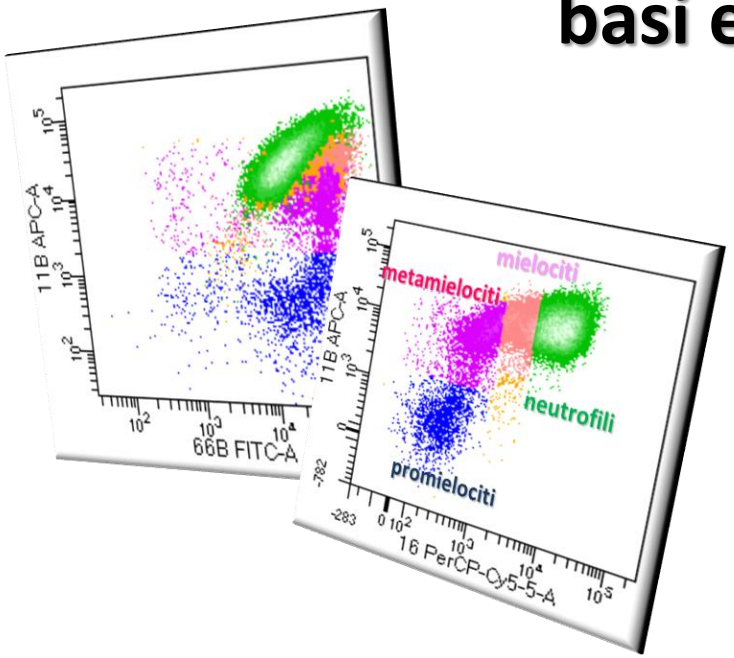
Università degli Studi di Padova
Dipartimento di Medicina - DIMED

Rare Disease Day in DIMED
Technical Advances
CORSO DI FORMAZIONE: Microscopia in fase fluida

Citometria a flusso e Next Generation Flow: basi ed applicazioni

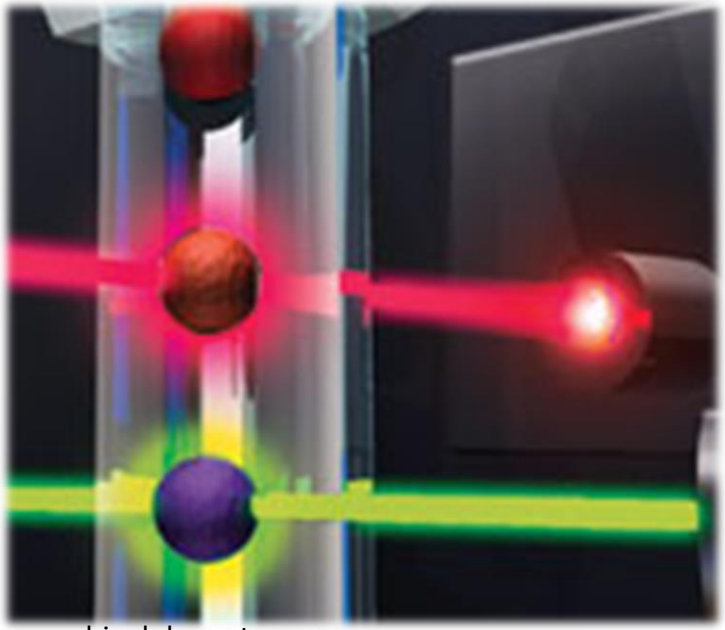


Dr. Valentina Trimarco
Ematologia e Immunologia Clinica, DIMED

1 Marzo 2019

FLOWCYTOMETRY

Laboratory method that allows an automatic analysis of monodisperse cell suspensions measuring cytological and/or biochemical characteristics within a laminar flow which intersects a light source, acquiring and storing more parameters (*volume, granularity, fluorescence*) for each acquired cell.



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FLOW CYTOMETRY YESTERDAY…

The appearance of flow cytometry occurs around the 70s, resulting in a fast and intense development of histological and cytochemical techniques.

Initially it was limited to the measurement of *1-2 parameters*: physical and fluorescence.

The great complexity of the immune system stimulated:

- **the development of MoAb more specific;**
- **the search for new fluorescent dyes;**
- **the creation of multi-parameter flow cytometers.**

…AND TODAY

In recent years, flow cytometry has achieved considerable diffusion, both in clinical and in research laboratories due to:

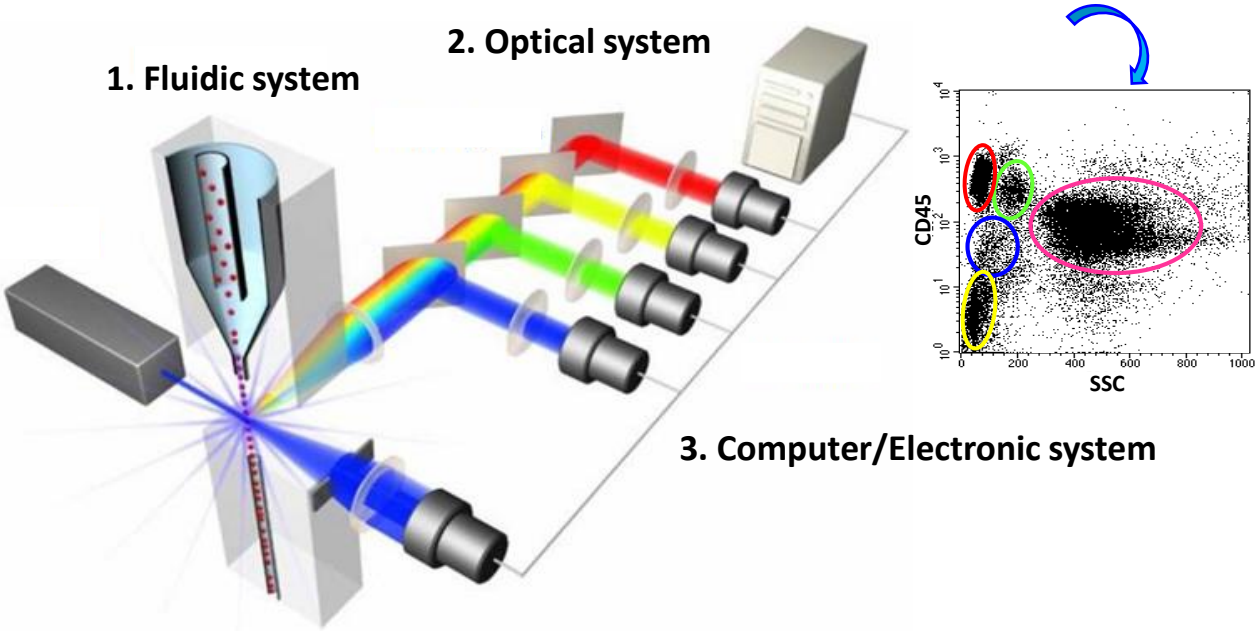
- **the possibility of using more lasers to analyze 6 and more colors;**
- **MoAb labeled with a wide range of fluorochromes and directed against a very wide variety of antigens;**
- **the reduction of costs and complexity in the use of the instrument.**

FACS Canto II

Cytomics FC500

FACS Aria

THE FLOW CYTOMETER



There are three main components:

1 *The Fluidic System*

Presentation of the sample to the laser.

2 *The Optical System*

Gathering information from the scattered light of the analysis.

3 *The Computer/Electronic System*

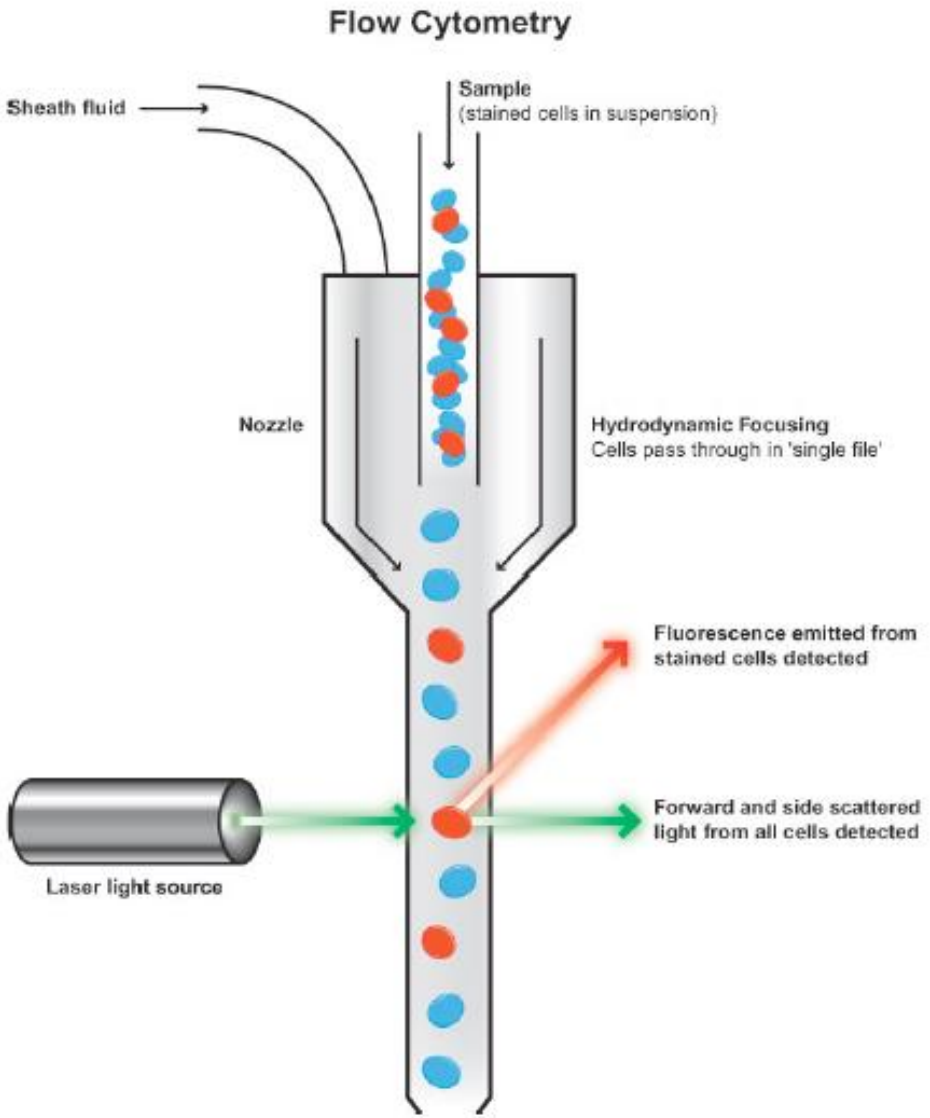
Conversion of optical to digital signals for display.

1. The fluidic system: the hydrodynamic focusing

The cell sample is injected into a sheath fluid.

- **By the laminar flow principle, the sample remains in the center of the sheath fluid.**
- **The cells in the sample are accelerated and individually pass through a laser beam for interrogation.**

This process is known as 'hydrodynamic focusing'.

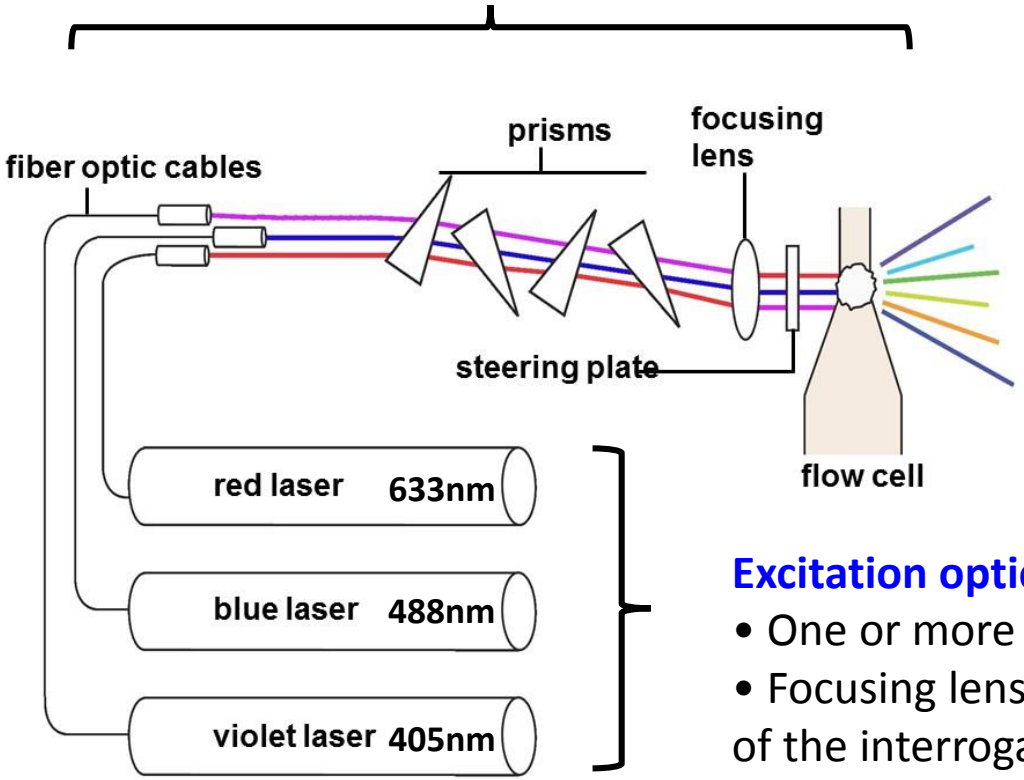


Adan A crit Rev Biotechnol 2016; 1-14

2. The optical system

Detection optics:

- Lens that collects signals generated from the light-single cell interaction.
- System of mirrors and optical filters able to separate the specific wavelengths to be collected from the different detectors.

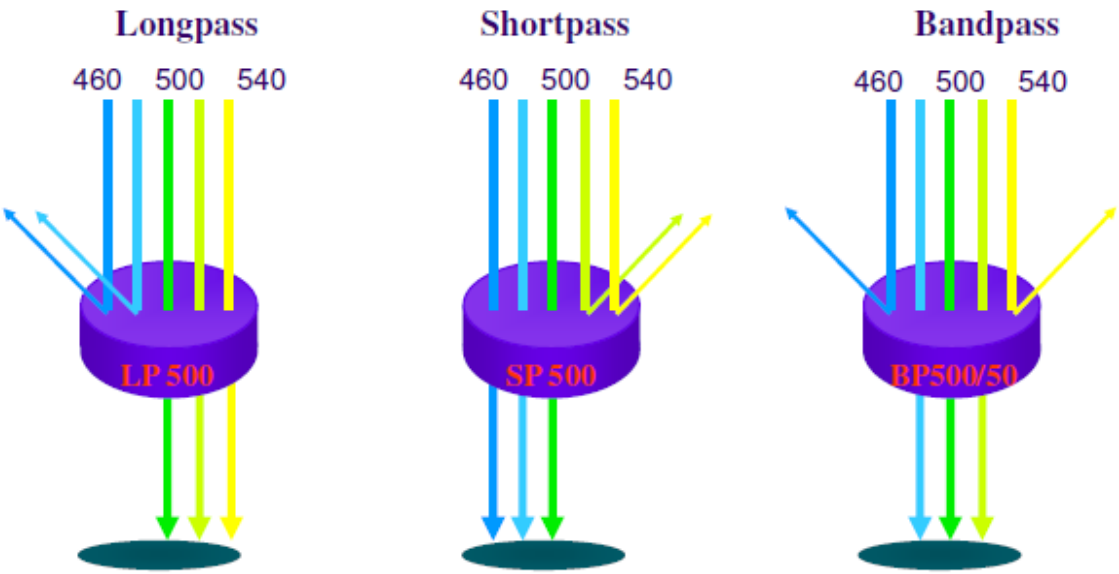


Excitation optics:

- One or more lasers as a light source.
- Focusing lenses to collimate the light beam first of the interrogation point.

2. The optical system

Shortpass, longpass, and bandpass optical filters are used to limit each fluorochrome emission to a desired wavelength.



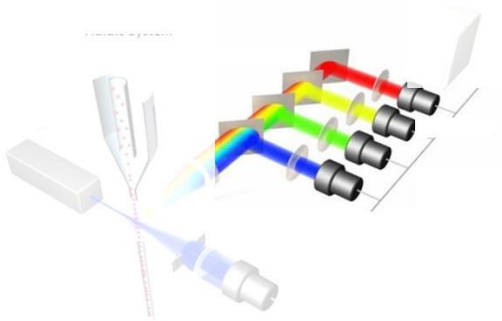
LONG PASS
wavelengths longer than the filter number pass

SHORT PASS
wavelengths lower than the filter number pass

BAND PASS
wavelengths in the range specified by the filter number pass

As a particle passes through the laser and fluoresces, it is detected by a photodetector (PMT).

2. The optical system

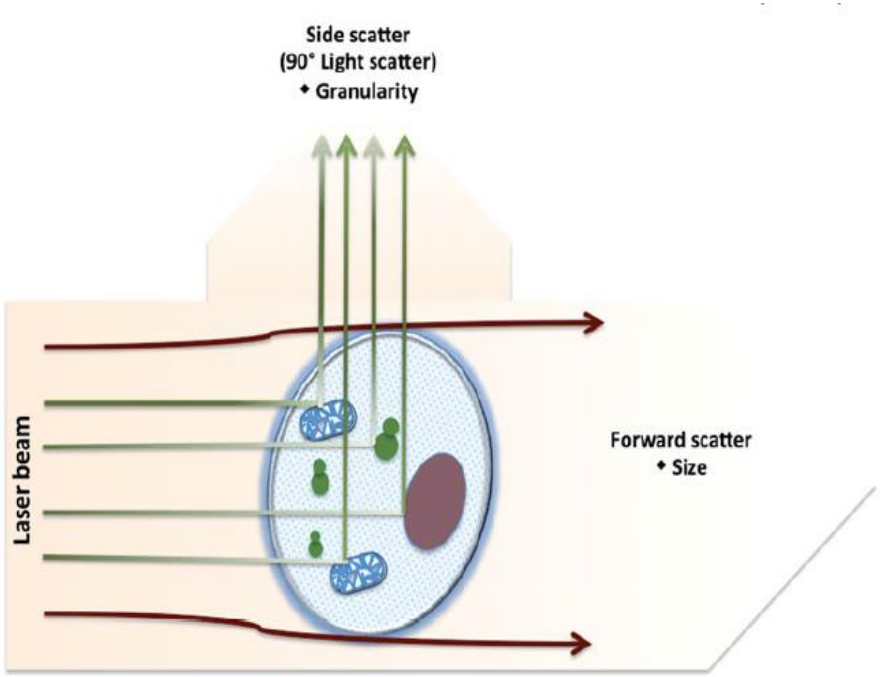


To characterize cells and their molecules, flow cytometry exploits two physical phenomena:

- **LIGHT SCATTERING**
- **FLUORESCENCE**

2. The optical system: light scattering

Generation of signals that allow identification of physical and morphological characteristics of cell populations.



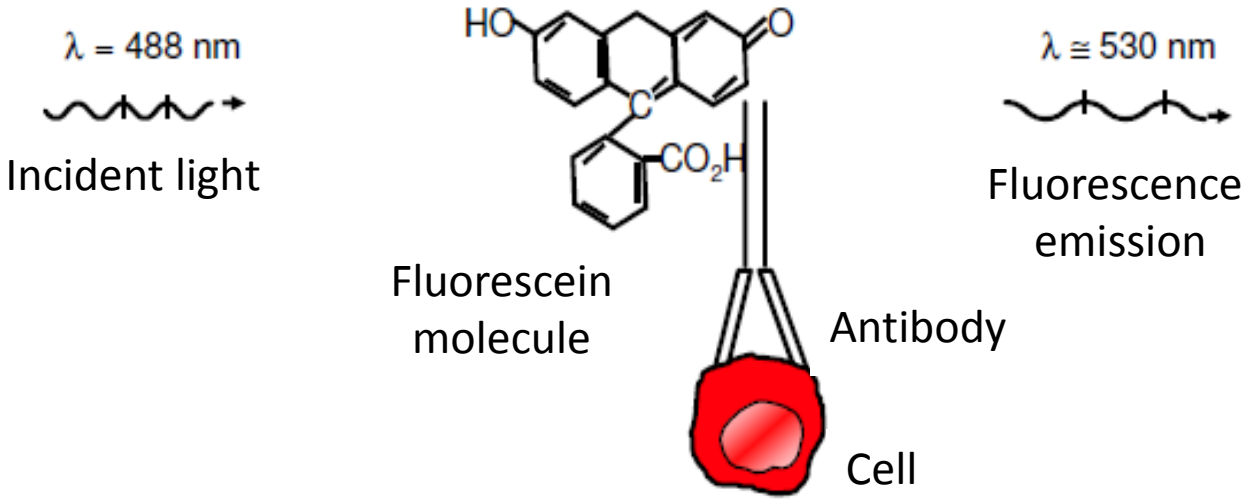
FORWARD SCATTER (FSC) **is proportional to the surface area or size of a cell.**
The linear scatter (0°) is related to the phenomenon of diffraction.

SIDE SCATTER (SSC) **is proportional to the granularity or internal complexity of a cell.**
The orthogonal scatter (90°) is related to reflection and refraction.

Adan A crit Rev Biotechnol 2016; 1-14

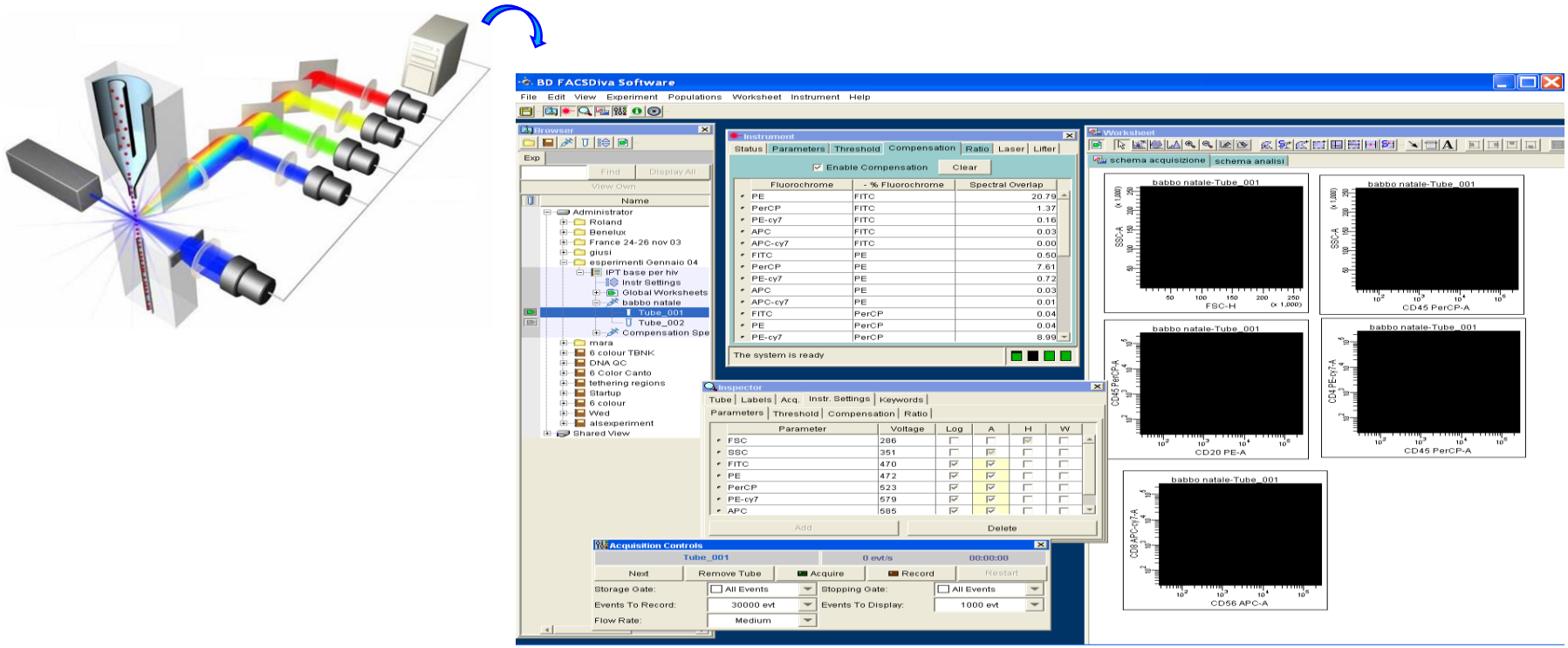
2. The optical system: fluorescence

It is the phenomenon whereby a molecule struck by a luminous radiation of a defined wavelength (λ) emits another one at a greater λ.



Fluorescence is typically "bestowed" upon a cell through the use of fluorescent dyes called fluorochromes.

3. The electronic system



The data processing is finally performed thanks to the computer connected to the instrument, which uses specific software to translate the signals into graphs on video display in real time.

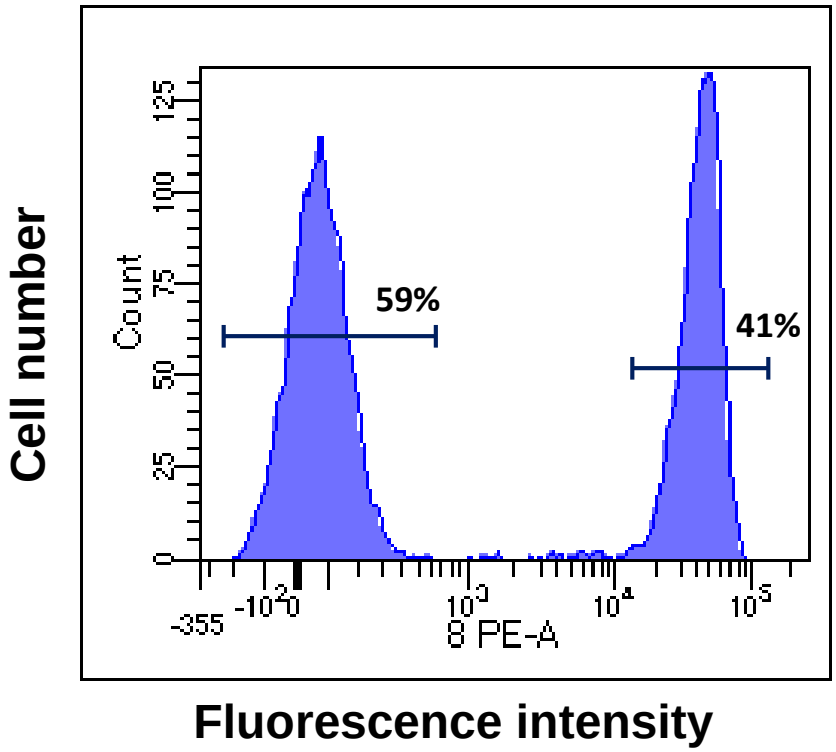
3. The electronic system

Data representation

The simplest representation of a flow cytometryc datum is the creation of an ISTOGRAM in which the acquired events provide a distribution diagram of the frequencies.

Abscissa: fluorescence intensity

Ordinate: cell number (events)

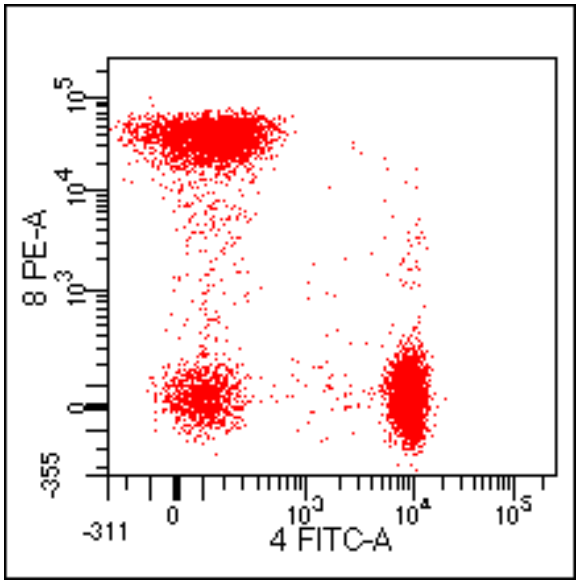


3. The electronic system

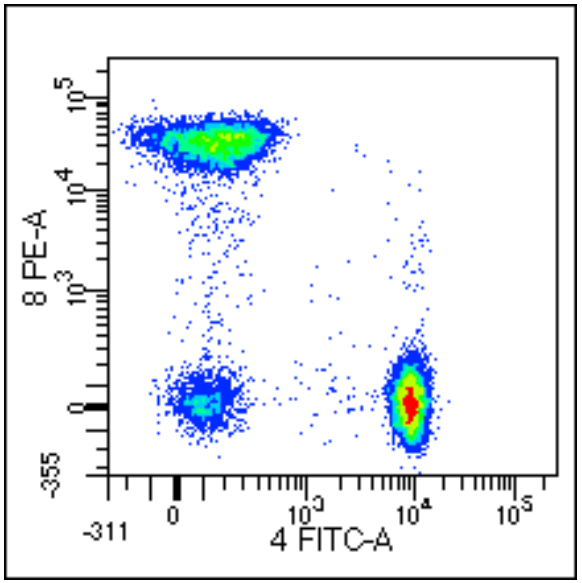
Data representation

Moreover, it is possible to use two-dimensional representations that allow to correlate two parameters to each other.
Each point represents an acquired event (cell), with a defined value and correlated to the measured parameters.

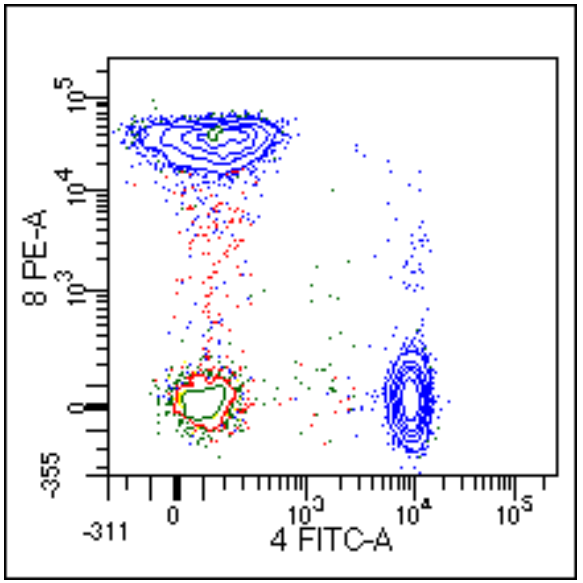
Dot-plot:
each point represent an event (cell)



Density-plot:
The colour intensity is proportional to the density of the events



Contour-plot:
display areas with the same density



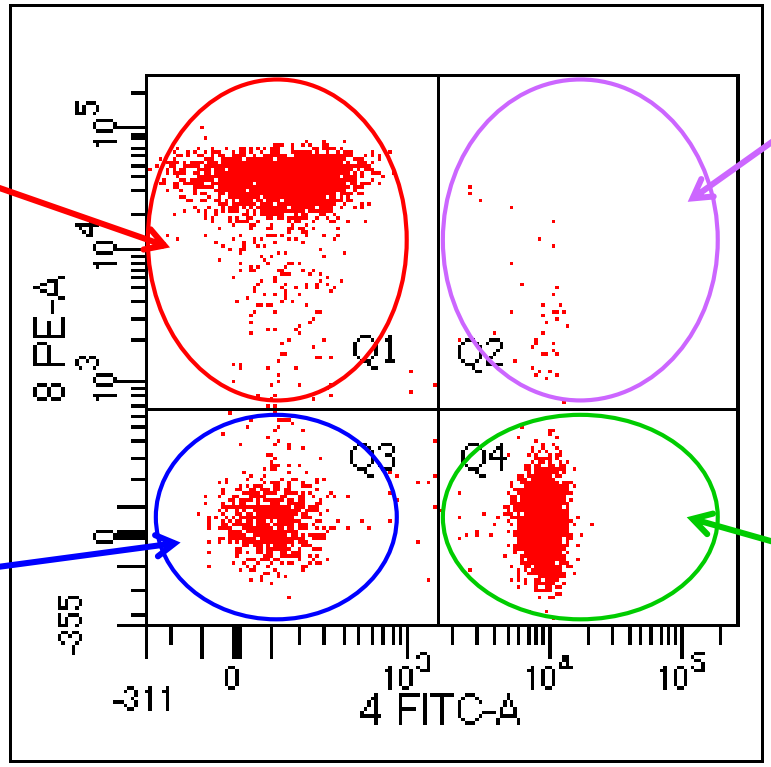
3. The electronic system

Data representation

In a two-dimensional plot it is possible to introduce QUADRANTS (Q) to detect 4 different populations

CD8 positive cells (CD8+)

Double positive cells (CD4+/CD8+)



Double negative cells (CD4-/CD8-)

CD4 positive cells (CD4+)

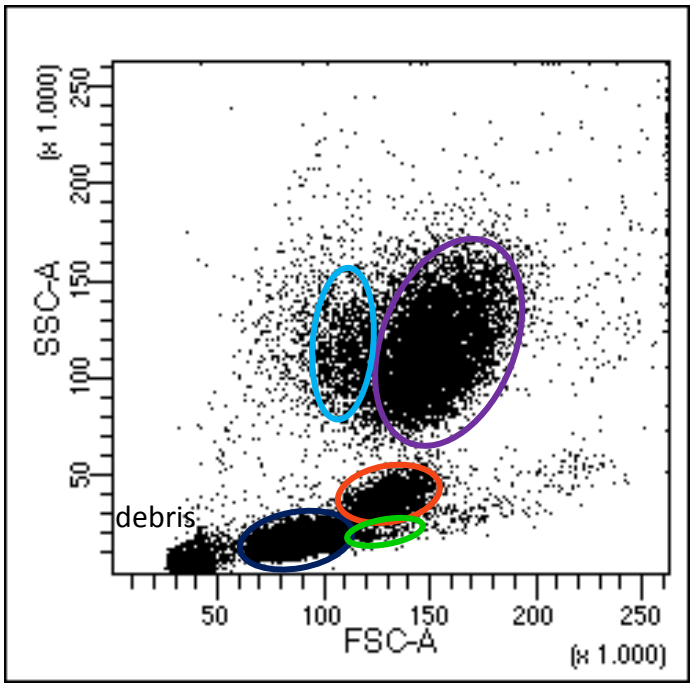
3. The electronic system

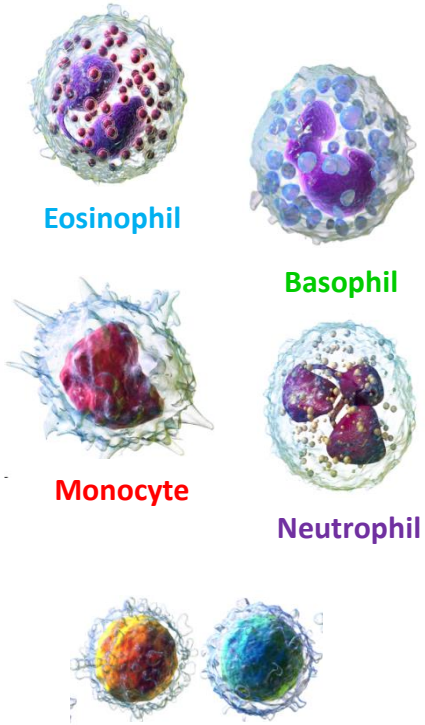
Data representation: GATING

Ability to isolate single populations of interest within a heterogeneous sample, basing on physical (morphological gate) or immunological (immunological gate) parameters.

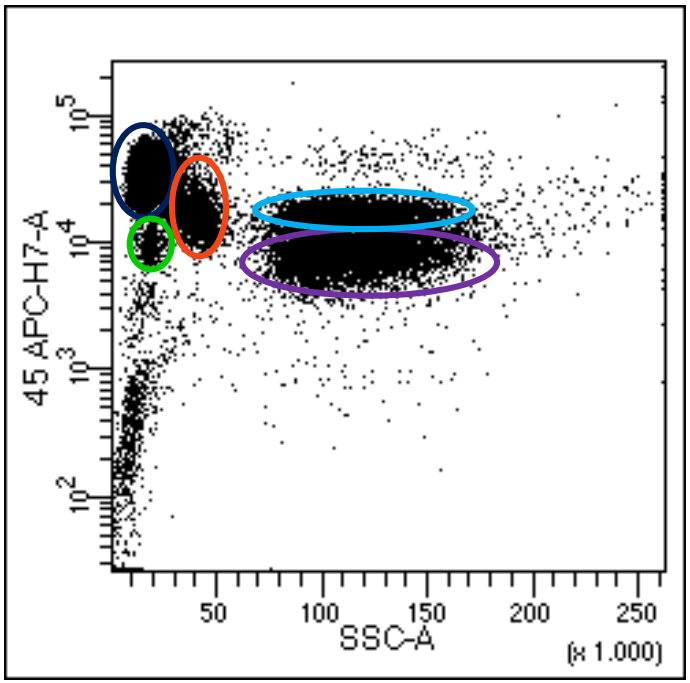
Example: leucocyte subpopulation in peripheral blood

morphological gate (FSC *vs* SSC)



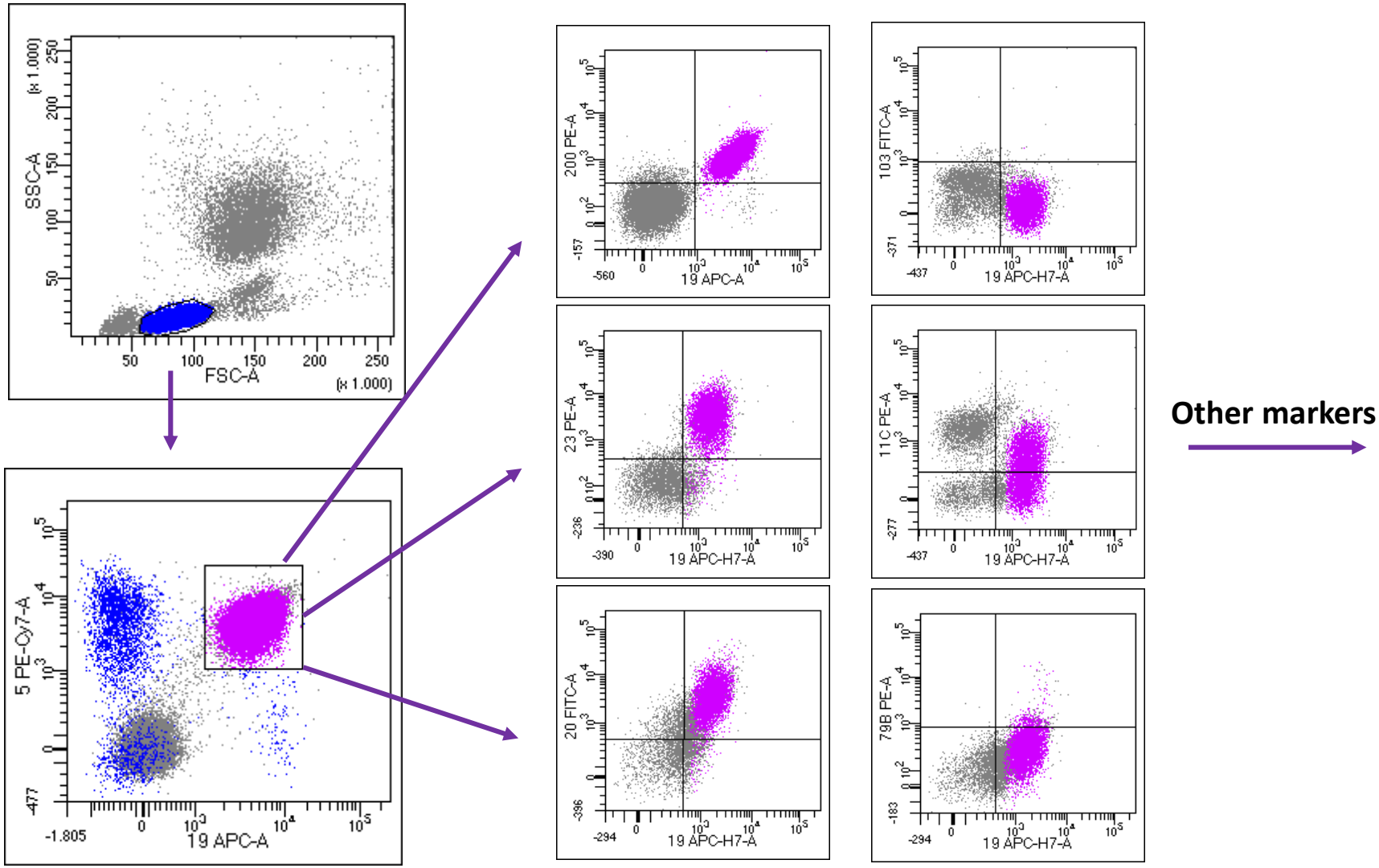


immunological gate (CD45 *vs* SSC)



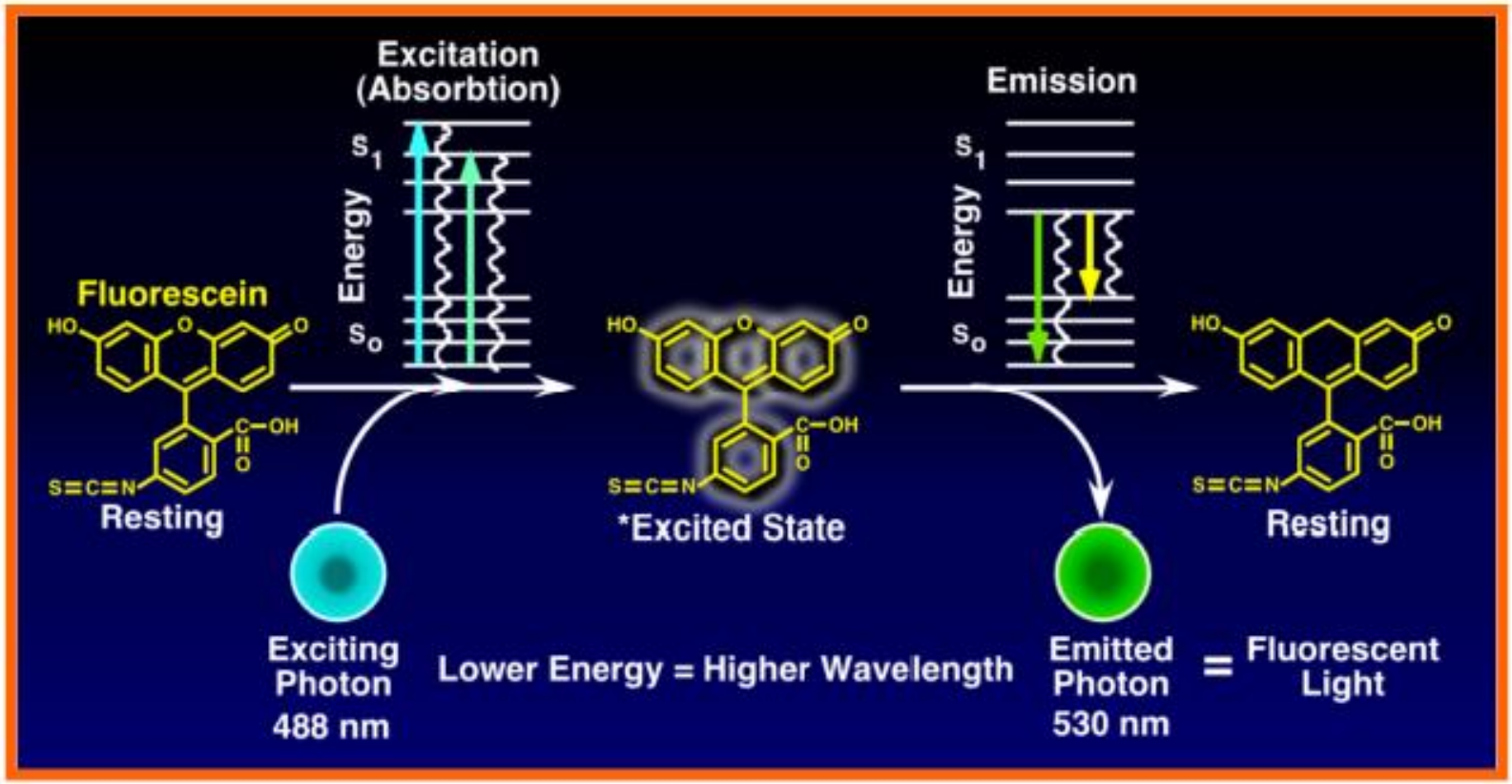
3. The electronic system

Data representation: GATING



FLUOROCHROMES

It will absorb the light, gaining energy, resulting in the excitation of electrons within the molecule; on returning to its unexcited state this excess energy is released as photons of light resulting in fluorescence.



Fluorochromes are excited by light of a wavelength that is characteristic for that molecule.

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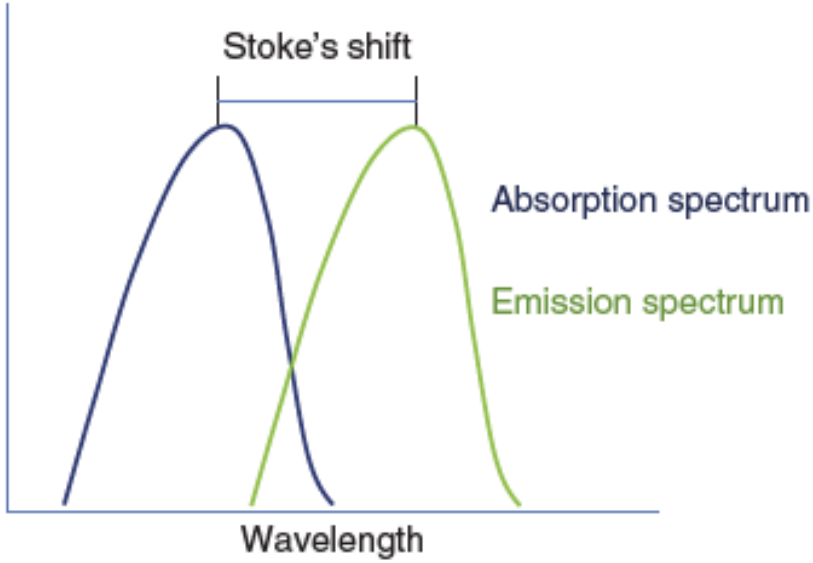
FLUOROCHROMES

The emission wavelength of any fluorochrome will always be longer than its excitation wavelength.

The difference between the maxima in the wavelengths of absorption and emission is known as the Stoke's shift that determines how good a fluorochrome.

The higher the Stoke's shift means, the greater the separation between the exciting and the emitted light.



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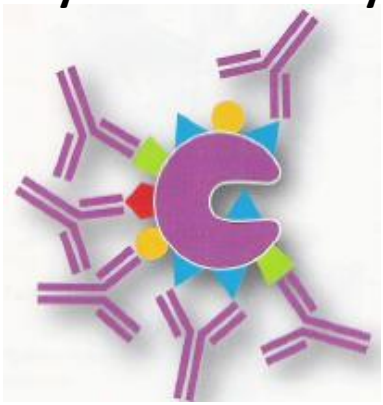
FLUOROCHROMES IN FLOW CYTOMETRY

Fluorochromes used in flow cytometry:

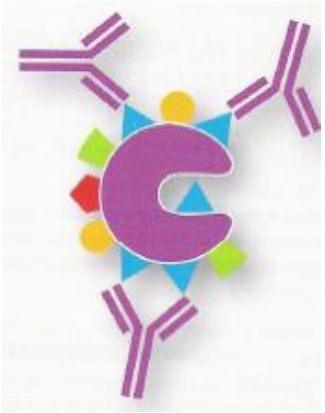
- **conjugated to specific monoclonal or polyclonal antibodies for molecules (with antigenic or receptorial significance) placed on the cell membrane, in the nucleus or in the cytosol;**
- **directly color other substances (DNA, RNA, proteins).**

Polyclonal antibody



Polyclonal antibodies bind to multiple aspects of the same antigen. Their heterogeneity causes problems with standardization when used in flow cytometry.

Monoclonal antibody



Monoclonal antibodies bind to only one aspect of an antigen and will reproducibly label cells.

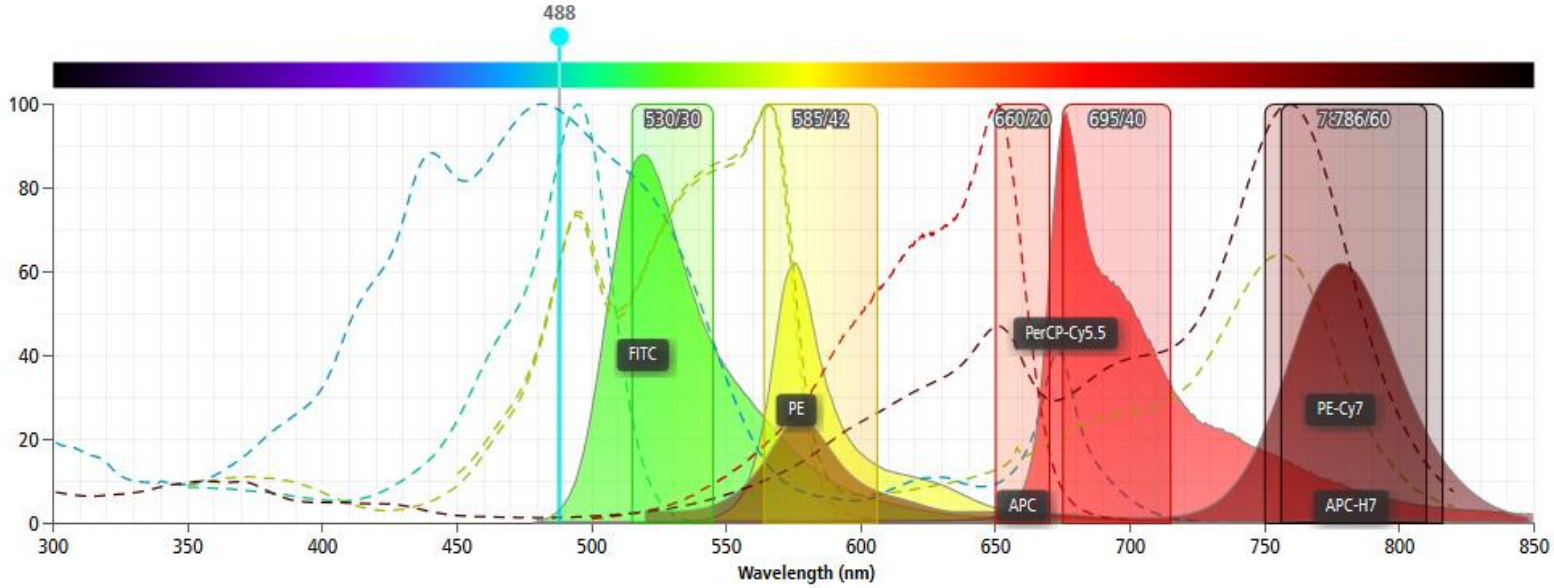
Cells present a weak measurable background fluorescence, autofluorescence.

Guide to Flow Cytometry; DakoCytomation

FLUOROCHROMES IN FLOW CYTOMETRY

Ultraviolet (355nm)	Violet (405nm)	Blue (488nm)	Green (532nm)	Yellow (561nm)	Red (635-855nm)	Far Red/IR (685-785nm)
• AMCA	• Atto 425	• Atto 488	• Atto 532	• Atto565	• APC	• DyLight755
• Atto 390	• Dylight 405	• Cy2	• Cy3	• Atto 594	• APC-Cy7	• DyLight 800
• Dylight 350		• FITC	• DyLight 550	• Atto 590	• Atto 633	• FluoProbes 752
		• HiLyte 488	• Dyomics 547	• Atto 610	• Atto 637	• HiLyte 750
		• PerCP	• FluorProbes 547H	• Cy3.5	• Atto 655	
		• PerCP-Cy5.5	• HiLyte 555	• Dylight 594	• Atto 680	
		• PerCP-Cy7	• PE-Cy5		• Atto700	
		• R-PE	• PE-Cy5.5		• Cy 5.5	
		• PE-Cy5	• PE-Cy7		• Cy5	
		• PE-Cy5.5	• Rhodamine		• Cy7	
		• PE-Cy7	• R-PE		• DyLight 633	
			• Texas Red		• DyLight 650	
			• TRITC		• DyLight 680	
					• Dyomics 647	
					• FluorProbes 647H	
					• FluorProbes 682	
					• HiLyte 647	

The use of antibody linked to fluorochromes with different wavelengths allows multiparametric analysis of single cells.



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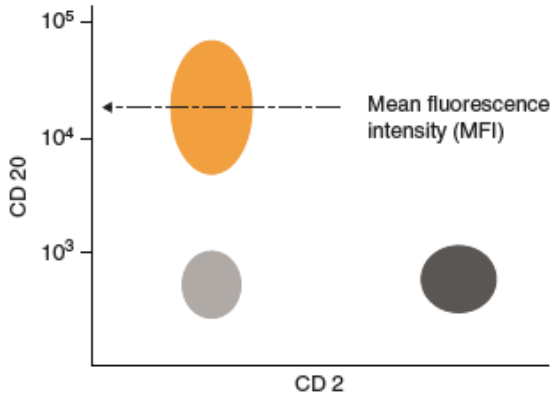
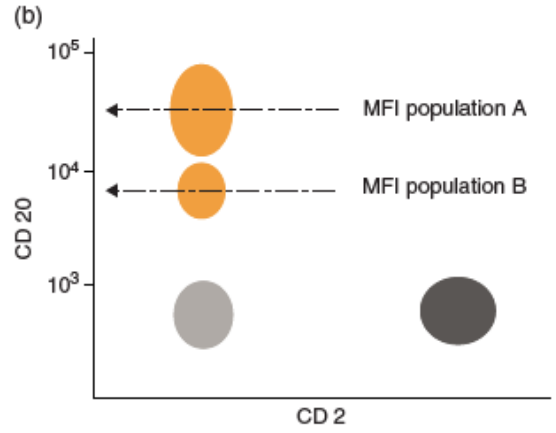
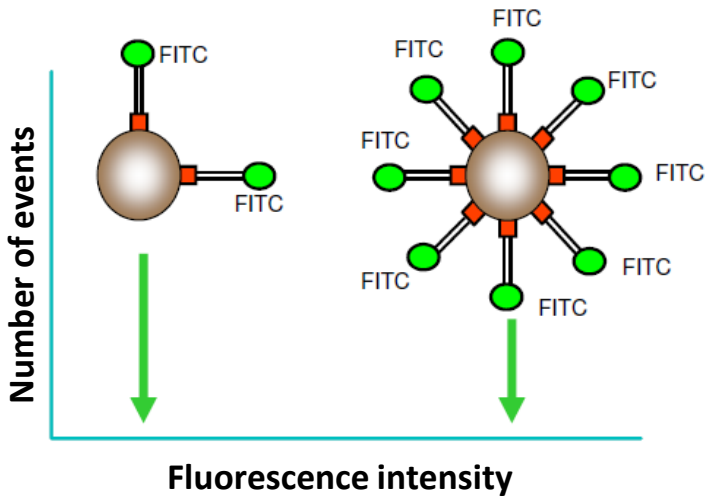
FLUORESCENCE INTENSITY

The brightness or fluorescence intensity of any captured event for a particular fluorochrome is recorded by the cytometer for that channel.







The mean fluorescence intensity (MFI) relates to not only the presence of the antigen, but also the strength of expression of that antigen in a given cell population.

Fluorescence intensity can also be used to identify dual populations.

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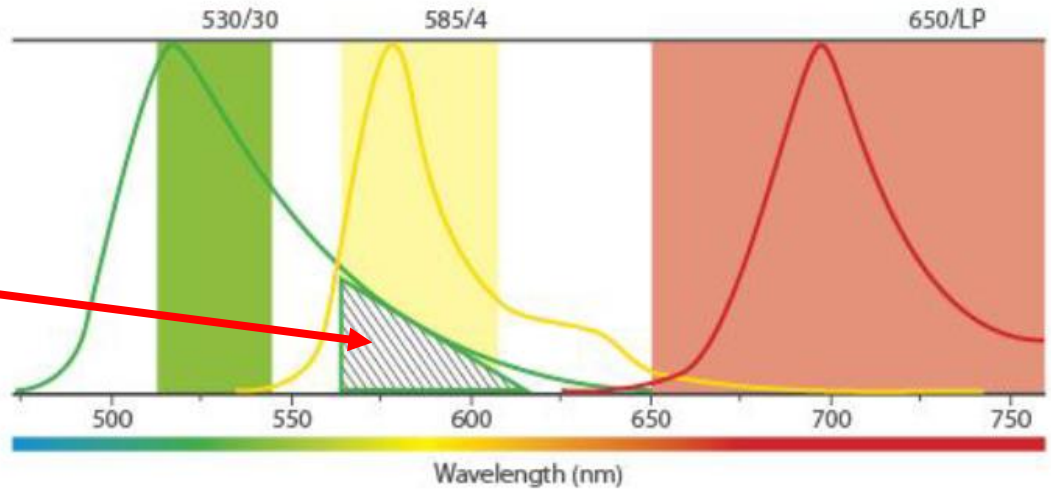
COMPENSATION

The emission spectra for a given fluorochrome can cover a range of wavelengths, allowing fluorescence spill over to a detector designed for a different fluorochrome.

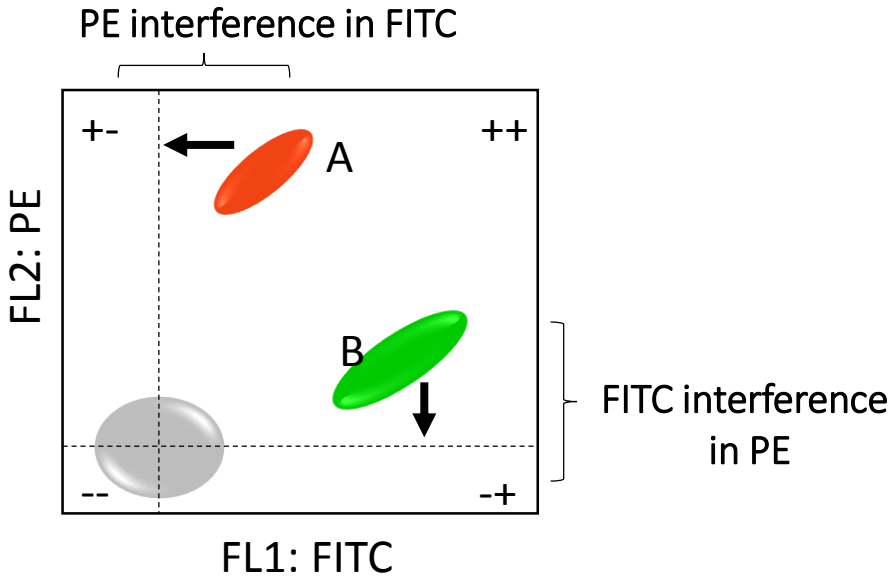
SPECTRAL OVERLAP

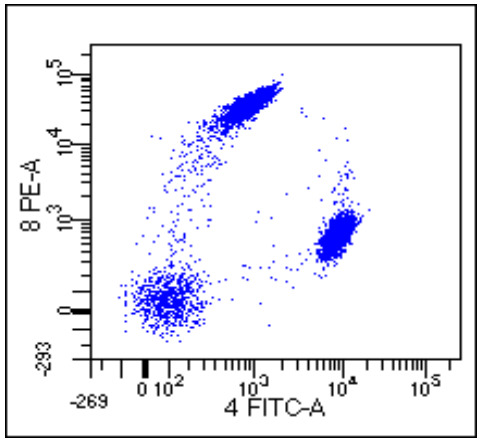


This fluorescence interference can be corrected by adjusting the measurement parameters of the flow cytometer (either manually or automatically).

COMPENSATION

COMPENSATION



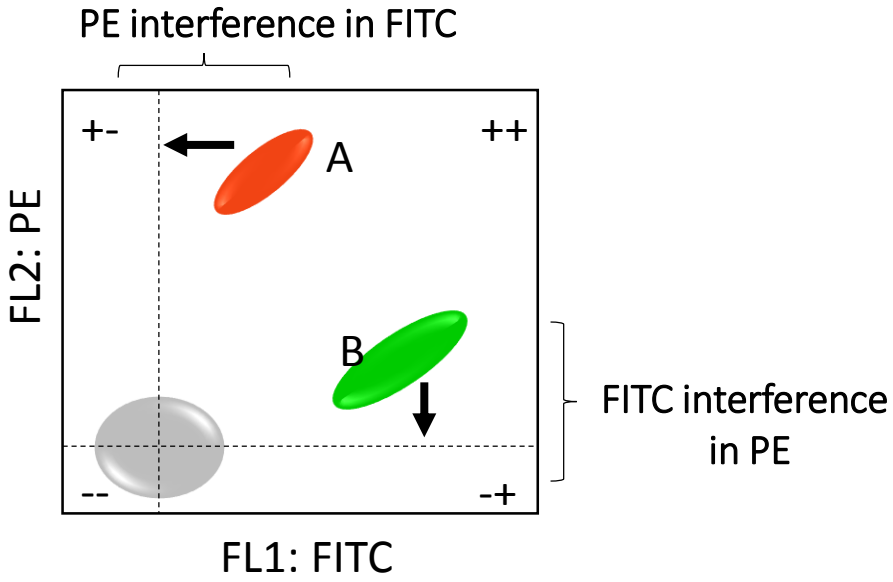


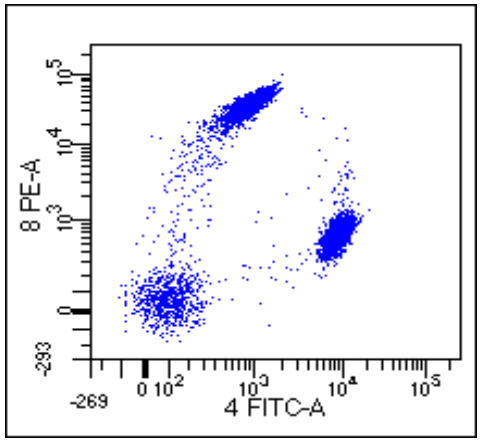
A and B population ~~positive~~ for both FITC and PE?

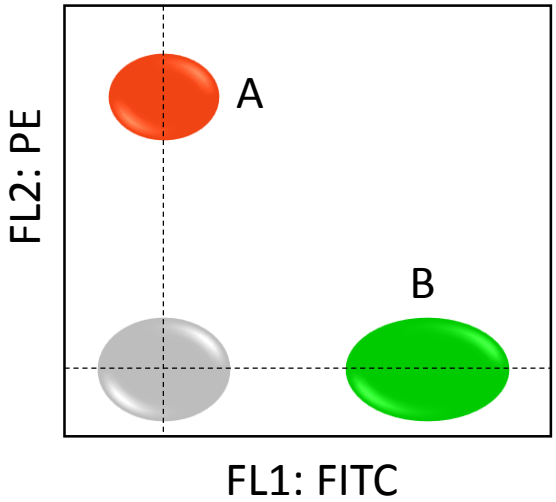
NO!

Need compensation!

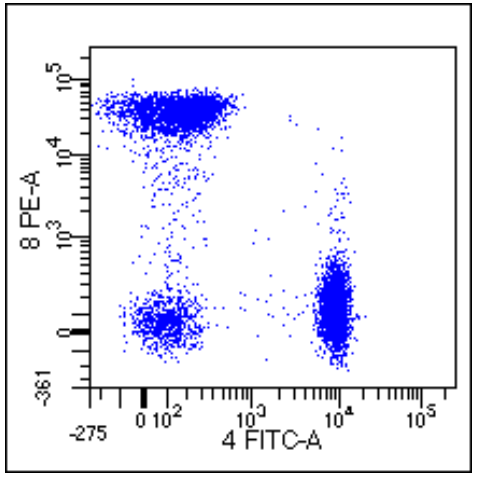
COMPENSATION







Correct Compensation!



ADVANTAGES OF FLOW CYTOMETRY

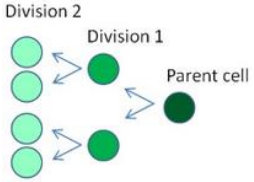
- **Multiparametric** analysis;
- possibility to relate **different** characteristics of a same single cell;
- analysis of a **large** number of cells;
- **reproducibility** and statistical reliability of acquisitions;
- great **sensibility**;
- **celerity** of analysis;
- possibility of further **retrospective** analysis.

LIMITATIONS OF FLOW CYTOMETRY

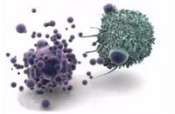
- Need to use single cell suspensions;
- requires viable cells;
- high costs;
- relative complexity of the instrumentation that requires extensive staff training and careful maintenance;
- no flow cytometer will give good results from bad samples; this depends on the scruple of the operator and on his ability to evaluate the quality of a preparation or mark.

FLOW CYTOMETRY: APPLICATIONS

- Cell proliferation

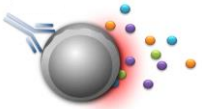


- Cell cytotoxicity

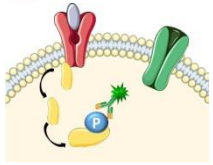


- Cell viability (apoptosis and necrosis)

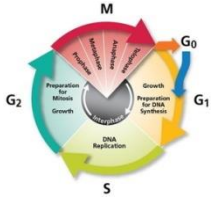
- Intracellular cytokine detection



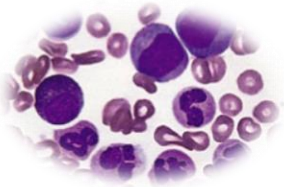
- Intracellular cell signaling analysis



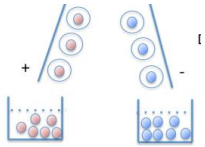
- Cell cycle



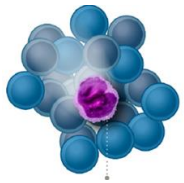
- Immunophenotyping of leucocyte subpopulations



- Cell sorting

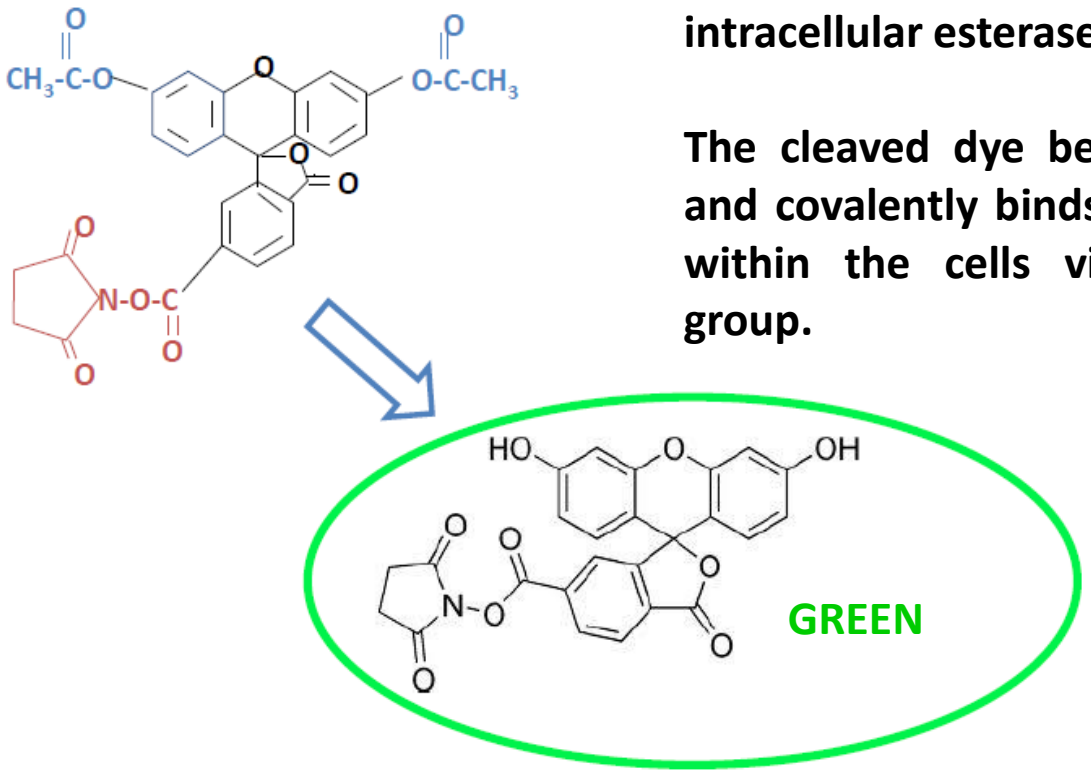


- Evaluation of rare events



CELL PROLIFERATION: CFSE STAINING

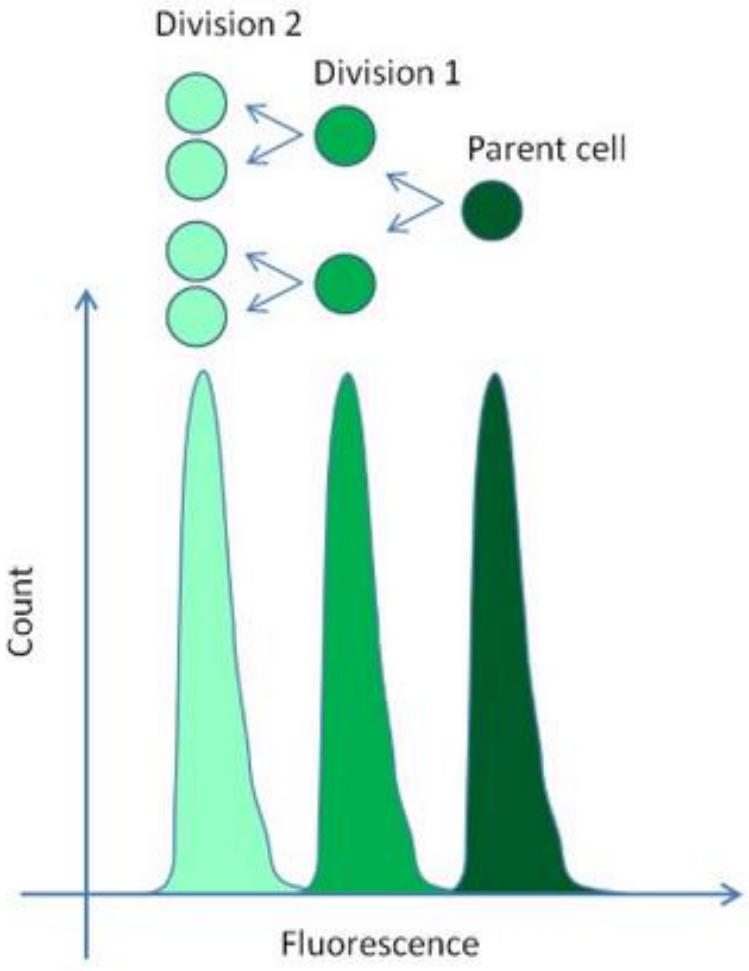
CFSE (carboxyfluorescein diacetate succinimidyl ester) is a blue laser excitable dye that can be used for flow cytometric monitoring of cell divisions.



The non-fluorescent dye passively diffuses across cell membranes and is cleaved by intracellular esterases within viable cells.

The cleaved dye becomes highly fluorescent and covalently binds to protein amine groups within the cells via its succinimidyl ester group.

CELL PROLIFERATION: CFSE STAINING

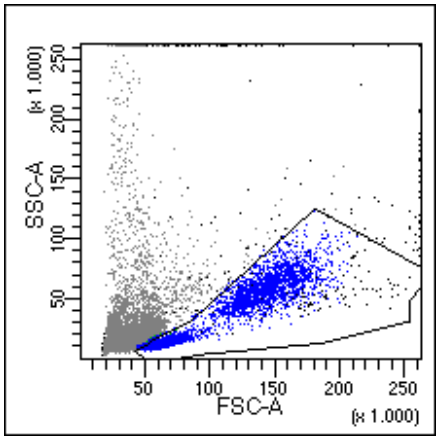


As viable cells divide, CFSE dye is distributed uniformly between daughter cells.

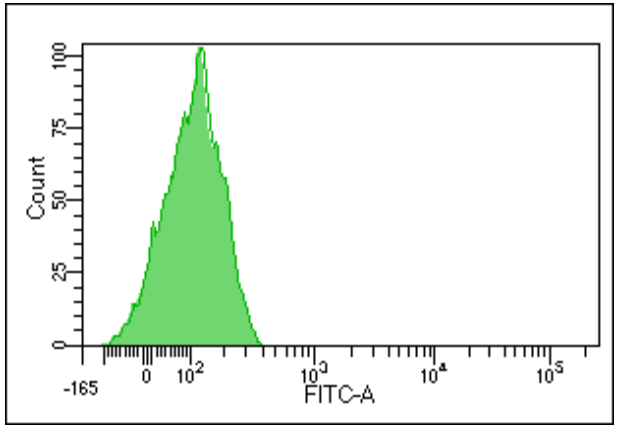
Each daughter cell retains approximately half of the CFSE fluorescence intensity of its parent cell.

www.chemometec.com

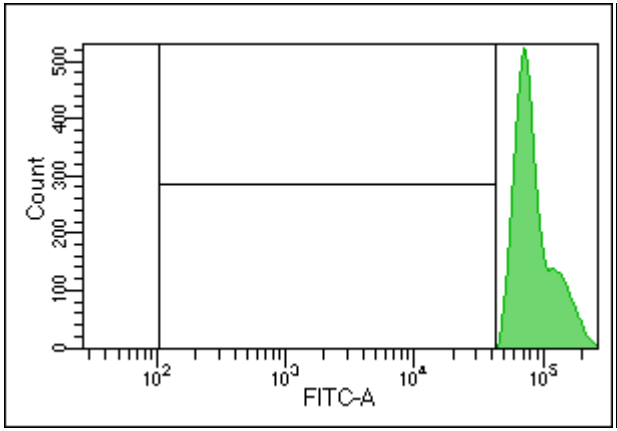
CELL PROLIFERATION: CFSE STAINING



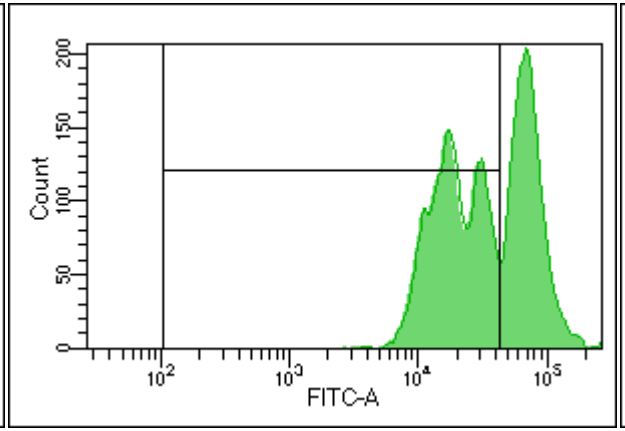
NO CFSE



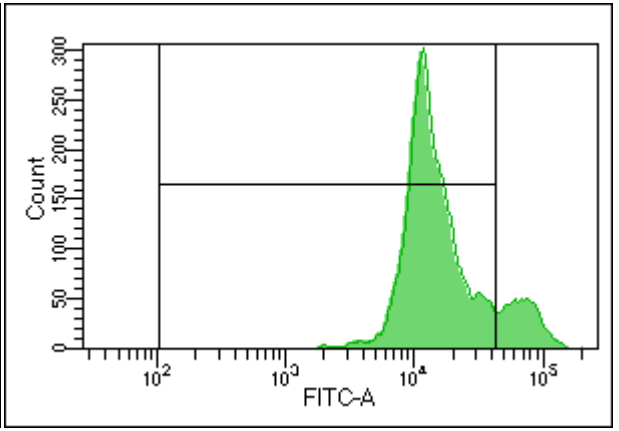
CFSE not stimulated



CFSE PHA 1.2 μg/ml

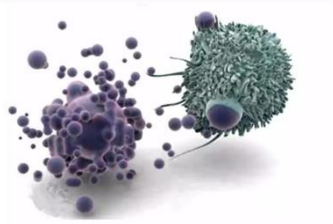


CFSE PHA 2.5 μg/ml

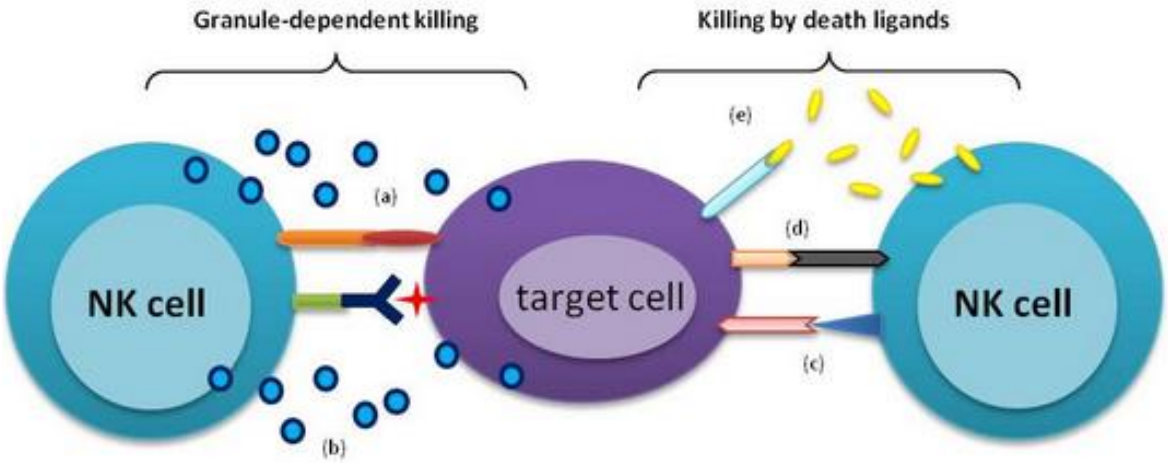


NO radioactive substances

NATURAL KILLER (NK) CELL CYTOTOXICITY



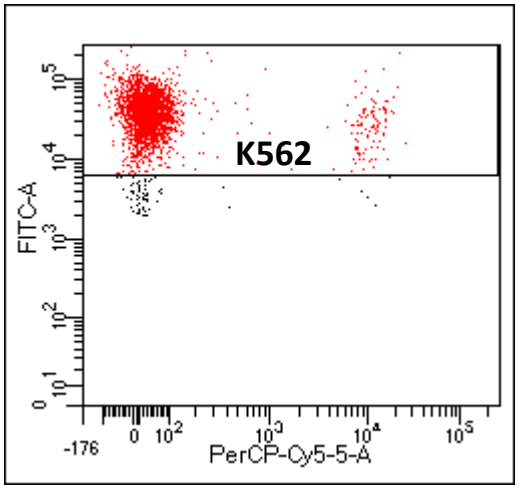
Depressed NK-cell mediated cytotoxicity is one of the many immunological defects observed in several disease.

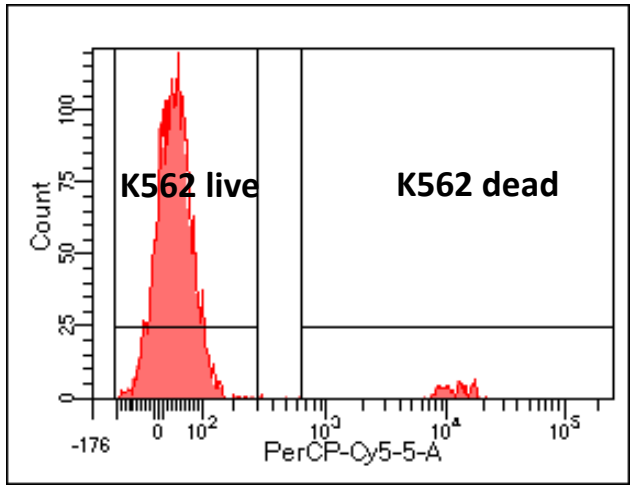


This test allows the quantitative determination of the cytotoxic activity of NK cells.
Target cells are labelled with a fluorescent dye in order to discriminate them from effector cells (NK).
The percentage of target cells killed can be performed by flow cytometry.

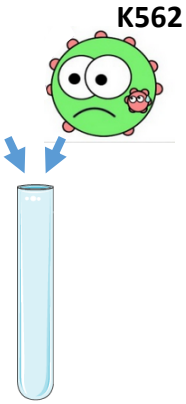
Mehta RS Frontiers in Immunology 2018; 9: 283

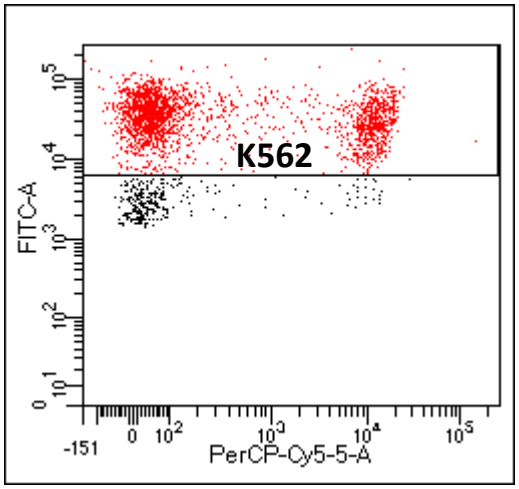
NATURAL KILLER (NK) CELL CYTOTOXICITY

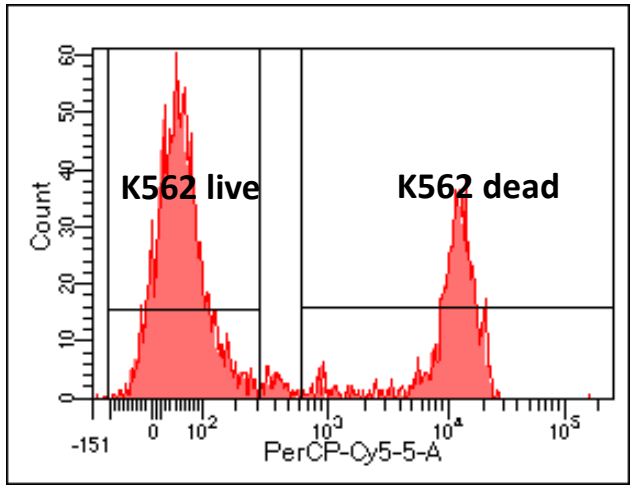




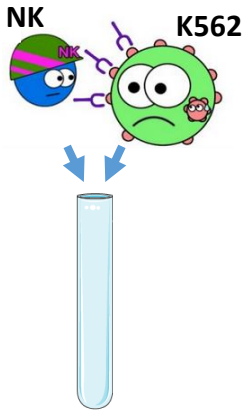
Control sample





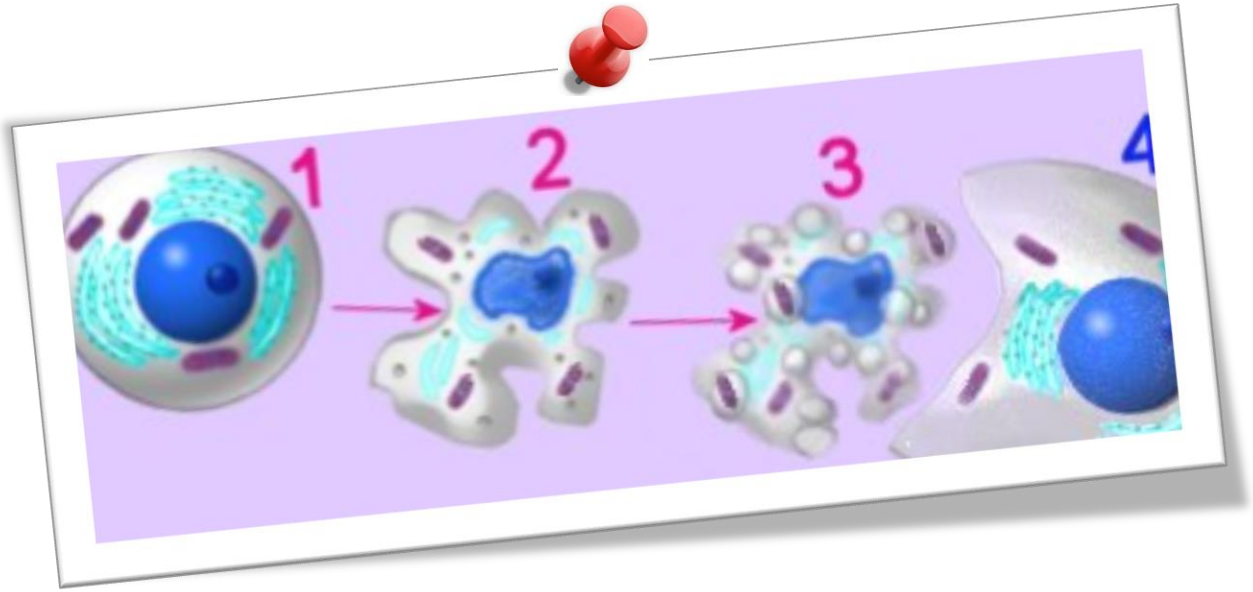


Mix effector
+ target cells



Killed target cells are identified by a DNA-stain, which penetrates the dead cells.

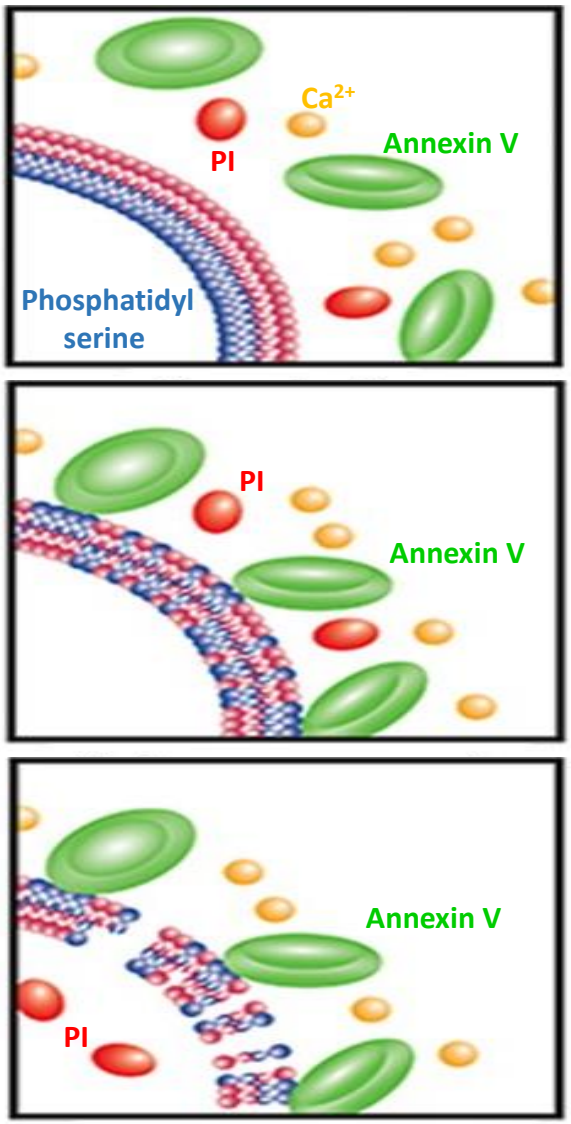
CELL VIABILITY: ANNEXIN V / PROPIDIUM IODIDE



Annexin V bind to phosphatidylserine, a component of the inner cell membrane that is exposed on cell surface during early stage of apoptosis.

Propidium Iodide fluorescent intercalating agent used as a DNA stain in flow cytometry to evaluate cell viability or DNA content in cell cycle analysis.

CELL VIABILITY: ANNEXIN V / PROPIDIUM IODIDE (PI)



www.leinco.com

Normal live cell

- intact cell membrane
- Phosphatidylserine on the inner side

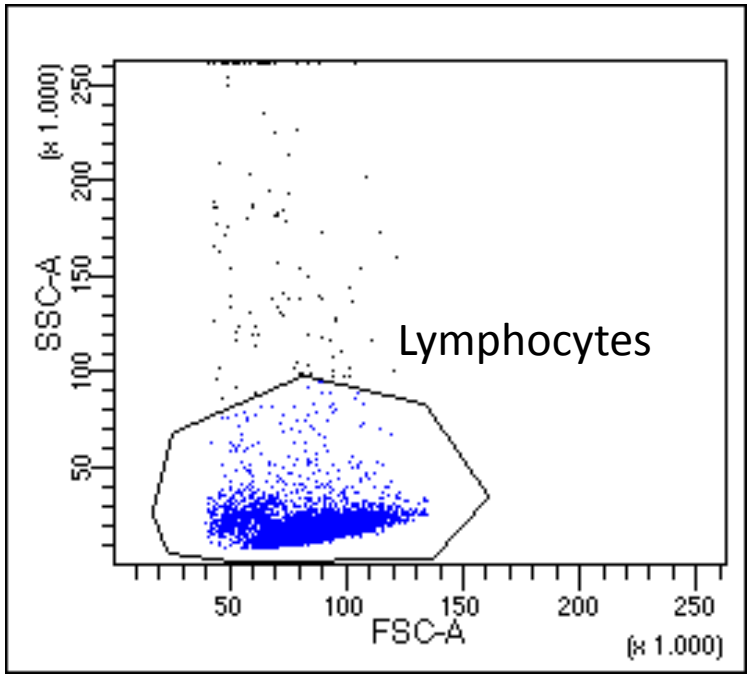
Early stage apoptosis

- intact cell membrane
- Phosphatidylserine exposure on membrane surface → Annexin V binding

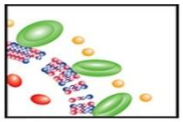
Late stage apoptosis

- Loss of cell membrane integrity → PI entry
- Phosphatidylserine exposure on membrane surface → Annexin V binding

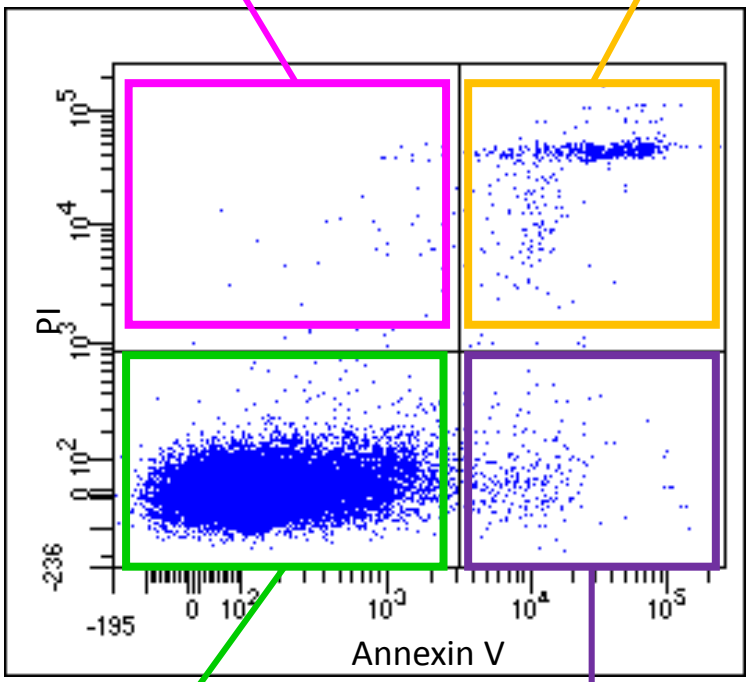
CELL VIABILITY: ANNEXIN V / PROPIDIUM IODIDE



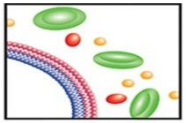
Necrosis
PI+/Annexin V-



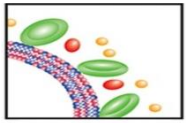
Late stage apoptosis
PI+/Annexin V+



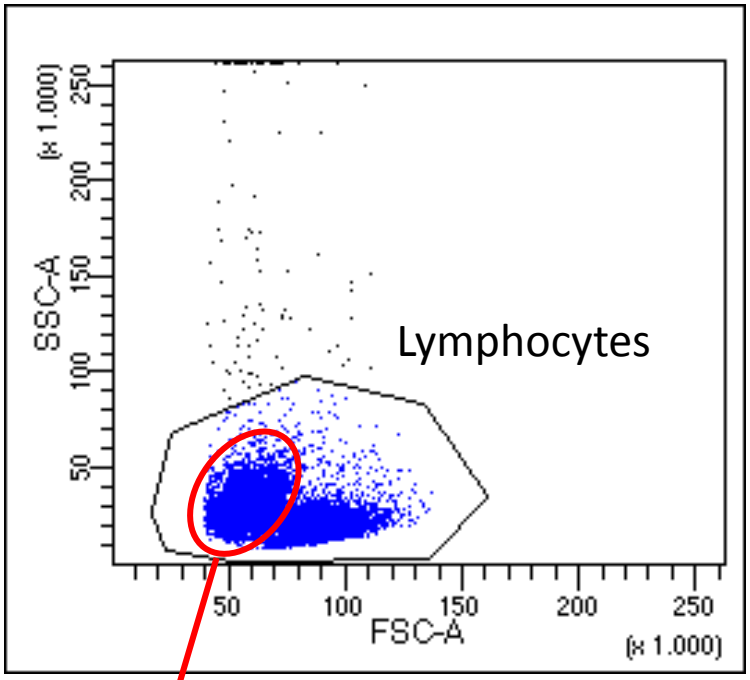
Live cells
PI-/Annexin V-



Early stage apoptosis
PI-/Annexin V+

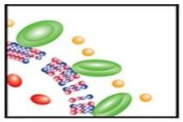


CELL VIABILITY: ANNEXIN V / PROPIDIUM IODIDE

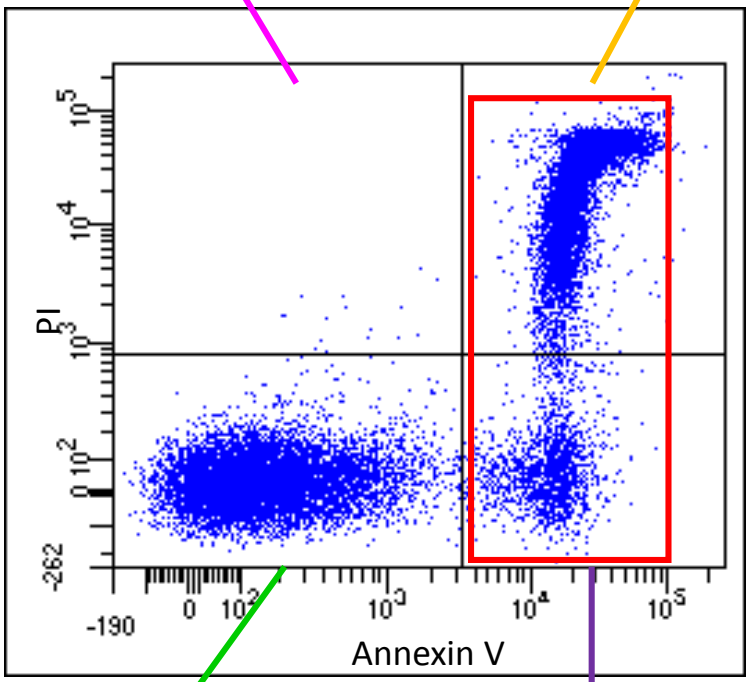


Modification of physical parameters due to cytoplasm condensation of apoptotic cells.

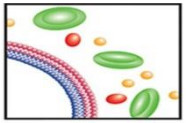
Necrosis
PI+/Annexin V-



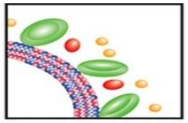
Late stage apoptosis
PI+/Annexin V+



Live cells
PI-/Annexin V-

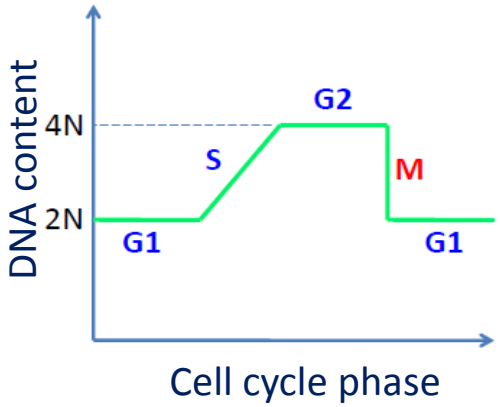


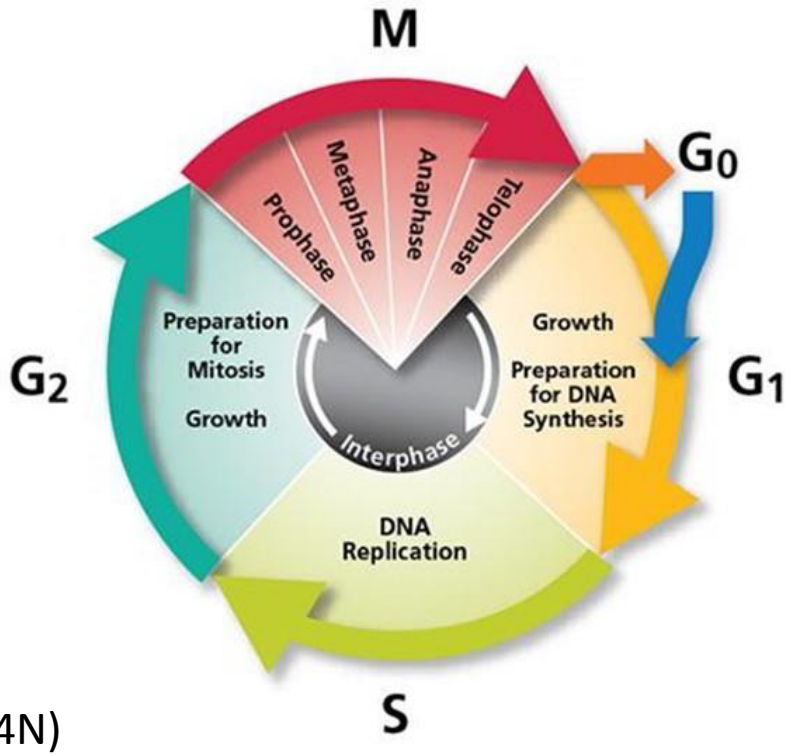
Early stage apoptosis
PI-/Annexin V+



CELL CYCLE

The Prodidium Iodide (PI) binds to the DNA in proportion to the amount of DNA present. It is not able to permeate the cell membrane, in order to evaluate the DNA content it is necessary to permeabilize the cells.
Fluorescent intercalation of DNA
- excitation 488 nm,
- emission 625 nm

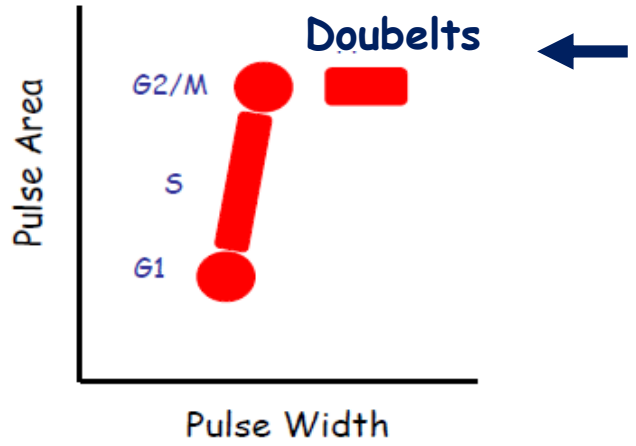




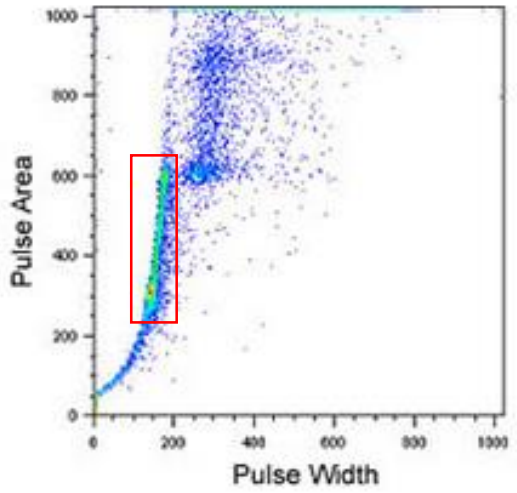
- **G_1**: the cell have a diploid DNA content (2N)
- **S**: the cell have a variable DNA content (2N-4N)
- **G_2**: the cell have a tetraploid DNA content (4N)
- **M**: mitosis, cell divides generating two daughter cells with diploid DNA content (2N)

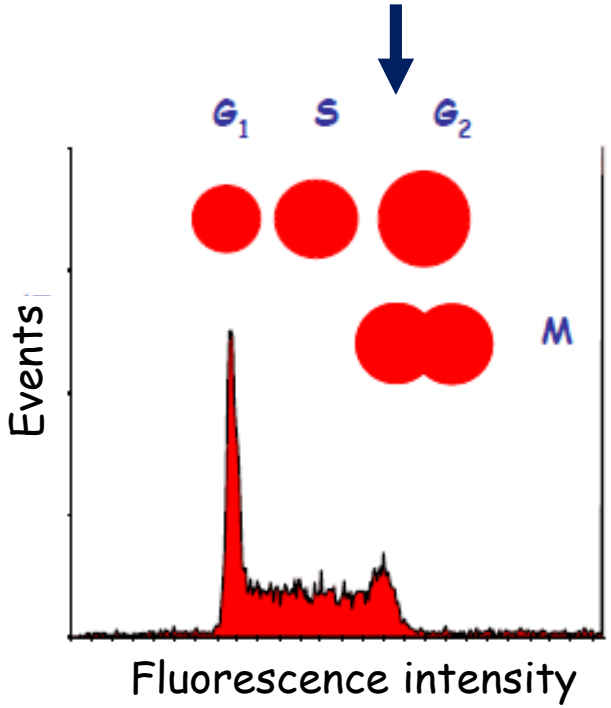
CELL CYCLE

Cells must be in single suspension, clumps can alter the cycle evaluation. Two paired cells could be interpreted as either a G2 or a phase M cell.



Cell doublets must therefore be excluded from the analysis: paired cells have a wider signal width than a single double-DNA cell because they take longer to cross the laser.



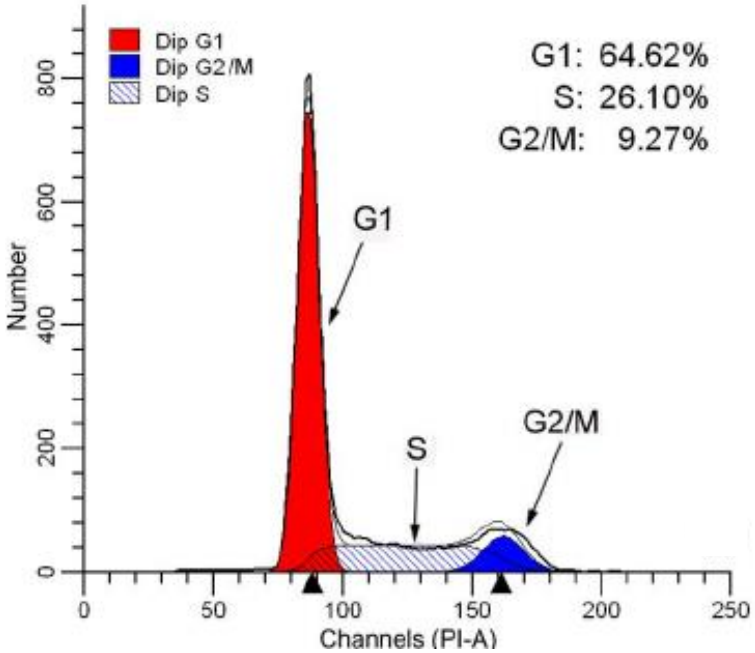


www.abcam.com

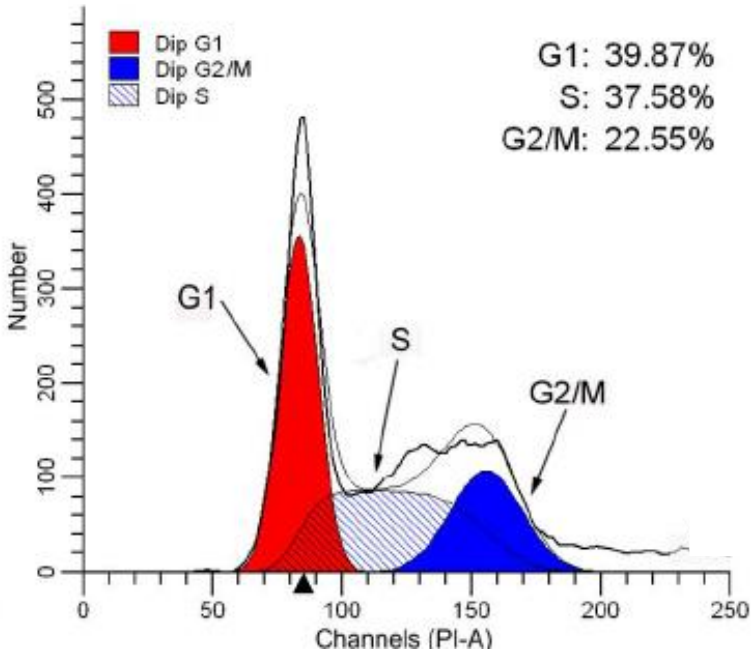
CELL CYCLE

It is possible to quantify the percentage of cells in each cell cycle and monitor the treatment effect.

Control



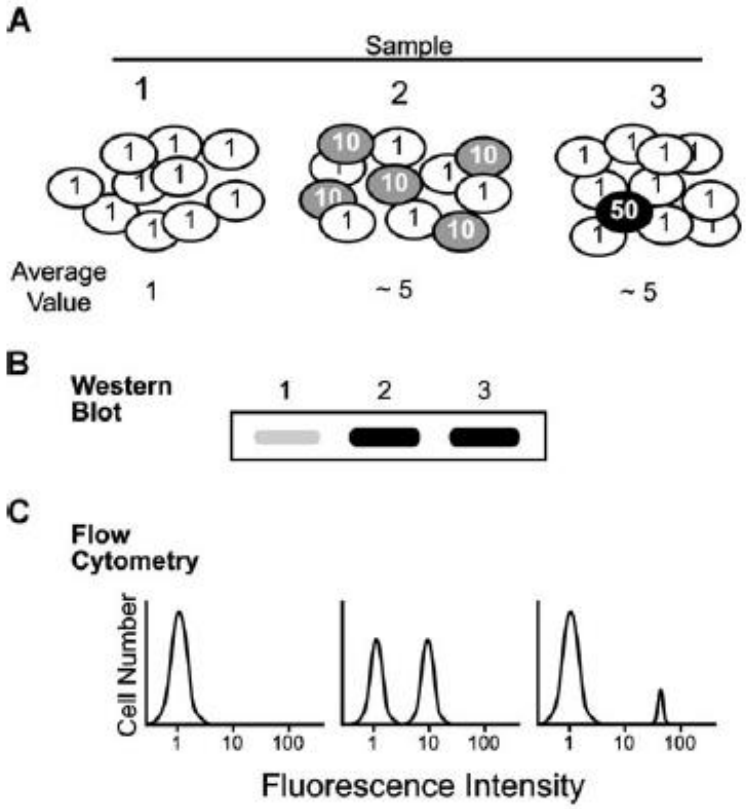
Treatment



Zhao S IEEE transactions on plasma science 2012; 40:2179 - 2184

INTRACELLULAR CELL SIGNALING ANALYSIS

Analysis of protein phosphorylation with flow cytometric techniques has emerged as a powerful tool in the field of immunological signaling.



Advantages

- Single cell analysis
- Multiparameter analysis
- Rare subsets within complex populations
- Rapid protocols

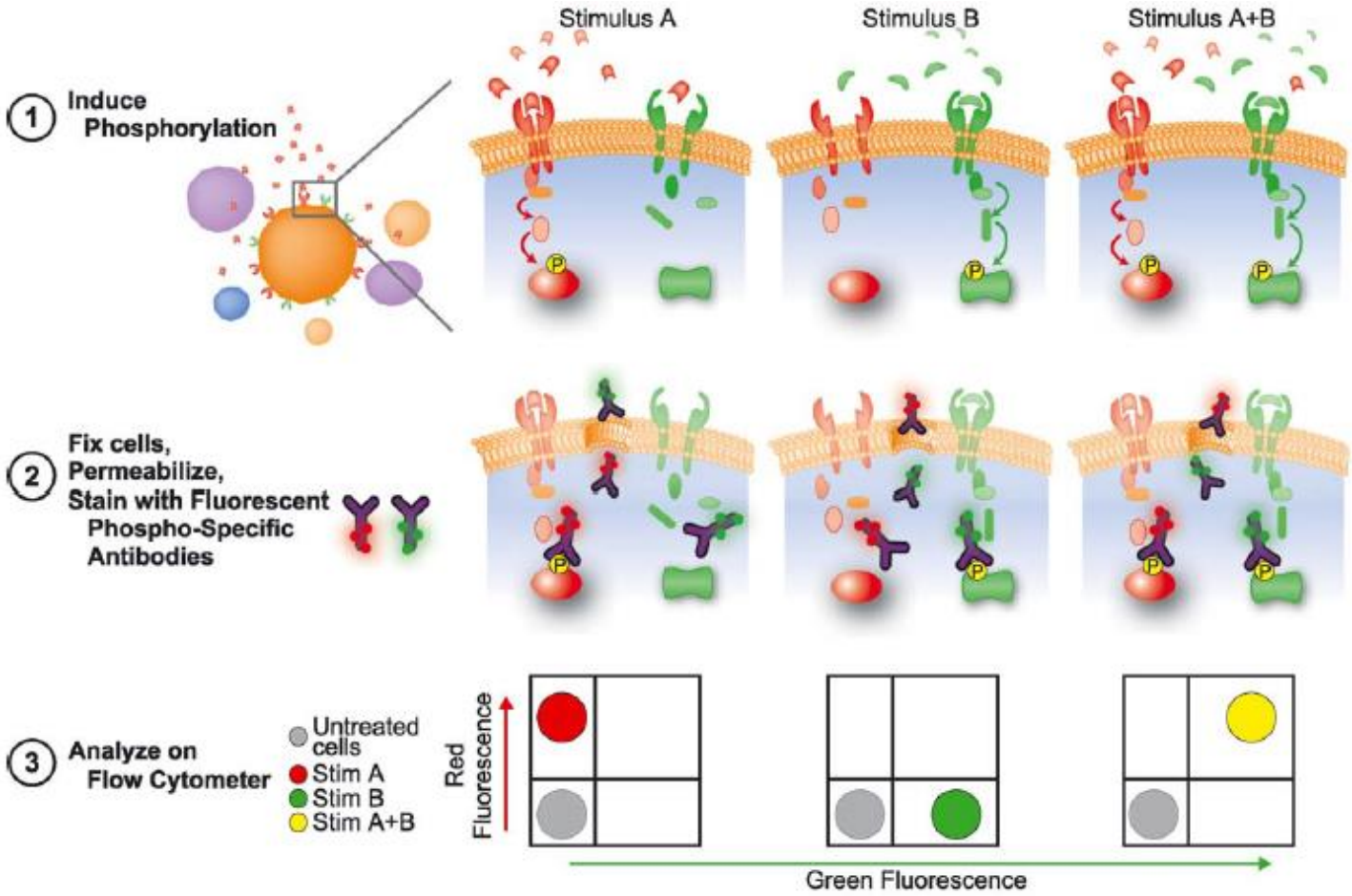
Limitations

- No information about protein interactions
- Antibody must have high affinity and selectivity

Krutzik PO Clin Immunol 2004: 206-221

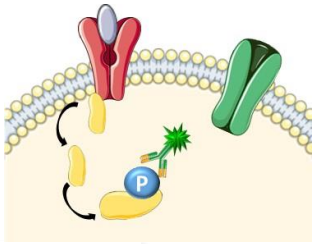
INTRACELLULAR CELL SIGNALING ANALYSIS

Generale phospho-protein staining technique for flow cytometry



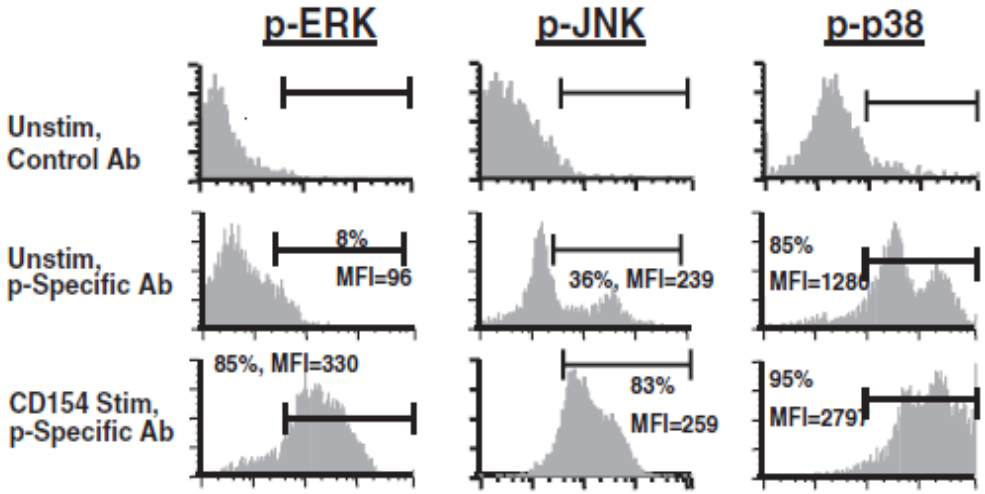
Krutzik PO Clin Immunol 2004: 206-221

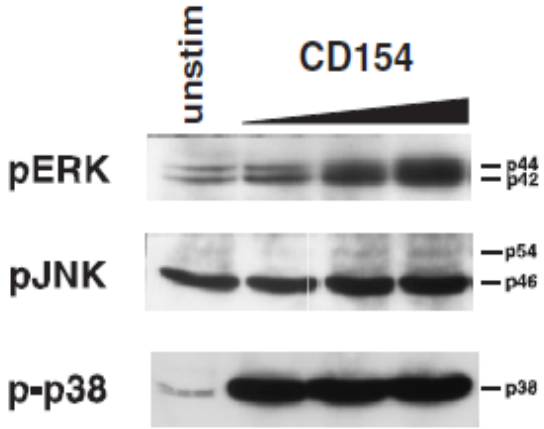
INTRACELLULAR CELL SIGNALING ANALYSIS



Flow cytometric assessment of the signaling status of human B lymphocytes from normal and autoimmune individuals

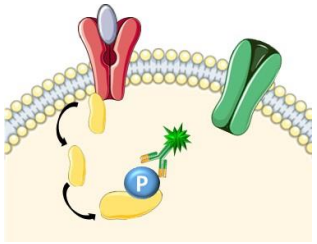
Amrie C Grammer[1], Randy Fischer[1], Olivia Lee[1], Xuan Zhang[1] and Peter E Lipsky[2]





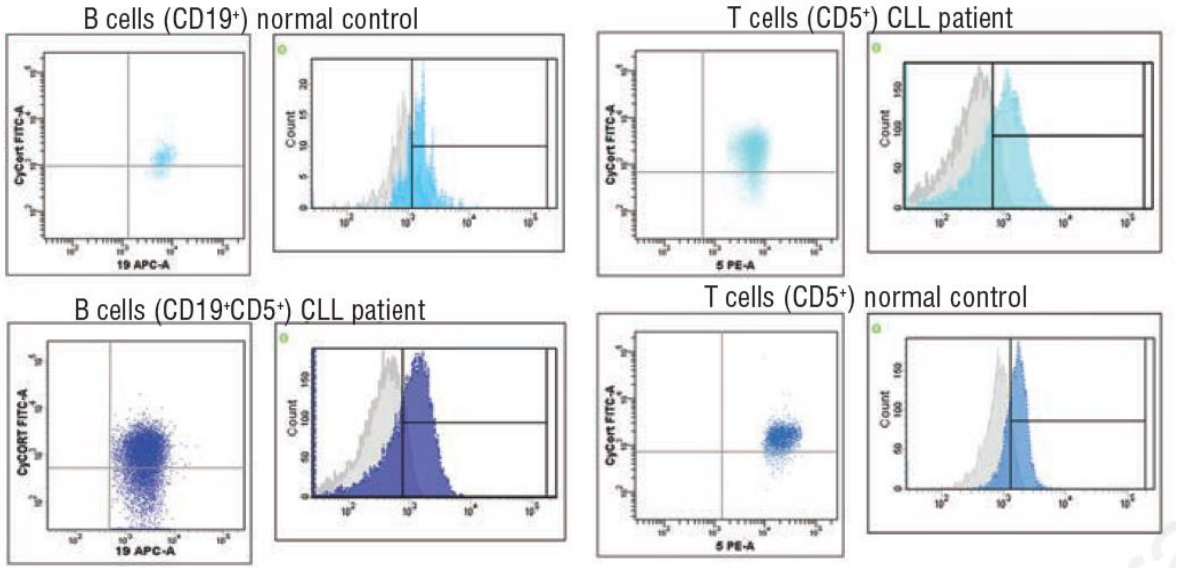
Grammer AC Arthritis Res Ther. 2004; 6:28-38

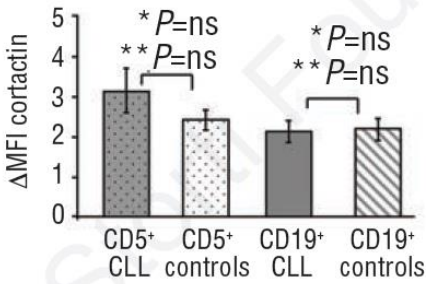
INTRACELLULAR CELL SIGNALING ANALYSIS

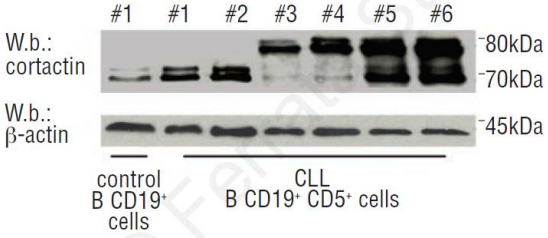


Cortactin, another player in the Lyn signaling pathway, is over-expressed and alternatively spliced in leukemic cells from patients with B-cell chronic lymphocytic leukemia

Cristina Gattazzo,[1,2] Veronica Martini,[1,2] Federica Frezzato,[1,2] Valentina Trimarco,[2] Elena Tibaldi,[3] Monica Castelli,[3] Monica Facco,[1,2] Francesca Zonta,[3] Anna Maria Brunati,[3] Renato Zambello,[1,2] Gianpietro Semenzato,[1,2*] and Livio Trentin[1,2*]







Gattazzo C Haematologica. 2014; 99:1069-77.

CELL SORTING

Fluorescence activated cell sorting (FACS) physically separates a cell population into sub-populations.

Cells stained using fluorochrome conjugated antibodies can be separated from one another depending on which fluorochrome they have been stained with.

MoFLO

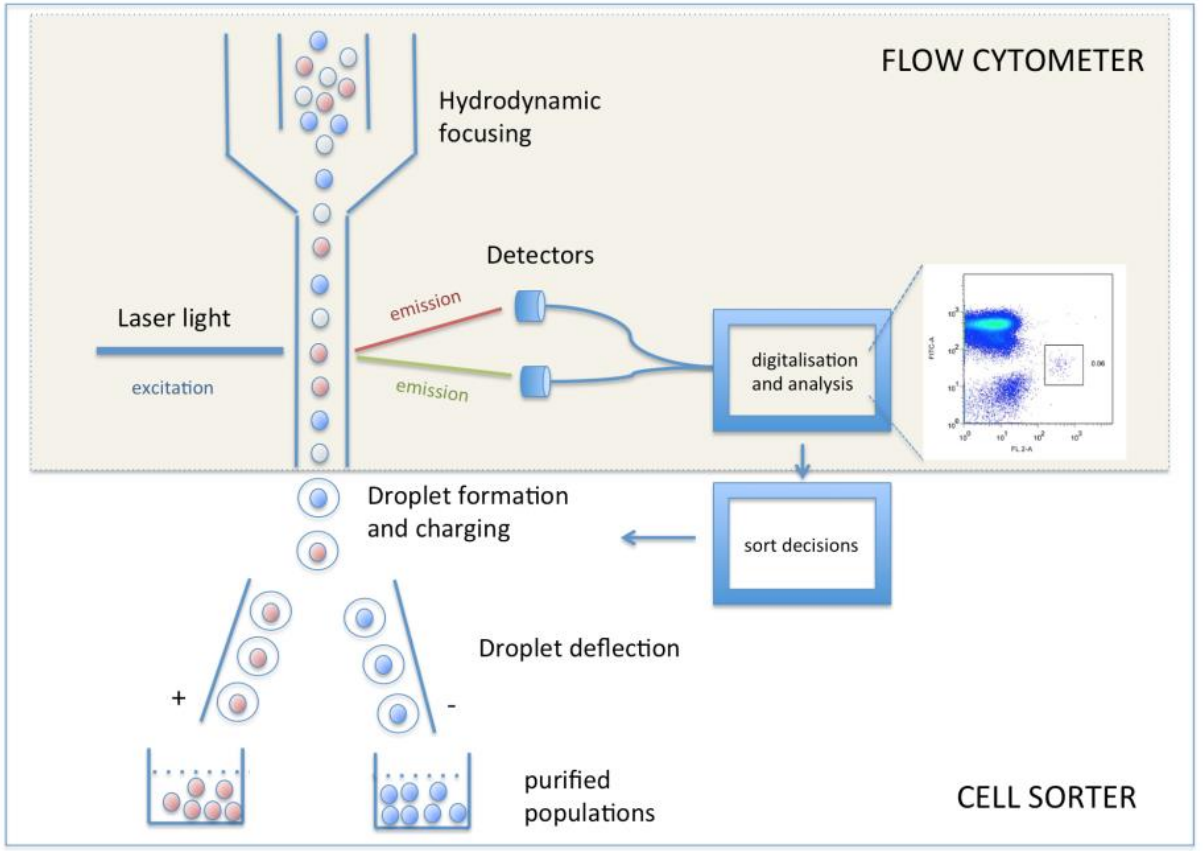
FACS Aria III

CELL SORTING

1. **Single cells are interrogated by the laser.**

2. **Each individual cell then enters a single droplet as it leaves the nozzle tip. This drop is given an electronic charge, depending on the fluorescence.**

3. **Deflection plates attract or repel the cells accordingly into collection tubes.**

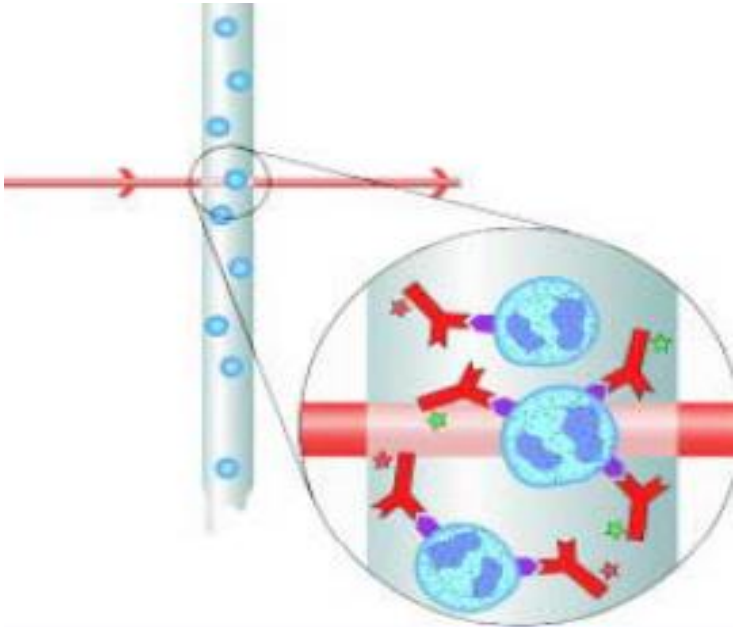


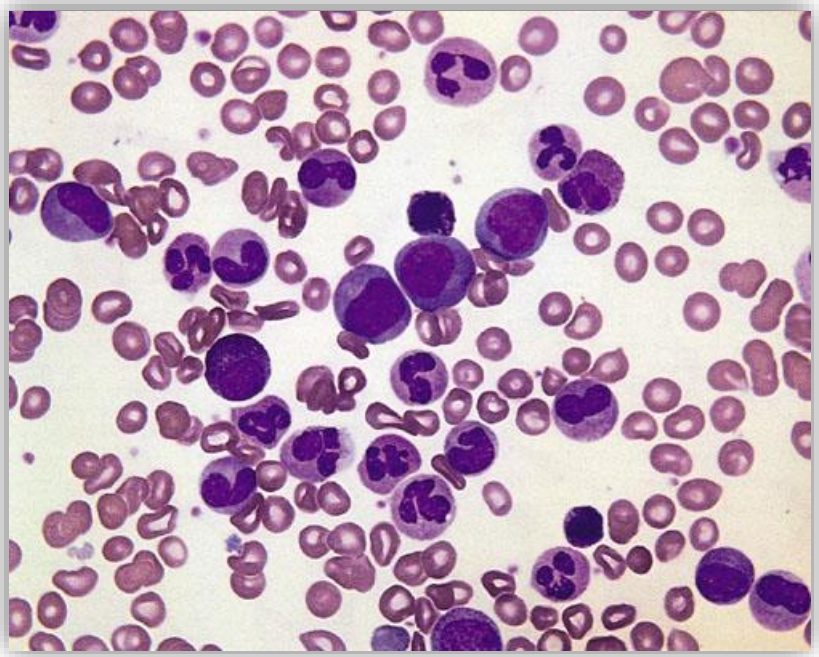
Sorted cells can then be cultured.

www.facs.ethz.ch/education

IMMUNOPHENOTYPING

Flow cytometric immunophenotyping represents an indispensable tool for the diagnosis, classification, staging, and monitoring of hematologic neoplasms.





Cells are labeled with appropriate antibodies directed against surface proteins on their membrane.

www.serotec.com

IMMUNOPHENOTYPING

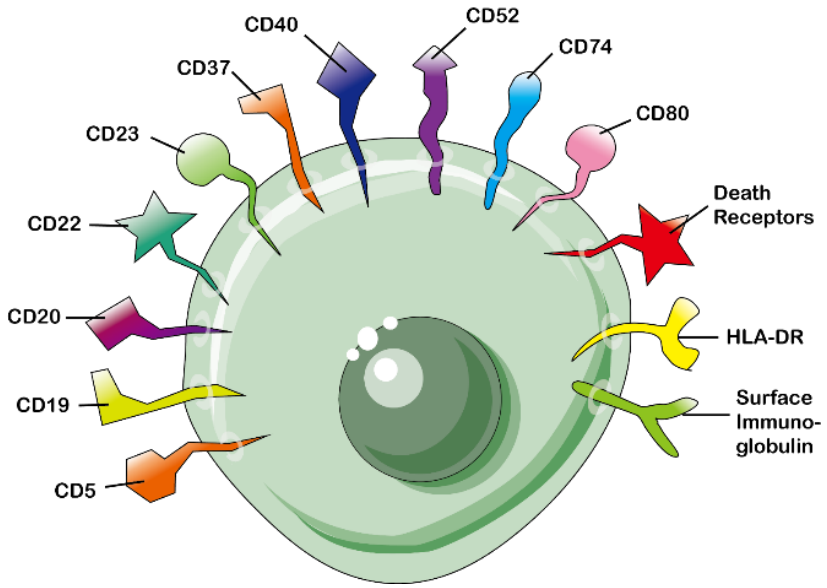
Samples analyzed

- Peripheral blood and bone marrow aspirates
- Cell lines
- Tissue cells, subject to suspension
- Ascitic, pleural, pericardial fluids
- Liquor
- Bronchoalveolar lavage (BAL)
- Vitreous humour, aqueous humour

IMMUNOPHENOTYPING

The leukocyte antigens that act as targets for the MoAb conjugated to fluorochromes for fluorescent staining are identified through a protocol called clusters of differentiation (CD).

The phenotypic characterization of a cell of hematopoietic origin may be made by examining the pattern of CD markers expressed.



IMMUNOPHENOTYPING
CLUSTER OF DIFFERENTIATION

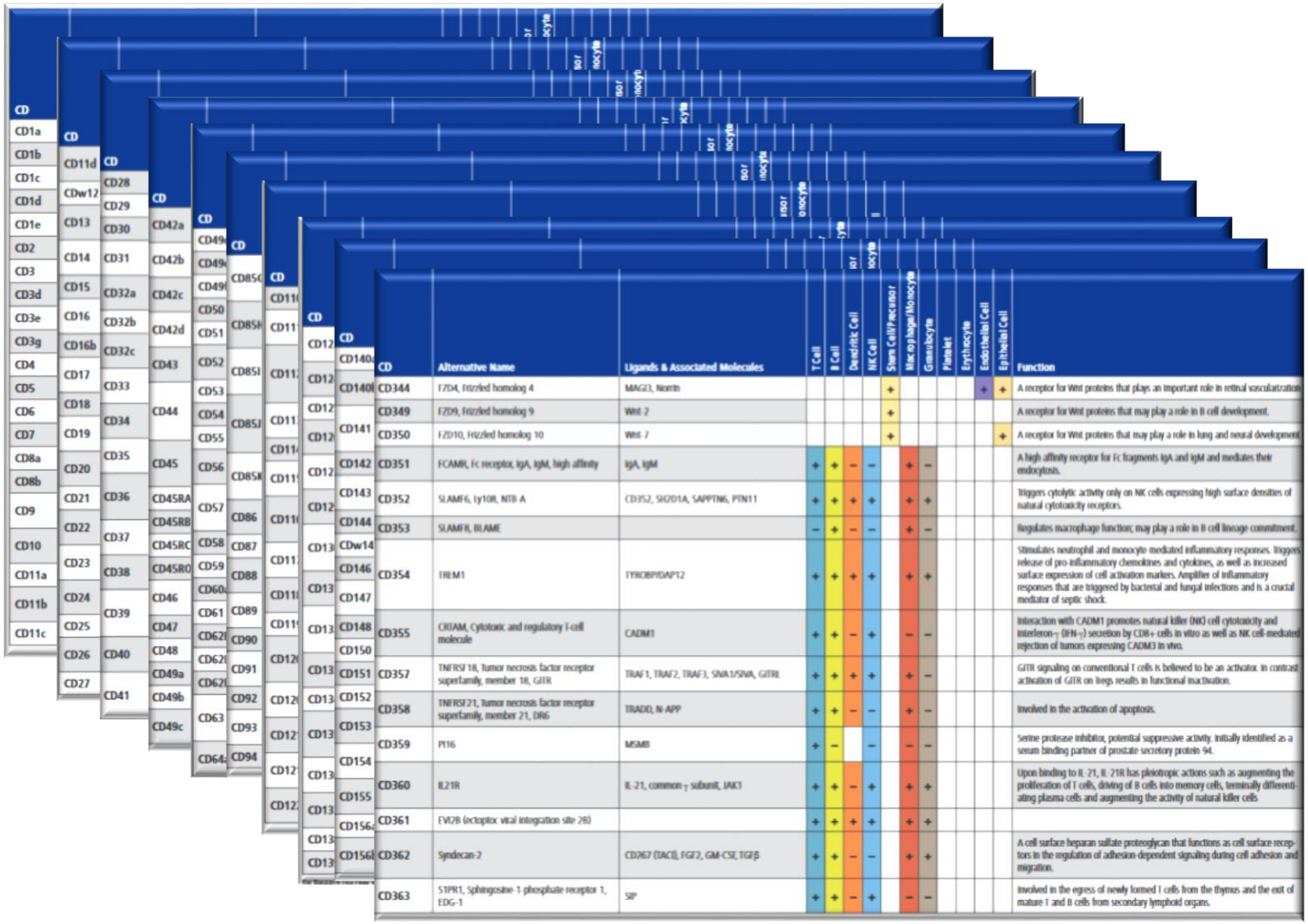
Main cluster of differentiation (CD) for each cell type

	Key Markers - Human
T Cell	CD3 CD4 CD8
B Cell	CD19 CD20
Dendritic Cell	CD11c CD123
NK Cell	CD56
Stem Cell/ Precursor	CD34 *hematopoetic stem cell only*
Macrophage/ Monocyte	CD14 CD33

	Key Markers - Human
Granulocyte	CD66b
Platelet	CD41 CD61 CD62
Erythrocyte	CD235a
Endothelial Cell	CD146
Epithelial Cell	CD326

bdbiosciences.com

IMMUNOPHENOTYPING
CLUSTER OF DIFFERENTIATION

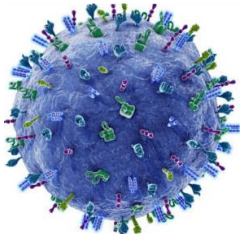


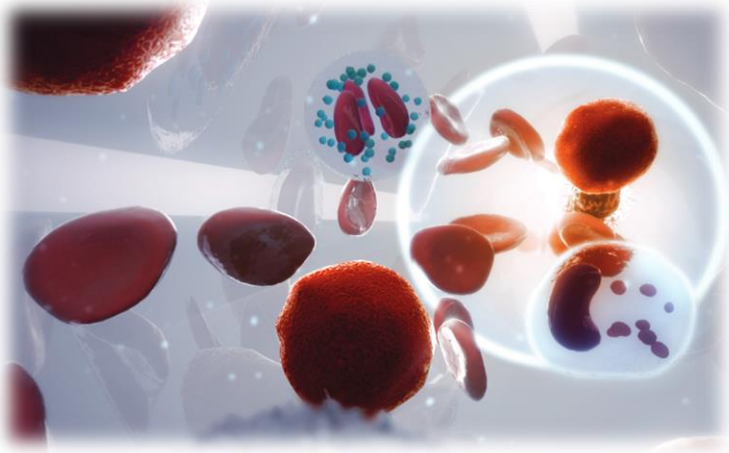
CD	Alternative Name	Ligands & Associated Molecules	T Cell	B Cell	Dendritic Cell	NK Cell	Stem Cell/Precursor	Macrophage/Monocyte	Granulocyte	Platelet	Erythrocyte	Endothelial Cell	Epithelial Cell	Function
CD344	FZD4, Frizzled homolog 4	MAGE3, Norrin					+					+	+	A receptor for Wnt proteins that plays an important role in retinal vascularization
CD349	FZD9, Frizzled homolog 9	Wnt-2					+							A receptor for Wnt proteins that may play a role in B cell development.
CD350	FZD10, Frizzled homolog 10	Wnt-7					+						+	A receptor for Wnt proteins that may play a role in lung and neural development
CD351	FCAMR, Fc receptor, IgA, IgM, high affinity	IgA, IgM	+	+	–	–		+	–					A high affinity receptor for Fc fragments IgA and IgM and mediates their endocytosis.
CD352	SLAMF6, Ly108, NTB-A	CD352, SH2D1A, SAPPTN6, PTN11	+	+	+	+		+	+					Triggers cytolytic activity only on NK cells expressing high surface densities of natural cytotoxicity receptors.
CD353	SLAMFR, BLAME		–	+	–	–		+	–					Regulates macrophage function; may play a role in B cell lineage commitment.
CD354	TREM1	TYROBP/DAP12	+	+	+	+		+	+					Stimulates neutrophil and monocyte mediated inflammatory responses. Triggers release of pro-inflammatory chemokines and cytokines, as well as increased surface expression of cell activation markers. Amplifier of inflammatory responses that are triggered by bacterial and fungal infections and is a crucial mediator of septic shock.
CD355	CRTAM, Cytotoxic and regulatory T cell molecule	CADM1	+	+	–	+		–	–					Interaction with CADM1 promotes natural killer (NK) cell cytotoxicity and interferon-γ (IFN-γ) secretion by CD8+ cells in vitro as well as NK cell-mediated rejection of tumors expressing CADM3 in vivo.
CD357	TNFRSF18, Tumor necrosis factor receptor superfamily, member 18, GITR	TRAF1, TRAF2, TRAF3, SNAI1/SNA, GITRL	+	+	+	+		+	–					GITR signaling on conventional T cells is believed to be an activator. In contrast activation of GITR on Tregs results in functional inactivation.
CD358	TNFRSF21, Tumor necrosis factor receptor superfamily, member 21, DR6	TRADD, N-APP	+	+	–	–		+	–					Involved in the activation of apoptosis.
CD359	PI16	M5MB	+	–		–		–	–					Serine protease inhibitor, potential suppressive activity. Initially identified as a serum binding partner of prostate secretory protein 94.
CD360	IL21R	IL-21, common γ subunit, JAK1	+	+	–	+		+	+					Upon binding to IL-21, IL-21R has pleiotropic actions such as augmenting the proliferation of T cells, driving of B cells into memory cells, terminally differentiating plasma cells and augmenting the activity of natural killer cells
CD361	EVI2B (ectoptoc viral integration site 2B)		+	+	+	+		+	+					
CD362	Syndecan-2	CD267 (TACI), FGF2, GM-CSF, TGFβ	+	+	–	–		+	+					A cell surface heparan sulfate proteoglycan that functions as cell surface receptors in the regulation of adhesion-dependent signaling during cell adhesion and migration.
CD363	S1PR1, Sphingosine-1-phosphate receptor 1, EDG-1	S1P	+	+	–	+		–	–					Involved in the egress of newly formed T cells from the thymus and the exit of mature T and B cells from secondary lymphoid organs.

bdbiosciences.com

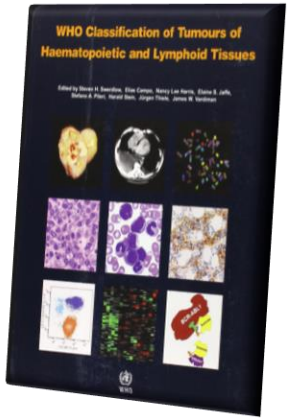
IMMUNOPHENOTYPING

- Evaluation of the cellular components of blood and biological fluids
- Evaluation of the stage of maturation of blood cells
- Demonstration of the presence in the sample of abnormal cells
- Possible demonstration of their clonality
- Analysis of peculiar phenotypes in order to identify a specific nosographic entity and/or to follow-up the presence of minimal residual disease (MRD)





IMMUNOPHENOTYPING
WHO classification of hematological disease



Myeloproliferative neoplasms (MPN)

- Chronic myeloid leukemia (CML), BCR-ABL1
- Chronic neutrophilic leukemia (CNL)
- Polycythemia vera (PV)
- Primary myelofibrosis (PMF)
  - prefibrotic/early stage
  - overt fibrotic stage
- Essential thrombocythemia (ET)
- Chronic eosinophilic leukemia, not otherwise specified (NOS)
- MPN, unclassifiable

- Mastocytosis

Acute myeloid leukemia (AML) and related neoplasms

AML with recurrent genetic abnormalities

- AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
- APL with PML-RARA
- AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A
- AML with t(6;9)(p23;q34.1);DEK-NUP214
- AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
- AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
- Provisional entity: AML with BCR-ABL1
- AML with mutated NPM1
- AML with biallelic mutations of CEBPA
- Provisional entity: AML with mutated RUNX1

AML with myelodysplasia-related changes

Therapy-related myeloid neoplasms

Myeloproliferative neoplasms (MPN)

- Chronic myeloid leukemia (CML), BCR-ABL1+
- Chronic neutrophilic leukemia (CNL)
- Polycythemia vera (PV)
- Primary myelofibrosis (PMF)
  - PMF, prefibrotic/early stage
  - PMF, overt fibrotic stage
- Essential thrombocythemia (ET)
- Chronic eosinophilic leukemia, not otherwise specified (NOS)
- MPN, unclassifiable

- Mastocytosis

Myelodysplastic syndromes (MDS)

- MDS with single lineage dysplasia
- MDS with ring sideroblasts (MDS-RS)
  - MDS-RS and single lineage dysplasia
  - MDS-RS and multilineage dysplasia
- MDS with multilineage dysplasia
- MDS with excess blasts
- MDS with isolated del(5q)
- MDS, unclassifiable
- Provisional entity: Refractory cytopenia of childhood

Myeloid neoplasms with germ line predisposition

Myeloid/lymphoid neoplasms with eosinophilia and rearrangement of

PDGFRA, PDGFRB, or FGFR1, or with PCM1-JAK2

- Myeloid/lymphoid neoplasms with **PDGFRA** rearrangement
- Myeloid/lymphoid neoplasms with **PDGFRB** rearrangement
- Myeloid/lymphoid neoplasms with **FGFR1** rearrangement
- Provisional entity: Myeloid/lymphoid neoplasms with **PCM1-JAK2**

Myelodysplastic/myeloproliferative neoplasms (MDS/MPN)

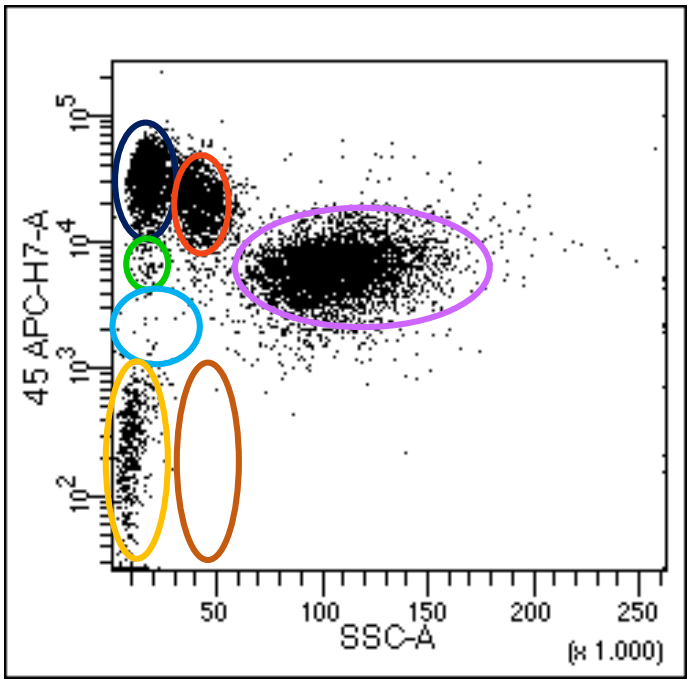
- Chronic myelomonocytic leukemia (CMML)
- Atypical chronic myeloid leukemia (aCML), BCR-ABL1 -ve
- Juvenile myelomonocytic leukemia (JMML)
- MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T)
- MDS/MPN, unclassifiable

- Precursor B cell
  - Acute lymphoblastic lymphoma
- Peripheral B cell
  - Small lymphocytic lymphoma SLL, Chronic lymphocytic leukemia CLL
  - Mantle cell lymphoma
  - Follicular lymphoma
  - Marginal zone lymphoma
  - Diffuse large B cell lymphom
  - Burkitt lymphoma

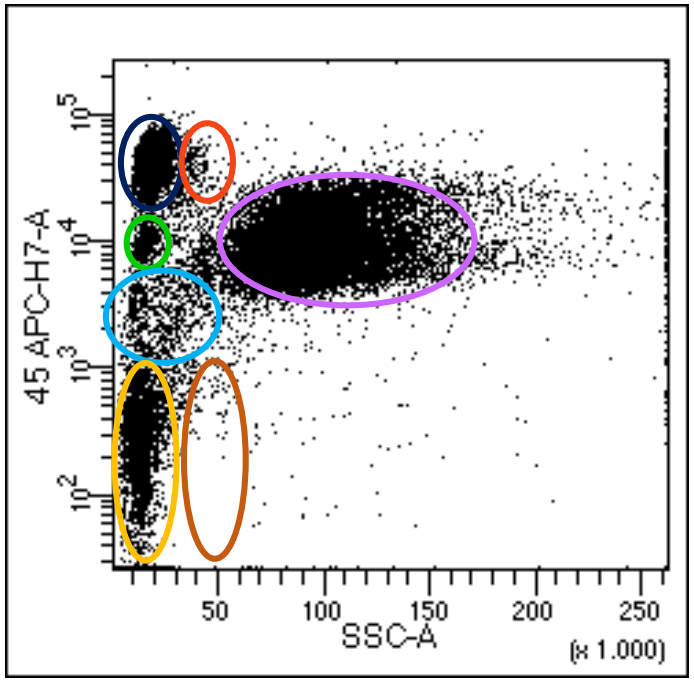
- Precursor T cell
  - Acute lymphoblastic lymphoma
- Peripheral T cell
  - Anaplastic large T cell lymphoma
  - Peripheral T cell lymphoma
  - Mycosis fungoides

IMMUNOPHENOTYPING
CD45 expression

Peripheral blood

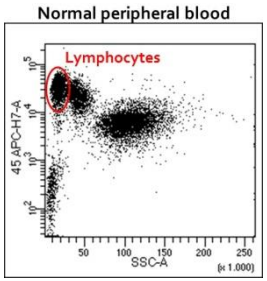


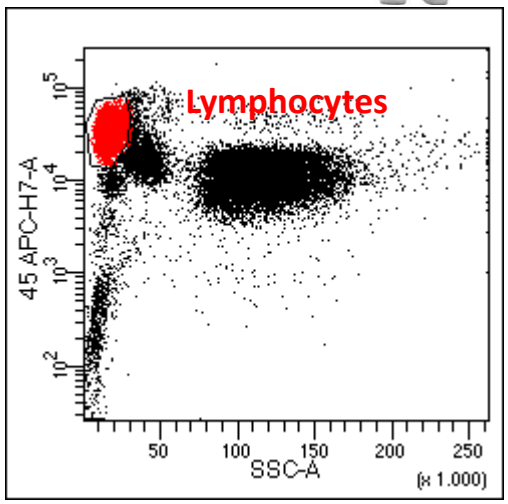
Bone marrow

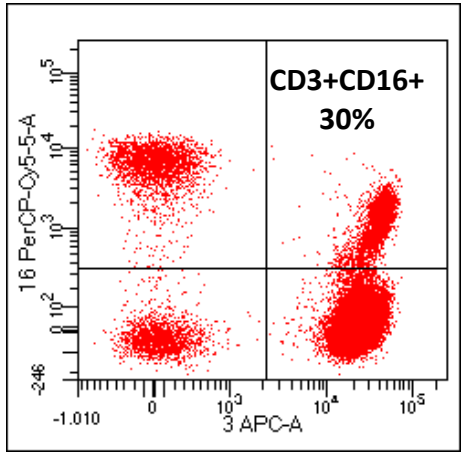


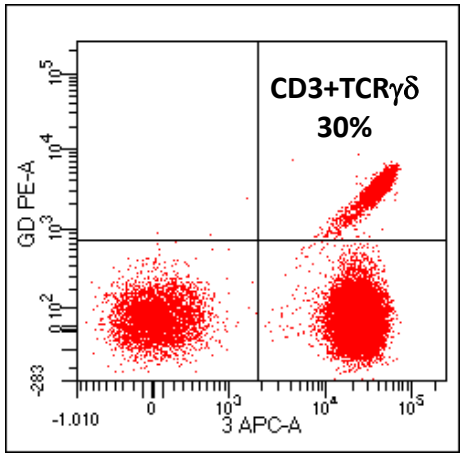
Lymphocytes **Monocytes** **Granulocytes** **Progenitor or basophil** **Blasts** **Eritrocytes** **Plasmacells**

IMMUNOPHENOTYPING
Mature cell neoplasm

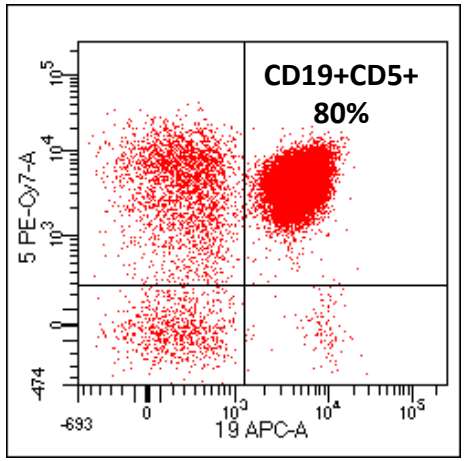


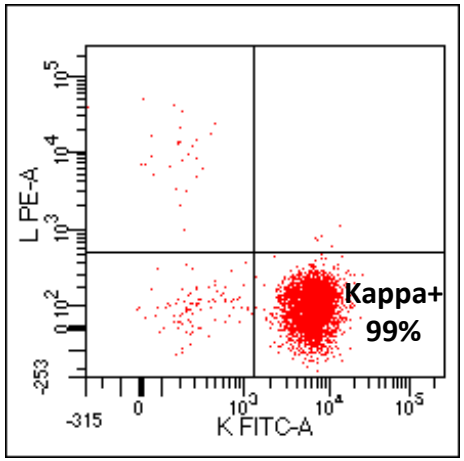






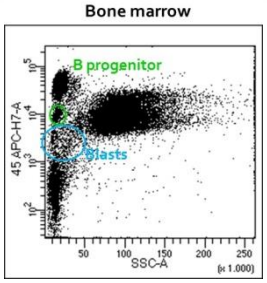
T cell leukemia



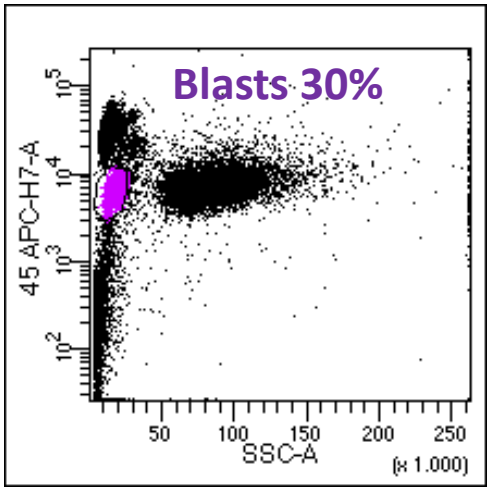


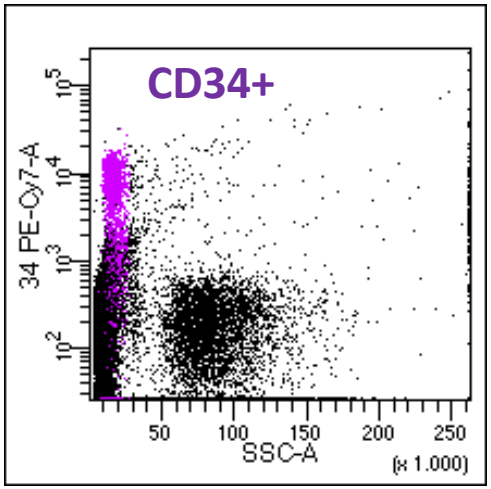
B cell leukemia

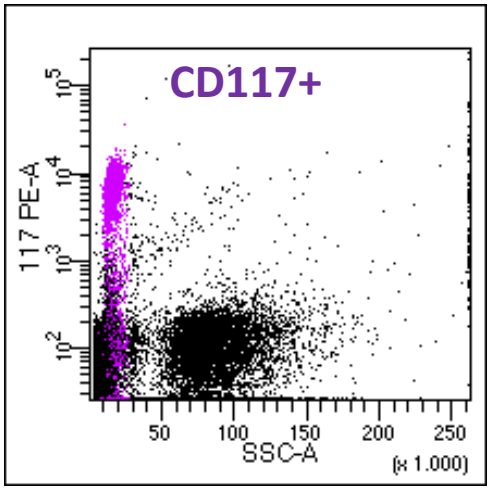
IMMUNOPHENOTYPING
Immature cell neoplasm



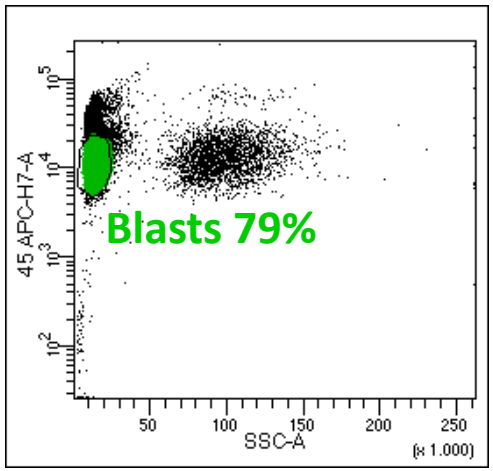
Acute Myeloid Leukemia

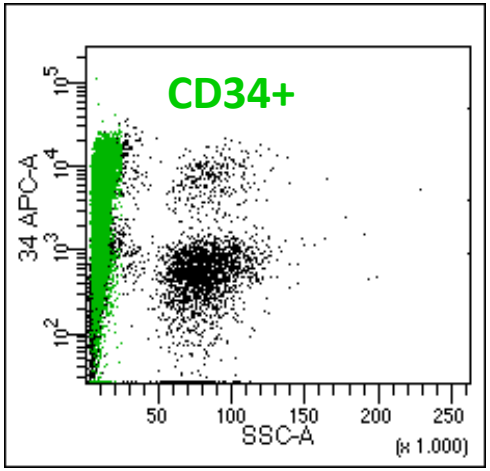


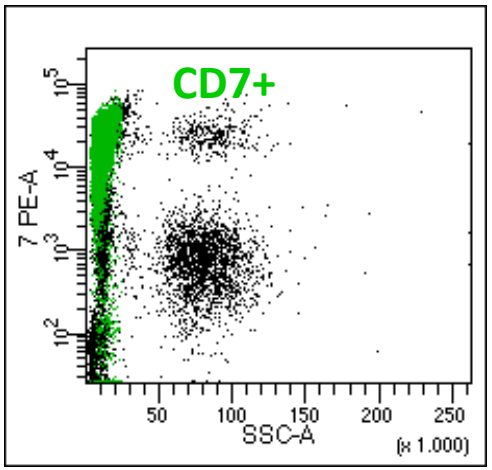




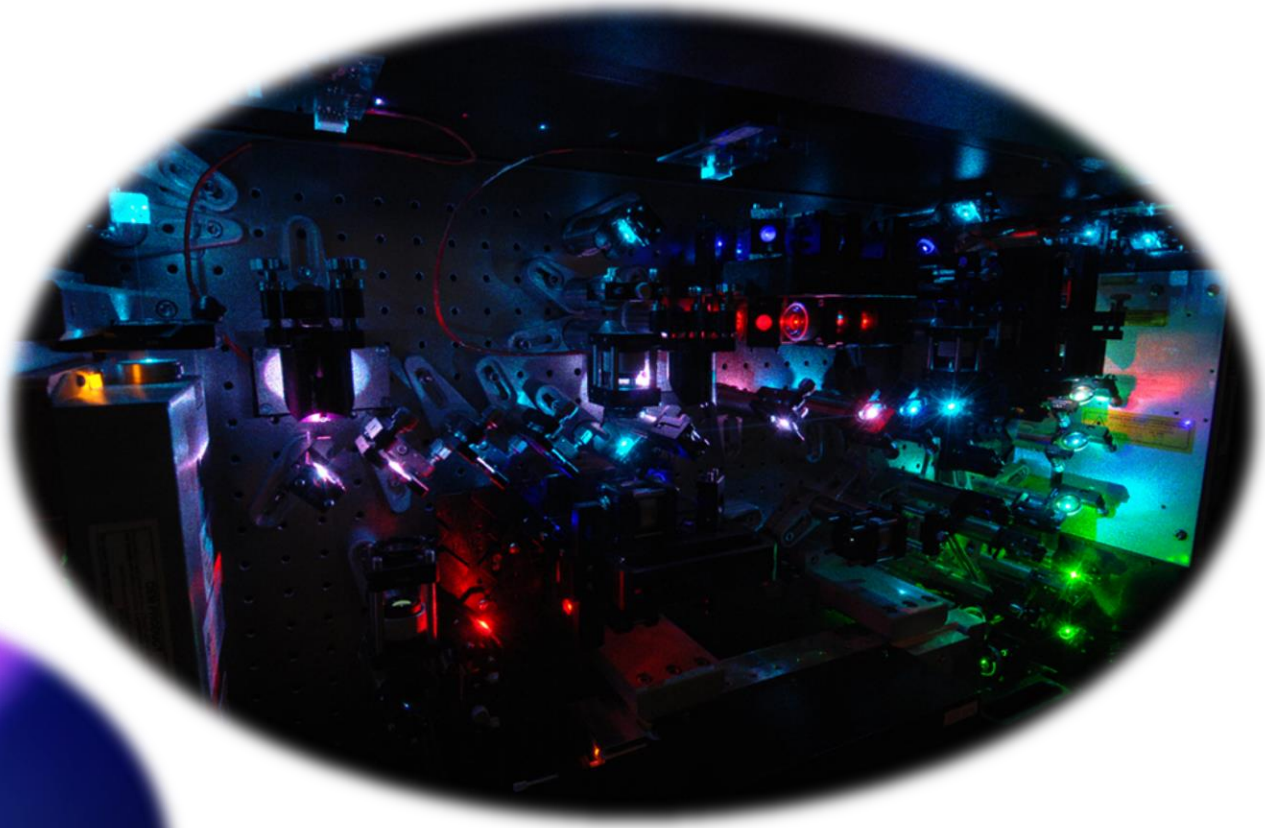
Acute Lymphoblastic Leukemia

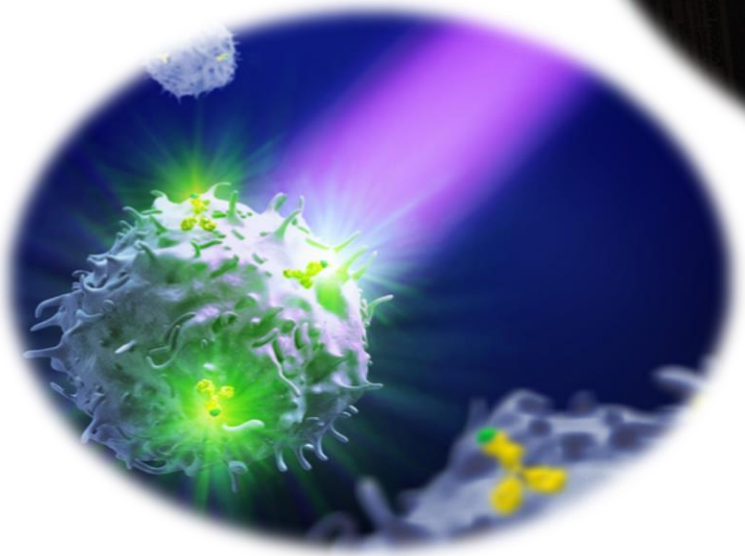






FROM CONVENTIONAL FLOW CYTOMETRY TO NEXT GENERATION FLOW





From conventional flow cytometry to next generation flow

Frequency of relevant cells	Total cell events to be acquired	Analytical context
10^{-2}	10,000	Conventional flow cytometry
10^{-3}	100,000	Second generation flow cytometry
10^{-4}	1,000,000	High resolution flow cytometry
10^{-5}	10,000,000	Next generation flow cytometry

High cell number acquired → High sensibility

Cytometric rare events can be defined as cell population represented at $\leq 10^{-3}$ over the total events.

From conventional flow cytometry to next generation flow

Flow cytometric analysis of *rare cell events* is an essential tool in a number of crucial applications:

- **detection and characterization of infrequent cell populations (i.e. dendritic cells, stem cells and precursors, circulating endothelial or neoplastic cells…);**
- **high resolution detection of PNH Clones;**
- **analysis of feto-maternal hemorrhage;**
- **minimal residual disease (MRD) in hematological disease.**

From conventional flow cytometry to next generation flow

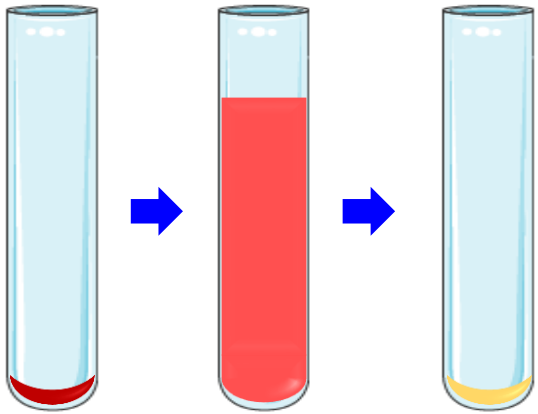
Rare event analysis technical pre-requisites

- Ensure a careful cleaning of the fluidic system to avoid carry over.
- Ensure the maximal specificity of the staining protocol (multicolor analysis).
- Set a well designed gating syntax aimed at eliminating non-specific events.
- Acquire the highest possible amount of total cell events (denominator).
- Acquire the highest possible amount of relevant cell events (numerator).
- Prepare a cell-rich sample to collect a high number of cell events.

From conventional flow cytometry to next generation flow

Cell concentration to collect a high number of events

Stain-and-lyse



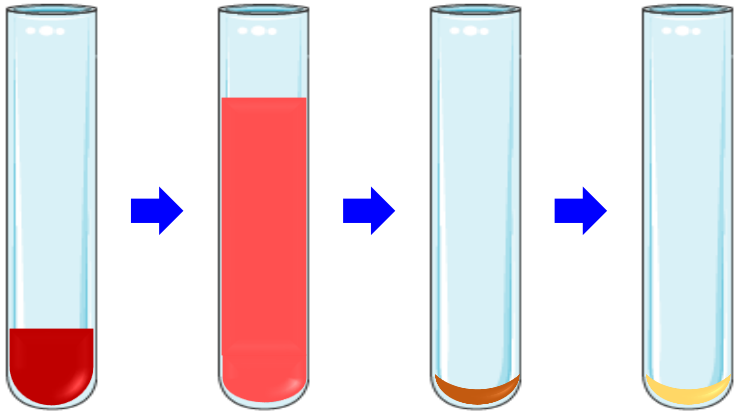
30-100 ul of blood, mix and incubate a RT for 10-15 min

Add 8-10 ml of lysing solution, mix and incubate a RT for 10 min

Resuspend the final pellet in 100-200ul PBS+0.5% BSA

VS

Bulk lysis



1-2 ml of blood

Add 8-10 ml of lysing solution, mix and incubate a RT for 10-15 min

Spin and wash twice with 10ml PBS+0.5% BSA

Resuspend the final pellet in 200-300ul PBS+0.5% BSA

Flores-Montero J. Leukemia 2017; 31: 2094-2103
Dahmani A. Am J Clin Pathol 2016; 145: 407-417

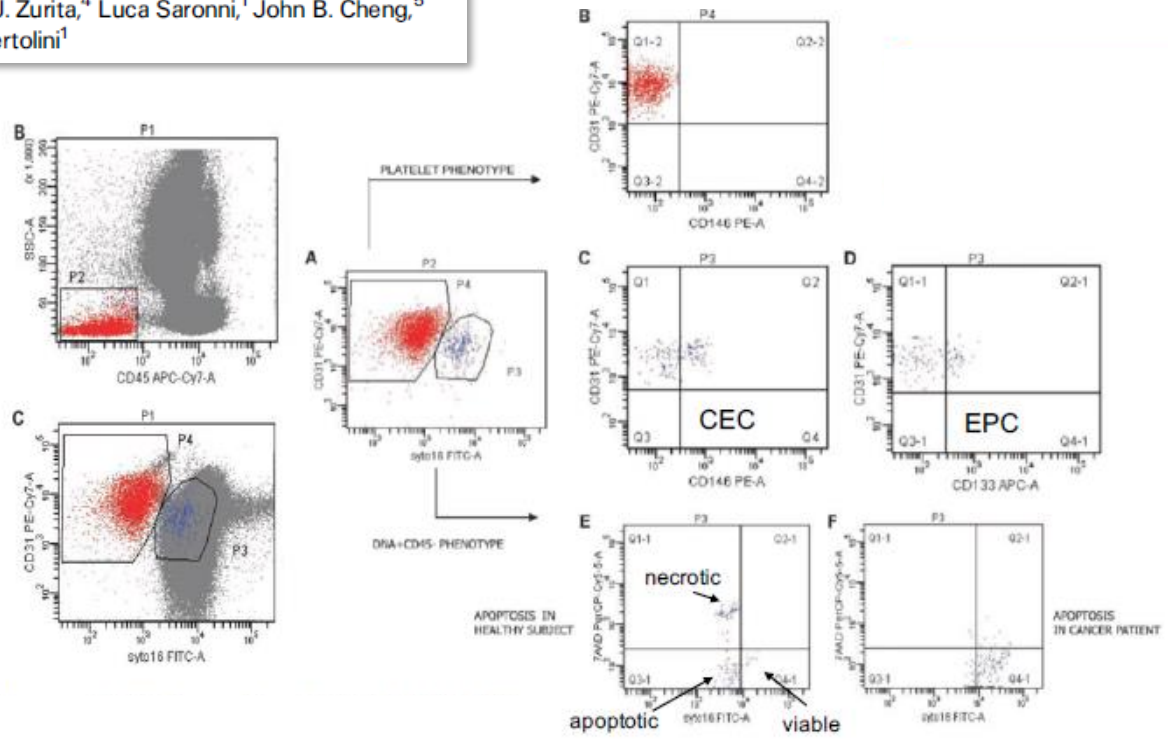
From conventional flow cytometry to next generation flow

Rare event analysis example: characterization of circulating cells

Validation of a Standardized Method for Enumerating Circulating Endothelial Cells and Progenitors: Flow Cytometry and Molecular and Ultrastructural Analyses

Patrizia Mancuso,[1] Pierluigi Antoniotti,[1] Jessica Quarna,[1] Angelica Calleri,[1] Cristina Rabascio,[1] Carlo Tacchetti,[2] Paola Braidotti,[3] Hua-Kang Wu,[4] Amado J. Zurita,[4] Luca Saronni,[1] John B. Cheng,[5] David R. Shalinsky,[5] John V. Heymach,[4] and Francesco Bertolini[1]

Acquisition of **1 MILLION** events to research the presence of epithelial progenitor cells (EPCs) and circulating epithelial cells (CECs).



Mancuso P. Clin Cancer Res 2009;15: 267-273

From conventional flow cytometry to next generation flow

Rare event analysis example: characterization of circulating cells

Plerixafor improves the endothelial health balance. The effect of diabetes analysed by polychromatic flow cytometry

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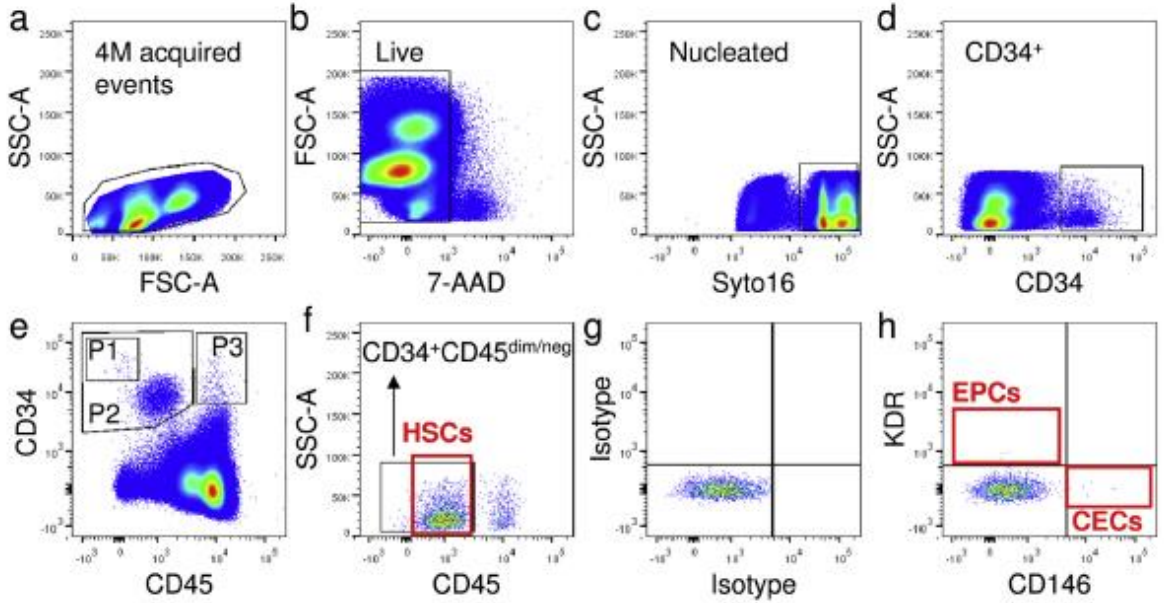


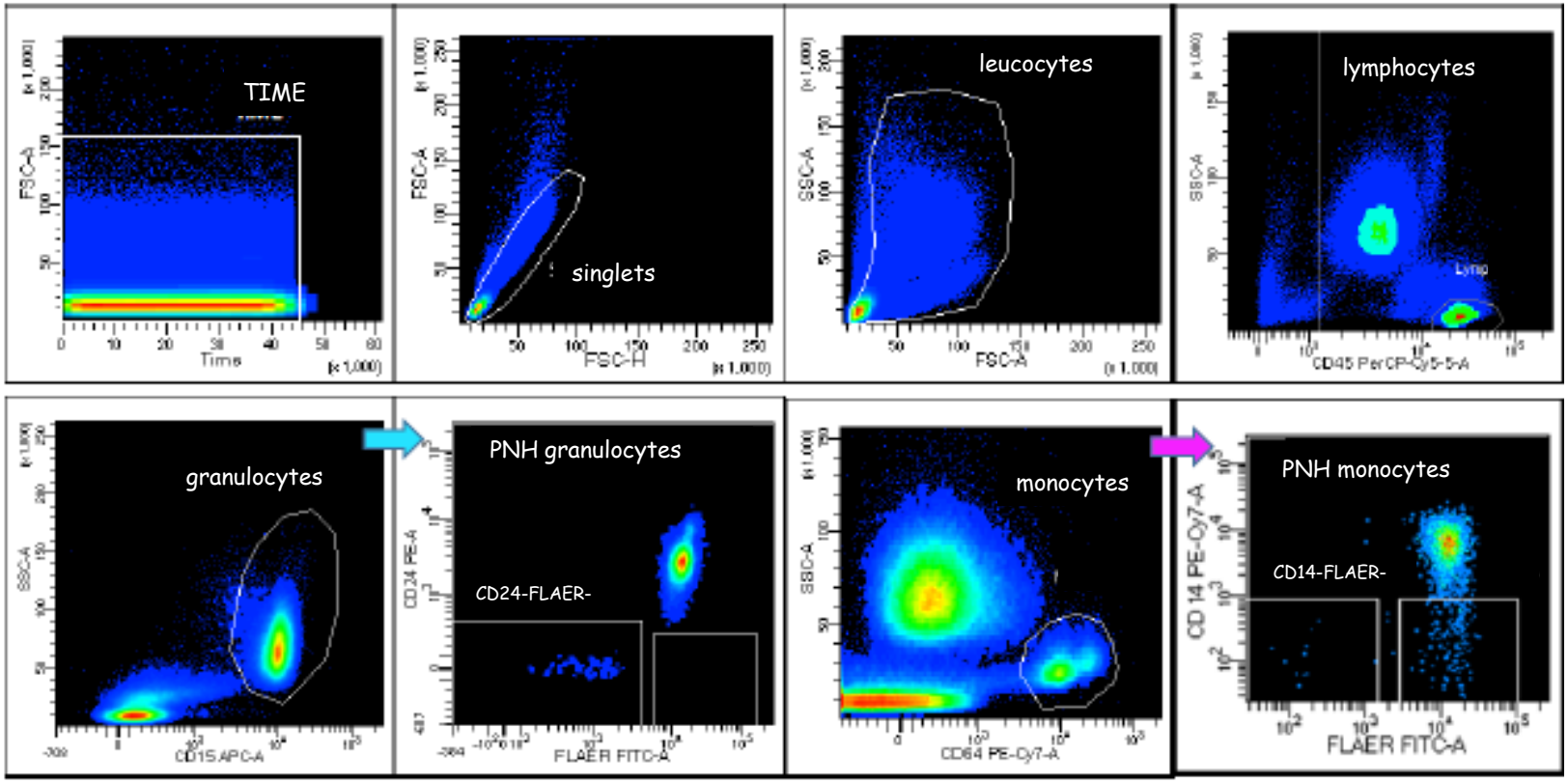
Acquisition of **4 MILLION** events to research the presence of Hematopoietic stem cells (HSCs), epithelial progenitor cells (EPCs) and circulating epithelial cells (CECs).

Cappellari R Atherosclerosis 2016; 251: 373-380

From conventional flow cytometry to next generation flow

Rare event analysis example: high resolution PNH detection and quantification

6-colour tube for white blood cells: FLAER/CD247 CD45/CD15/CD64/CD14



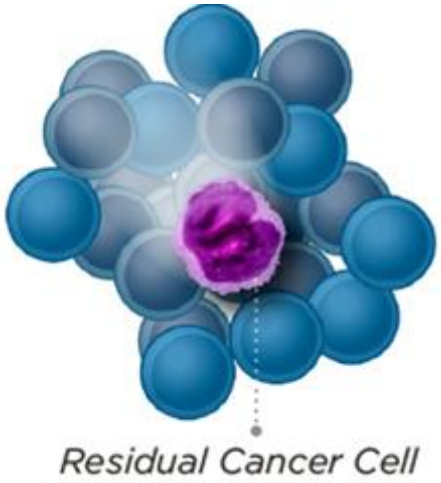
3.5 MILLION cell events acquired

Courtesy of Arianna Gatti, 2018

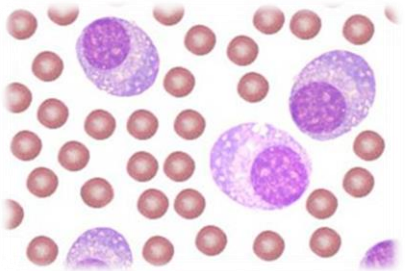
From conventional flow cytometry to next generation flow
MINIMAL RESIDUAL DISEASE

MRD is the presence of malignant cells below the detection of conventional methods.

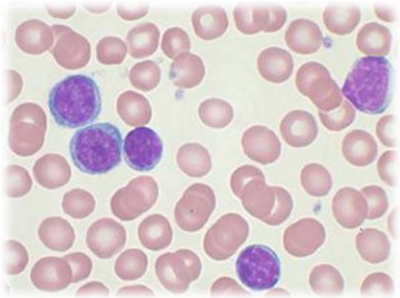
MRD assessments have become a key element in the management of patients with hematological disease



Multiple Myeloma (MM)

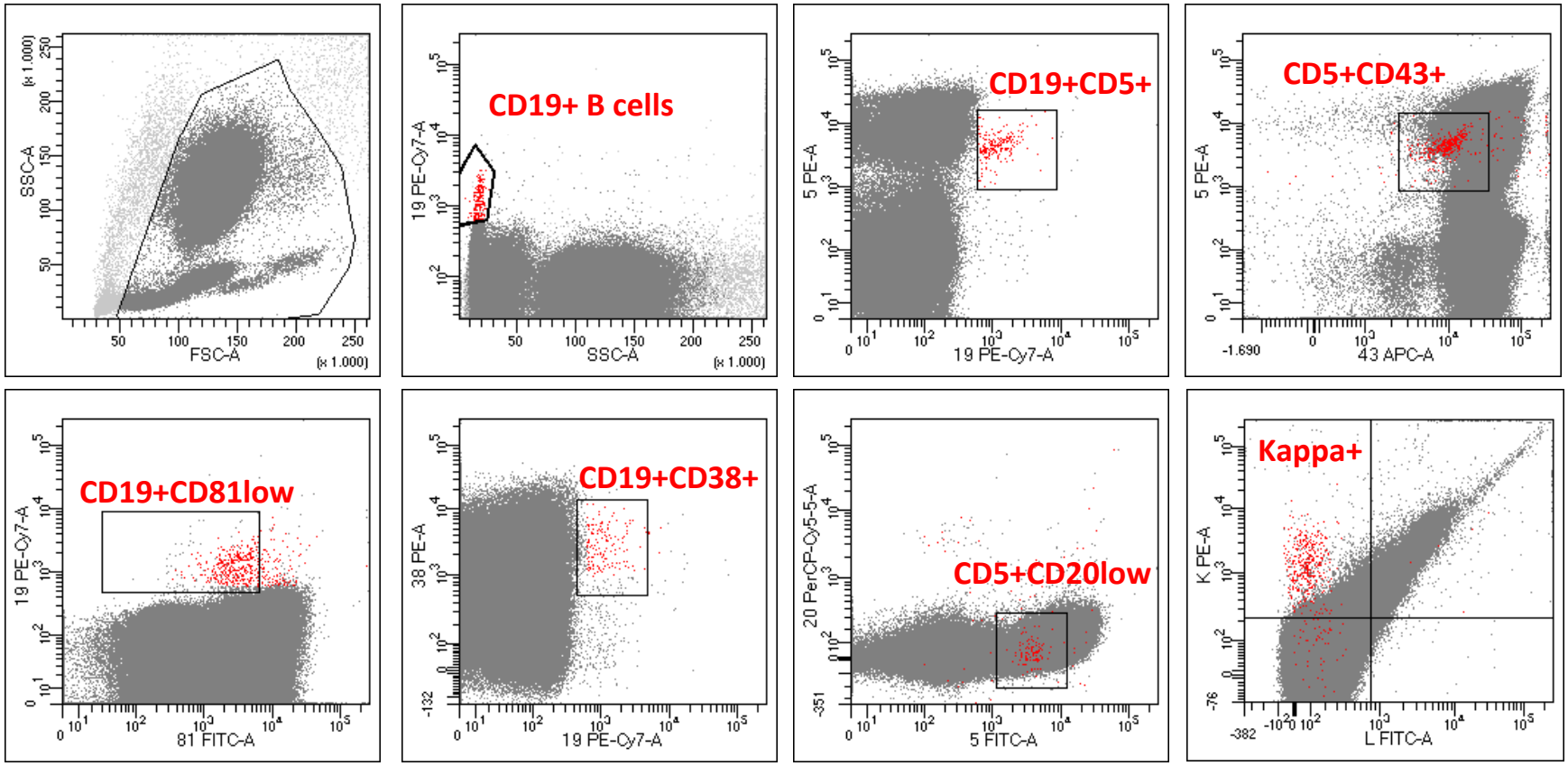


Chronic Lymphocytic Leukemia (CLL)



From conventional flow cytometry to next generation flow

MRD: the example of Chronic Lymphocytic Leukemia (CLL)



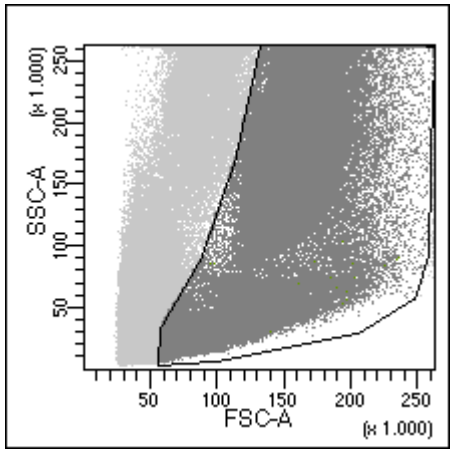
Number of events acquired: 1,000,000

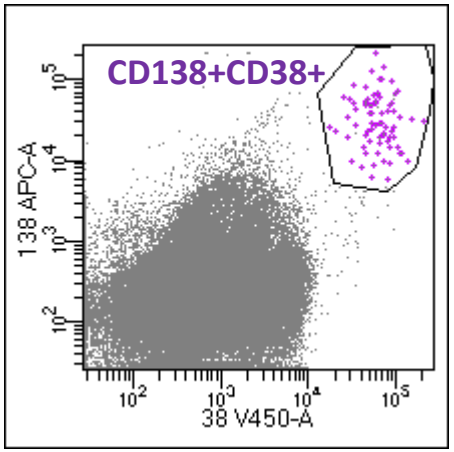
CLL B cells CD19+/CD5+/CD43+/CD81low/20low/CD38+/Kappa+: 0.032%

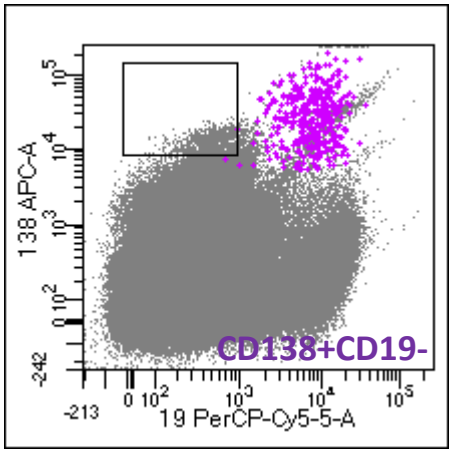
MRD POSITIVE

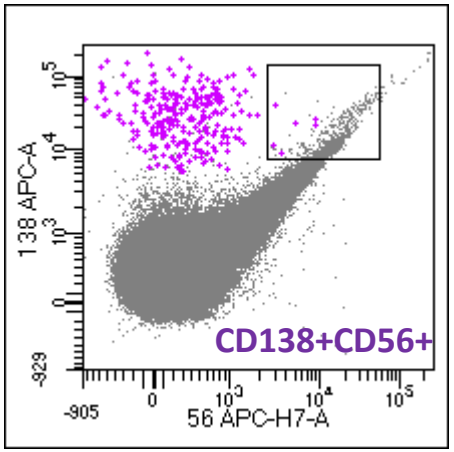
From conventional flow cytometry to next generation flow

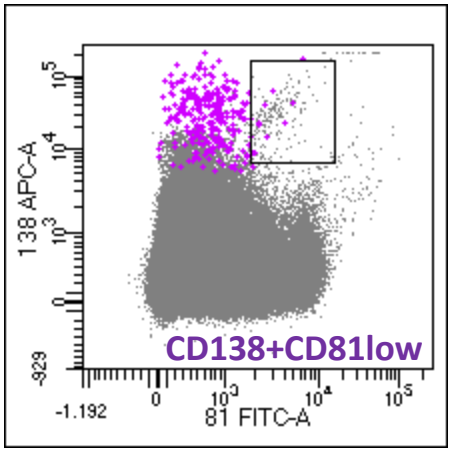
MRD deeper and deeper: the example of Multiple Myeloma (MM)

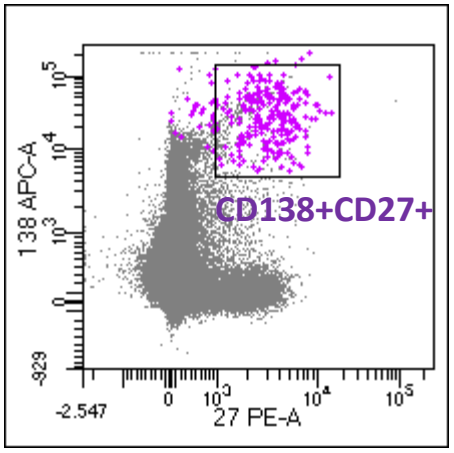


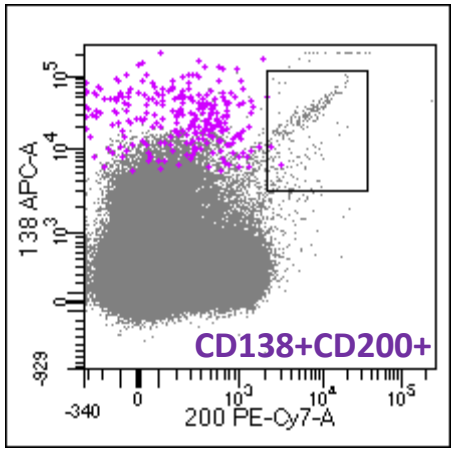












MRD NEGATIVE

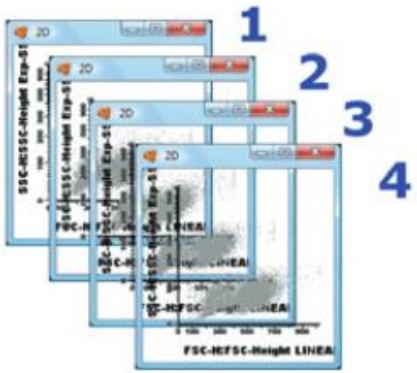
Number of events acquired: 10,000,000

MM plasmacells CD138+/CD38+/CD19-/CD56+/CD81low/CD27+/CD200+: 0.000%

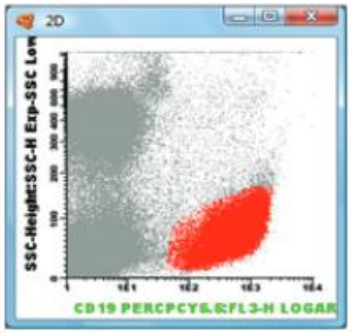
NEXT GENERATION FLOW CYTOMETRY

New multiparameter analysis

New software for data integration and multidimensional analysis of flow cytometry files.
The software is able to separate as many cluster as possible, analysing all the different choices of the parameter combinations.

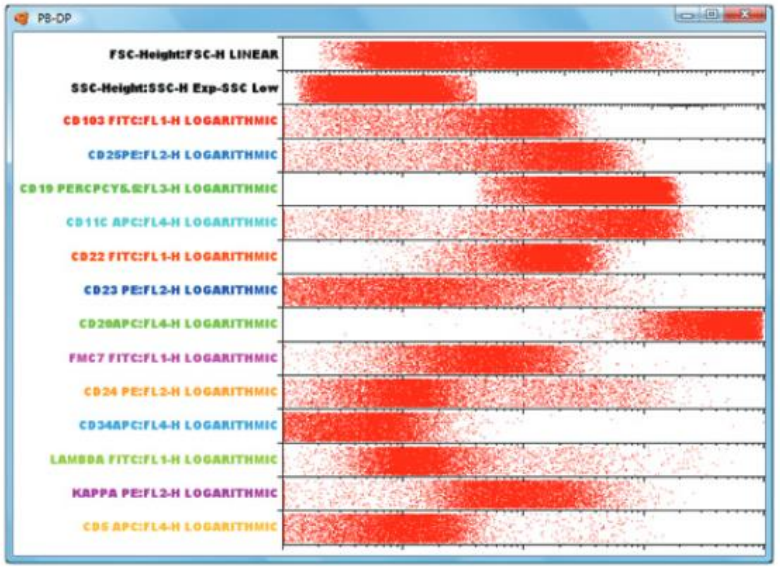


4 Single files



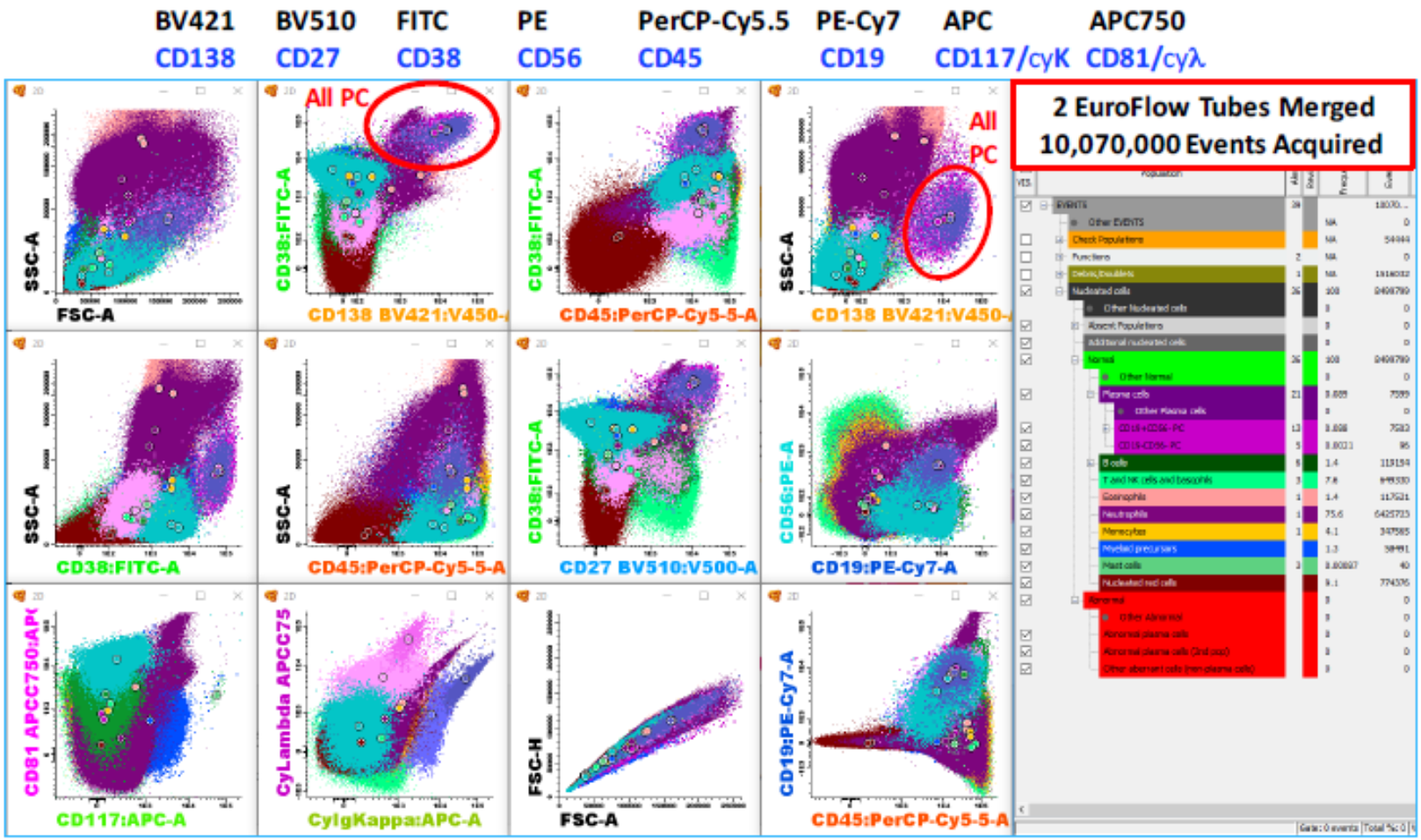
1 Merge file

Phenotypic description of cells population



NEXT GENERATION FLOW CYTOMETRY

New multiparameter analysis

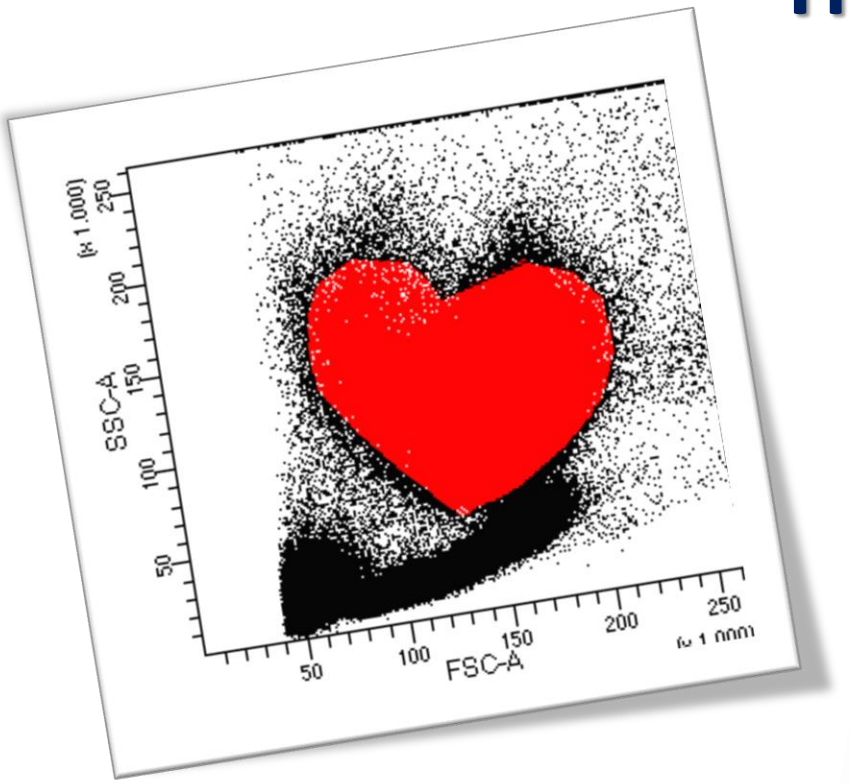


Courtesy of Arianna Gatti, 2018

TAKE HOME MESSAGES

- Flow cytometry measure cells based on their size, internal complexity, and fluorescence.
- Powerful technique for studying a variety of cellular characteristics at the single cell level (multiparametric analysis) with great sensibility.
- Results can be obtained in real time.
- Qualitative and quantitative analyses of cell populations have many different clinical and research applications.
- Successful experimental design depends on an understanding of flow cytometer instrumentation and basic immunological principles (operator dependent).

Thanks to



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Dr. S. Carraro
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And all of you for the attention