**dT d**U**TP **n**ick **e**nd **l**abeling (TUNEL) assay [20]. It should be mentioned, however, that the acronym TUNEL is inaccurate because the DNA double-strand breaks (ds) and not the "nicks" (single-strand breaks — ss) are being labeled and instead of dUTP other deoxynucleotides are used in most types of these assays [18, 19]. The alternative approach to label DNA strand breaks by the nick translation assay utilizing DNA polymerase I was less successful [18, 21]. Currently, the most widely used and also the most sensitive assay utilizes BrdUTP as the TdT substrate for labeling DNA strand breaks [22]. It should be noted that unlike in the case of detecting apoptotic cells based on their fractional DNA content (sub-G_1) the cells subjected to the TdT assay have to be pre-fixed in formaldehyde to crosslink DNA and thereby prevent its leakage out from the cell. Concurrent differential labeling of DNA strand breaks and DNA provides a convenient assay to correlate induction of apoptosis with the cell cycle phase [19, 23]. Analysis of such correlation is helpful in exploring mechanism of action of anticancer drugs that target cell cycle progression machinery [23].

Among other early cytometric methods identifying apoptotic cells was the one which recognizes them by the virtue of detection of intense chromatin condensation [24]. Chromatin condensation is characterized by the increased susceptibility of DNA *in situ* to undergo denaturation ("melting") when stressed by heat or acid. The extent of DNA denaturation *in situ* was assessed with the metachromatic fluorochrome acridine orange, which interacts with dsDNA yielding green-, and with ss (denatured) DNA, red-fluorescence [24].

ACTIVATION OF CASPASES

Development of the methods designed to detect activation cysteine-aspartic acid-specific proteases (caspases) was also among the early attempts to explore applications of cytometry in studies of apoptosis. Caspases activation, which occurs at relatively early stages of apoptosis, has been considered to be also a hallmark of this mode of cell death [25, 26]. As mentioned, however, it was later found that the process of apoptosis may also be caspase-independent [3–7]. Many different methods detecting caspase activation have been developed and widely used in basic and clinical studies. One of the early approaches utilized fluorogenic substrates of these enzymes. These peptide substrates are not fluorescent but following the caspase-induced cleavage the products show strong fluoresce [27–30]. A number of kits of these reagents having different color fluorochromes have been developed and became commercially available. Depending on the peptide specificity these substrates can be used to detect activation of either multiple or individual caspases, respectively.

Another approach to detect activation of caspases is based on the immunocytochemical detection of the cleaved poly(ADP-ribose) polymerase (PARP), one of the "death substrates" of caspases. In live cells the nuclear enzyme PARP becomes activated in response to DNA damage and functions during DNA repair [31]. At early stage of apoptosis, however, PARP is cleaved by caspases and the specific cleavage products are 89-kDa and 24-kDa PARP fragments [25, 32]. Detection of these protein products by Western blotting, in analogy to DNA "laddering" assay was considered to be a specific biochemical marker of apoptotic mode of cell death. However, the immunocytochemical detection of the 89-kDa product of the PARP cleavage provided the means to label apoptotic cells for their recognition by cytometry [33]. The multiparameter analysis of the cells differentially stained for PARP p89 and DNA made it possible to correlate the induction of apoptosis with the cell cycle phase or DNA ploidy [33].

Still another way to detect activation of caspases is based on immunocytochemical detection of the epitope of the activated (cleaved) caspases. Abs specific to several activated caspases have been developed and initially used for detection of apoptosis by Western blotting. Subsequently these Abs were adapted to cytometry to identify apoptotic cells. Detection of caspase-3, one of the "cell executioners" caspases, with its Ab was found to be particularly useful to reveal its activation and as an overall marker of apoptotic mode of cell death. In analogy to PARP p89 Ab, immunocytochemical detection of caspase-3 concurrent with counterstaining of DNA with another color fluorochrome made it possible to relate induction of apoptosis with the cell cycle phase by flow cytometry [34, 35].

Activation of caspases can also be detected with the use of **f**luorochrome-**l**abeled **i**nhibitors of **ca**spases (FLICA) [35, 36]. FLICAs are the affinity ligands to the active enzyme centers of caspases. Each FLICA has three functionally different domains: (I) the fluorochrome (fluorescein or sulforhodamine), (II) four amino-acid peptide providing the individual caspase recognition domain, and (III) either chloro- or fluoromethyl ketone (CMK or FMK) that binds irreversibly to cysteine of the active center of respective caspase. FLICAs are permeant to live cells and nontoxic. Exposure of live cells to FLICAs leads to the uptake of these reagents followed by their covalent binding to activated caspases; unbound FLICAs are removed from the cells that lack activated caspases by rinsing the cells. Combination of FLICA and propidium iodide (PI), the plasma membrane integrity probe, provides the possibility to distinguish three consecutive stages of apoptosis. FLICA may also be used concurrently with a probe of mitochondrial potential as well as with other probes that are applicable to either live or fixed cells [35]. It was recently observed that FLICAs (defined as FLIVO reagents) can be used *in vivo* to detect apoptosis in mouse tissues [37].

The use of the tandem molecules of green fluorescent protein (GFP) and blue fluorescent protein (BFP), or cyan- (CFP) and yellow-fluorescent protein (YFP), covalently linked by a short peptide that is the target of caspase, represents still another approach to detect caspases activation [38, 39]. The fluorescence resonance energy transfer (FRET) normally occurs between the pairs of these fluorescent proteins when they are linked by the peptide. However, after cleavage of the peptide linker by activated caspase the linkage is disrupted leading to loss of FRET fluorescence. Because in this case the marker of caspase activation is intrinsic, operating in the live cell, the method is simple, rapid and not requiring fixation or application of external reagents. The collection of cell lines stably transfected to constitutively express the tandems of FRET fluorescing proteins would be particularly useful for drug screening by monitoring apoptosis of the drug treated cells in real time.

MITOCHONDRIAL ELECTROCHEMICAL TRANSMEMBRANE POTENTIAL

Mitochondria play an essential role in induction of apoptosis by releasing cytochrome c, apoptosis-inducing factor (AIF) and variety of other apoptosis-associated molecules that initiate the cascade of caspases activation [40–42]. The release occurs as a consequence of mitochondrial outer membrane permeabilization (MOMP) resulting from change in equilibrium of pro- versus anti-apoptotic molecules of the Bcl-2 family of proteins. Dissipation of mitochondrial inner membrane electrochemical potential ($\Delta\Psi_m$) called also permeability transition (PT) appears to be associated with MOMP, although there is conflicting evidence as to whether it is a prerequisite for the release of cytochrome c and activation of caspases [42–46]. The conflicting data may be due to the fact that PT is a transient event that initially occurs con-

currently with MOMP, then mitochondria became re-charged, but subsequently with progression of the apoptotic process, the $\Delta\Psi_m$ dissipates again.

Different fluorescent probes reporting $\Delta\Psi_m$ have been adapted to flow cytometry. Historically, the first was rhodamine 123, which when used in combination with PI, provided a sensitive marker distinguishing two stages of cell death, revealed by (I) loss of $\Delta\Psi_m$ followed by (II) loss of plasma membrane ability to exclude PI. Through this approach it was possible to discriminate between apoptotic and necrotic mode of cell death [47]. Another, widely used probe, was the cationic lipophilic dye 3,3'-dihexiloxa-dicarbocyanine [$DiOC_6$ (3)] [48]. Subsequently, a series of MitoTracker™ fluorochromes (chloromethyltetramethylrosamine analogues) of different fluorescence emission color were developed by Molecular Probes Inc (Eugene, OR, USA). One of them, MitoTracker Red CMXRos, appears to be very sensitive probe of $\Delta\Psi_m$ and unlike $DiOC_6$ (3) or rhodamine 123, remains retained in the cell after fixation with formaldehyde [49]. It can be used therefore in combination with the markers that require cell fixation such as TUNEL or detection of intracellular protein(s) by immunocytochemical probes. Another useful probe of $\Delta\Psi_m$, analogous to MitoTracker Red CMXRos, is tetramethylrhodamine methyl ester perchlorate (TMRM). The latter, in combination with FLICA was shown to be able to detect the sequence of time-resolved apoptotic events, namely the dissipation of mitochondrial potential followed by activation of caspases [35].

Another type of $\Delta\Psi_m$ probes are lipophilic cationic fluorochromes that form so called J-aggregates, such as 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) and 3,3'-dimethyl-α-nafthoxacarboxycyaninine iodide (JC-9). Their uptake by charged mitochondria leads to dye aggregation which is reported by the metachromatic shift from green to orange/red fluorescence. The loss of $\Delta\Psi_m$ during apoptosis leads to dissociation of the J-aggregates and in the monomeric form these probes show green fluorescence [50].

The point to be considered while measuring $\Delta\Psi_m$ is that the distribution (concentration gradient) of the cationic mitochondrial probes reflects the electrochemical potential differences both across the plasma membrane and across mitochondrial membranes. The probes thus accumulate in mitochondria as well as in cytosol. The relative proportion in their distribution between cytosol and mitochondria thus depends not only on mitochondrial potential but also on charge of plasma membrane and abundance of cytosol. The contribution of fluorescence from cytosol is therefore affecting mitochondria-related specificity of the measured $\Delta\Psi_m$. The J-aggregates probes (JC-1, JC-9) which do not form the aggregates in cytosol, appear thus to have an advantage being more specific mitochondrial probes than rhodamine 123, $DiOC_6$ (3) or MitoTracker™ dyes [51].

ANNEXIN V BINDING

In live cells distribution of the phospholipids phosphatidylcholine and sphingomyelin, constituents of plasma membrane, is asymmetrical across the membrane. While phosphatidylcholine is exposed externally phosphatidylserine is located on the inner surface the lipid bilayer. This asymmetry disappears during apoptosis when, to make apoptotic cells more attractive targets to be engulfed by macrophages, phosphatidylserine becomes exposed on the outside leaflet of the membrane [51, 52]. The anticoagulant protein annexin V shows high affinity in binding to phosphatidylserine. The methodology was developed, therefore to use the fluorochrome-conjugated annexin V as a marker of apoptosis [52, 53]. After induction of apoptosis the expression of phosphatidylserine on external surface of plasma membrane occurs prior to the loss of its ability to exclude cationic dyes such as PI. At early stages of apoptosis therefore cells bind annexin V but exclude PI. At later stages the cells show both, annexin V binding and PI fluorescence.

The virtue of this methodology is simplicity. Currently, this is perhaps the most widely used cytometric method to detect apoptosis. The results obtained by this method, however, may be biased by the presence of non-apoptotic cells having damaged plasma membrane with the exposed phosphatidylserine that binds annexin V. Specifically, cell isolation by mechanical or enzymatic disaggregation of tissues, extensive use of proteolytic enzymes to detach adherent cells, mechanical removal of cells from tissue culture flasks (e.g. by "rubber policeman"), cell electroporation or transfection, all may affect the asymmetry of plasma membrane phospholipids and lead to binding of annexin V by non-apoptotic cells. It should also be stressed that otherwise live and healthy macrophages or monocytes, after ingestion of apoptotic bodies or fragments of apoptotic cells become annexin V positive and thus may be misidentified as apoptotic cells [54]. This problem, however, is not unique for the methodology based on the use of annexin V. It was observed that using TUNEL or FLICA assays as well the engulfment of apoptotic cells by macrophages makes them "false positive apoptotic cells" during flow cytometric analysis [55, 56].

SYTO PROBES

Progress has recently been made by the development of cell permeant, cyanine SYTO stains and their exploitation for quantification of apoptosis using flow cytometry [49, 57–59]. This novel class of cyanine probes spans a broad range of visible excitation and emission spectra: 1) SYTO blue (*Ex/Em 419–452/445–484 nm*); 2) SYTO green (*Ex/Em 483–521/500–556 nm*); 3) SYTO orange (*Ex/Em 528–567/544–583 nm*) and 4) SYTO red (*Ex/Em 598–654/620–680 nm*) [59, 60]. Their low cost offers an advantage for broad application in the first line screening of novel therapeutics with cytotoxic properties, particularly antitumor drugs targeting

apoptotic pathways, whereas spectral characteristics make them highly amenable for development of new multiparameter/multiplexed assays [57–60]. Following initiation of caspase-dependent apoptosis cells loaded with selected SYTO fluorochromes exhibit gradual reduction in fluorescence signal intensity to dim values [57–60]. This phenomenon substantially precedes plasma membrane permeability changes. When progression towards the terminal stages of cellular demise advances, loss of SYTO fluorescence intensifies, and this usually coincides with the increased plasma membrane permeability to PI. The assay requires only a short incubation (20 min, at room temperature) to supravitally discriminate viable cells ($SYTO^{high}$ / PI^- events) [57–61]. Early apoptotic cells are characterized by initial loss of SYTO fluorescence and preservation of plasma membrane integrity ($SYTO^{dim}$ / PI^- events) [57–60]. Cells in late stages of apoptosis feature progressive loss of SYTO fluorescence and gain bright PI staining ($SYTO^{neg}$ / PI^+ events).

Interestingly, our knowledge on the fundamental mechanisms behind differential staining of SYTO labeled apoptotic versus viable cells still remains ambiguous [57, 59]. To date several hypotheses have been put forward and include: (I) self-quenching of SYTO molecules that follows changes in inter-probe proximity during apoptotic chromatic condensation; (II) decrease in the number of SYTO binding sites as the chromatin condensation and/or RNA degradation advances in the process of apoptosis; (III) alterations in binding of SYTO to mitochondrial DNA; and (IV) decrease in $\Delta\psi_m$-driven mitochondrial uptake of SYTO molecules [59]. Despite this SYTO 16/PI assay has recently been reported to allow discrimination between primary and secondary necrotic cells [57]. Therefore SYTO 16/PI assays provides substantial enhancement over the standard PI exclusion assay in discerning cell demise mode by flow cytometry [59, 61]. Recently published data also indicate that sensitivity of SYTO probes in detection of early apoptotic events is equal or in some models higher as compared to Annexin V, YO-PRO 1 or FLICA assays [49, 59]. A caution should be, however, exercised as SYTO probes are effective substrates for P-glycoprotein (P-gp) efflux pump [57, 59, 62]. Hence there is a need to confirm "multi drug resistance" (MDR) status of studied cell population. In cells with confirmed high P-gp activity its inhibition (e.g. by verapamil hydrochloride) is advisable to avoid masking of apoptotic $SYTO^{dim}$ subpopulation by $SYTO^{dim}$ subpopulation resulting from active dye efflux [57, 59, 62]. It should be mentioned that high efflux activity driven by P-gp in certain cells may also complicate interpretation of other assays that are based on the intake of fluorescent probes by live cells.

REAL-TIME ANALYSIS OF APOPTOSIS

Most contemporary cell death assays are performed in the "end-point" fashion that only reveals frequency of live versus dead cells at the time of harvesting or at the intermittent and subjective time-points during cell culture [63]. Cell death within cell populations is, however, a stochastic process where cell-to-cell variability in time between the varying stages of cell death arises from the subtle fluctuations in the concentrations or states of regulatory proteins, protein oscillations, the induction of multiple compensatory mechanisms (e.g. autophagy), or "molecular noise" [63, 64]. The possibility to continuously track cells from the time of encountering stress signal, through the execution phases, up to the point of demise, can provide valuable data on kinetic quantification of pharmacologically-induced cell death. It would also offer a superior sensitivity and accuracy allowing one to assess dynamics of the apoptotic process and enhance the understanding of the molecular processes underlying the apoptosis and other cell death modes [63, 64]. Limitations imposed by conventional analytical techniques and bioassays are, however, often prohibitive for the kinetic analysis of single-cell responses to therapeutic compounds [63].

Only recently substantial progress has been made in development of real-time fluorescent assays to provide cost-effective solution for automated and kinetic analysis of investigational anti-cancer agents in hematopoietic cancer cells [65–70]. There is a mushrooming evidence that cancer cells can be continuously grown in the presence of inert fluorescent probes and imaged in a time-lapse mode or intermittently sampled to provide the multiparameter and kinetic profile of anti-cancer drug action [65–70]. A substantial reduction of sample processing steps and avoidance of washing protocols achieved with such kinetic protocols greatly facilitates the preservation of fragile cell subpopulations [65–70].

In this context cyanine SYTO probes discussed under the previous subtitle of this review have been recently reported to be inert and safe for tagging tumor cell over extended periods of time [65]. When preloaded into cells or continuously present in medium, SYTOs do not interfere with cell viability while their intracellular retention can be easily monitored for kinetic analysis of caspase-dependent apoptosis. SYTO 16-based sorting of intact apoptotic cells provides also an innovative approach to supravitally track progression of apoptotic cascade [65]. It has also been reported that many organic fluorescent probes such as PI, SYTOX Green, SYTOX Red and YO-PRO 1 allow for a non-invasive tracking of cell death events over extended periods of time without compromising cell viability or interfering with cytotoxic drug action [66–69]. Fluorescent annexin V conjugates represent yet another promising class of markers that do not adversely affect normal cellular physiology and can be used in real-time protocols [70]. The low-dose, continuous labeling procedure not only provides similar results to a standard end-point staining protocol, but also allows a straightforward adaptation for innovative Lab-on-a-Chip microfluidic platforms with minimal protocol modifications [65, 67–70].

As discussed earlier in this review several approaches to track caspase activation in living cells have been de-

veloped. Among them are cell permeable fluorogenic caspase substrates such as PhiPhiLux, NucView 488 and FLICA [27, 35–37, 71, 72]. They are all relatively nontoxic to live cells but identify cells with activated caspases. By virtue of slowing down the process of disintegration of apoptotic cells ("stathmo-apoptosis") FLICA can be used to estimate kinetics apoptosis in cell populations [73]. Because of paucity of fluorescent probes that can be applied to measure extent of apoptosis in living organisms the use *in vivo* FLICA reagents (defined "FLIVO") is of particular interest [74, 75].

Yet another group of the probes applicable to live cells are also already mentioned tandem molecules exploiting FRET of fluorescent proteins [38, 39, 76–78]. These probes can be reportedly used in real-time or near real-time assays featuring superior sensitivity and wealth of information as compared to standard end-point assays [76–78].

In closing the recent reports on innovative real-time bioassays provide a rationale for the development of a new generation of bioanalytical techniques with vast applications for tracking of differences in stochastic cell death sensitivity that often remain undetectable with conventional end-point analysis.

CONCLUSIONS AND FUTURE OUTLOOK

During the past decade mechanisms underlying cell death have entered into a focus of interest of many researchers in diverse fields of biomedicine and biotechnology. Considerable progress is currently being made in our understanding of a diversity of existing modes of programmed cell death. Burgeoning data show that although the elimination of many cells relies heavily on classical apoptotic pathways, the alternative, quasi-apoptotic and non-apoptotic mechanisms, may also be involved in a plethora of biological processes. In this context, there is an ever-increasing demand for convenient analytical tools to rapidly quantify and characterize diverse cell demise modes. Since cell death is a stochastic process, high-throughput single-cell analysis platforms are of essence to deliver meaningful insights into intrinsically heterogeneous cell populations. Development of virtually inert fluorescent probes with enhanced photostability would open up new horizons for many functional and real-time cytometric assays. There is also a strong reason to believe, that progress in novel technologies like imaging cytometry including laser scanning cytometry (LSC) and flow imaging cytometry, multispectral imaging cytometry and spectroscopic cytometry is just a prelude to a major transformation that the field will experience in the coming years.

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