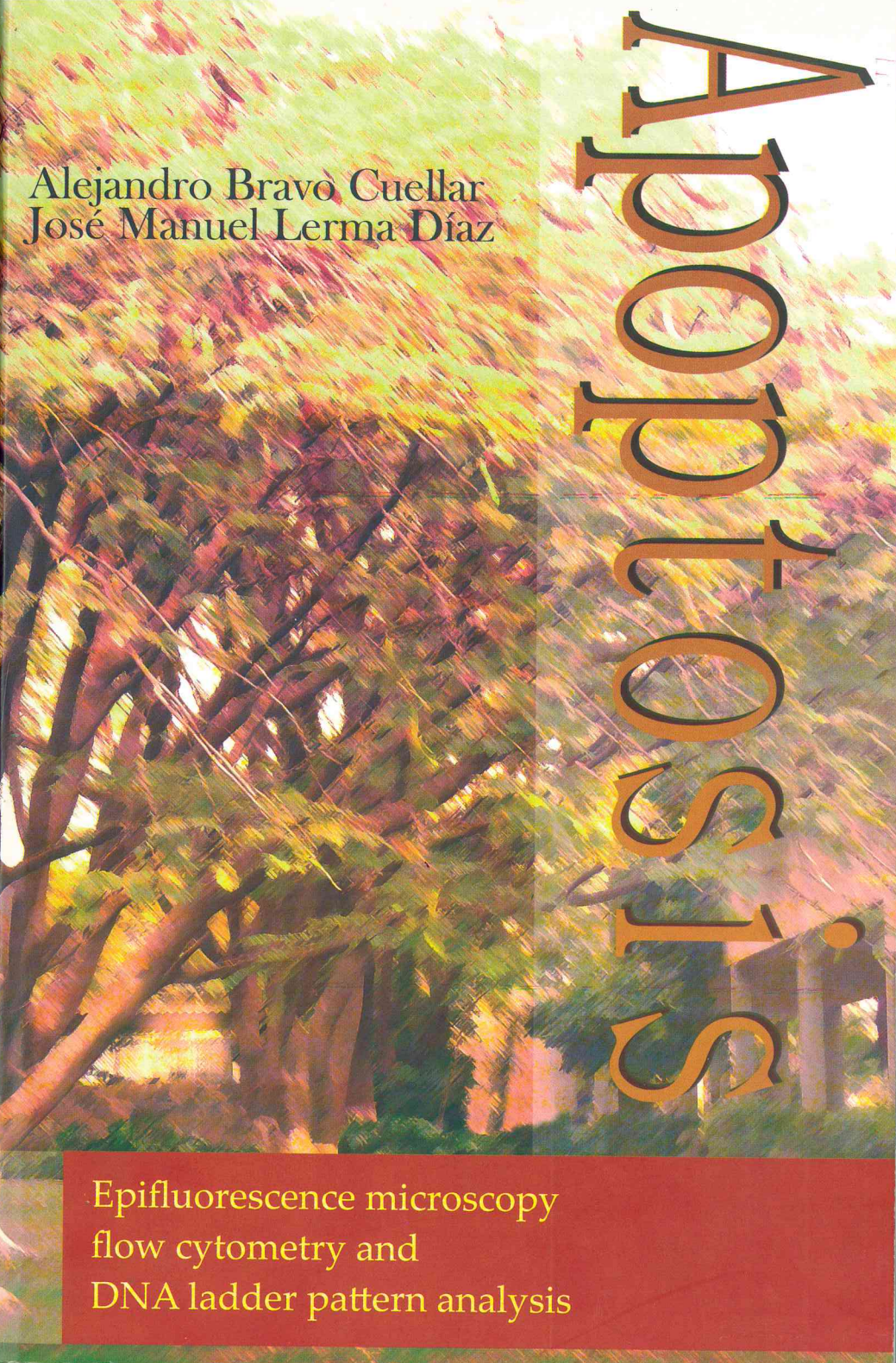
Alejandro Bravo Cuellar
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Apoptosis
Epifluorescence microscopy
flow cytometry and
DNA ladder pattern analysis

Apoptosis

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To our professors

To our students

Preface

Detection of apoptotic cell death in cells and tissues has become of crucial importance in many fields of modern Biology, including studies of embryonic development, degenerative diseases, and Cancer Biology. The need for reliable methods to evaluate death of cells, especially in clinical and experimental Oncology, has led to the development of several techniques. Most of these methods take advantage of properties of dying cells that are more or less specific for the apoptotic process. However, considerable debate exists over the interpretation of some of these methods and their usefulness in all settings. There are many ways of detecting apoptosis and the methods used in the laboratories are mainly based on the identification of apoptotic cell populations by epifluorescence microscopy, flow cytometry and DNA-laddering assay. Here we discuss some of the practical utility of such techniques according to our experience.

Georgina Hernández Flores
Luis Felipe Jave Suárez

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Figures and Tables

1 Apoptotic cell death

Georgina Hernández Flores
Pablo Cesar Ortiz Lazareno
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Martha Barba Barajas
Alejandro Bravo Cuellar

At present, it is well-known that anticancer treatment (chemotherapy and/or radiotherapy) can induce cell death by either of two major mechanisms: necrosis or apoptosis [1]. Necrosis is the death of cells through external damage, usually mediated by destruction of the plasma membrane or the biochemical supports of its integrity. The necrotic cell exhibits a swollen morphology and lysis of plasma membrane, releasing cytoplasmic components into the surrounding tissue spaces. This release of necrotic debris attracts inflammatory cells, leading to the tissue destruction characteristic of inflammation. The death of single cells by this mechanism might be resolvable in some tissues, but a large number of cells dying by necrosis usually results in inflammation and subsequent repair and scarring, leading to compromise and permanent alteration of tissue architecture. Necrosis can occur in a matter of seconds [2].

The other major form of cell death is based on the concept of programmed cell death or apoptosis. Apoptosis can be defined as "gene-directed cellular self-destruction".

Apoptotic cells can be recognized by a characteristic pattern of morphological, biochemical and molecular changes. These changes can be broadly assigned to three stages: An early stage, characterized by decreased cell size (cell dehydration), altered cell membrane, large (50 kilobase) DNA strand breaks and an increase in cellular calcium levels; an intermediate stage of apoptosis that show DNA cleavage into 180-200 base pairs (bp) fragments, which give the characteristic "laddering" on a DNA agarose gel, further decrease in cell size, alterations in plasma membrane symmetry, decreased pH and finally a late stage of apoptosis where typically are present loss of membrane function and apoptotic bodies [3,4].

1.1 Apoptotic nuclear morphology

An early observation in relation to apoptosis is that cells undergoing apoptosis showed dramatic and characteristic changes in nuclear shape and organization. Probably, the typical change in nuclear morphology is the most precise indicator of the involvement of apoptosis in the death of a cell. Nevertheless, some authors have observed that nuclear

segmentation is not essentially required for other aspects of apoptosis. To be precise, cells that have been enucleated still undergo the other changes associated with apoptosis [5]. This highlights the fact that the cytosolic events play an important role in the effector systems of the apoptotic machinery. Conversely, in nucleated cells, the change in nuclear morphology is still an early and relatively unequivocal hallmark of apoptosis. This nuclear shape change occurs at an early point in the series of apoptotic morphological events, usually soon after the beginning of surface blebbing [2].

1.2 DNA ladder pattern

Additionally to changes in nuclear morphology, fragmentation of DNA characterizes apoptosis as well. It was understood that these two events are correlated. However, there is no convincing evidence to suppose that they are correlated. When DNA extracted from apoptotic cells is analyzed using agarose gel electrophoresis, a characteristic internucleosomal "ladder" of DNA fragments is observed. Although apoptotic DNA consists in short fragment lengths of 180-200 bp, larger DNA

fragments have also been seen at earlier times in apoptotic cell cultures [6,7]. This DNA laddering pattern has been used as a hallmark of apoptotic detection. However, many authors assume that apoptosis must be relatively synchronous for this analysis, a synchrony that it is not always present. Those authors, suppose that the internucleosomal breaks in DNA may occur after or during DNA extraction procedures because of the potential fragility of caspase treated DNA in removing other components of chromatin structure [2]. Furthermore, the detection of strand breaks may be so sensitive that only a small number of apoptotic cells may be needed in a population to produce a detectable signal. That is, the entry of the majority of cells into apoptosis might occur at a much later time point than the first detection of "ladders." Even so, these observations led to the development of *in situ* assays for the presence of single strand or double-strand breaks in DNA. The interpretation and application of these methods have been somewhat controversial. Regardless of the specific methods used, the change in nuclear morphology frequently does not coincide with the appearance of detectable strand breaks in every cell [2].

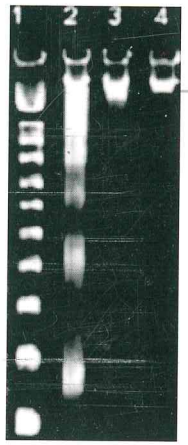

Fig. 1.– Electrophoretic analysis of L5178-Y lymphoma cell DNA. Lane 1, DNA molecular weight marker with 100 bp incremental fragmentation as the positive control; lane 2, lymphoma cells from tumor-bearing mice treated with doxorubicin (5mg/kg) show a DNA ladder pattern; lane 3, lymphoma cells from tumor-bearing mice treated with doxorubicin (2.5mg/kg) show no DNA ladder pattern; lane 4, lymphoma cells without treatment as the negative control.

In relation to this area under discussion, in the field of clinical and experimental oncology, it is recognized that many chemotherapeutic agents and radiotherapy have genotoxic effects either in cancer cells and/or in normal cells [8,9]. These anticancer therapies exert their cytotoxic action through some common pathways to induce damage in the DNA. However, in our experience we determined that it is necessary to obtain apoptotic indices ≥ 50% to visualize DNA laddering pattern (Fig. 1), *in vivo* and/or *in vitro* models, when cells under treatment are evaluated by fluorescence microscopy studies [8]. Moreover, we have observed that apoptosis is present in the late stages in an important number of these cells. Therefore, we may deduce that DNA fragmentation is a very late event in this process. Also, we have observed a considerable correlation

between the results of fluorescence microscopy studies, DNA laddering analysis and survival studies in a lymphoma bearing-mice model [10]. These data show that fluorescence microscopy and DNA laddering assay together represent reliable methods with high specificity to study apoptosis induction in cancer cells. In any case, more *in vivo* and survival studies are necessary to corroborate the biological importance of these findings.

1.3 The importance of mitochondria in cell death

Since mitochondria play important roles in cell death pathways, these organelles are potentially key targets for therapeutic intervention [8,10]. It is well known that chemotherapy and/or radiotherapy induce mitochondrial dysfunction and failure leading to apoptotic and necrotic cell death. Despite apoptosis and necrosis are two forms of cell death with clearly distinguishing morphological and biochemical features [11], there are reports suggesting, however, that apoptosis and necrosis have some common steps. BCL-2 and caspase inhibitors had been believed to specifically inhibit apoptosis,

but preventive effects of BCL-2 on necrosis were also proved by the demonstration that BCL-2 and its relative, BCL-XL, inhibit necrotic cell death induced by oxygen depletion, respiratory chain inhibitors such as KCN and actinomycin A, or by glutathione depletion [12,13].

Necrotic cell death can be also retarded by caspase inhibitors, including tetrapeptide inhibitors and a serpin, CrmA, derived from cowpox virus [14]. Moreover, the mitochondrial permeability transition (MPT) represents a pathway that is shared both in apoptosis and necrosis [15,16].

There is a general agreement that apoptosis, in contrast to necrosis, it is an active, energy-requiring process. Richter et al. (1996) were the first to propose that the cellular ATP level is an important determining factor for cell death, either by apoptosis or necrosis. They hypothesized that a cell stays alive as long as a certain ATP level is maintained. When ATP falls below this level apoptosis ensues, provided that enough ATP is still available for energy-requiring apoptotic processes such as enzymatic hydrolysis of macromolecules, nuclear condensation, and bleb formation. Only low ATP concentrations can switch an apoptotic death towards a necrotic fate [17].

2 Epifluorescence microscopy

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Fluorescent dyes are a vital component of the fluorescence microscopy systems because they allow for specific and sensitive determination of the localization of molecules (i.e., proteins and DNA) within the cell. Simply, fluorescent dyes are themselves molecules that are able to absorb light of one wavelength and then emit light of another, longer wavelength. For fluorescence microscopy, these dyes are usually bound to the cellular molecule under study. By illuminating a dye-labeled specimen with light matching the excitation spectrum of the dye, and then collecting the emitted light, it is possible to visualize only the location of the dye molecules (providing specificity). The use of a vertical illuminator and dichroic mirrors offers some advantages over fluorescence microscopy using transmitted illumination [18]. Practically all fluorescence microscopes use the objective lens to perform two functions: a) Focus the illumination (excitation) light on the sample. In order to excite fluorescent species in a sample, the optics of a fluorescent microscope must focus the illumination (excitation) light on the sample to a greater extent than is achieved using the simple condenser lens system found in the illumination

light path of a conventional microscope; b) Collect the emitted fluorescence. This type of excitation-emission configuration, in which both the excitation and emission light travel through the objective, is called epifluorescence. The key to the optics in an epifluorescence microscope is the separation of the illumination (excitation) light from the fluorescence emission emanating from the sample. In order to obtain either an image of the emission without excessive background illumination, or a measurement of the fluorescence emission without background "noise", the optical elements used to separate these two light components must be very efficient [19,20].

2.1 Fluorescent dyes for cell viability assessment

Acridine orange, ethidium bromide, and propidium iodide are among the most used fluorescent dyes to analyze cell culture viability. In fact, they respectively show specificity for apoptotic, living, and late apoptosis/necrosis states. In our laboratory, we commonly use double or triple dye combinations in both fluorescence microscopy and flow cytometry assays [7,16,21].

2.1.1 Acridine orange

Acridine orange, is a specific stain for the two types of nucleic acid. With this stain, DNA in the nuclei gives a green fluorescence; RNA in the cytoplasm fluoresces brown, reddish-brown, orange or bright red, depending upon whether small or large amounts of RNA are present. This specific histochemical reaction is a manifestation of the fact that RNA is closely associated with protein synthesis of the cell. Non dividing or rarely dividing cells, containing little or nothing RNA, show a green to brown fluorescence, while cells dividing at physiological rates contain moderate amounts of RNA and give a reddish brown fluorescence. Malignant cells, which have a very high content of RNA for protein synthesis during rapid formation of daughter cells, fluoresce bright orange to flaming red. Malignant cells, whether they happen in the respiratory, digestive, urinary or female genital tract, body fluid or other material, are characterized by bright orange to flaming red fluorescence of the cytoplasm and often by greenish-yellow hyperchromatic nuclei. In some chronic inflammatory conditions, samples of respiratory material and

body fluids may contain epithelial cells and macrophages which have an increased red fluorescence of the cytoplasm. In cells suffering chronic and acute inflammatory processes may similarly exhibit red fluorescence. It is thought, that a long-standing irritation stimulates the cells to proliferate at rates faster than normal and therefore they contain more RNA. Regardless of their increased fluorescence, such cells can be distinguished from malignant cells by their normal morphological features. Since acridine orange is a dye which quantitatively demonstrates the amount of RNA in all cells, any increase in the amount of RNA in normal cells will also be shown by the dye. Except for changes in nucleic acid content, there is little cytochemical difference between normal and malignant cells. Hence, it is not feasible to develop a specific stain for cancer cells [22].

2.1.2 Ethidium bromide

Ethidium bromide is a well-known and widely used fluorescent dye in biotechnology research. It is a mutagenic compound which intercalates double-stranded DNA and

RNA. The fluorescence of ethidium bromide increases 21-fold upon binding to double-stranded RNA, 25-fold on binding double-stranded DNA (although histones block binding of ethidium bromide to DNA). Ethidium bromide has been used in a number of fluorimetric assays for nucleic acids. It has been shown to bind to single-stranded DNA (although not as strongly) and triple-stranded DNA. Because of the binding to DNA, ethidium bromide is a powerful inhibitor of DNA polymerase [23].

2.1.3 Propidium iodide

Propidium iodide binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. Propidium iodide also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold, the fluorescence excitation maximum is shifted 30–40 nm to the red and the fluorescence emission maximum is shifted ~15 nm to the blue. Propidium iodide

is appropriate for fluorescence microscopy, confocal laser scanning microscopy, flow cytometry and fluorometry. Propidium iodide is membrane impermeant and generally excluded from viable cells. Therefore, propidium iodide is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques [24,25].

2.2 Assessment of cellular death

In our laboratory, we commonly utilized a stain mixture composed by both acridine orange and ethidium bromide (100 µg/mL each suspended in phosphate buffer saline) to assessment of apoptotic, live and necrotic indices. Acridine orange intercalates into DNA and makes it appear green. It also binds to RNA, but because it cannot intercalate, the RNA stains red-orange. Thus a stained cell will have a green nucleus and may have red orange-cytoplasm. Acridine orange is used to determine how many cells within a giving cell population have undergone apoptosis, but it can not differentiated between viable and nonviable cells. To do this, the above-mentioned mixture of acridine orange and ethidium bromide

is needed. The differential uptake of these two dyes allows the identification of viable and nonviable cells (Fig. 2) . With this method, we are able to measure the following cellular states [8,10]:

- Live cells with normal nuclei, bright green chromatin and organized structure (LN).
- Apoptotic cells with highly condensed or fragmented bright green-yellow chromatin (A).
- Dead cells with normal nuclei, bright red chromatin and organized structure (DN).
- Dead cells with apoptotic nuclei, and bright orange chromatin that was highly condensed and fragmented (DA).

Indices of these cellular states can be calculated as follows:

Apoptotic index (AI)

$$AI = 100 \left(\frac{A+DA}{LN+A+DN+DA} \right)$$

Live cell index (LCI)

$$LCI = 100 \left(\frac{LN}{LN+A+DN+DA} \right)$$

Necrotic cell index (NCI)

$$NCI = 100 \left(\frac{DN}{LN+A+DN+DA} \right)$$

We are agreement with other authors, that this method used to evaluate AI has a high degree of reproducibility and a sufficient sensitivity to clearly detect statistical differences between diverse cell populations, whether *in vitro* and/or *in vivo* models [8,10,26].

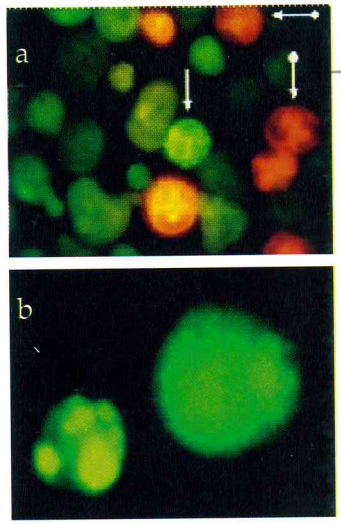

Fig. 2.– Epifluorescence microscopy with acridine orange/ethidium bromide stains. **(a)** L-5178Y cells from tumor-bearing mice treated with doxorubicin were scored as follows: A= apoptotic cell (normal-arrow), DN= dead cell with normal nuclei or necrotic cell (rhomb-arrow) and, dead cell with apoptotic nuclei (oval-arrow). **(b)** Cultured U937 cells treated with doxorubicin, note the dramatic decrease in the cell size and the morphological characteristic changes in cell shape of the apoptotic cell (left side) in comparison with a live cell (right side).

2.3 Mitochondrial monitoring

Disruption of mitochondrial membrane potential ($\Delta\Psi m$) is one of the earliest intracellular events that occur following induction of apoptosis. Currently, available kits provide a simple, fluorescent based method for differentiating between healthy and apoptotic cells by detecting the changes in the

mitochondrial transmembrane potential (Fig.3). Usually, these methods use a cationic dye (i.e. MitoCapture™ dihexyloxacarbocyanine iodide, $DiOC_6$) that fluoresces differently in healthy or in apoptotic cell populations. With the MitoCapture™ assay, we can recognize the following two cellular states [8,16]:

- **Live cells** (LC), dye accumulates and polymerizes in the mitochondria, giving a bright red fluorescence.
- **Apoptotic cells** (AC), dye cannot aggregate in the mitochondria because of the lost mitochondrial transmembrane potential and thus remains in the cytoplasm in its monomeric form, fluorescing green.

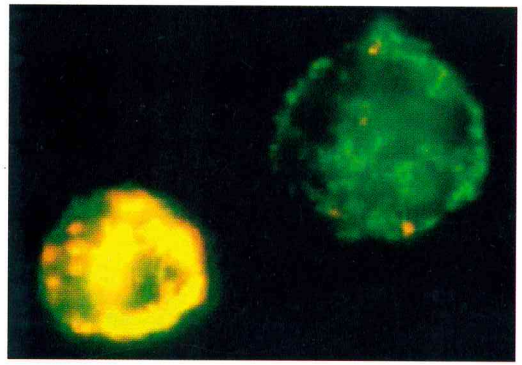

Fig. 3.– U937 human monocytic leukemia cell line treated with doxorubicin. A fluorescent-based method (MitoCapture™) for distinguishing between healthy and apoptotic cells by detecting the changes in the mitochondrial membrane potential was used. Live cell (left side) and apoptotic cell (right side).

The fluorescent signals can be easily detected by fluorescence microscopy using a band-pass filter. Indices of these cellular states can be calculated as follows:

Mitochondrial apoptotic index (MAI)

$$MAI = 100\left(\frac{AC}{LC+AC}\right)$$

Mitochondrial live cell index (MLCI)

$$MLCI = 100\left(\frac{LC}{LC+AC}\right)$$

3 Flow cytometry

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Flow cytometry is a technique used for measuring parameters in cells, such as size, complexity, and other characteristics including expression of cell surface receptors, intracellular molecules, and phosphorylation states. We can count and isolate since microscopic particles until cells suspended in a stream of fluid [27-31].

A flow cytometer consists of fluidic, optic, and electronic systems. The fluidic system is responsible of moving a particle or cell through all the system, from the injection site of the sample to the point of examination by the laser and subsequent removal to waste container or collection during the cell separation (sorting) [32-37].

The optic system consists of an excitation complex composed of laser, optic wires, and lens. In addition, there is a collection system made up of a photomultiplier tube (PMT), bandpass filters, longpass filters, optical cables and lens. The electronic system converts analogical signals to digital ones; these signals are capable of being stored in a computer. Several laser types are available that can be used in flow cytometers;

these include helium-neon, argon, and helium-cadmium [34,38-40]

3.1 Flow cytometry basic principles

When the laser collides with the individual particles or cells this interaction create light dispersion and fluorescence signals [41-43].

Dispersion signals result from the interaction of laser light with a particle or cell that produces a change of direction in all directions of space. In flow cytometry, there are measured two fractions from this dispersion:

Forward Scatter (FSC) is a measure relationated with the size of the particle or cell, is a parameter measuring light scattered less than 10° angle when a particle or cell passes through the laser beam [44-47].

The Side Scatter (SSC) is a measure related with the complexity or granularity of the internal structure of the particle or cell (nuclei, organelles), and the light scattered at a 90° angle when a cell passes through the laser beam [45,48-50].

Fluorescent signals result from the interaction of laser beam with a fluorochrome, the later is a molecule with the property to absorb light in a specific wavelength and emit light at a longer wavelength. Flow cytometers detect fluorescence at a 90° angle from the exciting light beam. Some fluorochromes are conjugated to antibodies, this allows us to measure several keystone parameters in cells such as expression of cell surface receptors, intracellular molecules, enzymatic activity, transcription factors, cell cycle, cell proliferation, apoptosis and loss of the mitochondrial membrane potential ($\Delta\psi m$) among others [51-62]. A list of different fluorochromes that can be used in flow cytometry are shown in Table 1.

Applications for flow cytometry and cell sorting are becoming increasingly important in clinical diagnosis and biology research. Phenotypic surface markers, physiological responses, functional expression of intracellular products and cell apoptotic status are attributes that can be measured independently or simultaneously by flow cytometry using different fluorochrome-conjugated antibodies. It is important to note that depending on the performance of the cytometer we can use from one to up to seven different fluorochromes

reaching a very high level of specificity [63,64].

Table 1.- Fluorochromes that can be used in flow cytometry

Fluorochrome	Ex (nm)	Em (nm)
7-AAD	546	647
Acridine Orange	503	530/640
Alexa Fluor 430	430	545
Alexa Fluor 488	494	517
Alexa Fluor 532	530	555
Alexa Fluor 680	679	702
Allophycocyanin (APC)	650	660
APC-Cy7 conjugates	650; 755	767
Cy2	489	506
Cy3	(512); 550	570; (615)
Cy3.5	581	596; (640)
Cy5	(625); 650	670
Cy5.5	675	694
Ethidium Bromide	493	620
Fluorescein	495	519
Hoechst 33258	345	478
Hoechst 33342	343	483
PE-Cy5 conjugates	480; 565; 650	670
PE-Cy7 conjugates	480; 565; 743	767
PerCP	490	675
Propidium Iodide (PI)	536	617
R-Phycoerythrin (PE)	480; 565	578
SYTOX Green	504	523
SYTOX Orange	547	570
Texas Red	589	615
X-Rhodamine	570	576

Ex = Excitation spectrum; Em = Emission spectrum.

3.2 Flow cytometry and apoptosis

Apoptosis is an interesting phenomenon that can be studied by flow cytometry. During the apoptotic process, there are

cell permeability and membrane integrity changes, both in early and late apoptotic states that can be detected with this technology.

Cell death can be analyzed by flow cytometry by observing changes in size and granularity. Cells in the early stage of apoptosis suffer a decrease in their forward light scatter, while those in late apoptosis decrease in their side scatter as seen in Figure 4.

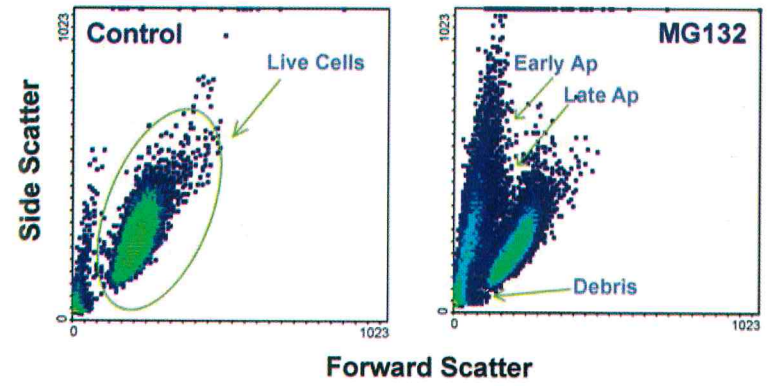

Fig 4.– Light scattering properties of apoptotic cells. The figure shows a representative example of the light scattering properties of apoptotic cells; U937 cells were exposed for 16 h to 2 μM of proteasome inhibitor MG132.

It is well known that in early apoptotic stages the plasmatic membrane involves externalization of phosphatidylserine (PS) residues. These can be detected directly using annexin-V labeled with fluorescein isothicyanate (FITC) or with other fluorochrome directed against the phosphatidylserine

residues. For the detection of the late apoptotic states, dyes such as propidium iodide (PI) are suggested. PI binds to double stranded nucleic acids by intercalating in the double helix and analyzed by flow cytometry [67-69]. PI cell death is illustrated with a red coloration, while live cells do not capture the dye. Such analysis always allows us to know the percentages of cells that are live, dead and their respective apoptotic populations (early and late) [70-72].

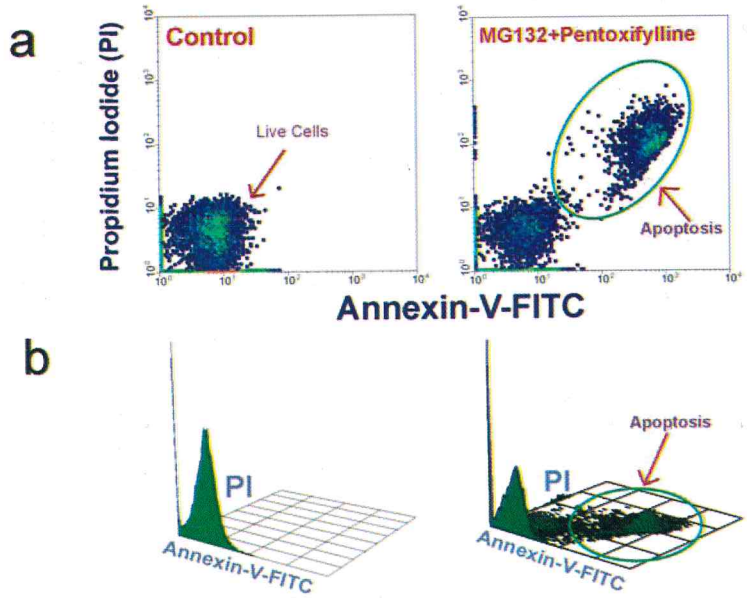

Fig. 5.– Representative example of apoptosis in K562 cells treated with pentoxifylline and MG132. The figure shows a representative example of apoptosis in K562 cells, which were treated for 24h with 8 mM pentoxifylline and 1 μM MG132. (a) Density plot: We can observed cells alive in the lower left, cells in the upper rigth are in apoptosis. (b) We show the same experiment in 3D plot.

In Figure 5 a representative example with K562 cells is shown. The leukemic cells were treated with pentoxifylline and proteasome inhibitor (MG132) for 24h, and apoptosis was measured by flow cytometry using annexin-V-FITC/IP [10]. We represent the same data in density plot (a), and 3D plot (b).

The natural toxin called gossypol is a polyphenol aldehyde found in cotton seeds which can induce apoptosis both in normal and tumor cells. In Figure 6, an example of

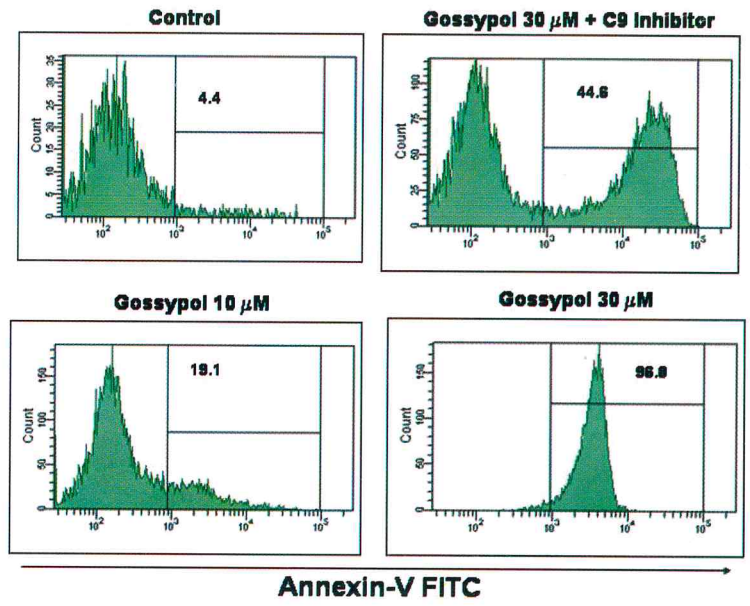

Fig. 6.– Apoptosis in monocytes from peripheral blood of healthy volunteers pretreated with caspase inhibitor-9 before gossypol treatment. The cells were exposed for 15 min to caspase-9 inhibitor, then monocytes were exposed for 15 min to 10 and 30 μM of gossypol and apoptosis was measured by flow cytomery using annexin-V-FITC.

enzymatic activity determined by flow cytometry is shown; we illustrate peripheral blood monocytes from normal volunteers pretreated with caspase inhibitor-9 before gossypol treatment. Apoptosis was measured by flow cytometry using annexin-V-FITC (Fig. 6).

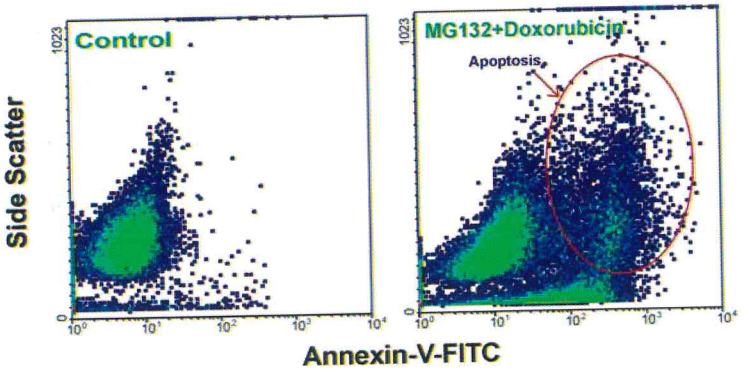

Fig. 7.– Apoptosis in U937 cells treated with doxorubicin and proteasome inhibitor MG132. U937 cells were exposed for 16 h to 2 μM MG132 and 1 μM doxorubicin after that apoptosis was measured by flow cytomery using annexin-V-FITC.

In Figure 7 we show U937 cells treated with doxorubicin, an antitumor agent of the anthracycline family and proteasome inhibitor MG132, apoptosis was measured by flow cytometry using annexin-V-FITC.

We can measure the Δψm by flow cytometry using different fluorochromes or dyes like rhodamine, $DiOC_6$, 5,5′,6,6′tetrach loro1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-

1). In Figure 8, we show human peripheral blood monocytes treated with gossypol, subsequently the loss of the Δψm was measured by flow cytometry using $DiOC_6$ [73-77].

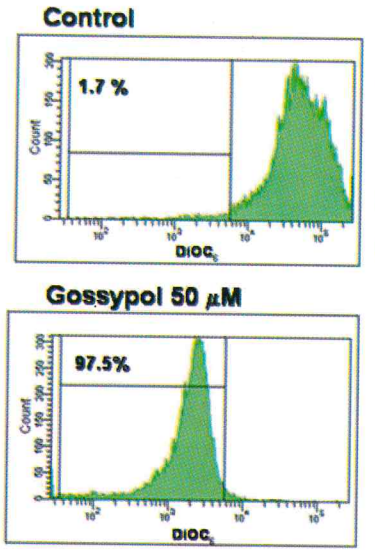

Fig. 8.– Loss of the Δψm in monocytes treated with gossypol. The figure shows a representative example. Cells ***in vitro*** were exposed for 15 min to gossypol, then, we measured apoptosis by flow cytometry using $DiOC_6$. Normal cells bright green, cells with loss of the Δψm show decreased the intensity of the bright for $DiOC_6$. We observed 97.5 % of monocytes after gossypol treatment show a disruption of the Δψm.

4 Concluding and remarks

Alejandro Bravo Cuellar
José Manuel Lerma Díaz
Georgina Hernández Flores
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Since its introduction, flow cytometric assays have been widely used for the evaluation of apoptosis in different experimental models. It is based on the principle that apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Use of a fluorochrome, such as propidium iodide, that is capable of binding and labeling DNA, makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis. This technique is a quick, easy but not very specific method of detecting apoptosis, although it can give valuable clues to what exactly is happening in a population of cells, apoptosis of cells in late S phase or from G2 may be missed [78]. One of the membrane changes in the early/intermediate stages of apoptosis is the translocation of phosphatidylserine from the inner of the cell membrane to the outside. It is possible to detect phosphatidylserine by using FITC-labeled annexin-V, which is a Ca^{++} dependent phospholipid-binding protein. By combining staining of annexin-V-FITC with propidium iodide, we can obtain a

comparable profile *in vitro* models to the acridine orange/ethidium bromide method, where live cells are negative for both dyes, dead cells are positive for both, while apoptotic cells are positive only for annexin-V-FITC. The advantage of this method is that it can be used on an analytical flow cytometer but it is more applicable to suspension cells. In our experience, fluorescence microscopy together with DNA leadering pattern give good results in survival studies, although they are not automated methods.

It is documented, that phosphatidilserine may be a reversible marker of apoptosis, since there is evidence that DNA repair is activated early in p53-induced apoptosis and may be involved in its reversibility. Furthermore, it has been confirmed that early p53-induced apoptotic cells can be rescued from the apoptotic program, and DNA repair can modulate that cell death process [79]. During apoptosis induced by various stimuli, cytochrome c is released from mitochondria into the cytosol where it participates in caspase activation. This process has been proposed to be an irreversible consequence of mitochondrial permeability transition pore opening, which leads to mitochondrial swelling and rupture

of the outer mitochondrial membrane. However, there is evidence demonstrating that nervous growth factor (NGF)-deprived sympathetic neurons protected from apoptosis by caspase inhibitors possess mitochondria which, though depleted of cytochrome c and reduced in size, remained structurally intact as viewed by electron microscopy. After re-exposure of neurons to NGF, mitochondria recovered their normal size and their cytochrome c content, by a process requiring *de novo* protein synthesis. These data suggest that depletion of cytochrome c from mitochondria is a controlled process compatible with function recovery [80]. This may be an inconvenience if we decide to utilize mitochondrial monitoring assays. The major advantage of the fluorescence method is that it enables an untrained observer to readily ascertain whether a sample contains cells which exhibit a green or red fluorescence and hence may be viable or non-viable cells, respectively. The technician then can be easily trained to discriminate the cells which show typical morphological features of apoptosis. In addition, categorizing the cells by live, necrotic and apoptotic cell populations, the observer can note ultimely when either a toxic or inappropriate therapeutic effects appear. A second

advantage is that the solutions used in this technique keep indefinitely. A third advantage is that a fluorescence lamp with a high pressure mercury burner and proper filters, which can be attached to any laboratory microscope, is the only equipment required for the microscopy. Such equipment is available at major optical and scientific instruments firms and represents an economical and efficient procedure. Thus, microscopic examination of acridin orange/ethidium bromide stained cells can be recommended as a reliable methods. Finally, it is important to stress that flow cytometry is a very versatile and economic method that allows a comprehensive study of apoptosis in each of its stages and pathways.

Each technique possesses variable levels of specificity and sensibility and they are dependent of the biological features of the cells, the type of apoptotic stimulus, the amount of cells undergoing apoptosis at the moment of the apoptosis evaluation, and the progression of apoptosis from early to late stages in the cell populations.

Bibliography

[1] Hernandez-Flores G, Gomez-Contreras PC, Dominguez-Rodriguez JR, Lerma-Diaz JM, Ortiz-Lazareno PC, Cervantes-Munguia R, Sahagun-Flores JE, Orbach-Arbouys S, Scott-Algara D, Bravo-Cuellar A. Gamma-irradiation induced apoptosis in peritoneal macrophages by oxidative stress. Implications of antioxidants in caspase mitochondrial pathway. Anticancer Res 2005;25:4091-4100.

[2] Collins JA, Schandi CA, Young KK, Vesely J, Willingham MC. Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem 1997;45:923-934.

[3] Ouyang XL, Liu JH, Luo Q, Shi Q, Han W, Li XJ, Gao D. [Flow cytometry combined assay for phosphatidylserine and CD62p expressed by preserved platelets]. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2002;10:66-69.

[4] Ghibelli L, Maresca V, Coppola S, Gualandi G. Protease inhibitors block apoptosis at intermediate stages: a compared analysis of DNA fragmentation and apoptotic nuclear morphology. FEBS Lett 1995;377:9-14.

[5] Schulze-Osthoff K, Walczak H, Droge W, Krammer PH. Cell nucleus and DNA fragmentation are not required for apoptosis. J Cell Biol 1994;127:15-20.

[6] Lesauskaite V, Ivanoviene L. [Programmed cell death: molecular mechanisms and detection]. Medicina (Kaunas) 2002;38:869-875.

[7] Lerma-Diaz JM, Hernandez-Flores G, Dominguez-Rodriguez JR, Ortiz-Lazareno PC, Gomez-Contreras P, Cervantes-Munguia R, Scott-Algara D, Aguilar-Lemarroy A, Jave-Suarez LF, Bravo-Cuellar A. In vivo and in vitro sensitization of leukemic cells to adriamycin-induced apoptosis by pentoxifylline. Involvement of caspase cascades and IkappaBalpha phosphorylation. Immunol Lett 2006;103:149-158.

[8] Bravo-Cuellar A, Gomez-Contreras PC, Lerma-Diaz JM, Hernandez-Flores G, Dominguez-Rodriguez JR, Ortiz-Lazareno P, Cervantes-Munguia R, Orbach-Arbouys S. In vivo modification of adriamycin-induced apoptosis in L-5178Y lymphoma cell-bearing mice by (+)-alpha-tocopherol and superoxide dismutase. Cancer Lett 2005;229:59-65.

[9] Sala-Trepat M, Cole J, Green MH, Rigaud O, Vilcoq JR, Moustacchi E. Genotoxic effects of radiotherapy and chemotherapy on the circulating lymphocytes of breast cancer patients. III: Measurement of mutant frequency to 6-thioguanine resistance. Mutagenesis 1990;5:593-598.

[10] Ortiz-Lazareno PC, Hernandez-Flores G, Dominguez-Rodriguez JR, Lerma-Diaz JM, Jave-Suarez LF, Aguilar-Lemarroy A, Gomez-Contreras PC, Scott-Algara D, Bravo-Cuellar A. MG132 proteasome inhibitor modulates proinflammatory cytokines production and expression of their receptors in U937 cells: involvement of nuclear factor-kappaB and activator protein-1. Immunology 2008.

[11] Helewski KJ, Kowalczyk-Ziomek GI, Konecki J. [Apoptosis and necrosis--two different ways leading to the same target]. Wiad Lek 2006;59:679-684.

[12] Tsujimoto Y, Shimizu S, Eguchi Y, Kamiike W, Matsuda H. Bcl-2 and Bcl-xL block apoptosis as well as necrosis: possible involvement of common mediators in apoptotic and necrotic signal transduction pathways. Leukemia 1997;11 Suppl 3:380-382.

[13] Gomez-Contreras PC, Hernandez-Flores G, Ortiz-Lazareno PC, Del Toro-Arreola S, Delgado-Rizo V, Lerma-Diaz JM, Barba-Barajas M, Dominguez-Rodriguez JR, Bravo Cuellar A. In vitro induction of apoptosis in U937 cells by perillyl alcohol with sensitization by pentoxifylline: increased BCL-2 and BAX protein expression. Chemotherapy 2006;52:308-315.

[14] Tsujimoto Y. Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes. Cell Death Differ 1997;4:429-434.

[15] Lemasters JJ, Qian T, Bradham CA, Brenner DA, Cascio WE, Trost LC, Nishimura Y, Nieminen AL, Herman B. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. J Bioenerg Biomembr 1999;31:305-319.

[16] Dominguez-Rodriguez JR, Chaparro-Huerta V, Hernandez-Flores G, Gomez-Contreras PC, Lerma-Daz JM, Ortiz-Lazareno PC, Cervantes-Munguia R, Orbach-Arbouys S, Scott-Algara D, Cuellar AB. In vivo adriamycin-induced apoptosis in peritoneal murine macrophages: partial participation of a caspase cascade. Anticancer Res 2004;24:2689-2696.

[17] Richter C, Schweizer M, Cossarizza A, Franceschi C. Control of apoptosis by the cellular ATP level. FEBS Lett 1996;378:107-110.

[18] Sawano A, Hama H, Saito N, Miyawaki A. Multicolor imaging of Ca(2+) and protein kinase C signals using novel epifluorescence microscopy. Biophys J 2002;82:1076-1085.

[19] Lang E, Baier J, Kohler J. Epifluorescence, confocal and total internal reflection microscopy for single-molecule experiments: a quantitative comparison. J Microsc 2006;222:118-123.

[20] Lowy RJ. Evaluation of triple-band filters for quantitative epifluorescence microscopy. J Microsc 1995;178:240-250.

[21] Dominguez-Rodriguez JR, Gomez-Contreras PC, Hernandez-Flores G, Lerma-Diaz JM, Carranco A, Cervantes-Munguia R, Orbach-Arbouys S, Bravo-Cuella A. In vivo inhibition by antioxidants of adriamycin-induced apoptosis in murine peritoneal macrophages. Anticancer Res 2001;21:1869-1872.

[22] Blackshaw AW. Proceedings: Fluorescence microscopy of damage to the germinal epithelium of the rat testis. J Reprod Fertil 1974;36: 439.

[23] Pauluhn J, Naujok A, Zimmermann HW. [On the quantitative fluorometric determination of cellular DNA by ethidium bromide and the constancy of the quantum yield of the ethidium DNA-complex in the biological environment (author's transl)]. Z Naturforsch [C] 1980;35:585-598.

[24] Giordano PA, Mazzini G, Riccardi A, Montecucco CM, Ucci G, Danova M. Propidium iodide staining of cytoautoradiographic preparations for the simultaneous determination of DNA content and grain count. Histochem J 1985;17:1259-1270.

[25] Wulf M, Bosse A, Voss B, Muller KM. Improvement of fluorescence in situ hybridization (RNA-FISH) on human paraffin sections by propidium iodide counterstaining. Biotechniques 1995;19:368-370, 372.

[26] Baskic D, Popovic S, Ristic P, Arsenijevic NN. Analysis of cycloheximide-induced apoptosis in human leukocytes: fluorescence microscopy using annexin V/propidium iodide versus acridin orange/ethidium bromide. Cell Biol Int 2006;30:924-932.

[27] Romano AC, Espana EM, Yoo SH, Budak MT, Wolosin JM, Tseng SC. Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. Invest Ophthalmol Vis Sci 2003;44:5125-5129.

[28] Brown M, Wittwer C. Flow cytometry: principles and clinical applications in hematology. Clin Chem 2000;46:1221-1229.

[29] Wojcik T, Bereta M, Faron-Gorecka A, Dziedzicka-Wasylewska M, Kiec-Kononowicz K. Flow cytometry application for studies on adenosine A2A receptors expression. Acta Pol Pharm 2008;65:65-70.

[30] Bardsley WG, Kyprianou EK. A statistical theory for flow cytometry profiles in terms of the binding of ligands to cell surface receptors and changes in gene expression. J Math Biol 1996;34:271-296.

[31] Krutzik PO, Irish JM, Nolan GP, Perez OD. Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. Clin Immunol 2004;110:206-221.

[32] Goddard GR, Sanders CK, Martin JC, Kaduchak G, Graves SW. Analytical performance of an ultrasonic particle focusing flow cytometer. Anal Chem 2007;79:8740-8746.

[33] Holmes D, She JK, Roach PL, Morgan H. Bead-based immunoassays using a micro-chip flow cytometer. Lab Chip 2007;7:1048-1056.

[34] Wietzorrek J, Plesnila N, Baethmann A, Kachel V. A new multiparameter flow cytometer: optical and electrical cell analysis in combination with video microscopy in flow. Cytometry 1999;35: 291-301.

[35] Shvalov AN, Surovtsev IV, Chernyshev AV, Soini JT, Maltsev VP. Particle classification from light scattering with the scanning flow cytometer. Cytometry 1999;37:215-220.

[36] Noguchi Y, Kashima S, Aikata T. Beam-shaping optics for a slit-scan flow cytometer. Cytometry 1993;14:819-825.

[37] Sebestyen Z, Nagy P, Horvath G, Vamosi G, Debets R, Gratama JW, Alexander DR, Szollosi J. Long wavelength fluorophores and cell-by-cell correction for autofluorescence significantly improves the accuracy of flow cytometric energy transfer measurements on a dual-laser benchtop flow cytometer. Cytometry 2002;48:124-135.

[38] Shapiro HM, Glazer AN, Christenson L, Williams JM, Strom TB. Immunofluorescence measurement in a flow cytometer using low-power helium-neon laser excitation. Cytometry 1983;4:276-279.

[39] Stewart CC, Woodring ML, Podniesinski E, Gray B. Flow cytometer in the infrared: inexpensive modifications to a commercial instrument. Cytometry A 2005;67:104-111.

[40] Bigler RD. A comparison of low-power helium-cadmium and argon ultraviolet lasers in commercial flow cytometers. Cytometry 1987;8: 441-444.

[41] Ramirez S, Aiken CT, Andrzejewski B, Sklar LA, Edwards BS. High-throughput flow cytometry: validation in microvolume bioassays. Cytometry A 2003;53:55-65.

[42] Lepesteur M, Blasdall S, Ashbolt NJ. Particle dispersion for further Cryptosporidium and Giardia detection by flow cytometry. Lett Appl Microbiol 2003;37:218-229.

[43] Haraguchi M, Kakeji Y, Orita H, Korenaga D, Maehara Y, Sugimachi K. Absorption cytometric DNA analysis of sections of gastric carcinomas and a comparison with cytofluorometry of single-cell suspensions and flow cytometry. J Surg Oncol 1995;60:201-206.

[44] Robertson BR, Button DK, Koch AL. Determination of the biomasses of small bacteria at low concentrations in a mixture of species with forward light scatter measurements by flow cytometry. Appl Environ Microbiol 1998;64:3900-3909.

[45] Petriz J, Tugues D, Garcia-Lopez J. Relevance of forward scatter and side scatter in aneuploidy detection by flow cytometry. Anal Cell Pathol 1996;10:243-252.

[46] Thompson JM, Gralow JR, Levy R, Miller RA. The optimal application of forward and ninety-degree light scatter in flow cytometry for the gating of mononuclear cells. Cytometry 1985;6: 401-406.

[47] Cordier G, Dezutter-Dambuyant C, Lefebvre R, Schmitt D. Flow cytometry sorting of unlabelled epidermal Langerhans cells using forward and orthogonal light scatter properties. J Immunol Methods 1985;79:79-88.

[48] Lavigne S, Bosse M, Boulet LP, Laviolette M. Identification and analysis of eosinophils by flow cytometry using the depolarized side scatter-saponin method. Cytometry 1997;29:197-203.

[49] Nicholson JK, Hubbard M, Jones BM. Use of CD45 fluorescence and side-scatter characteristics for gating lymphocytes when using the whole blood lysis procedure and flow cytometry. Cytometry 1996;26:16-21.

[50] Papa S, Zamai L, Cecchini T, Del Grande P, Vitale M. Cell cycle analysis in flow cytometry: use of BrdU labelling and side scatter for the detection of the different cell cycle phases. Cytotechnology 1991;5 Suppl 1:103-106.

[51] Li CS, Chia WC, Chen PS. Fluorochrome and flow cytometry to monitor microorganisms in treated hospital wastewater. J Environ Sci Health A Tox Hazard Subst Environ Eng 2007;42:195-203.

[52] Telford W, Murga M, Hawley T, Hawley R, Packard B, Komoriya A, Haas F, Hubert C. DPSS yellow-green 561-nm lasers for improved fluorochrome detection by flow cytometry. Cytometry A 2005;68: 36-44.

[53] Chen PS, Li CS. Real-time quantitative PCR with gene probe, fluorochrome and flow cytometry for microorganism analysis. J Environ Monit 2005;7:257-262.

[54] Jaye DL, Geigerman CM, Fuller RE, Akyildiz A, Parkos CA. Direct fluorochrome labeling of phage display library clones for studying binding specificities: applications in flow cytometry and fluorescence microscopy. J Immunol Methods 2004;295:119-127.

[55] Malickova K, Sedlakova Z, Rerichova M, Mareckova H, Fucikova T. [Study of leukocyte phagocytic activity using flow cytometry and hydrophilic fluorochrome-labeled microspheric particles]. Epidemiol Mikrobiol Imunol 1999;48:106-110.

[56] Badolato R, Bond HM, Valerio G, Petrella A, Morrone G, Waters MJ, Venuta S, Tenore A. Differential expression of surface membrane growth hormone receptor on human peripheral blood lymphocytes detected by dual fluorochrome flow cytometry. J Clin Endocrinol Metab 1994;79:984-990.

[57] Gelman R, Cheng SC, Kidd P, Waxdal M, Kagan J. Assessment of the effects of instrumentation, monoclonal antibody, and fluorochrome on flow cytometric immunophenotyping: a report based on 2 years of the NIAID DAIDS flow cytometry quality assessment program. Clin Immunol Immunopathol 1993;66:150-162.

[58] Troiano L, Ferraresi R, Lugli E, Nemes E, Roat E, Nasi M, Pinti M, Cossarizza A. Multiparametric analysis of cells with different mitochondrial membrane potential during apoptosis by polychromatic flow cytometry. Nat Protoc 2007;2:2719-2727.

[59] Guthrie HD, Welch GR. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J Anim Sci 2006;84:2089-2100.

[60] Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum Reprod 2002;17:1257-1265.

[61] Darzynkiewicz Z, Juan G, Bedner E. Determining cell cycle stages by flow cytometry. Curr Protoc Cell Biol 2001;Chapter 8:Unit 8 4.

[62] Gerashchenko BI. Quantitative assessment of cell proliferation in the co-culture of mixed cell populations by flow cytometry. Cytometry A 2008;73:492-493.

[63] Al-Mawali A, Gillis D, Hissaria P, Lewis I. Incidence, sensitivity, and specificity of leukemia-associated phenotypes in acute myeloid leukemia using specific five-color multiparameter flow cytometry. Am J Clin Pathol 2008;129:934-945.

[64] Chen BG, Zhang L, Li BL, Luo WD. [Sensitivity and specificity analysis of the lineage related antibodies in acute leukemia immunophenotyping by flow cytometry.]. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2005;13:329-331.

[65] Martinez M, Ricart JM, Ruiz-Aja S, Rus A, Todoli J, Calvo J, Vaya A. Platelet activation and red blood cell phosphatidylserine exposure evaluated by flow cytometry in patients with Behcet's disease: are they related to thrombotic events? Pathophysiol Haemost Thromb 2007;36:18-22.

[66] Stewart A, Urbaniak S, Turner M, Bessos H. The application of a new quantitative assay for the monitoring of integrin-associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry. Transfusion 2005;45:1496-1503.

[67] O'Brien MC, Healy SF, Jr., Raney SR, Hurst JM, Avner B, Hanly A, Mies C, Freeman JW, Snow C, Koester SK, Bolton WE. Discrimination of late apoptotic/necrotic cells (type III) by flow cytometry in solid tumors. Cytometry 1997;28:81-89.

[68] Riccardi C, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 2006;1:1458-1461.

[69] Aguilar-Lemarroy A, Romero-Ramos JE, Olimon-Andalon V, Hernandez-Flores G, Lerma-Diaz JM, Ortiz-Lazareno PC, Morgan-Villela G, Del Toro-Arreola S, Bravo-Cuellar A, Jave-Suarez LF. Apoptosis induction in Jurkat cells and sCD95 levels in women's sera are related with the risk of developing cervical cancer. BMC Cancer 2008;8:99.

[70] Goldberg JE, Sherwood SW, Clayberger C. A novel method for measuring CTL and NK cell-mediated cytotoxicity using annexin V and two-color flow cytometry. J Immunol Methods 1999;224:1-9.

[71] Rasola A, Geuna M. A flow cytometry assay simultaneously detects independent apoptotic parameters. Cytometry 2001;45:151-157.

[72] Telford WG, Komoriya A, Packard BZ. Detection of localized caspase activity in early apoptotic cells by laser scanning cytometry. Cytometry 2002;47:81-88.

[73] Lopez-Amoros R, Castel S, Comas-Riu J, Vives-Rego J. Assessment of E. coli and Salmonella viability and starvation by confocal laser microscopy and flow cytometry using rhodamine 123, DiBAC4(3), propidium iodide, and CTC. Cytometry 1997;29:298-305.

[74] Porter J, Pickup R, Edwards C. Membrane hyperpolarisation by valinomycin and its limitations for bacterial viability assessment using rhodamine 123 and flow cytometry. FEMS Microbiol Lett 1995;132:259-262.

[75] Lizard G, Roignot P, Maynadie M, Lizard-Nacol S, Poupon MF, Bordes M. Flow cytometry evaluation of the multidrug-resistant phenotype with functional tests involving uptake of daunorubicin, Hoechst 33342, or rhodamine 123: a comparative study. Cancer Detect Prev 1995;19:527-534.

[76] Juan G, Cavazzoni M, Saez GT, O'Connor JE. A fast kinetic method for assessing mitochondrial membrane potential in isolated hepatocytes with rhodamine 123 and flow cytometry. Cytometry 1994;15:335-342.

[77] Lizard G, Chardonnet Y, Chignol MC, Thivolet J. Evaluation of mitochondrial content and activity with nonyl-acridine orange and rhodamine 123: flow cytometric analysis and comparison with quantitative morphometry. Comparative analysis by flow cytometry and quantitative morphometry of mitochondrial content and activity. Cytotechnology 1990;3:179-188.

[78] Gunji H, Hass R, Kufe D. Internucleosomal DNA fragmentation during phorbol ester-induced monocytic differentiation and G0/G1 arrest. J Clin Invest 1992;89:954-960.

[79] Geske FJ, Lieberman R, Strange R, Gerschenson LE. Early stages of p53-induced apoptosis are reversible. Cell Death Differ 2001;8:182-191.

[80] Martinou I, Desagher S, Eskes R, Antonsson B, Andre E, Fakan S, Martinou JC. The release of cytochrome c from mitochondria during apoptosis of NGF-deprived sympathetic neurons is a reversible event. J Cell Biol 1999;144:883-889.


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