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Flow cytometry: a literature review

Citometria de fluxo: uma revisão da literatura

Paolo Ruggero Errante[1*], Pâmela Carolina Cruz Ebbing [2], Francisco Sandro Menezes Rodrigues[3], Renato Ribeiro Nogueira Ferraz[4], Neusa Pereira da Silva[2]

[1]Department of Immunology, Institute of Biomedical Sciences, Universidade de São Paulo. USP; [2]Department of Rheumatology, Universidade Federal de São Paulo. UFS; [3]Department of Pharmacology, Universidade Federal de São Paulo. [4]Pós-Doutor e Doutor em Ciências Básicas pela Universidade Federal de São Paulo.UNIFESP. Professor Permanente do Programa de Mestrado Profissional em Administração – Gestão em Sistemas de Saúde (PMPA-GSS) da Universidade Nove de Julho. UNINOVE.

Abstract

Introduction: *flow cytometry is a technique that employs an optical-electronic detection apparatus to analyze the physical and chemical properties of microscopic particles suspend in a liquid medium.* ***Objective****: to review the literature in search of the main studies that used flow cytometry as the main methodology.* ***Method:*** *Articles were selected according to their impact factor in the Journal of Citation Reports.* ***Literature review:*** *a light beam is direct to a continuous flow of suspended particles marked with fluorescent substances. The light is scattered differently from the beam by the particles and is captured by sensors in line and perpendicular to the light beam. These microscopic particles are conjugated with fluorescent substances that, once excited, emit light of lower frequency than the light source. The emitted light is captured by sensors and the particles are analyzed according to fluctuations in brightness of each detector and/or fluorescence emission. The result of this process is the formation of images in real time for each cell fluorescence, scattering and transmission of light. A major problem of flow cytometry is to determine whether a subset of cells labeled with fluorochrome-conjugated monoclonal antibodies is positive or negative. Gains compensation should be determined and applied correctly, and controls should be conducted concisely with the adoption of a biological control, isotype control or Fluorescence Minus One (FMO). None of these controls are considered ideal, and must be chosen according the number of different labeling done, rarity of molecule expression on surface or intracellularly in certain cell subsets, overlap of wavelengths or unspecific binding of the fluorochrome-conjugated antibodies.* ***Conclusion:*** *due to its great potential, flow cytometry has been expanded to diverse fields of biological sciences, and is routinely used in clinical diagnostic, biotechnology, and basic and applied research.*
Keywords: *Flow cytometry. Monoclonal antibodies. Fluorochromes.*

Resumo

Introdução: a citometria de fluxo é uma técnica que utiliza um aparelho de detecção óptico-eletrônico para analisar as propriedades físicas e químicas das partículas microscópicas suspensas em meio líquido. **Objetivo**: revisar a literatura em busca dos principais estudos que utilizaram a citometria de fluxo como a metodologia principal. **Método**: os artigos foram selecionados de acordo com seu fator de impacto no Journal of Citation Reports. **Revisão da literatura**: um feixe de luz é dirigido para um fluxo contínuo de partículas suspensas marcadas com substâncias fluorescentes. A luz é difundida de forma diferente a partir do feixe de partículas e é capturada por meio de sensores em linha e perpendiculares ao feixe de luz. Tais partículas microscópicas são conjugadas com substâncias fluorescentes que emitem luz de frequência mais baixa do que a fonte de luz. A luz emitida é capturada por meio de sensores e as partículas são analisadas de acordo com flutuações de brilho de cada um dos detectores e/ou de emissão de fluorescência. O resultado deste processo é a formação de imagens em tempo real para cada célula da fluorescência, dispersão e transmissão de luz. Um dos principais problemas de citometria de fluxo é determinar se um subconjunto de células marcadas com anticorpos monoclonais conjugados com fluorocromos é positivo ou negativo. Ganhos de compensação devem ser determinados e aplicados corretamente, e os controles devem ser realizados de forma concisa, com a adoção de um controle biológico, controle de isotipo ou fluorescência menos uma (FMO). Nenhum desses controles é considerado ideal, e deve ser escolhido de acordo com o número de rotulagem, paucidade da expressão de moléculas na superfície ou no meio intracelular em determinadas subpopulações de células, sobreposição de comprimentos de onda ou ligação inespecífica dos anticorpos conjugados com fluorocromo. **Conclusão**: devido ao seu grande potencial, o uso da citometria de fluxo foi expandido para diversos campos das ciências biológicas, e é utilizada rotineiramente em clínicas de diagnóstico, e laboratórios de pesquisa básica e aplicada.
Palavras-chave: Citometria de fluxo. Anticorpos monoclonais. Fluorocromos.

Corresponding/Correspondente:*Paolo Ruggero Errante. Department of Immunology. Institute of Biomedical Sciences, University of São Paulo. Avenida Lineu Prestes, 1730 – São Paulo, SP – 05508-000, Brazil. E-mail: errantepr@yahoo.com.

INTRODUCTION

Flow cytometry is a technique that uses an optic-electronic detention device capable of analyzing the physical and chemical characteristics of microscopical particles in

suspension in a liquid medium. Flow cytometer, differently from a microscope that produces a particle image, quantifies a set of parameters from a particle in suspension (WALLER et al., 2001). A beam of light of a single wave length is directed to a continuous flow of suspended particles marked with fluorescent chemical stains. Each particle in suspension that passes by this light beam disperses light in a distinct form caught by sensors on-line with the light beam and perpendicular to it.

These microscopical particles are conjugated with fluorescent substances that, when excited, emit light of lower frequency than that from the light source. The emitted light caught by detectors and analyzed later according to the brightness fluctuations of each detector or fluorescence emission (LOKEN; PARKS; HERZENBERG, 1977). The result is the formation of real time images of each fluorescence cell, dispersion and transmission of light (TELFORD, 2015).

The main components of a flow cytometer are a flow chamber; a light source; a detector and a digital analogical converter that generates fluorescent parameters of size, complexity and signals; a system of amplification of linear or logarithmic signal; and a computer for the signal analysis (KUREC, 2014).

Flow cytometry is generally used in the diagnosis of infectious and neoplasic diseases, cellular phenotyping, cellular cycle studies, normal or abnormal content of DNA, chromosome analysis, organ transplant, immunodeficiency studies, animal and vegetal physiology and pharmacology (CURTIS; WALKER; DENNY, 2011).

HISTORICAL ASPECTS

The first automated cell counter was developed by Moldavan, and consisted of a capillary tube mounted under an optical microscope with an objective provided with a photoelectric detector which recorded the passage of cells stained according to the light change (MOLDAVAN, 1934).

In 1949, Wallace Coulter developed a cell counter based on the voltage change caused by the passage of a particle through the interior of a needle (CARTER; CROSLAND-TAYLOR; STEWART, 1968) and Mellors and collaborators used a microfluorimetric scanner for differential cell detection, improving the capacity of detection of technique (MELLORS; SILVER, 1951).

The evolution of technique was obtained when Crosland-Taylor developed a flow chamber based on the passage of cells in a liquid stream (sheath fluid) through a capillary where samples were detected by a light source (CROSLAND-TAYLOR; STEWART; HAGGIS, 1958).

In that same year, using the technology of automated cell counter, Parker and Horst developed the first differential hematology counter using cells stained in red (RBCs), in blue (leukocytes), and visible light and detectors for the blue and red light (HORST, 1959).

This methodology of automated cell counter was used by Mendelsohn (MENDELSOHN, 1958) and (CASPERSSON, 1964) to study malignant cells of the uterine cervix, expanded the potential use of apparatus.

A year later, Katmentsky built the Rapid Cell Spectrophotometer (RSC), equipment in which the cell suspension passed through a channel in a microscope slide capable of measuring the size and quantity of cellular DNA. This new procedure expanded the capacity of cell count based in DNA quantity, important to study of malignance cells.

In that same year, Katmentsky used spectrophotometry (absorbed light) to measure and quantify the DNA and the multiparametric measurement of dispersed light, introducing the biparametric histograms and the pneumatic sorter (KAMENTSKY; MELAMED; DERMAN, 1965).

Van Dilla described the first flow cytometer with orthogonal configuration using the chamber created by Crosland and Taylor, and demonstrated the relationship between fluorescence intensity and ploidy in DNA quantification, creating histograms to describe the cell cycle phases G0/G1, S and G2M (VAN DILLA; FULWYLER; BOONE, 1967). This discovery increases the knowledge of normal and malignance cell cycle study. Two years late, Dittrich & Gohde (DITTRICH; GÖHDE, 1969) used ethidium bromide for DNA labeling in cells analyzed by flow cytometry, contributing to study of cell cycle study.

In late 60s, Bonner, Sweet, Hullet and Herzenberg created the Fluorescence Activated Cell Sorter (FACS), which began to be commercialized in the 70s by Becton and Dickinson (BD) (HERZENBERG, et al., 2002). The equipment was able to add electric charge to individual cells with specific markers and separate those using electrostatic field, and presented an advantage of improved signal to noise ratio when compared to cytometers based on dyes and absorption.

In 1974, the first flow cytometer capable of identifying light scattering and the different wavelengths emitted by enzyme substrates used in the detection of leukocyte esterase and peroxidase was commercialized. Subsequently Gratzner used bromodeoxyuridine (BrdU) and monoclonal antibodies in the study of cell cycle phases (GRATZNER et al., 1975); Salzman used FACS in the identification and separation of leukocytes (SALZMAN et al., 1975) while Merril and collaborators investigated light scattering using lung tumor cells (MERRILL; DEAN; GRAY, 1979) and Darzynkiewicz and collaborators marked DNA and RNA with acridine orange (DARŻYNKIEWICZ et al., 2011).

In 1977, Loken and collaborators measured simultaneously two cell surface antigens with a single laser beam using electronic compensation to distinguish the signals from fluorescein isothiocyanate (FITC) and tetramethylrhodamine (RD) (LOKEN; PARKS; HERZENBERG, 1977).

In the late 70s, the spectrum study was applied to flow cytometry to others fields, such measure intracellular pH (VISSER; JONGELING; TANKE, 1979), electrical charge of red blood cells surface (VALET et al., 1979) and the redox state of human glial cells (THORELL; KOHEN; KOHEN, 1978).

A important application flow cytometry technology is the use of monoclonal antibodies to identify human T cell

subsets according to their surface antigens (HOFFMAN et al., 1980) and for B cell phenotyping (HARDY et al., 1982), before limited to immunohistochemistry study.

In the early 80s, new fluorochromes are add to the monoclonal antibodies technology, and phycobiliproteins were introduce as fluorochromes. Expanded the use of flow cytometer technique, was used to study DNA from nuclei of samples preserved in paraffin (HEDLEY et al., 1983), before restrict to live cells, and karyotyping (COLLARD et al., 1984).

In recent decades, technological advances have led to the development of smaller instruments with basic devices for use in laboratory routine and more complex instruments, capable of measuring different properties of particles with higher sensitivity, available for the academic community, government agencies and research department of multinational industries. In an exponential growth, we observed the development of reagents and fluorochromes, making the flow cytometry technology effective, accurate and precise in seeking quantitative and qualitative information from individual cells (WALLER et al., 2001).

However, a major problem has been the high cost of equipment's and reagents involved added to the need for training qualified people for its use (SHAPIRO, 2005). Due to its great potential, the use of flow cytometry has been expanded to diverse fields of biological sciences, and is routinely used in clinical diagnostics, biotechnology, and basic and applied research.

Flow cytometry

Flow cytometer is an instrument that analyzes individual particles or cells by employing a hydrodynamic, optical, electronic and computational system (TUNG et al., 2007).

It comprises a pneumatic pump that propels particles and cells in suspension in a continuous flow into a hydrodynamic focusing chamber (flow cell), which due to its conical shape forces the accommodation of particles or cells into a capillary or nozzle of 250 microns in diameter producing a thin jet of fluid at an average speed of 10 m/s.

This aligns the particles or cells one by one in the nozzle to be analyzed individually (SHAPIRO, 2005). For characterization each particle need to be excited by a light source such as laser, which has a monochromatic emission, low signal to noise ratio, high spectral purity and is stable (POZAROWSKI; HOLDEN; DARZYNKIEWICZ, 2006).

In sequence, the light source excites each aligned particle that scatters the light and the photoelectric transducers convert the scattered light signals into electrical signals (MARTI et al., 2001; SHAPIRO, 2004).

The light is scattered according to morphological and structural features of the cell; the frontal or forward scatter (FSC) is associated with cell size, and the orthogonal or lateral scatter called side scatter (SSC) is dependent of the cytoplasmic granularity (SALZMAN, 2001; GOLIM et al., 2007).

The optical detection transducer for the forward scatter is the photodiode and that for the side scatter is the photomultiplier tube (PMT), which presents a better signal to noise ratio, since the PMT sensor is more sensitive to light and has high signal-noise ratio (TUNG et al., 2007).

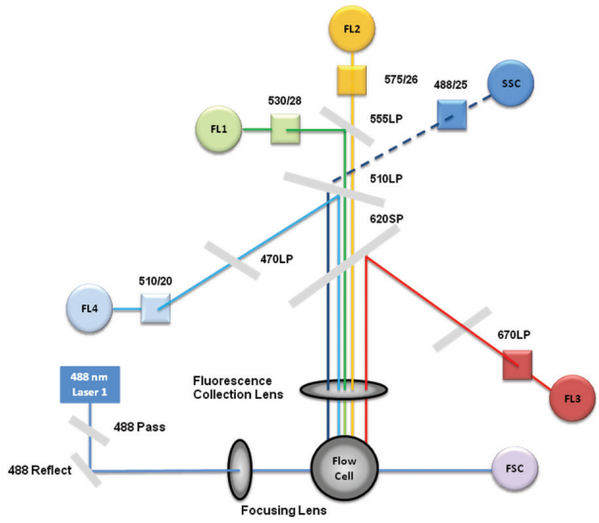
Since the cells are label with monoclonal antibodies conjugated to fluorochromes, the laser excitation results in light emission according to the fluorescent characteristics of the labels. A series of lenses near the interception area (cell-laser) collects the scattered light and sends it to the PMT which converts the light signal into electrical pulses proportional to the amount of scattered light or fluorescent light captured by the PMT (TUNG et al., 2007).

The selection and collection of these light signals are performed by optical filters that block certain wavelengths of incident light and let through only light of the desired wavelength (WOOD, 2006).

There are different types of filters that both reflect a given wavelength and allow others to pass through (dichroic mirror or filter) or absorb the unwanted wavelengths (barrier). Some filters let longer wavelengths to pass and reflect or block shorter wavelengths (long pass/LP), others let shorter wavelengths to pass and reflects or block longer wavelengths (short pass/SP), and some transmit a relatively narrow range or band of light (band pass / BP) wavelengths (MARTI et al., 2001).

*The electrical signals generated by the PMT are amplified and converted digitally to a computer connected to the flow cytometer, which has specific software, able to translate simultaneously parameters such as cell size (FSC), cell granularity (SSC), green fluorescence (FL1) orange fluorescence (FL2), red fluorescence (FL3) and so on (GREEN; WACHSMANN-HOGIU, 2015; HERZENBERG et al., 2006) (***Figure 1***). This panel color analysis has expanded increasingly coming so far to 18 different colors (http://www.ebioscience.com).*

Figure 1 – *Principles of flow cytometer.*



Subtitle – A laser beam excites the fluorochrome whose light is scattered frontally (FSC) and sideways (SSC). Argon laser is directed laterally by a mirror 488Pas and 488Reflect. Filter 530/28 BP capture the emission of FL1 and filter 555LP reflects the emission of FL1 and

passes the emission of FL2. Filter 575/26BP eliminate interference from FL1 to FL2, and FL3 on FL2. Filter 620SP reflects the emission of FL3 and 670LP filter lets through the emission of FL3. Filter 510/20 captures FL4 and 470LP/510LP filter eliminates interference from FL3 on FL4 (adapted from www.cnbc.pt).

General characteristics of flow cytometers

Some flow cytometers are equipped with low power air-cooled argon laser, analyze up to four colors and others, perform physical separation of selected cells in a suspension (sorting), and are equipped with high power water cooled and air cooled lasers, and may detect up to 18 colors simultaneously (BAUMGARTH; ROEDERER, 2000).

Most equipment's use lasers as stable, monochromatic, high potency light source, which can be cooled by air or water. The mostly used light source is the low intensity (10-15 mW) air-cooled argon laser, which has an emission at 488 nm, that can excite the FITC, PE, ECD, PerCP, CyCrome, PI, 7-AAD, annexin V, ethidium bromide, acridine orange, pyronin Y, fluo-3 and rhodamines fluorochromes (HERZENBERG, 2002).

The helium-neon laser (He-Ne) has an emission of 633 nm and is used to excite the fluorochromes Hoechst, mithramycin and APC (SHAPIRO, 2005; POZAROWSKI; HOLDEN; DARZYNKIEWICZ, 2013).

A key component in the flow cytometer is the flow chamber (flow cell). The mostly flow chamber used flow cell is the sense-in-quartz, which has a quartz tip at the laser-cell intercession. There are three different types of flow chambers; the sort sense, the fast quartz and the hypersort sense quartz (ZHU; OZCAN, 2015).

The sort sense is a low speed hemodynamic flow chamber has low signal to noise ratio, and may have holes of 76 or 100 µm in diameter. They are use in cell sorting devices. The fast quartz has low signal to noise luminous ratio and greater speed passage of samples than the sort sense type being use in high-speed cell sorting devices and its laser is air-cooled or cooled lasers. The hypersort sense quartz has low signal to noise ratio, greater passage velocity and a lens to enhance the sensitivity under high flow pressure (NUNEZ, 2001).

The commercialized cytometers are either bench-top flow cytometers used in clinical laboratories for multiparametric cell analysis (www.millipore.com/flowcytometry) or cell separator (cell sorter) equipment's displaying a larger number of resources (JOCHEM, 2005).

Fluorochromes used in Flow Cytometry

*Fluorochrome or fluorophore is a molecule that absorbs luminous energy of a specific wavelength and subsequently emits light in a longer wavelength with lower energy (***Table 1***).*

Table 1 – *Fluorochromes commonly used in flow cytometry and emission spectrum depending on the type of laser used.*

Fluorochrome	Laser (nm)	Emission (nm)	References
FITC	488	519 – 525	IBÁÑEZ-PERAL *et al.,* 2008
Alexa Flúor	488	519	DAVIS *et al.,* 2013b
R-PE (PE)	488	575 – 578	DAVIS *et al.,* 2013a
PE -Cy5 (TC1)	488	667	KALINA *et al.,* 2012
PerCP (BD2)	488	675 – 678	DAVIS *et al.,* 2013a
PerCP/Cy5.5 (BD)	488	695	DEGHEIDY *et al.,* 2015
PE – Cy7	488	795	PREIJERS *et al.,* 2011
7 – AAD	488	655 (620 – 675)	DAVIS *et al.,* 2013b
HOECHST 3342	488	461	BARNETT *et al.,* 2013
PI	488	570 – 617	DE ROSA *et al.,* 2001
Rodamine 123	488	528	ADLER; SCHMITT-JANSEN; ALTENBURGER 2007
Texas Red	595	615	KAPOOR *et al.,* 2008
APC	633, 635, 640	660	TJIOE *et al.,* 2001
Cy5	633, 635	667	KALINA *et al.,* 2012
DAPI	488	461 (DNA), 500 (RNA)	DARZYNKIEWICZ *et al.,* 2011.

Subtitle – FITC = Fluorescein isothiocyanate; PE = Phycoerythrin; 7-AAD 7-Aminoactinomicina = D; PI = propidium iodide; APC = allophycocyanin; PerCP/Cy5.5 = piperidin chlorophyll protein cyanine dye 5.5; Höchst 3342 = 2'-[4-ethoxyphenyl] -5 – [4-methyl-1-piperazinyl] -2,5 '-bi-1H-benzimidazole trihydrochloride trihydrate; Texas Red = sulforhodamine 101 acid chloride; DAPI = 4 ',6-diamidino-2-phenylindole.

This phenomenon is dependent on the type of fluorochrome and its chemical environment. The fluorochromes used in flow cytometry may be linked to antibody molecules that will bind specifically to cellular components; they may attach directly to specific cellular components in a non-covalent way or may present light emission variation depending on some microenvironment characteristics (BARNETT et al., 2013; WOOD, 2006).

The fluorochromes that perform covalent binding are those that react and are use for labeling proteins, lipids and other organic molecules. Included in this group are the isoticyanate, clorotrirzinil, succinimide esters, cyanines and Texas Red, which presents great excitement, yield, and photostability (MAECKER et al., 2004).

The fluorochromes that do not perform covalent binding to cellular structures are used as markers of DNA and RNA content, as Hoescht 33342 that binds to AT bases, DAPI (AT), DIPI (AT), chromomycyn A3 (GC), Olivomycins (GC), mithramycin (GC) and acridine orange that binds to DNA and RNA, emitting different wavelengths depending on the nucleic acid to which is attached to (COLLARD et al., 1984; DAVIS et al., 2013a).

Fluorescent markers sensitive to the microenvironment vary its excitation spectrum or emission characteristics depending on the microenvironment, and are used for the determination of functional states such as pH (6-carboxy-fluorescein, 2,3-dicyano-1, 4-dihydroxidencen, hydroxypyrene trisulfato), calcium (II quin, fura-2, Indo-1), redox potential (dichlorofluorescein, NADH, NADPH), enzymatic activity, viscosity/fluidity, and polarity (anilino-naftalina-sulfato/ANS) (NAKAGE; SANTANA, 2006; DAVIES et al., 2013b).

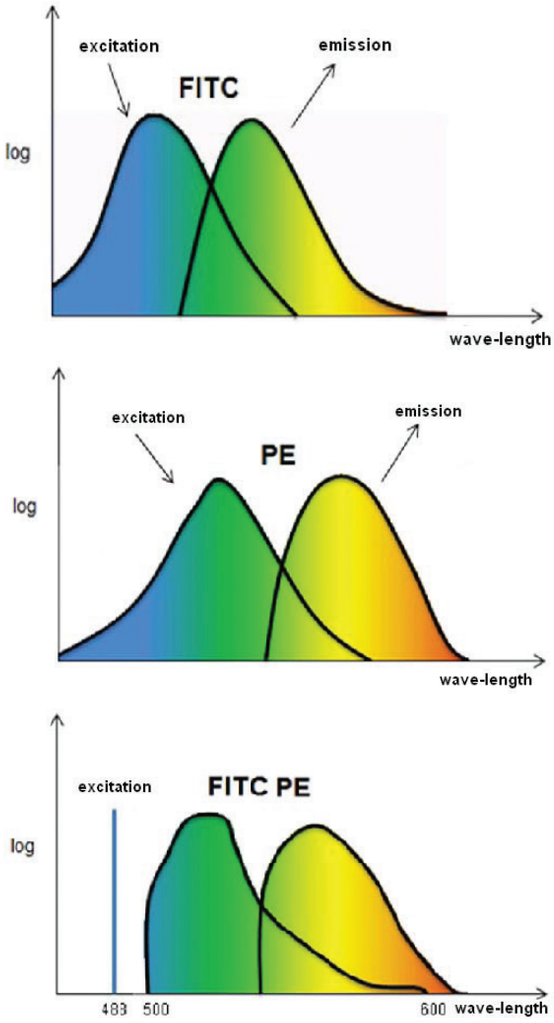
The fluorescence is increase in proportion to the increase in the number of molecules of fluorochrome added to the antibody, and to the number of molecules that can be conjugate to the antibody without substantial loss of fluorescence. This depends on the size and distance of the protein functional group in which the fluorochrome is attach. The degree of conjugation is dependent on the reactivity of the antibody with a fluorochrome, molecular weight and number of reactive amines (GOLIM et al., 2007).

Configuration of commands

When conducting an experimental test using a flow cytometer, we assume that the device is in good condition, correct laser alignment, time delay correction of the laser and its sensitivity. However, each experiment requires a specific configuration for the markers and fluorochromes used, which varies from cytometer to cytometer (analog versus digital) and use of colors (one color versus several colors).

*It should be noted that experiments with the use of several colors in which there are spectral overlap should include two elements: the gain of the device (PMT voltage) and the degree of spectral overlap, which must be corrected by calculating the compensation values dependent of tension (***Figure 2***).*

Figure 2 – *Color separation.*



Lenses *placed near this interception zone collects the scattered light that is sent to photomultiplier tubes (PMT) that converts the light signal into an electrical pulse proportional to the amount of scattered light captured by the PMT(adapted from www.cnbc.pt).*

This does not occur when the flow cytometer presents software to recalculate the compensation when the PMT voltages are modified (HERZENBERG et al., 2006).

Control standards

Control standards are stable materials that produce known or expected results, used to monitor the reproducibility and quality of the cell preparation reaction methods. Are important for aligning or calibrating flow cytometers and in control and immunofluorescence analyzes (HURLEY, 2001).

The goal of the immunofluorescence analysis is to determine cell subpopulations, and provide sensitivity and specificity to the experiment (TUNG et al., 2007). They are use to detect changes in optical configuration and/or signal, which damage the emission of fluorescence.

Flow cytometers provide relative information rather than absolute obtained from the correlation between

channels voltage with the results of known samples, and by adjusting the voltage of the PMT for peak intensity determined in a stable pattern (SHAPIRO, 2005).

As fluorochromes which have emission spectra may overlap beyond the capacity of sensitivity of filters, there is a computer system subtraction or compensation signal, which is set individually (for example, FL1, FL2, FL3-FL2, FL3-FL1) (HERZENBERG. et al., 2006).

In flow cytometry there should be three types of control, instrument controls (set up); specificity controls or biological spread and; biological controls for comparison. The instrument controls are those used to verify or create proper configuration of the instrument, either through PMT or gains of voltage/tension and compensation (HERZENBERG, 2002).

The specificity controls (gate) are use to distinguish one specific binding of non-specific binding and are used to define the location of gates or graphical regions for cell classification. In other words, they are used to determine the positivity or negativity for specific cell markers (e.g., CD3 for T lymphocytes, CD19 for B lymphocytes) (TUNG et al., 2007).

The biological controls are those which provide biologically relevant comparison conditions such as stimulated samples or samples from healthy donors (WALLER et al., 2001).

Instrument controls (set up)

Voltage Gain

The voltage gain or tension of voltage (TV) is adjusted so that the cells not stained with fluorochromes or isotype control appear in the lower left quadrant (biparametric histogram) or at the left side of a two axes graph (histogram monoparametric) with logarithmic scale for each analyzed fluorochrome (MARTI et al., 2001). The fluorescence intensity of unstained cells is close to zero, and any attempt of tension adjustment based on these channels visual placement of an unlabeled sample is difficult and subjective, and not correlated with the detection of actual signals of each channel (HERZENBERG et al., 2002).

Generally, this procedure works well, however, is not universally ideal, as for longer fluorochrome emission wavelength such as APC Cy7 and Alexa 700. Thus, it is prudent to determine the minimum tension of voltage required to ensure that each detector has a gain sufficient to boost negative signals above the underlying electronic noise (WOOD, 2006).

This initial configuration may serve as a basis for subsequent adjustments in preparing the experiment. In each fluorescence detection, the greater the average population fluorescence is, the greater the electronic noise reduction, and in the case of populations with weak labeling the opposite occurs, being the compensation performed by the mean of fluorescence population versus the applied tension (ROEDERER, 2001).

The TV decrease as the compensation gain is increase, so that the sufficient voltage tension is apply to stabilize the TV and the presence of electronic minimal noise. The adjustment of fluorescence for unstained cells or low-fluorescence is performed through the minimum tension obtained, demonstrated through the inflection point of the fluorescence curve, ensuring that the apparatus does not mislabel not stained from weakly stained events (HERZENBERG, 2002).

This phenomenon is performed in digital flow cytometry apparatuses, but not analog, since compensation of the electronic baseline circuit does not report values below zero. What can be done is a mixture of noise signal measurement (positive population median/negative population median), generating a series of inverted curves compared to the previous situation, and the inflection point of the curve in this case is the minimum voltage of resolution of higher sensitivity from the point of lowest fluorescence (MARTI et al., 2001).

The tensions of initial voltage are the starting point for the achievement of a flow cytometry experiment; the adjustments are compensate according to the fluorescent antibody panel, activated cells, marking surface or intracellular components. If positive signals are very high, the PTM tension should be reduced to bring all events into the range of detection of the equipment, since the extent of fluorescence scale below the detection range cannot be measured by the equipment, leading to error during the acquisition of positive samples.

However, if the sample has high negative scale of fluorescence, the most common is the reduction of tension voltage, bringing the negative population more to lower and left in a biparametric histogram, which may not be desirable as it may cause an increased error in measurements impacting on compensated populations (ROEDERER, 2001).

On this situation, it is advisable to search for other causes of increased measurement error, such as antibody titer, number of washes, use of isotype controls or block of Fc receptors that might be interfering with the marking (TUNG et al., 2007).

Compensation

Compensation corresponds to a mathematical process which occurs correction of multiparametric flow cytometry data in order to decrease the spectral overlap (or spillover overlap) caused by the use of fluorescent conjugates which are measurable by more than one detector (SHAPIRO, 2005).

Once the appropriate gains of fluorescence have been determined for each fluorescence channel by adjusting the voltage, it is important to adjust the compensation of channels to avoid the overlap of fluorochromes with a very close wavelength. This compensation can be accomplished through the equipment or software and is always performed after PMT tension correction, since the degree of compensation required is gain-dependent (HERZENBERG et al., 2006).

This can be achieved by using an extra source of cells which will not necessarily participate directly with the results of the experiment, which increases the cost of materials and time. Another problem to be considered are the weak markings or observed in a rare subset of cells, which makes it difficult the accuracy in the compensation to be used in antibodies against these markers (MAECKER; TROTTER, 2006).

Some researchers to work around this problem use a different antibody conjugated with the same fluorochrome originally used for the experiment as a compensating control. The use of a tri-test image as CD3/CD4/CD8 which has peculiar and characteristic CD3/CD4 and CD3/CD8, in addition to high expression of these molecules in a subset of cells, is perfectly acceptable in practice since the use of a signal which is equal to or brighter in signal intensity in test samples allows accurate calculation of compensation for a given fluorochrome (MAECKER et al., 2004).

This protocol can be used in research of leukocytes of patients with severe combined immunodeficiency (SCID), to classify the different subtypes of disease. This protocol is extensity use in our laboratory to study the SCID and others primary immunodeficiency disease.

However, this is not valid for the fluorochromes used in tandem, such as Cy7, and APC-Cy7-PE, which exhibit fluorescence variability from batch to batch, overlapping fluorescence and altered fluorescence by handling and storage (WOOD, 2006).

Therefore, the use of these fluorochrome dyes such as substitution dyes can lead to the emergence of inaccurate compensations. Another possibility would be to use beads labeled with fluorochrome, or that may be label with antibodies of user's choice labeled with fluorochrome.

The beads labeled with fluorochrome exhibit stable fluorescence emission and are used in clinical laboratories for routine compensation of equipment's or tests. But when used in tandem with APC-Cy7 and PE-Cy7 fluorochromes, it needs to be used with caution (MAECKER et al., 2004).

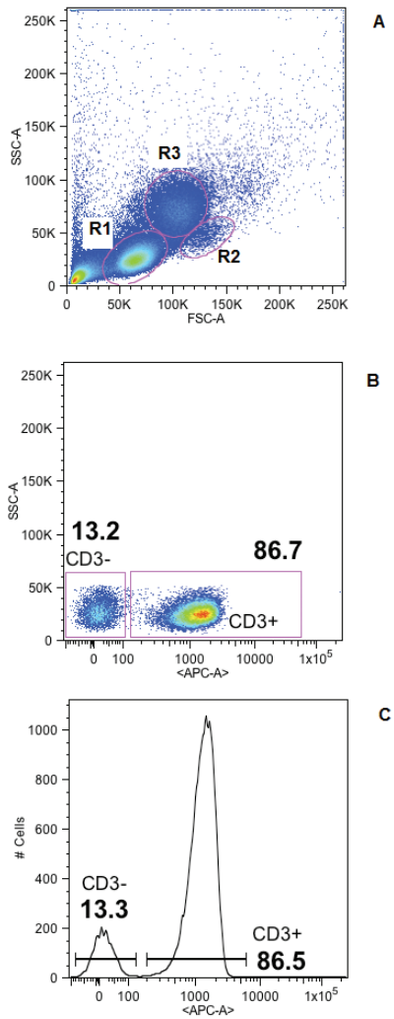
Beads which may be labeled with antibodies conjugated to fluorochromes provide a uniform fluorescent signal for each antibody used, and are safe since it uses the same class of antibody light chain and same species of origin (rat, mouse) and can be subjected to the same treatment conditions as the samples to be tested (fixation, permeabilization, change in temperature, pH, exposure to light), which validates the test (TUNG et al., 2007). This procedure is very common in measure of cytokines produced by leukocytes stimulate in vitro with polyclonal or protein antigens.

Data acquisition strategy (gate)

From the moment that the PMT tension is define and compensation is performed, experimental samples can be collected and analyzed. However, it may be necessary to formulate a strategy for the data acquisition, which includes the use of gates or acquisition gate to define positive/negative cell populations for a particular marker, or the proportion of positive cells (WOOD, 2001).

*The gate corresponds to the selection of areas to be selectively analyzed, determining the intensity of fluorescence only in cells located in this region (***Figure 3***). The population of cells from the gate corresponds to a specific fraction of the total cells present in the sample selected by the operator of the flow cytometer for acquisition or analysis. From this, it is possible to determine the percentage of cells in each strategically chosen region (HERZENBERG et al., 2006). This is important to verify the number of different subtypes of leukocytes, such mononuclear and polymorphonuclear cells in different pathological situations, like autoimmune disease, allergy, primary and secondary immunodeficiency and malignance.*

Figure 3 – *Acquisition gates. (A)*



Subtitle *-Densit (FSC/SSC) provides acquisition gates for lymphocytes (31%), monocytes (0.43%) and neutrophils (5.7%) obtained of patient with systemic lupus erythematous. (**B**) Biparametric representation CD3-APC-Cy7 (86.7%), demonstrate the percentual number of lymphocytes. (**C**) Monoparametric representation of CD3-APC-Cy7 (86.5%), demonstrate the percentual number of lymphocytes.*

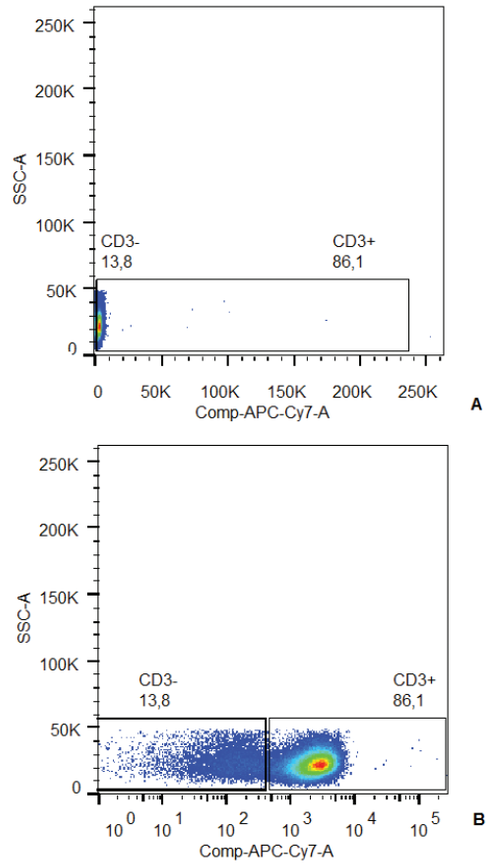
Thus, the gate is of great importance in the interpretation of data, since some gates can be draw unequivocally, without reference to any control sample. Markers with bimodal expression without overlapping positive and negative populations, such as CD4 and CD8 lymphocyte population T, do not require this control (KASINRERK, 2003).

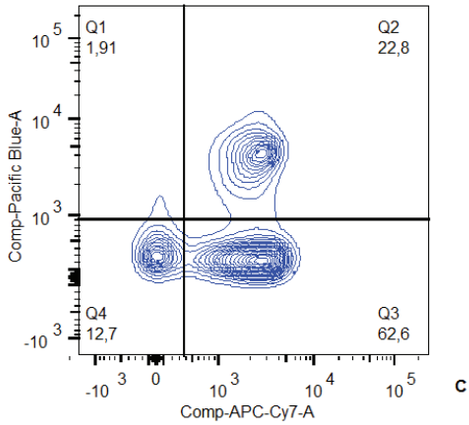
One should be careful for the marking of a small subset of cells weakly stained or with low fluorescence pattern, or in populations where there is no clear division between positive and negative populations as early markers of cell activation or cytokines. In this case, the control for these biological markers may be an unstipulated cell sample, a isotype control or the Fluorescence Minus One (FMO) strategy (MAECKER et al., 2004).

Visualization of the acquired images: linear, logarithmic and Logicle

*Images acquired by flow cytometry can be expressed in linear scale, logarithmic or logicle (***Figure 4***).*

Figure 4 – *Linear, logarithmic or logicle expression of acquired images.*





Subtitle -**(A)** *Biparametric linear representation SSC vs CD3-APC-Cy7 cells (86.1%). This image was obtaine from densit plot, with initial gate of lymphocytes population. Note the truncate figure of cells in image.*

(B) *Biparametric logarithmic representation SSC vs CD3-APC-Cy7 cells (86.1%). With this kind of acquisition, lymphocytes obtained from densit plot, are separate in positive (Q2 and Q3) and negative cell marker.*

(C) *Biparametric logicle representation Pacific Blue vs APC-Cy7. In this protocol, separation of cells is easier to visualize, when compared with biparametric linear representation. Q1 = Pacific Blue (1.91%); Q2 = Pacific Blue-APC-Cy7 (22.8%); Q3 = APC-Cy7 (62.6%); Q4 = Pacific blue/APC-Cy7 negative (12.7%).*

The linear scale has been use for years for visualization, data collection and analysis. However, this type of image does not separate populations of interest very well in density plot (FSC versus SSC), whereas logarithmic scale defines better these populations (lymphocytes, neutrophils and monocytes), in addition is able to separate dead cells and debris (MUIRHEAD; SCHMITT; MUIRHEAD, 1983).

The logarithmic scale cannot be used to correctly represent the values of cells whose fluorescence is below zero, as result of the subtracting background and fluorescence compensation. To correct this, there is the logicle scale (biexponential) which approximates the normal logarithmic scale at the high and at the same time allows data to be viewed close to zero in linear scale, allowing the visualization of the data below zero (data subtraction) (GRATAMA et al., 1988).

In this type of compensation, over-compensation centralizes the distribution of these cells below the level of autofluorescence, while the sub-compensation centers the distribution above zero (TUNG et al., 2007).

Specificity control and biological propagation

Isotype control

Flow cytometry is a technique that allows to us verifies physical-chemical characteristics of cells or particles suspended in a fluid medium. In this technique monoclonal

antibodies conjugated to fluorochromes are used, which requires isotype control for defining negative regions (background) (MAECKER et al., 2004).

These isotype controls consist of the same immunoglobulin's isotype, light chain antibody and fluorochrome used in the test, and serves to define nonspecific fluorescence (negative cells) and regions of fluorescence (positive cells)(NUNEZ, 2001).

Nonspecific fluorescence can occur as the product of the absorption of antibody to the cell surface without occurring antigen-antibody specific reaction (either via binding Fab or Fc), which is considered a false event. In order for this to not occur, it is necessary to eliminate the nonspecific interference evaluating the samples with the isotype control, an antibody of the same tested antibody isotype, labeled with the same fluorochrome, but that is not specific to the antigenic sites of test sample (Figure 5) (TUNG et al., 2007).

Thus, the fluorescence emitted by this control does not recognize any of the cell surface molecules of the sample under analysis, allowing the establishment of the background, where values lower than this are not recognized or nonspecific. This non-specificity may also be diminished by Fc site blocking before performing the specific staining (MAECKER et al., 2004; TUNG et al., 2007).

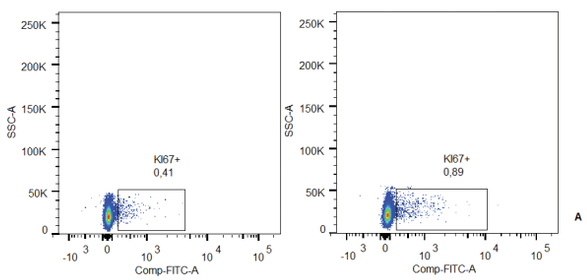
The limitations for the use of isotype control are the various levels of background for each individual antibody, according to its specificity, concentration, degree of aggregation and proportion of antibody and fluorochrome. Thus, for each particular antibody an appropriate isotype control is required (SHAPIRO, 2005).

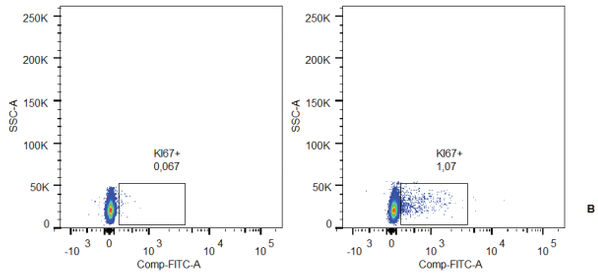
Another observed problem is that the isotype controls do not take into account the fluorescence exhaust of other channels, which may be overcome by including all relevant antibodies on other channels together with the antibody isotype for each channel of interest, which increases the number of cells tested and time to perform the experiment (HERZENBERG et al., 2006).

Fluorescence Minus One (FMO)

*Besides compensating controls for staining and isotype control, there is the Fluorescence Minus One (FMO) (***Figure 5***), which is a way to identify limits of propagation, coloring problems and not marked controls, which can be confused with the background or autofluorescence of the system used in the analysis (TUNG et al., 2007).*

Figure 5 – *Isotype and FMO.*





Subtitle *– **(A)** The figure on the left shows analysis of the sample of peripheral blood mononuclear cells (PBMC) not stimulated with mitogen (PHA), with absence in the expression of marker proliferation (Ki67) and labeled with FITC, and the figure on the right shows analysis of the sample stimulated with mitogen (PHA), with expression of Ki67 and labeled with FITC, using FMO gate for analysis.*

(B) *The figure on the left shows analysis of the sample not stimulated with mitogen (PHA), with absence in the expression of marker proliferation (Ki67) and labeled with FITC, and the figure on the right shows analysis of the sample stimulated with mitogen (PHA), with expression of proliferation marker Ki67, using isotype gate for analysis.*

In FMO control, all colors except one is used, for example, a control FMO FITC contains all fluorochromes except FITC. Since isotype controls do not have propagation controls, since each antibody has specific properties and non-specific binding, the isotype controls are good to circumvent problems of coloring, particularly if primary and secondary antibodies are used (MAECKER et al., 2004).

However, isotype control do not identify with certainty what is negative and what is positive when there is a large amount of fluorochrome-conjugated antibody, which tends to show a low background staining (SHAPIRO, 2005).

In experiments with more than four colors, the main source of coloration tends to be the escape of fluorescence. The FMO provides a means of measuring the effects of overlapping fluorescence of populations in a particular experiment channel (TUNG et al., 2007).

Comparison of biological controls

Although some researchers believe that it is possible to perform a test of flow cytometry without the presence of specificity control as an isotype control, there are biological controls that are most appropriate for establishing the threshold of positivity/negativity (MAECKER; TROTTER, 2006).

In assays using stimulated cells, unstimulated cells can be used as control to discern positive from negative events being more relevant than an isotype control or FMO, since the level of background subtraction is determined more accurately (ROEDERER, 2004). Various examples can be cited, for example, PBMC not stimulated with mAb anti-CD3, anti-CD28 (MARTI et al., 2001), dendritic cells not stimulated with cytokines (ALAWIEH et al., 2009), and intracellular parasite kill (KRAM et al.,2008).

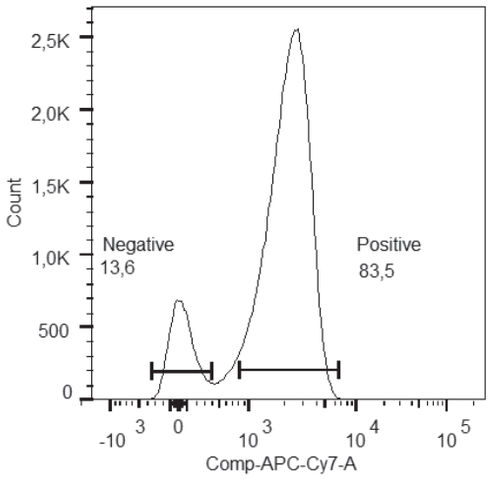
This protects the test from misinterpretation of negative data where a reagent is not added or if the cells were not viable (MAECKER et al., 2004).

Processing and analysis

The information obtained after the passage of samples in a flow cytometer may be stored in the form of histograms (monoparametric histograms 256 or 1024 channels; biparametric histograms 64x64) or in List mode, which consists in storing all the information obtained for each event so that one can modify the regions of analysis and reconstruct the histograms in function of new selection parameters (BERNAS et al., 2008).

*For univariable data monoparametric histograms are recommended (***Figure 6***), which calculates the percentage of events in selected regions, taking into account the resolution of the histogram for the discrimination of signal and voltage channel. The great advantage of this procedure is absence of more than use one cell marker. If the choose of gate is not correct, the monoparametric histogram not demonstrate this (MARTI et al., 2001).*

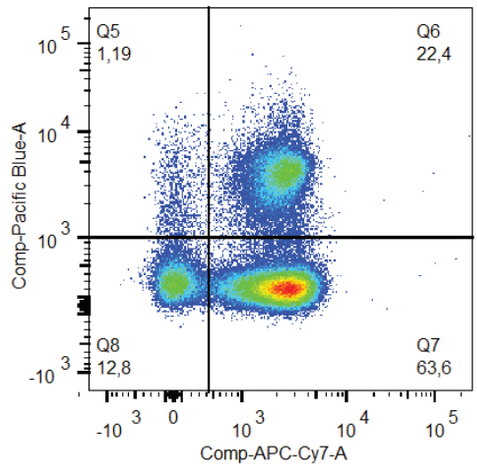
Figure 6 – *Monoparametric graph or histogram.*



Subtitle -Monoparametric representation of labeled cells with APC-Cy7. Image obtained from densit plot (FSC/SSC) with single marker APC-Cy7. With this protocol, is possible the visualization of only one marker in cell population. On the left, negative (13.6%), and right, positive population (83.5%).

*For this problem, the bivariable data (dot plot or histogram biparametrics) is better, because results are plotted as biparametric histograms with two axes and the numbers of events are represented as dot density, color or isometric lines (***Figure 7***) (RONGEAT et al., 2011). If the gate is not correct, the figure of biparametric representation is truncate (WOOD, 2006).*

Figure 7 – *Biparametric graph or dot plot.*



Subtitle -Biparametric representation of labeled lymphocytes obtained from densit plot (FSC/SSC), marked with Pacific blue and APC-Cy7. With this protocol, is possible the visualization of cell population with double marker. In Q5= Pacific blue (1.19%); Q6= Pacific blue/APC-Cy7 (22.4%); Q7= APC-Cy7 (63.6%); Q8= Pacific blue/APC-Cy7 negative cells marker (12.8%).

Use

Flow cytometry is a technique that simultaneously analyzes, mark and separates (sorts) cells of interest from small blood samples (WOOD et al., 2007) and other biological fluids for human diseases diagnostic confirmation (CURTIS; WALKER; DENNY, 2011), and in studies with animal models (HENSELEIT et al., 2012).

Through this assay, cells can be studied for expression of several surface molecules, in the identification of human naive T cells by phenotype, function, and T-cell receptor diversity (DE ROSA et al., 2001) or phenotyping leukocytes in primary and secondary immunodeficiency's (OLIVEIRA; FLEISHER, 2010).

Can be used to check cellular proliferation and in vitro cytotoxicity (WANG; ZHENG, 2002) or permeabilized for the quantification of intracellular components such as cytokines, hormones and other molecules produced after stimulation of cells in vitro, important in the development of basic and applied research (PALA; HUSSELL; OPENSHAW, 2000; FREER, RINDI, 2013).

Its applications have been described in DNA content analysis, karyotyping and genome research in vegetal models to estimation of nuclear genome size (DOLEZEL; BARTOŠ, 2005) and animal study of pluripotent cells to development of therapeutic protocols (NAKAGE; SANTANA, 2006).

Flow cytometry technique is important in neonatal and postnatal diagnosis of diseases, favoring the implantation of early treatment and possible cure of diseases (CURTIS; WALKER; DENNY, 2011); and applications to diagnostic hematopathology, such as pancytopenia and lymphocytosis (DUNPHY, 2004).

The use of technique is relevant in the quantification and analysis of lymphocyte subpopulations (CURTIS; WALKER; DENNY, 2011); reticulocyte quantification, monitoring and diagnosis of leukemia/lymphoma, multiple myeloma, myelodysplasia and myeloproliferative diseases (CRAIG; FOON, 2008); quality control of leukodepletion (BRATOSIN et al., 2001); chemotherapy and minimal residual disease monitoring (AL-MAWALI; GILLIS; LEWIS, 2009). The results obtained are important to check the efficacy of treatment, retreatment and research of minimal residual disease in different malignances (AL-MAWALI; GILLIS; LEWIS, 2009).

The flow cytometry technique can be use in the quality control of leukodepletion, important in patients with risk development of concomitant anemia, or to verify the platelet count and/or dysfunction by genetic cause (Glanzmann's thrombasthenia, Bernard-syndrome Sollier) (BRATOSIN et al., 2001; CURTIS; WALKER; DENNY, 2011; ISRAELS, 2015).

The quantification of hematopoietic stem cells ($CD34^+$) in bone marrow aspirates for cellular therapy, aphaeresis and umbilical cord products are check by the flow cytometry technique, important to the efficacy of reconstitution of X-SCID recipients after in utero transplantation of lymphoid-primed multipotent progenitors (LIUBA et al., 2009).

The evaluation of immune system of transplanted individuals and lymphocyte infusion, HLA and cross match testing (ALAWIEH et al., 2009), and analysis of primary and secondary immunodeficiency's (CURTIS; WALKER; DENNY, 2011), is dependent of accurate technique, since diagnosis, treatment and development of better life quality of patients.

The application of flow cytometry technique in clinical pathology expanded the spectrum diagnosis, before limited to histopathology evaluation, using peripheral blood cells, pleural effusion and cavity fluids (VIRGO; GIBBS, 2012).

The use can be extended to detection of parasitic diseases, such leishmania, normality reserved to serological or histopathologic diagnosis (KRAM et al., 2008); or apoptosis of lymphocyte T CD4 by HIV infection (LECOEUR et al., 2008).

The use can be extended to verify the toxicity due to nanomaterials TiO_2 in cells (ZUCKER et al., 2010) and heavy metals on Acinetobacter sp. with potential for bioremediation of contaminated wastewaters (BOSWELL; HEWITT; MACASKIE, 1998); multiple drug resistance of parasites such Leishmania amazonensis associated with overexpression of a Leishmania mdr1 gene (GUEIROS et al., 1995) and antibiotic susceptibility testing of different bacteria (PORE, 1994) or bacterial analysis (NEBE-VON-CARON et al., 2000).

The technique can be use to detection of fusion proteins for the introduction of new targeted therapies of diseases like specific leukemia (WEERKAMP et al., 2009); and selective cloning of hybridoma cells for enhanced immunoglobulin production (MARDER et al., 1990), biotechnology applied to microbiology (RIESEBERG et al., 2001); and animal sperm sexing technology to genetic improvement, before restrict to genetic crossing (MAXWELL et al., 2004). The flow cytometry, associate a cytogenetic and molecular markers enhance the genetic variation of vegetable with commercial use to industries, for example, the cotton to vestments industries (JIN et al., 2008).

Also, flow cytometry plays an important role in elucidating the processes related to differentiation and maturation of a particular cell line and expression of their functions in different stages of maturation of leukocytes in ocular disease (KIM et al., 2012).

Conclusions

Flow cytometry is a technique able to analyze the physical and chemical properties of microscopic particles in suspension in a liquid medium. This technique is commonly used in different area of basic and applied research. The major problem in flow cytometry is weather a subset of cells labeled with fluorochrome-conjugated monoclonal antibodies is positive or negative, and if this proportion of positive or negative cells for a given marker is reliable. The choice of these populations can follow subjective parameters, which often make the results invalid or misinterpreted. To avoid inaccurate results the correct adjustments of the detectors are mandatory, so that the compensation gains are fairly fixed for better resolution of sensitivity. The compensation gain must be properly determined and applied to avoid false or ambiguous results. The controls should be carried out in a concise manner, especially when labeling has no bimodal distribution. In our laboratory experience, this can be accomplished by the adoption of a biological control, isotype control or FMO. None of these controls is considered ideal, and should be selected according to the number of labelings made, rarity of expressed molecule on the surface or intracellular within certain cell subsets, overlap of wavelengths or unspecific binding of the fluorochrome-conjugated antibodies.

This implicates in carrying out many pilot experiments and controls to determine the positive and negative populations. The judgment of which is the best choice must be done individually in each case. We should keep in mind that the results, whether in basic or applied research, seek to answer relevant questions or new diagnostic and therapeutic options. Due to its great potential, the use of flow cytometry has been expanded to diverse fields of biological sciences and various health areas and is routinely used in clinical diagnostics, biotechnology, and basic and applied research.

REFERENCES

1. ADLER N. E.; SCHMITT-JANSEN M.; ALTENBURGER R. Flow cytometry as a tool to study phytotoxic modes of action. **Toxicol. Environ. chem**., New York, v. 26, n.2, p.297-306, Feb. 2007.

2. ALAWIEH, M. et al. Injection of donor-derived OX62+ splenic dendritic cells with anti-CD4 monoclonal antibody generates CD4+ CD25+ FOXP3+ regulatory T cells that prolong allograft skin survival indefinitely and abrogate production of donor-specific antibodies in a rat model. **Transplant. proc**.,New York, v.41, n. 8, p. 3363-3366, Oct. 2009.

3. AL-MAWALI, A.; GILLIS, D.; LEWIS, I. The role of multiparameter flow cytometry for detection of minimal residual disease in acute myeloid leukemia. **Am. j. clin. pathol**., Baltimore, v. 131, n. 1, p. 16-26, 2009.

4. BARNETT, D. et al. Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS – part IV – postanalytic considerations. **Cytometry, Part B. Clin. cytom**., HoboKen, n. 84, n. 5, p.309-314, Sept.2013.

5. BAUMGARTH, N.; ROEDERER, M. A practical approach to multicolor flow cytometry for immunophenotyping. **J. immunol. methods,** Amsterdan,v. 243, n. 1, p. 77-97, 2000.

6. BERNAS, T. et al. Quadratic form: a robust metric for quantitative comparison of flow cytometric histograms. **Cytometry,** New York, v.73, n.8, p.715-726, 2008.

7. BOSWELL, C. D.; HEWITT, C. J.; MACASKIE, L. E. An application of bacterial flow cytometry: evaluation of the toxic effects of four heavy metals on Acinetobacter sp. with potential for bioremediation of contaminated wastewaters. **Biotechnol. lett.,** Dordrecht, v. 20, n. 9, p. 857-863, 1998.

8. BRATOSIN, D. et al. Improved storage of erythrocytes by prior leukodepletion: flow cytometric evaluation of stored erythrocytes. **Cytometry**, New York, v. 46, n. 6, p. 351-356, 2001.

9. CARTER, A.; CROSLAND-TAYLOR, P. J.; STEWART, J. W. Measurement of mean corpuscular and packed cell volumes with a Coulter cell counter. **J. clin. pathol.**, London, v. 21, n. 2, p. 222, 1968.

10. CASPERSSON, O. Quantitative cytochemical studies on normal, malignant, premalignant and atypical cell populations from the human uterine cervix. **Acta cytol.,** Baltimore,v. 8, p. 45-60, Jan./Feb.1964.

11. COLLARD, J. G. et al. Separation and analysis of human chromosomes by combined velocity sedimentation and flow sorting applying single-and dual-laser flow cytometry. **Cytometry**, New York, v. 5, n. 1, p. 9-19, 1984.

12. CRAIG, F. E.; FOON, K. A. Flow cytometric immunophenotyping for hematologic neoplasms. **Blood**, v. 111, n. 8, p. 3941-3967, Apr.2008.

13. CROSLAND-TAYLOR, P.; STEWART, J. W.; HAGGIS, G. An electronic blood-cell-counting machine. **Blood**, v. 13, n. 4, p. 398-409, 1958.

14. CURTIS, M. G.; WALKER, B.; DENNY, T. N. Flow cytometric methods for prenatal and neonatal diagnosis. **J. immunol. methods.**, Amsterdam, v. 363, n. 2, p. 198-209, Jan. 2011.

15. DARŻYNKIEWICZ, Z. et al. Conformation of RNA in situ as studied by acridine orange staining and automated cytofluorometry. **Exp. cell. res.**, New York, v. 95, n. 1, p. 143-153, 1975.

16. DARZYNKIEWICZ, Z. et al. Cytometry of DNA replication and RNA synthesis: Historical perspective and recent advances based on "click chemistry". **Cytometry, Part A**., Hoboken, v. 79, n.5, p.328-337, May. 2011.

17. DAVIS, B. H. et al. ICSH/ICCS WORKING GROUP. Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS – part II – preanalytical issues. **Cytometry, Part B Clin. cytom**., Hoboken, v.84, n. 5, p.286-290, Sept./oct.2013a.

18. _____. Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS – part I – rationale and aims. **Cytometry, Part B Clin. cytom**., Hoboken, v.84, n. 5, p. 282-285, 2013b.

19. DEGHEIDY, H. et al. Consistent, multi-instrument single tube quantification of CD20 in antibody bound per cell based on CD4 reference. **Cytometry, Part B Clin. cytom**., Hoboken, v. 26, May. 2015.

20. DE ROSA, S. C. et al. 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. **Nat. med.,** New York, v. 7, n. 2, p. 245-248, 2001.

21. DITTRICH, W.; GÖHDE, W. [Impulse fluorometry of single cells in suspension]. **Z.Naturfosche B**., São Paulo, v. 24, n. 3, p. 360-361, Mar.1969.

22. DOLEZEL, J; BARTOŠ, J. Plant DNA flow cytometry and estimation of nuclear genome size. **Ann. bot.,** London, v. 95, n. 1, p. 99-110, Jan. 2005.

23. DUNPHY, C. H. Applications of flow cytometry and immunohistochemistry to diagnostic hematopathology. **Arch. pathol. lab. med.**, Chicago, v. 128, n. 9, p. 1004-1022, Sept. 2004.

24. FREER, G.; RINDI, L. Intracellular cytokine detection by fluorescence-activated flow cytometry: basic principles and recent advances. **Methods**, v.15, n. 61, p.30-38, may. 2013.

25. GOLIM, M. A. et al. Conjugation and validation of IgG1-FITC isotype control to be used in flow cytometry. **Rev. bras. hematol. hemoter**., São Paulo,. v. 29, n. 4, p. 361-368, 2007.

26. GRATAMA, J. W. et al., Flow cytometric quantitation of immunofluorescence intensity: problems and perspectives. European Working Group on Clinical Cell Analysis. **Cytometry,** New York, v. 33, n. 2, p.166-178, 1988.

27. GRATZNER, H. G. et al. The use of antibody specific for bromodeoxyuridine for the immunofluorescent determination of DNA replication in single cells and chromosomes. **Exp. cell res.**, New York, v. 95, n. 1, p. 88-94, 1975.

28. GREEN, R.; WACHSMANN-HOGIU, S. Development, history, and future of automated cell counters. **Clin. lab. med**., Philadelphia, v. 35, n.1, p.1-10, Mar. 2015.

29. GUEIROS, F. J. et al. Leishmania amazonensis: multidrug resistance in vinblastine-resistant promastigotes is associated with rhodamine 123 efflux, DNA amplification, and RNA overexpression of a Leishmania mdr1 gene. **Exp. parasitol.**, New York, v. 81, n. 4, p. 480-490, Dec. 1995.

30. HARDY, R. R. et al. B-cell subpopulations identifiable by two-color fluorescence analysis using a dual-laser FACS. **Ann. N. Y Acad. sci.**, New York, v. 399, n. 1, p. 112-121, 1982.

31. HEDLEY, D. W. et al. Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. **J. histochem. cytochem.**, v. 31, n. 11, p. 1333-1335, Nov. 1983.

32. HENSELEIT, K. D. et al. Characterizing the Phenotype of Murine Epidermal Progenitor Cells: complementary Whole-Mount Visualization and flow cytometry strategies. **Methods mol. biol.,** Totowa, p. 243-261, 2012.

33. HERZENBERG, L. A. et al. Interpreting flow cytometry data: a guide for the perplexed. **Nat. immunol**., New York, v. 7, n. 7, p. 681-685, July. 2006.

34. _____. The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. **Clin. chem.**, New York, v. 48, n. 10, p. 1819-1827, Oct. 2002.

35. HOFFMAN, R. A. *et al.* Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood. **Proc. Natl. Acad. Sci. U. S. A**., Washington, v. 77, n. 8, p. 4914-4917, Aug. 1980.

36. HORST, W. R. **Method of simultaneously counting red and white blood cells US Patent 2,875,666**. Google Patents, 1959. Disponível em: <http://www.google.com/patents/US2875666>. Acesso em: 20 Nov. 2014.

37. HURLEY A. A. Principles of quality control. **Curr. Protoc. Cytom**., Chapter, v. 3, n.1, p.3, May. 2001.

38. IBÁÑEZ-PERAL, R. et al., Potential use of quantum dots in flow cytometry. **Int J. Mol. Sci.,** v. 9, n. 12, p. 2622-2638, Dec. 2008.

39. ISRAELS, S. J. Laboratory testing for platelet function disorders. **Int. j. lab. hematol**., Oxford, v.37, Supl. 1, p.18-24, May. 2015.

40. JIN, S. et al. Detection of somaclonal variation of cotton (Gossypium hirsutum) using cytogenetics, flow cytometry and molecular markers. **Plant cell rep**., Berlin, v. 27, n. 8, p. 1303-1316, Aug. 2008.

41. JOCHEM, F. J. Short-term physiologic effects of mechanical flow sorting and the Becton-Dickinson cell concentrator in cultures of the marine phytoflagellata Emiliania huxleyi and Micromonas pusilla. **Cytometry, Part A**., Hoboken, v. 65, n. 1, p. 77-83, May. 2005.

42. KALINA, T. et al. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. **Leukemia**. n. 26, p.1986-2010, 2012.

43. KAMENTSKY, L. A.; MELAMED, M. R.; DERMAN, H. Spectrophotometer: new instrument for ultrarapid cell analysis. **Science,** v. 150, n. 3696, p. 630-631, Oct. 1965.

44. KAPOOR, V. et al. Solid state yellow and orange lasers for flow cytometry. **Cytometry** A. v.73, n. 6, p.570-577, 2008.

45. KASINRERK, W. A flow cytometric method for enumeration of lymphocyte sub-populations in sample containing lysis-resistant red blood cells. **Immunol. lett**., Amsterdam, v. 86, n. 3, p.259-264, May. 2003.

46. KIM, T. W. et al. Maturation profiles of peripheral blood dendritic cells in patients with endogenous uveitis. **Immunol. lett.,** Amsterdam, v. 142, n. 1/2, p. 14-19, Feb. 2012.

47. KRAM, D. et al. Intracellular parasite kill: flow cytometry and NO detection for rapid discrimination between anti-leishmanial activity and macrophage activation. **J. immunol. methods.**, Amsterdam, v. 333, n. 1/2, p. 79-88, Apr. 2008.

48. KUREC, A. Flow cytometry: principles and practices. **Med lab. obs**., Oradell, v. 46, n.5, p. 30-31, may. 2014.

49. LECOEUR, H. et al. Chapter Three Analysis of Apoptotic Pathways by Multiparametric Flow Cytometry: application to HIV Infection. **Methods enzymol.**, New York, v. 442, p. 51-82, 2008.

50. LIUBA, K. et al. Polyclonal T-cell reconstitution of X-SCID recipients after in utero transplantation of lymphoid-primed multipotent progenitors. **Blood,** v. 113, n. 19, p. 4790-4798, may. 2009.

51. LOKEN, M. R.; PARKS, D. R.; HERZENBERG, L. A. Two-color immunofluorescence using a fluorescence-activated cell sorter. **J. histochem. cytochem**., Baltimore, v. 25, n. 7, p. 899-907, July. 1977.

52. MAECKER, H. T. et al. Selecting fluorochrome conjugates for maximum sensitivity. **Cytometry, Part A**., Hoboken, v. 62, n. 2, p. 169-173, Dec. 2004.

53. MAECKER, H. T.; TROTTER, J. Flow cytometry controls, instrument setup, and the determination of positivity. **Cytometry, Part A**., Hoboken, v. 69, n. 9, p. 1037-1042, Sept. 2006.

54. MARDER, P. et al. Selective cloning of hybridoma cells for enhanced immunoglobulin production using flow cytometric cell sorting and automated laser nephelometry. **Cytometry**, New York, v. 11, n. 4, p. 498-505, 1990.

55. MARTI, G. E. et al. **Introduction to flow cytometry**. WB Saunders, 2001. p.93-99.

56. MAXWELL, W. M. C. et al. Integration of sperm sexing technology into the ART toolbox. **Anim. reprod. sci.,** Amsterdam, v. 82/83, p. 79-95, July. 2004.

57. MELLORS, R. C.; SILVER, R. A microfluorometric scanner for the differential detection of cells; Application to exfoliative cytology. **Science**. v. 114, n. 2962, p. 356-360, Oct. 1951.

58. MENDELSOHN, M. L. The Two-Wavelength Method of Microspectrophotometry II. A Set of Tables to Facilitate the Calculations. **J. biophys. biochem. cytol.,** New York, v. 4, n. 4, p. 415-424, July.1958.

59. MERRILL, J. T.; DEAN, P. N.; GRAY, J. W. Investigations in high--precision sorting. **J. histochem. cytochem.**, Baltimore, v. 27, n. 1, p. 280-283, 1979.

60. MOLDAVAN, A. Photo-electric technique for the counting of microscopical cells. **Science,** v. 80, n. 2069, p. 188-189, Aug. 1934.

61. MUIRHEAD, K. A.; SCHMITT, T. C.; MUIRHEAD, A. R. Determination of linear fluorescence intensities from flow cytometric data accumulated with logarithmic amplifiers. **Cytometry,** New York, v. 3, n. 4, p.251-256, Jan. 1983.

62. NAKAGE, A. P. M.; SANTANA, A. E. Células-tronco hematopoéticas em cães. **Ciênc. rural.,** Santa Maria, v. 36, n. 1, p. 325-329, 2006.

63. NEBE-VON-CARON, G. et al. Analysis of bacterial function by multi--colour fluorescence flow cytometry and single cell sorting. **J. microbiol. methods,** Amsterdam, v. 42, n. 1, p. 97-114, Sept. 2000.

64. NUNEZ, R. Flow cytometry: principles and instrumentation. **Curr. issues mol. biol (Print).,** Wymondham, v. 3, n. 2, p. 39-45, Apr. 2001.

65. OLIVEIRA, J. B.; FLEISHER, T.A. Laboratory evaluation of primary immunodeficiencies. **J. allergy clin. immunol**., St. Louis, v.125, p.297-305, 2010.

66. PALA, P.; HUSSELL, T.; OPENSHAW, P. J. Flow cytometric measurement of intracellular cytokines. **J. immunol. methods**., Amsterdam, v. 243, n. 1/2, p. 107-124, Sept. 2000.

67. POZAROWSKI, P.; HOLDEN, E.; DARZYNKIEWICZ, Z. Laser Scanning Cytometry: principles and applications. **Methods mol. biol**., Totowa, v. 391, p.165-192, 2006.

68. _____ . Laser scanning cytometry: principles and applications-an update. **Methods mol. biol.,** Totowa, v. 931, p.187-212, 2013.

69. PORE, R. S. Antibiotic susceptibility testing by flow cytometry. Journal of Antimicrobial **Chemotherapy,** Basel, v. 34, n. 5, p. 613-627, 1994.

70. PREIJERS, F. W. et al., The new violet laser dye, Krome Orange, allows an optimal polychromatic immunophenotyping based on CD45-KO gating. **J. immunol. Methods**., Amsterdam, v. 372, n.1/2, p.42-51, 2011.

71. RIESEBERG, M. et al. Flow cytometry in biotechnology. **Appl. microbiol. biotechnol**., Berlin, v. 56, n. 3/4, p. 350-360, 2001.

72. ROEDERER, M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. **Cytometry**, New York, v. 45, n. 3, p. 194-205, Nov. 2001.

73. ROEDERER, Mario et al. Flow cytometric analysis of vaccine responses: how many colors are enough?. **Clin. immunol.**, Orlando, v. 110, n. 3, p. 199-205, 2004.

74. RONGEAT, N. et al. Automatic cytometric device using multiple wavelength excitations. **J. biomed. opt.,** v. 16, n. 5, p. 057006, May. 2011.

75. SALZMAN, G. C. et al. Cell classification by laser light scattering: identification and separation of unstained leukocytes. **Acta cytol**., Baltimore, v. 19, n. 4, p. 374-377, July./Aug. 1975.

76. SALZMAN, G.C. Light scatter: detection and usage. **Curr. Protoc. Cytom**., Chapter, v. 1, p. 1-13, May. 2001.

77. SHAPIRO, H.M. **Practical flow cytometry.** Wiley – Liss, 2005.

78. _____ . Scanning laser cytometry. **Curr. Protoc. Cytom.** Chapter, v. 2, p. 2-10, May. 2004.

79. TELFORD, W.G. Near infrared lasers in flow cytometry. **Methods**, v. 82, p. 12-20, July, 2015.

80. THORELL, B.; KOHEN, E.; KOHEN, C. Metabolic rates and intercellular transfer of molecules in cultures of human glia and glioma cells. **Med. biol.**, Helsinki, v. 56, n. 6, p. 386-392, 1978.

81. TJIOE, I. et al. Phycoerythrin-allophycocyanin: a resonance energy transfer fluorochrome for immunofluorescence. **Cytometry**, New York, v. 44, n. 1, p. 24-29, May, 2001.

82. TUNG, J. W. et al. Modern flow cytometry: a practical approach. **Clin. lab. med.,** Philadelphia, v. 27, n. 3, p. 453-468, Sept. 2007.

83. VALET, G. et al. Flow cytometry as a new method for the measurement of electrophoretic mobility of erythrocytes using membrane charge staining by fluoresceinated polycations. **J. histochem. cytochem**., Baltimore, v. 27, n. 1, p. 342-349, 1979.

84. VAN DILLA, M. A.; FULWYLER, M. J.; BOONE, I. U. Volume distribution and separation of normal human leucocytes. **Exp. biol. med.,** Basel, v. 125, n. 2, p. 367-370, June. 1967.

85. VIRGO, P. F.; GIBBS, G. J. Flow cytometry in clinical pathology. **Ann. clin. biochem.,** London, v. 49, n. 1, p. 17-28, 2012.

86. VISSER, J. W.; JONGELING, A. A.; TANKE, H. J. Intracellular pH-determination by fluorescence measurements. **J. histochem. cytochem.,** Baltimore, v. 27, n. 1, p. 32-35, 1979.

87. WALLER, A. et al. Validation of flow cytometric competitive binding protocols and characterization of fluorescently labeled ligands. **Cytometry**, New York, v. 45, n. 2, p. 102–114, 2001.

88. WANG, YAN-YI.; ZHENG, XIAO-XIANG. A flow cytometry-based assay for quantitative analysis of cellular proliferation and cytotoxicity in vitro. **J. immunol. methods.**, Amsterdam, v. 268, n. 2, p. 179-188, 2002.

89. WEERKAMP, F. et al. Flow cytometric immunobead assay for the detection of BCR–ABL fusion proteins in leukemia patients. **Leukemia,** New Jersey, v. 23, n. 6, p. 1106-1117, 2009.

90. WOOD, B. 9-color and 10-color flow cytometry in the clinical laboratory. **Arch. pathol. lab. med**., Chicago, v. 130, n. 5, p. 680-690, May. 2006.

91. WOOD, B. L. et al. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: optimal reagents and reporting for the flow cytometric diagnosis of hematopoietic neoplasia. **Cytometry, Part B Clin. cytom**., Hoboken, v. 72, n. 1, p. 14-22, 2007.

92. WOOD, J. C. Principles of gating. **Curr. Protoc. Cytom**., Chapter, v. 1, p. 1-8, May. 2001.

93. ZHU, H.; OZCAN, A. Opto-fluidics based microscopy and flow cytometry on a cell phone for blood analysis. **Methods mol. biol**. Totowa, n. 1256, p.171-190, 2015.

94. ZUCKER, R. M. et al. Detection of TiO2 nanoparticles in cells by flow cytometry. **Cytometry, Part A.**, Hoboken, v. 77, n. 7, p. 677-685, 2010.

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