

Flow cytometry in the analysis of cellular populations

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Abstract. Flow cytometry has become a valuable tool in cell biology. By analyzing large number of cells individually using light-scatter and fluorescence measurements, this technique reveals both cellular characteristics and the levels of cellular components. Flow cytometry has been developed to rapidly enumerate cells and to distinguish among different cell stages and structures using multiple staining. In addition to high-speed multiparametric data acquisition, analysis and cell sorting, which allow other characteristics of individual cells to be studied, have increased the interest of researchers in this technique. This chapter gives an overview of the principles of flow cytometry and examples of the application of the technique.

1. Introduction

Flow cytometry (FCM) is a technique used to rapidly detect and count microscopic particles such as cells, suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. FCM is used in the diagnosis of health disorders, and has many applications in both research and clinical practice. Some instruments can physically sort particles based on their properties, so as to purify populations of interest. An impedance-based flow cytometry device was patented in 1953; the first fluorescence-based flow cytometry device was developed in the late 1960's [Shapiro, 2003].

1.1. Principle of flow cytometry

One or more beams of light (usually laser light) is directed onto a hydrodynamically- focused stream of fluid. A number of detectors are placed at the intersection of the stream with the light beam, to detect scattered light (forward scatter or FSC, in line with the light beam, and side scatter or SSC, perpendicular to it) and one or more fluorescent detectors. Each suspended particle -from 0.2 to 150 micrometers- passing through the beam scatters the light, and fluorescent chemicals found in the particle or attached to it may be excited, emitting light at a longer wavelength than the light source.

This combination of scattered and fluorescent light is recorded by the detectors and, by analyzing changes in brightness at each detector, information about the physical and chemical structure of each individual particle is obtained. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Fluorescence detected is related to the presence of specific cellular structures (antigens, nucleic acid) or functions (viability, enzyme activity, membrane potential, intracellular ion concentration).

Modern flow cytometers are able to analyze several thousand particles every second, in "real time", and can actively isolate particles having specified properties. A flow cytometer is similar to a microscope, but instead of producing an image of the cell, flow cytometry offers "high-throughput" (for a large number of cells) automated quantification of set parameters. To analyze solid tissues, a single-cell suspension must first be prepared [Robinson, 1993; Darzynkiewicz, 1994.]

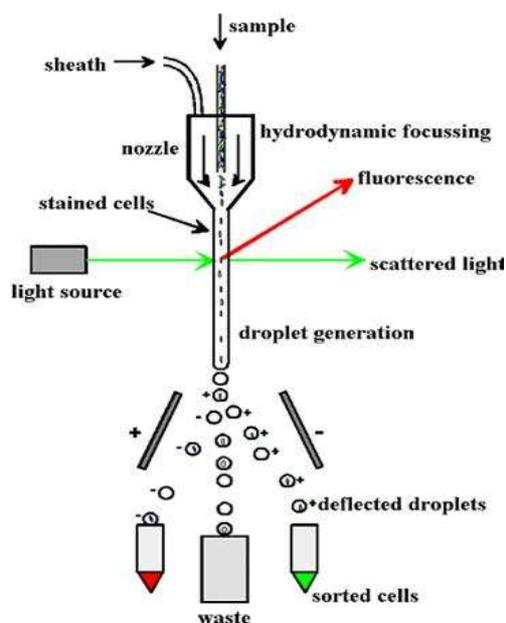


Figure 1. Schematics of a flow cytometer

2. Methodology

A flow cytometer has several components: 1) a flow cell - liquid stream (sheath fluid), which carries and aligns the cells so that they pass aligned through the light beam; 2) an optical system, with at least one light source (usually laser) and light collection devices (photodetectors or photomultiplier tubes); 3) an electronic system which generates and amplifies electrical signals from light. Acquisition and analysis of data is done by a computer connected to the flow cytometer. [Ormerod 1999, Ormerod 2000, Ormerod 2008].

Modern instruments usually have multiple lasers and fluorescence detectors. Increasing the number of lasers and detectors allows for multiple labelling. Certain instruments can even take digital images of individual cells, allowing for the analysis of fluorescent signal location.

Data generated by flow-cytometers can be plotted in a histogram, or in two-dimensional dot plots or even in three dimensions. The regions on these plots can be sequentially separated by creating a series of subset extractions ("gates"). Because different fluorescent dyes' emission spectra overlap, signals at the detectors have to be compensated. Data accumulated can be analyzed once the data are collected using appropriate software.

2.1. Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of signals from individual cells as well as physical separation of cells of particular interest. The first cell sorter was invented in 1965 [Shapiro, 2003]

The cell suspension is entrained in the centre of a narrow stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter; a vibrating mechanism causes the stream of cells to break into individual droplets. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. Charge is applied directly to the stream, and the droplet retains charge of the same sign as the stream; the stream is then returned to neutral. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge [Shapiro, 2003; Longobardi, 2001].

2.2. Fluorescent labels

A wide range of fluorophores can be used as labels in flow cytometry, with a characteristic peak excitation and emission wavelength. Also, the emission spectra of the labels often overlap. Consequently, the combination of labels which can be used depends on the wavelength of the light used to excite the fluorochromes and the detectors available [Haugland, 2010].

Depending on the specificity of the fluorochromes detected, very different measurable parameters can be analyzed by flow cytometry: pigments content (chlorophyll, phycoerythrin...), total DNA or RNA content (cell cycle analysis, cell kinetics, proliferation, etc.), chromosome analysis and sorting (library construction, chromosome paint), protein expression and localization, phospho-proteins, transgenic products in vivo (particularly the green fluorescent protein (GFP) or related fluorescent cell surface antigens (Cluster of differentiation (CD) markers), intracellular antigens (various cytokines, secondary mediators, etc.), nuclear antigens, enzymatic activity, pH, intracellular ionized calcium, membrane potential, membrane fluidity, apoptosis (measurement of DNA degradation, mitochondrial membrane potential, permeability changes, caspase activity), cell viability, monitoring electro-permeabilization of cells, oxidative burst, multidrug resistance (MDR) in cancer cells. These parameters can be detected individually or combined, as far as fluorochromes used in detection can be spectrally resolved.

3. Applications

3.1. Clinical applications

3.1.1. Haematology

The distributed nature of the hematopoietic system makes it amenable to flow cytometric analysis. Applications in haematology include DNA content analysis, leukemia and lymphoma phenotyping, immunologic monitoring of HIV-infected individuals, and assessment of structural and functional properties of erythrocytes, leukocytes, and platelets. Antibodies against intracellular proteins such as myeloperoxidase and terminal deoxynucleotidyl transferase are also commercially available and permit the analysis of an increasing number of intracellular markers [Bollum, 1979].

- *Cluster of differentiation antigens on leukocytes.* Cell identification is involved in flow cytometry. In order to identify blood cells, it is necessary to define sets of cellular parameters, which include intrinsic (such as cell size, cytoplasmatic granularity...) and extrinsic parameters. The cluster of differentiation (CD) is a nomenclature used for the identification of cell surface molecules present on leukocyte cells. CD molecules can act in numerous ways, often acting as receptors or ligands. The International Workshop on Human Leukocyte Differentiation Human Cell Differentiation Molecules names and characterises CD molecules [Zola et al., 2006].
- *Reticulocytes.* The reticulocyte count reflects the erythropoietic activity of the bone marrow and is thus useful in both the diagnosis of anemias and in monitoring bone marrow response to therapy. Starting in the mid-1990s, automated flow-cytometric analysis has replaced traditional microscopic quantitation of reticulocytes. Reticulocyte analysis now includes measurements of the mRNA content and the maturity of reticulocytes, cell volume, hemoglobin concentration and content [Piva et al. 2010].
- *Hematopoietic stem cells.* The use of flow cytometry in stem cell research requires high levels of stringency and as “fool-proof” techniques as possible, because often the number of cell populations of interest are relatively small, compared to those in other fields of study. Because of these small numbers of cells, it is useful to take advantage of the benefits of contemporary instruments and multiple reagent combinations to learn the maximum possible about these populations. CD34, Hoeschst 33342, CD105, CD90, CD117, CD133, Sca-1, CD150 are some of the antigens and functional markers which have primarily been useful in the study of human and murine hematopoietic and other stem or progenitor cells [Preffer et al. 2009]. CD34 is a stage-specific antigen that is expressed on human hematopoietic stem and progenitor cells whose expression decreases with differentiation of the cell. Side population (SP) cells are stem and early progenitor cells identified as a subpopulation by their low Hoechst blue and red fluorescent emission signature. The characteristic Hoechst fluorescence SP “tail” [Goodell et al. 2005; Petriz 2006] is attributed to a highly active efflux of Hoechst 33342, via a p-glycoprotein multidrug/ATP binding cassette transporter Bcrp/ABCG2 protein.

3.1.2. Immunology

Characterization of lymphocyte subtypes and cytokine signalling is essential for understanding the complex nature of the immune system. Activation by antigens, suppression of normal immune activation, and disease states can affect the phenotypes of lymphocytes. Multiparametric phenotypic analysis by flow cytometry allows to distinguish one subpopulation of cells within a heterogeneous mixture, and thereby enables the study the dynamics of immune signalling in intact cells.

- *HIV infection.* Infection with the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) represent the most common clinical application of flow cytometric immunophenotyping of peripheral blood lymphocytes. CD4+ T cells are the main cellular targets of HIV infection; they are destroyed by several mechanisms and their number decreases with progression of disease. According to certain organizations, the clinical decision concerning initiation of antiviral therapy or prophylaxis of opportunistic infections are based on the numbers of CD4+ T cells, therefore, accuracy and precision in measuring lymphocyte subsets are crucial during the follow-up of these patients [Chattopadhyay et al., 2010].
- *Lymphocyte activation.* Lymphocyte response to mitogens is a habitually-used test in the evaluation of the immune system. The old usual method with [3H]thymidine yielded results which were difficult to interpret. The identification of cell surface structures

(e.g., CD69), minimally expressed on resting PBMC and broadly expressed on activated lymphocytes very early following the addition of stimuli, enhances the potential for detection of low-frequency responses [Vernon et al. 1995]. CD69 is expressed on all activated lymphocytes, so it represents a generic marker to monitor individual subset responses to provocative stimuli. This assay allows the study of the activation at the level of specific T, B, and natural killer (NK) cells. CD69 expression is a good marker of cells capable of cytokine production, which itself is an indicator of activation.

To monitor an immune response, it is important to have procedures that can follow lymphocyte proliferation, both in vitro and in vivo. The intracellular fluorescent dye, CFSE, has been found to be particularly effective at monitoring lymphocyte division in many experimental situations. The method is based on the ability of CFSE to covalently label long-lived intracellular molecules. Following each cell division, the equal distribution of these fluorescent molecules to progeny cells results in halving of the fluorescence of daughter cells [Parish et al., 2001].

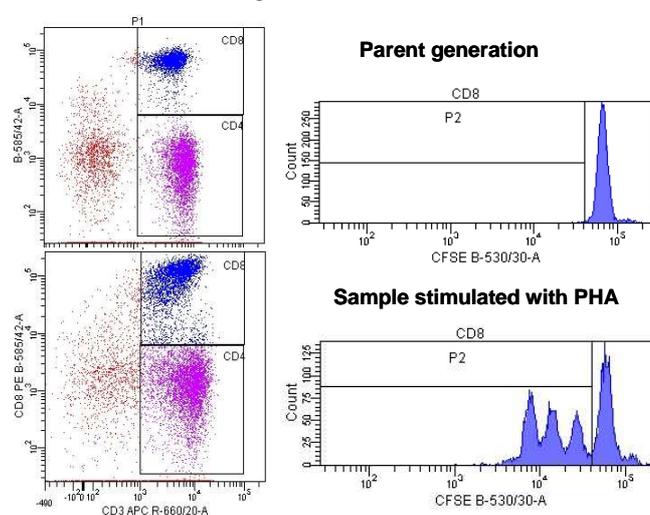


Figure 2. Cell proliferation indicated by dilution of CFSE in stimulated and unstimulated CD8 lymphocytes

3.1.3. Cancer biology and clinical oncology

In the last decade the main oncology application of cytometry has been the measurements of abnormalities of DNA content in tumor cells and with perturbation of the cell cycle by chemotherapeutic agents. By measurements with high resolution, the demonstration of abnormal cellular DNA content in several types of tumors can be of definite diagnostic value when combined with conventional diagnostic procedures.

Attempts to monitor cancer treatment by studying altered cell cycle distribution have not been successful, although some applications are of potential value. The main reasons for this are the complexity of tumor tissue as well as difficulties with interpretation of altered cell cycle distribution caused by drug combinations. For further progress in this field more emphasis on other cell constituents than DNA measured by flow cytometry is desirable, either as single or as multiparameter measurements. Interest remains in determination of drug sensitivity of tumor cells, or determinations of kinase activities in cells. The appreciation that various oncogenes are expressed in many human cancers has led to the development of cytometric techniques for detecting oncogenes by immunofluorescence.

3.2. Non-clinical applications

3.2.1. Flow cytometry of plant cells.

Flow cytometry on plant material is usually done on protoplast or cell suspensions. Chromosomes and plant subcellular organelles such chloroplasts and mitochondria have also been subjected to flow cytometric analysis.

The main area of application of flow cytometry is the measurement of DNA content for systematic, evolutionary or breeding purposes [Garcia et al., 2006; Torrel et al., 2001]. A reference standard of known DNA content is used (Fig. 3, *Pisum sativum* 'express long', where $2C=8.37$ pg). Propidium iodide, which has no bias for AT or G-C-rich sequences within the genomes is commonly used as fluorescent stain. The measured fluorescence is directly proportional to DNA content. Using this procedure, up to 3.7% differences in DNA content has been resolved [Dolezel et al., 1995].

Measurements of the fluorescence of chlorophyll and other natural pigments as well as measurements of protein and nucleic acid content and specific antigens have been made in plant cells. Galbraith [Galbraith et al., 1995] has used GFP and its variants to study gene expression in plants.

3.2.2. Microbiology

Detection of microorganisms has been done from very different perspectives: biotechnology, environmental, clinical, food, sanitary or even military; the interest of a technique such as flow cytometry in microbial detection is clear since it allows the detection of microbes at a single-cell level.

DNA or protein content, membrane potential and immunofluorescence measurements of algae, bacteria, fungi and protozoa using flow cytometry had been reported in the literature by the mid-1980's, as had autofluorescence due to photosynthetic and other pigments. Differences in cell wall composition and in the outer membrane of bacteria, which are the basis of classical stains like the gram staining, can be detected by FCM. Commercial detection kits like the LIVE/ DEATH[®] BacLight[™] bacterial counting and viability kit provides rapid and reliable assessment of bacterial viability in multiple applications [Haugland et al., 2010].

- *Environmental applications and marine biology.* Marine microorganisms detected by FCM include bacteria, phytoplankton, zooplankton and viruses. New species like *Prochlorococcus*, which is responsible almost of a third of the total bacterioplankton turnover of aminoacids, have been discovered using FCM (fig 4). DNA content and photosynthetic pigments are the most common parameters measured in oceanography. Specific organism (like the infective protozoa *Giardia* or *Microsporidium*) had been detected on water by flow using immunofluorescence techniques.
- *Clinical microbiology.* Blood, urine or spinal fluid have been used as clinical specimens when searching pathogens by flow. But FCM provides a tool not only for detection and identification of microbes, but measuring antimicrobial susceptibility testing, providing results in 2 to 7 hours, rather than in 15 to 24 hours, as it is the case when traditional culture methods are used. Bactericidal and the effect of bacteriostatic agents can be monitored by flow on cultures, measuring cell membrane permeability or membrane potential.

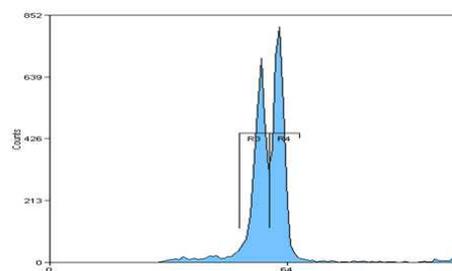


Figure 3: fluorescence distribution of propidium iodide stained nuclei fo standard (*P. sativum*, mode=57) compared to problem plant (mode=62)

- *Food industry.* Food microbial analysis is routinely performed by colony counting. However, plate-culturing techniques are time consuming and they only reveal a small proportion (viable and cultivable) of the true microbial population. The application of FCM to foods is related to a correct setting of discriminator gates to select microbial counts from background particles. FCM has been applied on milk, brewing and oenology industries, and has been used for measurement of spoilage and pathogen microorganism contamination, starters and probiotics. [Comas and Rius, 2009]. Reported sensitivity of flow cytometry is $\leq 10^4$ total bacteria/mL, and 103 cells/mL for yeast.

3.2.3. Pharmacology and toxicology

Flow cytometric analysis of drug effects on cells *in vivo* has been considered as an alternative to animal testing. DNA content analysis has been used to determine effects of anticancer drugs on the cell cycle. In other cases, it is possible to study drug uptake directly, using fluorescent drugs such as anthracyclines or labeled analogs. Even when direct assessment of drug uptake on a cell-by-cell analysis is not possible, FCM can provide quantitative analyses of the effects of different drug doses on cell's metabolism over time courses. The measurement of apoptosis –the genetic controlled ablation of cells- is routinely done by flow cytometry using multiparametric approaches.

Standard tests of clastogenic agents such as bone marrow and blood erythrocyte micronucleus assays are performed by FCM detecting DNA-containing micronuclei in relative immature cells, and distinguishing them from immature erythrocytes which contain RNA [Derringer et al., 1997].

3.2.4. Biotechnology

Industrial uses of flow cytometry include on-line monitoring of bioreactors or selection of yeast cells expressing antibodies. Flow cytometers without sorting capability are used for high-throughput screening; sample handling hardware has reduced the time needed to process a single sample to under two seconds. Sorters are used to isolate fractions from highly heterogeneous mixed populations, based on ligand binding and metabolic activity [Hewitt et al., 2001].

4. Practical examples

4.1. Human Regulatory T-cell analysis.

Regulatory T cells (Tregs) play a critical role in maintaining immune modulation and are present in normal peripheral blood in low numbers. Cell cytometry can be used to evaluate the proportion of Tregs and memory T cells in peripheral blood. A description of the method used to identify and isolate these cells by flow cytometry is provided here.

Recipient peripheral heparinized blood samples were collected and PBMC were obtained by standard Ficoll density gradient centrifugation. Recipient PBMC were stained with the following monoclonal Abs: APC anti-CD3, PerCP-Cy5.5 anti-CD8, FITC anti-CD4, APC anti-CD25, PerCP-Cy5.5 anti-CD127, PE anti-Foxp3, APC anti-CD45RA), all stains were carried out following the instructions of the manufacturer.

Flow cytometric experiments were carried out using a FACSCalibur (BD Bioscience). Excitation of the sample was done using a standard 488nm and 635nm laser. The instrument was set up with the standard configuration. FSC and SSC were used to gate lymphocytes cell population. As negative control, samples were incubated with corresponding isotype controls. A representative dot plot is shown in Fig. 5.

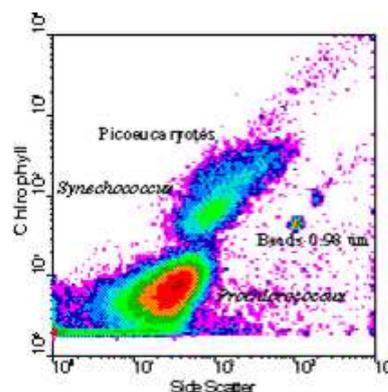


Figure 4. *Prochlorococcus*: FCM detection according to scatter and chlorophyll content. (D. Vaultot, CNRS).

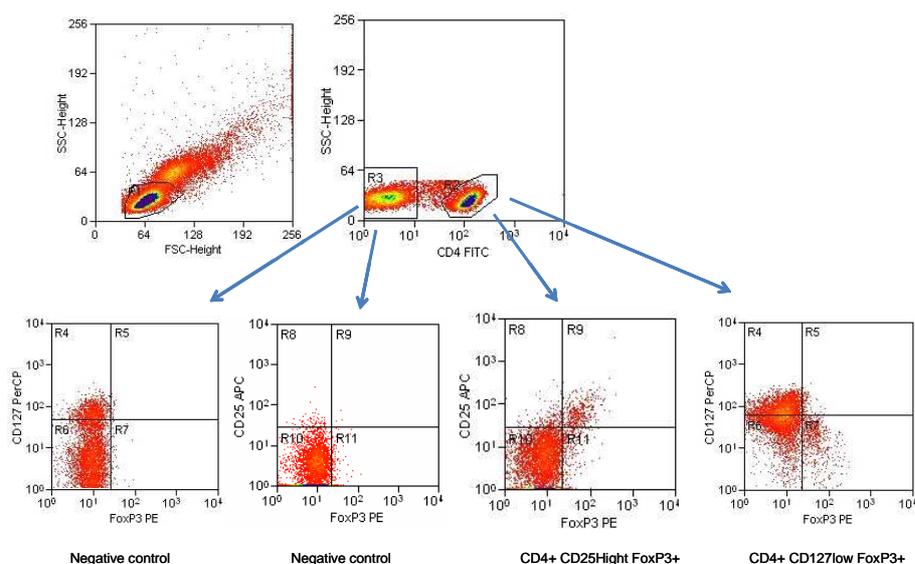


Figure 5. Representative dot plot used for the cytometric analysis of Foxp3+Tregs. Negative controls are also shown. Lymphocytes are identified using a routine scatter gate procedure. Events from the later gate are then transposed to the SSC vs. CD4 dot plot to obtain CD4+cells (R2) and CD4- cells (R3). CD4- (R3) cells were used as negative control. Finally, the cells from R2 or R3 gate are transposed to the CD127 vs. FoxP3 dot plot and the CD25 vs. FoxP3 dot plot.

In conclusion, the evolution of memory/effector and regulatory T cells can be analyzed by cell cytometry in response to different treatments. It is possible identify and sort Tregs within a CD4+ lymphocyte population based upon high expression levels of CD25. As an alternative, CD127 can be used to identify Tregs as CD127low events within the CD4+CD25+ lymphocyte population. More details can be found elsewhere [Bestard 2011]

4.2. Detection of apoptosis induction in B-cell chronic lymphocytic leukemia cells without effect in T lymphocytes

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of long-lived B lymphocytes. Most of the circulating cells appear to be nondividing and the clonal excess of B cells is mainly caused by defects that prevent programmed cell death rather than by alterations in cell cycle regulation. Glucocorticoids and other chemotherapeutic agents used clinically, including the nucleoside analogues 2-chloro-2-deoxyadenosine and fludarabine, induce apoptosis in B-CLL lymphocytes, suggesting that apoptosis is the mechanism of their therapeutic action. New apoptotic drugs can be alternative therapeutic option for CLL patients.

Experimental procedures: Seventy samples from patients with B-CLL who had not received treatment in the previous 6 months were studied. B-CLL was diagnosed according to standard clinical and laboratory criteria. Cells were obtained from the Hospital Clinic, Barcelona, Spain. Mononuclear cells from peripheral blood samples were isolated by centrifugation on a Ficoll-Hypaque (Seromed, Berlin, Germany) gradient and cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide (DMSO).

Apoptosis was measured by annexin V binding. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca²⁺-dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is used in conjunction with a vital dye such as propidium iodide (PI) to identify early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI.

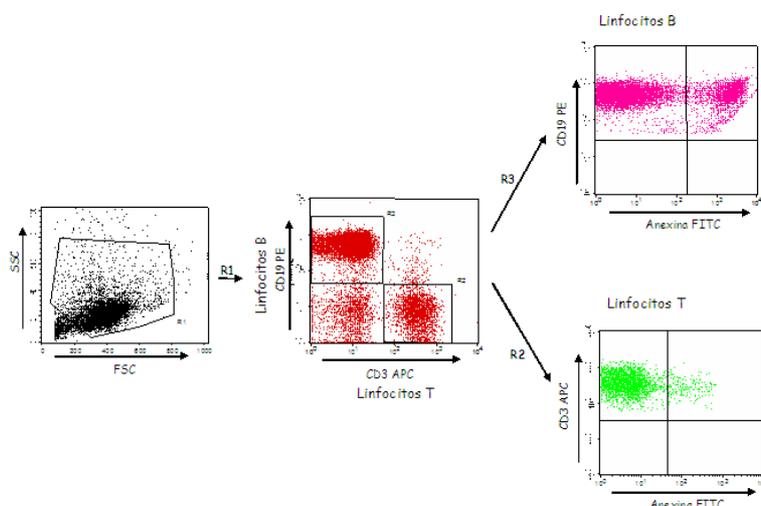


Figure 6: Representative dot plot used for the cytometric analysis of annexin-V+ in T (CD3+) and B (CD19+) cells. Lymphocytes are identified using a routine scatter gate procedure. Events from the later gate are then transposed to the CD19 vs. CD3 dot plot to obtain CD3+ CD19- cells (T lymphocytes) and CD3- CD19+ cells (B lymphocytes). Finally, the B or T cells are transposed to the CD19 or CD3 vs Annexin dot plot.

To analyze apoptosis in T cells from the samples, 500 000 cells were incubated for 24 hours with the indicated factors. Cells were then washed in PBS, and incubated in annexin-binding buffer with allophycocyanin (APC)-conjugated anti-CD3 and phycoerythrin (PE)-conjugated anti-CD19 for 10 minutes in the dark. Cells were then diluted with annexin-binding buffer and incubated with annexin V-FITC for 15 minutes in the dark. Then, annexin-binding buffer and PI were added just before flow cytometric analysis. Data were analyzed using Cell Quest software (Becton Dickinson, Mountain View, CA).

The precursor of nucleotide biosynthesis acadesine or 5-aminoimidazole-4-carboxamide (AICA) riboside has various effects in several types of eukaryotic cells. Because acadesine blocks glucocorticoid-induced apoptosis of quiescent thymocytes, it was examined whether glucocorticoid induced apoptosis in B-CLL cells was also blocked by acadesine. The effect of several doses of acadesine on the viability of B-CLL cells was analysed (data not shown). Unexpectedly, acadesine induced apoptosis. To determine whether the induction of apoptosis in B-CLL cells was selective to B population, the number of apoptotic T cells (CD3- cells) in CLL blood samples was analysed.

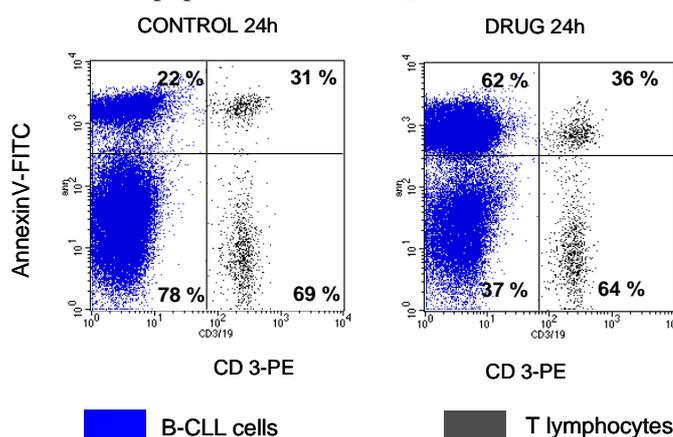


Figure 7: Acadesine effect on lymphocytes viability. Lymphocytes are identified using a routine scatter gate procedure (data not shown). Lymphocytes are then transposed to the CD3 vs AnnexinV dot plot to quantify apoptosis in B cells (CD3-) and in T cells (CD3+)

In conclusion, acadesine, 5-aminoimidazole-4-carbox-amide (AICA) riboside, induced apoptosis (62% in treated cells vs 22% in control) in B-cell chronic lymphocytic leukemia (B-CLL) cells, whereas T cells (CD3+) from these patients were not affected (31% in treated cells vs 36% in control). Flow cytometry is a practical and easy technique to analyse the induction of apoptosis in treated cells.

4.3. Flow cytometric detection of Plasmodium Falciparum in malaria-infected erythrocytes

Malaria is a parasitic disease caused by unicellular organisms of the genus *Plasmodium*. A large part of the life cycle of these parasites takes place in the blood circulation, where *Plasmodium* invade red-blood cells (RBC) in which they grow and multiply; this blood stage infection causes clinical symptoms and are targets for a number of drugs. The demonstration of the presence of parasites in the blood is used for diagnostics and treatment of the disease.

The analysis and detection of malaria infection by flow cytometry makes almost exclusive use of fluorescent dyes which are specific for nucleic acids. Since RBC do not contain DNA, DNA-specific fluorescence from infected RBC can only be due to the parasite DNA. In addition, since parasites multiply within the RBC, the fluorescence intensity of stained particles increases during the development of the parasites. As long as the DNA content of a parasite is 100-200 times less than the nucleated blood cells, they can be easily distinguished on the basis of their fluorescence intensity.

Most of the reported flow cytometric studies on malaria parasites used A/T specific DNA dyes (Hoechst 33258, Hoechst 33342). Others non DNA-specific stains (Acridine orange, Propidium iodide) had also been successfully used. In the presented example, the Syto-11[®] fluorescent dye (Molecular Probes) has been used because 1) its high cell membrane permeability, that allows to stain samples without fixation or permeabilization procedures, 2) its high-intensity green fluorescence, which is strongly enhanced when bounded to DNA or RNA. Syto-11 is excited at 488nm, which is the most commonly light source found in the commercial flow cytometers.

Cultures of *Plasmodium falciparum* 3D7 were grown in vitro in group B human RBCs (provided by the Banc de Sang i Teixits at the Vall d'Hebron Hospital in Barcelona) using previously described conditions. Briefly, parasites (thawed from glycerol stocks) were cultured in RPMI complete medium in a gas mixture of 94% N₂, 5% CO₂, and 3% O₂. The cultures were contained in Petri dishes, and parasites were cultured at intervals of 2 days at ca. 3% hematocrit. For flow-cytometry analysis, non-infected RBCs and *Plasmodium*-infected RBCs samples were diluted to a final concentration of 1-10x10⁶ cel/ml. The cell suspension is stained with 1 uL Syto 11 (0.5 mM in DMSO) to a final concentration of 0.5 μM. Samples were incubated from 5-10 minutes prior to flow cytometric analysis.

Flow cytometric experiments were carried out using an Epics XL flow cytometer (Coulter Corporation, Miami, Florida). Excitation of the sample was done using a standard 488nm air-cooled argon-ion laser at 15mW power. The instrument was set up with the standard configuration. FSC and SSC were used to gate red blood cell population. Green (525 nm) fluorescence for syto-11 was collected in a logarithmic scale. The single-cell population was selected on a forward-side scatter scattergram, and the green fluorescence from this population was analyzed.

Figure 8 shows the gating on RBC to exclude aggregates and cell debris (A), and how fluorescence of infected RBC can be detected in the gated population (B).

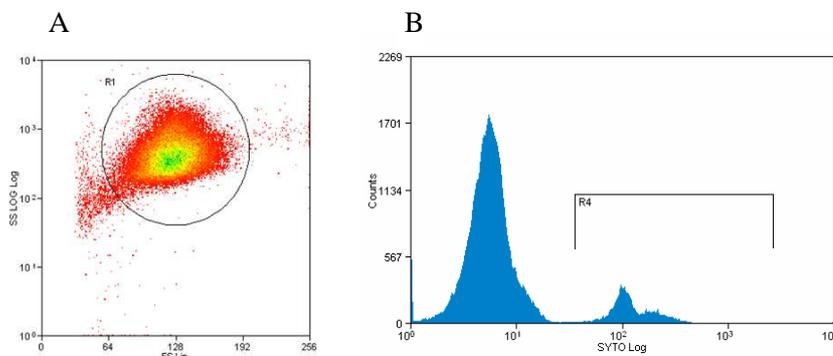


Figure 8. A: RBC population gated according to its scatter signal (horizontal, Forward Scatter; vertical: Side Scatter). B: Fluorescence histogram of Syto-11 obtained from an infected sample (horizontal axis: intensity of green fluorescence; vertical axis: frequency of cells). R4: region defined on *Plasmodium* infected cells.

In Fig. 9, a typical dotplot obtained combining FSC vs. green fluorescence can be seen; A has been obtained from a non-infected sample, while B has been obtained from an infected sample. Region R2 is used to quantify the percentage of *Plasmodium* infected cells. Its position is fixed according to the control (uninfected) sample, where R2 must be <0.1%.

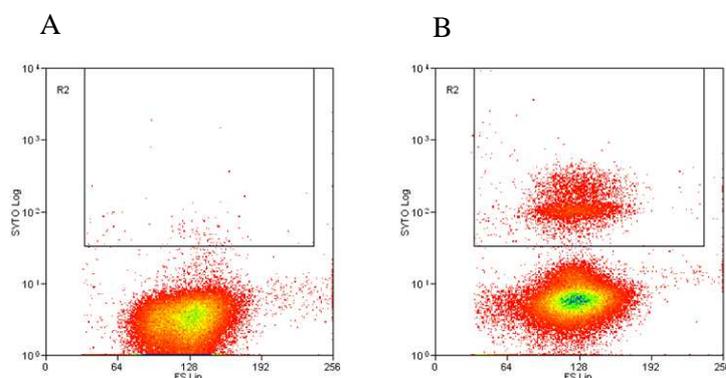


Figure 9: Comparison between a non-infected sample (a) (where false positive < 0.1%) vs. an infected (11% parasitemia) sample (b). (Horizontal axis: Forward Scatter; vertical axis: green fluorescence intensity). R2: infected cells.

In conclusion, detection of *Plasmodium falciparum* by flow cytometry using syto11 has proven to be a simple and fast procedure. As long as fixation and washing steps are not necessary when using this stain, the total time needed for staining and analyzing 50.000 RBC is less than 10 minutes. Flow cytometry has been a valuable tool especially for long batches of samples, where the alternative technique of microscopic counts is long and tedious. High correlation were found between microscopic and flow cytometry counts; minimum values of parasitemia detected by flow cytometry were about 0.5%

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4.4. Stem cell identification and isolation on mouse intestine

Colorectal cancer (CRC) is the second cause of death by cancer; a frequent complication is regeneration of the tumour after therapy. There is an evidence that specific gene signature of intestinal stem cells (ISCs) predicts disease relapse in CRC patients. A description of the method used to identify and isolate these cells by flow cytometry is provided.

Small mouse intestine samples were disaggregated combining enzymatic and mechanical methods; population of single, live cells were selected according to their scatter parameters and Propidium Iodide exclusion. A FACSARIA I SORP (Beckton Dickinson, Ca, USA) was used for cytometric analysis and sorting. ISCs were identified by their high expression of the EphB2 receptor, which becomes gradually silenced as cells differentiate. Using EphB2 expression, mouse ISCs, crypt proliferative progenitors and differentiated cells were FACS-purified to define a gene program specific for normal ISCs. Epithelial cells (EpCAM+, CD45-, CD31-, CD 11b-) were sorted according to their different EphB2 surface expression; Paneth cells were removed using lectin marker (Fig. 10).

In conclusion, flow cytometry and sorting is a powerful technique to identify and purify intestinal stem cells and the stem-like cell population of the tumour, population which has been suggested to play a central role in colorectal cancer relapse.

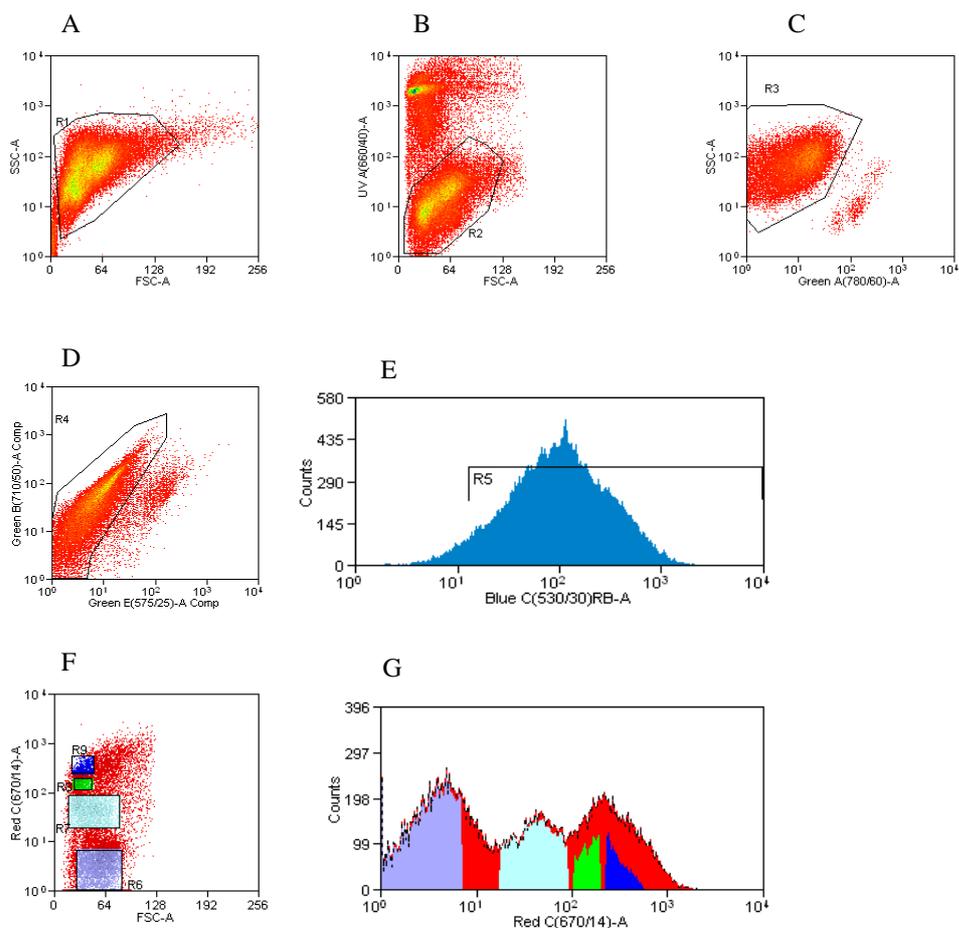


Figure 10: identification of intestinal stem cells by flow cytometry: live, single cells were identified combining scatter parameters (A) and viability by propidium iodide exclusion (B). Epithelial cells were then selected according to their pattern of antigen expression: exclusion of CD45+ CD11b+ CD31+ cells (C), and expression of EpCam (E); Paneth cells were also removed from the analysis (D). Remaining cells were then sorted according to their FSC and EphB2 expression (F); the profile of the sorted fractions according on their EphB2 expression can be seen on G. The profile of high EphB2, low FSC (dark blue color) corresponds to the stem cell population.

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