Chapter 8

ANALYSIS OF APOPTOSIS ON CHIP

Why the move to chip technology?

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Abstract: Apoptosis refers to a specific form of programmed cell death, which guarantees the welfare of the whole organism through the elimination of unwanted cells. The duration of apoptosis is short, involves single cells with morphological changes only after the point of no return, ending with phagocytosis without reaction in the neighbour cell. A number of techniques exist to measure cell death, but we still looking for a simple, specific and sensitive technique which offers the possibility to measure apoptosis on single cell level, without staining, in real time, with high-throughput. The Lab-in-a-Cell concept by using chip technology offers such a tool.

Keywords: apoptosis, necrosis, apoptotic cascade, morphology, biochemistry, measurements, chip technology, Lab-in-a-Cell technology

1. APOPTOSIS

All living organisms from unicellular bacteria to multicellular animals are products of cell division. Most scientist traditionally have studied proliferation and it was a given that cells survive. The role of cell death for development, growth and survival of individuals was left outside of consideration. Only after Kerr, Wyllie and Currie [1] had discovered on basis of morphological appearance the existence of two different forms of cell death, researchers have become aware that death is the inevitable complement to cell division. To discriminate the natural cell death from accidental cell death they introduced the term apoptosis. This term is derived

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from the Greek: apo “apart” and ptosis “fallen” meaning the shedding of leaves from trees during the autumn.

1.1 Physiological versus Pathological Cell Death

There are many ways to die, but from cell biological point of view only two forms exist: physiological and pathological cell death (Figure 8-1).

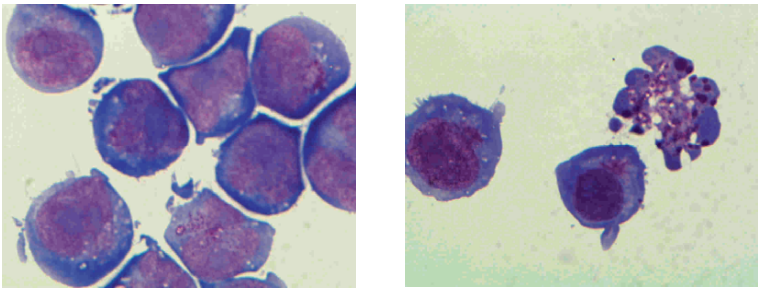

Figure 8-1. Apoptosis versus necrosis: Light microscopy of untreated control HL60 cells (left panel) and cells treated with apoptotic inducer campotothecin (right panel) and cultured 6 h in vitro.

Necrosis of cells occurs after a physical, chemical or osmotic injury, including hypoxia and complement attack [1-3]. During accidental cell death, the cell membrane loses its selective permeability and ion-pumping capacity. This leads immediately to swelling of the cell and its organelles and to leaking of the cellular contents into the extracellular space, eliciting an inflammatory reaction in the adjacent viable tissues.

Apoptosis is a physiological active bioenergy-saving cell elimination mechanism by which aged, unwanted or sublethal damaged cells are abolished and their contents are reutilised by macrophages or by phagocytosing adjacent cells. Physiological cell death occurs as "programmed cell death" (PCD) during the period of embryogenesis and goes on during post-embryonic life as "apoptosis", thus controlling cell numbers and organ size in a dynamic balance between cell proliferation and cell death [4-6]. Without continuous signalling by growth factors, hormones or cytokines, cells undergo apoptosis.

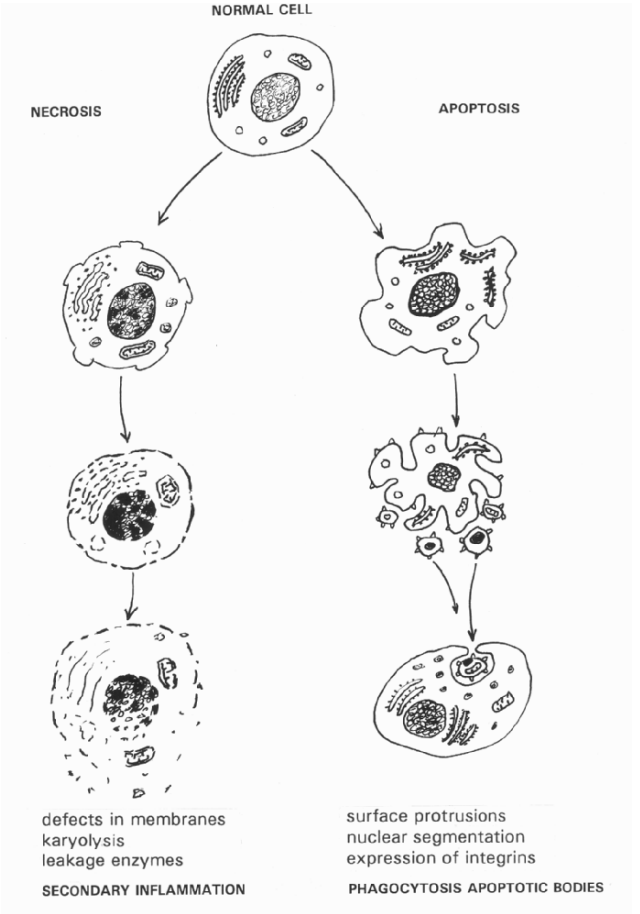

Figure 8-2. The most prominent differences between apoptosis and necrosis. From Vermes and Haanen [3] with permission of Academic Press.

During apoptosis a specific pattern of cell abolition takes place. The earliest changes include the loss of cell junctions and specialised membrane structures such as microvilli. The integrity of the cell membrane and of the mitochondria remains initially intact, the cytoplasm condenses and the nucleus coalesces into large masses, which then break up into fragments. The endoplasmatic reticulum transforms into vesicles that fuse with the cytoplasmic membrane. These processes result in contraction of the cytoplasmic volume. The cell adopts a convoluted outline and subsequently the cell breaks up into small vesicles enclosing parts of the cellular contents and apparently intact organelles. These apoptotic bodies end up in the extracellular space, where they are phagocytosed by nearby cells and

macrophages. The whole process takes only a few hours and the cell remnants do not elicit any inflammatory reaction (Figure 8-2).

1.2 Apoptosis and the Plasma Membrane

After external or internal death pathways have become activated and the decision to die has been made, signalling routes are activated to inform the environment about the cell death decision. The environment responds with removal of the dying cell by phagocytosis before the hydrolytic eruption inside the cell compromises the plasma membrane barrier integrity and causes leakage of inflammatory compounds into the surroundings [7-8]. In response to the cell death commitment, the plasma membrane changes its structure such that phagocytes can identify the cell as suicidal and can engulf and degrade it rapidly. Amongst these 'eat me signals' on the cell surface of the apoptotic cell are sugars, thrombospondin binding sites and phosphatidylserine (PS). Phagocytes bear receptors on their cell surface, which can recognise these 'eat me signals' [9]. The most investigated signal so far is the exposure of PS. The living cell keeps PS stringently located in the inner membrane leaflets that face the cytosol [10]. During apoptosis a phospholipid translocase is inhibited and a scramblase becomes activated [11]. The PS exposed on the cell surface is recognised by phagocytes as an 'eat me signal' [12,13].

This phenomenon is also exploited to detect and measure apoptosis by using Annexin V, which is a phospholipid binding protein with high affinity for PS [14,15]. In most cases cell surface exposure of PS was found to precede the other features of apoptosis like DNA fragmentation [16]. The molecular link between the executioner proteins and the plasma membrane has not been resolved. It appears that like the other themes of the molecular biology of apoptosis, this part of the apoptotic machinery is conserved during the evolution [17].

1.3 The Role of the Mitochondrion in Apoptosis

The mitochondrion has been suggested to be fundamental to the biochemistry of cell death by apoptosis for it might form the nidus where the decision of life and death is being made [18]. A crucial event of the role of the mitochondrion is the formation of permeability transition pores in its outer membrane leaflet allowing mitochondrial proteins to flux into the cytosol [19]. Amongst these proteins are Apoptotic Protease Activating Factor 2 (Apaf-2 or cytochrome c) and Apoptosis Inducing Factor (AIF) [20,21]. AIF is a protease, which may be responsible for the apoptosis typical nuclear features such as chromatin condensation and

internucleosomal DNA fragmentation. It was shown that Apaf-2, with the cofactors Apaf-1, Apaf-3 and dATP, can activate caspase 3 [22]. Apaf-3 was identified as caspase-9 [23]. Apaf-1, 2 and 3 and dATP form a complex, which is capable of activating caspase-3. The activated caspase-3 forms part of the executioner of apoptosis [22-24]. The unravelling of this mitochondrial switch from a state of reversibility into a state of irreversibility offered insights into the mechanism of action of the Bcl-2 like proteins (see: Bcl-2 family proteins). By blocking the release of Apaf-2 and AIF from the mitochondrion Bcl-2 prevents the formation of the caspase-3 activating complex. It has also been suggested that Bcl-2 interferes with this complex formation by binding to Apaf-1 and 2 directly.

1.4 Caspases

The proteins executing the apoptotic program belong to a family of proteases, called the caspases, members of a family of cysteine proteases, bearing an active site, which cleaves specifically following aspartate residues. These proteases are indicated caspases functioning as C(ysteine) dependent ASP(artate cleaving prote)ASEs. These proteins exist as inactive pro-enzymes in all cells. The caspases can be activated to execute apoptosis under a variety of conditions including receptor-ligand coupled signal transduction, DNA damage, lack of growth factors, oxidative stress and breakage of cell-cell and cell-matrix interaction [25].

Functionally caspases divide in two major subfamilies: 1) those related to ICE (caspase-1, caspase-4, caspase-5) function in cytokine maturation, 2) the remainder mediate apoptosis. Among these latter a further subdivision exists: ‘initiator caspases’ (caspase-8, caspase-9, caspase-10), which respond to pro-apoptotic stimuli and subsequently catalyse the activation of the ‘effector’ caspases (caspase-3, caspase-7).

The information obtained about the structures and mechanisms of caspases has been exploited for development of small-molecule inhibitors of caspases. A fluorochrome-labeled inhibitor of caspases FAM-VAD-FMK (FLICA) has been developed to estimate the rate of cell entrance to apoptosis and reveal the cumulative apoptotic turnover during this interval [26-30]. Exposure of cells to FLICA results in the uptake of this inhibitor followed by their covalent binding to activated caspases within the cells that undergo apoptosis. FLICA binds to activated caspases within the cell and irreversibly inactivates them, which causes the arrest of the apoptotic cascade [26]. The arrested apoptotic cells, labeled with FLICA, can be followed through the apoptotic cascade and identified by flow cytometry [27,28]. Although various pathways for activating caspases may exist, two mechanisms have now been elucidated in detail (Figure 8-3).

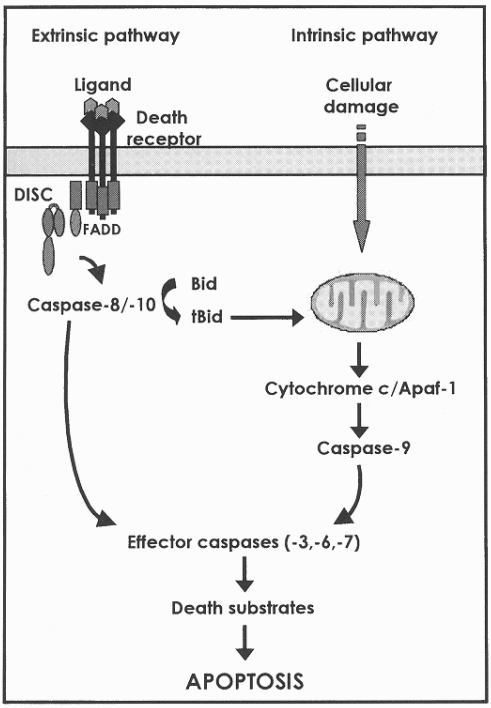

Figure 8-3. The extrinsic and intrinsic apoptotic pathways (From Frumalora and Guidotti [31] with permission of Kluwer Acad Publ.)

1.4.1 Caspase Activating Mechanisms

One caspase activating mechanism is mediated by death receptors, present on the Tumor Necrosis Factor Receptors (TNFR) family, which use caspase activation as a signalling mechanism, thus connecting ligand binding at the cell surface to apoptosis induction [32-34]. This form of caspase activation has been indicated as 'the extrinsic pathway'. The other caspase activation mechanism, indicated as the 'the intrinsic pathway', involves the participation of mitochondria, which release caspase-activating proteins into the cytosol, thereby triggering the apoptotic machinery [31,35,36].

The Extrinsic Pathway

With regard to the extrinsic pathway, the ligand binding causes the cytosolic domain of TNFR to recruit pro-caspase-8 and -10. Caspase-8 serves in the intrinsic pathway as the apical caspase [37-39].

The Intrinsic Pathway

In the intrinsic pathway permeabilization of the mitochondrial membrane (MMP) causes the release of cytochrome c from the mitochondria. Protein c binds to Apaf-1 (Apoptotic Protease Activating Factor) present in the cytosol. This complex triggers activation of pro-caspase-9, which apparently serves as the apical caspase in the intrinsic pathway [40].

1.4.2 Proteins Controlling Caspase Activation

A number of proteins have been recognised that control the intrinsic, extrinsic and other pathways of caspase activation and in this way are associated with apoptosis regulation. Domains, including caspase-associated recruitment domains (CARDs), death domains (DDs), death effector domains (DEDs), Bcl-2 homology (BH) domains of Bcl-2 family proteins, and the inhibitor of apoptosis proteins (IAP) commonly mediate the interaction of these proteins. All these proteins can be recognised based on their amino acid sequence and structural similarity [41].

Death Domain Proteins (DDs)

Members of the TNF family of cytokine receptors contain DDs in their cytosolic regions, including TNFR1, Fas (Apo1), DR3 (Apo2), DR4 (TrailR1), DR5 (TrailR2), DR6, Tradd, Fadd and DAP kinase. The death domain protein Fadd links the TNF receptors to caspases [42]. Several cytoskeleton-associated proteins contain DDs, which are involved in activation of caspase-8 after detachment of adherent cells. This may explain the phenomenon of anoikis, apoptosis induced by integrins, when the cytoskeleton of cells becomes detached from its extracellular matrix [43]. Non-caspase-activating DDs regulate apoptosis by suppressing the effect of NF-kB, which enhances the occurrence of apoptosis [44].

Defects in the function of DDs are associated with several human diseases. Inappropriate expression of Fas and Fas ligand (FasL) on immune cells has been implicated in the loss of lymphocytes in patients with HIV infection [45]. Hereditary mutation in de DD of the FAS (Apo1) gene causes an autoimmune lymphoproliferative syndrome [46]. Mutations and deletions of the FAS gene have been observed in various malignancies, affording resistance of cancer cells to immune-mediated attack. A soluble version of Fas, interfering with FasL-mediated apoptosis, is associated with autoimmune lupus and resistance of cancer against immune attack of cytolytic T-cells [47]. Trail (DR4, DR5) decoy receptors have been discovered, which interfere with Fas ligand binding and by which normal cells become resistant to apoptosis [48].

Death Effector Domain (DED) Proteins

DEDs are present in the initiator caspases, caspase-8 and caspase-10. Multiple DED-containing modulators of apoptosis have been identified, such as Fadd, pro-caspase-8, pro-caspase-10, Dredd, c-Flip, DEDD, Flash a.o. [41]. Some DED proteins enhance caspase-8 activation by Fas. During Fas-induced apoptosis DEDD is translocated from the cytosol to the nucleolus [49]. Other DED proteins like Flip suppress caspase-8 activation by competing with pro-caspases 6 and -10 for binding to Fadd. Such mechanism is used by tumors to escape apoptosis induction by cytotoxic lymphocytes [50].

Inhibitor of Apoptosis Proteins (IAPs)

The IAPs represent a family of apoptosis suppressors. IAPs bind and potently inhibit activated caspases [51]. Alterations in the expression of IAPs have been discovered in patients suffering from spinal muscular atrophy [52]. Overexpression of IAPs has been observed in various types of cancer and lymphomas [53].

Bcl-2 Family Proteins

The mitochondrial pathway for apoptosis is modulated by Bcl-2 family proteins. The Bcl-2 family includes at least 20 different members with both pro-apoptotic (Bax, Bak, Bok, Bad, Bid, Bim, Bik, Bcl-Xs) and anti-apoptotic (Bcl-2, Bcl-XL, Mcl-1, Bfl-1, Bcl-W, Boo) effects [54]. The relative ratio of anti- and pro-apoptotic Bcl-2 proteins dictate the ultimate sensitivity or resistance of cells to apoptotic stimuli, like growth factor deprivation, hypoxia, radiation, anti-cancer drugs, oxidants and Ca^{++} overload.

Alterations in the amounts of these proteins are associated with a variety of pathological conditions, such as cancers, malignant lymphomas, autoimmune diseases, immunodeficiency syndromes, ischemia-reperfusion injury after stroke and myocardial infarction, degenerative diseases such as Alzheimer, age related macula degeneration a.o. [41].

Bcl-2 family proteins are constitutively localised to the membranes of mitochondria. Some of these proteins insert to the endoplasmatic reticulum and the nuclear envelope. What can be stated for sure is that Bcl-2 family proteins regulate the sequestration versus the release of cytochrome c from the mitochondria [18,35,54]. Bcl-2 family proteins control also the release of certain caspases (caspase-2, -3, -9), of AIF and of Smac/Diablo, the inhibitor of AIF, in some types of cells [55,56].

The proforms of cytochrome c, AIF, Smac/Diablo are inactive in the apoptotic process, requiring modifications such as attachment of prosthetic groups (heme for cytochrome c; flavin adenine dinucleotide (FAD) for AIF)

and/or proteolytic processing (AIF, Smac/Diablo), which occurs only within the mitochondria. In this way, apoptosis is avoided during biosynthesis of the apoproteins and is functionally linked to disruption of the mitochondrial membrane, providing cells with a suicide mechanism that can be triggered in response to mitochondrial damage [41].

2. CONVENTIONAL TECHNIQUES TO MEASURE APOPTOSIS

There are a variety of techniques for detection of the two forms of cell death, apoptosis and necrosis. However, these tools either are not specific or lack quantitative values. In fact the very nature of apoptosis can explain the technical difficulty to measure programmed cell death. The duration of apoptosis is short, involves single cells with morphological changes only after the "point of no-return", ending in phagocytosis without reaction in the neighbouring cell. Therefore, it is no wonder that we are still far from a reference technique to measure apoptosis in a sensitive, specific and quantitative way. We can only briefly review here the methods, which have been described to demonstrate the cellular changes during the apoptotic cascade according to the sequence at which they occur.

2.1 Techniques Based on Morphological Changes

2.1.1 Measurement of Apoptotic Indices with Light Microscopy

Morphological evaluation is still the reference method for the detection of apoptosis [57, 58]. One of the most characteristic features of apoptosis is cell shrinkage, the loss of contact with neighbouring cells as the apoptotic cell shrinks and detaches from adjacent cells. Apoptotic cells are characterised based on their specific morphological features such as bud formation, chromatin condensation and appearance of apoptotic bodies containing remnants of cell organelles and nuclei. Quantification of the number of apoptotic cells requires scoring of great numbers of cells, since the execution phase of apoptosis is relatively short and therefore the relative frequency of apoptotic cells is expected to be low. The proportion of apoptotic cells in a population can be quantified by counting cells visualised by light microscopy and accordingly expressed as the apoptotic index (AI), being defined as the number of microscopic features per 100 cells that can be recognised in tissue or malignant tumors, exhibiting the morphological characteristics of apoptosis.

Very recently photothermal microscopy was used for detection and monitoring of apoptosis in single cells [59]. Photothermal microscopy is based on optical registration of a cell response to the thermal impact that is induced in a cell due to absorption of a short laser pulse by cellular hem-proteins. For hem-proteins dissolved in cytosol, the increase in their concentration may result from a decrease in the cytosol volume due to apoptotic cell death. In this way the early stage of apoptosis can be detected directly in a single cell without any exogenous agent and with a sensitivity which exceed the sensitivity of fluorescent methods [59].

2.1.2 Electron Microscopy

Electron microscopy is the method of choice when making detailed examination of the structural changes within cells but hardly a method for routine scoring of apoptosis. Hence this technique is primarily used to obtain qualitative information on ultrastructural changes during cell death [58, 60, 61].

2.1.3 Changes in Cell Scatter Pattern Measured by FCM

The integrity of the cytoplasmic membrane is lost immediately during necrosis but remains largely intact during the early stage of apoptosis. Later, during the process of cell death, cytoskeletal changes occur which, in the case of apoptosis, result in the formation of apoptotic bodies. These phenomena can be exploited with flow cytometry (FCM) by the measurement of changes that end up in the cell scatter pattern. Forward light scatter reflects the cell diameter, while right angle scatter is a measure of inner cellular structures. During the initial stages of apoptosis, the cell membrane remains intact but the cell shrinks, while during necrosis cell swelling occurs immediately as a result of early failure of the cell membrane. This means that during the initial phases of apoptosis, forward light scatter diminishes, while right angle scatter temporally increases or remains stable [62-65]. Unfortunately these parameters can only be evaluated on native cells in suspension and which have not underwent any mechanical handling.

2.2 Techniques Based on DNA Fragmentation

2.2.1 Measurement of DNA Content by FCM

As a result of the activation of an endonuclease, apoptotic cells exhibit [62,64-69] a low DNA stainability as measured by flow cytometry, below

the normal G_0/G_1 region, resulting in a sub G_0/G_1 peak designated as A_0 cells. There is circumstantial evidence that this reduced DNA stainability may be the consequence of progressive loss of DNA from nuclei due to the activation of endogenous endonuclease and subsequent leakage of the low-molecular weight DNA product prior to measurement. In contrast to apoptotic cells, necrotic cells do not show an immediate reduction in DNA stainability. In contrast, by 3H-thymidine labelling of the fragmented DNA (JAM-assay) one can measure the apoptotic cell death in reverse based on the detection of free DNA fragments [70].

2.2.2 Labelling of DNA Strand Breaks

Activation of the apoptosis-associated endonuclease results in extensive DNA cleavage and thus generates a large number of DNA strand breaks. The presence of 3'hydroxyl-termini of the strand breaks can be detected by labelling with modified nucleotides (e.g. biotin-dUTP, digoxigenin-dUTP, fluorescein-dUTP) in a reaction catalysed by exogenous enzymes like terminal desoxynucleotidyl transferase (TdT) [71-73] or DNA polymerase [74]. Fluorochrome conjugated avidin or digoxigenin antibodies are used in a second step of the reaction to make individual cells suitable for detection. Commonly used techniques for the detection of apoptosis are the **i**n **s**itu **n**ick (ISN) labelling technique or the **T**dT-mediated X-d**U**TP **n**ick **e**nd **l**abelling (TUNEL). Both techniques are applicable for conventional histological sections [75] and for cell-suspensions using flow cytometry (Figure 8-4) [65,72]. A simplified, single-step procedure has been developed recently, utilising desoxynucleotides directly conjugated to fluorochromes [65,76]. This single-step procedure utilises BrdUTP instead of digoxygenin or biotin conjugated triphosphodeoxynucleotides, which increases the sensitivity of the assay by giving a four-fold higher signal.

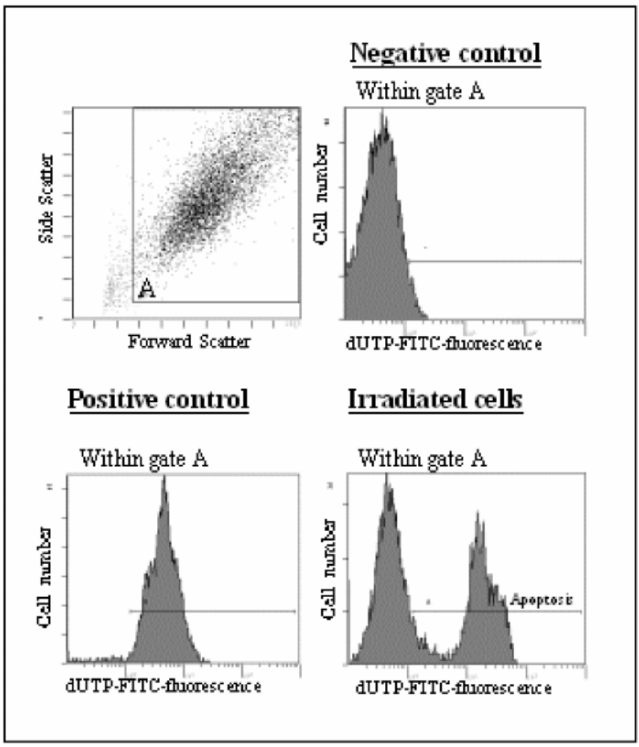

Figure 8-4. FCM of DNA double-stranded breaks: TUNEL assay. TUNEL was performed according to Gavrieli et al. [71] and Gorczyca et al. [72]. One million HSB2 cells were washed twice with 1 ml PBS. The HSB2 cells were fixed with 4% (w/v) paraformaldehyde during 30 minutes on ice. After two washing steps with PBS the pellet was resuspended in 100 µl permeabilization solution (1% (v/v) Triton (Merck, Darmstadt, Germany) and 0.1 % (w/v) TriSodium Citrate dihydrate (Sigma, Deisenhofen, Germany) and incubated on ice during 2 minutes. After these incubation two wash steps with PBS followed. The cells were labelled by adding 50 µl TUNEL mix [Terminal Deoxy nucleotidyl Transferase (TdT): Deoxy Uridine triphosphate (dUTP) = 1:9] (Boehringer Mannheim, Mannheim, Germany) followed by incubation during 60 minutes at 37 °C. The samples were washed with PBS and the pellet was resuspended in 250 µl PBS. The samples were analysed by flow cytometry. Cells incubated without TdT used as negative control (right upper panel) and cells incubated with DNase (left lower panel) used as positive control. Activation of the cell death program was induced by 10 Gray irradiation. 8 hours after irradiation samples were harvested (right lower panel). From Vermes et al. [68] with permission of Elsevier Sci.

2.3 Techniques Based on Membrane Alterations

2.3.1 Measurement of Dye Exclusion

During the initiating phase of apoptosis the fine architecture of the cell membrane is changed, but in contrast to necrosis, during apoptosis the integrity of the cytoplasmic membrane and a number of its basic functions remain intact. One of these functions is the active membrane transport. Accordingly, apoptotic cells exclude dyes such as Trypan Blue or PI (Propidium Iodide) while necrotic cells do not [14,58,62,66]. Recently, a two colour, fluorescence-based microplate assay has been published by using DNA intercalating dyes [77]. This assay is particularly suitable for high-throughput applications but unfortunately is not quantitative and specific enough.

2.3.2 Probing for Phospholipid Redistribution: Annexin V Assay

A change of the architecture of the plasma membrane during apoptosis involves the redistribution of the various phospholipid species between the two leaflets of the membrane. Under viable conditions the cell maintains lipid asymmetry over these two leaflets. The most pronounced feature of this asymmetry is the almost complete absence of phosphatidylserine (PS) in the outer leaflet of the plasma membrane. Fadok and co-workers were the first to show that cell surface exposure of PS occurs in nucleated cell types during apoptosis [12]. The observations of Fadok triggered us to study the interaction of Annexin V with apoptotic cells [14]. The rationale for this study came from the knowledge that Annexin V binds specifically to the phospholipid membrane in the presence of Ca^{++}-ions when PS is exposed [78].

Annexin V appears to be a potent discriminator between viable and apoptotic cells [14,79]. Using Annexin V as a FITC conjugate, in combination with the propidium iodide, one can distinguish between viable, apoptotic and secondary necrotic cells (Figure 8-5). The state of art arising from using this technology indicates that PS exposure is a universal phenomenon of apoptosis occurring in all cell types, independent of the initiating trigger [14-16].

Due to its high affinity for PS containing membranes, the Annexin V assay is easy to perform. Cells of interest and Annexin V-FITC are mixed in the presence of calcium.

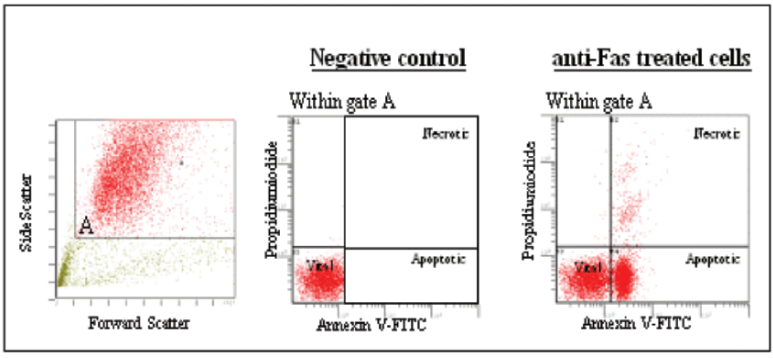

Figure 8-5. FCM of phospholipid redistribution: Annexin V/ Propidium iodide assay. The technique was performed according to Vermes et al. [14]. Jurkat cells were cultured for 8 hours in the presence (right panel) and the absence (middle panel) of anti-Fas (100 ng/ml). One million cells were washed twice with 1 ml PBS. The pellet was resuspended in 740 µl calcium containing binding buffer (10 mM Hepes +140 mM NaCl + 2.5 mM CaCl2, pH = 7.4), 1.0 µg/ml (final concentration) FITC-Annexin V (APOPTEST^{TM}-FITC, NeXins Research B.V. Hoeven, The Netherlands) and 1.0 µg/ml (final concentration) PI (Sigma, St. Louis, Missouri, U.S.A.). The samples were analysed for green fluorescence (FITC) and for red fluorescence (PI) by flow cytometry. Cells incubated without calcium served as a negative control (middle panel). The assay gives not only information about the numbers of vital (AV-/PI-) versus apoptotic (AV+/PI-) cells, but concurrently provides also the number of secondary necrotic cells (AV+/PI+). From Vermes et al. [68] with permission of Elsevier Sci. See also Colour Plate Section page 355.

Propidium iodide (PI) may be added to this mixture in order to stain specifically the cells, which have compromised plasma membrane integrity. Annexin V-FITC will bind immediately to cells which have surface exposed PS. Hence, after having prepared the reaction mixture it can be analysed almost instantaneously requiring neither prolonged incubation periods nor washing steps. Analysis can be carried out using fluorescence microscopy and flow cytometry. By these means viable and dead cells can be recognised easily. Viable cells will contain neither stain. Cells in apoptosis with intact plasma membrane integrity are stained only by Annexin V-FITC, whereas cells in secondary necrosis, the phase consecutive to apoptosis *in vitro*, contain both stains [14,16,79]. A new flow-cytometry-based ratiometric method that uses an internal reference standard of microbeads combined with Annexin V-FITC binding has been recently published to measure apoptotic rate *in vitro* [80]. In an other modified assay cells are prefixed with methanol free formaldehyde and labelled with FITC-Annexin V and with PI in the presence of digitonin [81]. Formaldehyde crosslinks DNA and hence prevents leakage of fragmented DNA from apoptotic cells. This allows one to identify the cell cycle position of apoptotic cell. Therefore this assay is suitable to study cell cycle-specific apoptosis [81].

2.4 Techniques Based on Cytoplasmic Changes

2.4.1 Changes in Intracellular Enzyme Activity

Measurement of the Endonuclease Activity

Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptosis [69]. Molecular characterisation of this process identified a specific DNase (CAD, caspase-activated DNase) that cleaves chromosomal DNA [82,83]. This type of assays is the most common biochemical method used for the detection of apoptosis rate. As a substrate exogenous DNA, a relatively large nucleic acid substrate isolated from non-apoptotic tissue nuclei [84-86], or endogenous DNA, when the substrate is the chromatin of the apoptotic nuclei [87], can be used. The direct measurement of the endonuclease-induced endogenous DNA fragmentation in extracts of apoptotic cells which was until recently thought to be the specific hallmark of apoptosis, is the most common method to detect apoptosis [88]. It was believed that the linker regions between nucleosomes were the only DNA targets for the apoptotic-endonuclease attack, resulting in fragments of 180-200 bp and multiples of this unit length. This type of cleavage can be assessed by the appearance of a ladder of bands on a conventional agarose gel [87,89], by using pulsed-field gel-electrophoresis [90,91] or by 2D-electrophoresis [90]. Unfortunately this type of assay is not sensitive enough to detect apoptosis in individual cells and needs large number of cells which precludes usage of this assay to study apoptosis in vivo. An application of the Southern blot technique was described as an assay to improve the sensitivity of DNA fragmentation [89,92]. It is important to note that, although non-random DNA fragmentation is widely used as a marker for apoptosis, some exceptions have been observed. It is therefore important to verify the occurrence of apoptosis by other criteria such as cell morphology [87]. Accordingly, the DNA degradation detected by these techniques must be viewed as a marker of the apoptotic process rather than a critical component of the death process itself [91].

2.4.2 Measurement of Caspases

During the execution-phase of apoptosis, intracellular enzymes are playing a key role in the cell death program [25,36]. As we described previously the caspase activity is vital to their role in apoptosis. Each of the caspase family members is a cysteine protease that possesses the unusual ability to cleave substrates after aspartate residues. Recently, by mapping the

cleavage site of PARP, Nicholson *et al*. [93] have identified the tetrapeptide, Asp-Glu-Val-Asp (DEVD) as a consensus cleavage site for caspase-3. Conjugation of a fluorometric (7-amino-4trifluoromethyl coumarin, AFC) or a colorimetric (p-nitroanilide, pNA) moiety to DEVD provides a potential substrate for analysing caspase-3 activity [94]. This protease assay is simple, quick and sensitive to measure caspase-3 activity of crude cell lysate of 10^6 suspended or adherent cells [95,96]. Recently, more sensitive homogenous caspase-3 time resolved fluorescence assays suitable for high-throughput usage by screening small molecule compounds were published [97,98].

Exposure of cells to a fluorescent inhibitor of caspases FAM-VAD-FMK (FLICA) stains viable cells supravitally [27-29]. When these cells enter apoptosis, the intracellular FLICA blocks the activation of caspases and arrests further progress of the apoptotic cascade and prevents cellular disintegration. The arrested apoptotic cells, labelled with FLICA, can be followed through the apoptotic cascade and identified by flow cytometry [99-101] or by laser scanning cytometry [102]. The fluorescent labelling of cells that enter into apoptosis and the labelling of dead cells with propidium iodide offer the possibility to estimate the rate of cell entrance into apoptosis, to measure the cumulative apoptotic turnover in time and to follow the occurrence of cell death in time [100]. Accordingly, this assay allows to measure the rate-constants between the different stages of the apoptotic cascade and the pattern of the apoptotic process [101].

2.4.3 Measurement of Tissue Transglutaminase

It has been demonstrated that activation of tissue transglutaminase (tTG) is part of the apoptotic machinery [103]. tTG is activated in dying cells to form cross-linked protein polymers/envelopes, which can be extracted from cells with a significant rate of physiological cell death [104]. When the apopotic bodies are degraded after a rapid phagocytosis, the cross-link itself is not cleaved but released, and the end product can be measured in the extracellular space. Measurement of tTG activity can be done based on the incorporation of radioactive putrescin into casein [104], and with a sensitive enzyme-linked immunosorbent assay [105]. There are several antibody preparations raised against tTG which have been used to detect and localise the tTG protein in apoptotic cells by immunohistochemistry and by immunoelectronmicroscopy [104]. In addition, the detection and localisation of tTG mRNA expression has been demonstrated by using TaqMan-based real-time RT-PCR, a semiquantitative RT-PCR technique [106]. It has been shown that tTG mRNA expression increases significantly in response to apoptosis inducing treatment in a dose- and time-dependent manner.

Accordingly, tTG expression can be used as a trace marker for detection and quantification of apoptosis [106].

2.4.4 Measurement of Calcium Flux

Elevations in the cytosolic Ca^{++} level are also a result of the apoptotic process [107,108]. Energy-dependent Ca^{++} transport system maintain the cytosolic Ca^{++} concentration at 100 nM, at least four orders of magnitude below that found in the extracellular milieu under physiological conditions. The increase of the cytosolic Ca^{++} concentration, measured by use of Ca^{2+}-selective fluorescent probes may be used as a sensitive indicator of cell death [86,109].

2.4.5 Measurement of Mitochondrial Dysfunction

Although for a long time the absence of mitochondrial changes was taken as a hallmark of apoptosis, mitochondria are considered today as the central executioner of PCD [6,110]. Decrease in mitochondrial membrane potential (DeltaPsim) is an early universal event of apoptosis. A fall of the mitochondrial membrane potential occurs before the DNA fragmentation and this drop of the mitochondrial membrane potential marks the point of no-return of a cell condemned to die [111-112]. Several cell viability assays are based on the fact that fluorochromes like Rhodamine 123, $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide), CMXRos (chloromethyl-X-rosamine), JC-1(5,5',6,6'-tetrachloro-1,1'3,3'-tetraethyl-benzimidazolcarbocyanine iodide) accumulate in mitochondria of live cells as a result of transmembrane potential. An early event of apoptosis is a decrease of DeltaPsim, which is reflected by a loss of the cell's ability to accumulate these fluorochromes [113,114].

It has been shown that a mitochondrial membrane protein designated 7A6-antigen appears to be exposed on cells undergoing apoptosis [115]. Accordingly, the antibody against this 38-kDa mitochondrial protein, APO2.7 (anti-7A6) could be used as a probe for the quantification of apoptotic cells. Phycoerythrin-labelled monoclonal APO2.7 antibody can be used in a FCM assay to demonstrate anti-Fas or radiation induced apoptosis in Jurkat cells [116,117]. It has been demonstrated that APO2.7 identifies the early apoptotic response, but it is not specific for apoptosis because 7A6 protein becomes exposed also in necrotic cells [79,117].

The release of cytochrome c by mitochondria is an essential step in the cell death cascade [6,34,110,118,119]. In addition to the release of cytochrome c, mitochondrial alterations in apoptosis include the release of other proapoptotic factors, including SMAC/DIABLO, apoptosis-inducing-

factor (AIF), CIDE-B (cell death-inducing DFF45-like effector protein B) and several caspases. All of these events surrounding cytochrome c release have been investigated in intact cells by flow cytometry and fluorescence microscopy and in reconstituted systems using isolated mitochondria and recombinant proteins or cytosolic extracts [6,34,110,118,119]. Another method of detecting cytochrome c release that is gaining in popularity is the use of green fluorescent protein (GFP)-tagged cytochrome c. The advantage of this system is that cytochrome c release can be observed in living cells [119].

A new flow cytometric assay simultaneously detects independent apoptotic parameters in one single cytofluorometric assay [120]. Mitochondrial dysfunction is assessed by using mitochondrion-permeable, voltage-sensitive dyes that accumulate in the organelle matrix of healthy cells, but not in the matrix of depolarised mitochondria. Analysis of cell morphology changes is performed following variations of the forward and side light scatter parameters. Plasma membrane alterations are investigated by FITC-Annexin V and with PI staining. In this way the same cell sample can be used to visualise early apoptotic events, such as mitochondrial dysfunction, mid steps, such as cell shrinkage and PS externalisation, and the late hallmarks of apoptosis, such as plasma membrane permeabilisation to PI [120]

2.5 Why the Move to Chip Technology?

At present there are about 300 different apoptosis–related kits and techniques that are developed for apoptosis detection and quantification. But all of these techniques have number of limitations. First of all, cells must be stained, fixed or destroyed for analysis, so intact single cells cannot be analysed. This is a crucial point when one is studying apoptotic cell death. We just show above that minimal manipulation of cells (e.g. detachment of adherent cells with trypsin, which is a frequently used tool) can induce apoptosis. Staining kills the cells therefore one can not study single cell. Accordingly, number of techniques are dealing with artifact. In addition, cell preparation for analysis requires some additional time (at least 15-30 min) and therefore real-time monitoring of the cell death cascade is not available. All of these techniques reviewed here need highly sophisticated equipment and persons to perform these measurements which are very labour consuming and expensive. Hence one can think of many reasons why microtechnology is advantageous compared to the existing conventional analysis methods. For example, different cell manipulation methods (sorting, detachment, staining, fixing, lysis a.o.) can be integrated on one chip, which reduces the work for the analysts and increase performance. Further, optical

detection techniques can be automated and in some cases be replaced by electrical on-chip detection techniques. Specific for apoptosis, integration of different detection techniques (electrical properties, cell size/morphology, released cell content) can overcome the technical difficulties now existing to measure programmed cell death. The different stages of the apoptotic cascade can in this way be monitored with high specificity on one chip device. Moreover, development of cell arrays, which are analogous to DNA or protein arrays, offers the possibility for high throughput screening. Accordingly there is a real need for simple chip technology to study apoptosis in real time on single cell level with high-throughput.

3. APOPTOSIS ON CHIP

In the past decade, chip technology has shown its great value for chemical analysis in so-called Lab-on-a-Chip systems [121-123]. Recently, the use of microtechnologies for cell biology receives rapidly growing attention [124,125]. Main reason for this is the manipulation of single cells in microfluidic structures and the possibilities for electrical characterisation and detection using microfabricated devices [126-128].

The conventional methods, which are now available to detect apoptosis have many limitations. Apoptosis is one of the most important topics in the field of cellular science, however it is not till recently that research groups have become interested in developing chips convenient for detecting apoptosis. The advantages of microfluidic devices are numerous such as the possibilities for non-destructive real-time analysis of apoptosis. In the section below we will present the few examples of chips for apoptosis analysis that have been presented until today.

Tamaki *et al*. [129] developed a microsystem for cell experiments consisting of a scanning thermal lens microscope detection system and a cell culture microchip. This system is able to detect non-fluorescent biological substances with extremely high sensitivity whitout any labelling materials. They succeeded in monitoring the cytochrome c distribution during apoptosis in a single neuroblastoma-glioma hybrid cell cultured in a microflask (1 mm x 10 mm x 0.1 mm; 1μl), fabricated in a glass microchip. The absolute amount of cytochrome c detected with this system is estimated to be ~ 10 zmol. Kurita *et al*. [130] has also developed a chip-based biosensor enabling the continuous monitoring of neurotransmitters and metabolites. This microfabricated device consist of two glass plates and two glass capillaries, integrated with four electrodes, designed to evaluate the effect of an endocrine disrupter tributyltin (TBT) on the secretion of

glutamate and hydrogen peroxide. High concentrations of TBT show apoptosis like features.

Flow cytometry is seen as the conventional method to analyse the different stages of the apoptotic process. Nowadays, there has been growing interest in flow cytometry performed in microfluidic devices. Chan *et al*. [131] developed a microfluidic system allowing flow cytometric analysis of apoptosis and protein expression with a minimum number of fluorescently stained primary cells. The cells move by pressure driven flow (as in conventional flow cytometry) inside a network of microfluidic channels and are analysed individually by two-channel fluorescence detection. Results obtained with this microfluidic device are consistent compared to conventional flow cytometry, with having the advantages of working on a smaller scale.

Another hallmark of apoptosis is DNA fragmentation, resulting from the activation of a nuclear endonuclease, which selectively cleaves the DNA at sites located between nucleosomal units. Thus, mono- and oligonucleosomal fragments of sizes of 180 base pairs and their multiples are generated, showing a ladder-pattern with conventional gel-electrophoresis. Klepárník *et al*. [132] has developed a CD-like plastic disc for cell handling in a vacuum-driven flow, alkaline lysis and denaturing, and electrophoretic separation. The migration of fluorescently stained DNA fragments is monitored with confocal microscopy. They aimed to confirm the causal relation which might exist between doxorubicin and the extent of DNA fragmentation, which will give a better understanding of the development of chronic doxorubicin induced cardiomyopathy.

Our approach is somewhat different then the methods described above. A new assay is developed which uses autofluorescence (AF) intensity to discriminate viable from apoptotic cells [133]. Measurement of AF reduces sample preparation time and avoids cellular toxicity due to the fact that no labelling is required. This offers us the possibility to measure apoptotic cell death without manipulation of cells and monitor the apoptotic cascade in real time. Human promyelocytic leukemic HL60 cells were incubated with different inducers of apoptosis, e.g. tumour necrosis factor-α, camptothecin, or irradiated with 6 and 10 gray. The progress of the AF intensity in time shows the same pattern for all the inducers used. The AF intensity is increasing the first two hours after incubation followed by a decrease till 24 hours. Between 24 and 48 hours, the AF intensity is increasing again. An important cellular factor driving the cells to apoptotic cell death is the availability of cellular ATP, which results in the increase in AF intensity seen the first two hours after induction of apoptosis. However, later in the apoptotic process, the cell's ability to maintain cellular ATP levels is compromised, which is seen as a decrease in the AF intensity. Necrosis

probably causes the AF intensity to increase again after 24 hours. A microfluidic chip has been developed, enabling the capture of viable cells and measuring the apoptotic cell death kinetics. The mechanical properties (e.g. size) of cells change during the process of apoptosis, and these apoptotic cells will pass the capture position, confirmed by optical detection of a decrease in AF intensity. In future developments, the optical detection will be transferred to an electrical on-chip cell counter specific for apoptosis.

4. CONCLUSIONS

The references described above give a brief summarisation of what has been accomplished in the past few years for detecting apoptosis in a chip-based system. However, till now still little has been done. The development of new micro- and nanotechnological tools, better understanding of single cells and promoting interest among scientist will create new opportunities for realising new micro- or nanofluidic devices to detect apoptosis, which can replace the conventional analytical methods now available.

REFERENCES

1. Kerr J.F.R., Wyllie A.H., Currie A.R. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 26:239-257
2. Wyllie A.H., Kerr J.F.R., Currie A.R. Cell death: The significance of apoptosis. Int Rev Cytol 1980; 68:251-300
3. Vermes I., Haanen C. Apoptosis and programmed cell death in health and disease. Adv Clin Chem 1994; 31:177-246
4. Fadeel B. Programmed cell clearance. CMLS, Cell Mol Life SCi 2003; 60:2575-85
5. Ravichandran K.S. "Recruitment Signals" from apoptotic cells to a quiet meal. Cell 2003; 113:817-20
6. Daniel N.N., Korsmeyer S.J. Cell death: Critical control points. Cell 2004; 116:205-19
7. Savill J. Apoptosis in resolution of inflammation. J Leukoc Biol 1997; 61:375-80
8. Savill J., Gregory C., Haslett C. Cell biology. Eat me or die. Science 2003; 302:1516-7
9. Pearson A.M. Scavenger receptors in innate immunity. Curr Opin Immunol 1996; 8:20-8
10. Diaz C., Schroit A.J. Role of translocases in the generation of phosphatidylserine asymmetry. J Membrane Biol 1996; 151:1-9
11. Zhou Q., Zhao J., Stout J.G., Luhm R.A., Wiedmer T., Sims P.J. Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. J Biol Chem 1997; 272:18240-4
12. Fadok V.A., Voelker D.R., Campbell P.A., Cohen J.J., Bratton D.L., Henson P.M.. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992; 148:2207-16

13. Fadok V.A., Henson P.M. Apoptosis: Giving phosphatidylserine recognition an assist - with a twist. Curr Biol 2003; 13:R655-7
14. Vermes I., Haanen C., Steffens-Nakken H., Reutelingsperger C. A novel assay for apoptosis - Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 1995; 184:39-51
15. Van Engeland M., Nieland L.J.W., Ramaekers F.C.S., Schutte B., Reutelingsperger C.P.M. Annexin V-affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998; 31:1-9
16. Martin S.J., Reutelingsperger C.P., McGahon A.J., Rader J.A., Van Schie R.C., LaFace D.M., Green D.R. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. J Exp Med 1995; 182:1545-56
17. Van den Eijnde S.M., Boshart L., Baehrecke E.H., DeZeeuw C.I., Reutelingsperger C.P.M., VermeijKeers C. Cell surface exposure of phosphatidylserine during apoptosis is phylogenetically conserved. Apoptosis 1998; 3:9-16
18. Kroemer G., Reed J.C. Mitochondrial control of cell death. Nat Med 2000; 6:513-9
19. Marchetti P., Castedo M., Susin S.A., Zamzami N., Hirsch T., Macho A., Haeffner A., Hirsch F., Geuskens M., Kroemer G. Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med 1996; 184:1155-60
20. Kluck R., Bossy Wetzel M.E., Green D.R., Newmeyer D.D. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997; 275:1132-6
21. Reed J.C. Cytochrome c : Can't live with it; can't live without it. Cell 1997; 91:559-62
22. Zou H., Henzel W.J., Liu X., Lutschg A., Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c -dependent activation of caspase-3. Cell 1997; 90:405-13
23. Li P., Nijhawan D., Budihardjo I., Srinivasula S.M., Ahmad M., Alnemri E.S., Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997; 91:479-89
24. Vaux D.L. CED-4 - The third horseman of apoptosis. Cell 1997; 90:389-90
25. Degterev A., Boyce M., Yuan J. A decade of caspases. Oncogene 2003; 22:8543-67
26. Bedner E., Smolewski P., Amstad P., Darzynkiewicz Z. Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): Correlation with DNA fragmentation. Experimental Cell Research 2000; 259:308-313
27. Smolewski P., Grabarek J., Phelps D.J., Darzynkiewicz Z. Stathmo-apoptosis: Arresting apoptosis by fluorochrome-labeled inhibitor of caspases. International Journal of Oncology 2001; 19:657-63
28. Smolewski P., Grabarek J., Halicka H.D., Darzynkiewicz Z. Assay of caspase activation in situ combined with probing plasma membrane integrity to detect three distinct stages of apoptosis. J Immunological Methods 2002; 265:111-21
29. Grabarek J., Amstad P., Darzynkiewicz Z. Use of fluorescently labeled caspase inhibitors as affinity labels to detect activated caspases. Hum Cell 2002; 15:1-12
30. Teodori L., Grabarek J., Smolewski P., Ghibelli L., Bergamaschi A., De Nicola M., Darzynkiewicz Z. Exposure of cells to static magnetic field accelerates loss of integrity of plasma membrane during apoptosis. Cytometry 2002; 49:113-8
31. Fumarola C., Guidotte G.G. Stress-induced apoptosis: Toward a symmetry with receptor-mediated cell death. Apoptosis 2004; 9:77-82

32. Yuan J. Transducing signals of life and death. Curr Opin Cell Biol 1997; 9:247-51
33. Wallach D., Varfolomeev E.E, Malinin N.L., Goltsev Y.V., Kovalenko A.V., Boldin M.P. Tumor necrosis factor receptor and Fas signaling mechanisms. Ann Rev Immunol 1999; 17:331-67
34. Ashe P.C., Berry M.D. Apoptotic signaling cascades. Progr Neuro-psychopharmacol Biol Psychiatry 2003; 27:199-214
35. Green D.R., Reed J.C. Mitochondria and apoptosis. Science 1998; 281:1309-12
36. Boatright K.M., Salvesen G.S. Mechanisms of caspase activation. Curr Opin Cell Biol 2003; 15:725-31
37. Juo P., Kuo C.J., Yuan J., Blenis J. Essential requirement for caspase-8/Flice in the initiation of the Fas-induced apoptotic cascasde. Curr Biol 1998; 8:1001-8
38. Hakem R., Hakem A., Duncan G.S., Henderson J.T., Woo M., Soengas M.S., Elia A., de la Pompa J.L., Kagi D., Khoo W., Potter J., Yoshida R., Kaufman S.A., Lowe S.W., Penninger J.M., Mak T.W. Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell 1998; 94:339-52
39. Muzio M., Stockwell B.R., Stennicke H.R., Salvesen G.S., Dixit V.M. An induced proximity model for caspase-8 activation. J Biol Chem 1998; 273:2926-30
40. Saleh A., Srinivasula S., Acharya S., Fishel R., Alnemri E. Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. J Biol Chem 1999; 274:17941-5
41. Reed J. Mechanisms of apoptosis. Am J Pathol 2000; 157:1416-30
42. Kischkel F.C., Lawrence D.A., Chuntharapai A., Schow P., Kim K.J., Ashkenazi A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity 2000; 12:611-20
43. Frisch S. Evidence for a function of death-receptor-related, death-domain-containing proteins in anoikis. Curr Biol 1999; 9:1047-9
44. Liu Z.G., Hsu H., Goeddel D.V., Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kB activation prevents cell death. Cell 1996; 87:565-76
45. Gougeon M., Montagnier L. Apoptosis in Aids. Science 1993; 260:1269-70
46. Fisher G.H., Rosenberg F.J., Straus S.E., Dale J.K., Middelton L.A., Lin A.Y., Strober W., Lenardo M.J., Puck J.M. Dominant interfering fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell 1995; 81:935-46
47. Hohlbaum A.M., Moe S., Marshak-Rotstein A. Opposing effects of transmembrane and soluble fas ligand expression on inflammation and tumor cell survival. J Exp Med 2000; 191:1209-19
48. Gura T. How TRAIL kills cancer cells, but not normal cells. Nat Med 1997; 277:768
49. Stegh A.H., Schickling O., Ehret A., Scaffidi C., Peterhansel C., Hofmann T.G., Grummt I., Krammer P.H., Peter M.E. DEDD, a novel death effector domain-containing protein, targeted to the nucleolus. EMBO J 1998; 17:5974-86
50. Tschopp J., Irmler M., Thome M. Inhibition of Fas death signals by Flips. Curr Opin Immunol 1998; 10:552-8
51. Deveraux Q.L., Roy N., Stennicke H.R., Van Arsdale T., Zhou Q., Srinivasula S.M., Alnemri E.S., Salvesen G.S., Reed J.C. IAP's block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 1998; 17:2215-23
52. Roy N., Deveraux Q.L., Takashashi R., Salvesen G.S., Reed J.C. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J 1997; 16:6914-25

53. Ambrosini G., Adida C., Altieri D. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997; 3:917-21
54. Gross A., McDonnell J., Korsmeyer S. Bcl-2 family members and the mitochondria in apoptosis. Genes Dev 1999; 13:1899-911
55. Krajewski S., Krajewski M., Ellerby L.M., Welsch K., Xie Z., Deveraux Q.L., Salvesen G.S., Bredesen D.E., Rosenthal R.E., Fiskum G., Reed J.C. Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. Proc Natl Acad Sci USA 1999; 96:5752-7
56. Susin S.A., Lorenzo H.K., Zamzami N., Marzo I., Brenner C., Larochette N., Prevost M.C., Alzari P.M., Kroemer G. Mitochondrial release of caspase-2 and -9 during the apoptotic process. J Exp Med 1999; 189:382-94
57. Smyth P.G., Berman S.A. and Bursztajn S. Markers of apoptosis: Methods for elucidating the mechanism of apoptotic cell death from the nervous system. BioTechniques 2002; 32:648-65
58. Watanabe M., Hitomi M.,Vanderwee Krothenberg F., Fisher S.A., Zucker R., Svobada K.K.H., Goldsmith E.C., Heiskanen K.M., Nieminen A.L. The pros and cons of apoptosis assays for use in the study of cells, tissues, and organs. Microsc Microanal 2002; 8:375-91
59. Lapotko D. Monitoring of apoptosis in intact single cells with photothermal microscopy. Cytometry 2004; 58A: 111-9
60. Gorman A., McCarthy J., Finucane D., Reville W., Cotter T. Morphological assessment of apoptosis. In *"Techniques in apoptosis. A user's guide"* ed. T.G. Cotter, S.J. Martin, London, Portland Press Ltd., 1996
61. Otsuki Y. Various methods of apoptosis detection. Acta Histochem Cytochem 2000; 33:235-41
62. Darzynkiewicz Z., Bruno S., DelBino G., Gorczyca W., Hotz M.A., Lassota P., Targanos F. Features of apoptotic cells measured by flow cytometry. Cytometry 1992; 13:795-808
63. Darzynkiewicz, Z., Juan G., Li X., Gorczyca W., Murakami T., Traganos F. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). Cytometry 1997; 27:1-20
64. Darzynkiewicz Z., Bedner E., Smolewski P. Flow cytometry in analysis of cell cycle and apoptosis. Semin Hematol 2001; 38: 179-93.
65. Darzynkiewicz Z., Li X. Measurements of cell death by flow cytometry. In *"Techniques in apoptosis. A user's guide"* ed. T.G. Cotter, S.J. Martin, London, Portland Press Ltd., 1996
66. Nicoletti I., Migliorati G., Pagliacci M.C., Grignani F., Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth 1991; 139:271-9
67. Telford W.G., King L.E., Fraker P.J. Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. Cytometry 1992; 13:137-43
68. Vermes I., Haanen C., Reutelingsperger C. Flow cytometry of apoptotic cell death. J Immunol Meth 2000; 243:167-90
69. Zhang J., Xu M. Apoptotic DNA fragmentation and tissue homeostasis. Trends in Cell Biol 2002; 12:84-9
70. Hoves S., Krause S.W., Schölmerich J., Fleck M. The JAM-assay: Optimized conditions to determine death-receptor-mediated apoptosis. Methods 2003; 31:127-34

71. Gavrieli Y., Sherman Y., Ben-Sasson S.A. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 1992; 119:493-501
72. Gorczyca W., Gong J., Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res 1993; 53:1945-51
73. Gorczyca W., Bruno S., Darzynkiewicz R.J., Gong, J. Darzynkiewicz Z. DNA strand breaks occurring during apoptosis: Their early in situ detection by the terminal deoxynucleotidyl transferase and nick translation assays and prevention by serine protease inhibitors. Int J Oncol 1992; 1:639-48
74. Meyaard L., Otto S.A., Jonker R.R., Mijnster M.J., Keet R.P.M., Miedema F. Programmed death of T cells in HIV-1 infection. Science 1992; 257:217-9
75. Wijsman J.H., Jonker R.R., Keijzer R., Van de Velde C.J.H., Cornelisse C.J., Van Dierendonck J.H. A new method to detect apoptosis in paraffin sections: In situ end-labelling of fragmented DNA. J Histochem Cytochem 1993; 41:7-12
76. Li X., Darzynkiewicz Z. Labelling DNA strand breaks with BdrUTP. Detection of apoptosis and cell proliferation Cell Proliferation 1995; 28:571-80
77. Wronski R., Golob N., Grygar E., Windisch M. Two-color, fluorescence-based microplate assay for apoptosis detection. BioTechniques 2002; 32:666-8
78. Andree H.A.M., Reutelingsperger C.P.M., Hauptmann R., Hemker H.C., Hermens W.T., Willems G.M. Binding of vascular anticoagulant a (VACa) to planar phospholipid bilayers. J Biol Chem 1990; 265:4923-8
79. Overbeeke R., Yildirim M., Reutelingsperger C.P.M., Haanen C., Vermes I. Sequential occurrence of mitochondrial and plasma membrane alterations, fluctuations in cellular Ca2+ and pH during initial and later phases of cell death. Apoptosis 1999; 4:455-60
80. Prieto A., Diaz D., Barcenilla H., Garcia-Suārez J., Reyes E., Monserrat J., Sanantonio E., Melero D., Hera de la A., Orfao A., Alvarez-Mon M. Apoptotic rate: A new indicator for the quantification of the incidence of apoptosis in cell culture. Cytometry 2002; 48:185-93
81. Tao D., Wu J., Feng Y., Qin J., Hu J., Gong J. New method for the analysis of cell cycle-specific apoptosis. Cytometry 2004; 57A:70-4
82. Nagata S. Apoptotic DNA fragmentation. Exp Cell Res 2000; 256:12-8
83. Nagata S., Nagase H., Kawane K., Mukae N., Fukuyama H. Degradation of chromosomal NDA during apoptosis. Cell Death Diff 2003; 10:108-16
84. Compton M.M. Development of an apoptosis endonuclease assay. DNA Cell Biol 1991; 10:133-41
85. Compton M.M., Cidlowski J.A. Thymocyte apoptosis. A model of programmed cell death. Trends Endocrinol Metab 1992; 3:17-23
86. Eastman A. Assays for DNA fragmentation, endonucleases, and intracellular pH and Ca^{2+} associated with apoptosis. Methods in Cell Biology 1995; 46:41-55
87. Wolfe J.T., Pringle J.H., Cohen G.M. Assays for the measurement of DNA fragmentation during apoptosis. In *"Techniques in apoptosis. A user's guide"* ed. T.G. Cotter, S.J. Martin, London, Portland Press Ltd., 1996
88. Lecoeur H. Nuclear apoptosis detection by flow cytometry: Influence of endogenous endonucleases. Exp Cell Res 2002; 277: 1-14
89. Wu C.F., Bishopric N.H., Pratt R.E. Nonradioactive method for the determination of internucleosomal cleavage associated with apoptosis. BioTechniques 1997; 23:840-3
90. Walker P.R., Leblanc J., Smith B., Pandey S., Sikorska M. Detection of DNA fragmentation and endonucleases in apoptosis. Methods 1999; 17:329-38

91. Kaufmann S.H., Mesner P.W., Samejima K., Toné S., Earnshaw W.C. Detection of DNA cleavage in apoptotic cells. Methods in Enzymology 2000; 322:3-15
92. Facchinetti A., Tessarollo L., Mazzocchi M., Kingston R., Collavo D., Biasi G. An improved method for the detection of DNA fragmentation. J Immunol Meth 1991; 136:125-31
93. Nicholson D.W., Ali A., Thornberry N.A., Vaillancourt J.P., Ding C.K., Gallant M., Gareau Y, Griffin P.R., Labelle M., Lazebnik Y.A., et al. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1995; 376:37-43
94. Gurtu V., Kain S.R., Zhang G. Fluorometric and colorometric detection of caspase activity associated with apoptosis. Anal Biochem 1997; 251:98-102
95. Saunders P.A., Cooper J.A., Roodell M.M., Schroeder D.A., Borchert C.D., Isaacson A.L., Schendel M.J., Godfrey K.G., Cahill D.R., Walz A.M., Loegering R.T., Gaylord H., Woyno I.J., Kaluyzhny A.E., Krzyzek R.A., Mortari F., Tsang M., Roff C.F. Quantification of active caspase 3 in apoptotic cells. Anal Biochem 2000; 284:114-24
96. Zeng L., Smith L.D. Caspase-3 colorimetric assay. BioTechniques 2002; 33:1196-7
97. Karvinen J., Hurskainen P., Gopalakrishnan S., Burns D., Warrior U., Hemmilä I.A. Homogeneous time-resolved fluorescence quenching assay (LANCE) for caspase-3. J Biomol Screening 2002; 7:223-31
98. Préaudat M., Ouled-Diaf J., Alpha-Bazin B., Mathis G., Mitsugi T., Aono Y., Takahasi K., Takemoto H. A homogeneous caspase-3 activity assay using HTRF® technology. J Biomol Screening 2002; 7:267-74
99. King M.A., Radicchi-Mastroianni M.A. Effect of caspase inhibition on camptothecin-induced apoptosis of HL-60 cells. Cytometry 2002; 49:28-35
100. Smolewski P., Grabarek J., Lee B.W., Johnson G.L., Darzynkiewicz Z. Kinetics of HL60 cell entry to apoptosis during treatment with TNF-α or camptothecin assayed by the stathmo-apoptosis method. Cytometry 2002; 47:143-9
101. Wolbers F., Buijtenhuijs P., Haanen C., Vermes I. Apoptotic cell death kinetics in vitro depend on the cell types and the inducers used. Apoptosis 2004; 9:385-92
102. Smolewski P., Bedner E., Du L., Hsieh T., Wu J.M., Phelps D.J., Darzynkiewicz Z. Detection of caspase activation by fluorochrome-labeled inhibitors: Multiparameter analysis by laser scanning cytometry. Cytometry 2001; 44:73-82
103. Fesus L., Davies P.J.A., Piacentini M. Apotosis: Molecular mechanisms in programmed cell death. Eur J Cell Biol 1991; 56:170-7
104. Fesus L., Nemes Z., Piredda L., Madi A., di Rao M., Piacentini M. Measurement of the induction, activity and products of tissue transglutaminase in cells undergoing apoptosis. In *"Techniques in apoptosis. A user's guide"* ed. T.G. Cotter, S.J. Martin, London, Portland Press Ltd., 1996
105. Vermes I., Jansen Steur E.N.H., Jirikowski G.F., Haanen C. Elevated concentration of cerebrospinal fluid tissue transglutaminase in Parkinson's disease indicating apoptosis. Movement Disorders, 2004 (in press)
106. Volokhina E.B., Hulshof R., Haanen C, Vermes I. Tissue transglutaminase mRNA expression in apoptotic cell death. Apoptosis 2003; 8:673-9
107. Hajnóczky G., Davies E., Madesh M. Calcium signalling and apoptosis. Biochem Biophys Res Commun 2003; 304:445-54
108. Orrenius S., Zhivotovsky B., Nicotera P. Regulation of cell death: The calcium-apoptosis link. Nature Rev Mol Cell Biol 2003; 4:552-65

109. McConkey D.J. Calcium flux measurement in cell death. In *"Techniques in apoptosis. A user's guide"* ed. T.G. Cotter, S.J. Martin, London, Portland Press Ltd., 1996
110. Shi Y. A structural view of mitochonria-mediated apoptosis. Nature Structural Biol 2001; 8:394-401
111. Tsujimoto Y., Shimizu S. The voltage-dependent anion channel: An essential player in apoptosis. Biochimie 2002; 84:187-93
112. Ly J.D., Grubb D.R., Lawen A. The mitochondrial membrane potential ($\Delta\psi_m$) in apoptosis: An update. Apoptosis 2003; 8:115-28
113. Kroemer G. Zamzami N., Susin S.A. Mitochondrial control of apoptosis. Immunology Today 1997; 18:44-51
114. Mignotte B., Vayssiere, J.L. Mitochondria and apoptosis. Eur J Biochem 1998; 252:1-15
115. Zhang C., Ao Z., Seth A. Schlossman S.F. A mitochondrial membrane protein defined by a novel monoclonal antibody is preferentially detected in apoptotic cells. J Immunol 1996; 157:3980-7
116. Koester S.K., Roth P., Mikulka W.R., Schlossman S.F., Zhang C., Bolton W.E. Monitoring early cellular responses in apoptosis is aided by the mitochondrial membrane protein-specific monoclonal antibody APO2.7. Cytometry 1997; 29:306-12
117. Overbeeke R., Steffens-Nakken H., Vermes I., Reutelingsperger C., Haanen C. Early features of apoptosis detected by four different flow cytometry assays. Apoptosis 1998; 3:115-21
118. Arnoult D., Parone P., Martinou J., Antonsson B., Estaquier J., Ameisen J.C. Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome c release in response to several apoptotic stimuli. J Cell Biol 2002; 6:923-9
119. Gottlieb R.A., Granville D.J. Analyzing mitochondrial changes during apoptosis. Methods 2002; 26:341-7
120. Rasola A., Geuna M. A flow cytometry assay simultaneously detects independent apoptotic parameters. Cytometry 2001; 45:151-7
121. Reyes D., Iossifidis D., Auroux P., Manz A. Micro total analysis systems. 1. Introduction, theory, and technology. Anal Chem. 2002; 74:2623-36
122. Aroux P., Iossifidis D., Reyes D., Manz A. Micro total analysis systems. 2. Analytical standard operations and applications. 2002; 74:2637-52
123. Van den Berg A., Lammerink T. Micro total analysis systems: microfluidic aspects, integration concept and applications. Top Curr Chem. 1998; 194:21-49
124. Andersson H., van den Berg A. Microfluidic devices for cellomics: a review. Sensors Actuators B. 2003; 92:315-25
125. Andersson H., van den Berg A. Microtechnologies and nanotechnologies for single cell analysis. Curr Opin Biotechnol 2004; 15:44-9
126. Guijt R.M., Baltussen E., van der Steen G., Schasfoort R.B., Schlautmann S., Billiet H.A., Frank J., van Dedem G.W., van den Berg A. New approaches for fabrication of microfluidic capillary electrophoresis devices with on-chip conductivity detection. Electrophoresis. 2001; 22:235-41
127. Blom M.T., Chmela E., Oosterbroek R.E., Tijssen R., van den Berg A. On-chip hydrodynamic chromatography separation and detection of nanoparticles and biomolecules. Anal Chem. 2003; 75:6761-8

128. Vrouwe E.X., Luttge R., van den Berg A. Direct measurement of lithium in whole blood using microchip capillary electrophoresis with integrated conductivity detection. Electrophoresis. Accepted for publication 2004.
129. Tamaki E., Sato K., Tokeshi M., Sato K., Aihara M., Kitamori T. Single-cell analysis by a scanning thermal lens microscope with a microchip: Direct monitoring of cytochrome c distribution during apoptosis process. Anal Chem. 2002; 74:1560-4
130. Kurita R., Hayashi K., Torimitsu K., Niwa O. Continuous measurement of glutamate and hydrogen peroxide using a microfabricated biosensor for studying the neurotoxicity of tributyltin. Anal Sciences. 2003; 19:1581-5
131. Chan S.D.H., Luedke G., Valer M., Buhlmann C., Preckel T. Cytometric analysis of protein expression and apoptosis in human primary cells with a novel microfluidic chip-based system. Cytometry. 2003; 55A: 119-25
132. Klepárník K., Horky M. Detection of DNA fragmentation in a single apoptotic cardiomyocyte by electrophoresis on a microfluidic device. Electrophoresis. 2003; 24: 3778-83
133. Wolbers F., Andersson H., van den Berg A., Vermes I. Apoptosis induced kinetic changes in autofluorescence of cultured HL60 cells – possible application for single cell analysis on chip. In press, Apoptosis 2004.