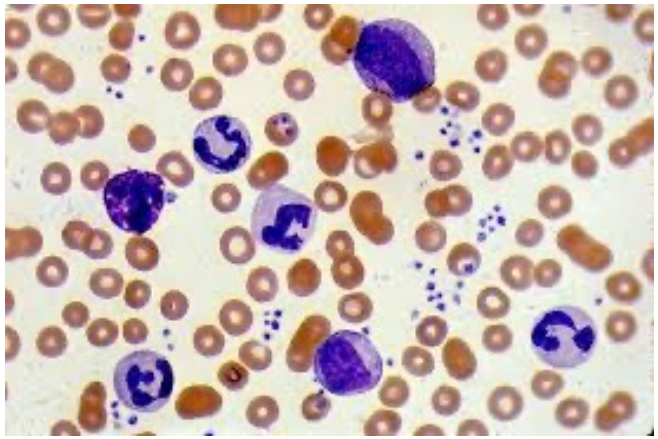
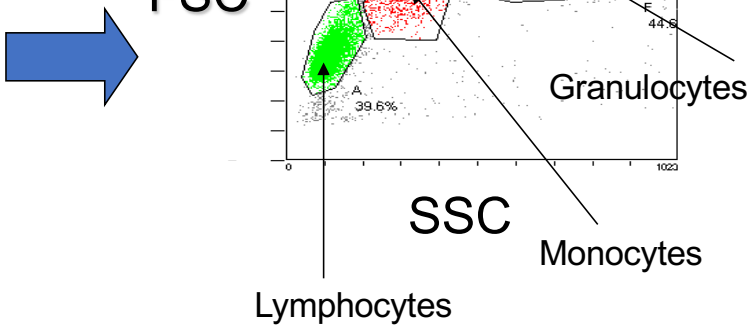
General principles: physical parameters

Flow cytometry measures optical (i.e. physical) and fluorescence characteristics of single cells (or any other particle..). Physical properties, such as size and internal complexity, can resolve certain cell populations.

Brown M and Wittwer , Clinical Chemistry, 2000



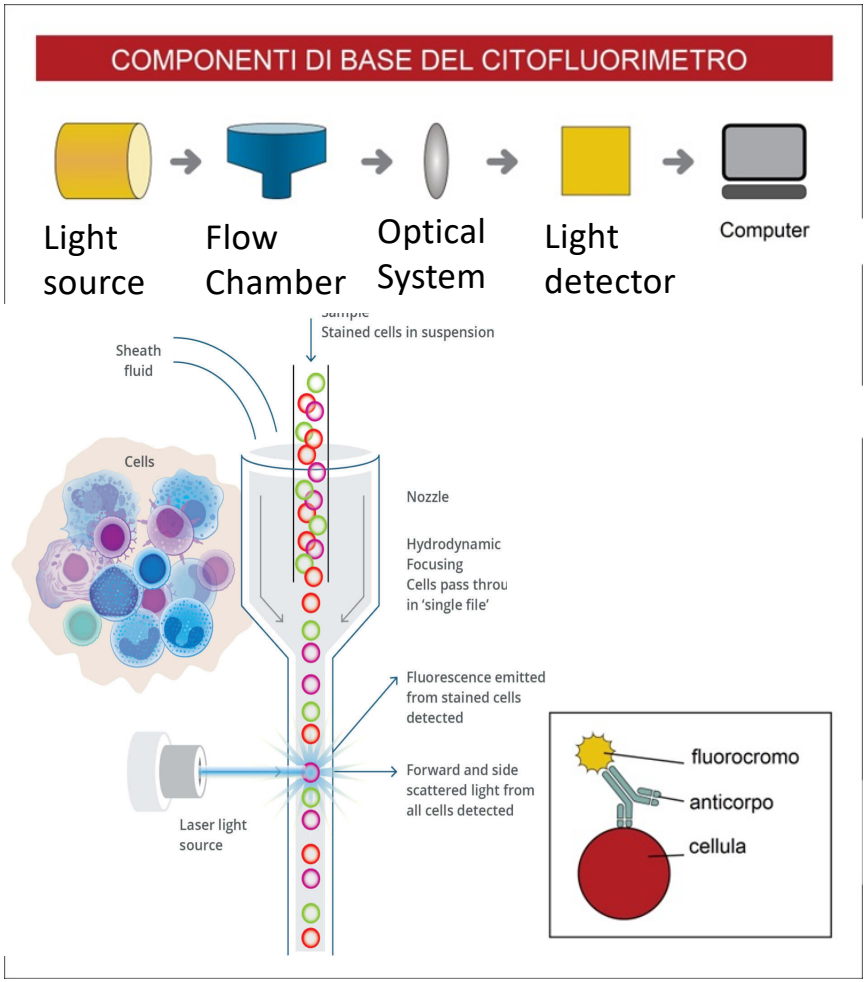
normal peripheral blood



Overview of a flow-cytometer

TO ANALYZE INDIVIDUALLY THE CELLS, LETTING THEM PASS IN A DETECTION CHAMBER, WITH A HIGH SPEED OF ANALYSIS.

The detection system is composed of: light sources, optical group, detector



Operating principles of a flow cytometer

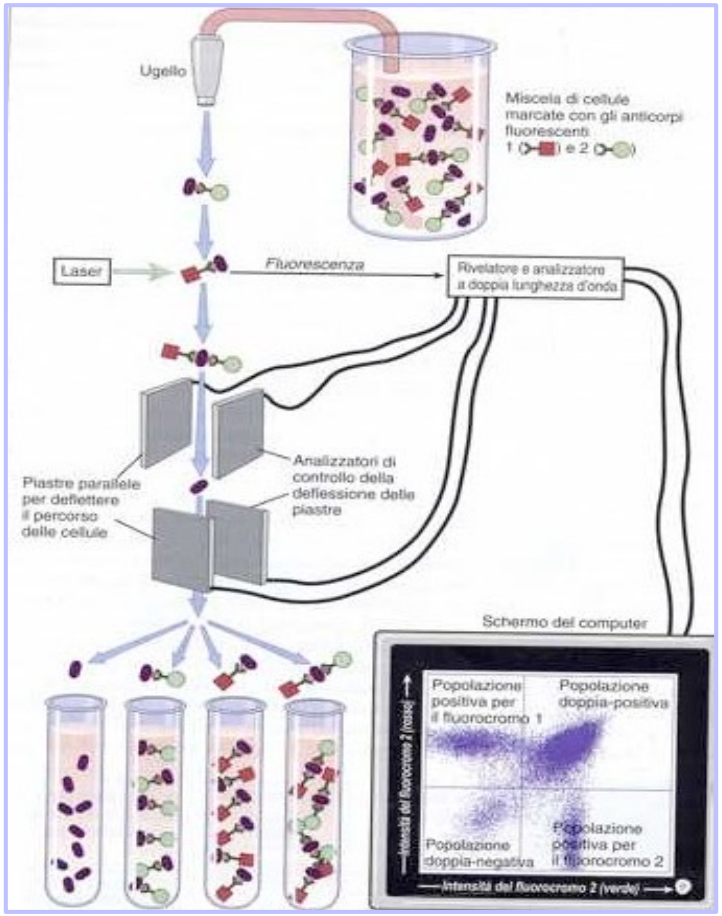
1)Cells from a heterogeneous population are aspirated from the tube and placed into a flow chamber where they are separated from each other.

2)Each single cell is then crossed by a beam of light which excites the fluorochromes and causes the emission of a fluorescent signal.

3)The signal passes through a system of filters and mirrors and reaches a detector.

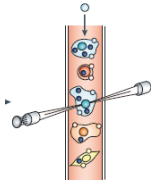
4)It is then processed electronically, transformed from analog to digital and sent to the analyzer, which processes the data and displays it via a graph.

5)Using deflection plates, the analyzed cells can be collected separately through a process called "sorting».



Main advantages of Flow-Cytometry

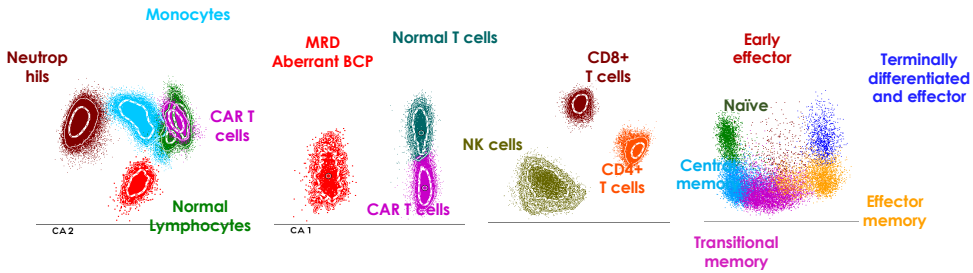
- Single cell analysis



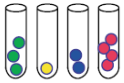
- Heterogenous populations of cells, gating of the population to be analyzed

- Analysis of rare events (even <1%)

- Simultaneous analysis of numerous parameters per cell



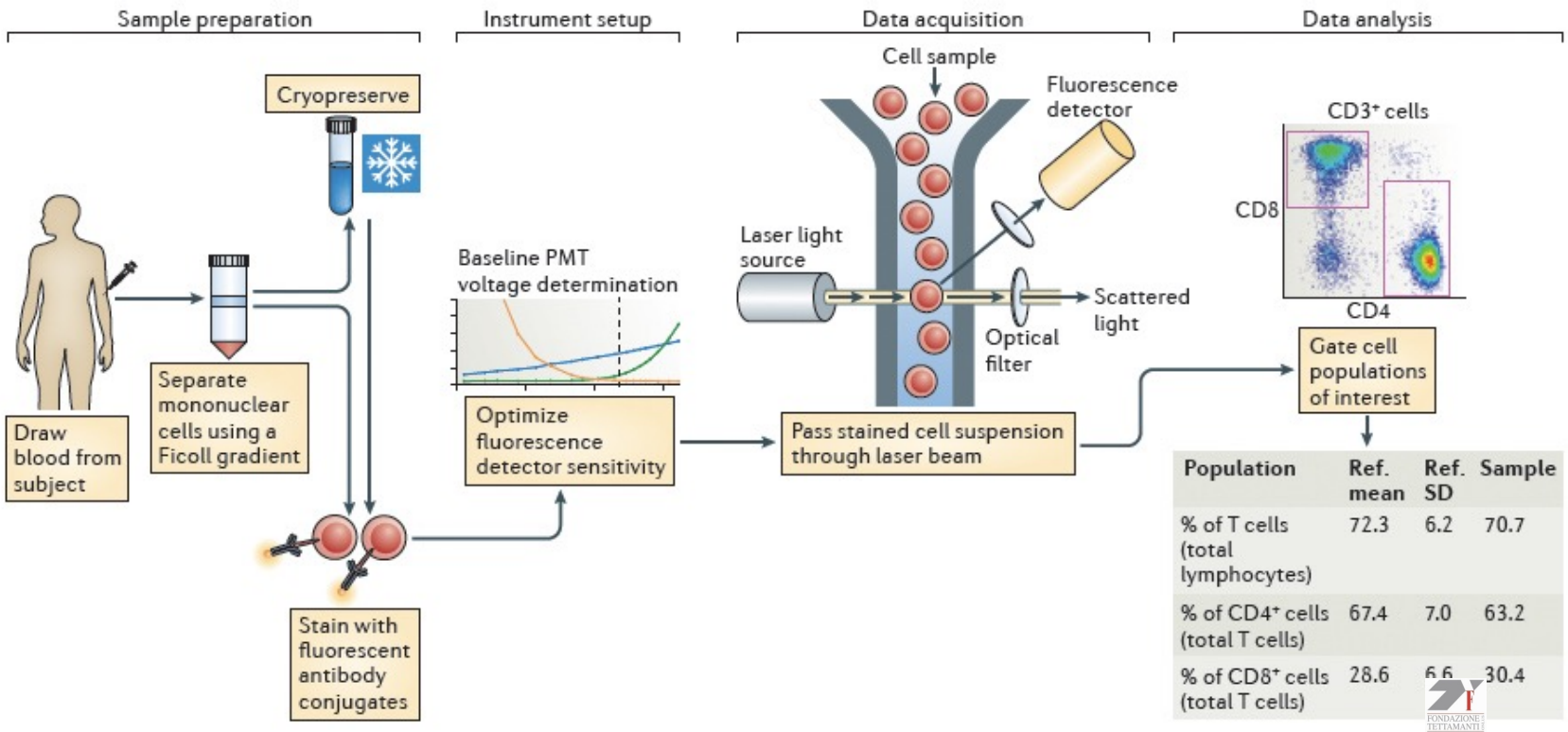
- Fast results from many samples



- Analysis of a high number of events (in the order of at least 10^6 cells)

- Low analysis time (sec)

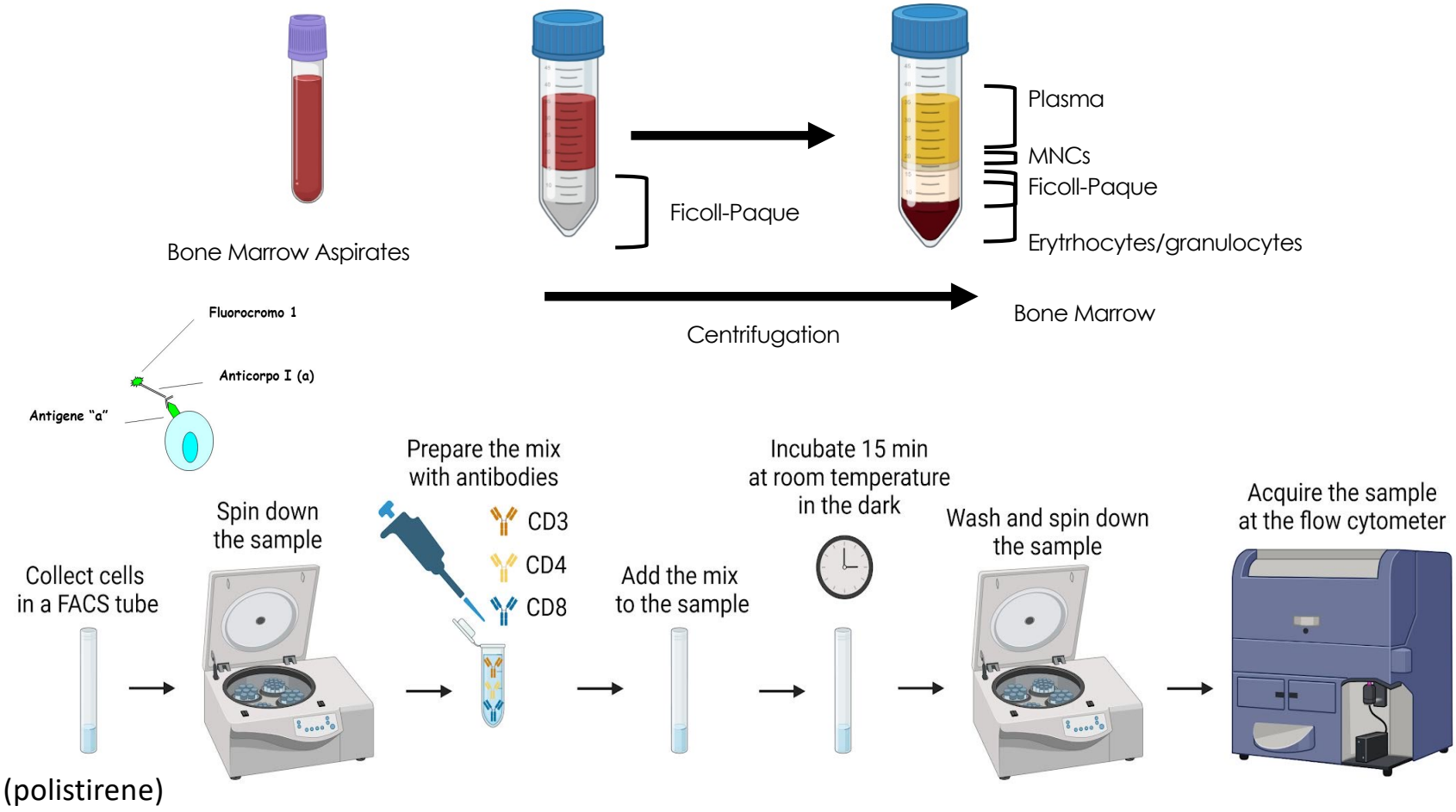
Complexity of measurements in Flow Cytometry: many variables



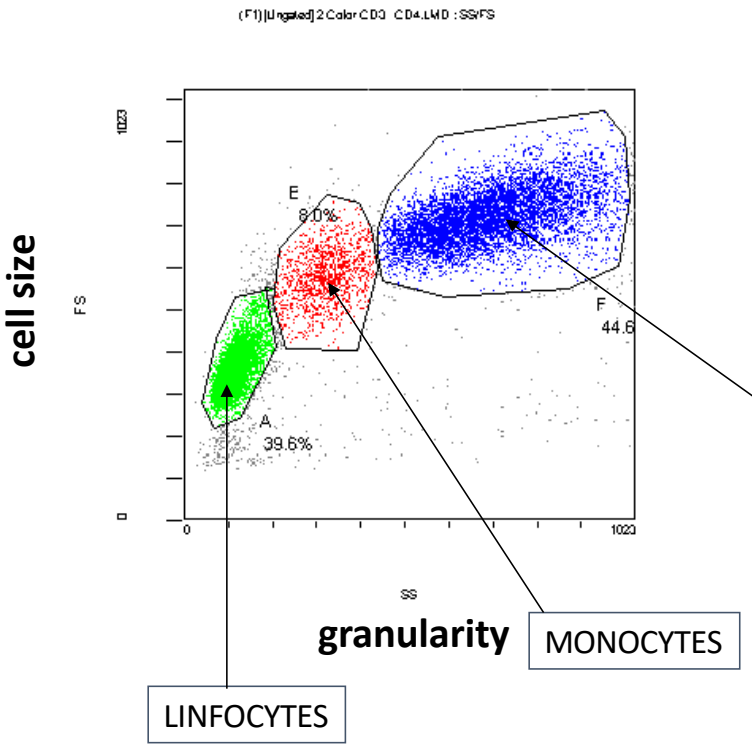
Population	Ref. mean	Ref. SD	Sample
% of T cells (total lymphocytes)	72.3	6.2	70.7
% of CD4⁺ cells (total T cells)	67.4	7.0	63.2
% of CD8⁺ cells (total T cells)	28.6	6.6	30.4

Maecker HT, McCoy JP, Nussenblatt R. Nat Rev Immunology 2012; 12: 191-200

Sample preparation



How to analyse a sample: side scatter *vs* forward scatter



Defining a gate means drawing a region around the population you plan to analyze

GRANULOCYTES

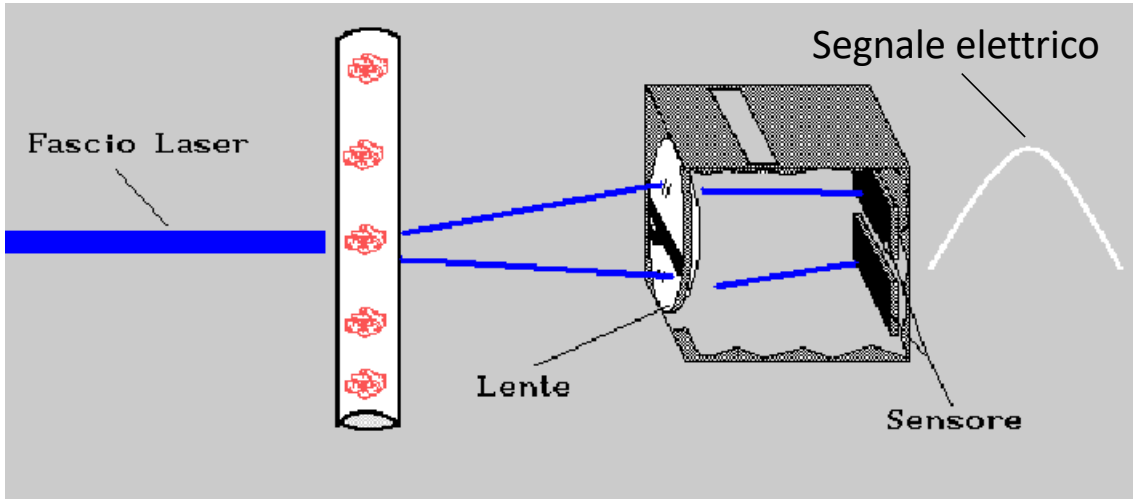
MORPHOLOGICAL GATE

MONOCYTES

LINFOCYTES

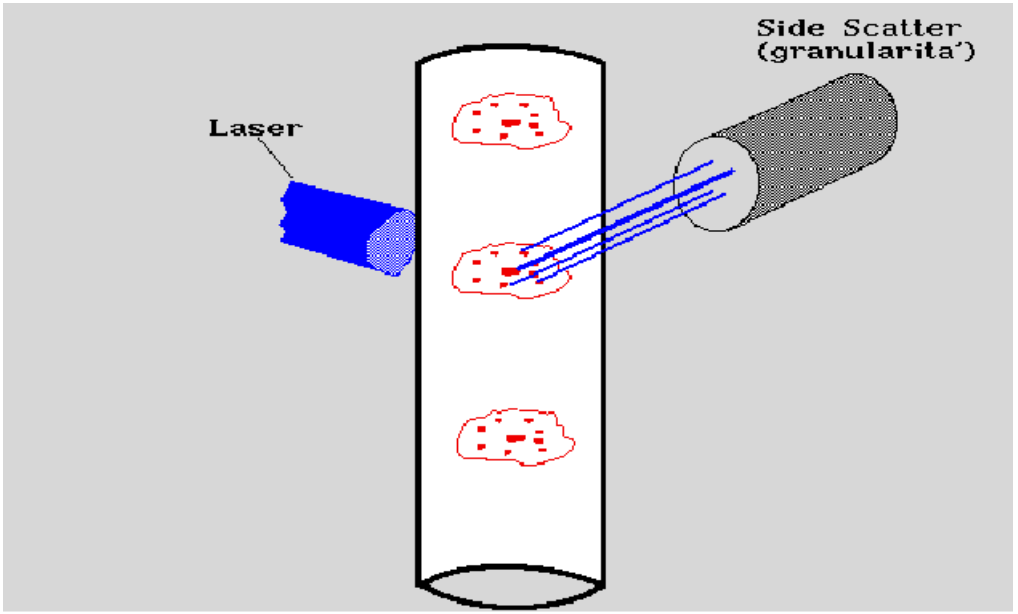
…distinguish the three major leukocyte populations as if looking at them under a microscope

Forward Scatter…cell size

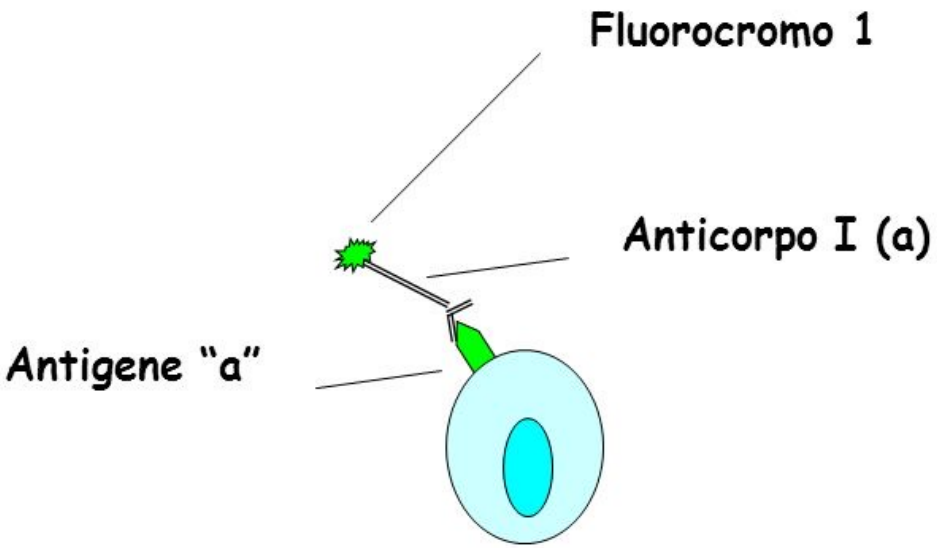


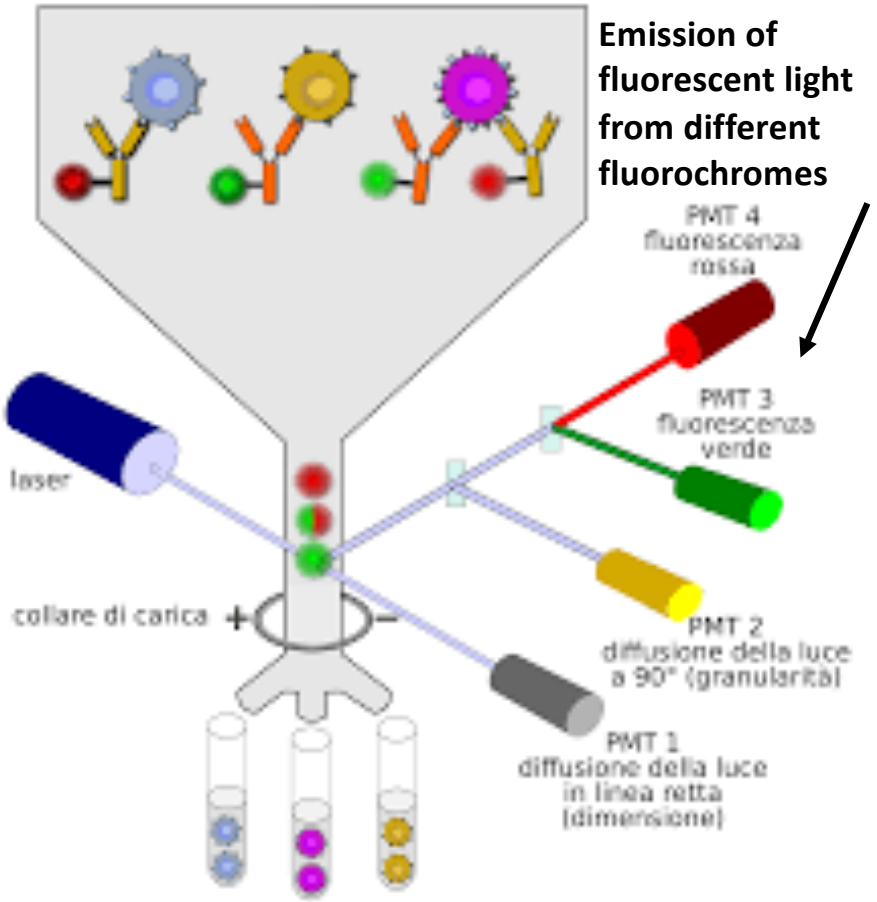
Forward scatter (FS) results from the refraction of light by the surface of the particles. Before being collected by the sensor, the light signal is filtered to reduce its intensity. The light signal is converted into an electrical pulse, this pulse is proportional to the size of the particles.

Side Scatter...granularity of the cells



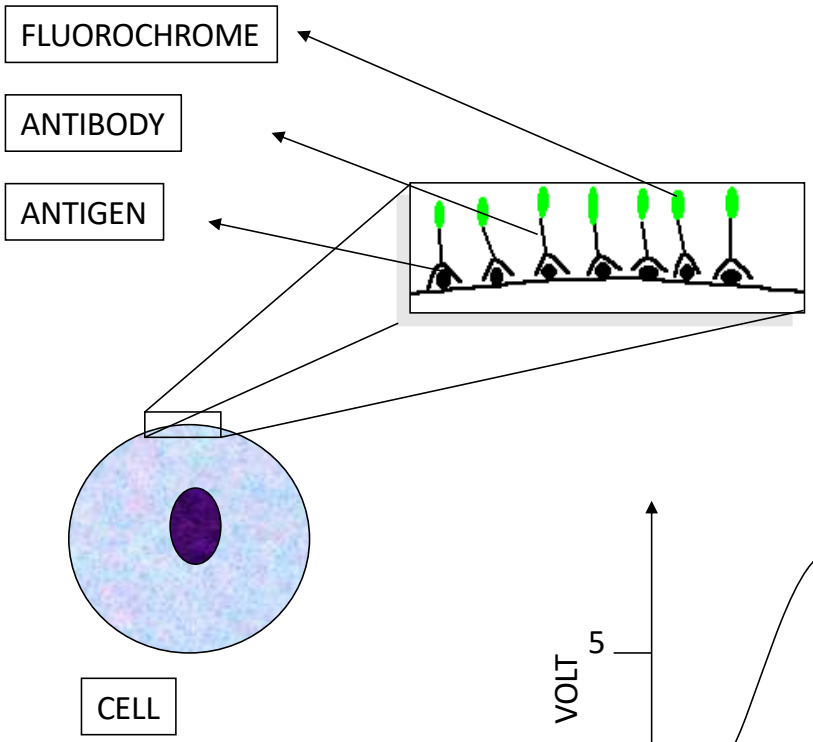
The laser light deflected by the granularity of the cells creates Side Scatter (SS) or 90° light scatter. The electrical signal produced will therefore be proportional to the structural complexity of the cell.





PMT=tubi fotomoltiplicatori

The intensity of fluorescence is directly proportional to the density of antigen receptors present on the cell



High antigenic density

⇓

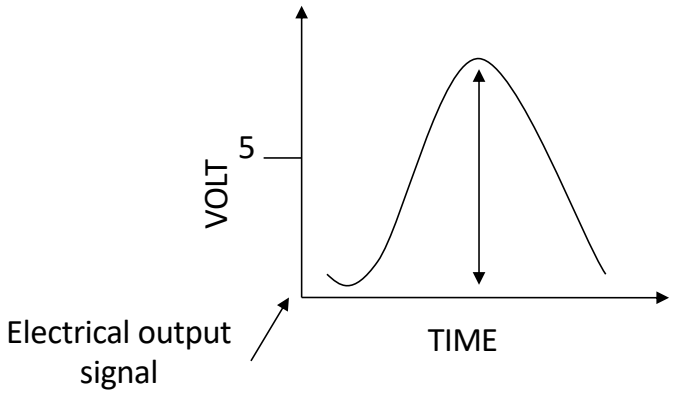
High number of bound antibodies

⇓

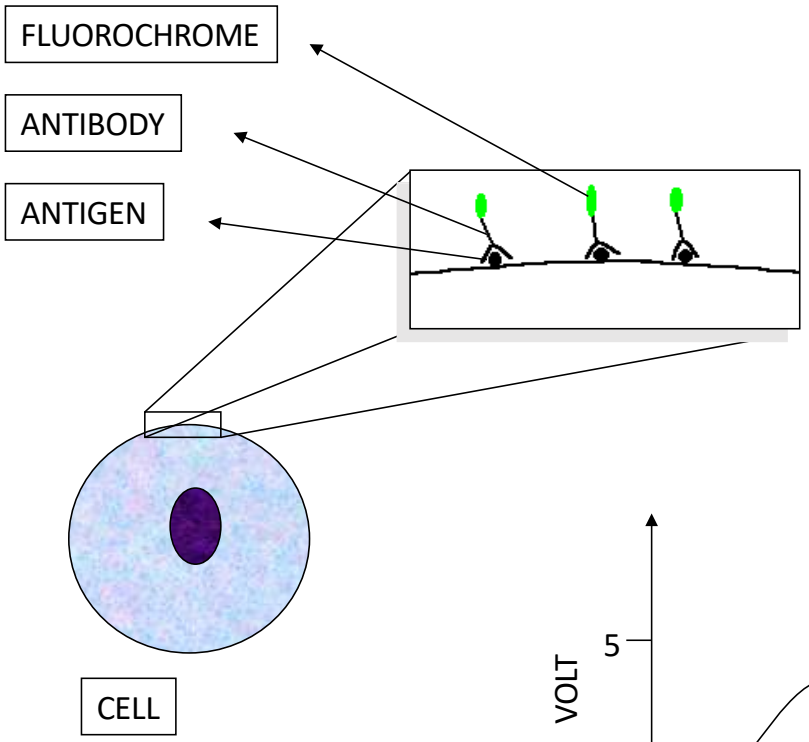
High number of light-emitting fluorochromes

⇓

High signal intensity



The intensity of fluorescence is directly proportional to the density of antigen receptors present on the cells



Low antigenic density

⇓

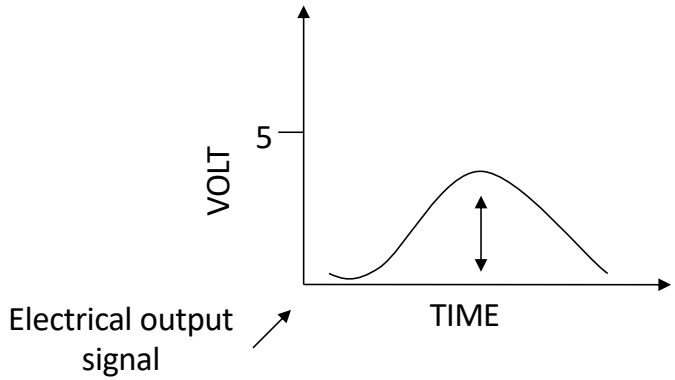
Low number of bound antibodies

⇓

Low number of light-emitting fluorochromes

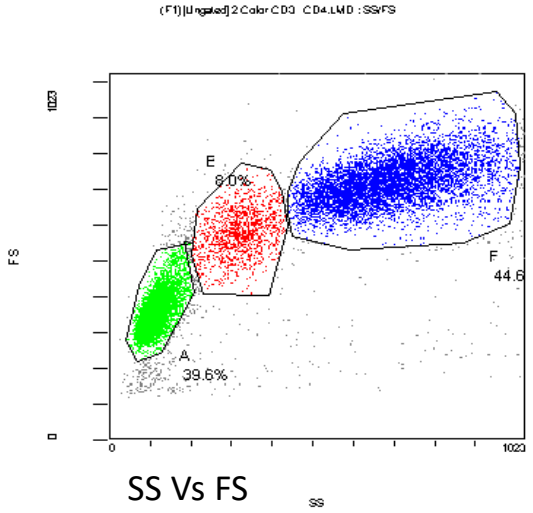
⇓

Low signal intensity

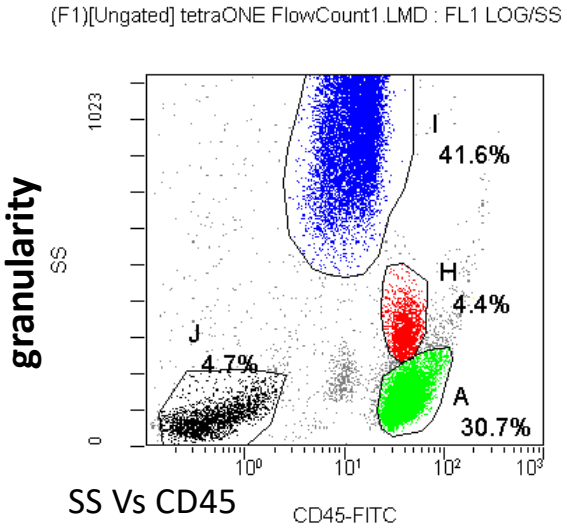


Electrical output signal

Morphological Gate and Immunologic gate



SS Vs FS



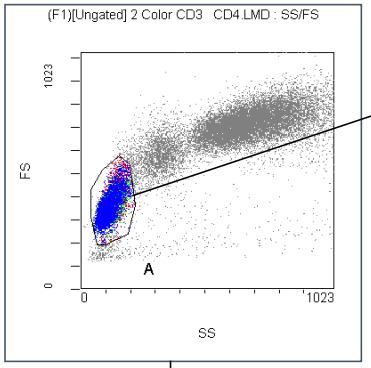
SS Vs CD45

MORPHOLOGICAL GATE

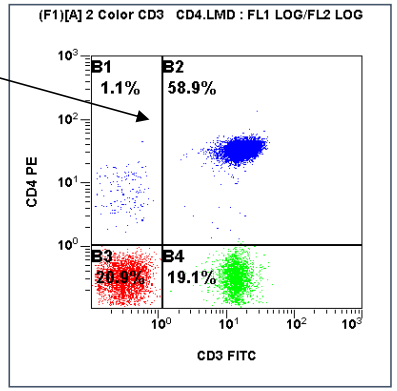
IMMUNOLOGICAL GATE

Using an antigenic parameter to determine the populations to be analyzed

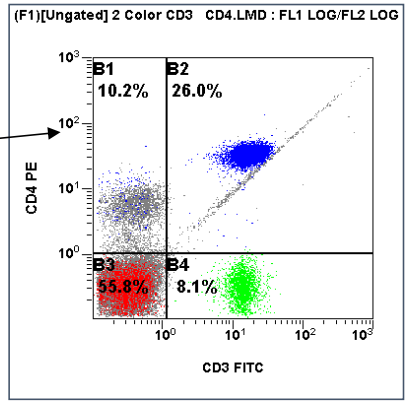
The importance of the gate



The fluorescences represented in the histogram take into consideration only the events that fall within region A



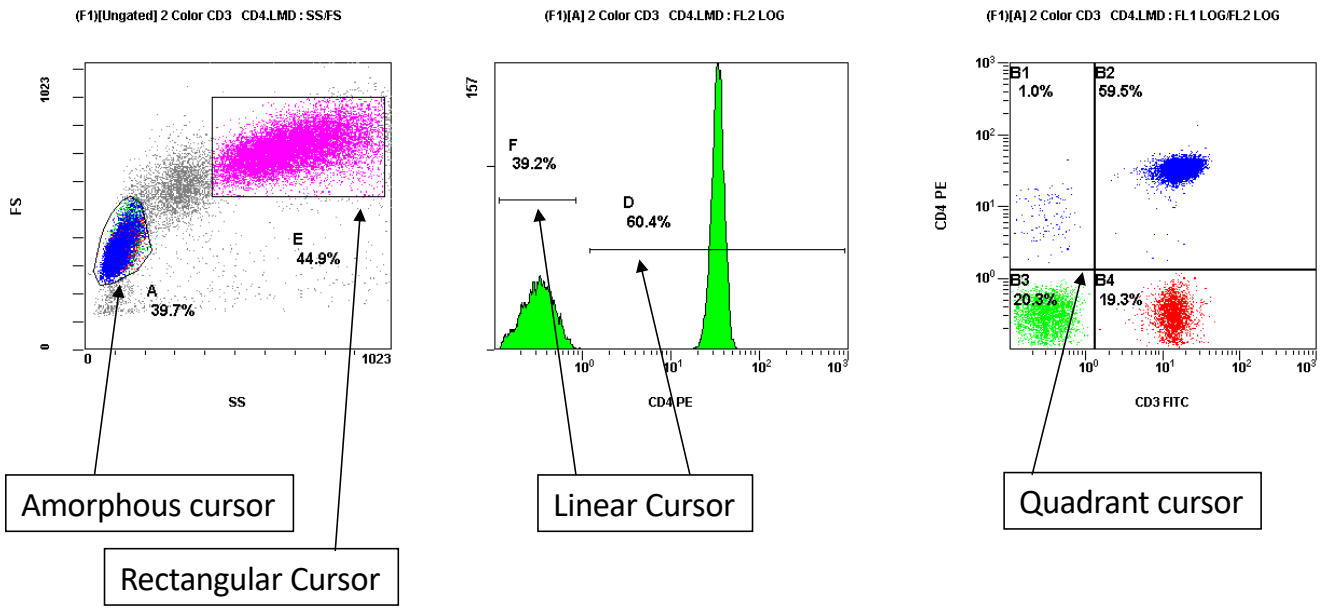
Fluorescences collected on the total sample: NOT IN GATE!



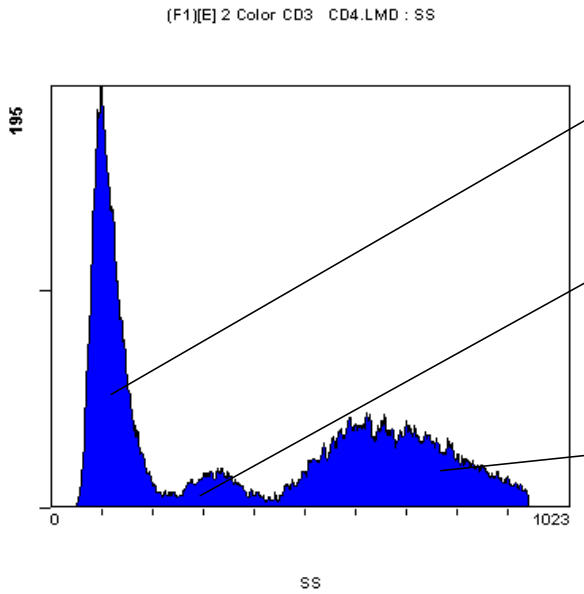
The statistical values of the populations are different

Quantification of the cellular populations

The cursors can be of different types and can be used both as statistical indicators and as gates:



Monoparametric histogram
Side scatter



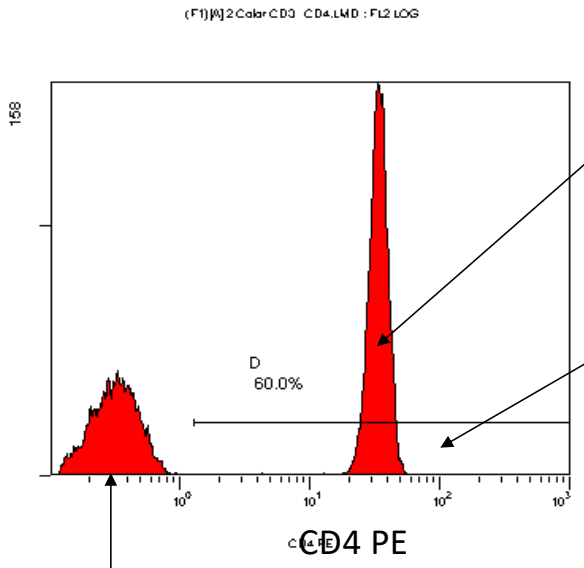
Peak determined by lymphocytes: internal structure not very complex

Peak determined by monocytes: medium complex internal structure

Peak determined by granulocytes: very rich in cytoplasmic organelles

The Side Scatter signal is directly proportional to the structural complexity of cells

Monoparametric histogram fluorescence FL2



Peak of CD4 positive cells, high fluorescence intensity

Cursor “D”: determining the statistic

The intensity of the fluorescence (average accumulation channel) is directly proportional to the density of the antigens present on the cell

Low intensity peak of fluorescence (Background), CD4 negative cells

Clinical application of flow cytometry techniques

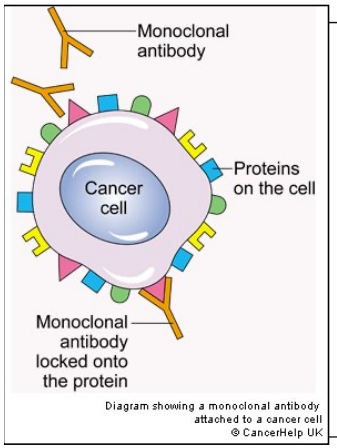
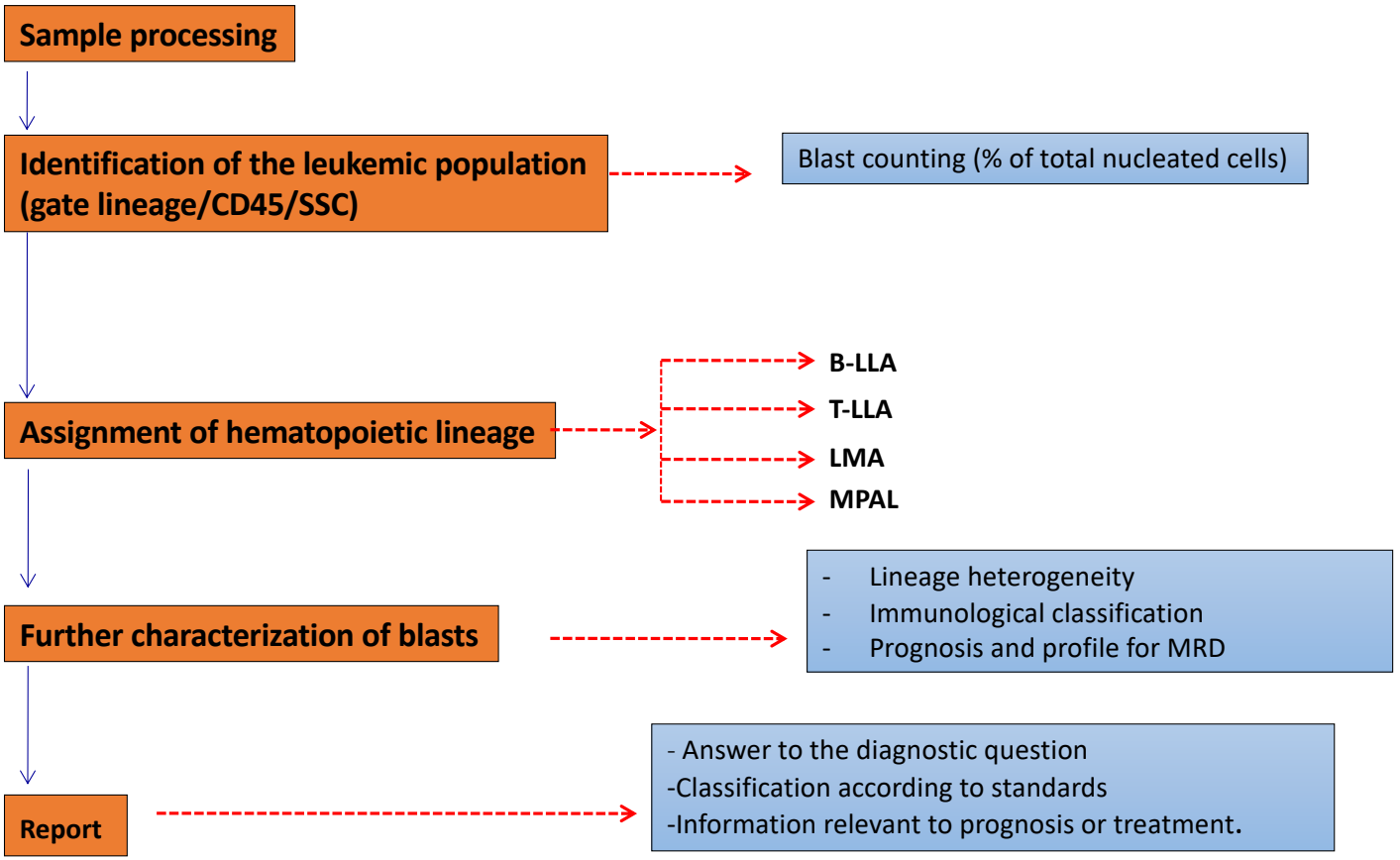


Table I. Summary of clinical applications of flow cytometry

	Clinical Situation	Cell/Analyte of Interest	Patient Specimen Types	Period of Clinical Use
Cancer	Diagnosis of hematolymphoid cancers (mostly leukemias/lymphomas)	Cancer cells	Blood, bone marrow, various tissues	Early 1980s
	Determine cell DNA content for prognosis (e.g. childhood lymphoblastic leukemia)	Cancer cells	Blood, bone marrow	Late 1980s
	Monitoring of hematolymphoid cancers after therapy	Residual cancer cells	Blood, bone marrow	Early 1990s
Immunologic diseases	Diagnosis and monitoring of HIV/AIDS	$CD4^+$ and $CD8^+$ T cell subsets	Blood	Early 1980s
	Diagnosis of primary immunodeficiencies	B cells, T cells, and T cell subsets	Blood, lymphoid tissues	Late 1980s
Cell therapy and transplantation	Determine adequacy of hematopoietic stem cell grafts (bone marrow transplantation) to repopulate bone marrow	$CD34^+$ stem cells	Stem cell graft	Early 1990s
	Risk assessment for graft rejection of solid organs and graft-versus-host disease after hematopoietic stem cell transplantation	Abs to HLA proteins	Recipient's serum	Early 2000s
		HLA genotype genomic DNA	Donor's blood cells	Middle 2000s

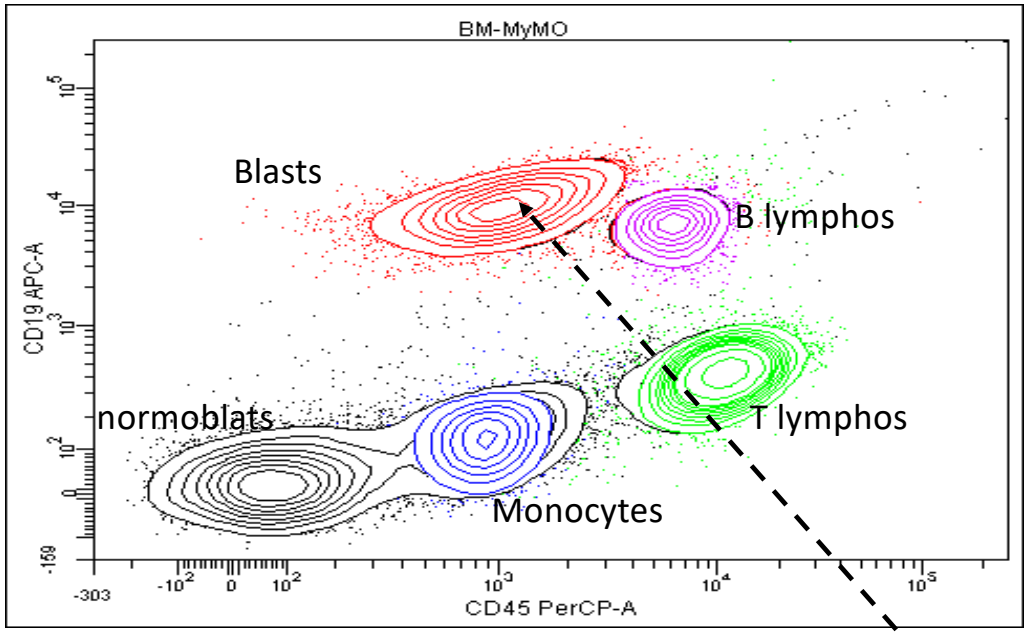
Jaye LA et al, J Immunol 2012

Analytical path for the diagnosis, classification and monitoring of acute leukemias using flow cytometry



The addition of a lineage marker together with CD45 improves the resolution of the tumor population

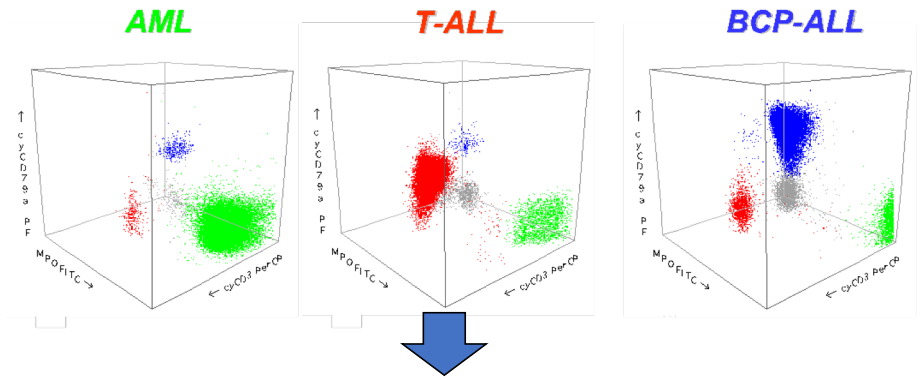
At least 30,000 nucleated cells for each tube in order to guarantee a sensitivity of 1% in the identification of leukemic cells and residual normal cells (cluster of 300 events).



Immature/abnormal cells

Borowitz MJ et al, Am J Clin Pathol, 1993

Lineage discrimination as a mandatory step



Appropriate treatment

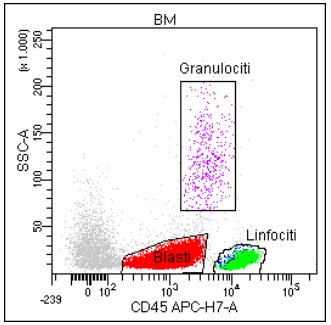
Lineage	Positive	Antigens
BCP-ALL	≥2 of:	§ CD19; CD10, (i)CD22, iCD79a
T-ALL	all 3 of:	# (i)CD3, CD7; iMPO negative or weak
AML	≥2 of:	CD13, CD33, CD64, CD65, CD117, iMPO and BCP-/T-ALL criteria not met

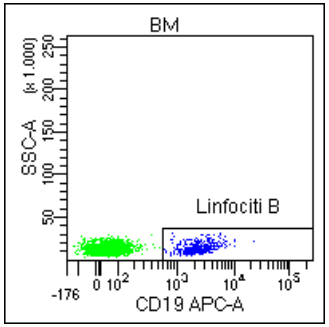
§ BCP-ALL needs strong positivity in ≥2 of the four antigens

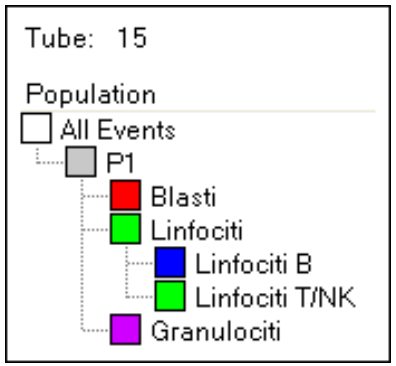
For T-ALL, (i)CD3 positivity must be either strong, or if rated weak at least one of two additional requirements must be fulfilled:

- (i)CD3 should be expressed on a separate blast population as bright or nearly as bright as on normal T cells and/or CD2 and/or CD5 should be any positive in addition.

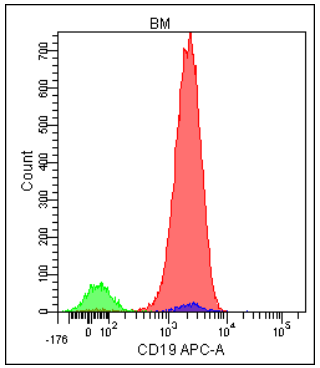
Gating Strategy and internal controls

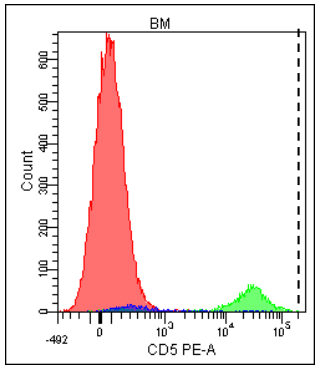






Blasts CD19

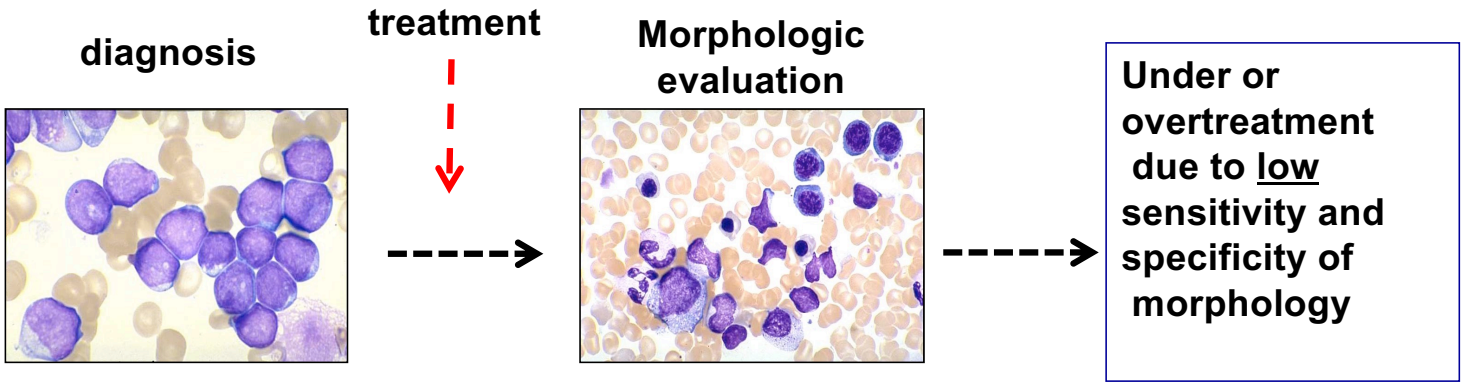




T lymphocytes are negative and serve as a negative internal control marker

If I use a marker like CD5 positive for T cells, B cells are negative

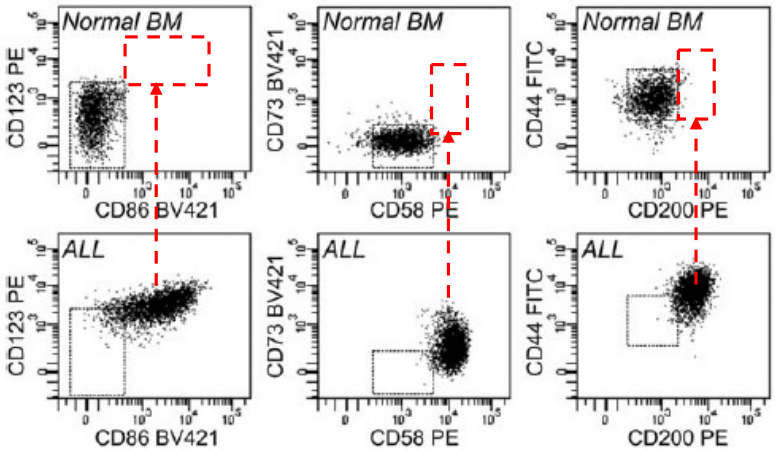
Minimal residual disease in acute lymphoblastic leukemia



Flow cytometry is a method used to study minimal residual disease

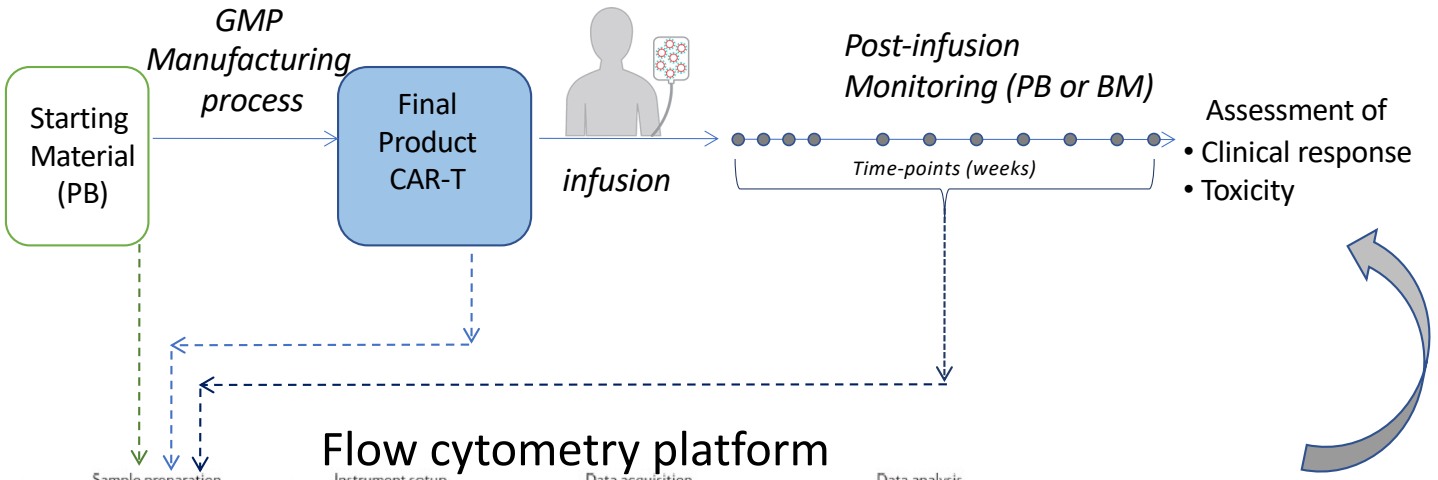
Table 1
Main Features and Applicability of the Methods Widely Used for Study MRD in Pediatric ALL

Method	Target	Applicability	Sensitivity	Advantages	Disadvantages
Flow cytometry	Leukemia-associated immunophenotypes	95% or more	10^{-4}	Widely applicable; rapid; accurate and direct MRD quantification	Operator-dependent; need for further standardization
Real-time quantitative (RQ) PCR	Fusion gene transcripts	40%	10^{-4}	Rapid; reflects the leukemic clone size	Uncertain quantitation of MRD; not completely standardized; potential false-positive results due to RNA degradation.
	Ig/TCR gene rearrangements	90–95%	10^{-5}	Widely applicable; highly sensitive; accurate MRD quantitation	Laborious and costly;need for experienced personnel and standardization; oligoclonality and clonal evolution may produce false-negative results

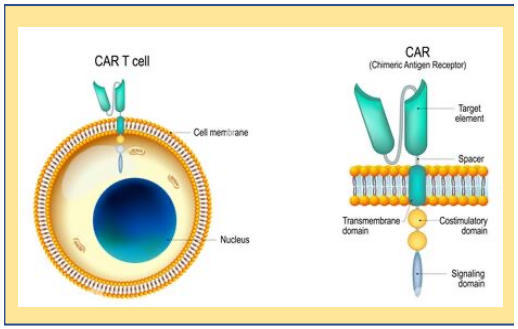


Gaipa et al Cytometry B, 2013

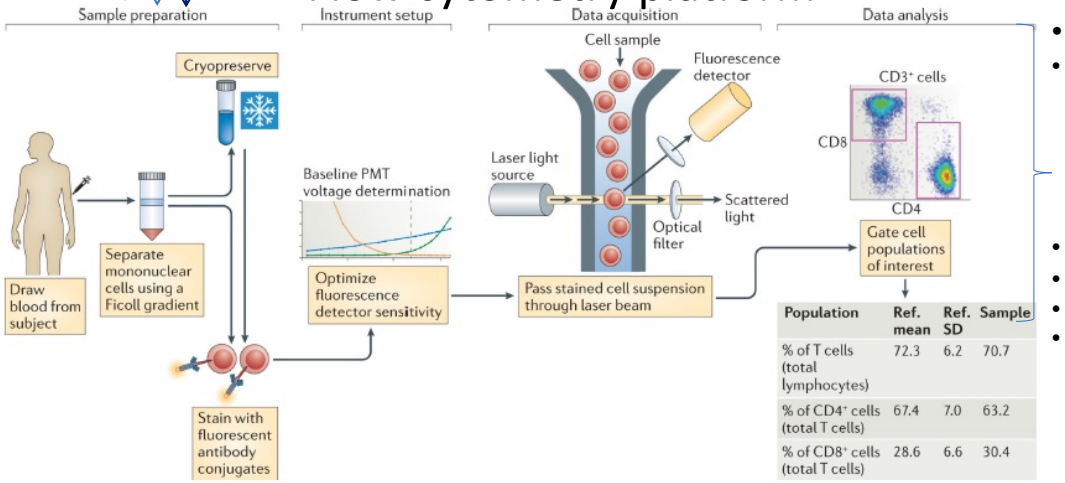
Flow cytometry in CAR-T therapy: an innovative application



Ability for simultaneous detection of multiple parameters on a single cell *(Kalos M et al,. Sci.Transl. Med. 2011, Kochenderfer JN et al, Nat. Rev. Clin. Oncol 2013)*



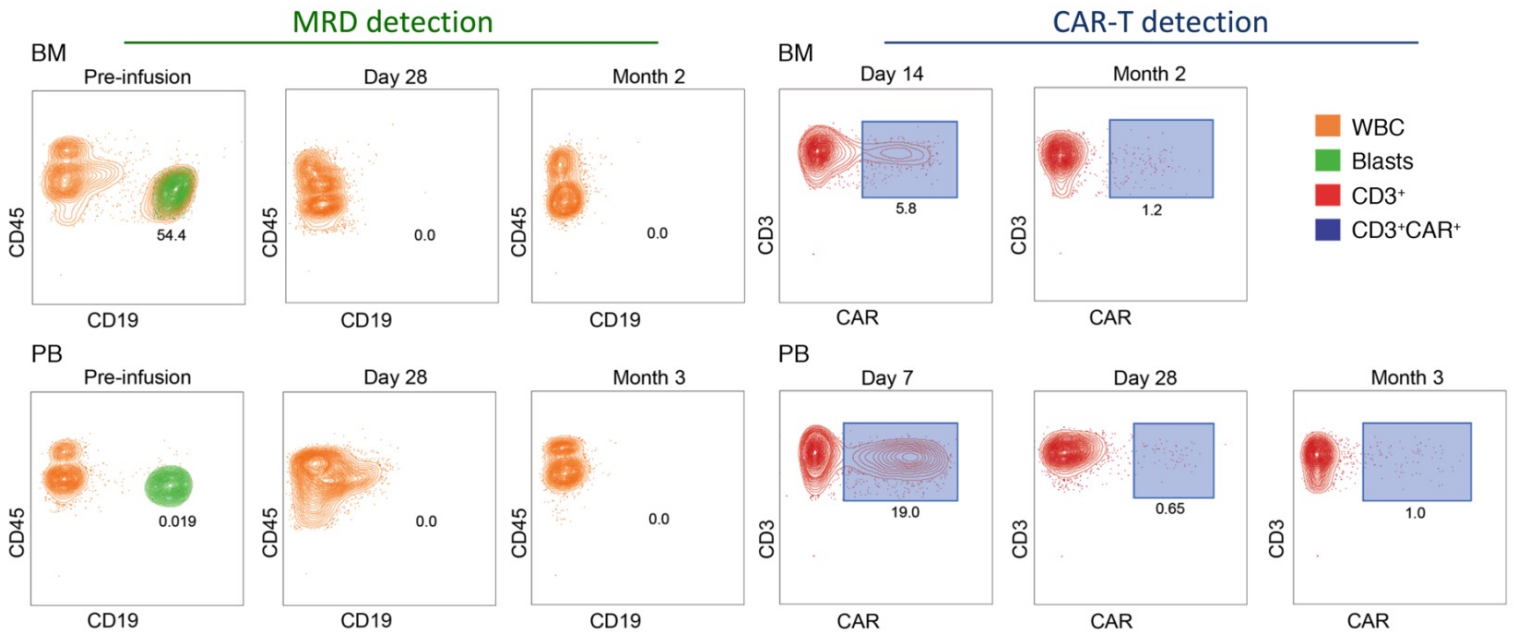
Flow cytometry platform



- CAR expansion kinetics
- CAR phenotype:
  - memory differentiation
  - helper/cytotoxic phenotype
  - activation markers
  - exhaustion/senescence markers

- MRD
- B-cell aplasia
- immune reconstitution
- cytokines secretion

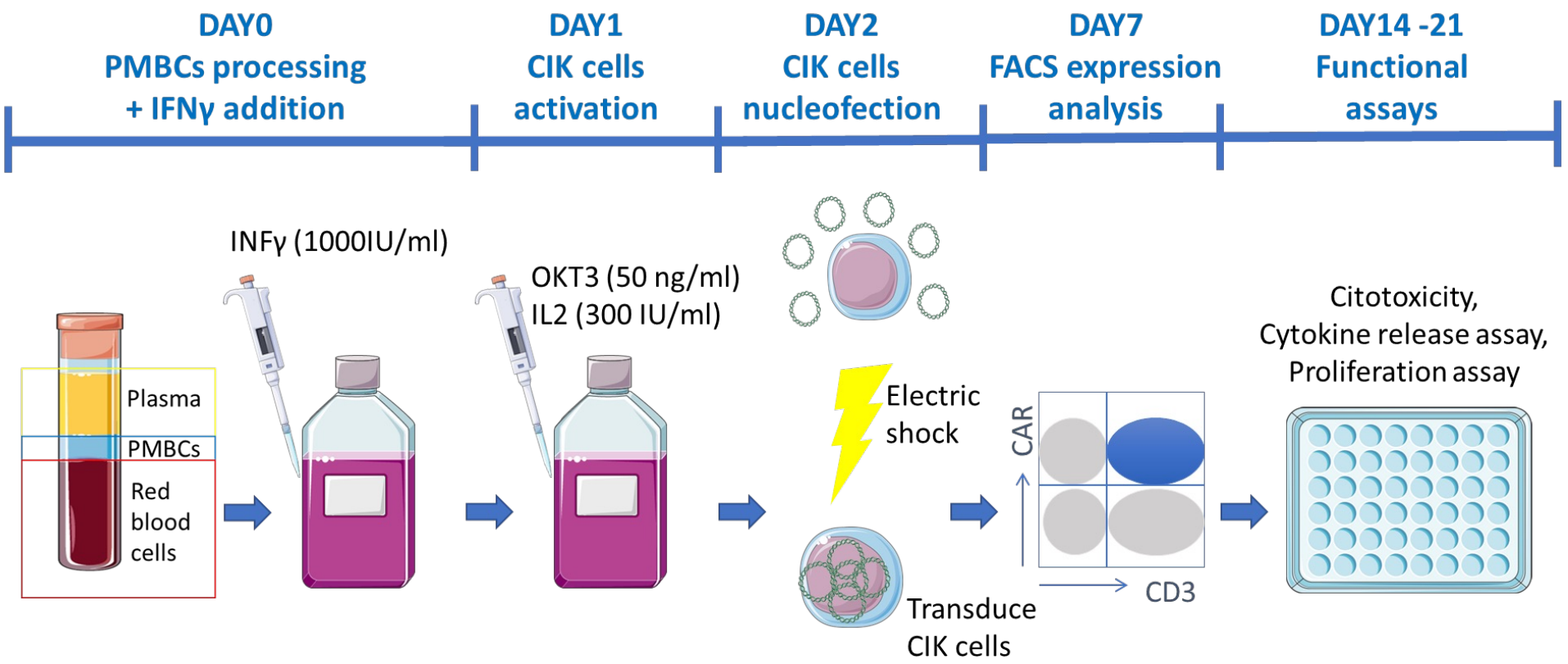
Monitoring simultaneous of MRD e CARCIK-CD19 cells



- CAR T cells were effective in promoting ablation of malignant CD19+ cells in PB and BM.
- CD19+ cell ablation was achieved in association with expansion and persistence of CAR T cells, even in a patient with massive BM blast infiltration persisting after lymphodepletion

C.F. Magnani et al, JCI 2020

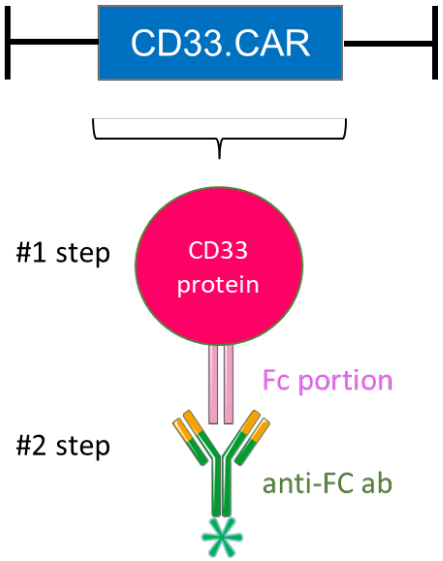
The production process of CAR cells

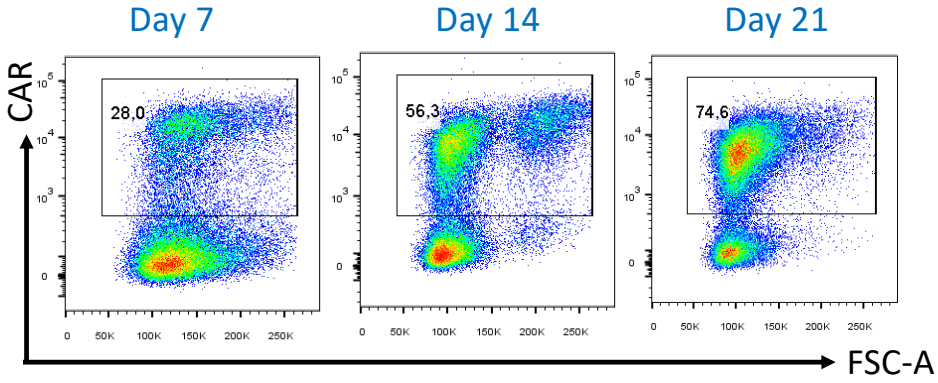


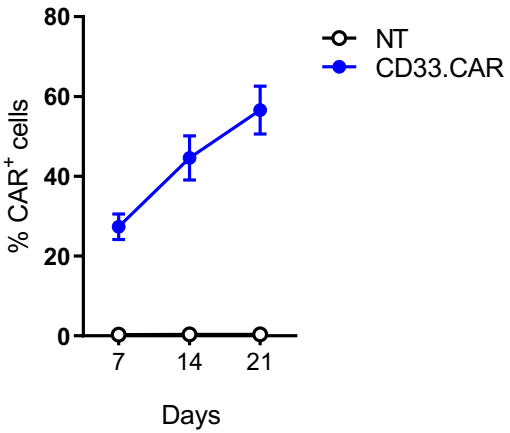
Monitoring of CAR expression

Staining a due passaggi

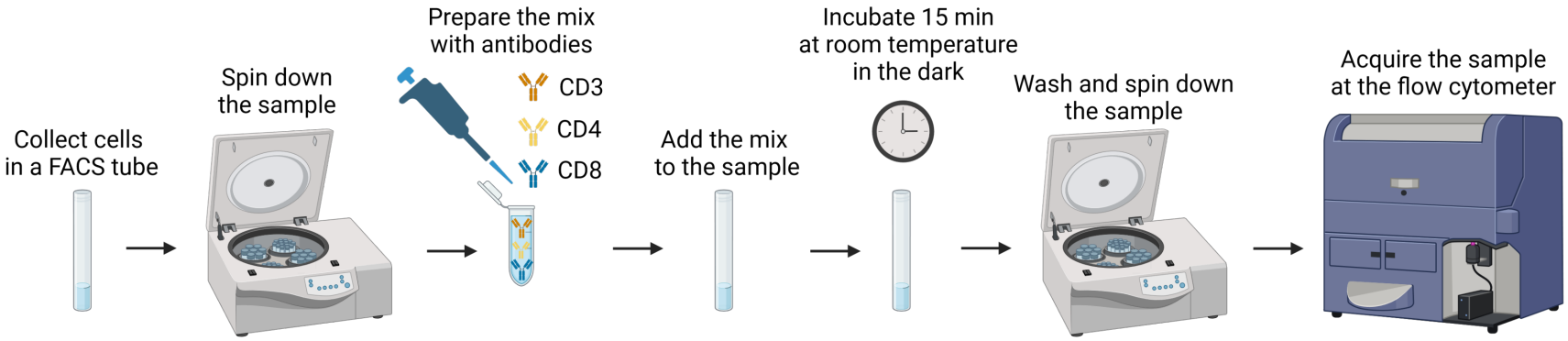
1. hCD33Fc
2. anti-FC coniugato con un fluorocromo(2-step)

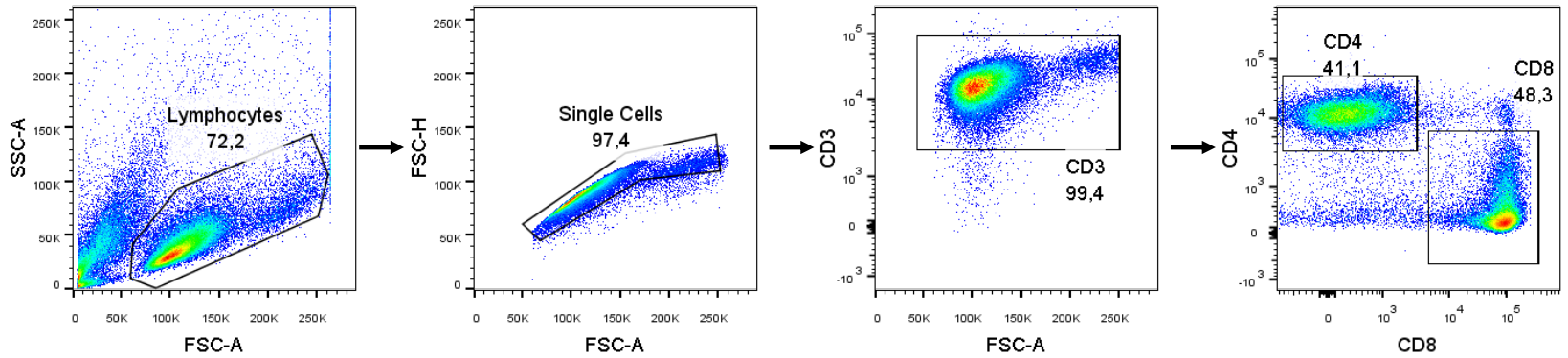




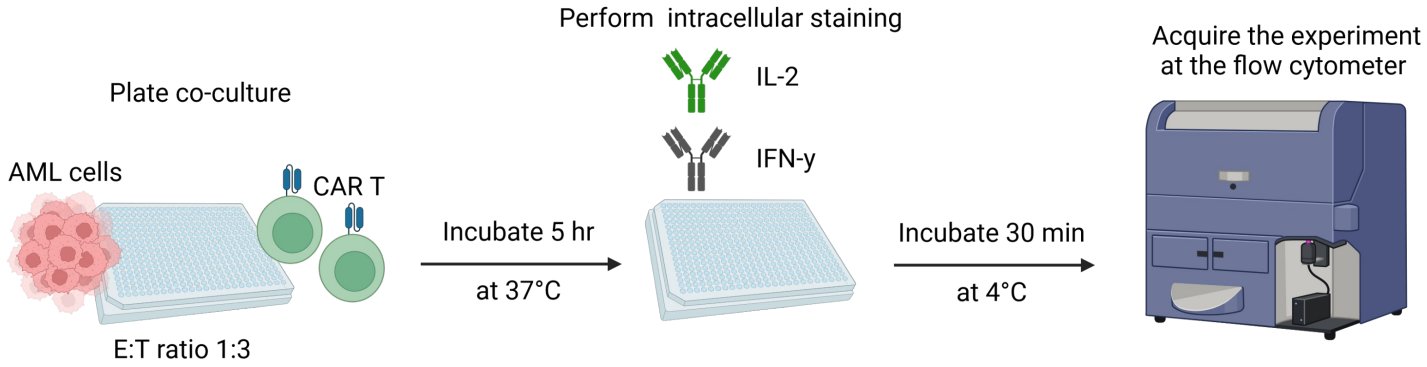


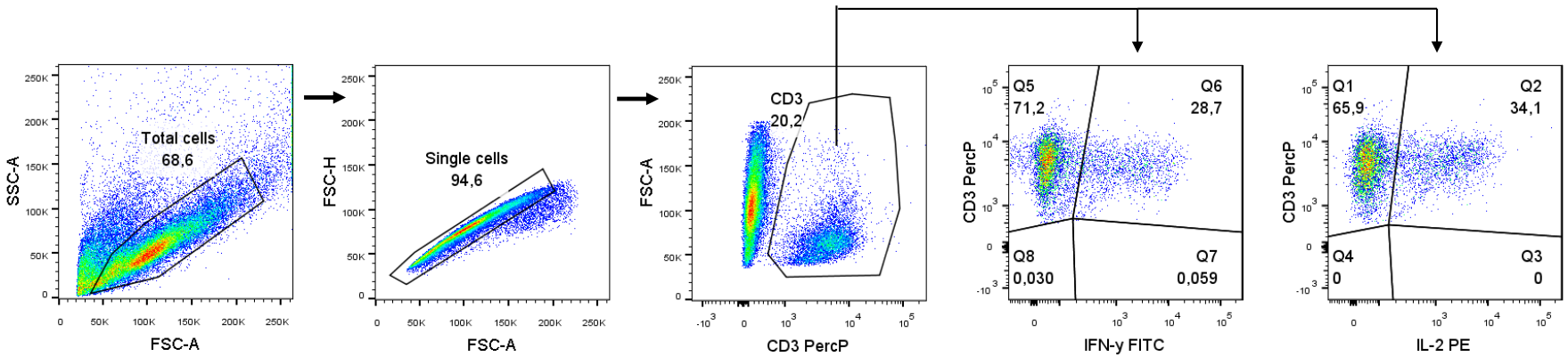
Evaluation of the phenotype of CAR T cells



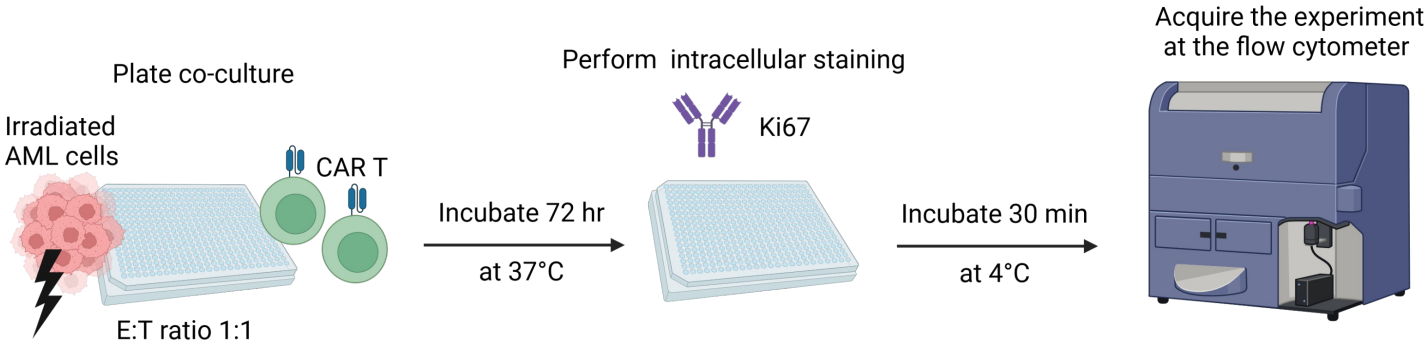


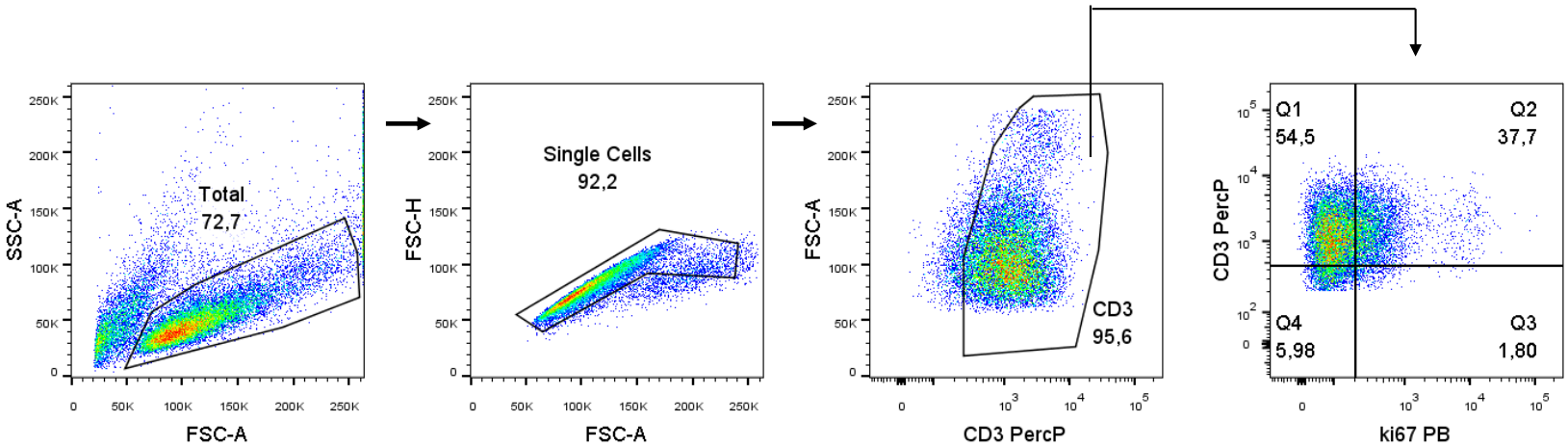
Cytokine release: intracellular test



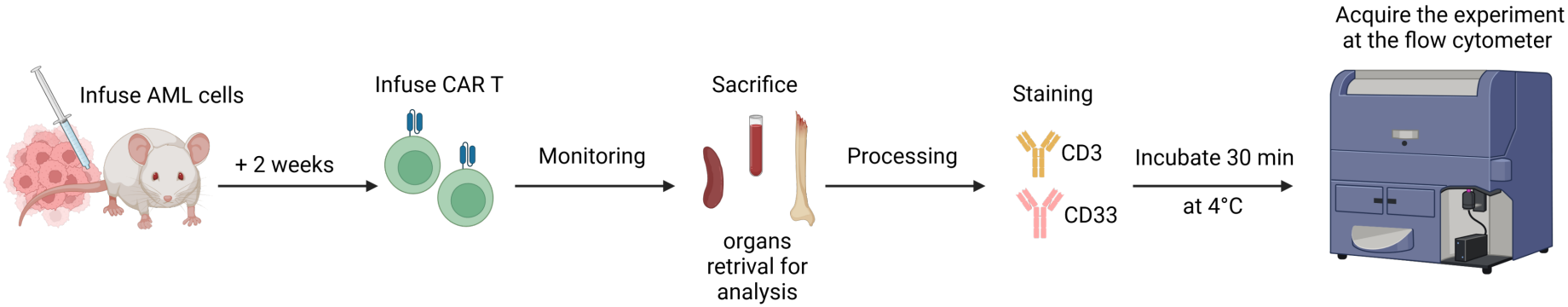


Proliferation test



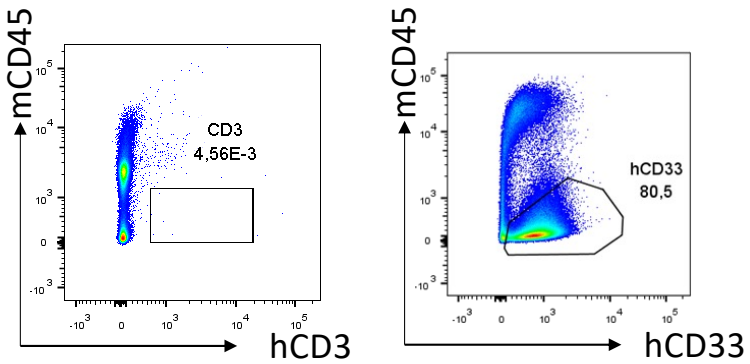


In vivo evaluation of the anti-tumor activity of CAR cells

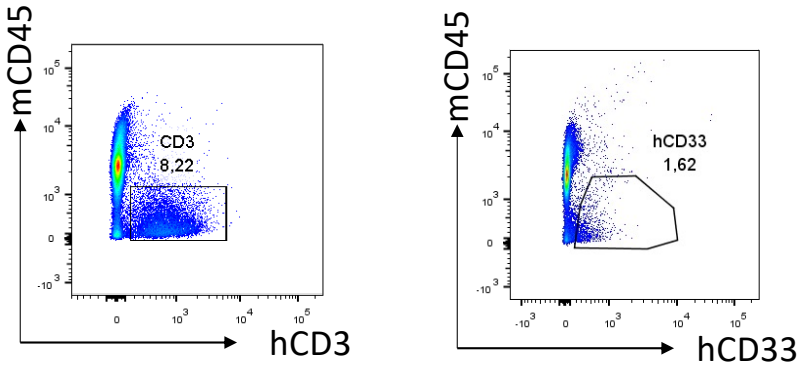


Bone marrow

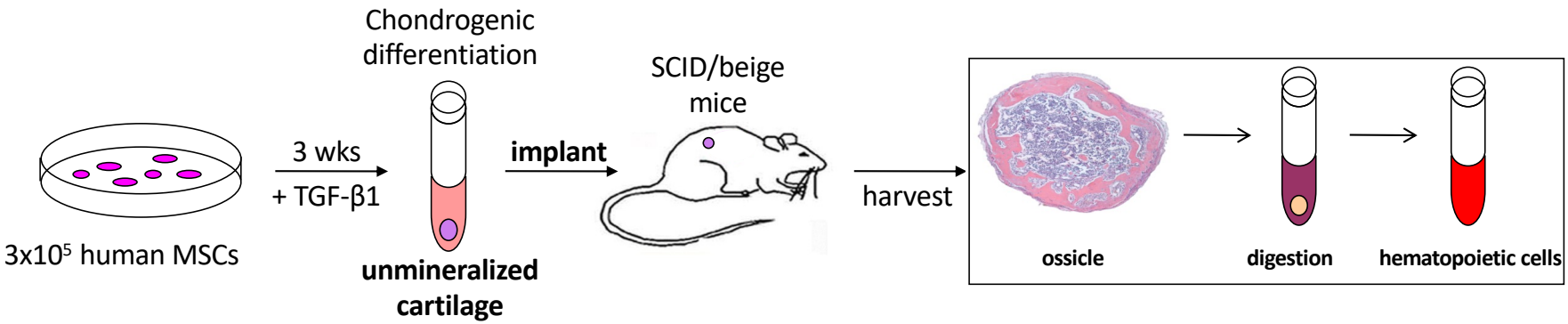
Control



Treated

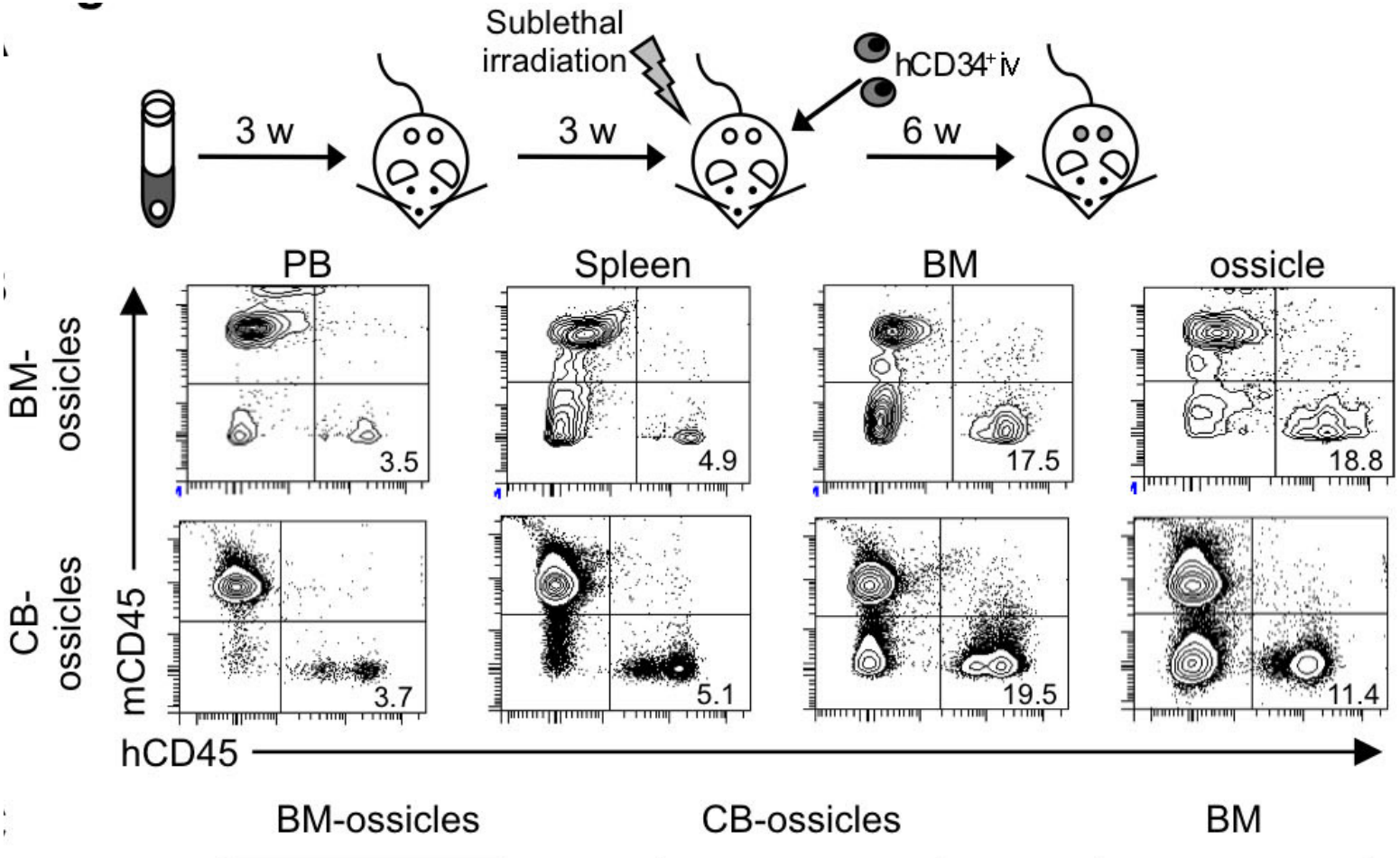


Flow cytometry in translational biotechnology(I)



Detectare cellule umane in un modello di trapianto eterotopico

Flow cytometry in translational biotechnology(II)



Flow Cytometry today

In recent years, CFM has achieved considerable diffusion, both in clinical laboratories and in research laboratories.

As we have seen, it is an extremely important tool also in the field of advanced therapies. Factors that contributed to this remarkable development: the possibility of using multiple emission lasers, which allows multiparametric analyzes to be carried out in 4 or more colours; the availability of MoAbs labeled with a wide range of fluorochromes and directed against a very wide variety of membrane and/or intracellular Ags; reducing costs and complexity in using the tool.