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FLOW CYTOMETRY REAGENTS

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FLOW CYTOMETRY



FLOW CYTOMETRY

1. INTRODUCTION TO FLOW CYTOMETRY.

Flow cytometry is undoubtedly one of the techniques that has undergone the greatest development in recent years. Using this technique it is possible to advance in the classification, diagnosis and treatment of many diseases.

The technique, which first appeared in the sixties of the last century for counting cell populations in suspension, is currently one of the methods most widely used for the analysis of cellular antigens, cell cycle studies, the separation of cell subsets, the determination of ploidy and also for studies of different cellular parameters, such as the concentration of intracellular calcium, intracellular pH, apoptosis and the uptake of thymidine analogues. Flow cytometry provides multiparametric data concerning the physical and chemical characteristics of cells in suspension.

The development of monoclonal antibodies and fluorochromes, together with the current excellence of flow cytometry, has allowed the technique to be used in clinical diagnosis.

2. THE FLOW CYTOMETER

The flow cytometer is a device able to measure the components and properties of cells and cellular organelles in suspension. The cells are conjugated with antibodies and excited with the laser beam generating fluorescent signals that allows different cellular subsets to be identified in a sample by their size and their granularity. This technique is widely used in the immunophenotyping of acute leukemia and lymphoproliferative syndromes.

THE COMPONENTS OF A FLOW CYTOMETER

A flow cytometer has three main systems:

The sample injection system

This system provides a stream of hydrodynamic fluid through a differential pressure between the sample container and the container of fluid. Normal flow rates range between 12 and 60 mL/minute. Both the sample fluid and the **enveloping** fluid enter into contact in the flow chamber, where a laminar flow is generated that drags the cells along at a rate between 1×10^3 and 1×10^6 cells per minute (currently there are cytometers under development that are able to analyse up to 1×10^5 events per second).

The optical system

This consists of a laser, lenses and detectors. The light source is normally produced by a laser (in some models, above all in research, several light sources are combined). In most cytometers, an air-cooled gas laser is used (commonly Argon), which produces monochromatic light at 488 nm. This light is used to excite the majority of fluorochromes and elicits the dispersion of light, which provides information about cellular characteristics. A prism placed at the end of the argon tube selects a single wavelength. Some cytometers incorporate another laser that emits at 630 nm. The most modern devices also have a third laser that emits at 407 nm.

The optical systems of cytometers consist of two light dispersion detectors and between 4 and 3 fluorescence detectors, although normally there are between 6 and 4. By means of the light dispersion detector it is possible to determine the size of cells (FSC) and by the right-angle dispersion detector it is possible to determine cellular granularity, that is, the internal complexity of the cell. The systems of fluorescence detection are located at right angles and, with them, cells labelled with fluorochromes can be determined. The emission of each fluorochrome is selected by an optical system comprising filters and dichroic mirrors and the signals are sent to a specific detector.

These detectors generate electrical pulses that are directly proportional to the area, height and width of the total amount of light detected, the maximum intensity detected and the duration of the luminous signal, respectively. The signals received are amplified, multiplying them by a linear or logarithmic factor. The amplified pulse is carried to an analogical/digital converter that changes the signal received into a digital signal that can be managed by a computer, with values on the linear scale ranging between 0 and 1024 increments, understanding one increment as a value of 0.01 volts. Normally, size and granularity measurements are taken on a linear scale and fluorescence measurements on a logarithmic scale.

Laser 407	Alexa Fluor 430
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Laser 633 nm	APC
	APC-Cy7
	Alexa Fluor 647

Laser 488 nm	FITC
	PE
	PECy5
	PECy7
	Alexa Fluor 488

The electronic-informatic (computer) system

This converts the dispersed light into electric signals and processes them for later analysis. Today, all flow cytometers have computer programs that are able to maintain the information about each of the cells separately, such that it is possible to contrast some parameters with others in two- or three-dimensional windows. Thus, from each cell two types of data are obtained: qualitative and quantitative; that is, information about the presence of a given epitope and its level of expression is obtained. Some flow cytometers are able to sort cells or sub-cellular elements according to some of the characteristics explained above.

The perfect integration of the system permits rapid and precise analysis of a large number of cells in a very short time.

PARAMETERS OF THE FLOW CYTOMETER

Sensitivity

The definition of sensitivity could be considered to be the number of markers detectable for fluorescence. Thus, sensitivity depends on many factors, such as the time elapsed, overlapping between excitation wavelengths and the absorption spectrum of the fluorochrome. ... Despite this, it can be said that the detection limit is of the order of 103 molecules of FITC per cell, although the most modern flow cytometers reach fewer than 100 molecules of FITC; or fewer than 50 molecules of PE per cell.

RESOLUTION

Resolution can be defined as the detection of the smallest particle possible. In the case of FSC, one is dealing with values close to 1mm and for SSC 0.5 mm. In the case of fluorescence, resolution is measured as the coefficient of variation; i.e., the standard deviation relative to the mean of the signal produced by the cells or particles analysed. In this case, resolution will depend on the stability of the light source, sample flow, its width, the intensity of the signal, and the background luminescence or noise.

Currently, flow cytometers have a resolution of less than 3%.

RATE OF MEASUREMENT

Cytometers have advanced considerably in this aspect and now offer really high analysis speeds. The normal measurement rate is 5,000 cells/second, although this depends on the parameters to be analysed. The most evolved cytometers can analyse 60,000 and 100,000 cells/second.

NUMBER OF MEASURABLE PARAMETERS IN A SAMPLE

Most flow cytometers are equipped with one or two lasers and four lenses, which permits the analysis of up to 6 parameters at once, including FSC and SSC. Some cytometers have 6 lenses, with which it is possible to measure 6 parameters, or have an additional laser, it then being possible to measure up to 15 parameters

SPECIFICITY

Flow cytometers offer relative, not absolute information. For quantification purposes, it is necessary to correlate the channels with the results of known samples; that is, calibrate and adjust the scales. To accomplish this, alignment standards are used that have already determined values of fluorescence or light dispersion and that are valid for the detection of changes and problems in the optical configuration and signal with time. These standards are based on calculation of the ratio between the doublets/singlets analysed of a control. The linearity offered by current flow cytometers is between 1.95 and 2.05 for each of the lasers.

QUANTITATIVE AND QUALITATIVE DATA

The interpretation of the microscopic image, even with the aid of techniques such as electron microscopy and immunohistochemical labelling, is based on the training and experience of the observer and is limited to the resolution of the human eye. Also, microscopic analysis is not appropriate for the rapid examination of a large number of cells.

Unlike the light and electron microscope, flow cytometers are rapid and objective. During analysis by flow cytometry, the cells are examined one by one, obtaining quantitative measurements of the physical parameters related to the biological properties of the cells. Additionally, it is possible to measure multiple parameters simultaneously and, on the basis of this, study different cellular subsets separately.

BIOLOGICAL PARAMETERS THAT CAN BE ANALYSED BY FLOW CYTOMETRY

	CELLULAR STRUCTURES	CELLULAR FUNCTIONS
WITHOUT CELL LABELLING	Cell size (FSC) Cell granularity (SSC)	Redox state Cell viability Intrinsic fluorescence
WITH CELL LABELLING	Cellular antigens Subcellular Organelles Parts of macromolecules Low-molecular weight metabolites Specific receptors	Cell viability Cell cycle Determination of ploidy Movements of metabolites (Ca ²⁺) Transport and internalization Integrity of the plasma membrane Enzymatic activities Membrane potentials Studies of apoptosis Intracellular pH Uptake of thymidine analogues

NUCLEAR PARAMETERS	Parameters based on DNA	DNA content	DNA ploidy
		Content of GC/AT pairs	Cell cycle
		DNA supercoiling	Apoptosis-necrosis
		Chromatin structure	Flow Karyotype
		DNA strand breakages	
	DNA and other parameters	DNA/RNA	DNA/Cellular antigens
		DNA/total proteins	Regulation of cell cycle
		DNA nuclear antigens	Cell cycle of specific populations
	Parameters not based on DNA	Nuclear receptors	Nuclear morphology
Expression of marker genes (reporters)		Nuclear components	

SURFACE PARAMETERS	Cell Surface structures	Surface receptors	Surface density
		Lectin binding sites	Cell wall
	Cell Surface dynamics	Ligand binding	Receptor exposure
		Transport/internalisation of ligands	Membrane potential
Solute efflux			
CYTOPLASMIC PARAMETERS	Intracellular components	Total proteins	Intracellular glycoproteins
		Structural proteins	Intracellular lipids
		Functional proteins	Free thiols
	Intracellular functions	Enzymatic activity	Membrane potential
		Protein synthesis	
	Intracellular milieu	Ion concentration	Oxidized lipids and proteins
		Ion movement	
EXTRACELLULAR PARAMETERS	Cell secretion dynamics	Uptake of secreted proteins	Cellular activation
		Quantification assays in serum	Cellular differentiation

CALIBRATION OF 3 COLOURS WITH LYMPHOCYTES FOR BD CYTOMETERS

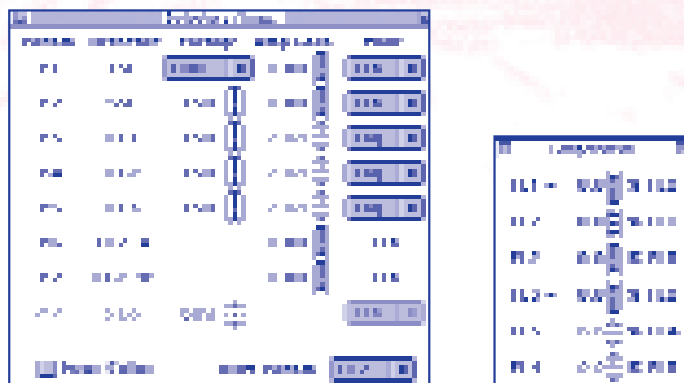
Set up the following panel for direct labelling with lysate:

1. A tube with lysed blood alone.
2. A tube with blood labelled with CD4 FITC/CD8 PE with nothing in FL3.
3. A tube with blood labelled only with CD3 PCy5.
4. A tube with blood labelled with CD4 FITC/CD8 PE/CD3 PCy5.

1. Switch on the cytometer and the Cell Quest program from BD with the usual working screen and the current calibration.
2. Set up the detectors/amplifiers and the compensations.

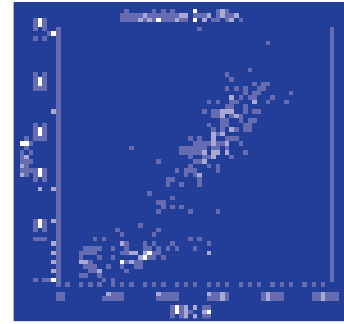


3. Lower all the voltages of the SSC, FL1, FL2 and FL3 detectors to 150 and the amplifiers of FSC gain to 1.00. Set all compensations to 0.

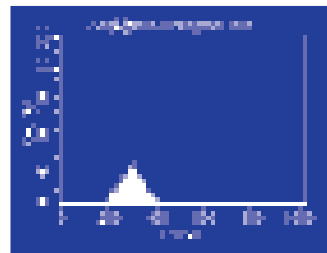
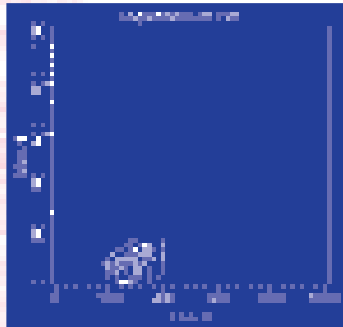


4. On a SSC/FSC dot/plot select the tube that contains the cells that have only been lysed.
5. With the detectors in view, raise the FSC gain amplifier and the SSC voltage until the cell sets appear on the dot/plot as shown in figure. The lymphocytes between channels 200 and 400.

Par. num.	Deflection	Yol. base	Base Volt	Mode
P1	130°	1000	0.800	Linear
P2	200°	400	0.800	Linear
P3	0.0°	1000	0.800	Linear
P4	0.0°	1000	0.800	Linear
P5	0.0°	1000	0.800	Linear
P6	10.0°		0.800	Lin
P7	0.0°		0.800	Lin
P8	0.0°	2000		Linear

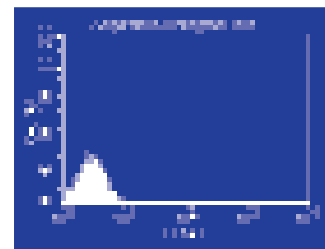


6. Paint a Gate in the lymphocyte region and with this window check on an SSC histogram that the lymphocyte peak is really between channels 200 and 400.



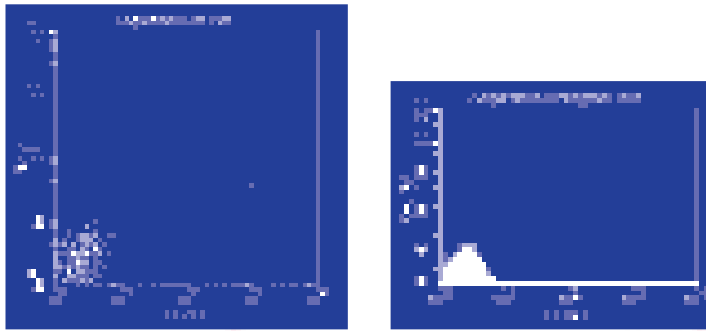
7. Open the FL1 histogram and, always acquiring the lymphocyte gate. Raise the voltage of FL1 until a peak on the histogram between channels 10^0 and 10^4 is obtained.

Par. num.	Deflection	Yol. base	Base Volt	Mode
P1	130°	1000	0.800	Linear
P2	200°	400	0.800	Linear
P3	0.0°	1000	0.800	Linear
P4	0.0°	1000	0.800	Linear
P5	0.0°	1000	0.800	Linear
P6	10.0°		0.800	Lin
P7	0.0°		0.800	Lin
P8	0.0°	2000		Linear



8. Open a FL2/FL1 dot plot and repeat previous step with FL2 in histogram, observing that the lymphocyte population is located between 10^0 and 10^4 , like its FL1 and FL2 negative control.

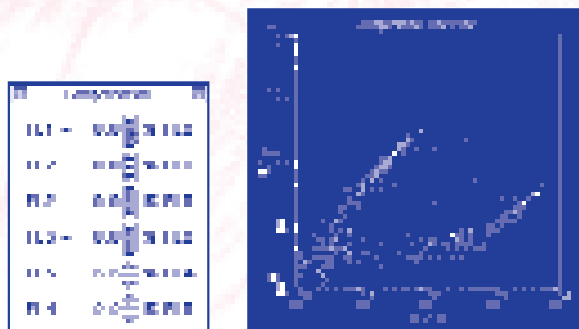
Par. num.	Deflection	Yol. base	Base Volt	Mode
P1	130°	1000	0.800	Linear
P2	200°	400	0.800	Linear
P3	0.0°	1000	0.800	Linear
P4	0.0°	1000	0.800	Linear
P5	0.0°	1000	0.800	Linear
P6	10.0°		0.800	Lin
P7	0.0°		0.800	Lin
P8	0.0°	2000		Linear



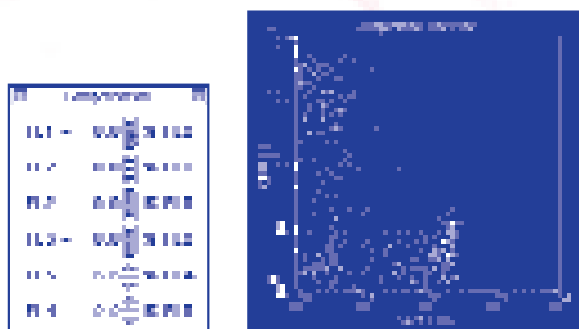
9. Repeat the previous two steps for FL3 with a FL2/FL3 dot plot to check the negative control.

channel	channel	range	amp gain	min
FL1	FL1	0.000000	0.000	0.000000
FL2	FL2	0.000000	0.000	0.000000
FL3	FL3	0.000000	0.000	0.000000
FL4	FL4	0.000000	0.000	0.000000
FL5	FL5	0.000000	0.000	0.000000
FL6	FL6	0.000000	0.000	0.000000
FL7	FL7	0.000000	0.000	0.000000
FL8	FL8	0.000000	0.000	0.000000
FL9	FL9	0.000000	0.000	0.000000
FL10	FL10	0.000000	0.000	0.000000
FL11	FL11	0.000000	0.000	0.000000
FL12	FL12	0.000000	0.000	0.000000
FL13	FL13	0.000000	0.000	0.000000
FL14	FL14	0.000000	0.000	0.000000
FL15	FL15	0.000000	0.000	0.000000
FL16	FL16	0.000000	0.000	0.000000
FL17	FL17	0.000000	0.000	0.000000
FL18	FL18	0.000000	0.000	0.000000
FL19	FL19	0.000000	0.000	0.000000
FL20	FL20	0.000000	0.000	0.000000

At this point, the minimum voltages have been adjusted for the photomultipliers.
 10) Close the histograms and the amplifier detectors. The next step is compensation.
 11) Acquire the tube with CD4 FITC/CD8 PE.

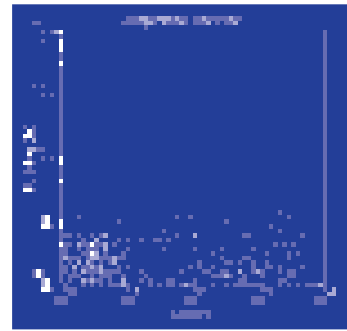
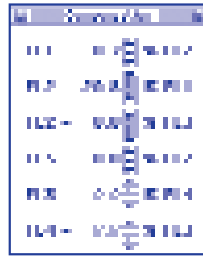


12) On an FL1/FL2 dot plot, raise the (FL1-%FL2) compensation until the CD8 lymphocytes are in place, and (FL2-%FL1) compensation for putting CD4 lymphocytes in place, avoiding undercompensations and overcompensations.

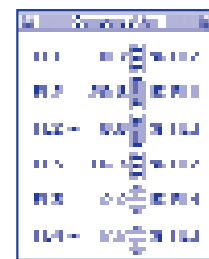
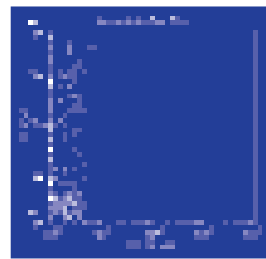
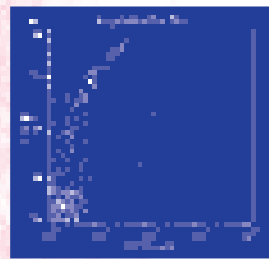


14) Increase the (FL3-%FL2) compensation to remove from FL2 what it detects on an FL3/FL2 dot plot.

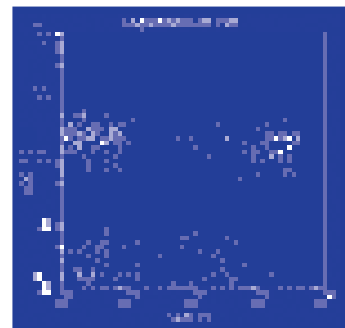
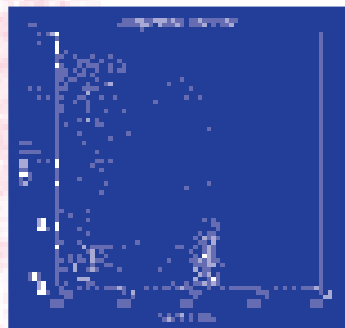
reagents against human antigens



- 15) Acquire a tube in which there is no FL1, no FL3 and there is CD8 in FL3.
- 16) Raise the FL2-%FL3 compensation to remove from FL2 what it detects from FL3.



- 17) Acquire the CD4 FITC/CD8 PE/CD3 PCy5 tube to check that all cell sets are in place.



- 18) To save this calibration, on the general menu of Cell Quest click on Cytometer; go to Settings and click on Save to identify the file with the name of the calibration.

Information courtesy of Dr. García Vela.

REQUIREMENTS OF A FLOW CYTOMETER

Today's flow cytometers do not have any special requirements since the lasers are cooled by air and not by water and do not need an entrance and exit for either air or water. The supply of a stabilised uninterrupted electric current is the most critical requirement, with a voltage of 100/115/230 V AC and 50-60 Mz, with a maximum of 1500 watts. The temperature ranges should lie between 15 and 30°C, with a relative humidity between 5 and 80% that is guaranteed not to condense.

It should also be noted that the surface on which the cytometer is placed should be ample and free of vibrations.

QUALITY GUARANTEE

STANDARDISATION.

To be able to compare the results among different institutions or with the passage of time, it is necessary to ensure that the cytometer functions correctly on a continuous basis, such that it is crucial to make daily adjustments to the voltage of the photodetector and/or the gains in order to be able to obtain peaks of a standard of a given intensity and generate follow-up curves. To monitor the performance or reproducibility of the reagents and the quality of the methods of cell preparation, controls are used; these are a material that produces expected or known results. Controls are important in the analysis of DNA (ploidy controls are used) and in immunofluorescence techniques (negative controls are used to rule out specific binding of the fluorochrome and to select the autofluorescence threshold, and positive controls are employed to check the correct functioning of the technique). The parameters that are assessed in the standardisation of the technique include: a) amplification of the logarithmic and linear signal; b) colour compensation; c) resolution; d) sensitivity; e) fluorescence quantification.

3. AREAS OF APPLICATION OF FLOW CYTOMETRY.

At general level, it could be considered that the four main areas of application of flow cytometry are:

RESEARCH Immune function Cancer Molecular Biology Basic microbiology	PHARMA Lead compound identification/validation Drug safety studies Biomarker discovery
CLINICAL Diagnostic (IVD) Emerging diagnostic Transplantation/Transfusion QC for cell therapy Clinical microbiology	INDUSTRIAL /BIOTECHNOLOGY Environmental Agriculture Bioreactor monitoring

In some disciplines such as immunology, haematology, oncology... flow cytometry is used mainly in routine clinical work, although it is true that recent years have witnessed a considerable expansion of the use of flow cytometry in basic research, as occurs in microbiology for biotechnological, ecological or clinical applications, for the detection of microorganisms, mainly their cell cycle, metabolic state, the detection of certain enzymes, culture viability....

Within the field of foods, flow cytometry is also beginning to be used widely for both the study of toxicity and for quality analysis in certain foods.

Immunophenotyping

Since flow cytometry is a multiparametric analytical technique, is rapid, specific and sensitive, it has become a powerful tool in cell analysis for the diagnosis, classification and prognostic assessment and evaluation of minimum residual disease.

There are uncountable areas in which flow cytometry has contributed to the development of knowledge about them. Examples are the diagnosis and classification of primary immunodeficiencies; the monitoring of autoimmune disease such as multiple sclerosis, systemic lupus erythematosus, or rheumatoid arthritis, and the follow-up of immunological values after bone marrow transplantation or the early identification of graft rejection.

General clinical applications of flow cytometry

Diagnosis based on cell analysis	Characterisation of normal cells	Structural parameters	Size
		Surface antigens	Complexity
			Identity
			Lineage
			Function
			Activation
		Nuclear content Ploidy	Ploidy
			Proliferative state
			Maturation state
			Gene expression
	Intracellular biochemistry	Basal conditions	
		Biochemical response due to controlled stimuli	
	Identification and detection of normal cells	Alterations in cell morphology	Changes in size
			Changes in granularity
		Alterations in the immunophenotype	Immunoproliferation / Immunodefense
		Alterations in the genotype	Changes in DNA content (ploidy)
			Changes in gene expression
		Alterations in intracellular biochemistry	Changes in the synthesis and concentration of molecules
			Changes in enzyme activities and in flow rates through metabolic pathways
			Alterations in the number and function of subcellular organelles
Changes in biochemical response to controlled stimuli			
Detection of low-frequency		Detection of circulating tumour cells	
	Detection of foetal cells in maternal blood		
	Detection of activated circulating cells		

reagents against human antigens

Prognostic applications	Correlation of cytometric parameters	Retrospective studies with archive material Prospective studies
	Parameters directly involved in pathological processes	Analysis of samples specific to the pathology Analysis of parameters specific to the pathology
	Parameters indirectly involved in pathological processes	Analysis of indicator samples Analysis of pathology-indicating markers Analysis of parameters predicting risk
Evaluation and monitoring of treatment	Cell selection in cell therapy	Analysis of progenitors in autologous transplantation Cross- tests of heterologous transplants Detection and quantification of residual leucocytes in blood preparations
	Analysis of therapeutic action at cellular level	Changes in structural parameters Changes in functional parameters
	Analysis of therapeutic action at patient level	Detection of relapse and minimum residual disease Establishment of prognostic patterns of therapeutic success
	Detection and analysis of resistance to therapy	Analysis of the uptake and retention of drugs Analysis of drug metabolism
Analysis of lesion and cell death	Detection of sublethal damage cell	Changes in structural parameters Changes in functional parameters
	Quantification of cell viability	Determination of global cytotoxic effects Quality control of cell preparations
	Characterisation of mechanisms involved in cell death	Identification and analysis of apoptotic cells Identification of necrotic cells
Applications in immunohaematology	Analysis of antigens	Surface immunophenotyping Detection of intracellular antigens Detection of circulating antigens
	Analysis of antibodies	Detection of circulating antibodies Detection of cell-bound antibodies
	Analysis of cellular function	Analysis of cellular proliferation Analysis of cellular biochemistry Analysis of the index of cell death

BASIC AND APPLIED RESEARCH

Microbiology, haematology, immunology, cytology, pathology, cell and molecular biology are just some of the fields in which today flow cytometry is used in widespread applications. The following tables attempts to classify some of these applications.

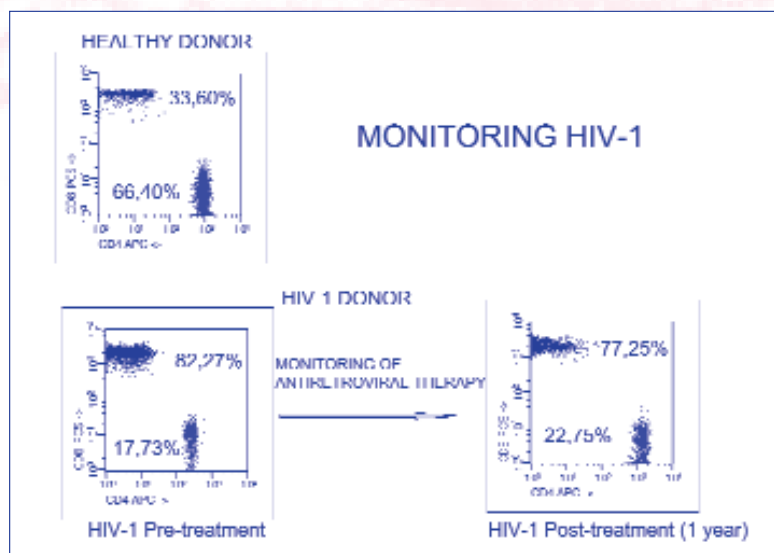
General application	Analysis parameters	Information provided by applications
Surface immunophenotyping	Identification of cell subsets by monoclonal antibodies	Cell lineage State of haematopoietic maturation Involvement in the immune response Degree of cellular activation
	Identification of cell subsets in haematopoiesis	Diagnosis and classification of haematological diseases Immunodeficiencies Nocturnal paroxistic haemoglobinuria Detection of minimal residual disease Enumeration of haematopoietic precursors CD34+ Diagnosis of congenital platelet diseases Glanzmann's thromboasthaenia Bernard- Soulier syndrome
Intracellular antigens	Detection of intracellular proteins	Production of intracellular cytokines Detection of circulating tumour cells
	Expression of cell cycle regulators; Cyclin D1	Intracellular Ig light chains: κ y λ . Non-expressed surface antigens: CD3 y CD22 Intracellular enzymes
	Expression of anti-apoptotic proteins; BCL-2, RB	
	Expression of tumour-suppressing proteins; p53	
	Analysis of intracellular cytokines	Phenotype Th0, Th1 and Th2

General application	Analysis parameters	Information provided by applications
Circulating antigens	Detection of circulating Autoantibodies	Antiplatelet antibodies Antineutrophil antibodies
	Detection of circulating alloantibodies	Pre-transplantation cross tests Use of spheres coated with specific HLA
	Detection of cell-bound antibodies in blood	
Immunoproliferation	Monoparametric analysis of the cell cycle	DNA content
	Multiparametric analysis of the cell cycle	Surface phenotype and NA content
	Multiparametric analysis of the cell cycle	Content of DNA and antigens related to the cycle
	Analysis of the cell division history	Cell tracers and surface phenotype
Intracellular biochemistry	Biochemical functions of specific immunity	Synthesis and secretion of cytokines Expression of adhesion molecules Ion movements following cellular activation
	Biochemical functions of innate immunity	Phagocytosis and destruction of microorganisms Intracellular generation of reactive O ₂ species Intracellular proteolytic activation Chemotactic responses Expression of adhesion molecules Oxidative explosion
	Biochemical functions of platelets	Platelet activation and aggregation
Cell death in the immune system	Analysis of cytosolic effector cells	Phenotypic identification of CD8 and NK subsets
	Analysis of cell-mediated cytotoxicity	Interaction between target and effector cells
	Analysis of antibody-dependent cytotoxicity	Monitoring of therapy with monoclonal antibodies
	Analysis of the process and mechanism of cell death	Identification and quantification of apoptotic cells Identification and quantification of necrotic cells Analysis of cellular parameters during apoptosis
Functional analysis	<i>in situ</i> kinetic analysis	Detection of early or transient effects
	Sequential analysis	Evaluation of dynamic processes

ANALYSIS OF HIV

It is interesting to point out the important role of flow cytometry in the monitoring of lymphocyte subsets in acquired immunodeficiency syndrome (AIDS). Currently, it is routinely used at many laboratories. Today, AIDS is the main cause of hospital deaths in people aged between 18 and 45.

The CD4/CD8 ratio appears useful in identifying HIV-infected. The development of lower cost and more robust flow cytometric methods that provide both CD4/CD8 ratio and %CD4 may be cost-effective for HIV-1 diagnosis and identification of highly active antiretroviral therapy.



CLASSIFICATION OF HAEMATOLOGICAL PATHOLOGIES ACCORDING TO THE WHO

A, B-CELL NEOPLASMS

1. PERCURSOR B CELL NEOPLASM.

- Precursor B lymphoblastic leukemia/lymphoma.

2. MATURE B-CELL NEOPLASMS

- **Chronic B cell lymphatic leukaemia/small cell lymphocytic lymphoma**
- B-cell prolymphocytic leukaemia
- Lymphoplasmacytic lymphoma/immunocytoma
- Splenic marginal zone lymphoma
- Hairy cell leukemia
- **Plasma cell neoplasms (multiple myeloma)/Plasmocytoma**
 - Multiple myeloma
 - Solitary plasmocytoma of bone
 - Extraosseous plasmocytoma
- **Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue**
- Ganglionic marginal zone B-cell lymphoma, monocytoid cells.
- Follicular lymphoma
 - Grade 1
 - Grade 2
 - Grade 3
 - Grade 4
- Diffuse follicular center lymphoma
- Mantle cell lymphoma
 - Variants: Blastic or blastoid, pleomorphic, small cell and monocytoid
- Diffuse large B-cell lymphoma
 - Variants
 - Centroblastic
 - Immunoblastic
 - T-cell /histiocyte rich
 - Large B cell anaplastic
 - Plasmablastic
 - With expression of full length ALK (IgA+)
- Mediastinal large B cell lymphoma
- Intravascular large B cell lymphoma
- Primary lymphoma of the serosa
- Burkitt's lymphoma
 - Variant: with plasmocytoid differentiation (associated with AIDS)

3. B-CELL PROLIFERATION OF UNCERTAIN MALIGNANT POTENTIAL

- Lymphomatoid granulomatosis
- Post-transplant lymphoproliferative disorder, polymorphic

B. T/NK CELL NEOPLASMS

1. PERCURSOR T-CELL AND NK-CELL NEOPLASMS

- Precursor T lymphoblastic leukaemia /lymphoma
- Blastic NK cell lymphoma

2. MATURE T AND NK CELL NEOPLASMS

2A. PREDOMINANTLY LEUKAEMIC/DISSEMINATED FORMS

- T-cell prolymphocytic leukaemia
 - Variants:
 - Small cell
 - Cerebriform cells
- T cell large granular leukaemia
- Aggressive NK cell leukaemia
- Sézary syndrome
- Adult T-cell lymphoma / leukaemia
 - Variants:
 - Acute
 - Lymphomatous
 - Quiescent
 - Hodgkin-type.

2B. PREDOMINANTLY GANGLIONARY FORMS

- **T angioimmunoblastic lymphoma**
- **Peripheral T-cell lymphoma (unspecified)**
 - Variants:
 - Lymphoepithelioid
 - T-zone
 - Pleomorphic, of small cells, mixed and of large cells
- **Large cell Anaplastic T lymphoma**
 - Variants:
 - Lymphohistiocytic
 - Small cell
- **Gamma/delta hepatosplenic T lymphoma**

2.C PREDOMINANTLY EXTRANODAL FORMS

- **T/NK cell extranodal lymphoma, nasal type**
- **Cutaneous T lymphoma**
 - Mycosis fungoides /epidermotropic cutaneous T lymphoma
 - T CD30+ cutaneous lymphoproliferative disease
 - Subcutaneous T lymphoma of the panniculitic type

3. T CELL PROLIFERATION OF UNCERTAIN MALIGNANCY POTENTIAL**C HODGKIN LYMPHOMA (HODGKIN'S DISEASE)**

- Hodgkin lymphoma, nodular lymphocytic predominance (paragranuloma)
- Classic Hodgkin's disease.
 - **Nodular sclerosis (grade I and II)**
 - Classic Hodgkin lymphoma, lymphocyte rich
 - **Mixed cellularity**
 - Lymphocyte-depleted

D. NEOPLASMS OF HISTIOCYTES AND DENDRITIC CELLS AND RELATED DISEASES**1. RELATED TO MACROPHAGES/HISTIOCYTES**

- Histiocytic sarcoma
- Malignant histiocytosis (related to acute monocytic leukaemia)

2. RELATED TO DENDRITIC CELLS

- Langerhan's cells histiocytosis
- Langerhan's cells sarcoma
- Sarcoma/tumour of interdigitating dendritic cells.
- Sarcoma/tumour of follicular dendritic cells
- Sarcoma of dendritic cells, unclassifiable.

E. ACUTE LEUKAEMIAS AND MYELODISPLASTIC SYNDROMES**1. ACUTE MYELOBLASTIC LEUKAEMIA**

- Acute myeloblastic leukaemia with recurrent genetic anomalies
 - Acute myeloid leukaemia with t(8;21)(q22;q22),(AML1/ETO)
 - Acute myeloid leukaemia with abnormal eosinophils in bone marrow and /MYH11)binv(16)(p13;q22) or t(16;16)(p13;q22),(CBF)
 - Acute promyelocytic leukaemia with t(15;17),) and variantsa(PML/RAR)
 - Acute myeloid leukaemia with anomalies in 11q23
- Acute myeloid leukaemia with multilineage dysplasia
 - Secondary to myelodysplastic syndrome (MDS) or myelodysplastic syndrome /myeloproliferative syndrome (MDS/MPS)
 - With no background of MDS or MDS/MPS, but with dysplasia in at least 50% of cells in two or more myeloid lines.
- Acute myeloid leukaemia and myelodysplastic syndromes, secondary to treatment
 - Secondary to treatment with alkylating agents/radiation
 - Secondary to treatment with topoisomerase II inhibitors
 - Others
- Acute myeloid leukaemia not belonging to any category
 - Acute myeloid leukaemia, minimally differentiated
 - Acute myeloid leukaemia without maturation
 - Acute myeloid leukaemia with maturation
 - Acute myelomonocytic leukaemia
 - Acute erythroid leukaemia (erythroid/myeloid and pure erythroleukaemia)
 - Acute megakaryoblastic leukaemia
 - Acute basophile leukaemia
 - Acute panmyelosis with myelofibrosis
 - Myeloid sarcoma

2. ACUTE BILINEAR/BIPHENOTYPIC LEUKAEMIA**3. MYELOYDYSPLASTIC SYNDROMES.**

- Refractory anaemia
- Refractory anaemia with ringed sideroblasts Refractory cytopenia with multilineage dysplasia
- Refractory cytopenia with multilineage dysplasia and ringed sideroblasts
- Refractory anaemia with excess blasts-1
- Refractory anaemia with excess blasts-2
- Myelodysplastic syndrome associated with del(5q) with no other chromosomal anomaly (5q- syndrome)
- Unclassifiable myelodysplastic syndrome

4. MYELOYDYSPLASTIC/MYELOPROLIFERATIVE DISEASES

- Chronic myelomonocytic leukaemia
- Atypical chronic myelomonocytic leukaemia
- Juvenile myelomonocytic leukaemia
- Unclassifiable myelodysplastic/myeloproliferative syndromes

5. CHRONIC MYELOPROLIFERATIVE DISEASES

- Chronic myeloid leukaemia t(9;22)(q34;q11) BCR-ABL- positive
- Acute neutrophilic leukaemia
- Chronic eosinophilic leukaemia/hypereosinophilic syndrome
- Polycythaemia vera
- Chronic idiopathic myelofibrosis (with extramedullary haematopoiesis)
- Essential thrombocythaemia
- Unclassifiable chronic myeloproliferative syndrome

6. MAST CELL DISEASE (MASTOCYTOSIS)

- Cutaneous mastocytosis
- Indolent systemic mastocytosis
- Systemic mastocytosis with an associated hematologic non-mast cell lineage disease
- Aggressive systemic mastocytosis
- Mast-cell leukaemia
- Extracutaneous mastocytoma

FLOW CYTOMETRY TECHNIQUES

A. SAMPLES.

PERIPHERAL BLOOD: The blood must contain HEPARIN as an anticoagulant agent for functional studies of EDTA for phenotypic studies. It is appropriate to make an initial count of the blood cell subsets with a haematological counter (haemogram).

BONE MARROW: As in the case of peripheral blood, the bone marrow must contain an anticoagulant agent: heparin or EDTA, depending on the type of study to be performed. In this case, before acquiring a haemogram the sample must be diluted in PBS and then passed through an insulin needle to break down aggregates.

LYMPHATIC GANGLION: Once the biopsy has been collected, the sample must undergo processing immediately after extraction and must be done in physiological saline, never in formaldehyde. For flow cytometry study of the samples, the cells must be in suspension; to accomplish this, first the lymphatic gland is cleaned completely of adhering tissue and the remains of blood are removed by washes in physiological serum. The sample is then minced with a scalpel and placed in a Petri dish, placing the sections in RPMI, HANK's or PBS and then extracting the cells from inside the ganglion by pressing this gently against the dish with tweezers. After mixing well, the liquid part containing the cells in suspension is removed with a Pasteur pipette, avoiding the presence of pieces of tissue. Following this, the liquid is washed twice with PBS at 2000 rpm for 5 min. Finally, the cell pellet is resuspended and medium is added until a concentration of $4-5 \times 10^6$ cells/mm³ is obtained.

PLEURAL FLUID OR CEREBROSPINAL FLUID: The samples must be extracted in heparinized tubes. In the event of the sample containing erythrocytes, these must be removed before hand. To accomplish this, any non-fixing lysis solution can be used; if the concentration is very high, it is possible to use the Lymphoprep® reagent to separate mononucleate cells. After several rounds of centrifugation, adding 10% BSA to stabilise the cells, they are left at a final cell concentration sufficient to start antigen determination.

B. DENSITY GRADIENT MONONUCLEAR CELL SEPARATION TECHNIQUE

Removal of platelets: This is carried out when the peripheral blood contains a high number of platelets or when the sample of bone marrow is strongly contaminated with blood. Initially, the sample is centrifuged at 900 rpm for 10 minutes at room temperature. Following this, the supernatant containing plasma and platelets is removed with a Pasteur pipette. The amount of liquid removed must be replaced by PBS or physiological saline. The precipitate contains the leukocytes and erythrocytes.

Dilution: When working with samples with a high leukocyte concentration, for example in the case of hyperleukocytotic leukaemias, the samples should be diluted between 5 and 10 times with PBS.

Separation of the mononuclear cell fraction. 4 mL of Lymphoprep® is added to a 15-mL tube, and a volume of 5 mL of sample is carefully overlaid, allowing it to run down the side of the tube but without blending with the Lymphoprep®. Two well-defined layers should be formed. The amount may be modified but maintaining the Lymphoprep/sample ratio of 4/5.

Following this, the tube is centrifuged at 1500 rpm for 35 minutes in an oscillating centrifuge without a breaking system. Once the centrifugation has been completed, four well-defined layers will appear, in the following order from bottom to top: erythrocytes and segmented cells, Lymphoprep®, mononucleate cells and finally, the diluting agent at the top. To prevent the Ficoll of the Lymphoprep from damaging the cells, the layer of mononucleate cells is removed with a Pasteur pipette. After centrifugation at 1200 rpm for 10 min at room temperature, the liquid is removed and the cells are resuspended.

Washing: two or three rounds of washing are made with culture medium or PBS (approx 4-5 mL) at 1200 rpm at room temperature for 10 minutes. At the end of each centrifugation, the supernatant is removed and the cells are resuspended. After the final wash, the cells are resuspended and a solution of 100 mL of medium supplemented with 10% inactivated SBF is added, if necessary.

C. MEMBRANE ANTIGENS IN LEUKOCYTES.

1. DIRECT IMMUNOFLUORESCENCE

1. The starting material is peripheral blood (PB) or another sample of cells in suspension, permanently anticoagulated with EDTA (this should be kept at 4°C until processing). If processing is to be carried out within a few hours, it may be more appropriate not to subject the cells to temperature changes and leave the samples at room temperature.
2. Before starting the technique, a white blood cell count is made by analysing a small amount of sample on a haematological counter.
3. Generally, 100 µL of PB are taken when the number of leukocytes is 10×10^3 cells/µL and 200/ mL when the number of leukocytes is equal to 5×10^3 cells/µL.
4. The McAbs conjugated at saturation concentration are added and the cells are incubated for 15 minutes in the darkness at room temperature.
5. Once the incubation period has been completed, the erythrocyte lysing solution is added at the amount recommended by the manufacturer and this is incubated at room temperature in the darkness (the blood should be well mixed with the lysis solution).
6. The tubes are centrifuged at 2000 rpm for 5 min. The supernatant is removed with a Pasteur pipette or a vacuum pump.
7. The cell pellet is resuspended and a final wash is made with 3-5 mL of PBS at 2000 rpm for 5 min.
8. After removing the supernatant and resuspending the cell pellet, some 300 µL of PBS is added and the recordings of the flow cytometer are read.

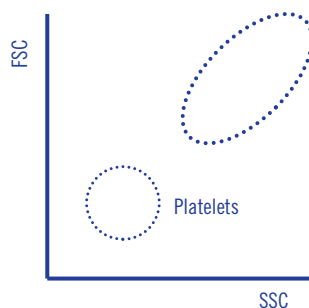
* Note: in the case of immunofluorescence of membrane antigens also present in erythrocytes, as is the case of CD59 or CD55, the erythrocytes must be lysed before labelling with the antibody when expression in leukocytes is to be analysed.

2. INDIRECT IMMUNOFLUORESCENCE.

1. The starting material is peripheral blood (PB) or another sample of cells in suspension. This is permanently anticoagulated with EDTA (until the time of processing it should be stored at 4°C). If processing is to be carried out within a few hours, it may be more appropriate not to subject the cells to changes in temperature and leave the samples at room temperature.
2. Before starting the technique.
3. Generally, 100 µL of PB are taken when the number of leukocytes is 10×10^3 cells/µL and 200/µL when the number of leukocytes is equal to 5×10^3 cells/µL.
4. 1 µg of the McAb is pipetted into each tube and the tubes are incubated for 15 min at room temperature in the darkness.
5. A washing is made with centrifugation at 2000 rpm for 5 min with 5 mL of PBS in order to remove the McAb not bound to antigens.
6. 10 µL of mouse anti-Ig antibody conjugated with some fluorochrome is added and the mixture is incubated at room temperature for 15 min in the darkness. The absence of light is necessary so that the fluorochrome will not deteriorate since it shows a high degree of photostability.
7. After the incubation period, an erythrocyte-lysing solution is added at the amount recommended by the manufacturer and the mixture is incubated at room temperature in the darkness (the blood should be well mixed with the lysing solution).
8. The tubes are centrifuged at 2000 rpm for 5 min. The supernatant is removed with a Pasteur pipette or with a vacuum pump.
9. The cell pellet is resuspended and a final wash is made with 3-5 mL of PBS at 2000 rpm for 5 min.
10. After removing the supernatant and resuspending the cell pellet, some 300 µL of PBS is added and the readings on the flow cytometer are recorded.

D. MEMBRANE ANTIGENS IN PLATELETS AND ERYTHROCYTES.

1. The starting material is peripheral blood or another sample of cells in suspension. This is permanently anticoagulated with EDTA (up to the time of processing it should be stored at 4°C). If processing is to be carried out within a few hours, it may be more appropriate not to subject the cells to changes in temperature and leave the samples at room temperature.
2. 5 µL of PB are dissolved in 95 µL of PBS.
3. The conjugated McAbs are added and the mixture is incubated for 15 min in the darkness at room temperature.
4. To increase the volume, a further 200 µL of PBS is added, and the readings of the flow cytometer are recorded, bearing in mind that the FSC and SSC light dispersion parameters must be on a logarithmic scale.



E. SURFACE IMMUNOGLOBULINS: κ/λ

Sample: Whole peripheral or medullary blood or cells obtained from lymphoid organs and placed in suspension.

Washing: The immunoglobulins must be removed from the plasma in order to prevent them from binding to the anti-kappa and anti-lambda McAb. When working with previously separated cells, this step is not necessary. For washing, the tube containing the sample is filled with PBS and incubated at 37°C in a bath for 30 min. After this time, the sample is centrifuged at 2000 rpm for 5 min and the supernatant is removed with a Pasteur pipette or vacuum pump.

Human anti-Ig antibody: The sample is added plus 10 µL of κ/λ antibody and is incubated for 15 min at room temperature in the darkness.

Lysis: Once the incubation period has finished, the erythrocyte lysis solution is added at the amount recommended by the manufacturer and the mixture is incubated at room temperature in the darkness (the blood should be well mixed with the lysis solution).

Centrifugation: The tubes are centrifuged at 2000 rpm for 5 min. The supernatant is removed with a Pasteur pipette or using a vacuum pump. The cell pellet is resuspended and a final washing is made with 3-5 µL of PBS at 2000 rpm, 5 min.

After the supernatant has been removed and the pellet has been resuspended, some 300 µL of PBS are added and readings on the flow cytometer are recorded.

F. TECHNIQUE FOR THE SIMULTANEOUS DETERMINATION OF MEMBRANE ANTIGENS AND CYTOPLASMIC ANTIGENS BY DIRECT IMMUNOFLUORESCENCE WITH THE FIX & PERM KIT

Samples: Peripheral or medullary blood.

Membrane labelling: The monoclonal antibodies conjugated with fluorochromes are added at saturation concentration and incubation is performed for 15 min at room temperature in the darkness.

Washing: Between 3 and 5 µL of PBS are added to each tube and the tubes are centrifuged at 2000 rpm for 5 min. After the centrifugation, the supernatant is removed with a Pasteur pipette or a vacuum pump.

Membrane fixing: 100 µL of Fix and Perm solution A, or another solution with fixing ability, are added, then incubating for 15 min at room temperature in the darkness. After a new wash in PBS the tubes are centrifuged for 5 min at 2000 rpm and the supernatant is removed.

Lysis and cell permeabilisation: The cells are well resuspended and 100 µL of solution B or another permeabilising solution is added. Immediately thereafter, the monoclonal antibodies against cytoplasmic molecules are added at saturation concentration. After a 15 min

incubation at room temperature in the darkness, between 3 and 5 μL of PBS is added and the tubes are centrifuged at 2000 rpm for 5 min. Finally, the supernatant is removed with a Pasteur pipette or vacuum pump, and the cell pellet is well resuspended in 0.5 μL of PBS. Data are acquired on the cytometer.

G. TECHNIQUE FOR THE DETERMINATION OF CYTOPLASMIC ANTIGENS BY INDIRECT IMMUNOFLUORESCENCE WITH THE FIX & PERM KIT.

Samples: Peripheral or medullary blood.

Membrane fixing: The cells are well resuspended and 100 μL of Fix & Perm solution A or another solution with fixing capacity is added, incubating for 15 min at room temperature in the darkness. Then, a wash is made with PBS; the tubes are centrifuged for 5 min at 2000 rpm, and the supernatant is removed.

Lysis and cell permeabilisation: The cells are well resuspended and 100 μL of solution B from Fix & Perm, or any other permeabilising solution is added. Immediately thereafter, the McAb against cytoplasmic molecules are added at saturation concentration. After a 15 min incubation at room temperature in the darkness, between 3 and 5 μL of PBS is added and the tubes are centrifuged for 5 min at 2000 rpm. Mouse anti-Ig antibody conjugated with some fluorochrome is added and a new incubation is carried out for 15 min, followed by a wash with 3-5 μL of PBS at 2000 rpm for 5 min. Finally, the supernatant is removed with a Pasteur pipette or a vacuum pump and the cell pellet is well resuspended in 0.5 mL of PBS. Data acquisition is carried out on the cytometer.

H. DETERMINATION OF CELLULAR CYTOKINES BY FLOW CYTOMETRY.

Samples: 1 mL of peripheral blood (PB), obtained in a tube containing an anticoagulant that will not chelate calcium (usually, PB samples in sodium heparin are used).

Two tubes are labelled for each test: NEGATIVE CONTROL and ACTIVATED.

Procedure:

A. ACTIVATION:

The following are added to the **NEGATIVE CONTROL** tube:

1. 500 μL of culture medium (RPMI 1640) supplemented with 2 mM L-Glutamine.
2. 10 μL of Brefeldin A is added per mL of culture with PB to block the secretion of proteins to the extracellular space.
3. 500 μL of heparinized blood.

The following are added to the **ACTIVATED** tube

1. 500 μL of culture medium (RPMI 1640) supplemented with 2 mM L-glutamine.
2. Cytokine secretion stimulants:
 - a. Lymphocyte activation:
 - I. 20 ng per mL of Phorbol Myristate Acetate (PMA)
 - II. 1 μg per mL of Ionomycin.
 - b. Activation of dendritic cells and monocytes:
 - I. 100 μg per mL of lipopolysaccharide (LPS)
 - II. 10 ng per mL of recombinant human interferon-gamma (IFN-g).

3. Brefeldin A: This is added at a total concentration of 10 μg per μL of culture to block the secretion of proteins to the extracellular space.

4. 500 μL of heparinized blood

B. INCUBATION

The tubes are incubated at 37°C in 5% CO₂ and 95% humidity in a sterile atmosphere.

The incubation time is 4 hours for lymphocytes and 6 hours for monocytes and dendritic cells.

C. MEMBRANE LABELLING

For membrane labelling, which will allow the identification of the different cell subsets to be studied, the direct immunofluorescence technique is used.

1. 200 μL of membrane is added to previously labelled tubes.
2. The recommended amount of McAb is added for the identification of the different cell types to be studied.
3. The samples are shaken with a vortex mixer and are incubated for 5 min in the darkness at room temperature.

D. WASHING

Two mL of PBS is added to each tube and the mixture is centrifuged at 2000 rpm for 5 min at room temperature. The supernatant is removed with a Pasteur pipette or a vacuum pump.

E. LABELLING OF THE CYTOPLASM

1. 100 mL of FIXING SOLUTION (Solution A from the Fix & Perm kit) is added. The mixture is shaken well with a vortex mixer and incubated for 15 min in the darkness at room temperature.
2. Washing is performed with 2 mL of PBS and the tubes are centrifuged at 2000 rpm for 5 min at room temperature. The supernatant is removed with a Pasteur pipette or a vacuum pump.

3. 100 μ L of PERMEABILISING SOLUTION (Solution B from the Fix & Perm kit) is added, together with the McAb directed specifically against each cytokine to be studied. The mixture is shaken well with a vortex mixer and incubated for 5 min in the darkness at room temperature.
4. A wash with 2 mL of PBS is performed and the tubes are centrifuged at 2000 rpm for 5 min at room temperature. The supernatant is removed with a Pasteur pipette or a vacuum pump.
5. The cells are resuspended in 0.5 mL of PBS.

F. ACQUISITION.

The samples are acquired on a flow cytometer in a maximum time of one hour. In the case of dendritic cells, the protocol of the analysis of different cells in general is followed; in a first step, some 30,000 cells corresponding to the total cellularity are acquired and in a second step those cells included in the HLA-DR fraction are acquired, up to a total of 500,000.

I. STAINING CELLS PROTOCOL WITH ANNEXIN-V FITC. FLOW CYTOMETRY

1. Prepare Annexin V Binding Buffer: 10 mM HEPES/NaOH (pH 7,4) 140 mM NaCl, 25 mM CaCl_2 .
2. Induce apoptosis in cells using the desired method. A negative control should be prepared by untreated cells, that is used to define the basal level of apoptotic and necrotic or dead cells.
3. Harvest the cells after the apoptosis induction and wash in temperate phosphate-buffered saline (PBS).
4. Wash cells twice with temperate PBS and resuspend cells in 1 X Annexin-binding buffer at a concentration 1×10^6 cells/ml.
5. Add 5 μ l of the Annexin V-FITC and 10 μ l of PI, to each 100 μ l of cell suspension.
6. Incubate the cells at room temperature for 15 minutes at room temperature (25°C) in the dark.
7. After incubation period, add 400 μ l of 1X Annexin-binding buffer. Analyze by flow cytometry within one hour.