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FLOW CYTOMETRIC APPLICATIONS IN BIOMEDICAL RESEARCH, CELL SORTING AND BIOTECHNOLOGY

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INTRODUCTION

Flow cytometry is a general method for the rapid individual analysis of a large numbers of cells using light-scattering, fluorescence and absorbance measurements. In flow cytometry, single cells or particles pass through a laser beam in a directed fluid stream. The interaction of the cells with the laser beam – their absorption, scattering, and/or fluorescence – can be monitored for each individual cell. The forward-scattered light provides information on the size of the cells and can be detected without further manipulation. The sideways-scattered light is affected by several parameters, including granularity, cell size and cell morphology. These data can be correlated with different cell characteristics and cell components, and thus, distributed data about a cell population can be obtained easily.

Flow cytometry was first used in medical sciences such as oncology (e.g., for diagnosis of cancer, chromosomal defect diagnosis) and hematology and subsequently in biology, pharmacology, toxicology, bacteriology, virology, environmental sciences, and bioprocess monitoring. Medical and clinical applications of flow cytometry still account for the vast majority of publications on this technique.

EVOLUTION OF FLOW CYTOMETRY

The evolution of flow cytometry can be divided in four distinct phases – the development of the microscope, the development of dye chemistry, the development of electronics andthe development of computers. All four of these fields had to reach sufficient maturity - coincident with a global biomedical need - to produce a prototype instrument.

The first light-based flow cytometers were little more than a fluid path, an excitation source and a photodetector. Early sorters used technology not dissimilar to that present in ink jet printers. As with many instruments, modern flow cytometers are becoming increasingly sophisticated devices that combine fluidics, optics, electronics and software to enable measurements to be performed on microscopic particles at event rates of many tens of thousands per second. These instruments have taken advantage of many technological developments in other sectors (such as semiconductor and telecommunications) to provide increased accuracy and reliability with significant size reduction.

PRINCIPLES OF THE FLOW CYTOMETERS

The prepared sample of particles under investigation begins its journey through the flow cytometer from within a sample vessel (test tube, multiwell plate or similar container). At the desired time for analysis, sample is aspired from this vessel and is transported through tubing to a flow cell or nozzle. At the flow cell, the sample is introduced to the center of a faster flowing carrier fluid and is in turn presented to one or more light sources for excitation. Light scatter and/or fluorescence are captured, spectrally filtered and directed to appropriate photodetectors for conversion to electrical signals. Electronic circuitry is used to process these signals for analysis, classification, sorting and data storage. For sorters, an individual particle is traced through the instrument as it breaks free from the continuous jet into a charged droplet for electrostatic deflection. The droplet passes through an electric field and is ultimately captured in a suitable container for further processing or study, or is disposed of as waste.

1. Fluidics

The fluidic system of a flow cytometer is used to transport particles from a random three-dimensional sample suspension to an orderly stream of particles traveling past one or more illuminating beams. In sorters these particles are transported in an electrically conductive carrier fluid. The fluidic system often uses air pressure regulation for stable operation and consists of at least one sheath line and a sample line feeding the flow cell.

As the sample enters the flow cell chamber, the outer, faster flowing sheath fluid hydrodynamically focuses this fluid into a narrow core region within the jet and presents a single file of particles to excitation sources. This geometry provides increased positioning accuracy at the laser interrogation point for consistent excitation irradiance and greatly reduced particle blockage of the flow cell. Depending on instrument design, particles are either interrogated when they are in the flow cell, or

once they have exited and are contained within the jet in air. After interrogation, the sample-sheath fluid mix is flushed to waste unless particle sorting is desired. To facilitate instrument start-up and cleaning, a vacuum line is usually provided.

2. Optics

Optics are central to flow cytometry for the illumination of stained and unstained particles and for the detection of scatter and fluorescent light signals.

Most flow cytometers utilize one or more laser sources for cell excitation. Emission, in the form of light scattering, occurs when excitation light is absorbed and then re-radiated by the particles under investigation, with no change in wavelength. Scatter is typically strongest in the forward direction with respect to the incident excitation source. Fluorescence occurs when a molecule excited by light of one wavelength returns to a lower state by emitting light of a longer wavelength. The excitation and emission light, being of sufficiently removed wavelengths, can be blocked or differentiated by optical filtering. Several cellular constituents can be measured simultaneously by using several excitation sources and several cell stains that emit at different wavelengths. This key ability to detect fluorescence simultaneously from several different compounds bound to one or many cells has resulted in flow cytometry being a technique of choice for multiparameter fluorescence analysis of cell populations.

In conventional flow cytometers, light is collected by two lenses termed the forward and side collection lenses, depending on their orientation as viewed from the entering laser beam. The forward collection lens gathers scattered light over a region centered on the laser beam axis. Forward scatter can be used to obtain information of particle size. The side scatter lens has a high numerical aperture (NA) for maximum fluorescence collection efficiency and collects light at 90 degrees to the laser beam axis. Side scatter can be used to differentiate particle populations based on morphology.

Once the fluorescence light from a cell has been captured by the collection optics, the spectral component of interest for each stain must be separated spatially for detection. This separation of wavelengths is achieved using dichroic (45 degree) and emission (normal incidence) filters. Longpass filters permit longer wavelength transmission, while shortpass filters allow shorter wavelength transmission. Bandpass filters only allow a selected wavelength band of interest to be transmitted while blocking unwanted wavelengths.

The two main photodetector types used in flow cytometry are silicon photodiodes and photomultiplier tubes (PMTs). Silicon photodiodes are typically used for forward scatter detection where signal levels are high. PMTs have high gain and sensitivity and are, therefore, assigned to side scatter and fluorescence detection. The principle of function of flow cytometry is briefly illustrated in Figure 1.

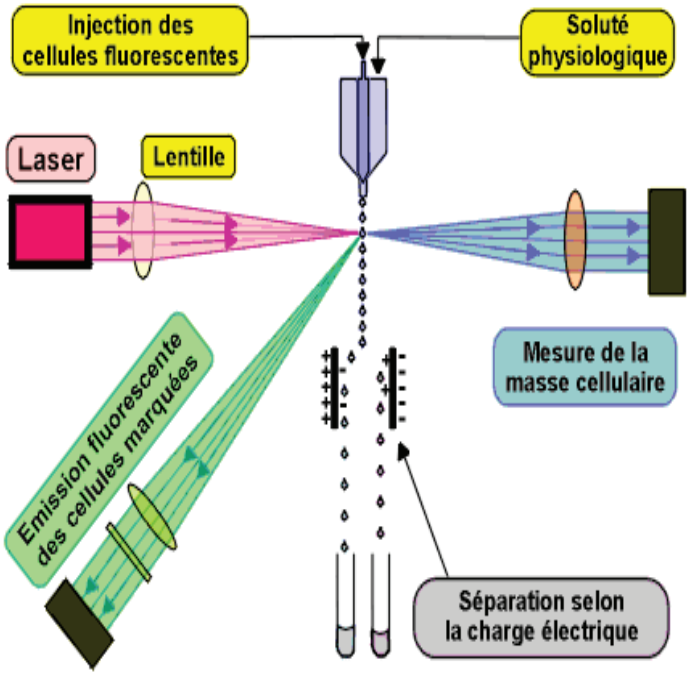

Fig.1. The analyse principle by flow cytometry (after a document provided by Service d'Anatomie Pathologique-Centre François Baclesse)

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3. Electronics

The role of the electronics is to monitor and control the operation of the flow cytometer, from detection of a particle event as it passes through the laser focus, to physical deflection of that particle into a collection flask. As a particle of interest passes through the focus, fluoresces and is detected by a photodetector, an electrical pulse is generated and presented to the signal processing electronics. The instrument is triggered when this signal exceeds a predefined threshold level. The threshold is primarily used to reject non-particle events such as debris or noise from optical and electronic sources. Pulse characteristics are determined by particle speed and size, width of the illumination beam, and in the case of fluorescence, distribution of fluorochrome within the particle.

Various pulse processing modes enable different measurements to be made from the same signal, such as peak height, integral (area), width, skew, etc.

In some instances, linear amplification and analysis of cell fluorescence or scatter is adequate for particle characterization, such as in DNA studies. In many instances, such as immunophenotyping studies, the measurement of both stained and unstained (bright and dimly fluorescent) particles requires measurement over several orders of magnitude. To achieve this broad dynamic range, a variety of analogue, digital or hybrid electronic systems can be used for signal processing. Analogue devices, such as logarithmic amplifiers, provide very large amplification to small signals and near unity amplification for large signals, while digital signal processing devices (DSPs) can be used to digitize pulse parameters. Some instruments use a combination of analogue and digital electronic elements in order to capture advantages of both devices. Real time calculation and decision-making capabilities enable sort functions to be performed at high speed.

4. Software

Early flow cytometers used an oscilloscope for simple pulse height display and analysis. Today, most instruments use software for instrument control, data acquisition, analysis and display. Many instruments provide software control for many or all instrument hardware functions including fluidics, lasers, electronics and sorting. Further, operations such as start-up and shutdown sequences, cleaning cycles, calibration cycles and self-test functions are monitored and regulated by software, so that instrument operation can be simplified and optimized for the user.

FLUOROCHROMES AND LIGHT

Fluorochromes are essentially dyes, which accept light energy (e.g. from a laser) at a given wavelength and re-emit it at a longer wavelength. These two processes are called excitation and emission. The process of emission follows extremely rapidly, commonly in the order of nanoseconds, and is known as fluorescence.

When light is absorbed by a fluorochrome, its electrons become excited and move from a resting state to a maximal energy level called the "excited electronic singlet state". The amount of energy required will differ for each fluorochromeand is depicted as $E_{excitation}$. This state only lasts for 1-10 nanoseconds because the fluorochrome undergoes internal conformational change and releases some of the absorbed energy as heat. The electrons subsequently fall to a lower, more stable, energy level called the "relaxed electronic singlet state". As electrons steadly move back from here to their ground state they release the remaining energy ($E_{emission}$) as fluorescence. As $E_{emission}$ contains less energy than was originally put into the fluorochrome it appears as a different colour of light to $E_{excitation}$. Therefore, the emission wavelength of any fluorochrome will always be longer than its excitation wavelength. The difference between $E_{excitation}$ and $E_{emission}$ is called Stokes Shift and this wavelength value essentially determines how good a fluorochrome is for fluorescence studies. After all, it is imperative that the light produced by emission can be distinguished from the light used for excitation. This difference is easier to detect when fluorescent molecules have a large Stokes Shift.

FluorochromeS selection

Knowing the excitation and emission properties of fluorescent compounds makes it possible to select combinations of fluorochromes that will work together optimally on a specific flow cytometer with specific lasers. However, for a fluorochrome to be useful in a biological application, it must attach to or be contained within a particle of biological significance. Some fluorochromes are useful because they bind to specific chemical structures, such as antibodies or the nucleic acids in DNA or RNA. Fluorochromes that are used most often in flow cytometry are ones that attach in some way to biologically significant molecules and are excitable by the lasers that are commonly found on commercial flow cytometers. Many fluorochromes can be attached to antibodies, which will then bind to specific chemical structures on or inside of cells. If these chemical structures are unique to a specific type of cell, then the fluorochrome will identify that cell type. This technique of identifying cells using fluorescent antibodies is called immunophenotyping. Some other common applications of fluorochromes in flow cytometry include the detection of intracellular calcium, measurement of the relative amount of cellular DNA or RNA, and measurement of transcription levels using a fluorescent protein as a reporter gene.

There are many other chemical and physical properties of fluorochromes that determine where and when these dyes are useful in various biological assays. For example, some of the fluorochromes that bind to DNA, such as Hoechst 33342, can get into living cells, but most DNA-binding fluorochromes cannot get past the

cell membrane. The fluorescent dyes that cannot get past a viable cell membrane, such as propidium iodide, are often used to distinguish live from dead or dying cells. In order to select the best fluorochromes for use in a specific application on a particular flow cytometer, it is necessary to know the laser configuration of the flow cytometer and the physical and chemical characteristics of the fluorochromes that are available for that application.

ANALYSIS AND PRESENTATION OF DATA OBTAINED BY FLOW CYTOMETRY

Depending on the device, the software used and the needs of the researcher, the results of analysis from flow cytometry may be represented differently. All graphics are called citograms. In Figure 1 are highlighted the main ways of representing the data in flow cytometry. A first model of representation is the double parametric analysis (dot-plot), where each point represents a cell. Thus, cell subpopulations can be separated, occurring in a cloud of points representing cells with similar characteristics for the two parameters represented. With the software, we can define these subpopulations, drawing "windows" or "regions", which then can be analyzed separately, each region, as shown in Figure 2D. Dimensional representation of the number of cells add to the double parametric analysis the Z axis (Figure 2F). If we look at one parameter, the data representation is made as a histogram and in this case we have information on an average parameter. This indicator is called "mean", eg "mean" fluorescence or MFI (Mean Fluorescence Intensity). In the histograms, single parametric analysis, subpopulations can be separated by areas, for example M1, M2, which can be compared by overlaying, by making the difference between the two areas of the parameter of interest (Figure 2H) or by their arrangement in space, if we are talking about comparing more histograms (Figure 2I). Thanks to software analysis and interpretation of results, we can, for example, ask the system to layout on a double parametric (Figure 2 K and L) a region defined on a mono parametric histogram (Figure 2J), this process being called "Paint the gate."

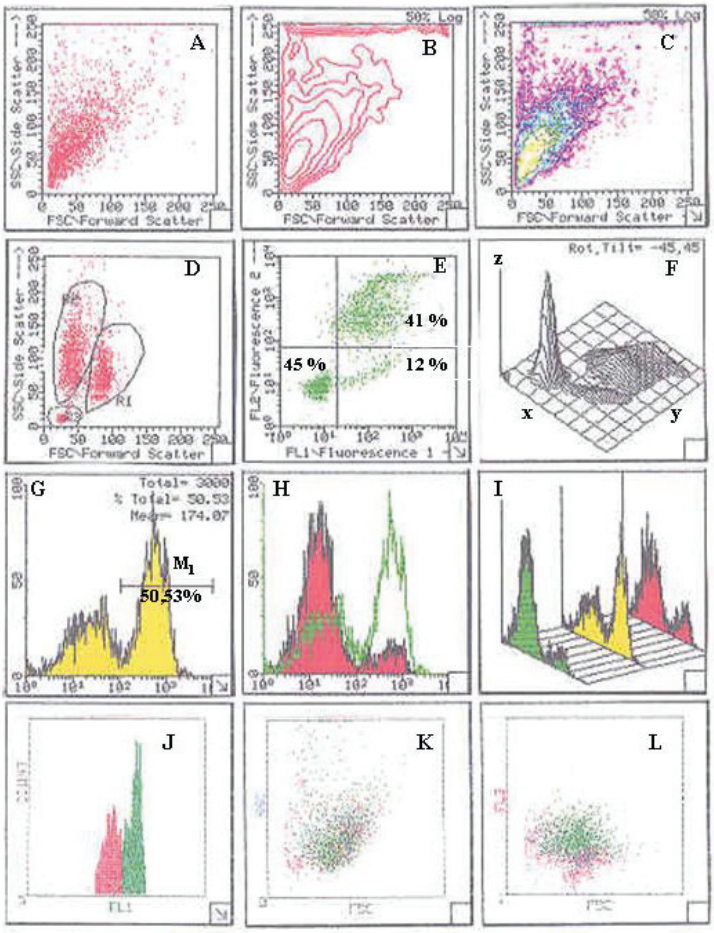

Fig. 2. Different types of representations used in flow cytometry analysis

AE: Different types of double parametric citograms (dot-plot): FSS/SSC (AD) or FL1/FL2 (E)
A: Cellular distribution citogram (dot-plot);
B: Contour citogram (contour dot plot);
C: Distribution citogram after the distribution of cell density (density dot-plot);
D: Example of delineation of regions of interest (R1, R2, R3) for further statistical analysis
E: "Quadrants" statistical analysis technique (Quadrant stats) for various subpopulations highlighted in FL1/FL2 system;
F: Three-dimensional representation of the double parametric citograms (x / y), where z is the number of cells;
GI: Examples of single parametric citograms for FSC, SSC, FL1, FL2 and FL3, known as histograms.
G: Example of a histogram analysis: M1 = region that delimits an area of interest and for which can be obtained the % from the total population and the average of analyzed parameter;
H: Comparative analysis by overlay of the histograms for two samples analyzed;
I: Comparative analysis of several three-dimensional single parametric histograms;
J-L: Analysis in the "Paint the gate" system. A subpopulation defined in a color in the J histogram is later identified in the double parametric citograms K (FSC / SSC) and L (FSC/FL1) through points of the same color, which allows finding the other parameters that characterize the sub-population.

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FLOW CYTOMETRY APPLICATIONS

1. Sorting

Sorting is an elegant use of flow cytometric technology that is attracting new attention from diverse segments of biology and industry. This technology provides the powerful yet unique ability to rapidly isolate pure populations of cells or particles with a desired set of biological characteristics. Two methods exist for sorting particles by flow cytometry: electrostatic and mechanical. Historically, this technology was developed to facilitate sorting of cells and other particles and it quickly caught on in the study of immunology for purification of lymphocyte subsets. Applications have continued to broaden since then, so that they currently range from ecological monitoring to industrial enzyme production.

2. Absolute cell counting

In the research and especially the clinical setting, there are situations that require not just the percentage positive of a particular cell population but the actual absolute number positive cells. There are three main areas in the clinical setting that require the precise and accurate determination of absolute cell counts.

For example, the absolute CD4+ T lymphocyte count has long been recognized as a useful laboratory tool for the staging of HIV infected patients. It can also be used to assess the timing of the administration of preventative treatment and, more recently, for monitoring the effect of new antiviral therapies.

Mobilization, harvesting and transplantation of CD34+ progenitor cells are now well-recognized techniques. By monitoring absolute CD34+ cell levels after growth factor-induced mobilization, it is possible to ensure the maximum number of cells can be collected with the minimum number of time-consuming and expensive harvesting procedures.

3. Immunophenotyping

Immunophenotyping of biological samples refers to the use of immunological tools (e.g. monoclonal and/or polyclonal antibodies) for the specific detection of antigens, most frequently from proteins, expressed by cells or other particles (e.g. apoptotic bodies) localized either on their surface or inside them. In the past, antibody/antigen reactions on cells were revealed using either enzymatic approaches (immunocytochemistry) or fluorescence (immunofluorescence), both analyzed by microscopy. More recently, flow cytometry has been increasingly used for immunophenotyping purposes. As compared to other techniques, flow cytometry immunophenotyping is more sensitive and simple, and it provides a better way for the simultaneous quantitative assessment of multiple antigens in large numbers of cells. At present, the applications of immunophenotyping by flow cytometry are numerous and almost infinite. In general, staining of cells with antibodies may be used to identify, count and characterize any type of individual cells or subcellular components. In the last two decades, many of the applications of immunophenotyping have shown to be of great clinical utility, mainly in the area of hematology and immunology. For exemplification, in Figure 3 are presented results from an imunofluorescence reaction for CD 34 identification realized on cells from umbilical cord blood.

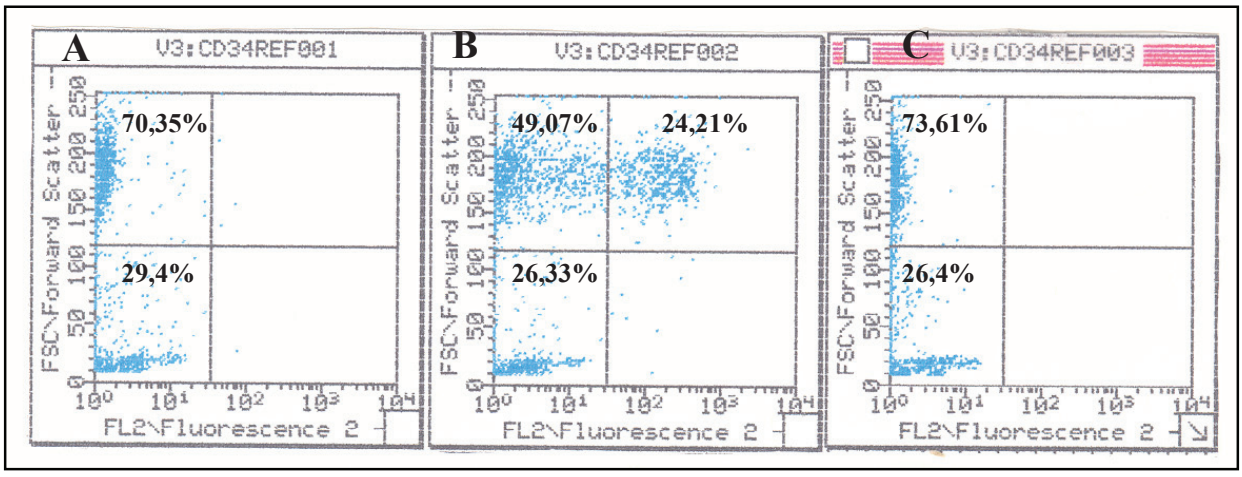

Fig. 3. Identification of blood stem cells isolated from umbilical cord with anti-CD 34 antibody. A: autofluorescence control, B: cells incubated with anti-CD-34 antibody; C: negative control with γ1p mouse antibody. (Bratosin & Heron, unpublished results)

4. Signal transduction

It should be understood that the field of signal transduction is very diverse. The study of signal transduction events by flow cytometry is in a state of relative infancy compared to other means of studying signal transduction cascades, but the insights to be gained by the application of flow cytometry to signal transduction events can be very powerful. Like microscopy-based systems, the flow cytometer interrogates individual cells, but unlike microscopy, the analysis of thousands of cells per second by modern flow cytometers enables powerful statistical results and the recognition of very rare events at rates not possible by microscopy-based systems. Secondly, the flow cytometer is designed to sort, or recover, those cells exhibiting a phenotype of interest to the scientist – a process that is not currently available on microscopy-based systems, with the exception of certain instruments with laser ablation technologies still under development. Thus, in the case of signal transduction events, sorting capabilities allow the investigator to isolate those cells from a population exhibiting the signal transduction response of interest for further growth or biochemical or molecular analysis.

5. Quantifying interactions between proteins and membrane lipids

The flow cytometric method to detect protein-lipid interactions allows for quantitative measurements of interactions between multiple fluorescently labeled proteins and membrane lipids reconstituted in lipid bilayers. The assay can be used to quantify binding efficiencies and to determine kinetic constants. The method is further characterized by a short sampling time of only a few seconds that allows for high-content screening procedures. Finally, using light scatter measurements, the described method also allows for monitoring changes of membrane curvature as well as tethering of liposomes evoked by binding of proteins.

Flow cytometric analysis of liposomes has so far mostly been used to determine size and volume properties but it may be used also as a quantitative method to detect interactions between membrane lipids and peripheral membrane proteins in suspension, both interactions partners are presented in their natural environment. In addition to measuring direct interactions on a quantitative basis, flow cytometry method is also suited to detect membrane surface effects that may be the result of proteins binding to specific membrane lipids. This includes clustering of liposomes by multivalent interactions and changes of membrane curvature. The experimental system is a single-step assay without the need for extensive washing procedures. It does not require large amounts of purified proteins and, provided that different fluorescent tags are used, allows for competitive binding studies.

6. Analysis of morphological changes by scattered light flow cytometry in the mode FSC/SSC

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter/SSC), on the other hand, correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light. The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies.

Focusing on the RBCs side of the crosstalk, we investigated Gaucher RBCs by means of flow cytometric analyses. A dramatic change in the morphology of Gaucher RBCs was suggested by flow-cytometric analyses wich revealed important variations in the size/ density distribution of RBC populations in untreated patients compared with control. The results are correlated with the scanning electronic microscopy,as shown in Figure 4.

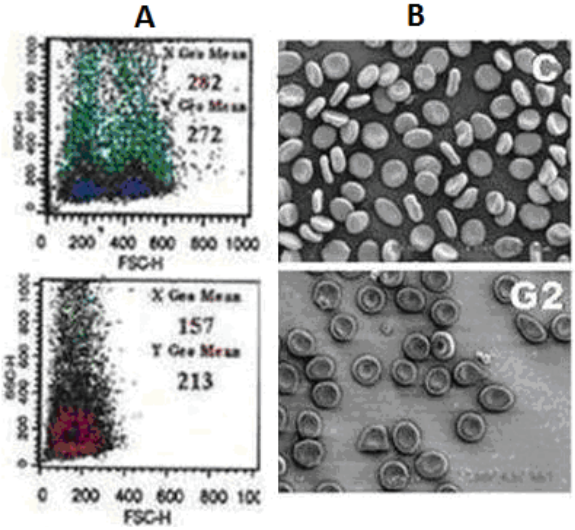

Fig. 4. A: Dot-plot analysis FSC/SSC of cells shape changes of normal (C) and Gaucher erythrocytes (G2) Abscissae: forward scatter (cell size); ordinates: side scatter (cell density, granularity and refractiveness). Number of counted cells: 10,000. Results presented are from one representative experiment of three performed. B: Scanning electron microscopy of of normal (C) and Gaucher erythrocytes (G2).

(Gheorghe et al., 2010)

7. Flow cytometric measurement of reactive oxigen species

Oxidative stress is considered in tissues one of important general toxicity mechanisms of many xenobiotics. Induction of oxidative stress after exposure to numerous xenobiotics was revealed experimentally by anthropogenic contaminants as persistent organic pollutants, heavy metals, by pesticides and also by toxins produced during massive blooms of cyanobacteria.

Measurement of reactive oxigen species (ROS) is extremely difficult, because of the short lifetime of theses species and methods such as electron spin resonance and spin trapping are complicated and provide average values that can skew results when heterogeneous populations are being studied. Flow cytometry has been used to measure oxidative stress in various types of cells, including human normal and thalassaemic erythrocytes.

Intracellular level of reactive oxygen species may be measured using an oxidation-sensitive fluorescent probe, 2′,7′-dichlorofluorescein-diacetat (DCFH-DA) after the methods of Bass *et al.,* 1983. In the presence of various intracellular reactive oxygen species, 2′,7′-dichlorofluorescein-diacetat (DCFH-DA) is oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). The appearance of reactive oxygen species are measured by flow cytometry using an excitation and emission settings of 488 and 530 nm respectively, compared to a positive control that had been incubated with 2mM H_2O_2 for stimulating ROS production in RBCs and consequently for a positive fluorescence. In Figure 5, using flow cytometry, we evaluated how ROS generation during , on nucleated erythrocytes.

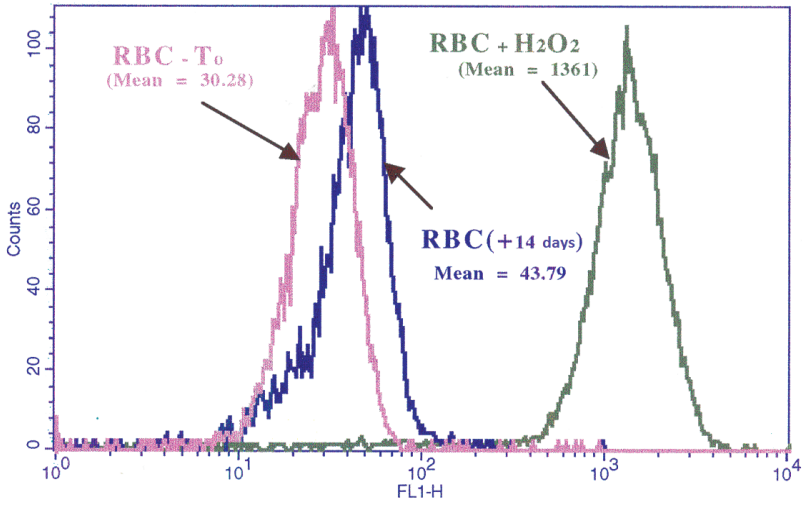

Fig. 5. Determination of free radical production during accelerated aging of human erythrocyte plasma by their conservation at 4 ° C. RBC-To: freshly isolated erythrocytes (reference standard), 14 days RBC: red blood cells stored at 4 ° C for 14 days in their own plasma, RBC + H2O2: positive control prepared according to the protocol.

(Bratosin D, unpublished results)

The calculated average of the MFI values for erythrocytes incubated with various concentration of aluminium showed a significantly higher ability of aluminium to generate ROS as compared to the normal sample . The MFI for unstimulated normal RBC sample was 34 comparative with 242 for normal H_2O_2-stimulated sample. The results showed that the presence of aluminium caused an increase in fluorescence, depending of aluminium concentrations.

8. Flow cytometry determination of viability using Calcein-AM

The assay is based on the use of acetoxymethyl ester of Calcein (Calcein-AM), a fluorescein derivative and nonfluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent Calcein, which is retained by cells with intact membranes and inactive multidrug resistance protein. The loss of calcein can be easily determined by flow cytometry, and the cytosolic localization of esterases was demonstrated by spectrofluorometric analyses.

Application of this assay for analysing the effect of nanomaterials practised on nucleated erythrocytes showed that two regions could be clearly and unambiguously defined: the region of fluorescent erythrocytes with intact membranes that is related to intracellular esterase activity and strongly correlated with the number of living cells (region M1) and the region of nonfluorescent dead cells with damaged cell membranes (region M2), as shown in Figure 6.

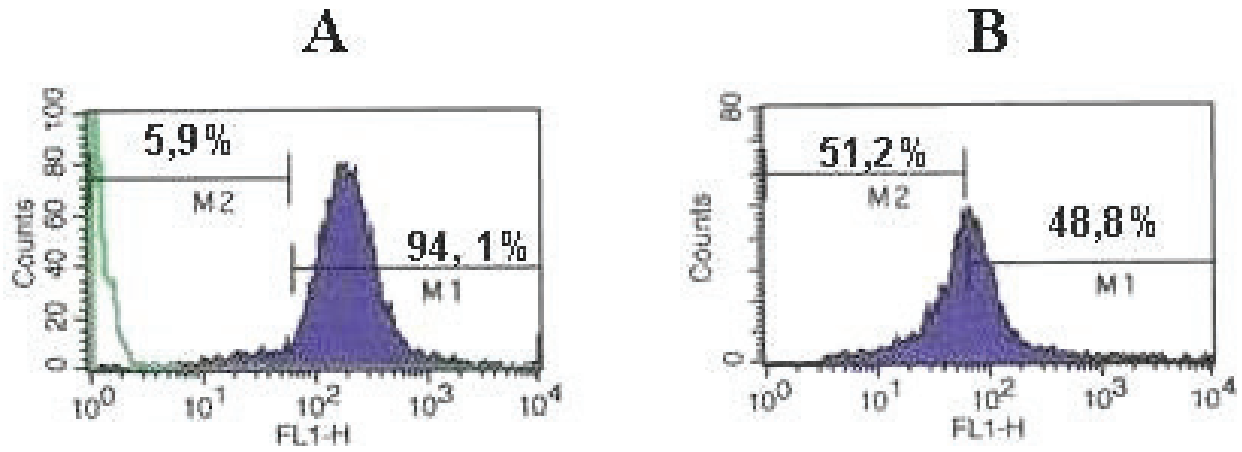

Fig. 6. Comparative flow cytometric histogram analysis of Calcein-AM cell viability of normal nucleated erythrocytes (A) and incubated for 24 h with a porfirinic matherial (B). M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged cell membranes (dead cells). Abscissae: log scale green fluorescence intensity of calceine (FL1). Ordinates: relative cell number. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

(Bratosin et al., 2011)

9. Flow cytometry in microbiological analyse

Flow cytometry capabilities to provide information on the size, intracellular content and to distinguish low represented subpopulations allowed the creation of new methods for measuring microbial analysis, determining the physiological state of cells and rapid identification of microorganisms compared to traditional methods, which are long and subjective. Microorganisms analyzed by flow cytometry include types of Gram negative (Escherichia, Legionella, Pseudomonas, Klebsiella), Gram positive (Bacillus, Streptococcus, Staphylococcus, Listeria), yeast (Saccharomyces, Candida, Filobasidiella), protozoa (amoeba, Trypanosoma, Plasmodium), algae or viruses. Most frequently analyzed samples are coming from pure cultures isolated and grown in laboratories, but also wastewater, water from columns of air conditioning, biological fluids as milk, blood, urine or samples of dental plaque.

10. Role of flow cytometry in cell necrobiology

The explosive growth in recent years of flow cytometry for the analysis of cell death in a variety of disciplines of biology and medicine is the best evidence of the value of this methodology in cell necrobiology. Two general directions characterize this geowth. One direction is the use of flow cytometry to quantify apoptotic cells (apoptotic index). A variety of flow cytometric methods currently exist to identify apoptotic cells and new methods are continuously being developed and proposed. One of these methods involves measurement of the phosphatidylserine externalisation, as a marker of apoptosis.

Phosphatidylserine residues are exposed in the external leaflet of cell membrane early during the process of apoptosis whereas the uptake of propidium iodide indicates a disrupted cellular membrane integrity generally observed during late apoptosis and cell necrosis. Cell death may be determined using an Annexin-V-FITC/ propidium iodide apoptosis kit. Annexin-V is a Ca^{2+} - dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS) and is useful for identifying apoptotic cells with exposed PS. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI whereas membranes of dead and damaged cells are permeable to PI. Cells that stain positive for Annexin V-FITC and negative for propidium iodide are undergoing apoptosis. Cells that stain positive for both Annexin-V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both Annexin-V-FITC and PI are alive and not undergoing measurable apoptosis, as you can see in Figure 7, which shows comparative flow cytometric analyses of normal (A) and incubated erythrocytes with a porphyrinic nonmaterial (B).

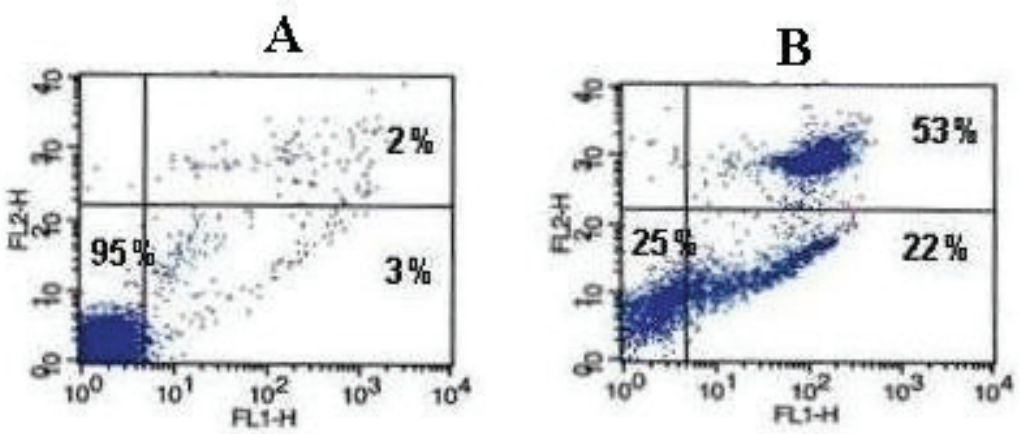

Fig. 7. Comparative flow cytometric quadrant analysis of Annexin-V-FITC/propidium iodide double-stained of normal nucleated erythrocytes (To) and exposed at 0,008 g/ml porphyrinic nanomaterial (P1). Abscissae: log scale green fluorescence intensity of annexine-V-FITC (FL-1). Ordinates: log scale red fluorescence intensity of propidium iodide (FL-2). Low left quadrant: viable cells (annexin-V and propidium iodide negative cells); low right quadrant: apoptotic cells (annexin-V positive and propidium iodide negative cells); upper right quadrant: dead cells (Annexin-V and propidium iodide positive cells). % refers to the cell percentage of each population. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.
(Bratosin et al., 2011)

Flow cytometric methods to quantify apoptotic cells have already found application in clinical oncology. Tumor apoptotic index prior to treatment appears to have predictive value, at least in some tumor types. Clearly, the knowledge of the rate of cell proliferation and cell death is more predictive of the rate of tumor growth than information on the rate of cell proliferation alone.

Furthermore, analyzing the rate of death of tumor cells during treatment offers a unique possibility to asses the efficiency of the treatment very early, before other clinical parameters of treatment efficiency can be measured. Another clinical application of these methods were apoptosis also appears to be of predictive value is in the analysis of spontaneous-or actiation induced-apoptosis of lymphocytes in the course of HIV infection. These routine clinical applications call for development of standardized procedures and for quality control that could be generally accepted in clinical practice. The second area of cell necrobiology where flow cytometry is already widely applied is in the study of molecular mechanisms associated with cell death. In this area, flow cytometry offers unique opportunities and is even more advantageous compared to alternative techniques. By vurtue of the possibility of correlating measurements of multiple parameters on the same cells, the methodology allows one to detect cells undergoing apoptosis with respect to their cell cycle position, without the need for cell synchronization, or to study the relationship between apoptosis and a particular function of the cell or cellular organelle.

11. Role of flow cytometry in biocompatibility

Biocompatibility interest has a long history (more than 100 years) and biological evaluation is one of the most important in assessing the potential benefit of implantable materials for human use. In transfusion medicine, blood and blood components, donors and patients are increasingly confronted with biomaterials. The need to understand the response of human blood to contact with these artificial surfaces has led to multiple studies on the biocompatibility of biomaterials. Up to this time, these investigations have predominantly been performed using physical, immunological and biochemical methods.Many of these approaches are useful in investigating the multiple factors involved in blood-biomaterial interactions. However, they always reflect the overall behaviour of whole cellular populations in local or systemic reactions. These application of multiparameter flow cytometry, on the other hand, provides insight into antigenic expression and changes at the single-cell level. Therefore, the technique of flow cytometry represents a new powerful way of analyzing and improving the biocompatibility of these materials in blood-contacting applications in this field.

In order to evaluate cell-biomaterials interactions we developed a new experimental cell system, the RBCs, and we have evaluated the toxic effects of metals released from differents implant materials as shown in Figure 8. Our results indicated that flow cytometry could provide a rapid and accurate means of measuring for effectively screening and evaluating biological responses to implant materials. The approach of RBCs interactions with foreign materials opens up a variety of new possibilities in biocompatibility assessing.

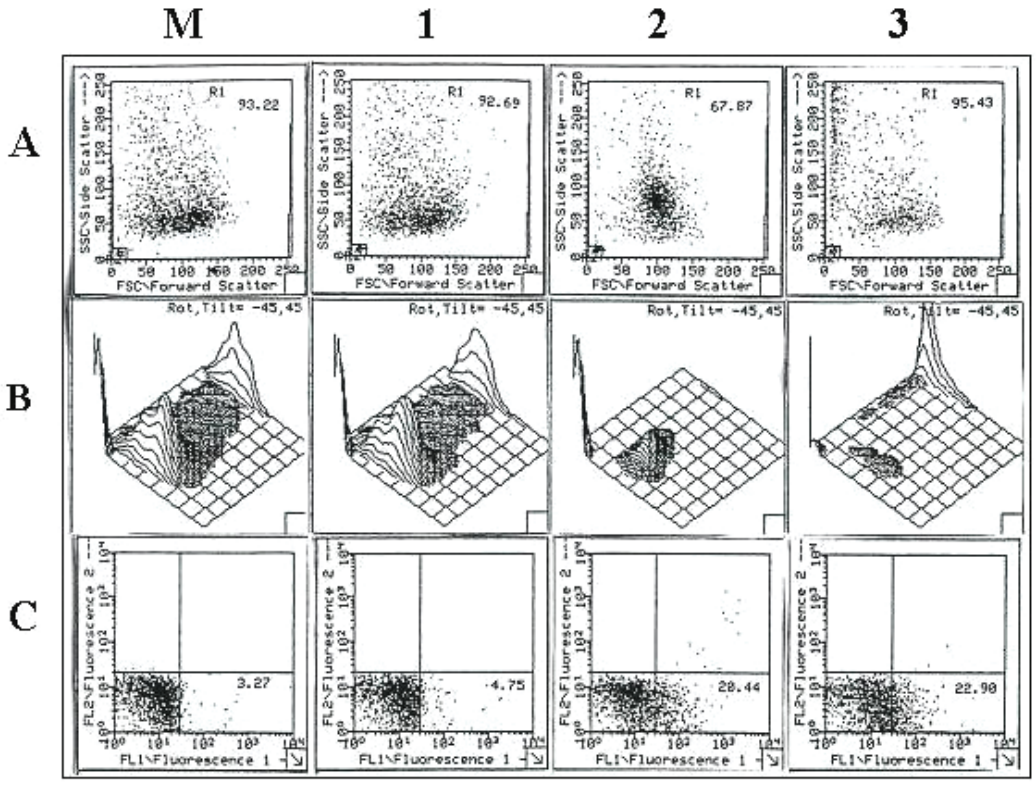

Fig.8. Flow cytometric analysis of RBCs incubated (37°C for 48h) in PBS buffer in absence (M) and in presence of 3 different implant materials (1 to 3); A: Dot-plot FCS/SSC; B: 3D histogram FSC/SSC; C: Annexin-V-FITC labelling for apoptosis analyses.

(Bratosin et.al., 2004)

12. Flow cytometry of plant cells

In recent years, there has been a significant upsurge in the application of flow cytometry to plant cells and plant cell cultures. As well as a range of uses in plant biology, flow cytometry offers many advantages for monitoring plant cell cultures used in large-scale bioprocessing operations. During the last ten years or so, these techniques have been adapted for application to plant cells, allowing rapid analysis of DNA and RNA contents, detection of transgene expression,karyotyping, cell counting, study of chloroplasts, cell membranes and cell wall regeneration, evaluation of mitochondrial activity, measurement of secondary metabolite accumulation, and selection of particular cells or subcellular organelles of interest. As a result, a wealth of useful information has been obtained about plant molecular biology, biochemistry, physiology, ecology and agronomy.

MODERN FLOW CYTOMETRY

Up to this point, cells could flow through an orifice and be counted; they could also be stained and identified microscopically in a somewhat automated fashion. The real beginning of modern flow cytometry occurred when Fulwyler at the U.S. Los Alamos National Laboratories built a cell sorter using the Coulter principal to size cells and electrostatic charging of droplets to sort them. Dittrich and Gohde then developed what they called the impulsecytophotometer (ICP), which became known as the "phywe." Cells were introduced into a flowing sheath stream located under a high power microscope objective that provided the optics for scatter measurements and fluorescence detection. Paul Mullaney, also at Los Alamos, introduced multiparameter flow cytometry by combining the measurement of volume, light scatter and fluorescence into a single instrument. Gary Salzman did extensive experiments that resulted in the addition of the ability to measure side scatter. By the mid 1970s, flow cytometers were entering the marketplace, and Leonard Herzenberg at Stanford coined the term, Fluorescence Activated Cell Sorter, or FACS. In the early 1980s, interest in immunophentoyping grew with the discovery that HIV, which caused a severe immunodeficiency syndrome, or AIDS killed CD4 T cells. Among the first characterized monoclonal antibodies were antibodies to the membrane proteins CD3, CD4 and CD8 expressed by two subsets of these T cells. AIDS began to spread through the populations of the world, and with it, the first major clinical application of immunophenotyping by flow cytometry. The measurement of CD4 T cells using monoclonal antibodies was developed into a

test to monitor the progression of AIDS. With this development, and the successful measurement of DNA content, it became clear that flow cytometry would be an important automated approach to cell analysis, and other clinical applications began to appear. These applications included ploidy and S phase fraction measurement in solid tumors, diagnosis and follow-up of hematopoietic malignancy and paroxysmal nocturnal hemoglobinuria, and monitoring of transplant rejection and hematopoietic regeneration. While immunophenotyping was initially directed to the measurement of membrane markers on cells, it became apparent that intracellular markers could also be measured. The ability to identify a cell population by membrane markers and simultaneously determine the cell's function had now become a powerful application of flow cytometry. Another application pioneered by Larry Deaven and Scott Cram at Los Alamos and Joe Gray and Ger van den Engh at Lawrence Livermore National Laboratory was chromosome staining and sorting for cloning of DNA to produce chromosome-specific sequences. This new application led to the construction of the first high-speed cell sorter for the purpose of sorting each human chromosome. This project, supported by the U.S. Department of Energy, was the first step in the Human Genome Project. As you can see, since a very slow beginning in the 17th century, the development of dye chemistry, the electronic and computer industries, and the means for producing monoclonal antibodies have all come together to produce the rapid and expanding field of flow cytometry. No other technology has been developed that can rapidly measure the number of correlated properties on a cell-by-cell basis. This book provides a compilation of applications that have been developed to properly utilize this complex technology.

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