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Apoptosis and Beyond: Cytometry in Studies of Programmed Cell Death

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Abstract

A cell undergoing apoptosis demonstrates multitude of characteristic morphological and biochemical features, which vary depending on the inducer of apoptosis, cell type and the “time window” at which the process of apoptosis is observed. Because the gross majority of apoptotic hallmarks can be revealed by flow and image cytometry, the cytometric methods become a technology of choice in diverse studies of cellular demise. Variety of cytometric methods designed to identify apoptotic cells, detect particular events of apoptosis and probe mechanisms associated with this mode of cell death have been developed during the past two decades. In the present review, we outline commonly used methods that are based on the assessment of mitochondrial transmembrane potential, activation of caspases, DNA fragmentation, and plasma membrane alterations. We also present novel developments in the field such as the use of cyanine SYTO and TO-PRO family of probes. Strategies of selecting the optimal multiparameter approaches, as well as potential difficulties in the experimental procedures, are thoroughly summarized.

I. Introduction

During the past decade mechanisms underlying cell death have entered into a focus of interest of many researchers in diverse fields of biomedicine. These mechanisms include a wide range of signaling cascades that regulate initiation, execution, and postmortem cell disposal mechanisms (Darzynkiewicz *et al.*, 1997, 2001b, 2004). The term *cell necrobiology* (biology of cell death) was introduced to collectively define all these cellular activities (Darzynkiewicz *et al.*, 1997; see *Cell Necrobiology* in Wikipedia). Particular interest in cell necrobiology comes from the appreciation of the multitude of complex regulatory circuits that control the cellular demise. Considerable progress is currently being made in our understanding of a diversity of existing modes of programmed cell death (Blagosklonny, 2000; Leist and Jaattela, 2001; Zhivotovsky, 2004). Burgeoning data show that although the elimination of many cells relies heavily on classical apoptotic pathways, the alternative, quasiapoptotic, and nonapoptotic mechanisms, may also be involved in a plethora of biological processes (Kroemer and Martin, 2005; Leist and Jaattela, 2001). Undoubtedly, the cell propensity to undergo classical apoptosis still remains a key mechanism in the pathogenesis of many human diseases (Brown and Attardi, 2005; Danial and Korsmeyer, 2004). Genetic alterations that affect circuitry of the apoptotic machinery are reportedly linked to many disorders that are characterized by either diminished (cancer) or excessive

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(neurodegeneration) proclivity of cells to suicide. Thus, the in-depth understanding of different regulators of apoptosis at molecular level offers vast opportunities for innovative pharmacological intervention (Brown and Attardi, 2005; Green and Kroemer, 2005).

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 often of essence to deliver meaningful insights into intrinsically heterogeneous cell populations (Darzynkiewicz *et al.*, 1997, 2004). Here, a gross majority of classical attributes of apoptosis can be quantitatively examined by flow and image cytometry, platforms that allow assessment of multiple cellular attributes on a single cell level (Darzynkiewicz *et al.*, 1997, 2001a, 2001b, 2004; Telford *et al.*, 2004). To date, diverse methods have been introduced that allow implementation of apoptotic assays on both live and/or fixed specimens (Darzynkiewicz *et al.*, 2001a, 2001b, 2004). Some of them have evolved toward commercially available kits supplied by countless vendors. Although kits offer an advantage of simplicity and easy step-by-step protocols, the information accompanying is generally enigmatic. Adequate information about chemistry of the components or even mechanistic principles of the kit is often lacking because of the proprietary nature of patented reagents (Darzynkiewicz *et al.*, 2004). Therefore, interpretation of the results and potential pitfalls may be particularly cumbersome for researchers unfamiliar with the biology of apoptosis. This chapter has been designed to complement the protocol-format literature by providing additional background information, methods' comparison, and discussion about advantages and limitations of commonly used assays. Some steps of individual methods are discussed to emphasize their critical role and avoid the likelihood of artifacts. We update also some earlier reviews on the application of cytometry in analysis of cell death (Darzynkiewicz *et al.*, 1992, 1994, 1997, 2001a, 2001b, 2004; Telford *et al.*, 2004).

II. The Biology of Apoptosis

Archetypically cells can disassemble in two morphologically and biochemically distinct processes: apoptosis and necrosis (Darzynkiewicz *et al.*, 1997; Kerr *et al.*, 1972; Lockshin and Zakeri, 2001). Both were initially identified based on characteristic changes in cell morphology (Kerr *et al.*, 1972). Despite subsequent development of numerous molecular markers, the morphological changes still remain the "gold standard" to define the mode of cell death (Darzynkiewicz *et al.*, 1997; Majno and Joris, 1995). Fig. 1 outlines major morphological and molecular changes occurring during apoptosis versus accidental cell death (herein termed necrosis). These were thoroughly discussed in some of our earlier reviews (Darzynkiewicz *et al.*, 1997, 2001b, 2004). Alterations in cellular parameters, as presented in Fig. 1, become a basis to development of specific markers for microscopy, cytometry, and molecular techniques (Darzynkiewicz *et al.*, 1997, 2001b, 2004). Importantly, however, constellation of apoptotic markers can vary depending on the stimuli and stress level, cell type, and unique cellular microenvironment that modulate cellular stress responses. In this context, some markers (such as oligonucleosomal DNA fragmentation) may not be detected in specimens challenged with divergent stimuli or microenvironmental conditions (e.g., cytokines, growth factor deprivation, heterotypic cell culture, etc.). It is, thus, always advisable to study several parameters at a time, which provide a multidimensional view of the advancing apoptotic cascade (Darzynkiewicz *et al.*, 1997, 2001b, 2004).

Noteworthy, recent reports have also provided closer insights into the mechanisms of cell death sentence and led to the characterization of several alternative demise modes (caspase-independent apoptosis-like PCD, autophagy, necrosis-like PCD, mitotic catastrophe) with serious connotations to disease pathogenesis and treatment (Edinger and Thompson, 2004;

Leist and Jaattela, 2001; Lockshin and Zakeri, 2002; Okada and Mak, 2004). These important discoveries also initiated an ongoing debate aiming at the definition and classification of different modes of cell death that is of particular importance for the development of novel cytometric assays (Blagosklonny, 2000; Zhivotovsky, 2004). The general term apoptosis, exploited commonly in many research articles, tends sometimes to misinterpret the actual mechanisms underlying cell suicide program (Leist and Jaattela, 2001; Zhivotovsky, 2004). Therefore, it has been postulated to restrict the term apoptosis to only the traditional cell demise program featuring all "hallmarks of apoptotic cell death," namely (i) activation of caspases as an absolute biomarker of cell death; (ii) condensation of chromatin; (iii) activation of endonucleases(s) causing internucleosomal DNA cleavage leading to extensive DNA fragmentation; (iv) appearance of distinctive cellular morphology with preservation of organelles; (v) cell dehydration leading to its shrinkage; (vi) plasma membrane blebbing; and (vii) nuclear fragmentation and formation of apoptotic bodies (Figs. 1 and 2; Blagosklonny, 2000; Leist and Jaattela, 2001; Zhivotovsky, 2004; Ziegler and Groscurth, 2004). The use of the general term apoptosis should be always accompanied by listing the particular morphological and/or biochemical apoptosis-associated feature(s) that was (were) detected. It is also advisable to exploit a plethora of different assays to cross-analyze action of, for example, novel anticancer compounds and bear in mind that the characteristic changes in cell morphology revealed by cell imaging (light or electron microscopy) still remain the gold standard in the ultimate classification of the cell demise mode (Darzynkiewicz *et al.*, 1997; King *et al.*, 2000; Smolewski *et al.*, 2003). Proper experimental approaches will help to avoid any potential misclassifications as the evidence accumulates that the roads to cellular disintegration represent a much more diverse and interconnected course than previously anticipated (Ferri and Kroemer, 2001; Leist and Jaattela, 2001).

Not surprisingly the development of novel functional probes for cell death and thorough understanding of the mechanisms underlying properties of existing ones are of utmost importance for the future progress in cell necrobiology (Darzynkiewicz *et al.*, 1997, 2001a, 2004). This is particularly relevant in view of the growing appreciation of the multitude of cell demise modes, and the need for sensitive and high-throughput cytometric assays capable to discriminate them.

III. Cytometry in Cell Necrobiology

The major advantages of flow cytometry include the possibility of multiparameter measurements (correlation of different cellular events at a time), single cell analysis (avoidance of bulk analysis), and rapid analysis of cell populations (thousand of cells per second) (Bonetta, 2005; Melamed, 2001). Flow cytometry overcomes, thus, a frequent problem of traditional bulk techniques such as fluorimetry, spectrophotometry, or gel techniques (e.g., Western blot, WB). These are based on analysis of a total cell population that averages the results from every given cell (Darzynkiewicz *et al.*, 1997, 2001a; Melamed, 2001). Moreover, by virtue of multiparameter analysis, cytometry allows correlative studies between many cell attributes based on both light scatter and fluorescence measurements (Darzynkiewicz *et al.*, 1997; Melamed, 2001; Robinson, 2006). For example, when cellular DNA content, the parameter that reports the cell cycle position, is one of the measured attributes, an expression of other measured attribute(s) can be then directly related to the cell cycle position without a need for cell synchronization (Darzynkiewicz *et al.*, 1997, 2004; Halicka *et al.*, 1997). Furthermore, the change in expression of particular cell constituents, or coexpression of different events, if correlated within the same cell, may yield clues regarding a possible cause–effect relationship between the detected events (Darzynkiewicz *et al.*, 1997, 2001b, 2004). It is why during the past two decades cytometric methodology has been applied in a gross majority of cell demise studies (Darzynkiewicz *et*

al., 1997, 2004; Halicka *et al.*, 1997; Huang *et al.*, 2005). Novel technologies such as cell imaging in flow and laser scanning cytometry (LSC) deliver even more sophisticated features that combine superior statistical power of cytometric analysis coupled with low-resolution imaging capabilities (Darzynkiewicz *et al.*, 1999; Deptala *et al.*, 2001; George *et al.*, 2004; Smolewski *et al.*, 2001). Finally, high-speed sorting capabilities of newly designed bench-top equipment expand further cytometric applications by allowing detailed studies on the purified cell subpopulations of interest (Eisenstein, 2006; Melamed, 2001). Expectedly, the current pace in the development of novel cytometric technologies will open up new horizons for future research on cell demise (Bernas *et al.*, 2006; Darzynkiewicz *et al.*, 2004; Robinson, 2004).

Applications of cytometry in cell necrobiology studies have archetypically two goals (thoroughly reviewed in Darzynkiewicz *et al.*, 2001a, 2004). One aim is to elucidate molecular mechanisms associated with cell death. Here cytometric assays have been applied to quantify the expression of cell constituents involved in apoptotic circuitry [such as members of the Bcl-2 protein family (caspases), inhibitors of caspases, etc.]. Cytometric methods have been also developed to study many changes in metabolic attributes, such as mitochondrial metabolism, redox status, intracellular pH or calcium fluxes. The second goal of cytometry in cell necrobiology is to estimate the viability of individual cells in a given population. This includes identification and quantification of dead cells and discrimination between apoptotic versus necrotic mode of death. Such discrimination is generally based on the change in cell morphology and/or on the presence of characteristic biochemical or molecular markers (Fig. 1). Most of these changes serve as markers to identify and quantify apoptotic cells by cytometry. To stress it again, morphological criteria (examined by the light, fluorescent, and electron microscopy) are still the “gold standard” to define the mode of cell death and confirm flow cytometric results (Darzynkiewicz *et al.*, 1997, 2004; Majno and Joris, 1995; Ziegler and Groscurth, 2004). Therefore, lack of microscopic examination may potentially lead to the misclassification and false-positive or -negative artifacts, and is a common drawback of the experimental design (Darzynkiewicz *et al.*, 1997, 2001a, 2004). The striking example of such misclassification is identification by flow cytometry of phagocytes that engulfed apoptotic bodies as individual apoptotic cells (Bedner *et al.*, 1999).

IV. Cytometric Methods to Detect Apoptosis

A. Light Scattering Changes in Apoptotic Cells

Flow cytometry allows quantitative measurements of laser light scatter characteristics that reflect morphological features of cells. Cell shrinkage due to the dehydration can be detected at early stages of apoptosis as a decrease in intensity of forward light scatter (FSC) signal (Ormerod *et al.*, 1995; Swat *et al.*, 1981). Either unchanged, or often increased side scatter signal (SSC, measured at 90° angle) is concomitantly observed as cell shrinkage; the condensation of nucleus and cytoplasm driven by cell dehydration leads to enhancement of light refraction and reflection (Fig. 3). When apoptotic cascade advances the cells become progressively smaller, and intensity of side scatter also decreases. Late apoptotic/secondary necrotic cells, therefore, are characterized by markedly diminished ability to scatter light in both, forward and right angle directions (Fig. 3). Necrosis, on the contrary, often proceeds through the simultaneous and rather drastic reduction in intensity of both light scatter parameters, which is believed to reflect rapid loss of the cell membrane integrity and leakage of cytoplasmic constituents. Primary necrotic cells fall, thus, into subpopulation similar to secondary necrotic cells and cannot be properly distinguished by light scattering measurements (Darzynkiewicz *et al.*, 1997, 2004; Majno and Joris, 1995).

It should be noted, however, that observable changes in light scattering are not a reliable marker of apoptosis or necrosis by themselves. Mechanically broken cells, isolated nuclei,

cell debris, and individual apoptotic bodies all display reduced light scatter properties and may be mistakenly accounted for as apoptotic cells. Furthermore, activation of tissue transglutaminase 2 (TGase 2) has recently been reported to influence light scattering properties detected by flow cytometry in some models of apoptosis (Darzynkiewicz *et al.*, 2004; Grabarek *et al.*, 2002). TGase 2 activity results here in protein crosslinking and enhancement of nuclear/cytoplasmic condensation. This is reflected by transient increase in intensity of the side scatter signal and moderate decrease in forward scatter signal. Conversely, apoptosis proceeding in absence of TGase 2 activation is reflected by the decrease in both forward and side scatter signals (Darzynkiewicz *et al.*, 2004; Grabarek *et al.*, 2002).

It should be stressed that morphological features revealed by laser light scattering in flow cytometry should be considered as auxiliary parameters and be used only in conjunction with more specific markers of cell death. However, novel platforms such as LSC and multispectral imaging cytometry (cell imaging inflow), by providing low-resolution imaging of individual cells and expanding analytical capabilities to morphometric analysis deliver substantial improvements over classical flow cytometry in cell necrobiology studies (Bedner *et al.*, 1999; Darzynkiewicz *et al.*, 1999; George *et al.*, 2004; Kamentsky, 2001; Pozarowski *et al.*, 2006).

B. Dissipation of Mitochondrial Transmembrane Potential ($\Delta\psi_m$)

The mitochondrion stands at the nexus of sensing and integrating diverse incoming stress signals, and mitochondrial disturbances often occur long before any marked morphological symptoms of apoptosis (Green, 2005; Skommer *et al.*, 2007). In recent years multiple mechanisms have been revealed that explain mitochondrial function in apoptosis, including release of apoptogenic proteins into the cytosol upon mitochondrial outer membrane permeabilization (MOMP), loss of mitochondrial physiological processes indispensable for cell survival and generation of reactive oxygen species (ROS). The MOMP is a fundamental event leading to a release of holocytochrome *c* (cyt *c*) and an array of cell death modulating small proteins such as AIF, EndoG, Omi/HtrA2, Smac/DIABLO, Smac *β*, normally enclosed in the intermembrane space of the organelle (Saelens *et al.*, 2004; van Gurp *et al.*, 2003). Dissipation of mitochondrial inner transmembrane potential ($\Delta\psi_m$) is frequently associated with MOMP (Kroemer, 1998; Zamzani *et al.*, 1996, 1998). There are, however, examples of divergence where loss of $\Delta\psi_m$ can precede, coincide, or follow MOMP (Li *et al.*, 2000; Skommer *et al.*, 2007). Interestingly, as described by us and others, dissipation of mitochondrial inner transmembrane potential may not be an ultimate point of no return for cell commitment to die (Milella *et al.*, 2002; Wlodkowic *et al.*, 2006).

The cytometric detection of $\Delta\psi_m$ loss is a sensitive marker of early apoptotic events. Procedures are based on lipofilic cationic probes that are readily taken up by live cells and accumulate in mitochondria according to the Nernst equation (Castedo *et al.*, 2002). The extent of their uptake, as measured by intensity of cellular fluorescence, is proportional to $\Delta\psi_m$ status (Fig. 4). Majority of $\Delta\psi_m$-sensitive probes are easily applicable for multiparameter detection with other apoptotic markers including caspase activation by fluorescently labelled inhibitors of caspases (FLICA), phosphatidylserine (PS) exposure by Annexin V and plasma membrane permeabilization by propidium iodide (PI) or YO-PRO 1 (Fig. 5; Castedo *et al.*, 2002; Pozarowski *et al.*, 2003; Wlodkowic *et al.*, 2006, 2007a).

In this context, lipophilic cationic fluorochromes rhodamine 123 (Rh123) or carboxycyanine dyes such as 3,3′-dihexiloxa-dicarbocyanine [$DiOC_6(3)$] can serve as markers of $\Delta\psi_m$ loss (Darzynkiewicz *et al.*, 1981, 1982; Johnson *et al.*, 1980). Historically, a combination of Rh123 and PI was introduced as a viability assay that discriminates between live cells that stain with Rh123 but exclude PI versus early apoptotic cells that lost ability to accumulate

Rh123 versus late apoptotic/necrotic cells that stain with PI only (Darzynkiewicz *et al.*, 1982, 1994). The specificity of Rh123 and $DiOC_6(3)$ as selective $\Delta\psi_m$-sensitive probes has been questioned (Salvioli *et al.*, 1997). The apparent controversy may be due to the fact that to be a specific marker of $\Delta\psi_m$ Rh123 or $DiOC_6(3)$ has to be used at low concentration (≤1 μM), which was not the case in many studies. The alternative probes such as chloromethyltetramethylrosamine analogues or tetramethylrhodamine esters have became now more widely used to detect mitochondrial depolarization during apoptosis. MitoTracker™ dyes (chloromethyltetramethylrosamine analogues) were introduced by Molecular Probes Inc. as new mitochondrial potential markers (Haughland, 2003). One of them is MitoTracker Red CMXRos, a probe considered to be highly sensitive and specific to $\Delta\psi_m$ and (Castedo *et al.*, 2002; Pendergrass *et al.*, 2004; Poot and Pierce, 1999). The previously reported retainability of CMXRos after fixation with formaldehyde was recently challenged by some authors (Ferlini *et al.*, 1998; Macho *et al.*, 1996; Poot and Pierce, 1999). It has been shown that although uptake of CMXRos by live cells is a function of mitochondrial gradient, its retention following fixation depends rather on the availability of intra-mitochondrial thiols (Poot and Pierce, 1999). Thus, it is not advisable to apply CMXRos with measurements of another cell attributes that require subsequent cell fixation such as DNA fragmentation detected by the TDT-mediated dUTP-biotin nick-end labeling (TUNEL) assay or immunocytochemical detection of intracellular protein (see below).

Other useful probes sensitive to $\Delta\psi_m$ changes are tetramethylrhodamine methyl ester perchlorate (TMRM) and tetramethylrhodamine ethyl ester perchlorate (TMRE). The application of TMRM combined with the marker of caspase activation (FLICA) and small cyanine cation YO-PRO 1 is illustrated in Fig. 5 (Pozarowski *et al.*, 2003; Wlodkowic *et al.*, 2006). Due to convenient spectral characteristics these probes are especially useful for multiparameter assays combining diverse apoptotic markers (Pozarowski *et al.*, 2003; Wlodkowic *et al.*, 2006, 2007a, 2007b).

Major improvements have recently been made by implementing ratiometric J-aggregate forming cationic fluorochromes: JC-1 (5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolcarbocyanine iodide) and JC-9 (3,3′-dimethyl-*αα*-naphthoxacarbocyanine iodide) (Cossarizza and Salvioli, 2001; Pritchard *et al.*, 2001). Their uptake by energized mitochondria leads to formation of aggregates in the mitochondrial matrix and emission of orange/red fluorescence. Loss of $\Delta\psi_m$ leads to dissociation of the J-aggregates and transition to monomeric, cytoplasmic form that exhibits green fluorescence (Fig. 4). The major disadvantage of J-aggregate probes lies in occupation of crucial fluorescent channels that makes it difficult to multiplex on single 488 nm laser instrumentation. Furthermore, poor solubility of these probes in aqueous media may occasionally lead to staining artifacts. Nevertheless, when used concurrently with violet or red excitable fluorochromes they offer substantial improvements over traditional mitochondrial probes.

Finally, probes such as 10-nonyl acridine orange (NAO), MitoFluor Green, and MitoTracker Green were previously advertised as markers of mitochondrial mass that are insensitive to $\Delta\psi_m$ changes (Pendergrass *et al.*, 1997; Ratinaud *et al.*, 1988). For increased sensitivity it was, thus, proposed to simultaneously measure both $\Delta\psi_m$ and mitochondrial mass with a combination of $\Delta\psi_m$-sensitive and $\Delta\psi_m$-insensitive probes (e.g., Petit *et al.*, 1995). Disappointingly, further observations revealed that all three probes are dependent on changes in $\Delta\psi_m$ and cannot be used as mere markers of mitochondrial mass (Keij *et al.*, 2000). NAO can be, however, conveniently applied to track peroxidation of mitochondrial cardiolipin by flow cytometry. This simple assay measures an early event that is a prerequisite for cytochrome *c* release during apoptosis (Castedo *et al.*, 2002; Garcia Fernandez *et al.*, 2004).

Measurement of $\Delta\psi_m$ is particularly sensitive to changes in cellular environment. Therefore, samples assigned for comparison should be incubated and measured under identical temperature, pH, time elapsed between the onset of incubation and fluorescence measurement. Moreover, according to Nernst equation, the intra-cellular distribution of any cationic mitochondrial probe reflects the differences in the transmembrane potential across both the plasma membrane (i.e., between exterior vs. interior of the cell) and the outer mitochondrial membrane (Castedo *et al.*, 2002; Shapiro, 2003). Thus, apart from mitochondria the probes can also accumulate in the cytosol. This is facilitated by both active and passive transport across the plasma membrane. To decrease the passive transport, mitochondrial probes should be used at their lowest possible concentration, that is, the minimal concentration that is still adequate to detect mitochondrial changes. This may, however, necessitate relatively high settings of the photomultiplier (photomultiplier voltage) and higher laser power outputs (Darzynkiewicz *et al.*, 2004). Caution should be also taken, as cationic probes may be targeted to other organelles like endoplasmic reticulum (ER) or lysosomes. Moreover, accumulation of some probes may be influenced by the activity of multidrug efflux pumps (MDR).

In each experiment it is advisable to assess probes' specificity by preincubation of cells for 20–30 min with 50–100 μM protonophores CCCP or FCCP. Both agents cause a collapse of the mitochondrial transmembrane potential and are used as positive controls (Castedo *et al.*, 2002; Darzynkiewicz *et al.*, 2004).

C. Activation of Caspases

One of the hallmarks of classical apoptosis is the activation of unique cysteine aspartyl-specific proteases having a conserved QACXG consensus site containing active cysteine, called caspases (from *c*ysteinyl *asp*artate-specific prote*ases*; Fig. 2) (Alnemri *et al.*, 1996; Kaufmann *et al.*, 1993; Thornberry and Lazebnik, 1998). In mammals there are probably at least 14 members of the caspase family proteins that form a closely related family of proteases (Boyce *et al.*, 2004; Zhivotovsky, 2003). Although individual caspases have specific functions, some degree of overlapping specificity and redundancy among them is apparent (Earnshaw *et al.*, 1999). At present only eight caspases are known to participate in execution of apoptotic cell dismantling (caspase-2, -3, -6, -7, -8, -9, -10, -12). Remaining members of the caspase family participates in cytokine processing and inflammatory responses (Boyce *et al.*, 2004; Lavrik *et al.*, 2005; Zhivotovsky, 2003). Under normal physiological conditions caspases are constitutively expressed in the cytoplasm as zymogens with very low intrinsic activity. They become activated upon transcatalytic cleavage followed by dimerization. Specifically, cleaved molecules assemble to form a single heterotetramer with two active enzymatic sites in head-to-tail configuration (Earnshaw *et al.*, 1999; Zhivotovsky, 2003). Once activated, caspases function in an orchestrated proteolytic cascade leading to self-amplification, cleavage of vital cell substrates, and ultimate cell disassembly (Fig. 2; Earnshaw *et al.*, 1999; Zhivotovsky, 2003). Several methods were developed to detect activation of caspases by flow and laser scanning cytometry (thoroughly reviewed in Darzynkiewicz *et al.*, 2001b, 2004; Telford *et al.*, 2004). Here, we outline two commonly used techniques based on the affinity labeling of the caspase active centers and cleavage of the poly (ADP-ribose) polymerase (PARP).

1. Fluorochrome-Labeled Inhibitors of Caspases (FLICA)—Use of *f*luorochrome-*l*abeled *i*nhibitors of *ca*spases (FLICA, recognized also under commercial names: CaspaTag, CaspACE, CaspGLOW, FLIVO) allows for a convenient estimation of apoptosis by both cytometry and fluorescence microscopy (Figs. 5B and 6A; Bedner *et al.*, 2000; Smolewski *et al.*, 2001). FLICAs were designed as affinity ligands to active centers of individual caspases (Bedner *et al.*, 2000; Pozarowski *et al.*, 2003; Smolewski *et al.*, 2001). Each

molecule has three functional domains: (i) the fluorochrome (carboxyfluorescein, FAM; fluorescein, FITC; or sulforhodamine, SR), (ii) the caspase recognition element comprising of a four amino-acid peptide, (iii) the chloro- or fluoromethyl ketone (CMK or FMK) binding moiety (Bedner *et al.*, 2000; Pozarowski *et al.*, 2003). The specificity toward individual caspases is provided by the recognition element. Currently, several FLICA kits are commercially available. The most common contains the valyl-alanyl-aspartic acid residue sequence (VAD). The VAD sequence allows binding to activated caspase-1, -3 -4, -5, -7, -8, and -9 providing a pan-caspase marker. Other inhibitors were subsequently developed and contain DVAD, DEVD, VEID, YVAD, LETD, LEHD, or AEVD peptide residues. They preferentially bind to activated caspase-2, -3, -6, -1, -8, -9, or -10, respectively. After docking of the FLICA molecule to the caspase active center, the FMK reacts with the active cysteine and forms a thiomethyl ketone (Thornberry *et al.*, 1997; van Noorden, 2001). This irreversible, covalent reaction is deemed to inactivate the target enzyme. Presence of the fluorescent tag (FITC or SR) allows detection of FLICA–caspase complexes inside the cells.

FLICAs are highly permeant to plasma membrane and relatively nontoxic. This provides a unique opportunity to detect caspase activation in living cells where uptake of these reagents is followed by covalent binding to activated caspases. To date, no interference resulting by MDR efflux pump activity has been reported for FLICA uptake. Unbound FLICAs are readily removed from the cells that lack caspase activity by rinsing with PBS buffer. When FLICAs are applied together with the plasma membrane permeability marker PI, several consecutive stages of apoptosis can be distinguished (Fig. 6A) (Pozarowski *et al.*, 2003; Smolewski *et al.*, 2001). Green fluorescent FLICAs (FAM, FITC) can also be used together with $\Delta\psi_m$ sensitive probes, such as MitoTracker Red CMXRos and TMRM as shown in Fig. 5B (Pozarowski *et al.*, 2003; Wlodkowic *et al.*, 2006). Moreover, other multiplexing combinations are compatible with both single and multilaser instrumentation.

Because intracellular binding is covalent, FLICAs withstand cell fixation (with formaldehyde) and subsequent cell permeabilization with ethanol and methanol. As a result, this assay can be combined with the analysis of cell attributes that can require prior cell permeabilization such as DNA content measurement, DNA fragmentation (TUNEL assay), and so on.

Recent reports shed, however, new light onto the FLICA binding mechanistic during apoptosis and cast doubts onto their absolute specificity toward caspase active centers (Kuzelova *et al.*, 2007; Pozarowski *et al.*, 2003). Namely, in apoptotic cells only a minor proportion of total FLICA binding was attributed to their FLICA–caspase interactions (Pozarowski *et al.*, 2003). Likewise there is also no significant competition for the binding sites between FLICAs and unlabelled caspase inhibitors (e.g., z-VAD-fmk, z-DEVD-fmk) that are based on the same principle (Pozarowski *et al.*, 2003). In the recent report, Kuzelova *et al.* (2007) confirmed that the overall fluorescence intensity of apoptotic cells labeled with FLICA does not reflect unique binding to caspase active centers. Moreover, FLICA appears to be incapable to arrest apoptosis a feature that initially formed the basis of “stathmo-apoptosis” assay (Pozarowski *et al.*, 2003; Smolewski *et al.*, 2002). These inconsistencies may be due to contamination of the early batches of FLICAs with the unlabelled caspase inhibitors. It remains evident, however, that other cellular constituents apart from caspase active centers contribute to FLICA staining.

As FLICA reagents withstand fixation, this strongly suggests covalent interactions with the intracellular targets becoming accessible in the course of apoptosis (Darzynkiewicz and Pozarowski, 2007; Kuzelova *et al.*, 2007; Pozarowski *et al.*, 2003). The reactivity of FMK moiety with intracellular thiols may provide some explanation of these interactions. In this

context, opening of the disulfide cysteine bridges (inter- and/or intramolecular) may provide as yet unidentified affinity sites (Darzynkiewicz and Pozarowski, 2007). Noncovalent hydrophobic interactions between fluorochrome domain and intracellular targets have also been postulated to play a role in FLICA retention (Pozarowski *et al.*, 2003). It should be stressed that the covalent labeling of apoptotic cells with FLICA make these probes, called FLIVO, the markers of choice for detection of apoptosis *in vivo*, both in real time and after fixation of the tissue (Griffin *et al.*, 2007).

In any case FLICA reagents have proven to be reliable and sensitive markers of apoptotic cell death. Necrotic cells do not exhibit FLICA staining and caspase-3 activation assay correlates well with results obtained by FLICA. Recently published data suggest also their superior applicability in a plethora of multiparametric applications. Nevertheless, in light of recent reports one should be aware that staining with FLICA apparently does not represent affinity labeling of individual caspase active centers (Darzynkiewicz and Pozarowski, 2007; Pozarowski *et al.*, 2003).

The alternative approach for assessment of caspases activation involves the use of caspase substrates that upon cleavage generate fluorescent products (Lee *et al.*, 2003; Telford *et al.*, 2002). Another assay is based on the use of substrates consisting of two variants of fluorescent protein that differ in emission spectrum connected with a peptide linker whose cleavage by caspase leads to a loss of fluorescence resonance energy transfer (FRET) between the respective fluorescent proteins (He *et al.*, 2004; Lee and Segal, 2004).

It should be underscored that the use of labeled or unlabeled caspase inhibitors as well as caspase substrates poses uncertainty with respect to their specificity. The tetrapeptide moiety of these reagents is designed to confer their specificity. However, in studies of live cells they are used at four orders of magnitude higher concentration (20–50 μM) than their binding constants (0.2–2.2 nM) estimated on isolated caspases (Thornberry *et al.*, 1997). Since their intracellular concentration and *in situ* accessibility to active caspase centers are unknown the published data on specificity of individual caspases detection should be in treated with a reservation.

Immunocytochemical detection of activated (cleaved) caspases essentially has no problems with specificity provided that the antibody does not cross-react with other proteins. Antibodies to different activated caspases are available from variety of vendors. Flow cytometric analysis of immunocytochemically detected caspase-3 activation concurrently with DNA content (cell cycle analysis) has been reported most frequently (e.g., Pozarowski *et al.*, 2003; Tanaka *et al.*, 2007).

2. Detection of PARP Cleavage—Another approach to study caspase activation is based on the analysis of the cleavage of specific caspase substrates. In this context, PARP is known as one of the characteristic endogenous “death substrates.” PARP is a nuclear enzyme involved in DNA repair that is activated in response to DNA damage (de Murcia and Menissier-de Murcia, 1994). Following initiation of proteolytic cascade, PARP is cleaved by executioner caspase-3 and -7, which is considered as hallmark of classical apoptosis (Alnemri *et al.*, 1996; Kaufmann *et al.*, 1993; Lazebnik *et al.*, 1994). The specific cleavage results in generation of 89- and 24-kDa fragments that can be easily detected on Western blots. An antibody that recognizes the 89-kDa product of PARP cleavage has been adapted to label apoptotic cells for detection by both flow and laser scanning cytometry (Li and Darzynkiewicz, 2000). Since measurement of DNA content provides valuable information about the cell cycle position and DNA ploidy, attempts have been made to combine PARP cleavage assay with DNA labeling. Multiparameter analysis of the cells

differentially stained for PARP p89 and DNA and correlating apoptosis with the cell cycle phase is shown in Fig. 6B (Li and Darzynkiewicz, 2000; Li *et al.*, 2000).

Because of the immunocytochemical detection principle, the assay requires prior cell fixation (with formaldehyde) and subsequent permeabilization (usually with ethanol). It should be stressed that the methanol-free formaldehyde obtained by hydrolysis of paraformaldehyde is often incorrectly named “paraformaldehyde.” Paraformaldehyde is the condensed, polymerized, solid state of formaldehyde. Since alcohol preserved samples may be stored for extended periods of time, this assay is particularly suitable for analysis of archive sample collections. Extensive kinetic studies are also straightforward as cells may be collected, fixed at the respective time intervals, and subsequently mass analyzed. Bias related to differential labeling conditions and/or progression of apoptotic cascade during the period of cell preparation is thus avoided. It should be noted that to enhance permeability of plasma membrane and to increase accessibility of the detected epitope (e.g., the cleaved 89-kDa form of PARP) to the primary Ab (and also to secondary Ab, if needed) a nonionic detergent (e.g., Triton X-100) at final concentration 0.1% into the solution containing Ab is often included, together with the blocking reagent (1% w/v bovine serum albumin).

D. Changes in the Plasma Membrane During Apoptosis

To preserve physiological functions each cell strives to maintain an intact plasma membrane. The preservation plasma membrane integrity until late stages of cellular disintegration is also a distinctive feature of apoptosis that differentiate this process from accidental cell death, necrosis (Darzynkiewicz *et al.*, 1997, 2004; Majno and Joris, 1995). Plasma membrane represents, thus, an active and dynamic organelle that plays an important part in the cascade of signaling events leading to a final removal of dying cell. However, alterations in both lipid composition and permeability to small cationic probes have been reported as relatively early signs of apoptotic cascade (Idziorek *et al.*, 1995; Koopman *et al.*, 1994; van Engeland *et al.*, 1998). These usually follow $\Delta\psi_m$ collapse, caspase activation and chromatin condensation but precede nuclear disassembly and DNA laddering (van Engeland *et al.*, 1998). Here, we describe common markers for both hallmarks that allow convenient analysis of live cells by flow cytometry.

1. Externalization of Phosphatidylserine—A characteristic feature of healthy cell is the asymmetric distribution of plasma membrane phospholipids between inner and outer leaflets. Under physiological conditions, choline phospholipids (phosphatidylcholine, sphingomyelin) are exposed on the external leaflet while aminophospholipids (phosphatidylserine, phosphatidylethanolamine) are exclusively located on the cytoplasmic surface of the lipid bilayer. This asymmetry is scrambled during apoptosis when PS, constituting less than 10% of the total membrane phospholipids, becomes exposed on the outside leaflet of the membrane (Fadok *et al.*, 1992; Koopman *et al.*, 1994; van Engeland *et al.*, 1998). Exposition of PS on cell surface provides signaling to macrophages, which then become attracted and initiate to phagocytize apoptotic cells and apoptotic bodies. The detection of exposed PS allows for a precise estimation of apoptotic incidence. The assay usually employs fluorochrome-tagged 36-kDa anticoagulant protein Annexin V (van Engeland *et al.*, 1998). This probe reversibly binds to PS residues in the presence of millimolar concentration of divalent calcium ions. Annexin V conjugated to fluorochromes of different absorption and emission wavelength has found many applications as a marker of apoptotic cells, in particular for their detection by flow cytometry and fluorescence microscopy (van Engeland *et al.*, 1998; Van Genderen *et al.*, 2006). Noteworthy, a C2A domain of Synaptotagmin I exhibits similar properties to Annexin V and was successfully used in cytometric applications (Jung *et al.*, 2004). The cells become reactive with Annexin V prior to the loss of the plasma membrane’s ability to exclude cationic dyes such as PI or

7-aminoactinomycin D (7-AAD). Thus, when using Annexin V in conjunction with plasma membrane permeability marker a distinction can be made between live, apoptotic, and late apoptotic/secondary necrotic cells. Live cells stained with fluorochrome-tagged Annexin V and PI, have minimal Annexin V fluorescence and minimal PI fluorescence. At the early stages of apoptosis, cells stain brightly with Annexin V but still exclude PI. Finally, when apoptotic cascade advances to later stages, the secondary necrotic cells stain intensely with both probes. Of note the primary necrotic cells will also fall into the last group as Annexin V will penetrate cells with ruptured membrane and stain PS residues displayed on the inner leaflet of the plasma membrane. Moreover, cells with severely damaged membranes and very late apoptotic cells stain rapidly and strongly with PI and may not exhibit Annexin V staining. It should also be mentioned that even intact and live cells may become permeable to PI upon prolonged incubation times. Therefore, cytometric analysis should be performed shortly after addition of this dye. Our recent studies revealed also that the time-window of apoptosis detected by FLICA binding is much wider than by the Annexin V binding (Pozarowski *et al.*, 2003). These data also suggest that activation of caspases is a prerequisite for externalization of PS since essentially no FLICA-negative cells that bind Annexin V are apparent (Pozarowski *et al.*, 2003).

Although commonly applied, the interpretation of results from Annexin V assay may be difficult after mechanical disaggregation of tissues to isolate individual cells, enzymatic (e.g., by trypsinization) or mechanic detachment (e.g., by “rubber policeman”) of adherent cells from culture flasks, cell electroporation, chemical cell transfection, or high-titer retroviral infections. All these conditions have been reported to influence PS flipping and introduce substantial experimental bias. Interestingly, a high surface expression of PS has also been detected on some healthy cells such as differentiating monocytes, activated T cells, positively selected B lymphocytes, activated neutrophils, or myoblasts fusing into myotubes (Callahan *et al.*, 2003; Elliott *et al.*, 2005; van den Eijnde *et al.*, 2001; Van Genderen *et al.*, 2006). Furthermore, as PS serves as “eat me” signal for professional phagocytes, healthy macrophages/monocytes, become Annexin V positive upon ingestion of apoptotic bodies. In all these instances Annexin V binding may be mistakenly identified as a marker of apoptotic cells leading to false-positive identification of nonapoptotic cells (Marguet *et al.*, 1999). Noteworthy, there are increasing examples of programmed cell death proceeding without exposure of PS, which may bring in false-negative bias when relying solely on Annexin V assay (King *et al.*, 2000).

Currently, a range of Annexin V conjugates with organic fluorescent probes is commercially available with the predominant popularity of FITC, PE, and APC conjugates. There is also a considerable interest in inorganic, semiconductor nano-crystals (Quantum Dots; QDs) conjugates (Dicker *et al.*, 2005; Le Gac *et al.*, 2006). Their significant advantages over currently available organic fluorochromes are rapidly attracting attention in both cytometric and imaging applications (Chattopadhyay *et al.*, 2006; Jaiswal and Simon, 2004; Jaiswal *et al.*, 2003). Moreover, a recent development from Alexis-Axxora introduced fluorescently labeled monoclonal antibodies against PS residues that can be used instead of Annexin V. This new class of reagents reportedly alleviates dependence on calcium-supplemented buffers without compromising sensitivity of detection. Progress is also being made in the field of inorganic zinc coordination complexes (fluorescent Zn^{2+}–dipicolylamines, DPA) that under Ca^{2+}-free conditions selectively bind to membranes enriched in anionic phospholipids (Hanshaw *et al.*, 2005; Koulov *et al.*, 2003). Finally, a small cationic molecule merocyanine 540 (MC540) can reportedly be used to detect apoptotic cells based on the altered phospholipids composition (Laakko *et al.*, 2002).

2. Changes in Plasma Membrane Permeability—Externalization of anionic phospholipids is not a sole hallmark occurring early during apoptosis at the cell surface.

Structural integrity and most of the plasma membrane transport function are preserved during the early phase of apoptosis. However, the permeability to certain fluorochromes, such as 7-AAD, Hoechst 33342, or Hoechst 33258 is increased (Ormerod *et al.*, 1993; Schmid *et al.*, 1992, 2007). Recent work by Idziorek *et al.* (1995) also revealed that following initiation of apoptotic cascade plasma membrane becomes selectively permeable to small, cationic molecules such as cyanine dyes. At the same time it remains impermeable to larger cations such as PI or 7-AAD. Live, noninduced to apoptosis cells, exclude both classes of probes. As a result, a new assay has been developed based on green florescent YO-PRO 1 and more recently violet fluorescent PO-PRO 1 cyanine probes (Idziorek *et al.*, 1995). Of note, violet excitable PO-PRO 1 probe features similar properties to YO-PRO 1 and can provide increased multiplexing capabilities on high-end analyzers. The assay is rapid and only short incubation (20 min, at RT) is required to supravitally discriminate viable cells (YO-PRO $1^{neg}/PI^{neg}$ events) from early apoptotic cells characterized by initial cell membrane permeabilization (YO-PRO $1^{+}/PI^{neg}$ events). Cells in late stages of apoptosis and primary necrotic cells are characterized by pronounced loss in cell membrane integrity, and are thus permeable to both YO-PRO 1 and PI probes (YO-PRO $1^{+}/PI^{+}$ events) (Idziorek *et al.*, 1995; Wlodkowic *et al.*, 2007a).

Some reports have recently postulated that entry of YO-PRO 1 cation (629 Da) into early apoptotic cell depends on the activation of P2X7 ion-gated channel, event concurrent with scramblase activation and PS externalization (Holme *et al.*, 2007). Early changes in lipid composition, structural relaxation, and/or impaired active dye efflux cannot, however, be also excluded as similar hypotheses have previously been raised for bisbenzimide dye, Hoechst 33342 (Idziorek *et al.*, 1995; Ormerod *et al.*, 1993; Schmid *et al.*, 2007). Interestingly, our recent studies revealed that the time-window of apoptosis detected by YO-PRO 1 when analyzed by multiparameter flow cytometry is substantially wider than assessed by Annexin V binding (Wlodkowic *et al.*, 2007a; unpublished data). Comparable results are also often achieved when $\Delta\psi_m$ selective probe TMRM is used in conjunction with YO-PRO 1 (Wlodkowic *et al.*, 2007a). These observations reinforce the notion that YO-PRO 1 is a convenient and sensitive marker of early apoptotic events. Caution should be, however, exercised as dye uptake and/or efflux may vary between different cell types and some cells may not exhibit differential staining with YO-PRO 1 and PI. This holds to be especially true in cells of murine origin such as FL5.12, BaF3, and primary fetal-liver progenitors (Wlodkowic *et al.*, unpublished data). Result achieved by means of YO-PRO 1 or PO-PRO 1 may, thus, introduce false-negative bias if not confirmed by other methods.

Finally, similar to the Annexin V binding assay, the interpretation of results from YO-PRO 1 assay may be difficult after enzymatic (e.g., by trypsinization) or mechanic detachment (e.g., by “rubber policeman”) of adherent cells from culture flasks, cell electroporation, chemical cell transfection, or high-titer retroviral infections. Furthermore, some drugs or culture conditions may distort lipid bilayer structure leading to enhanced permeability in the absence of apoptosis. It is always advisable to test selective uptake of cyanine dyes by apoptotic cells in every new experimental system.

E. Nuclear Hallmarks of Apoptosis

Upon initiation of executioner caspase-3 and -7, caspase-activated DNase (CAD/DFF40) becomes activated by the cleavage of its putative inhibitor (ICAD/DFF45) (Enari *et al.*, 1996). CAD translocates then to the nucleus where its activity leads to characteristic DNA fragmentation (Arends *et al.*, 1990; Kerr *et al.*, 1972; Nagata, 2000). Although CAD is the best-characterized enzyme, DNase-I, DNase-II, DNase-X, and AIF are also postulated in the execution of DNA degradation (Barry and Eastman, 1993; Los *et al.*, 2000; Peitsch *et al.*, 1993; Sussin *et al.*, 1999). Apoptotic DNA fragmentation proceeds in three consecutive steps: (i) type-I DNA fragmentation (high molecular weight fragmentation to 0.05–1 Mb

sections); (ii) type-II DNA fragmentation (intermediate fragmentation to 300 kb sections); and (iii) type-III DNA fragmentation (internucleosomal fragmentation to mono- and oligonucleosomal sections). The latter is often detected by a characteristic pattern during agarose DNA electrophoresis (DNA-ladder) and considered as a hallmark of apoptosis (Nagata, 2000; Nagata *et al.*, 2003). Of note, DNA fragmentation during classical apoptosis may be terminated at 50–300 kb fragments. As a result characteristic "DNA-ladder" is absent due to a lack of internucleosomal-sized fragments (Darzynkiewicz *et al.*, 1997; Oberhammer *et al.*, 1993).

Not surprisingly DNA fragmentation provided basis for two commonly used cytometric assays that allow identification of apoptotic cells: (i) estimation of fractional DNA content (sub-G_1 fraction; Gong *et al.*, 1994; Nicoletti *et al.*, 1991; Umansky *et al.*, 1981) and (ii) labeling of DNA strand breaks (DSBs) with fluorochrome-tagged deoxynucleotides by exogenous terminal deoxynucleotidyltransferase, TdT (TUNEL; Gorczyca *et al.*, 1992, 1993; Li and Darzynkiewicz, 1995; Li *et al.*, 1996) (Fig. 7).

1. Assessment of Fractional DNA Content (Sub-G_1 Fraction)—The fragmented, low molecular weight DNA can be extracted from cells during the process of cell staining in aqueous solutions. Such extraction takes place when the cells are treated with detergent and/or hypotonic solution instead of fixation, to make them permeable to fluorochrome. Alternatively fixation in precipitating fixatives such as ethanol can be used for the same purpose. Fixation with crosslinking fixatives such as formaldehyde, on the other hand, results in the retention of low molecular weight DNA in the cell as they become crosslinked to intercellular proteins. Therefore, a formaldehyde fixation is incompatible with the "sub-G_1" assay. As a result of DNA extraction, apoptotic cells exhibit a deficit in DNA content. Following staining with a DNA-specific fluorochrome they can be recognized by cytometry as cells having fractional DNA content. On the DNA content frequency histograms it is often characterized by a distinctive "sub-G_1" peak (Fig. 7A, Gong *et al.*, 1994; Nicoletti *et al.*, 1991; Umansky *et al.*, 1981). Interestingly, apoptotic DNA fragmentation detected by several distinctive sub-G_1 peaks has recently been reported as a discontinuous process, relaying on sequential activation of different deoxynucleases and also modulated by chromatin structure (Kajstura *et al.*, 2007).

Optimally, the "sub-G_1" peak representing apoptotic cells should be separated with little or no overlapping from the G_1 peak of the nonapoptotic cell population. However, the degree of low molecular weight DNA extraction varies markedly depending on the extent of DNA degradation (duration of apoptosis), the number of cell washings, pH, and molarity of the washing/staining buffers. Shedding of apoptotic bodies containing fragments of nuclear chromatin may also contribute to the loss of DNA from apoptotic cells. As a result, the separation of "sub-G_1" is not always satisfactory. On the other hand, when DNA degradation does not proceed to internucleosomal regions but stops after generating 50–300 kb fragments (Oberhammer *et al.*, 1993), little DNA can be extracted. This method fails, thus, to detect such atypical apoptotic cells. Furthermore, the loss of DNA from G_2/M and late S-phase cells undergoing apoptosis, may be inadequate to generate clear "sub-G_1" peak. In such situations cells often end up with DNA content equivalent to that of G_1 or early S phase and are indistinguishable during cytometric analysis. Noteworthy, a reduced stainability with DNA fluorochromes, that resembles fractional DNA content, may be present during cell differentiation or even necrosis (Darzynkiewicz *et al.*, 1984; Oberhammer *et al.*, 1993). Unfortunately numerous investigators still apply sub-G_1 analysis as the sole method for enumeration of apoptotic cells. Because without additional assays fractional DNA content cannot be used as decisive marker of cell death caution should be exercised interpreting such data.

It is a common practice to use detergents or hypotonic solutions instead of fixation in DNA staining protocols (Nicoletti *et al.*, 1991). This simple approach causes lysis of plasma membrane and nuclear isolation and yields excellent resolution for DNA content analysis. When used to quantify apoptotic cells, however, this method is poised to generate a significant bias. Namely, nuclei of apoptotic cells are often fragmented and upon cell lysis a multiplicity of chromatin fragments/nuclear bodies are released from a single cell. Lysis of mitotic cells additionally releases individual chromosomes and/or chromosome aggregates. Furthermore, after cell irradiation or treatment with clastogens the generated micronuclei are often released during hypotonic procedures. As a result, each nuclear fragment, chromosome or micronucleus is recorded by flow cytometer as an individual object with sub-G_1 DNA content. Such objects are then erroneously classified as individual apoptotic cells. This bias is particularly pronounced when logarithmic scale is used to display DNA content on the histograms, which allows one to detect objects with minute DNA content such as 0.1% of that of G_1 cells. These events certainly cannot be classified as individual apoptotic nuclei, and their percentage overestimates the actual percentage of apoptotic cells in the sample.

2. Assessment of DNA Strand Breaks (TUNEL Assay)—DNA fragmentation during apoptosis generates a multitude of DSBs in the nucleus (Arends *et al.*, 1990; Oberhammer *et al.*, 1993). The 3′-OH termini of the breaks may be marked by attaching a fluorochrome to them. This is generally done directly or indirectly (e.g., via biotin or digoxygenin) by using fluorochrome-labeled triphosphodeoxynucleotides in a reaction catalyzed preferably by exogenous terminal deoxynucleotidyltransferase (Gorczyca *et al.*, 1992, 1993; Li and Darzynkiewicz, 1995; Li *et al.*, 1996). The reaction is commonly known as TUNEL from "*T*DT-mediated d*U*TP-biotin *n*ick-*e*nd *l*abeling" (Gavrieli *et al.*, 1992). This acronym is a misnomer since the double strand breaks are labeled rather than the single strand *nick*s. Furthermore, other than dUTP deoxynucleotides are often used in this assay. Of all the deoxynucleotides BrdUTP appears to be the most advantageous to label DSBs, in terms of high sensitivity, low cost and simplicity of the assay (Li and Darzynkiewicz, 1995; see Fig. 7B). BrdU attached to DSBs (as poly-BrdU) is detected with an FITC-conjugated anti-BrdU Ab; the very same Ab that is used to detect BrdU incorporated during DNA replication (Fig. 7B). Poly-BrdU at the DSBs, however, is accessible to the Ab without acid- or heat-induced DNA denaturation, which otherwise is needed to detect the precursor incorporated during DNA replication.

The detection of DSBs by this assay requires cell prefixation with a crosslinking reagent such as formaldehyde, which unlike ethanol, prevents the extraction of small DNA fragments. Labeling DSBs in this procedure, which utilizes fluorescein-conjugated anti-BrdUAb, can be combined with staining of DNAwith the fluorochrome of another color (PI, red fluorescence). Cytometry of cells that are differentially stained for DSBs and for DNA allows one to distinguish apoptotic from nonapoptotic cell subpopulations and reveal the cell cycle distribution in each of these subpopulations (Fig. 7B; Gorczyca *et al.*, 1992, 1993). Since late apoptotic cells may have diminished DNA content because of prior shedding of apoptotic bodies or due to such extensive DNA fragmentation that small DNA fragments cannot be retained in the cell after fixation with formaldehyde such cells may have sub-G_1 DNA content and be TUNEL-positive. Several types of kits are commercially available, which utilize either directly fluorochrome-tagged triphospho deoxynucleotides or BrdUTP and BrdU Ab.

The extensive DNA fragmentation during apoptosis, similar to radiation-induced DNA breakage, leads to an early attempts by the cell to repair the damage that manifests by activation of *Ataxia Telangiectasia* mutated protein kinase (ATM) and phosphorylation of histone H2AX on *Ser*-139. Both ATM activation as well as H2AX phosphorylation can be detected immunocytochemically by phospho-specific antibodies (Huang *et al.*, 2005; Kurose

et al., 2005; Tanaka *et al.*, 2007). The extent of H2AX phosphorylation in early apoptotic cells is extremely high, by an order of magnitude higher than the maximal level that can be induced by the DNA damaging drugs or radiation (Kurose *et al.*, 2005). Multiparameter cytometric analysis of H2AX phosphorylated on *Ser*-139 concurrently with DNA content makes it thus possible to identify subpopulations of cells with primary DSBs induced by the drug or radiation versus the cells with secondary, apoptosis-associated DSBs, and characterize cells in each subpopulation with respect to cell cycle phase (Kurose *et al.*, 2005).

F. SYTO-Based Detection of Apoptosis

Progress in the modern field of cell necrobiology necessitates exploitation of novel methods that support high-throughput and multivariate analysis of critical cellular parameters at a single cell level (Darzynkiewicz *et al.*, 1997, 2001b, 2004). To this aim diverse cytometric assays have been introduced, as described in previous sections of this chapter. Some of them include cell permeant DNA selective stains, such as Hoechst 33342, DRAQ5, and, more recently, probes from Vybrant DyeCycle family (Haughland, 2005; Schmid *et al.*, 2007; Smith *et al.*, 2000). All allow staining of unfixed cells and restrict cumbersome procedures to a simple step. To date, however, live-cell assays based on cell permeant DNA selective probes suffered mostly from their unfavorable spectral characteristics that necessitate UV excitation source and dedicated optics. Excessive toxicity/phototoxicity precludes also long-term studies such as cell sorting with subsequent cell cultivation (Durand and Olive, 1982; Fried *et al.*, 1982; Martin *et al.*, 2005). Nevertheless, progress has recently been made by the development of cell permeant, cyanine SYTO stains. This novel class of 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) (Frey, 1995; Haughland, 2005). Exploitation of SYTO probes to cytometric detection of apoptosis started in 1990s (Frey, 1995; Poot *et al.*, 1997) and is slowly gaining appreciation as an easy to perform, live-cell assay (Poot *et al.*, 1997; Schuurhuis *et al.*, 2001). Although, the fundamental mechanism underlying differential staining of SYTO-labeled apoptotic versus viable cells still remains uncertain, several hypotheses have been raised in the recent years (reviewed in Wlodkowic and Skommer, 2007).

Following initiation of caspase-dependent apoptosis cells loaded with selected SYTO stains exhibit gradual reduction in fluorescence signal intensity to dim values. This phenomenon substantially precedes plasma membrane permeability changes (Fig. 9) (Frey, 1995; Poot *et al.*, 1997; Wlodkowic *et al.*, 2007b). Evidence from recently published data indicate an overall higher sensitivity of SYTO probes in detection of early apoptotic events as compared to Annexin V-based assays (Eray *et al.*, 2001; Schuurhuis *et al.*, 2001; Sparrow and Tippett, 2005). When progression toward the terminal stages of cellular demise advances, loss of SYTO fluorescence intensifies, and this usually coincides with the increased plasma membrane permeability to PI and 7-AAD (Poot *et al.*, 1997; Schuurhuis *et al.*, 2001; Wlodkowic *et al.*, 2007b). Fig. 8 illustrates results obtained from a green fluorescent SYTO 11 probe used in conjunction with plasma membrane permeability marker PI. Both probes are exited by 488 nm line permitting their concomitant application on single laser analyzers. The assay requires only a short incubation (20 min, at RT) to supravitally discriminate viable cells ($SYTO^{high}/PI^{neg}$ events; V) and early apoptotic cells. The latter population is characterized by initial loss of SYTO 11 fluorescence and preservation of plasma membrane integrity ($SYTO^{dim}/PI^{neg}$ events; A). Cells in later stages of apoptosis feature progressive loss of SYTO fluorescence and gain bright PI staining ($SYTO^{neg}/PI^{+}$ events; N) (Wlodkowic and Skommer, 2007; Wlodkowic *et al.*, 2007b). Of note, the primary necrotic cells will also fall into the last group with minimal SYTO 11 and bright PI fluorescence. We

have recently shown that yet another green fluorescent probe, SYTO 16 allows discrimination between primary and secondary necrotic cells (Wlodkowic *et al.*, 2007b). Therefore, SYTO 16 provides substantial enhancement over the standard PI exclusion assay in discerning cell demise mode by flow cytometry (Wlodkowic *et al.*, 2007b). Importantly, SYTO probes prove in many instances inert and safe for tracking cells over extended periods of time. This may open up new opportunities for single cell real-time analysis protocols by both fluorescent activated cell sorting (FACS) and Lab-on-a-Chip platforms.

Recent noteworthy reports provided strong evidence that at least some SYTO probes can be substrates for MDR efflux pumps (e.g., P-glycoprotein; P-gp) (Schuurhuis *et al.*, 2001; van der Pol *et al.*, 2003). Caution should be, thus, exercised when using SYTO probes in cells with active ABC-class transporters. It is always advisable to confirm MDR status of studied cell population. In cells with active P-gp its inhibition (e.g., by verapamil hydrochloride, PSC833, cyclosporin A) is required to avoid masking of apoptotic $SYTO^{dim}$ subpopulation by $SYTO^{dim}$ subpopulation engendered by an active dye efflux (Schuurhuis *et al.*, 2001; van der Pol *et al.*, 2003). Truly apoptotic reduction of SYTO fluorescence to dim values is not affected by the presence of P-gp inhibitors (Schuurhuis *et al.*, 2001). Moreover, one should always bear in mind that results obtained using SYTO-based assays may vary when compared to assays detecting different cellular processes. Results acquired with SYTO probes should, therefore, never be considered conclusive without verification by independent methods (Wlodkowic and Skommer, 2007; Wlodkowic *et al.*, 2007b).

V. Time-Window in Measuring Incidence of Apoptosis

Apoptosis is a stochastic event of a variable induction and execution kinetics. There is a short time-window when apoptotic cells display their characteristic features. Moreover, the induction and the onset of apoptosis vary strongly depending on the cell type. For instance HL-60 (human promyelocytic leukemia) and MCF-7 (human breast cancer) cells treated with the same DNA damaging agent can succumb to apoptosis between 2 and more than 24 h, respectively (Del Bino *et al.*, 1999). In general, the induction time in cells of hematopoietic lineage is much shorter compared to other cell types, such as fibroblasts of cells of solid tumors lineage. This induction-to-execution interval profoundly varies depending on the stimulus applied (Li and Darzynkiewicz, 2000). Furthermore, the length of apoptosis (i.e., from the initiation to complete cell disintegration) is cell type dependent parameter. *In vivo*, under homeostatic conditions when cell death rate balances proliferation rate mitotic index (MI) is often seen to exceed apoptotic index (AI). This is an indication that duration of apoptosis is actually shorter than that of mitosis (the latter is about of 1 h duration) (Darzynkiewicz *et al.*, 2004). In cell culture, however, apoptotic cells remain detectable for extended periods of time before complete disintegration. This reflects lack of phagocytic clearance that characterizes homotypic cell culture conditions.

Identification of apoptotic cells generally relies on a specific marker that is detectable in variable time intervals. Knowledge of time-windows when specific markers are being detected is, thus, essential for the rational use of the methodology. In this context, loss of the mitochondrial transmembrane potential appears to be initially a transient event, followed by permanent collapse later during apoptotic cascade (Li *et al.*, 2000). Depolarization of mitochondrial membrane is followed by activation of caspases while binding of fluorescently labeled inhibitors of caspases (FLICA) substantially precedes externalization of PS (Pozarowski *et al.*, 2003; Wlodkowic *et al.*, 2006). In HL-60 cells challenged with DNA damaging agents, for example, DNA fragmentation follows caspase activation indirectly detected by cleavage of PARP, by approximately 20 min (Li and Darzynkiewicz, 2000). Furthermore, at early stages of apoptosis cells negative for the fractional DNA

content (sub-G_1 fraction) may be positive in TUNEL assay and expose PS residues (Darzynkiewicz *et al.*, 2001a).

Common application of cytometry is a comparison made between incidences of apoptosis (AI) in different samples (Darzynkiewicz *et al.*, 1997, 2001a, 2004). This task is particularly problematic in view of the above-discussed variability and the snapshot measurement of AI may fail to target comparable time-windows of apoptosis. Observed AI indices may, thus, not reflect the actual differences in apoptosis incidence between samples (Darzynkiewicz *et al.*, 2004; Smolewski *et al.*, 2002). Attempts have recently been made to obtain the cumulative AI, by measuring the rate (kinetics) of cell entrance into apoptosis and preventing disintegration of apoptotic cells (Smolewski *et al.*, 2002). The alternative solution is to count the absolute number of cells in culture and account for cell loss while estimating the AI based on specific markers (Darzynkiewicz *et al.*, 2004; Pozarowski *et al.*, 2003).

VI. Multiparameter Detection of Apoptosis: Choosing the Right Method

As discussed before, in view of recent seminal discoveries, the universal term “apoptosis,” has a propensity to misinterpret the actual phenotype of cell suicide program (Leist and Jaattela, 2001; Zhivotovsky, 2004). Nevertheless, single cytometric assays such as the estimation of sub-G_1 fraction or Annexin V binding are still being exploited in many research articles. Moreover, data from such single assays are persistently referred to as “apoptotic cells.” One should remember, however, that positive identification of apoptotic cells is far from straightforward. Furthermore, the reliance on single cytometric readout without proper understanding of the underlying assay mechanistic may lead to profound artifacts. It was only recently proposed to define apoptosis as a “*caspase-mediated cell death*” (Blagosklonny, 2000). Logically, caspase activation would be the most specific marker of apoptosis (Shi, 2002). There are, however, many examples of cell death that resembles classical apoptosis yet there is no evidence of caspase activation (Joza *et al.*, 2001; Leist and Jaattela, 2001; Lockshin and Zakeri, 2002). Extensive DNA fragmentation is also considered as a specific marker of apoptosis. The number of DSBs in apoptotic cells is usually so large that intensity of their labeling in the TUNEL assay ensures their discrimination from the cells that underwent primary necrosis (Gorczyca *et al.*, 1992). As mentioned, the high degree of phosphorylation of histone H2AX on *Ser*-139 in apoptotic cells makes it also possible to positively identify them (Kurose *et al.*, 2005). There are, however, mushrooming examples where apoptotic or apoptotic-like cell death proceeds without extensive internucleosomal DNA degradation (Catchpoole and Stewart, 1993; Cohen *et al.*, 1992; Collins *et al.*, 1992; Knapp *et al.*, 1999; Ormerod *et al.*, 1994). In these instances, the intensity of cell labeling in TUNEL assay will be inadequate to positively identify apoptotic cells. Furthermore, estimation of the sub-G_1 fraction fails when DNA degradation does not proceed to internucleosomal regions but stops after generating 50–300 kb fragments (Oberhammer *et al.*, 1993). Little DNA can be extracted then from the cells and rigid reliance on this method provides false-negative results (Darzynkiewicz *et al.*, 2001a, 2004). Noteworthy, if G_2/M or even late S-phase cells undergo apoptosis, the loss of DNA from these cells may not produce the sub-G_1 peak. These apoptotic cells often end up with DNA content equivalent to G_1/early S phase and are, thus, indistinguishable (Darzynkiewicz *et al.*, 2001a, 2004). Finally, while nuclear fragmentation is commonly observed during apoptosis of hematopoietic lineage cells, it may not occur in cells of epithelial- or fibroblast-lineage. Likewise, cell shrinkage, at least early during apoptosis, is not a universal marker of the apoptosis or necrosis, which has been discussed earlier in this chapter.

There are many other difficulties and potential pitfalls in analysis of classical apoptosis by flow cytometry (thoroughly reviewed in Darzynkiewicz *et al.*, 2001a, 2001b, 2004). Cell harvesting by trypsinization, mechanical or enzymatic cell disaggregation from tissues, extensive centrifugation steps, may all lead to preferential loss of apoptotic cells. On the other hand, some cell harvesting procedures interfere with apoptotic assays as discussed earlier in this chapter. Because of cell shrinkage the density of apoptotic cells is markedly increased while volume is diminished. This change should be taken under consideration, for example, when isolating cells by density (ficoll-hypaque, percoll) gradient centrifugations or elutriation. The most common problem, however, is the inability to distinguish late apoptotic cells (called also “necrotic phase of apoptosis” or “secondary necrosis”) from the primary necrosis (accidental cell death). In both cases, the integrity of plasma membrane is lost and the cells cannot exclude cationic dyes such as propidium iodide or Trypan blue.

The loss of cell surface antigens during apoptosis creates another problem in the studies aimed to identify the lineage of apoptotic cells by their immunophenotype (Philippe *et al.*, 1997; Potter *et al.*, 1999; Schmid *et al.*, 1992). Antigen loss often occurs at early stages of apoptosis and selectively depends on the antigen and the inducer of apoptosis. Therefore, regardless of the apoptotic marker used, the attempts to identify lineage of apoptotic cells by immunophenotyping are prone to significant errors. All these potential pitfalls together with means to avoid them are discussed in extent elsewhere (Darzynkiewicz *et al.*, 2001a, 2001b).

Perhaps the most important feature of flow cytometry is the capability of multi-parameter gating analysis, which allows one to quantitatively correlate, within the same cell, expression of several measured attributes. Divergent cellular processes can be, therefore, simultaneously assessed, which has profound practical implications in cell necrobiology studies. For instance, since DNA content is the most frequently measured attribute, the expression of other parameters can be then directly related to the position in the cell cycle phase and/or to DNA ploidy of the tumor cell population. Thus, flow cytometry overcomes a limitation of traditional bulk techniques based on analysis of total cell population (such as fluorimetry, spectrophotometry, Western blots, etc.) that average the results from heterogeneous samples.

As discussed above, the preference of an optimal multiparametric method depends on the cell type, stimuli, desired information, and technical restrictions. For example, the need for sample transportation or prolonged storage prior to analysis requires cell fixation. This excludes the use of “supravital” methods such as the assays of plasma membrane integrity (exclusion of YO-PRO 1, PI, 7-AAD), PS exposure (Annexin V binding) or dissipation of mitochondrial membrane potential (JC-1, TMRM). At the same time, however, the cell fixation allows to obtain information on the cell cycle phase specificity of apoptosis by concurrent analysis of cellular DNA content. In this context considerable progress has recently been made by the introduction of amine reactive viability dyes (ViD) (Invitrogen-Molecular Probes; Prefetto *et al.*, 2006). These allow for a convenient discrimination of cells with intact and damaged plasma membrane in fixed specimens. Reportedly, ViD probes span a broad range of visible excitation and emission spectra. Their uptake by cells with permeabilized membranes is followed by covalent binding to cytoplasmic amine residues that withstand formaldehyde fixation and alcohol permeabilization procedures (Prefetto *et al.*, 2006).

Technical restrictions of the cytometer, such as a single laser excitation source, few fluorescence detectors may further limit number of multiplexing possibilities. Importantly, restricted number of organic fluorochromes that have nonoverlapping spectra hampers broader introduction of multiparameter approaches. This impasse has recently been

superseded by the development of semiconductor nanocrystals (QDs). Their unrivalled specifications such as prolonged stability, reduced photo-bleaching, broad excitation with narrow emission spectra are poised to profoundly transform multicolor cell analysis (Jaiswal and Simon, 2004; Jaiswal *et al.*, 2003). Successful attempts have already been made to implement semiconductor nanocrystals in multiparameter flow cytometry (Chattopadhyay *et al.*, 2006). Undoubtedly, future applications of QDs in multiplexed cytometric detection of apoptosis are of substantial commercial interest (Dicker *et al.*, 2005; Le Gac *et al.*, 2006).

VII. Beyond Apoptosis – Analysis of Alternative Cell Death Modes

Although detection of classical, caspase-dependent apoptosis is still the major ground for the advancement of cytometric techniques there is an increasing demand for novel analytical tools that can rapidly quantify noncanonical modes of cell death. Although still a matter of debate, these noncanonical pathways appear to have wide reaching connotations in pathogenesis and treatment of human diseases (Edinger and Thompson, 2004; Lockshin and Zakeri, 2001; Okada and Mak, 2004). Moreover, they present an increasingly complex network of molecular cross-talks reflecting in a diversity of phenotypes.

A. Autophagy

Autophagy is an intracellular bulk degradation system for long-lived proteins and whole organelles (Meijer and Codogno, 2009). Emerging evidence suggests that while autophagy may enhance survival of cancer cells exposed to nutrient deprivation, hypoxia or certain chemotherapeutics, it may also contribute to cell death when induced above an acceptable for cellular homeostasis threshold (Eisenberg-Lerner *et al.*, 2009). Accurate estimation of autophagosome formation and/or functional catabolic autophagy is, therefore, important for preclinical drug screening (Corcelle *et al.*, 2009; Vousden and Ryan, 2009). To date only a handful of methods have been introduced to quantify autophagy, including electron and fluorescent microscopy to follow steady-state accumulation of autophagosomes, and long-lived protein degradation assay to access the catabolic autophagic activity (Gurusamy and Das, 2009; Swanlund *et al.*, 2010). Fluorescent microscopy is generally used to follow autophagosome accumulation using markers such as LC3 protein tagged with fluorescent protein GFP. In this assay, after induction of autophagy, cytoplasmatically localized LC3-I is cleaved and lipidated to form LC3-II. The latter is associated with the formation of an isolation membrane (Gurusamy and Das, 2009). Using, for example, adenoviral delivery of LC3-GFP it is possible to follow the changes in LC3-GFP distribution from diffuse cytoplasmic into punctuate, the latter indicative of autophagosome accumulation with reasonable precision. Current methods designed to quantify autophagic activity using LC3 are, however, time consuming, labor intensive, and require substantial expertise in accurate data interpretation (Shvets and Elazar, 2009; Shvets *et al.*, 2008).

Several attempts have recently been made to quantify autophagy in cells stably expressing GFP-LC3 reporters using flow cytometry (Shvets and Elazar, 2009; Shvets *et al.*, 2008). Flow cytometry collects, however, only integrated fluorescence over each cell. This in turn is generally not sensitive enough to detect subtle redistribution at a subcellular level. More recently, a successful attempt has been made to employ the multispectral imaging flow cytometry to quantify autophagosome formation (Lee *et al.*, 2007). Authors utilized the “virtual sort” capability to enumerate cells exhibiting the bright, punctuate spots of GFP-LC3. The inflow imaging is the first example of an automated and unbiased detection of autophagy in rare subpopulations of cells (Lee *et al.*, 2007).

Surprisingly there have been no attempts to adapt Laser Scanning Cytometry (LSC^{TM}, CompuCyte Corp, Cambridge, MA, USA) for multivariate quantification of autophagosome formation. LSC has many attributes of both flow cytometry and low-resolution image

analysis that proved to be optimal for multiparameter studies of apoptotic cell death. We postulate that adaptation of LSC to detection of autophagy based on maximal pixel analysis of vesicular LC3-GFP protein might prove beneficial for high-throughput screening routines. By combining bivariate analysis of the DNA content and LC3-GFP redistribution one can potentially examine the cell cycle specificity of autophagosome formation, for example, in different tumor cell lines.

Recently a new elegant solution has been proposed by Farkas *et al.* (2009) to measure the dynamics of autophagic flux. The design of a luciferase-based reporter assay (RLuc-LC3) allows to measure an autophagic flux in real time. Particular advantage of the RLuc-LC3 assay lies in a broad dynamic range and applicability to a dynamic analysis on cell population. This system has already been validated by screening a small-molecule kinase inhibitor library and results demonstrated its applicability for tracking of dose- and stimulus-dependent differences in autophagy kinetics (Farkas *et al.*, 2009).

B. Necrosis

The recent discovery of alternative cell death modes such as necrosis-like PCD, necroptosis, and paraptosis calls for the development of new and robust markers to distinguish between molecularly divergent cell death processes (Bröker *et al.*, 2005; Galluzzi and Kroemer, 2008; Hetz *et al.*, 2005; Krysko *et al.*, 2008). As discussed previously in this article, the cell impermeant DNA binding dyes such as PI, YO-PRO 1, or SYTOX are very convenient markers for the detection of accidental cell death (primary necrosis) and late stages of apoptosis. They all fail, however, to distinguish whether the labeled population is of late apoptotic, primary necrotic, or necrosis-like PCD origin. Even in conjunction with other probes it is often a matter of speculation whether, for example, Annexin V^{neg}/PI^{+} or $FLICA^{neg}/PI^{+}$ population represents programmed necrotic phenomenon. This cannot be resolved by mere flow cytometric analysis. Recently, however, an innovative assay based on a high-mobility group B1 protein (HMGB1) has been proposed that can reportedly differentiate primary necrotic cells (Ito *et al.*, 2006; Scaffidi *et al.*, 2002). HMGB1 protein is an architectural chromatin-binding factor that bends DNA and promotes protein assembly on specific DNA targets (Scaffidi *et al.*, 2002). It normally resides in the nucleus and is passively released when cells die during necrotic cell death. HMGB1 remains, however, tightly sequestered in cells undergoing caspase-dependent apoptosis or autophagic cell death (Fig. 9) (Ito *et al.*, 2006; Scaffidi *et al.*, 2002). Interestingly, even during secondary necrosis that follows caspase-dependent apoptosis cells do not release HMGB1 (Scaffidi *et al.*, 2002). This unique process has been associated with the prevention of chromatin deacetylation during necrosis (Scaffidi *et al.*, 2002). As such immunohistochemical detection of HMGB1 can be readily applied in both flow cytometry and imaging cytometry to detect and quantify cells undergoing primary and necrosis, necroptosis, and/or necrosis-like PCD (Fig. 9) (Ito *et al.*, 2006; Krysko *et al.*, 2008).

C. Cell Senescence

In many solid tumors the anticancer treatment instead of apoptosis induces irreversible impairment of cell reproductive capacity, which is defined either as “reproductive cell death,” “senescence-like growth arrest,” “accelerated senescence,” “premature senescence,” or “drug- or radiation-induced senescence” (Gerwitz *et al.*, 2008; Ohtani *et al.*, 2009). Overexpression of certain oncogenes and excessive mitogenic signaling can also lead to cell proliferation arrest characterized by senescence-like features. Both the induction of apoptosis as well as senescence play important role as the barriers to tumor development (Campisi, 2001). Normal cells become senescent in the course of organismal aging and also after completion of certain number of cell divisions in cultures as a result of telomere shortening (Hayflick, 1985).

Several markers characterize senescent cells. The most characteristic are morphological alterations (Cristofalo and Pignolo, 1993). Senescent cells show low saturation density at the plateau phase of growth, “flattened” appearance, enlarged, often irregular nuclei and cytoplasmic granularities. Their increased overall size is paralleled by an increase in nuclear and nucleolar size. They have numerous vacuoles in the cytoplasm, increased number of cytoplasmic microfilaments, the presence of large lysosomal bodies, and prominent Golgi apparatus (Cristofalo and Pignolo, 1993; Funayama and Ishikawa, 2007). The prominent abnormality of nuclear chromatin of senescent cells is the presence of senescence-associated heterochromatic foci (SAHF) that are abundant in histone H3 modified at lysine 9 (K9M H3) and its binding partner heterochromatin protein 1 (HP1) (Li *et al.*, 2007). Senescent cells are also characterized by expression of CDKs inhibitors $p21^{WAF1}$, p16, and $p27^{KIP1}$; the feature common but not specific to these cells (Shen and Maki, 2010). Among all biomarkers of cell senescence the most specific are the characteristic changes in cell morphology and the induction of senescence-associated β-galactosidase activity, the latter considered to be the hallmark of cell senescence (Dimri *et al.*, 1995). An excellent review of the cytometric methods to identify senescent cells is provided by Hwang and Cho (Chapter 7).

Most recently, the imaging analytical capabilities of LSC have been used to assess morphological features considered to be typical of the senescent phenotype (Zhao *et al.*, 2010). The characteristic “flattening” of senescing cells was represented by the decrease in the density of staining (intensity of maximal pixel) of DNA-associated fluorescence (DAPI). This change was paralleled by an increase in nuclear size (area). The decline in ratio of maximal pixel to nuclear area was even more sensitive senescence biomarker than the change in maximal pixel or nuclear area, each alone (Fig. 10). Also, the saturation cell density at plateau phase of growth recorded by LSC was found to be dramatically decreased in cultures of senescent cells, thereby additionally serving as a convenient marker (Zhao *et al.*, 2010). This morphometric approach utilizing LSC complements other cytometric methods to identify senescent cells reviewed by Hwang and Cho (Chapter 7).

VIII. Future Outlook

Development of novel bioassays was the driving force for the immense progress in research in cell necrobiology field during the past two decades (Darzynkiewicz *et al.*, 1997, 2001b, 2004). Paradoxically, despite all the advances in flow cytometry the morphological changes defined by light and electron microscopy back in 1972 are still being considered to be the “gold standard” for the identification of cellular demise mode. Although detection of classical, caspase-dependent apoptosis is still the major ground for the advancement of cytometric techniques there is an increasing demand for novel analytical tools to rapidly quantify noncanonical modes of cell death.

It can be expected that novel technologies and instrumentation like LSC and cell imaging in flow (multispectral imaging cytometry) are just a prelude to a major transformation that cytometric field will experience in the coming years (Darzynkiewicz *et al.*, 1999; Deptala *et al.*, 2001; George *et al.*, 2004; Smolewski *et al.*, 2001). Here especially the LSC by having many attributes of both flow cytometry and low-resolution image analysis appears to be an optimal instrumentation for multiparameter studies on cell demise (Bedner *et al.*, 1999; Darzynkiewicz *et al.*, 1999; Kamentsky, 2001; Zhao *et al.*, 2010). Application of nanocrystal quantum dots (Qdots) as convenient multispectral markers (Alivisatos *et al.*, 2005) will also contribute toward expansion of cytometric methods in necrobiology. Furthermore, we expect to witness soon the massive rise of micro-and nanotechnologies that form a cornerstone for Lab-on-a-Chip platforms. Although still in their infancy the latter

technologies warrant a major “quantum leap” in studies of cell death at a single cell level (Chan *et al.*, 2003; El-Ali *et al.*, 2006; Huh *et al.*, 2005; Qin *et al.*, 2005).

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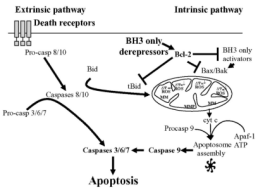

Fig. 1.
Simplified diagram of molecular pathways that regulate caspase-dependent apoptotic cell death.

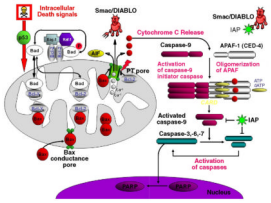

Fig. 2.
Mitochondrial pathway of caspase-dependent apoptosis.

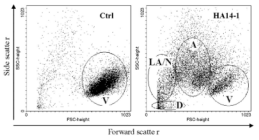

Fig. 3.

Changes in light scattering properties during apoptosis. Human B-cell lymphoma cells were untreated (left panel) or treated with small molecule Bcl-2 inhibitor HA14-1 (right panel) as described (Skommer *et al.*, 2006). Note that viable cell population (V) from the treated culture has similar light scattering properties as control cells. Apoptotic cells (A) have diminished forward scatter while their side scatter is enhanced. The late apoptotic/secondary necrotic cells (LA/N) have diminished both scatter parameters. Apoptotic bodies and cell debris exhibit extremely low light scatter values (D).

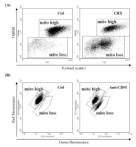

Fig. 4.

Dissipation of mitochondrial transmembrane potential ($\Delta\psi_m$). (A) Analysis by staining with tetramethylrhodamine methyl ester (TMRM). Human B-cell lymphoma cells were either untreated (Ctrl) or treated with cycloheximide (CHX) to induce apoptosis and supravitally loaded with TMRM as described (Castedo *et al.*, 2002; Wlodkowic *et al.*, 2006). Cells with collapsed mitochondrial transmembrane potential (*mito loss*) have decreased intensity of orange TMRM fluorescence. (B) Analysis by staining with the J-aggregate dye JC-9. Human B-cell lymphoma cells were either untreated (Ctrl) or treated with crosslinking anti-CD95 antibody (anti-CD95) to induce apoptosis and loaded with JC-9 as described (Pritchard *et al.*, 2001; Skommer *et al.*, 2006). Cells with collapsed mitochondrial transmembrane potential (*mito loss*) have decreased intensity of red fluorescence (mitochondrial J-aggregates) and increased intensity of green fluorescence (cytoplasmic J-monomers). Note that by only employing the $\Delta\psi_m$-sensitive probe there is no distinction between early, late apoptotic and necrotic cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of the chapter.)

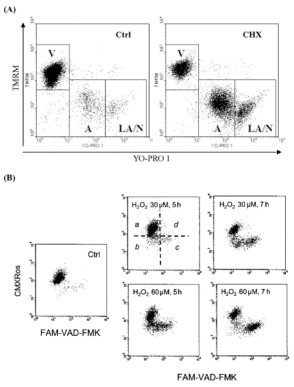

Fig. 5.

Multiparameter analysis employing mitochondrial potential sensitive probes. (A) Concurrent analysis of collapse of $\Delta\psi_m$ and early plasma membrane permeability during apoptosis. Cells were treated as in Fig. 4A and supravitally stained with both YO-PRO 1 and TMRM probes (Wlodkowic *et al.*, 2007a). Their green and orange fluorescence was measured by flow cytometry. Live cells (V) are both $TMRM^{high}$ and exclude YO-PRO 1. Early apoptotic cells (A) exhibit loss of $\Delta\psi_m$ ($TMRM^{low}$) and moderate uptake of YO-PRO 1. Late apoptotic/secondary necrotic cells (LA/N) are highly permeant to YO-PRO 1 probe. (B) Concurrent analysis of collapse of $\Delta\psi_m$ and caspase activation during apoptosis. Apoptosis of Jurkat cells was induced by oxidative stress (growth in the presence of 30 or 60 μM H_2O_2; Ctrl, untreated cells). The cells were then supravitally exposed to FAM-VAD-FMK and MitoTracker Red CMXRos, rinsed and their green and red fluorescence measured by flow cytometry (Pozarowski *et al.*, 2003). Two subpopulations of apoptotic cells can be detected. Subpopulation B represents the cells that lost mitochondrial potential but did not activated caspases, while cells in subpopulation C are characterized by both, collapsed $\Delta\psi_m$ and caspase activation (FLICA binding). At 60 μM H_2O_2 caspase activation was accelerated as reflected by the increased proportion of cells in C compared to B. Note that multiparameter analysis of $\Delta\psi_m$-sensitive probe with YO-PRO 1 or FLICA allows for excellent distinction between live, early, late apoptotic and necrotic cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of the chapter.)

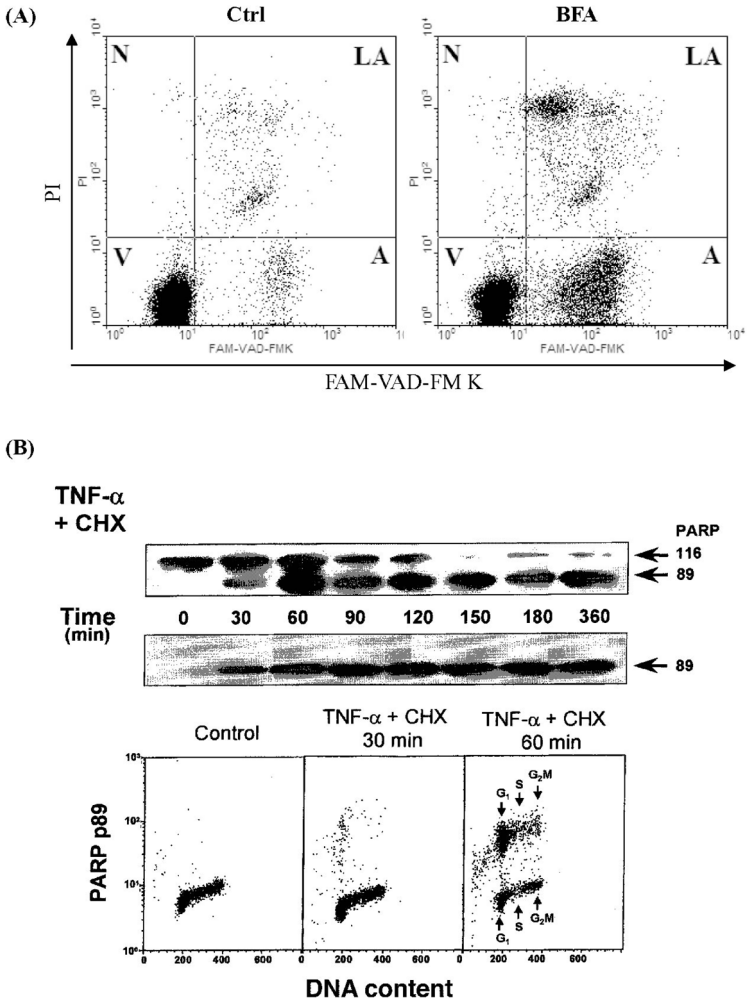

Fig. 6.

Activation of caspases. (A) Detection of caspases activation by fluorescently labeled inhibitors of caspases (FLICA) combined with plasma membrane permeability assessment (propidium iodide; PI). Human B-cell lymphoma cells were either untreated (Ctrl) or treated with Brefeldin A (BFA) to induce apoptosis as described (Wlodkowic *et al.*, 2007a). Cells were subsequently supravitally stained with FAM-VAD-FMK (pan caspase marker; FLICA) and PI. Their logarithmically amplified green and red fluorescence signals were measured by flow cytometry. Live cells (V) are both FAM-VAD-FMK and PI negative. Early apoptotic cells (A) bind FAM-VAD-FMK but exclude PI. Late apoptotic/secondary necrotic cells (LA) are both FAM-VAD-FMK and PI positive. Primary necrotic and some very late apoptotic cells (N) stain with PI only. (B) Detection of PARP cleavage combined with DNA content (cell cycle) analysis. To induce apoptosis, HL-60 cells were treated with TNF-*α* in the presence of CHX for 30–360 min (Li and Darzynkiewicz, 2000). Upper panel shows immunoblots of the treated cells, stained with PARP plus PARP p89 (upper gel) or PARP p89 only (lower gel)

Abs.LowerpanelsshowbivariatedistributionsofPARPp89versusDNAcontent(stained with PI)of the untreated (Ctrl) and treated for 30 and 60 min cells. Note the appearance of the first PARP p89 positive cells already after 30 min of treatment, coinciding in time with the detection of PARP cleavage on gels. There is no evidence of cell cycle phase specificity of

apoptosis induced by TNF-α. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of the chapter.)

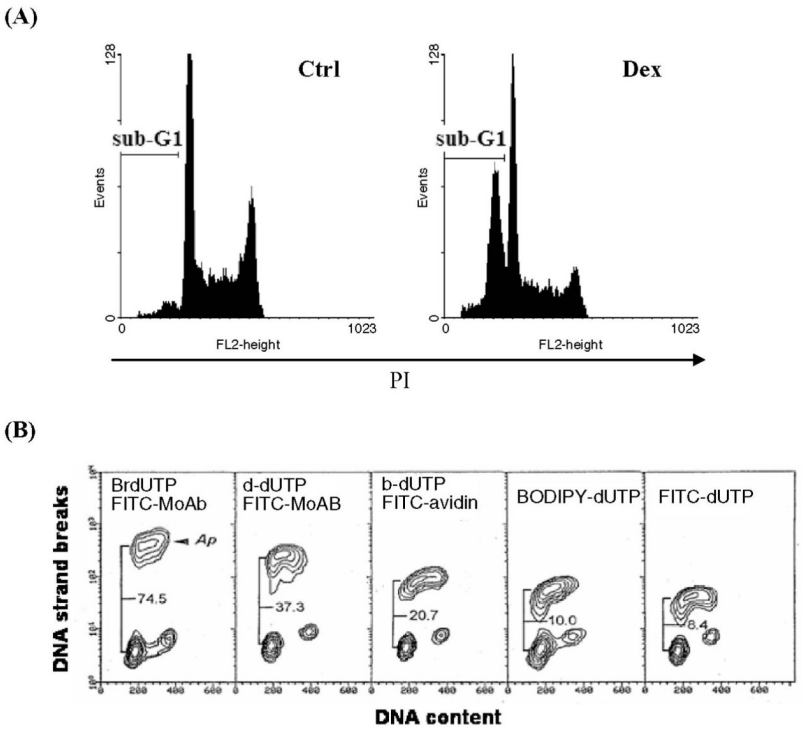

Fig. 7.

DNA fragmentation analysis. (A) Detection of fractional DNA content ("sub-G_1" peak). Apoptosis of human follicular lymphoma cells was induced with dexamethasone (Dex). Ethanol-fixed and propidium iodide (PI)-stained cells were analyzed on a flow cytometer. Red fluorescence of PI was collected using linear amplification scale. Debris were gated out electronically. Note distinctive sub-G_1 peak. For further details refer to text. (B) Detection of DNA strand breaks ("TUNEL" assay) using different deoxynucleotides. Apoptosis of HL-60 cells was induced by camptothecin, which selectively targets S-phase cells (Li and Darzynkiewicz, 1995). In the reaction catalyzed by terminal deoxynucleotidyl transferase, the DNA strand breaks were labeled (from right to left): directly with FITC and BODIPY-conjugated dUTP, or indirectly with biotinylated (biot) dUTP detected by FITC-avidin, digoxygenin-conjugated dUTP detected by digoxygenin-FITC Ab, and with BrdUTP detected by FITC-BrdU Ab. The highest resolution provides labeling with BrdUTP. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of the chapter.)

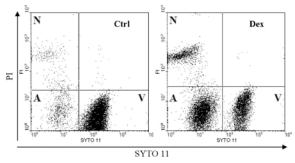

Fig. 8.

Detection of apoptosis by SYTO 11 probe. Human B-cell lymphoma cells were untreated (left panel) or treated with dexamethasone (right panel), as described (Wlodkowic *et al.*, 2007b). Cells were subsequently supravitally stained with SYTO 11 and PI probes. Their logarithmically amplified green and red fluorescence signals were measured by flow cytometry. Live cells (V) are SYTO 11^{bright} and PI negative. Early apoptotic cells (A) are SYTO 11^{dim} but still exclude PI. Late apoptotic/secondary necrotic cells (N) are both SYTO 11^{low} and PI positive. Primary necrotic cells do not exhibit $SYTO^{dim}$ staining pattern and rapidly take up propidium iodide while loosing SYTO to low values (not shown; Wlodkowic *et al.*, 2007b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of the chapter.)

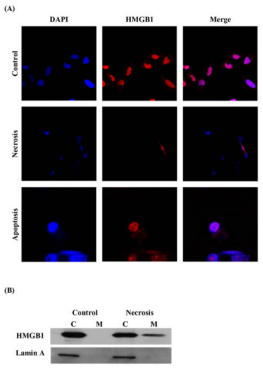

Fig. 9.

Selective detection of necrosis using monoclonal antibody against a high-mobility group B1 protein (HMGB1). (A) Human osteosarcoma U2OS cells cultured on glass coverslips were induced to undergo necrosis (freeze/thawing cycle) or apoptosis (Tet-On p53). Cells were then fixed and stained with DAPI (blue) and anti-HMGB1 antibody (red). Note that HMGB1 normally resides in the nucleus and is passively released when cells die during primary necrosis. It remains, however, tightly sequestered in cells undergoing caspase-dependent apoptosis. Immunohistochemical detection of HMGB1 can be readily applied in both flow cytometry and imaging cytometry to detect and quantify cells undergoing primary and necrosis, necroptosis, and/or necrosis-like PCD. (B) Specific release of HMGB1 from necrotic cells can be detected using Western blot (WB) analysis. Necrotic cell death was induced in U2OS cells by repeated freeze/thawing cycle. Both cells and medium were then harvested and protein extracts analyzed using WB. Note that HMGB1 band is detected in the medium. (See plate no. 1 in the color plate section.)

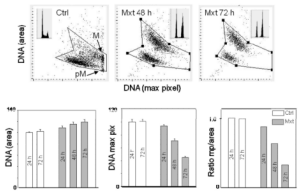

Fig. 10.

LSC-assisted morphometric analysis of nuclear changes of A549 cells in cultures treated with mitoxantrone (Mxt). The cells were untreated (Ctrl) or to induce cell senescence treated with 2 nM Mxt for 24, 48, or 72 h, their DNA was stained with DAPI. Intensity of maximal (max) pixel of DNA/DAPI reveals degree of chromatin condensation and in untreated cells has the highest value in mitotic (M) and immediately postmitotic (pM) cells (shown by the arrows). In the cells undergoing senescence while nuclear area increased, the intensity of maximal pixel decreased likely due to the “flattening” of the cell. The insets in the top left panels show DNA frequency histograms of cells from the respective cultures. The bar plots at the bottom panels show mean values (±SD) of nuclear DNA/DAPI area, DNA/DAPI maximal pixel, and the ratio of maximal pixel to nuclear area. The ratio of maximal pixel/ nuclear area of the Mxt-treated cells is expressed as a fraction of such ratio of the untreated cells (Ctrl = 1.0) (Zhao *et al.*, 2010).