BIOLOGÍA CELULAR Y CITOMETRÍA DE FLUJO

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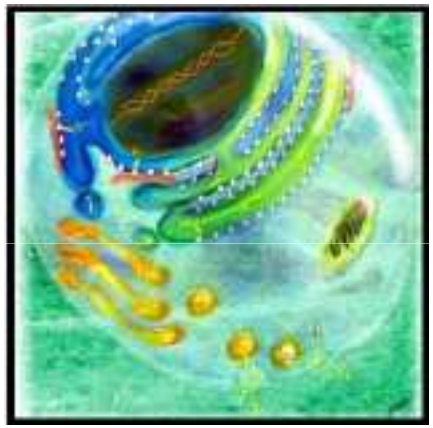
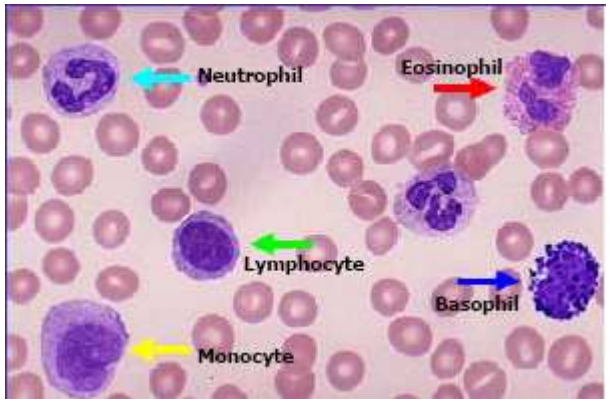
- Microscopía visualiza las células de acuerdo a la morfología y características de tinción.



- Citometría de flujo mide las células de acuerdo a características similares.
  - Una célula puede ser “vista” cualitativa y cuantitativamente.

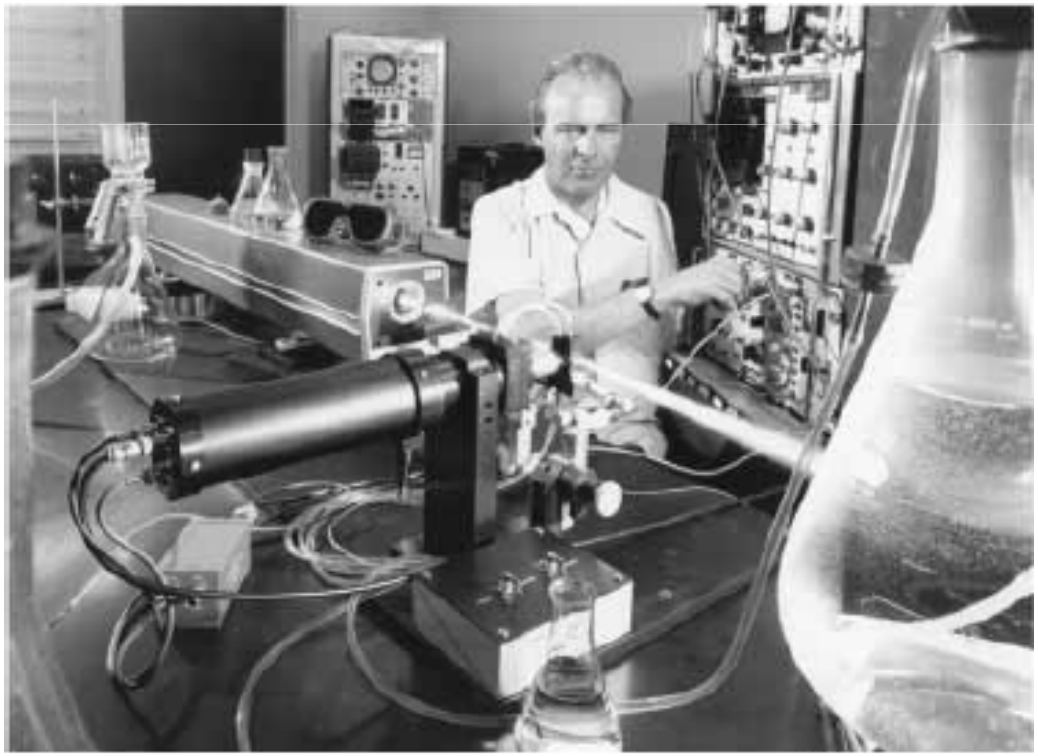
- Tamaño
- Expresión de proteínas en membrana (si-no, cuánto)
- Exp proteínas intracelulares
- Proteínas activadas
- Segundos mensajeros intracelulares (Ca++)
- Proliferación
- Ciclo celular
- Apoptosis

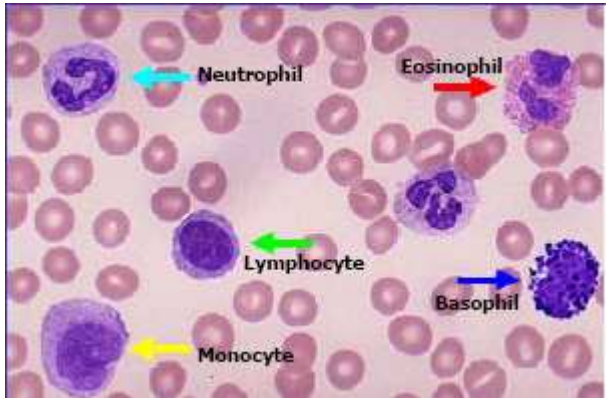
Célula --- Población ---- Subtipos ----- Diferentes estados



- Células sanguíneas
- 50´ contador automatizado Coulter basado en el tamaño.
- 60´ scaner sobre laminillas. Scaner, paso de células en fila, dispersión y absorción de luz.

- 1974 - Dr. Herzenberg (Stanford) patentó un aparato que colectaba células vivas en diferentes tubos – primer FACS (fluorescence-activated cell sorter).



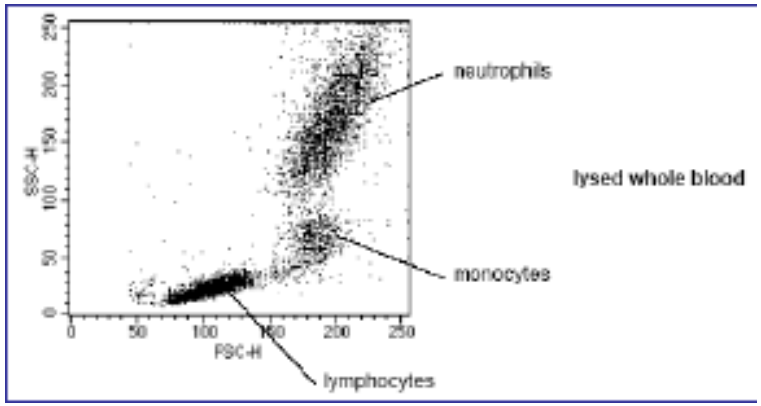


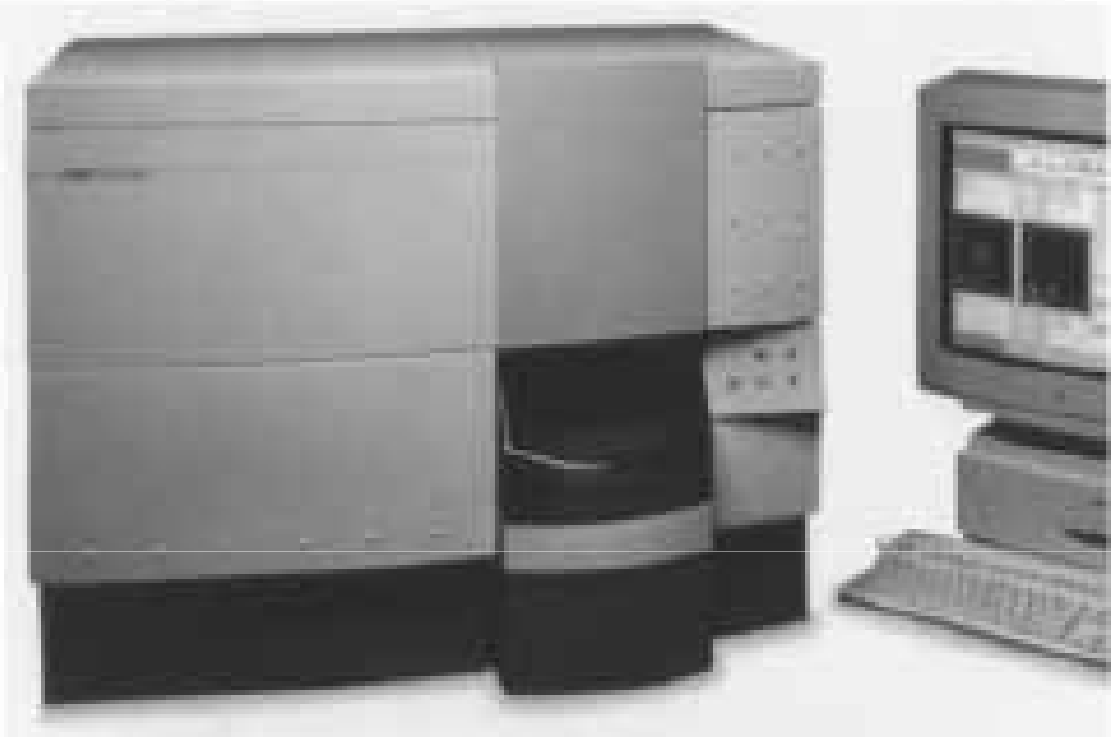
Lectura rápida de parámetros celulares

~Tamaño (FSC)

~ Complejidad (SSC)

Fluorescencia (3-4-6-9-15)





Sistema de flujo (corriente líquida con células en suspensión)

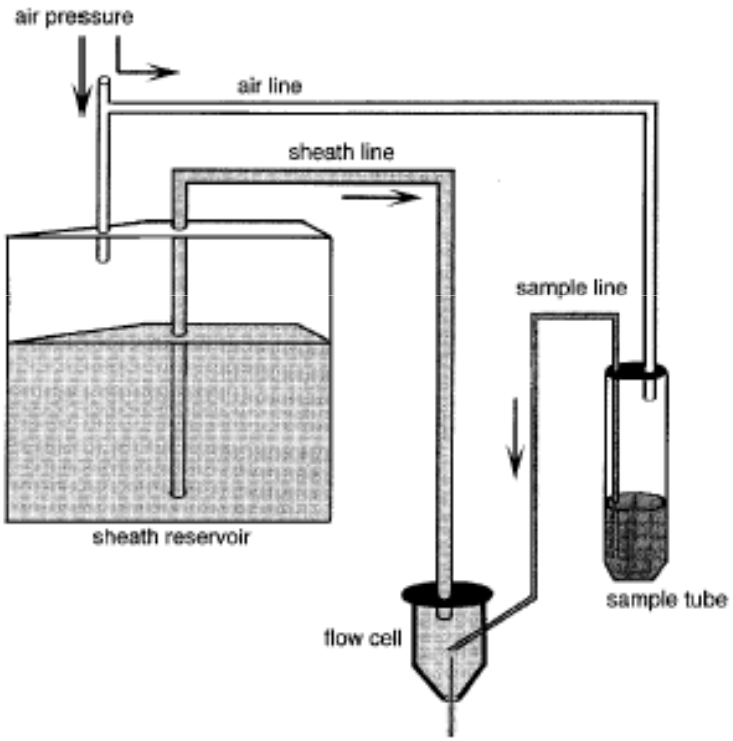
Sistema de excitación (LASER)

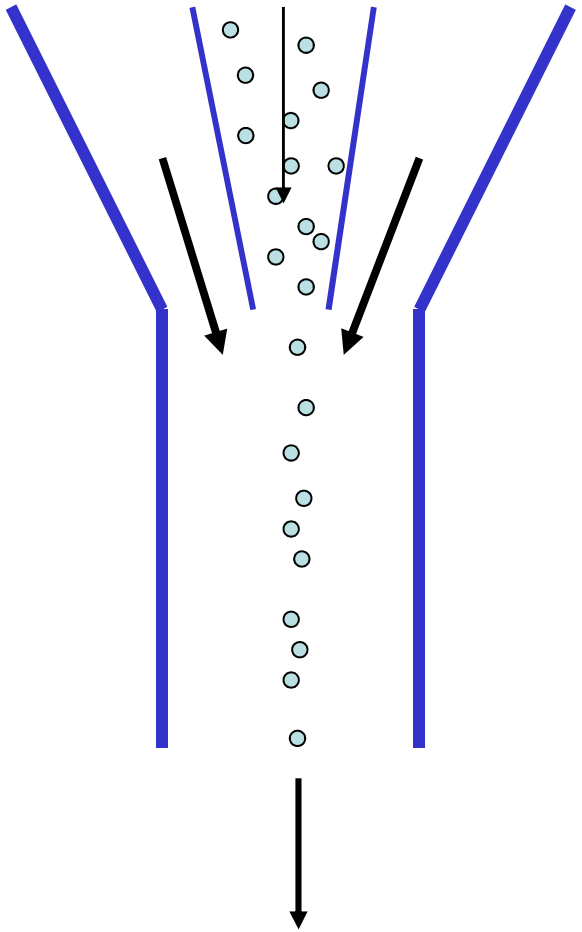
Sistema de detección (Filtros, fotomultiplicadores y diodos)

Sistema de recopilación y análisis de datos (BD: Cell Quest, DIVA)

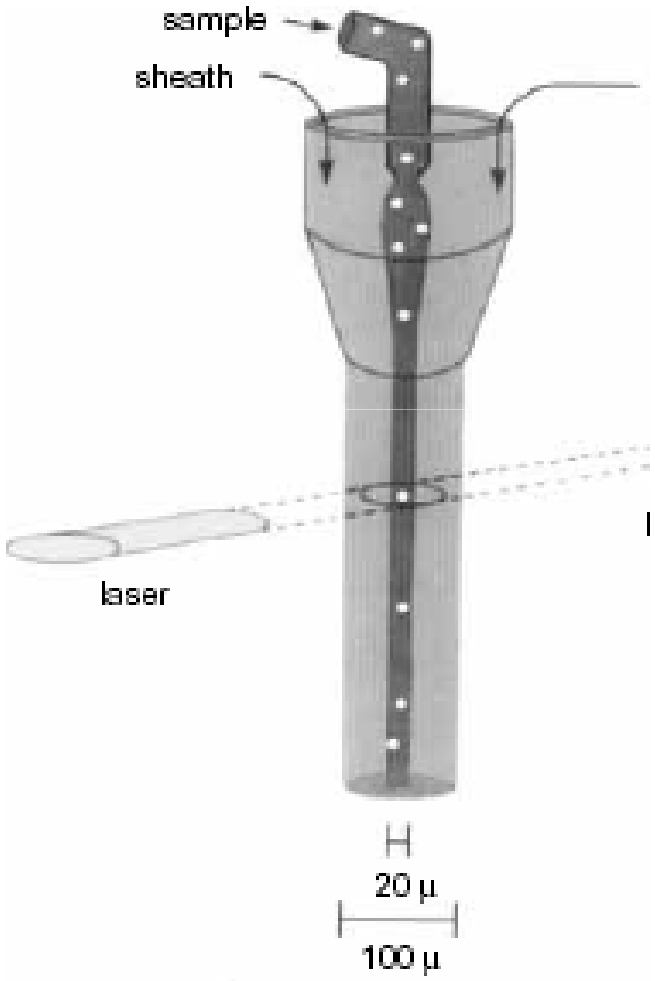
SISTEMA DE FLUJO

Paso de células de manera unitaria



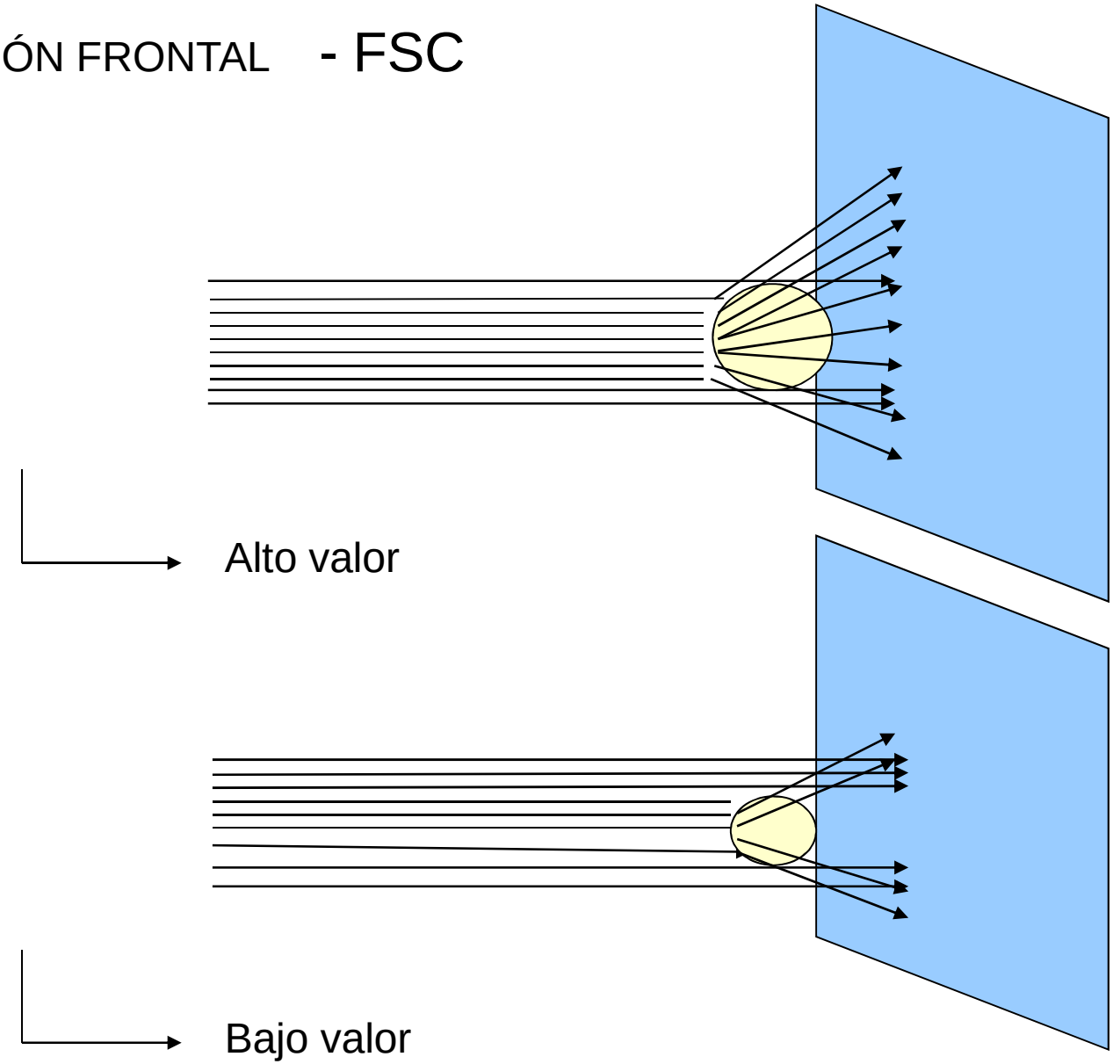


SISTEMA DE EXCITACIÓN

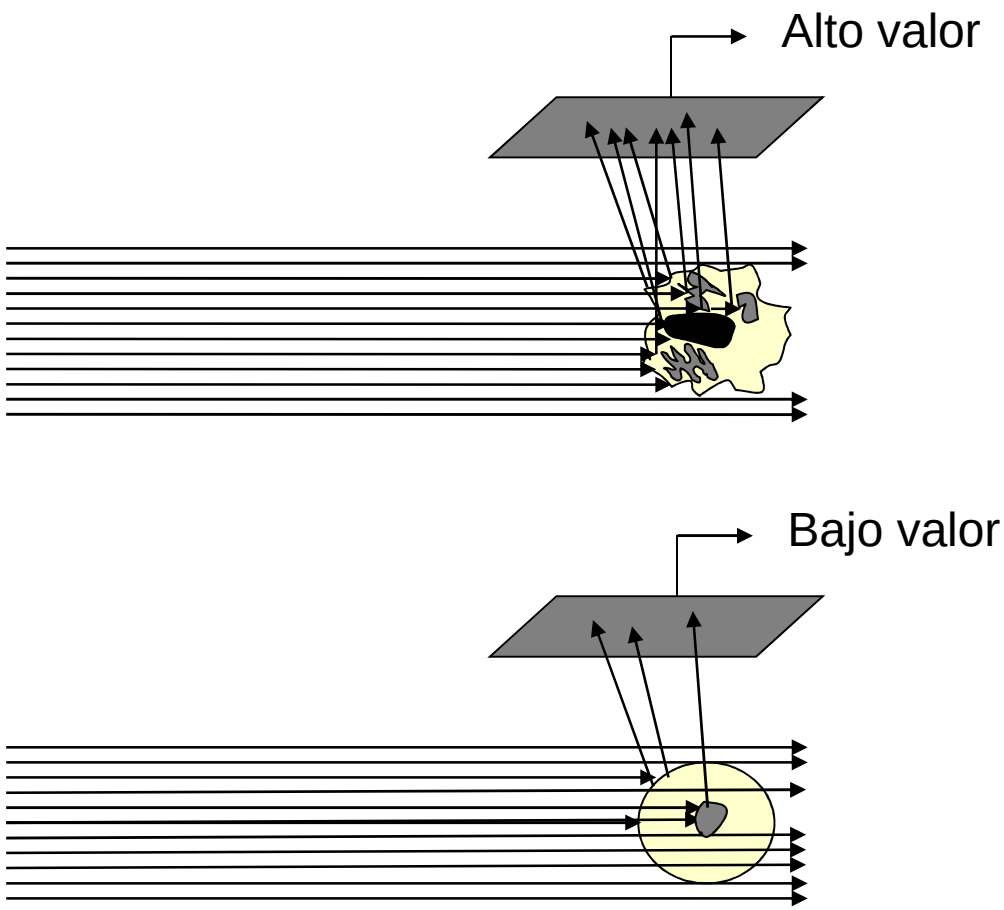


SISTEMA DE DETECCIÓN

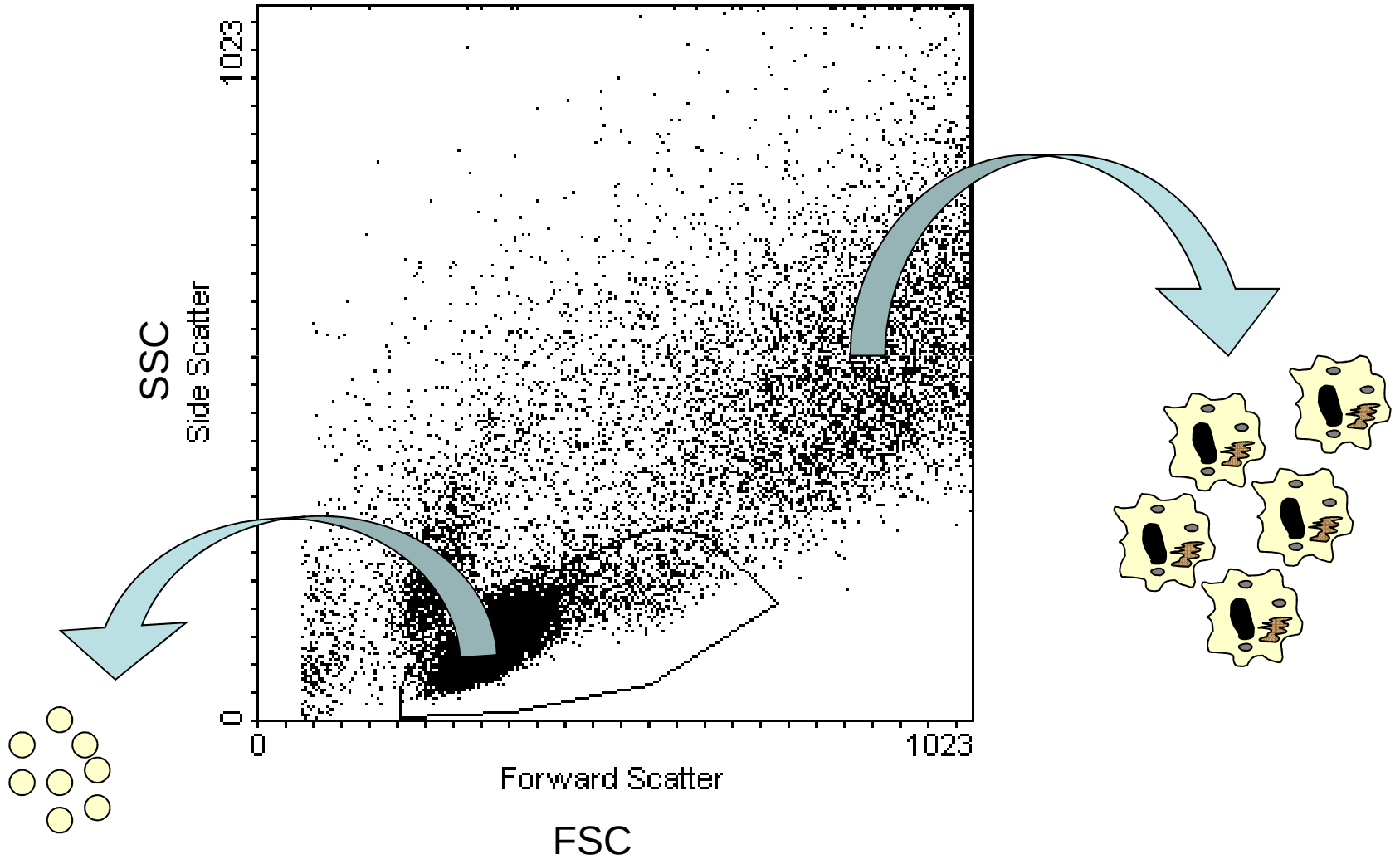
DISPERSIÓN FRONTAL - FSC



DISPERSIÓN LATERAL - SSC

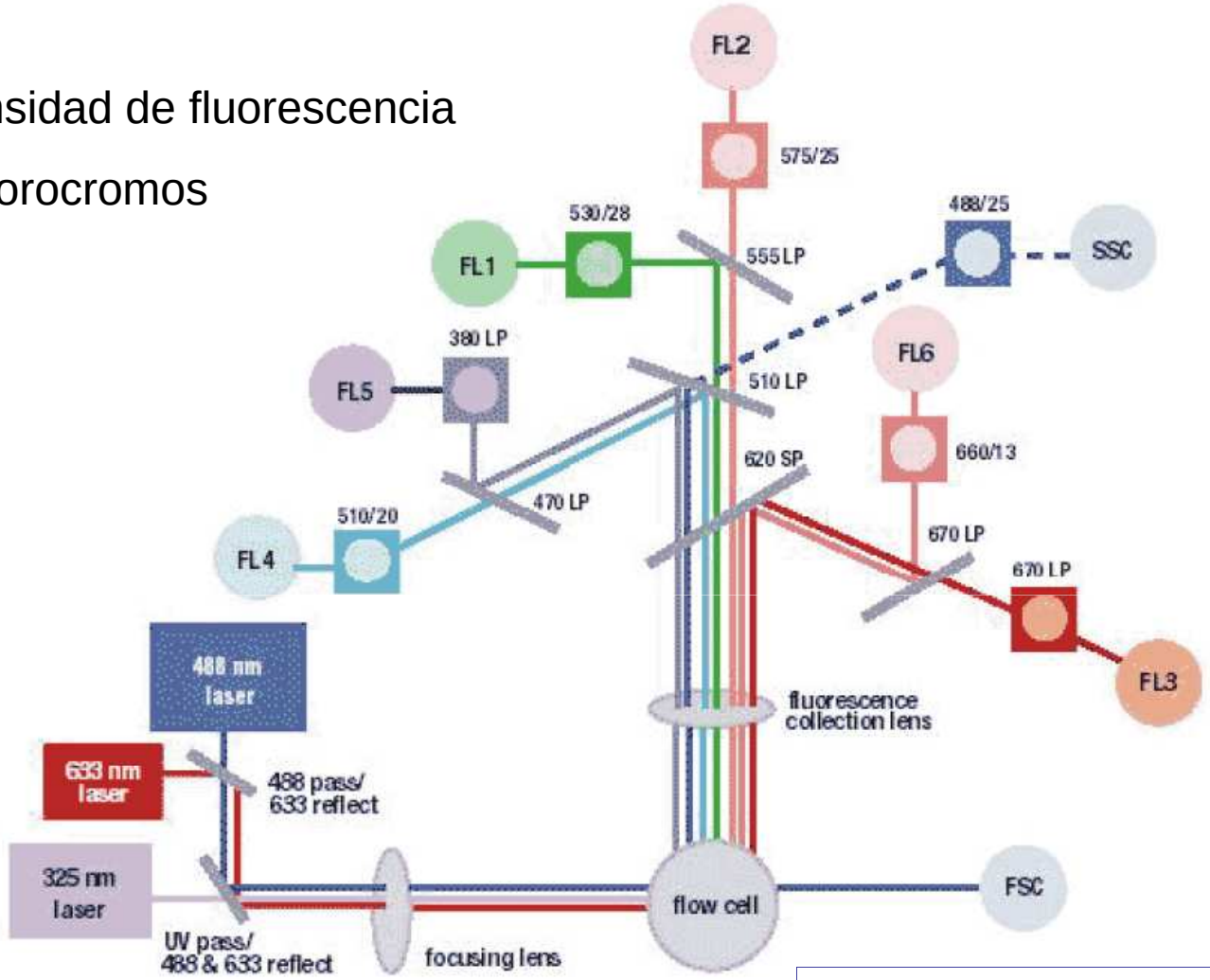


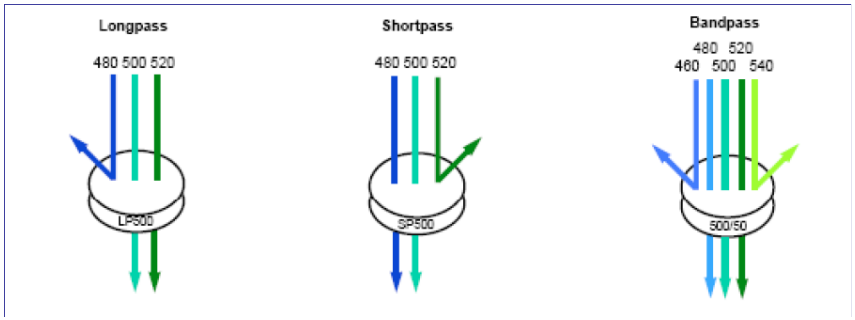
CÉLULAS MONONUCLEARES DE SANGRE PERIFÉRICA



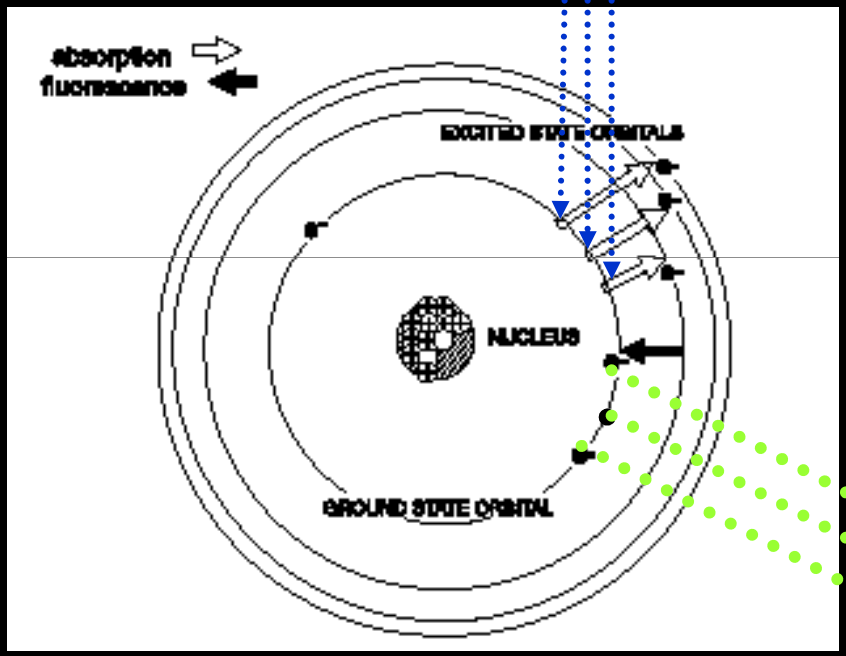
Intensidad de fluorescencia

- Fluorocromos



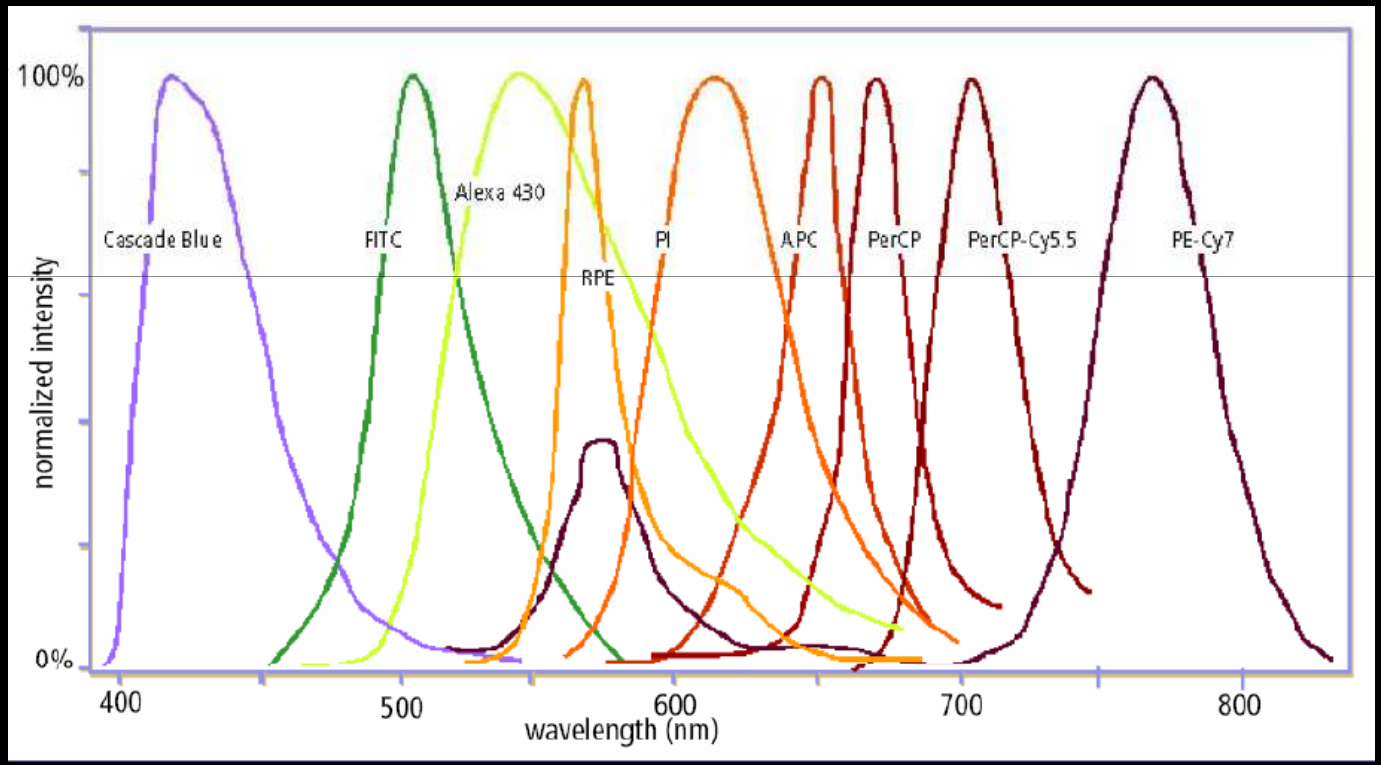


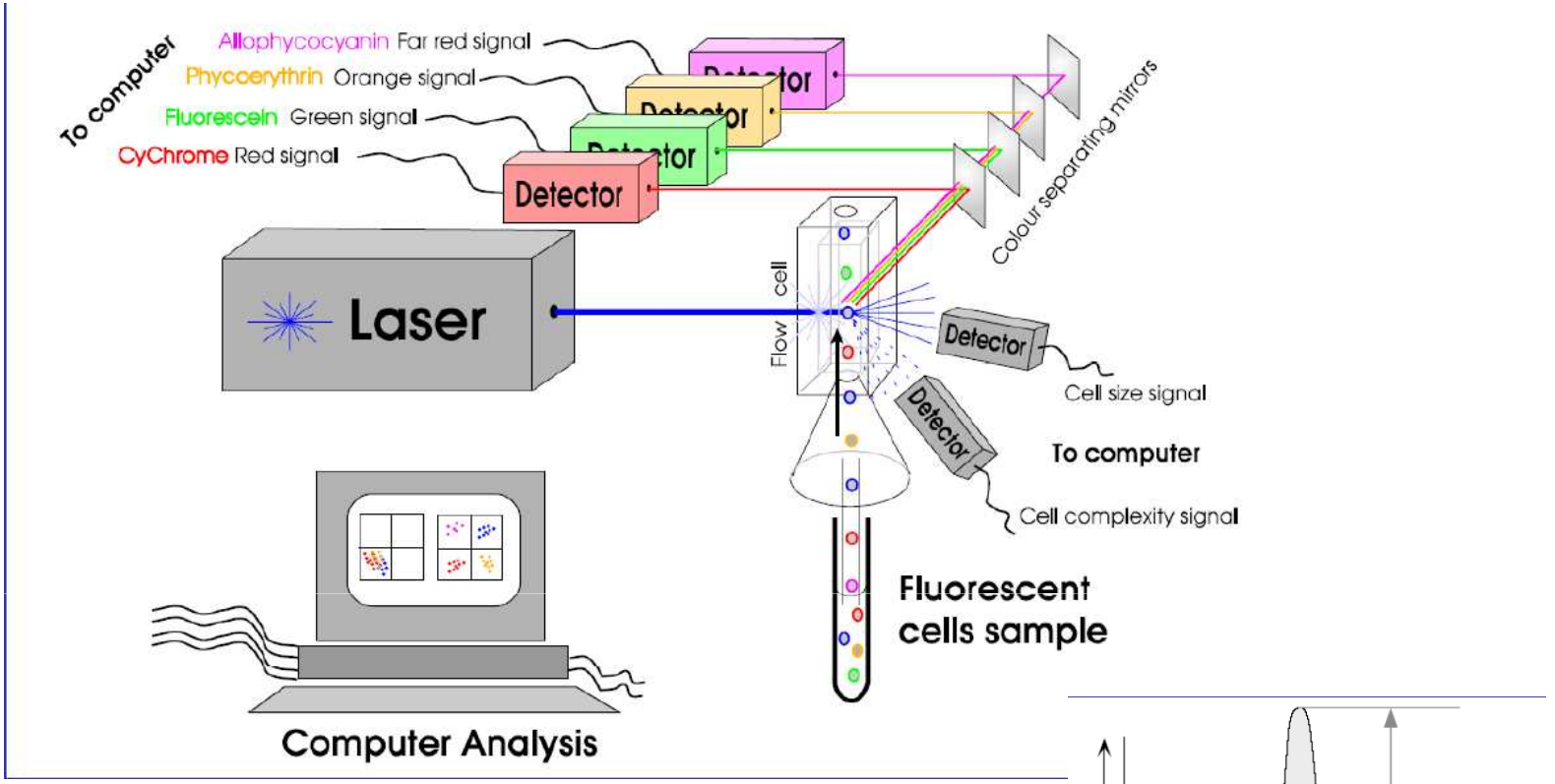
LASER DE
ARGÓN (488nm)

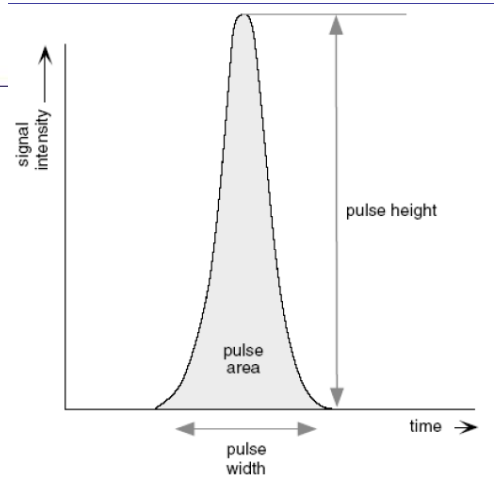


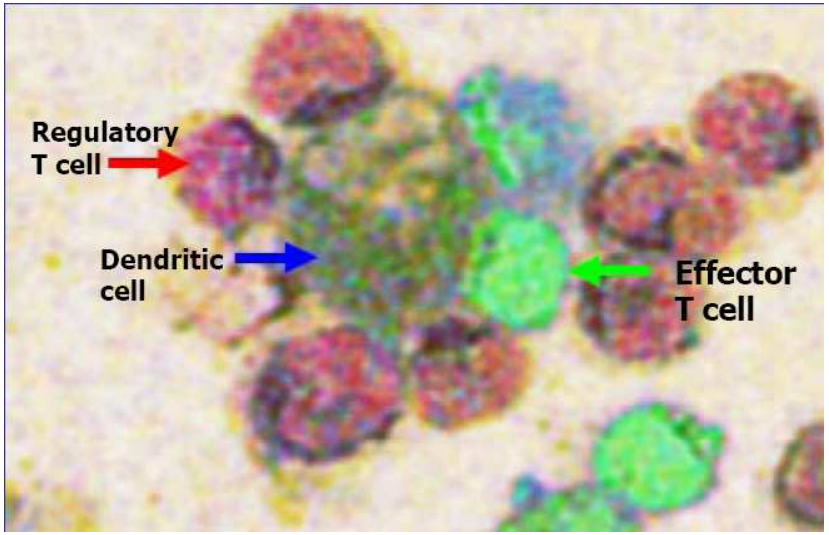
FLUORESCENCIA

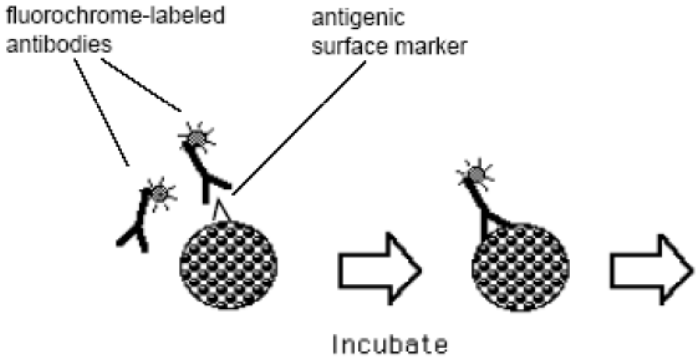
Espectro de emisión de diferentes fluorocromos

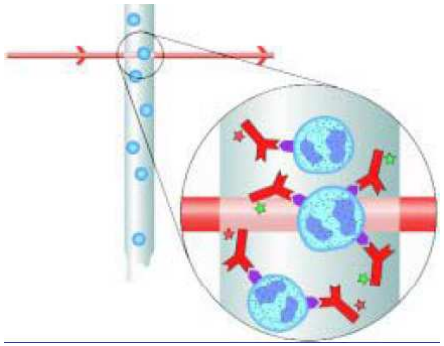


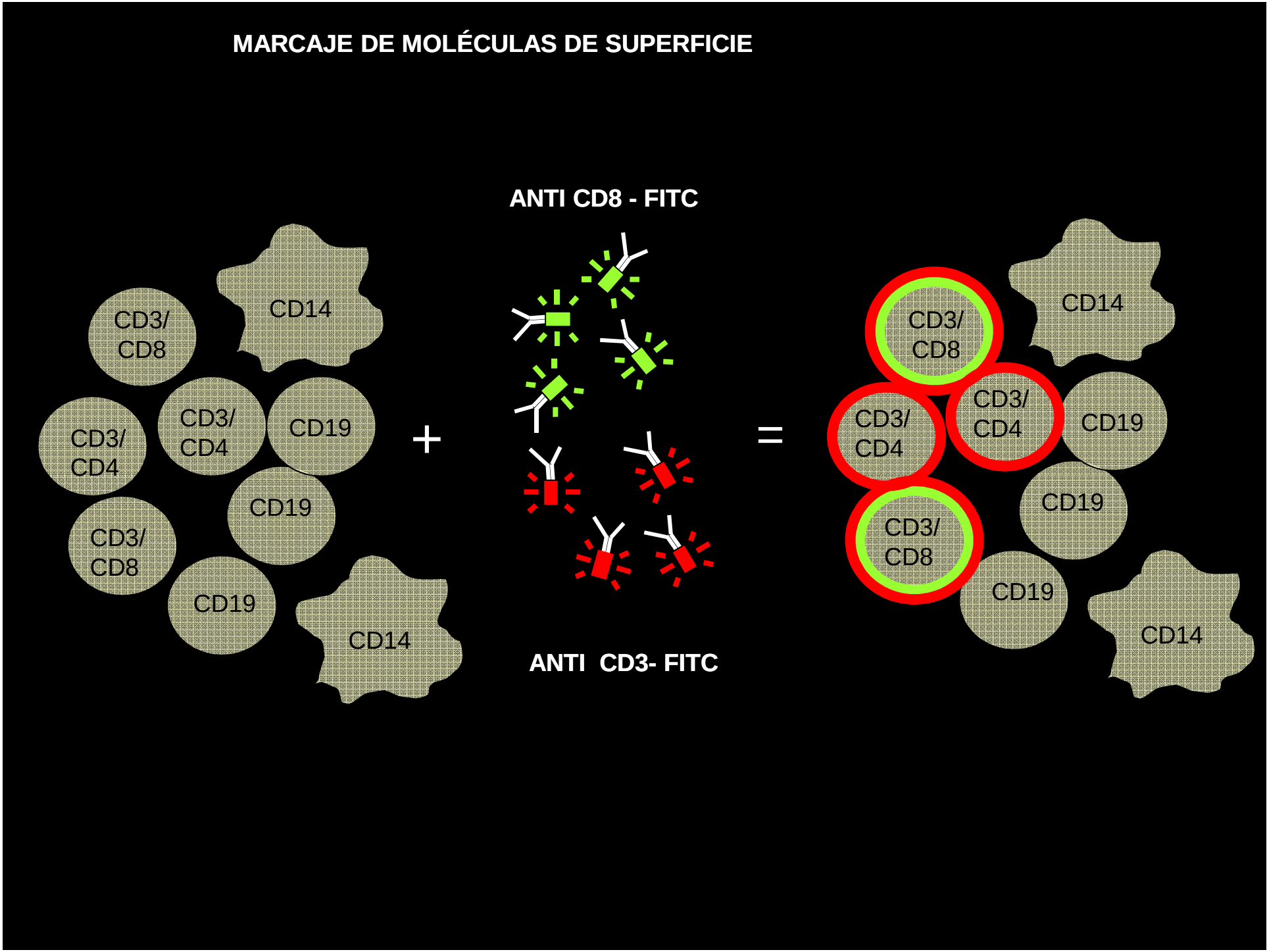

To computer
Allophycocyanin Far red signal
Phycoerythrin Orange signal
Fluorescein Green signal
CyChrome Red signal
Detector
Detector
Detector
Detector
Colour separating mirrors
Laser
Flow cell
Detector
Cell size signal
Detector
To computer
Cell complexity signal
Fluorescent cells sample
Computer Analysis


signal intensity
pulse height
pulse area
time
pulse width

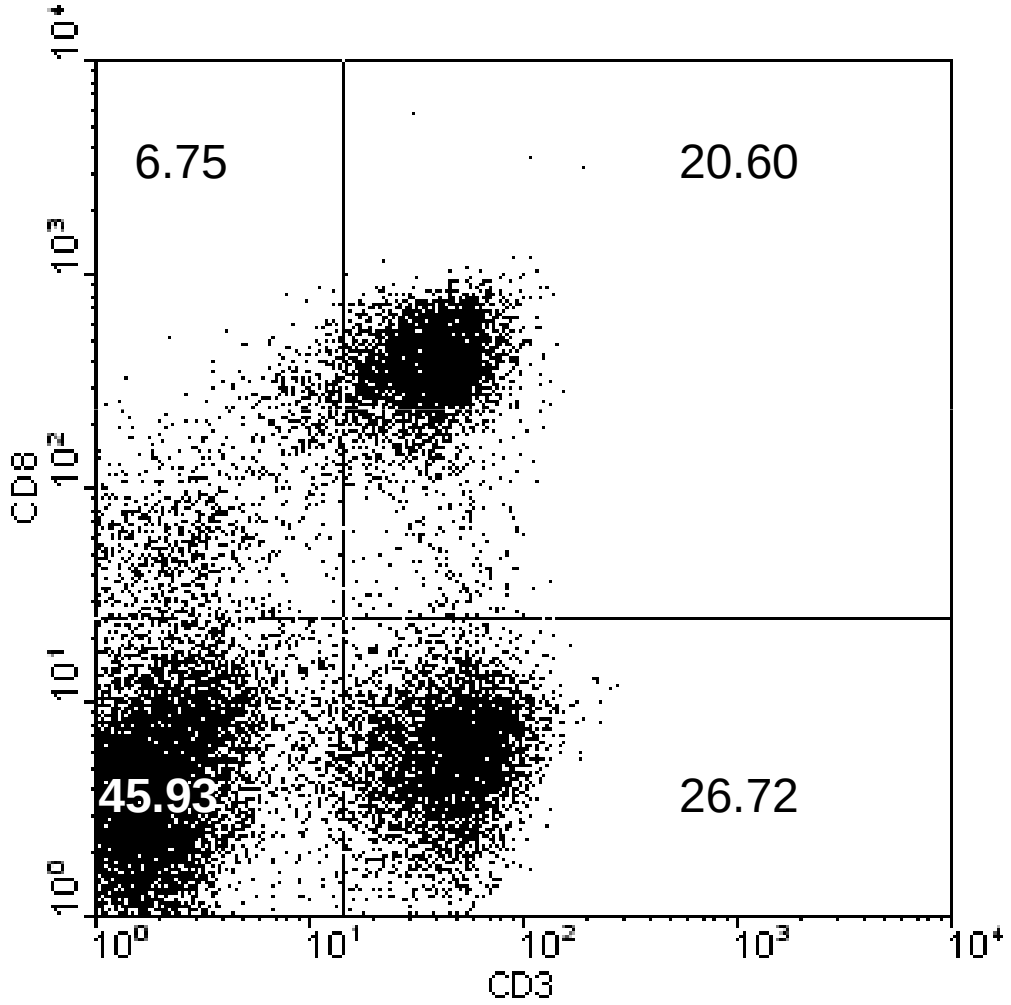

Regulatory
T cell
Dendritic
cell
Effector
T cell

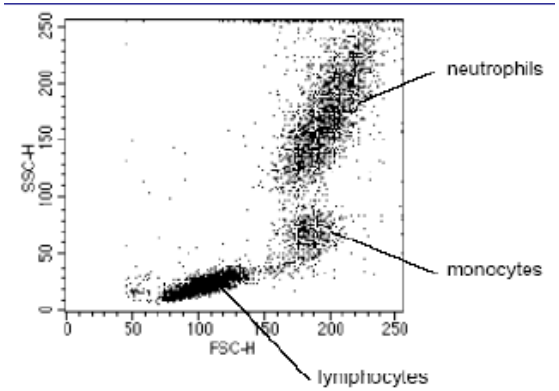

fluorochrome-labeled
antibodies
antigenic
surface marker
Incubate
Analyze

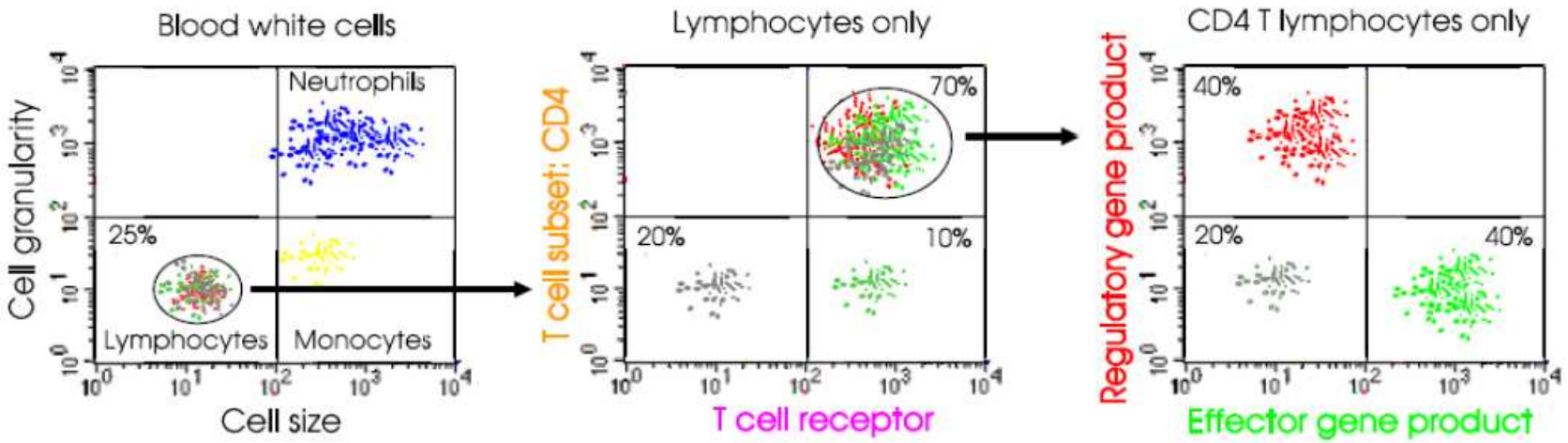



MARCAJE DE MOLÉCULAS DE SUPERFICIE
ANTI CD8 - FITC
CD3/
CD8
CD14
CD3/
CD4
CD19
CD3/
CD4
CD19
CD3/
CD8
CD19
CD14
+
ANTI CD3- FITC
=
CD3/
CD8
CD14
CD3/
CD4
CD3/
CD4
CD19
CD19
CD3/
CD8
CD19
CD14

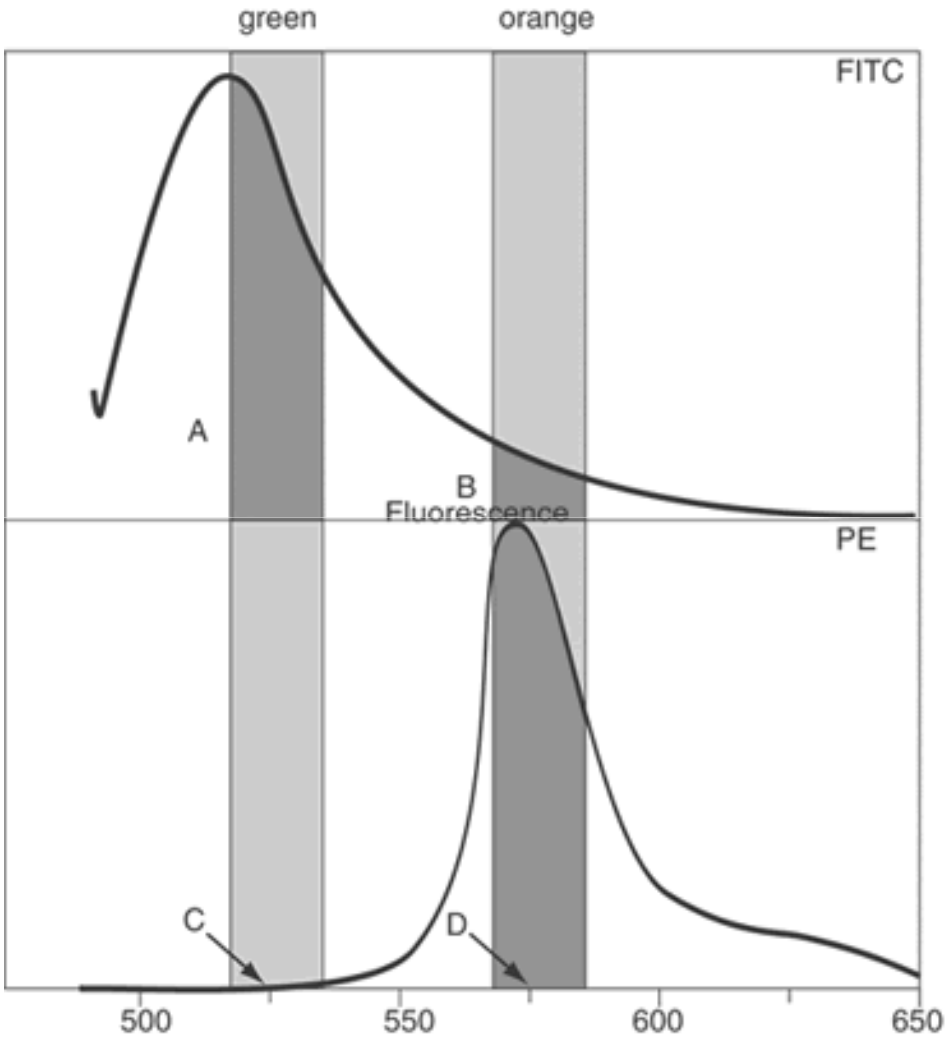
CONTEO DE LINFOCITOS T CD8+




neutrophils
monocytes
lymphocytes
SSC-H
FSC-H
0
50
100
150
200
250

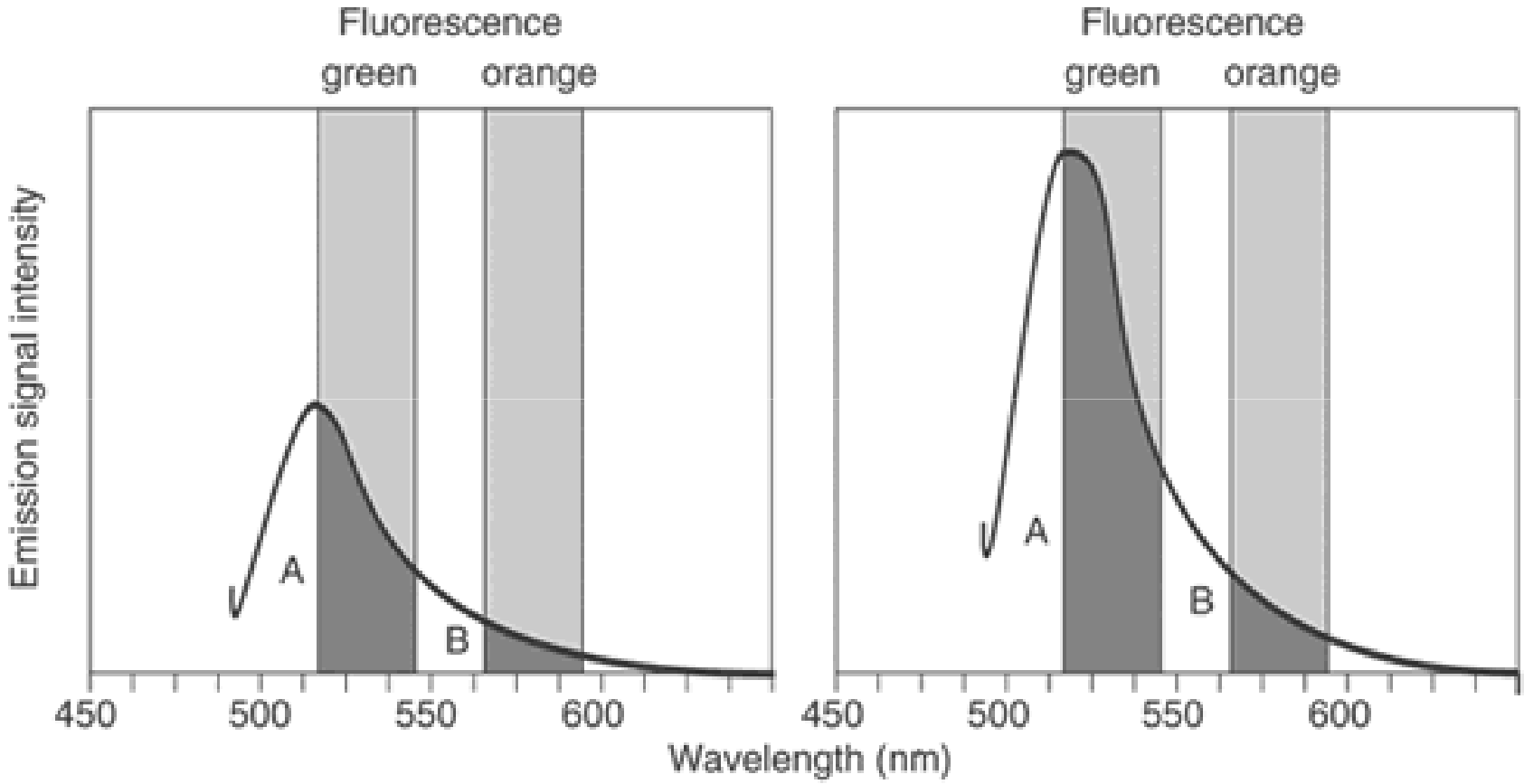

Blood white cells
Neutrophils
25%
Lymphocytes
Monocytes
Cell granularity
Cell size
Lymphocytes only
70%
20%
10%
T cell subset: CD4
T cell receptor
CD4 T lymphocytes only
40%
20%
40%
Regulatory gene product
Effector gene product

COMPENSACION

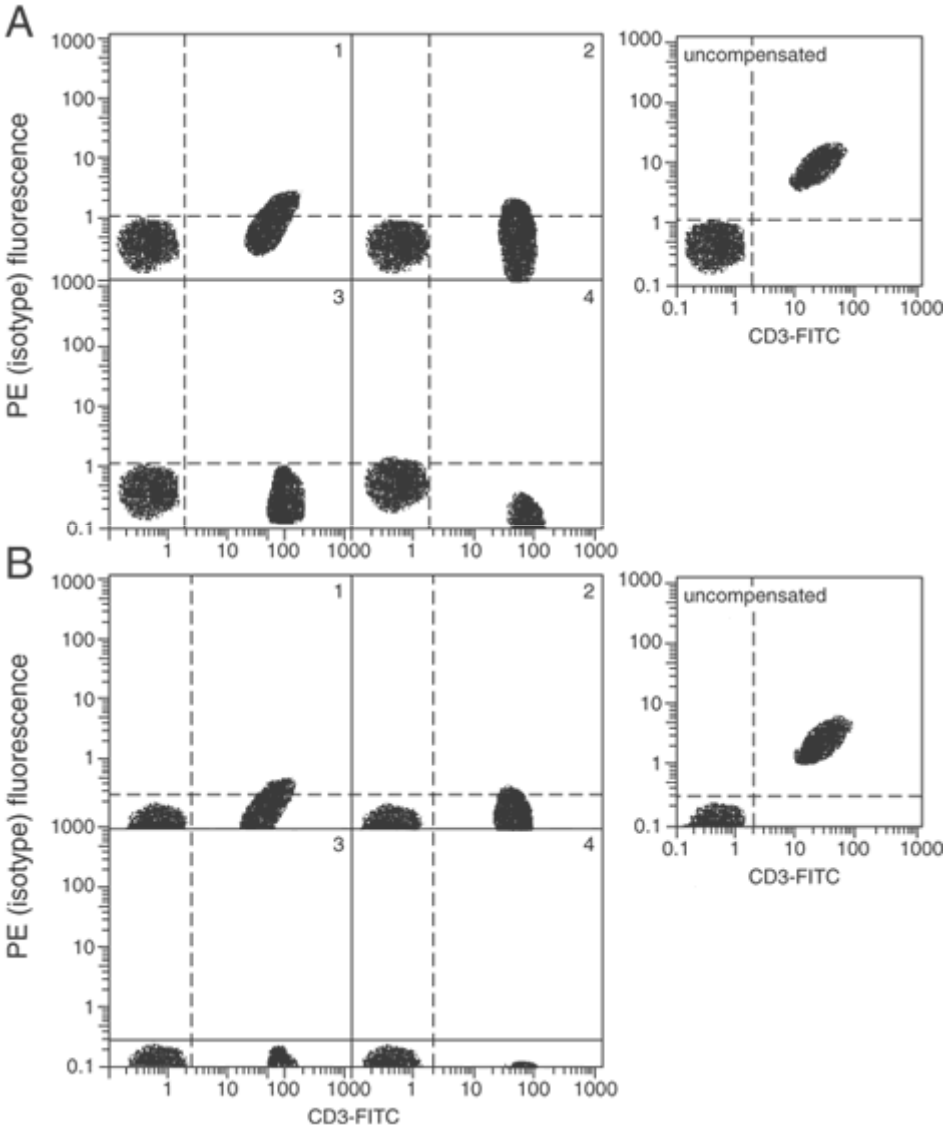


[X] FITC

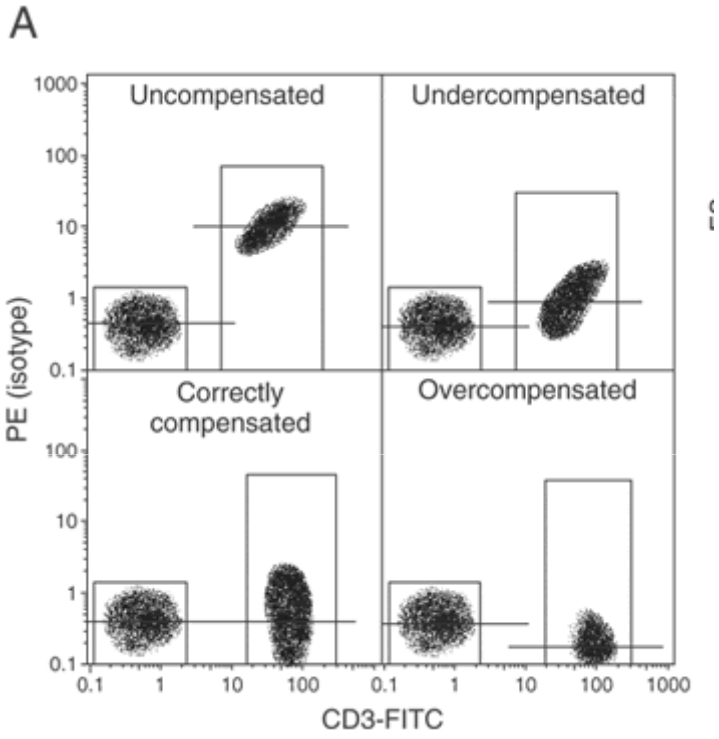
[2X] FITC



COMPENSACION



Compensación apropiada



In a hypothetical experiment, cells stained with CD3-FITC and PE isotype control were collected at different compensation settings. The horizontal line is drawn through the median of the population. The boxes indicate the analysis gates used when the median fluorescences were computed. Proper compensation is achieved when these centers align; note that the properly compensated positive population extends *above* the top of the negative population (i.e., above where an isotype gate would be set based on the negative population).

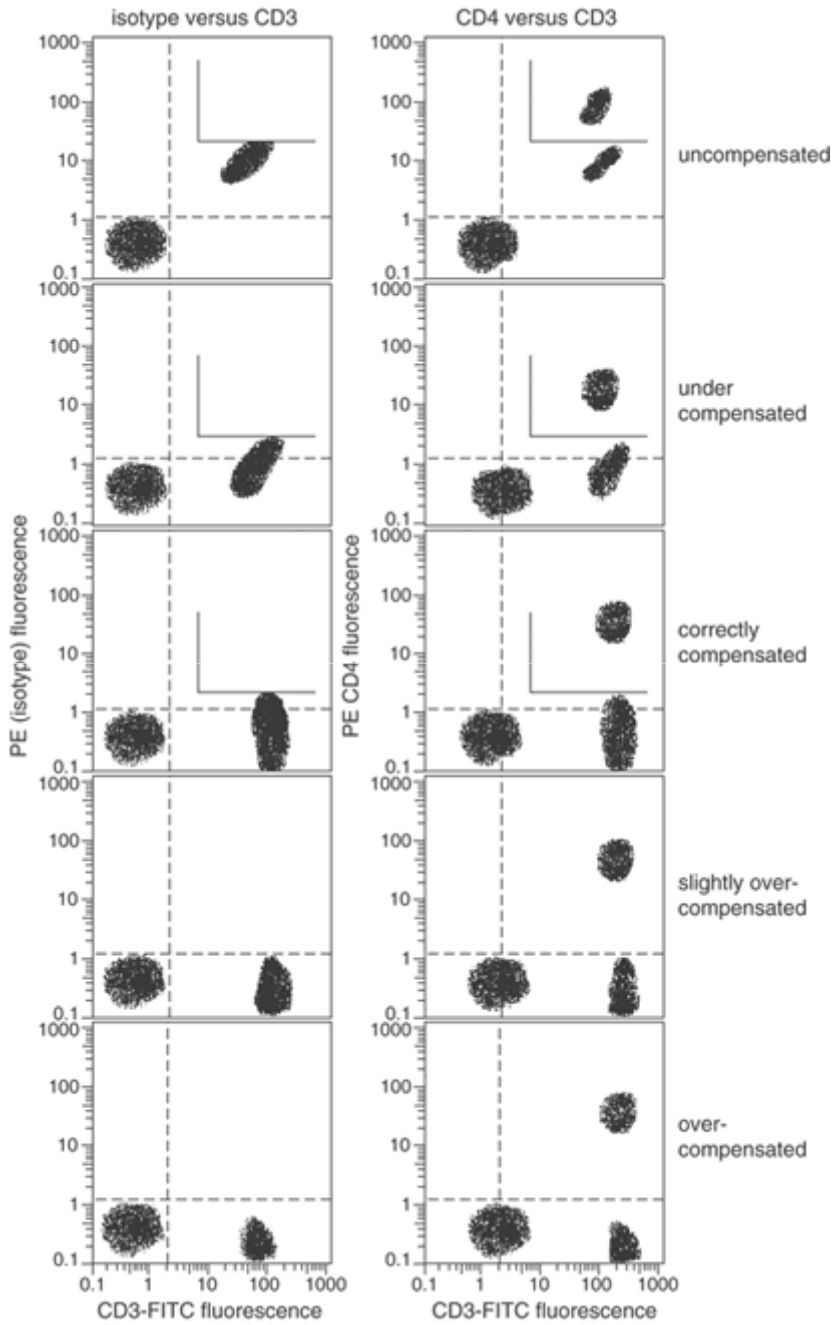
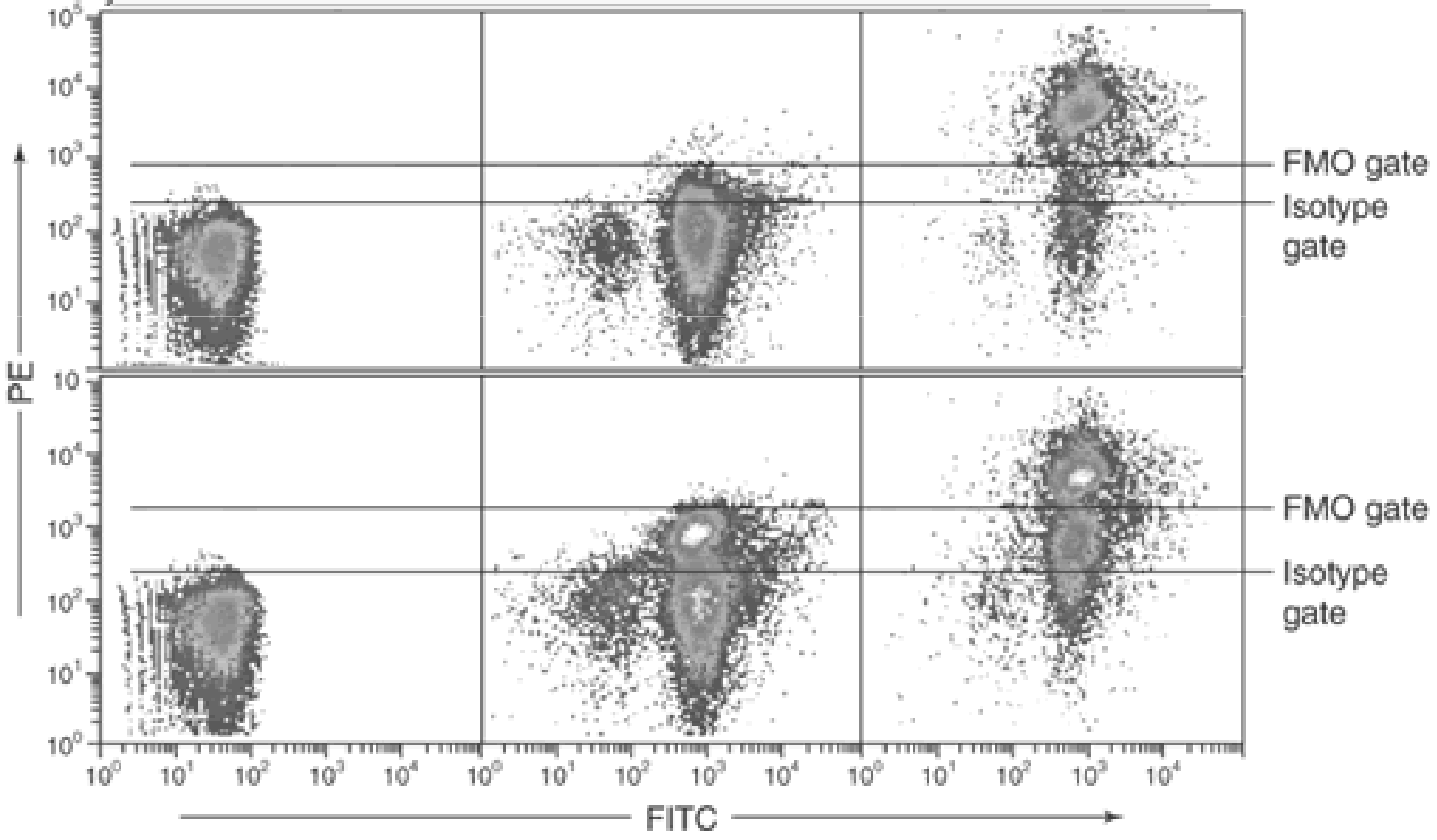

isotype versus CD3
CD4 versus CD3
uncompensated
under compensated
correctly compensated
slightly over-compensated
over-compensated
PE (isotype) fluorescence
PE CD4 fluorescence
CD3-FITC fluorescence
CD3-FITC fluorescence
0.1
1
10
100
1000

Table 1.14.1 Typical Spillover Matrix for a Three-Color Compensation

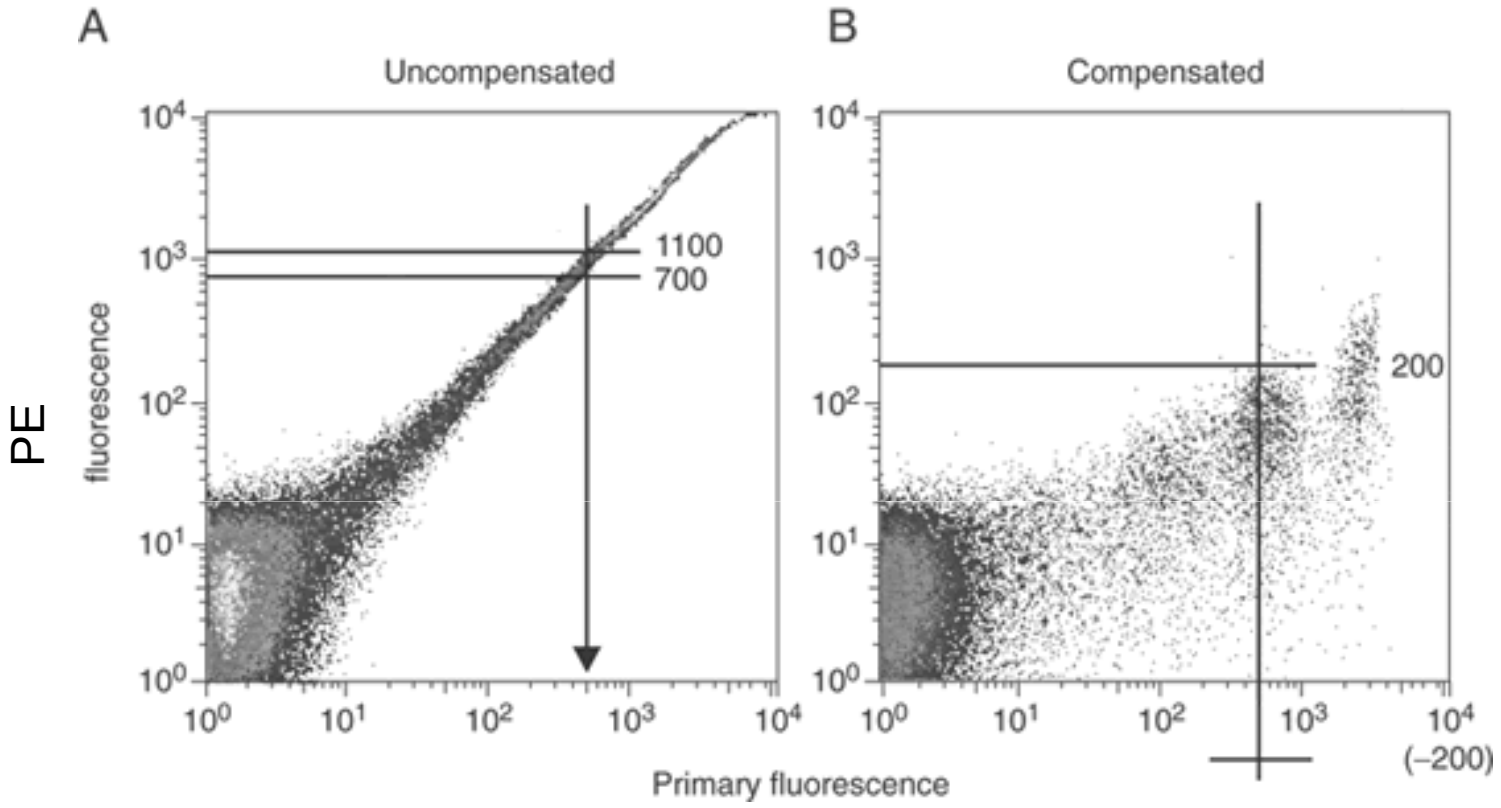
Fluorophore	Detector		
	Green	**Orange**	**Red**
FITC	1.000	0.180	0.040
PE	0.009	1.000	0.213
PE-Cy5	0.005	0.029	1.000

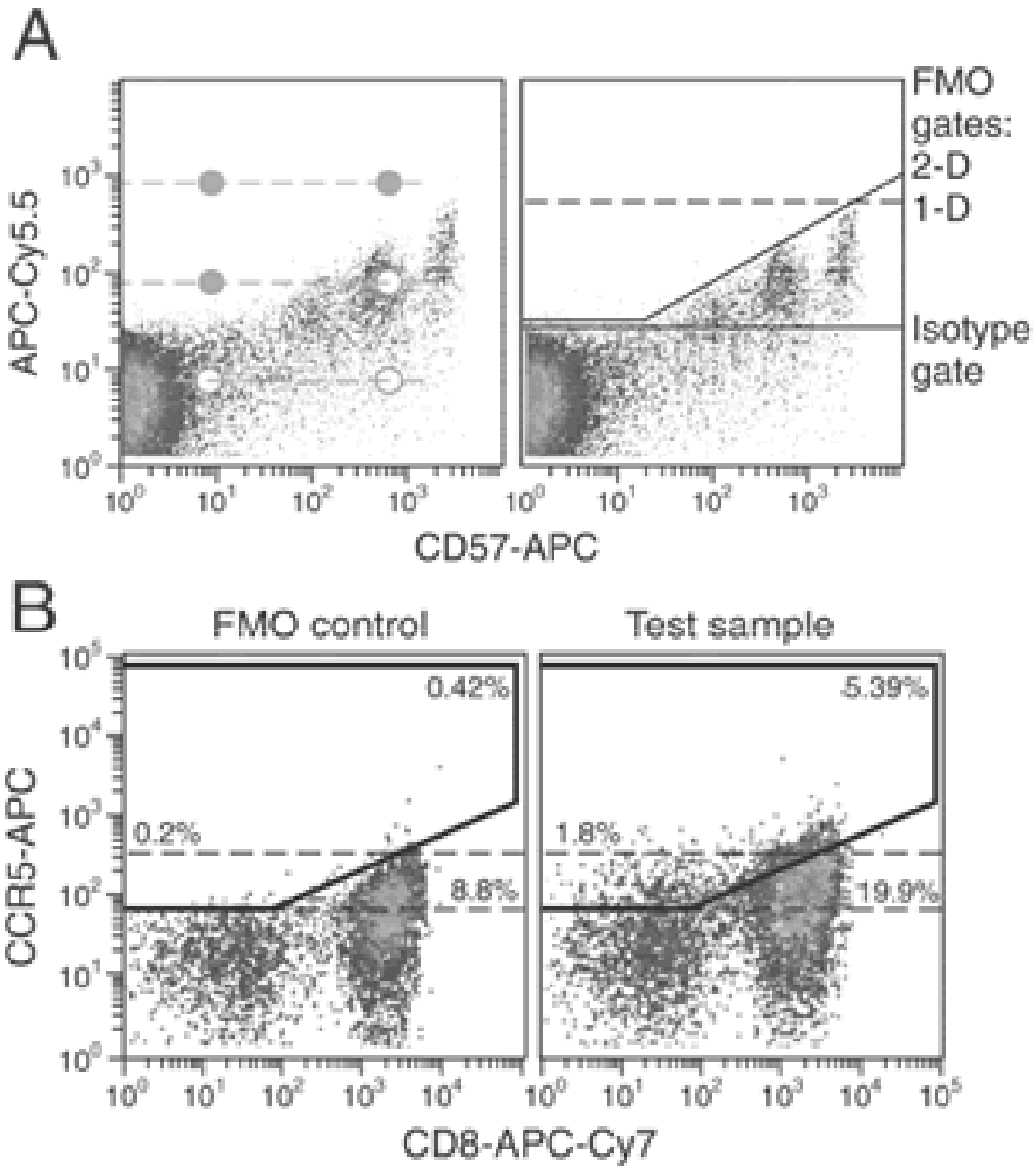
C

	Unstained control	FMO control	Fully stained
FITC	—	CD3	CD3
PE	—	—	CD4
Cy5PE	—	CD8	CD8
Cy7PE	—	CD45RO	CD45RO



PBMC + CD57-APC

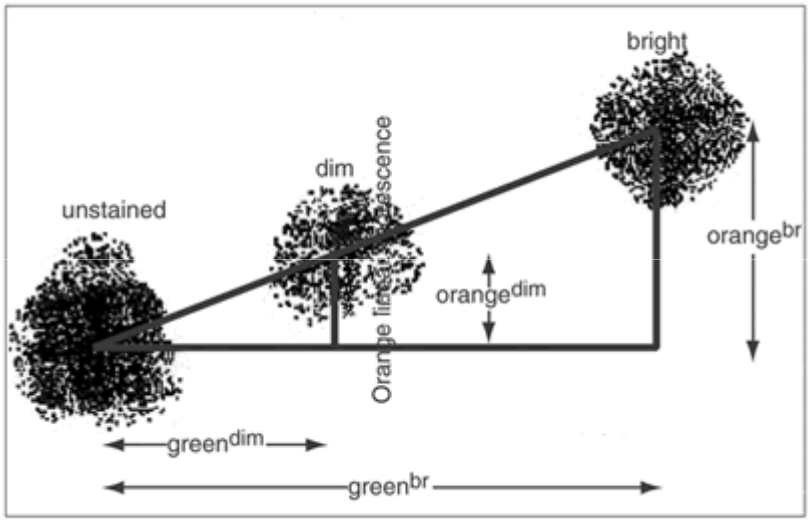




(A) stained only with CD57-APC.

The isotype gate is that defined by an unstained (or fully isotype-stained) sample. The 1-D FMO gate is defined by the CD57-APC-stained sample, examining only the distribution of APC-Cy5.5 and setting the threshold above all events. The 2-D FMO gate is defined by the limit of APC-Cy5.5 distribution when viewing this two-dimensional graph.

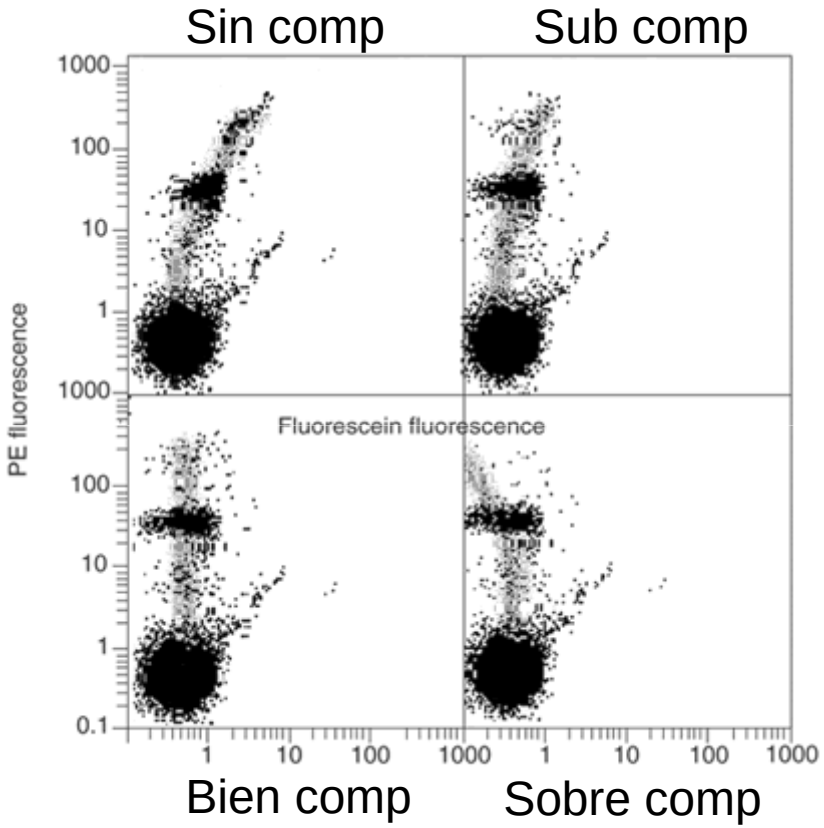
(B) Human PBMC were stained and gated for CD3 (not shown), CD8-APC-Cy7, with (right) and without (left) CCR5-APC. The expression of CCR5 is dim, and accurate discrimination of positive and negative events is necessary. Percentages show the fraction of CD8 T cells within each “positive” gate: the isotype gate (lower dotted line), the 1-D FMO gate (upper dotted line), and the 2-D FMO gate (solid polygon).



Green linear fluorescence

In an ideal world, this ratio is the same for dim or for bright cells. However, the orange and green values will have an inherently greater proportionate error for the dim cells than for the bright cells; therefore, the spillover coefficient will be less accurately determined on the basis of the dim population than on the bright population. For logarithmic scaling, this problem is exacerbated because dim populations can have orders of magnitude less fluorescence than bright populations (remember, the error of the measurement varies with the square root of the absolute value of the measurement).

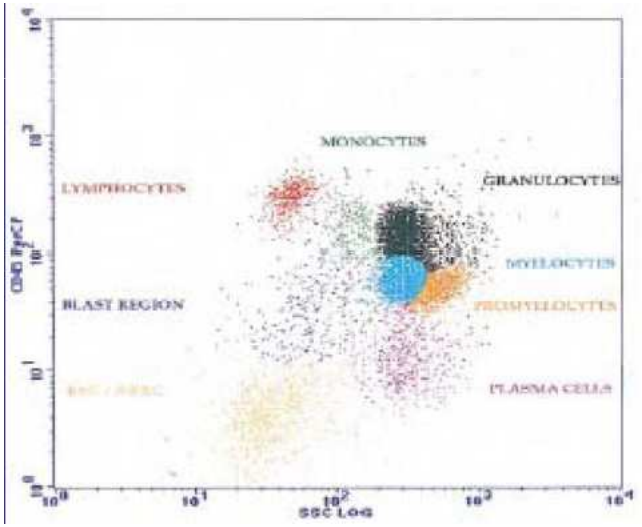
Note in particular that while the main PE+ population appears reasonably well compensated in all graphs where some compensation is set, the brightest cells clearly indicate the incorrect compensation level. This is a clear example of why only the brightest stain should be used to estimate correct compensation!



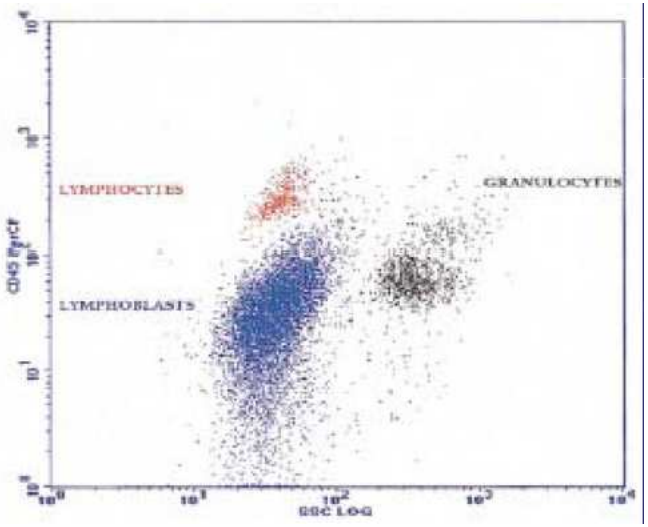
APLICACIONES

Células de médula ósea.
Diagnóstico de leucemia linfoblástica aguda (LLA)

NORMAL

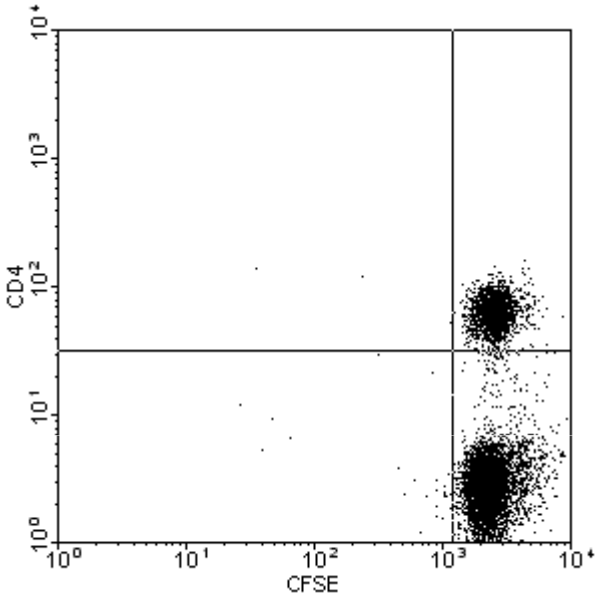


LLA

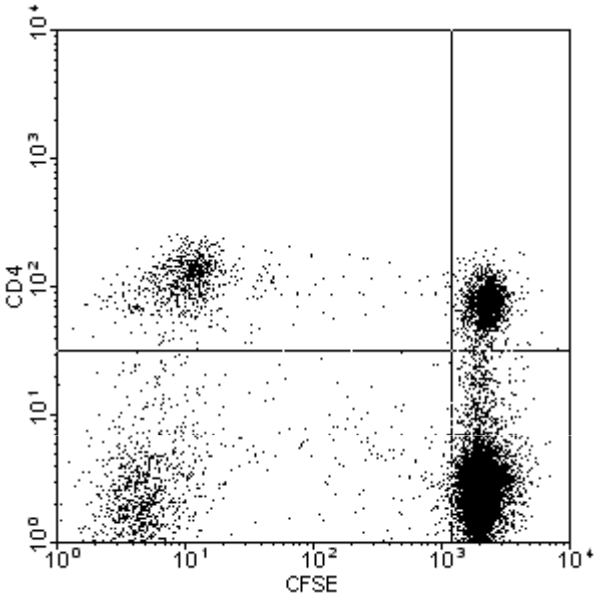


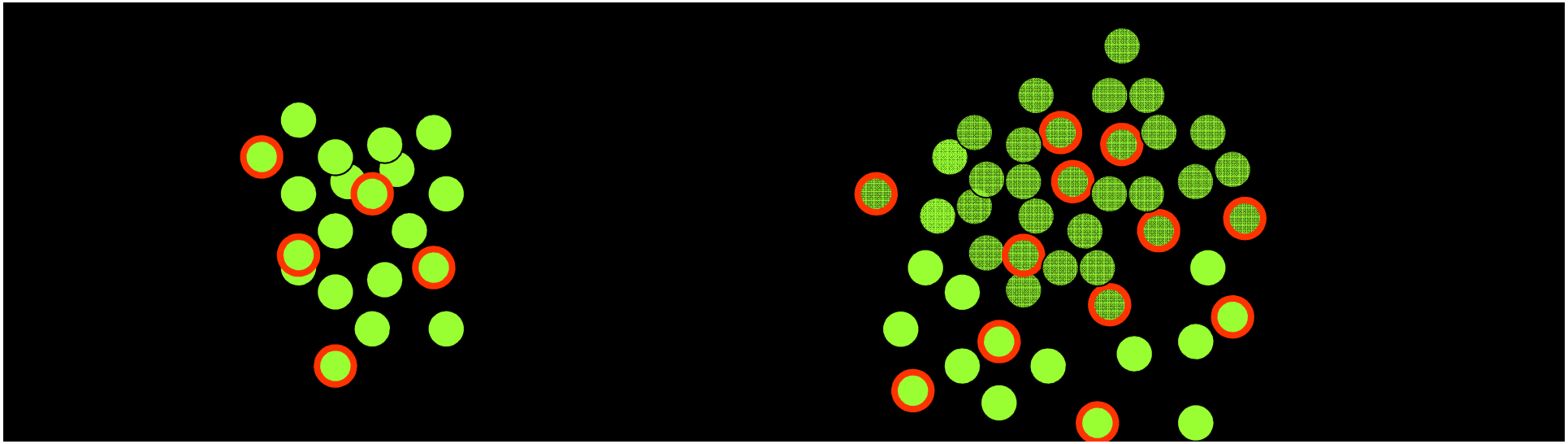
PROLIFERACION

PBMC control 7 d.



PBMC + MOi (*L. mex*) 7 d.





PROLIFERACION

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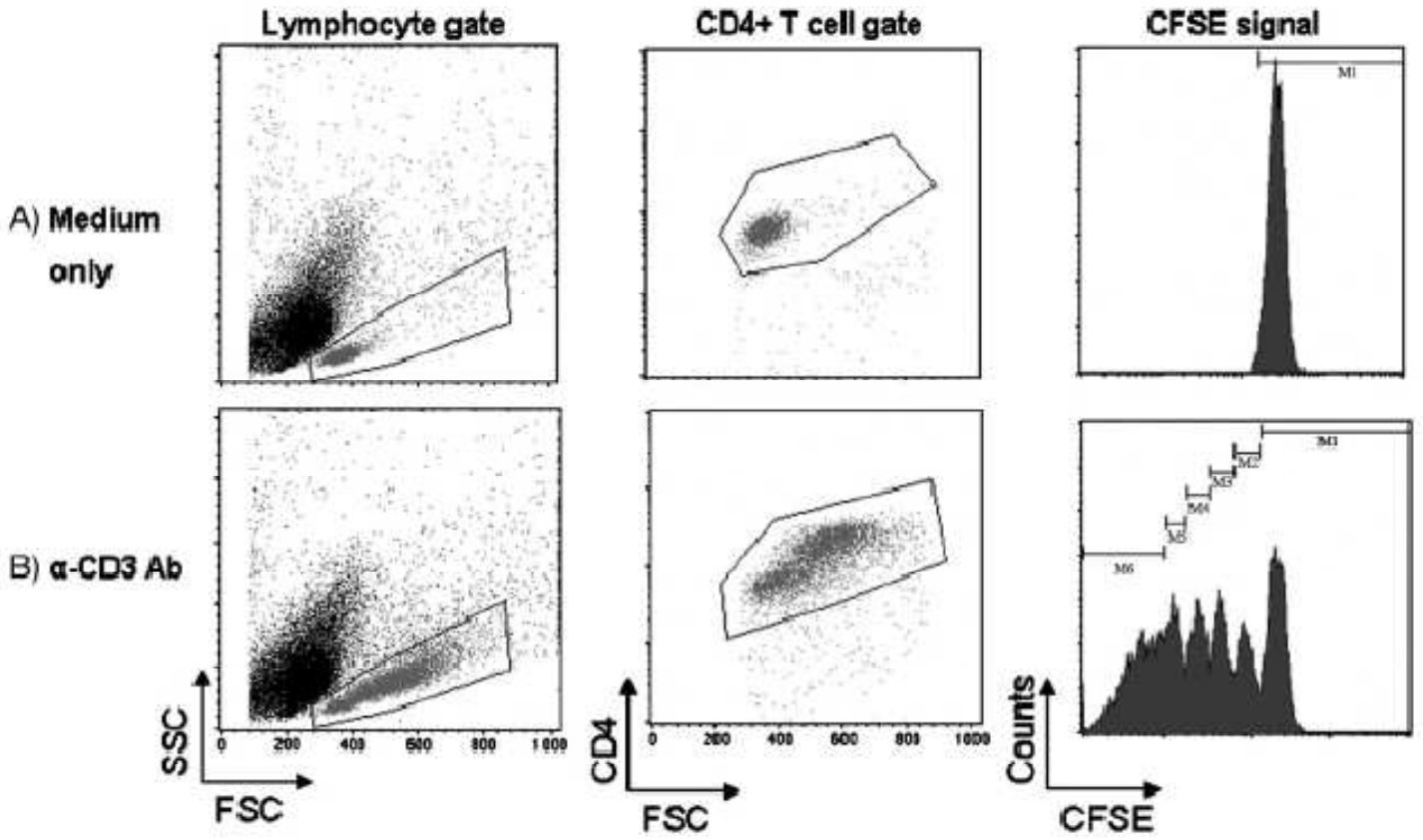
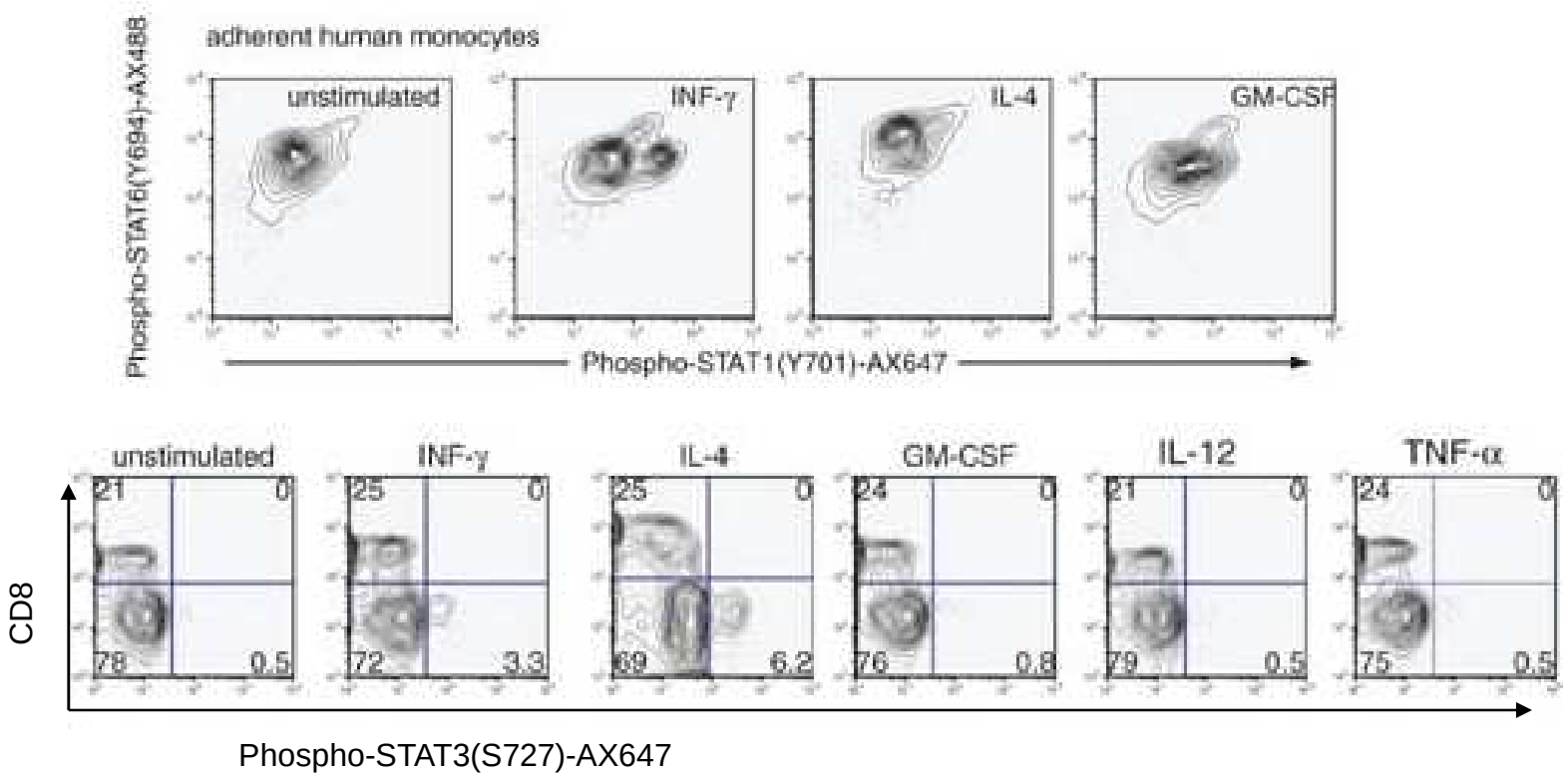
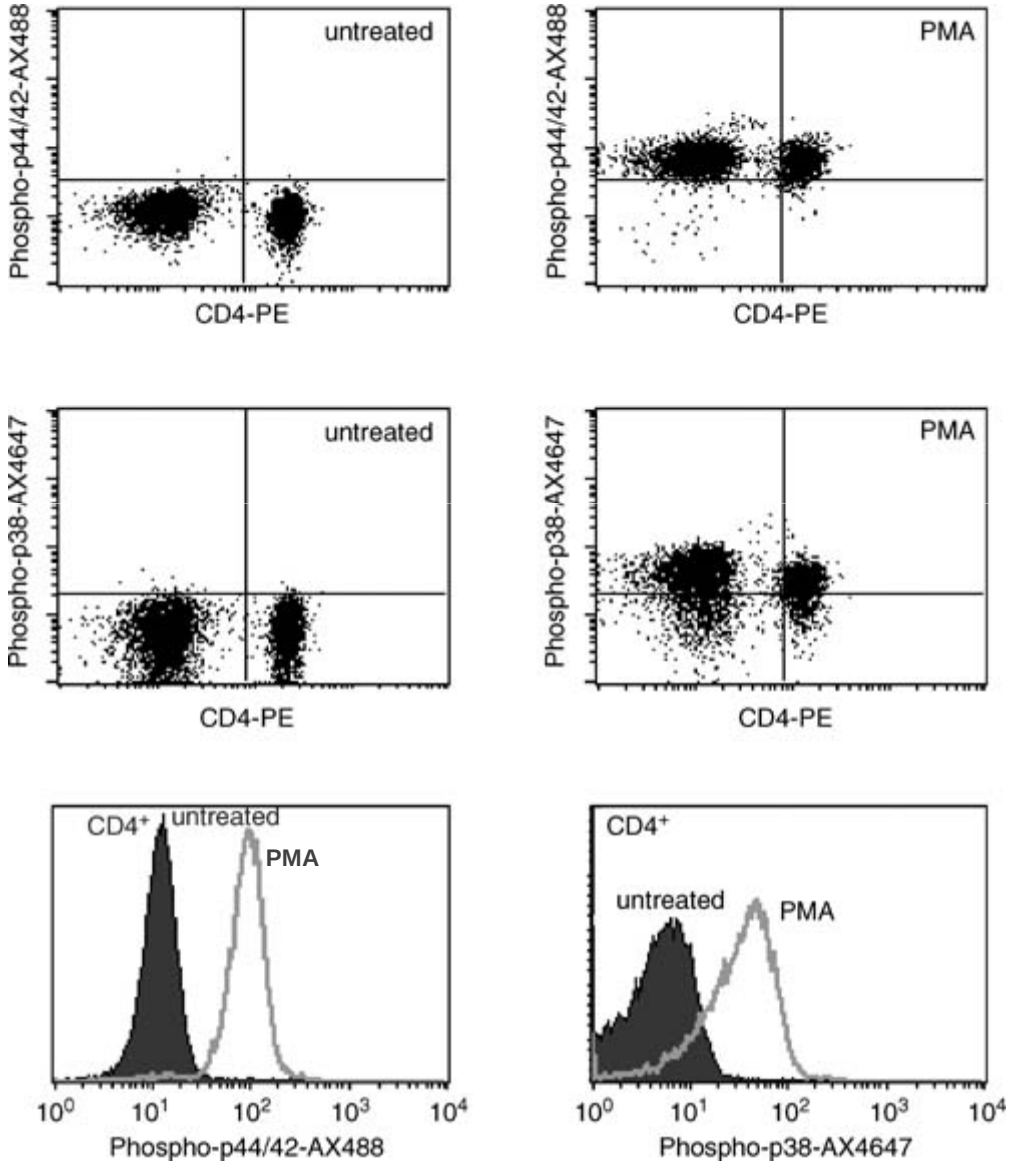


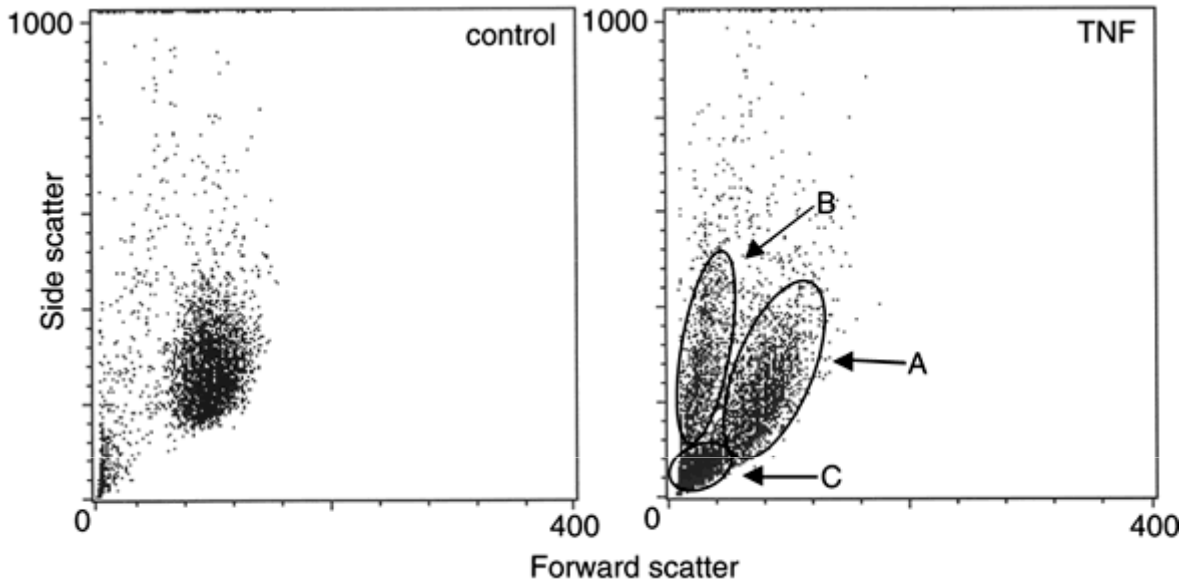
Análisis multiparamétrico de fosfo-epítopes



Multiparameter Analysis of Intracellular Phosphoepitopes in Immunophenotyped Cell Populations by Flow Cytometry



CELL DEATH



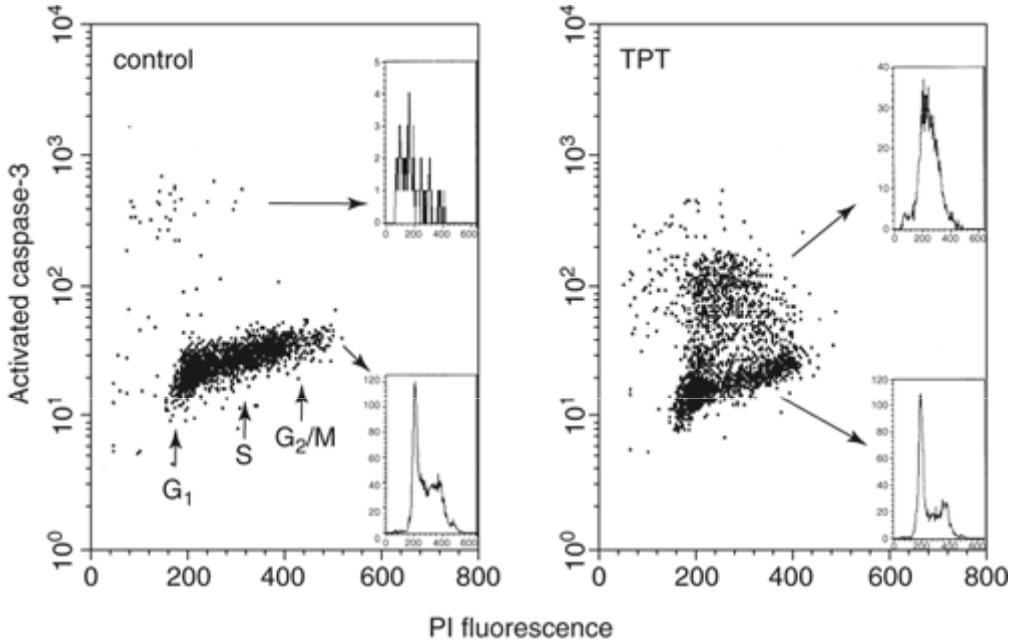
Changes in light scattering properties of cells undergoing apoptosis. HL-60 cells were untreated (left panel) or treated 3 hr with TNF- a and cycloheximide (CHX) to induce apoptosis (right panel).

(A) Cells that have light scattering properties similar to those of untreated cells.

(B) Early apoptotic cells have diminished forward scatter and are very heterogenous with respect to side scatter.

(C) Late apoptotic cells have both forward and side scatter diminished.

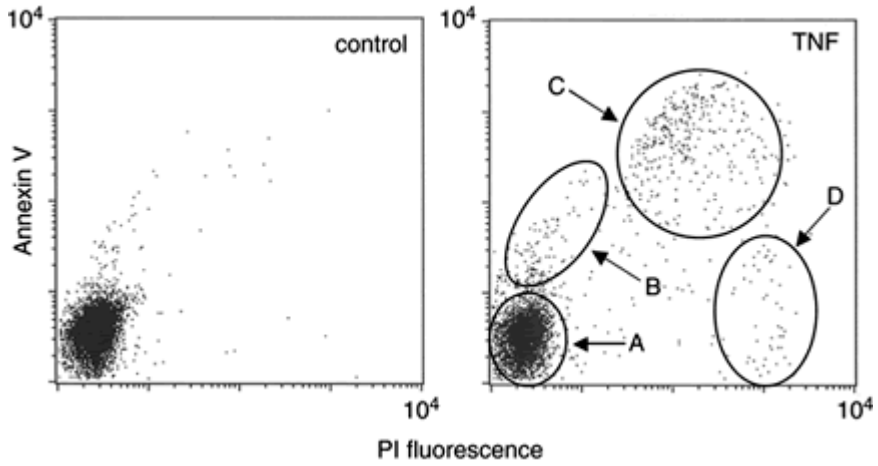
Immunocytochemical detection of caspase-3 activation using antibody reactive with the activated (cleaved) caspase-3.



Apoptosis of HL-60 cells was induced by topotecan (TPT). Top and bottom insets in each panel show cellular DNA content frequency histograms of cells with activated and nonactivated caspase-3, respectively.

Note that S-phase cells preferentially contain activated caspase-3 after induction of apoptosis by TPT.

Detection of early and late apoptotic cells after staining with annexin V–FITC and PI.



(A) Live nonapoptotic cells stained according to have minimal green (annexin V-FITC) fluorescence and also minimal red (PI) fluorescence.

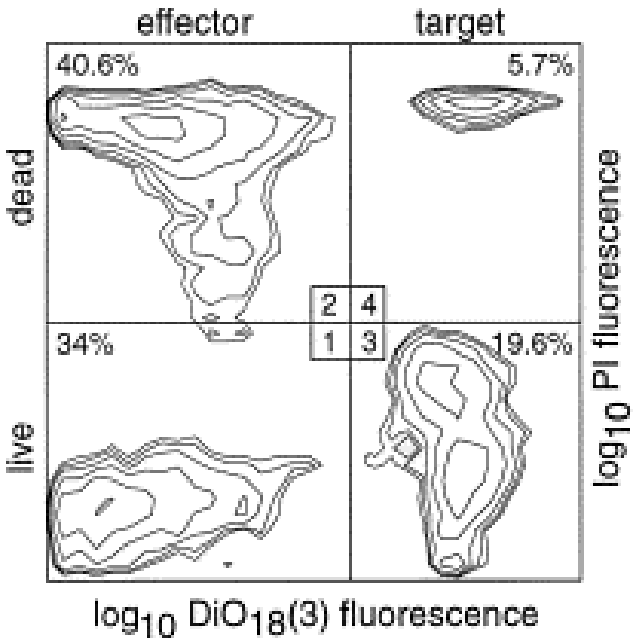
(B) At early stages of apoptosis, cells stain green but still exclude PI and therefore continue to have no significant red fluorescence.

(C) At late stages of apoptosis, cells show intense green and red fluorescence.

(D) It should be noted that isolated nuclei, cells with severely damaged membranes, and very late apoptotic cells stain rapidly and strongly with PI and may not bind annexin V.

Analyzing cytotoxic T lymphocyte activity: a simple and reliable flow cytometry-based assay

Anita E. Mattis, Günter Bernhardt, Martin Lipp and Reinhold Förster*



≈CFDA

Splenocytes were labeled with DiO18(3) and used in the CTL assay (4 h) employing an E:T ratio of 5:1 as described in Section 2. After counterstaining with propidium iodide to indicate dead cells, 4 cell populations could be distinguished by two-parameter flow cytometry:

(*1*) live effector cells;

(*2*) dead effector cells;

(*3*) live target cells; and

(*4*) dead target cells.

Numbers indicate the percentage of cells within each quadrant.

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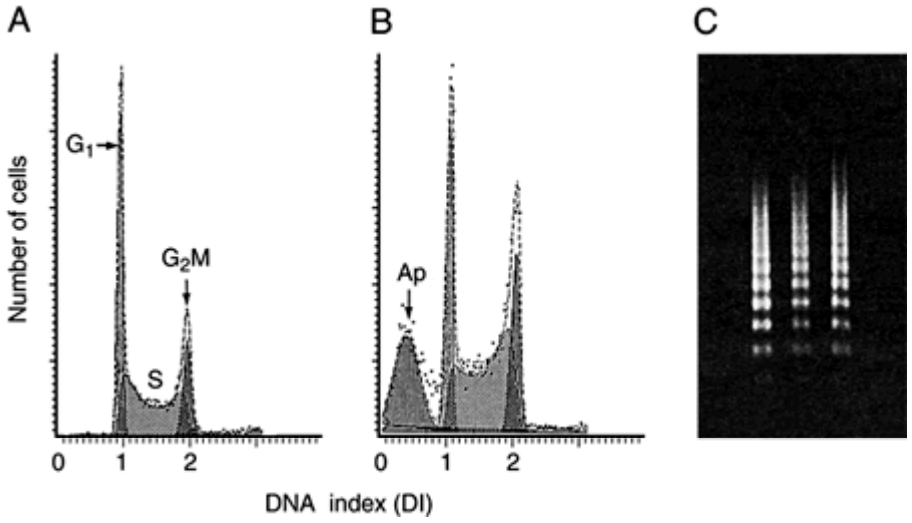
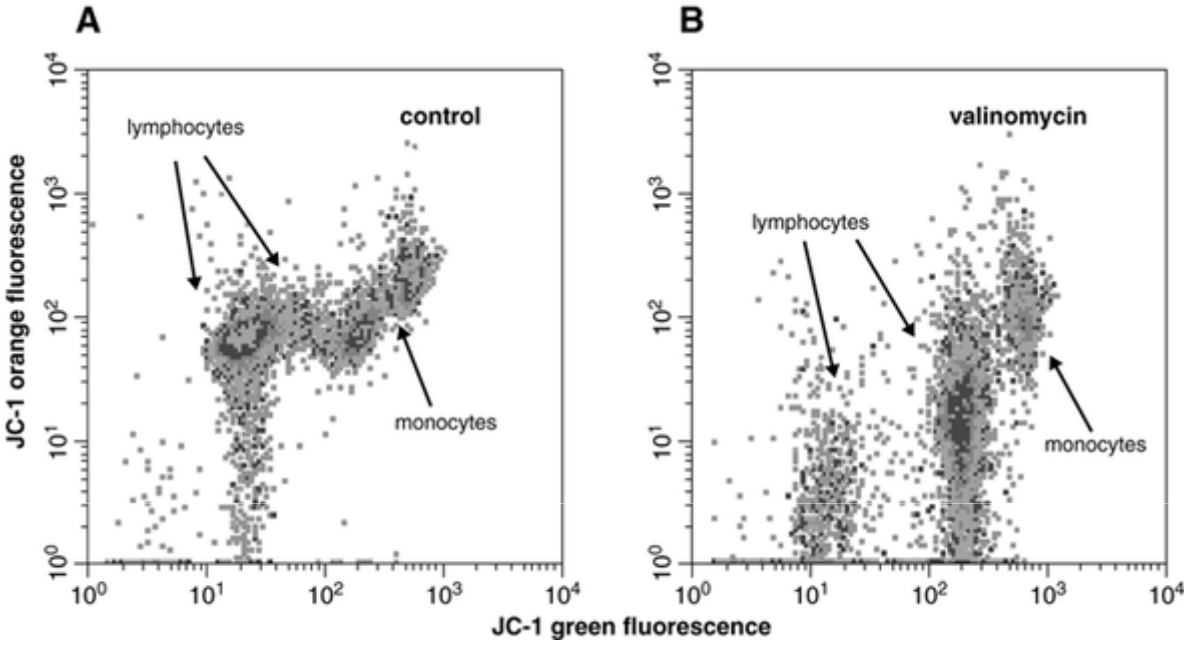
Volume 204, Issue 2, 26 May 1997, Pages 135-142

Detection of apoptotic cells by flow cytometry based on cellular DNA content analysis.



(A) Normal cell plot. (B) HL-60 cells were treated with the DNA topoisomerase II inhibitor fostriecin Cells were fixed in 70% ethanol, suspended in high-molarity phosphate buffer to extract fragmented DNA, and then stained with PI. A subpopulation of apoptotic cells (Ap) with fractional (sub-diploid) DNA content, i.e., with DNA index (DI) <1.0 (sub-G1 cells), is apparent. Note also the increase in the proportion of S-phase cells in the nonapoptotic population. (C) The fragmented DNA extracted from the apoptotic cells by the buffer was subjected to gel electrophoresis. Note “laddering” that reflects preferential DNA cleavage at internucleosomal sections, the characteristic feature of apoptosis

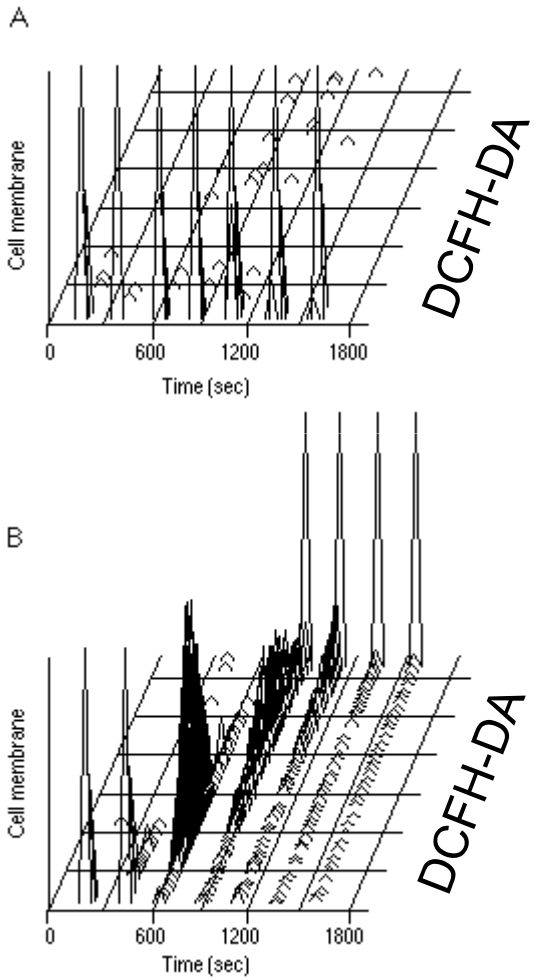
Flow Cytometric Analysis of Mitochondrial Membrane Potential



(A) After incubation with 2.5 µg/ml JC-1 at 37℃, PBM C usually show a single population of monocytes, while here lymphocytes give rise to two separate peaks. This could indicate the presence of a functional heterogeneity within lymphocytes. Such a phenomenon, however, is not always present.

(B) Treating cells with valinomycin results in a loss of orange signal either from lymphocytes or from monocytes.

Oxidative Metabolism



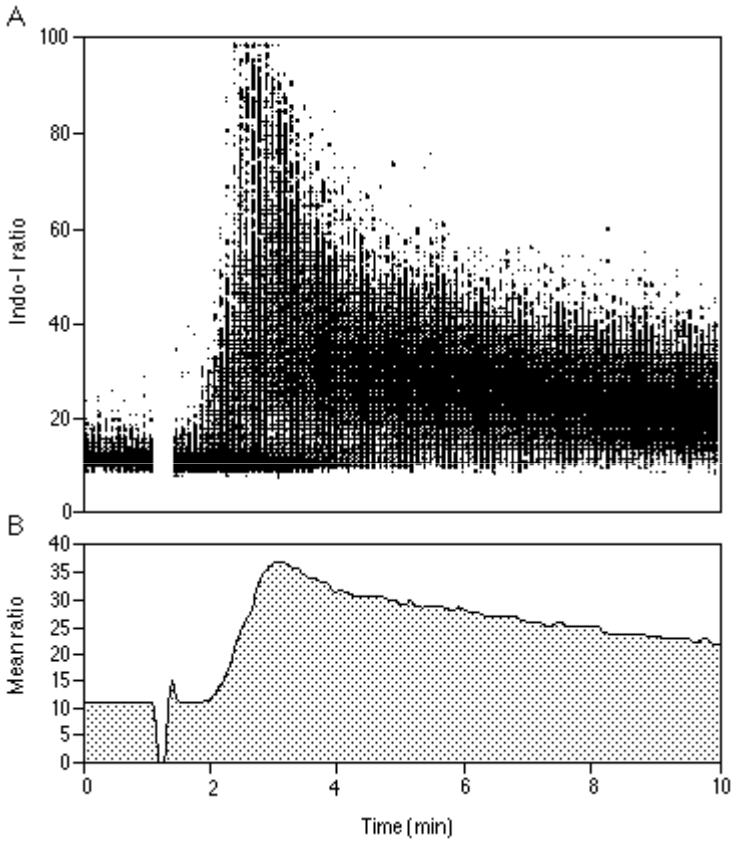
Stimulation of neutrophils by PMA over time. (A) Unstimulated cells, which show no increase in DCF fluorescence over 30 min. (B) Activated cells (stimulated with 50 ng/ml PMA), which show a rapid 10-fold increase in fluorescence following an initial time lag. Data were collected by sampling the same set of tubes multiple times over a 30-min period.

Measurement of Intracellular Calcium Ions

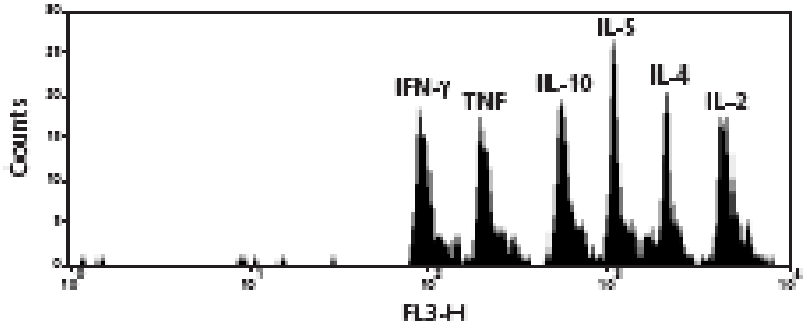
Effects of TCR stimulation on T cells ionized calcium concentration ([Ca2+]i). Lymphocytes were loaded with indo-1 AM and stained with PE-anti-CD8. The cells were maintained at 37℃; after obtaining a baseline for ~1 min, anti-CD3 MAb was added during the gap in analysis.

(A) The indo-1 ratio of 395 nm/500 nm fluorescence emission was calculated and the value for each cell displayed on the *y* axis versus time on the *x* axis. The results are displayed as a dot plot.

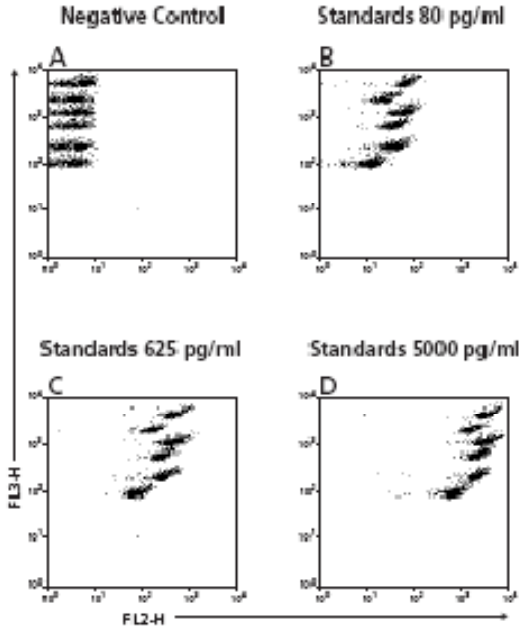
B) The mean response of the data from panel A plotted versus time.



BD™ Cytometric Bead Array (CBA)

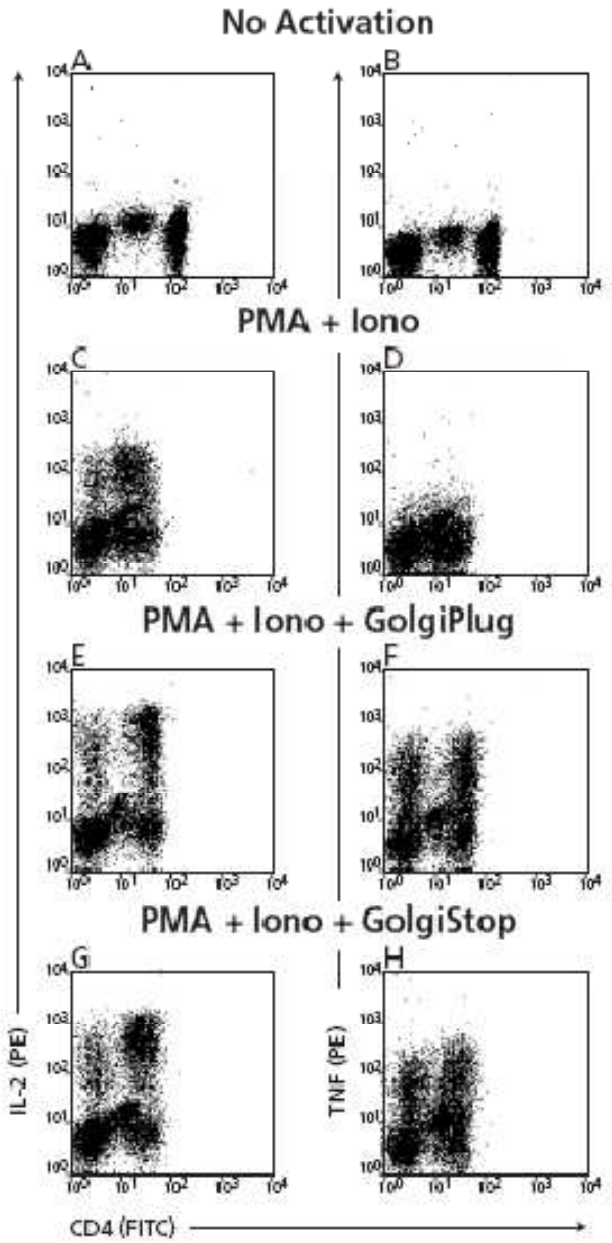


Fluorescencia de las esferas de detección



Standard dilutions analyzed in the BD CBA
Human Th1/Th2 Cytokine Kit.

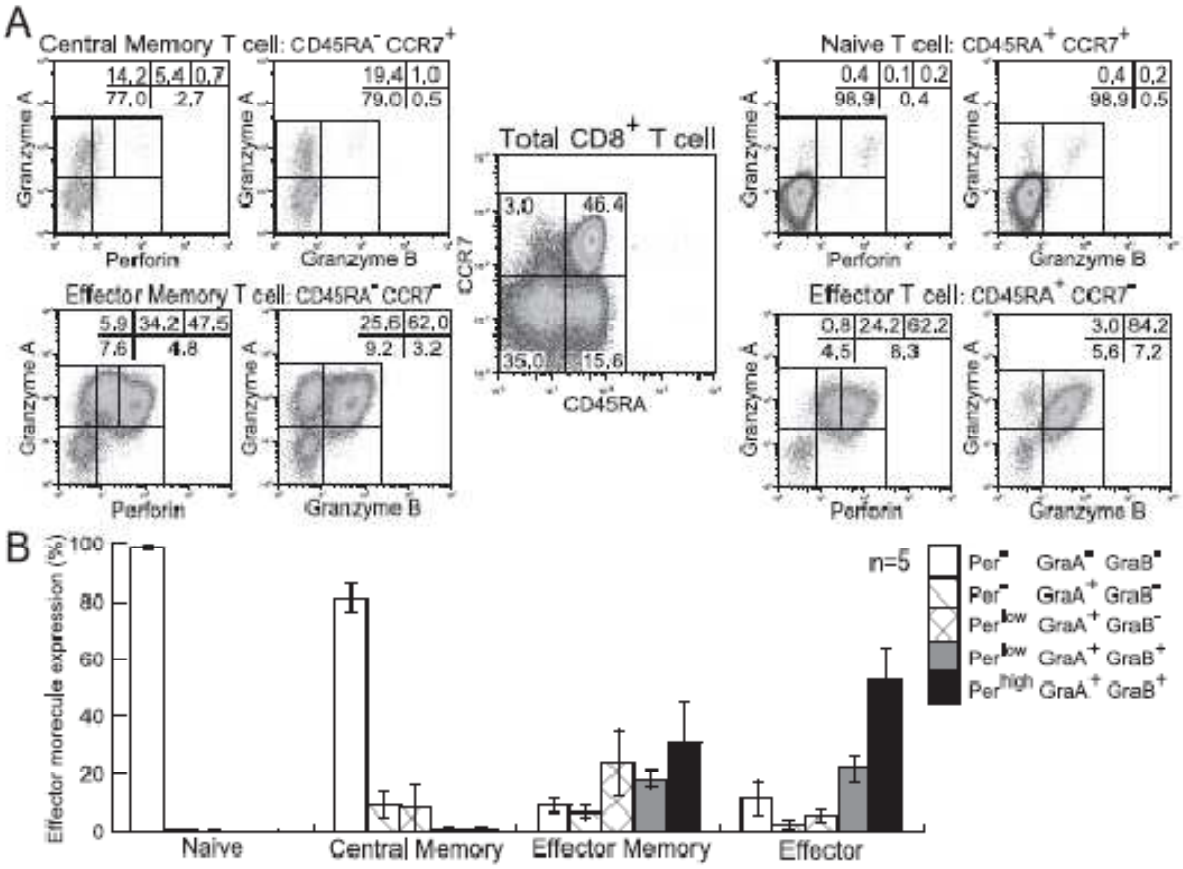
Proteínas intracelulares



Three Memory Subsets of Human CD8$^+$ T Cells Differently Expressing Three Cytolytic Effector Molecules[1]

Hiroshi Takata and Masafumi Takiguchi[2]

The Journal of Immunology, **2006, 177: 4330–4340.**



Ventajas

Velocidad
Análisis multiparamétrico
Eliminación de errores internos del exp.

Desventajas

Calidad de fluorocromos
Anticuerpos
Autofluorescencia
Compensación
Células en suspención