Citofluorimetria

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Flow Cytometry

1. *What is Flow Cytometry?*
2. *How does a Flow Cytometer work?*
3. *Fluidics and Optics*
4. *Dye and Single Color Compensation*
5. *Sample Preparation for Flow Cytometry*
6. *Applications*

Flow Cytometers

BD FACS Calibur

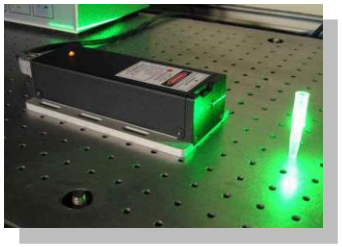
2 laser
4 colors

Beckman Coulter Epics Altra

BD FACS Canto II

> 2 laser
> 6-7 colors

BD LSRII and LSRII Green



4 laser
LSRII; 488, 633, 405, 355
LSRII Green; 405, 488, 532, 635
12 colors
*DPSS (diode-pumped solid state) 532 nm

What is Flow Cytometry?

- *Cytometry* refers to the measurement of physical/chemical characteristics of cells or other biological particles.

- *Flow Cytometry* is the process whereby such measurements are made upon cells/particles as *they pass through a measuring apparatus* (hopefully in single file) *suspended in a fluid stream*.

- *Flow Sorting (Flow Cytometric Cell Sorting)* extends flow cytometry with the additional capacity to divert and collect cells exhibiting an identifiable set of characteristics either mechanically or by electrical means *(Flow Cytometric Analysis)*.

- *FACS* - Fluorescence Activated Cell Sorting FACS is a trademark of Becton Dickinson Immunocytometry Systems (BDIS). All FACS instruments are BD systems, but not all cytometers are FACS.

Strength of FCM

- The strength of this technology lies in
  - its high throughput
    - measurement of high numbers of cells in short time
  - its ability to capture many parameters per cell, assessing them individually.
    - Multiparameter FCM

Multi-parameter flow cytometry

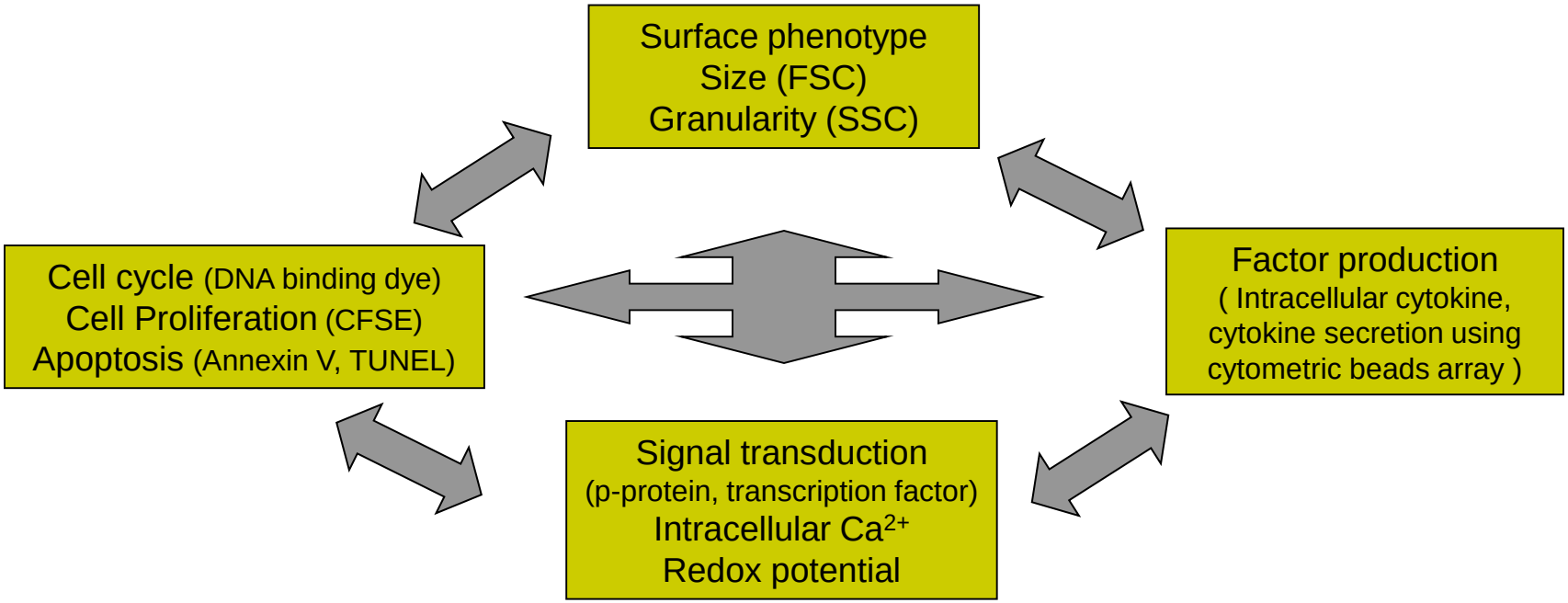
A technology that simultaneously measures multiple parameters of single cells at a rapid rate

relative Size : FSC,

relative Granularity or Internal Complexity : SSC

relative Fluorescence Intensity : FL1, FL2, FL3 ...

1. Permits the detailed analysis of markers of cellular differentiation;
2. Permits the *simultaneous* evaluation of cell phenotype and function;
3. Permits cell sorting



Advantages of MFC

- Increased accuracy.
  - Using large numbers of fluorochromes is associated with an exponential increase in the information obtained from a single combination of antibodies in the same tube, permitting a more reliable identification.
- Smaller sample size.
  - Increased number of antibodies per tube means fewer tubes and less sample needed but allows acquisition of more cellular events resulting in smaller coefficients of variation and increased data precision. This is of particular relevance to paucicellular samples such as cerebrospinal fluid (CSF) and fine needle aspirates (FNA) and also paediatric samples.
- Cost effectiveness.
  - Less usage of repeating backbone or gating antibodies.
- Increased efficiency.
  - Less time is required for sample processing and acquisition.
- Increased sensitivity for minimal residual disease monitoring.

Disadvantages

- Increased complexity of compensation.
  - Inaccurate compensation is probably the main source of erroneous data in MFC. This can be solved by applying compensation matrices but this requires expertise.
- Challenges of antibody panel validation.
  - It is crucial to run fluorescence minus one controls for all new antibody combinations and to check for stearic hindrance between antibodies used to label antigens that are in close proximity on the cell.
- Tandem dye conjugate issues.
  - Tandem dyes are conjugates of two fluorochromes, but this can lead to problems in resonance excitation transfer if exposed to light. Ideally a compensation matrix should be performed for each new tandem dye conjugate lot.
- Increased need for expertise in data analysis and interpretation.
- Human error associated with pipetting a high number of antibodies into a single tube.
  - This can be overcome by preparing in-house McAb cocktails, which have been shown be stable for up to 4 weeks or using commercial cocktails

Cellular Parameters Measured by Flow

Intrinsic

- No reagents or probes required (**Structural**)
  - Cell size
    - (Forward Light Scatter)
  - Cytoplasmic granularity
    - (90 degree Light Scatter)

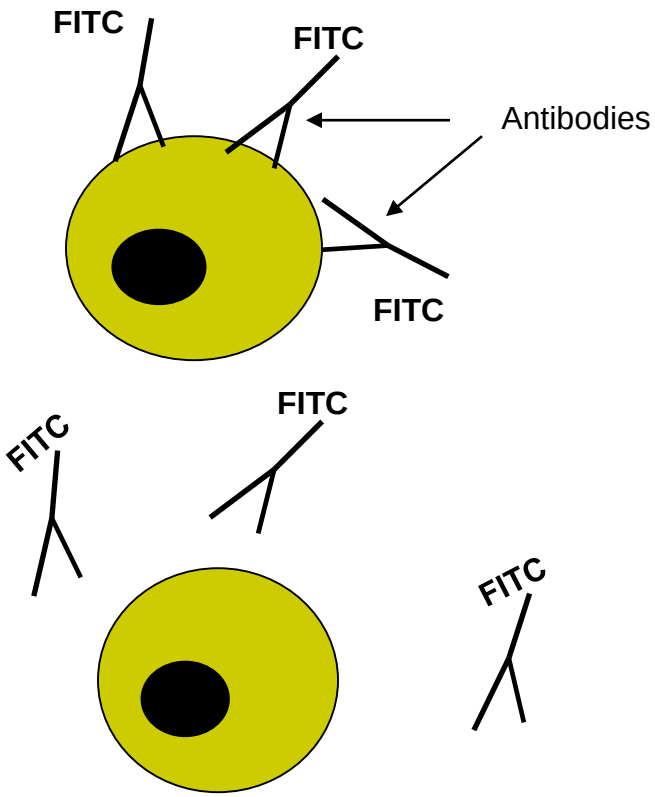
Extrinsic

- Reagents are required.
  - **Structural**
    - DNA content
    - DNA base ratios
    - RNA content
  - **Functional**
    - Surface and intracellular receptors.
    - DNA synthesis
    - DNA degradation (apoptosis)
    - Cytoplasmic Ca++
    - Gene expression

Fluorescence Activation Process (or Immunofluorescence)

Antibodies recognize specific molecules in the surface of some cells

Antibodies are artificially conjugated to **fluorochromes**



But not others

When the cells are analyzed by flow cytometry the cells expressing the marker for which the antibody is specific will manifest fluorescence.

Cells who lack the marker will not manifest fluorescence

Flow Cytometry Applications

- Immunofluorescence
- Cell Cycle Kinetics
- Cell Kinetics
- Genetics
- Molecular Biology
- Microbiology
- Biological Oceanography
- Parasitology
- Animal Husbandry (and Human as well)
- Bioterrorism

Flow cytometry in Hematology

- immunophenotyping by flow cytometry facilitates:
  - The identification and quantification of cell populations within a sample
  - The differentiation of normal from abnormal cells
  - The differentiation of reactive from neoplastic cells
  - The identification of the differentiation or maturation stage of a cell population
  - The quantification of tumour infiltration.

Flow cytometry in Hematology

- However it is the interpretation of the data provided by the this techniques which poses the greatest challenge and the reliable diagnosis of leukemia relies on:
  - Knowledge of physical characteristics/antigen expression on normal cells
  - The ability to distinguish between different patterns of expression of antigens
  - The ability to identify aberrant antigen expression
  - The identification of a robust leukaemia-associated immunophenotype (LAIP).

How does a Flow Cytometer work?

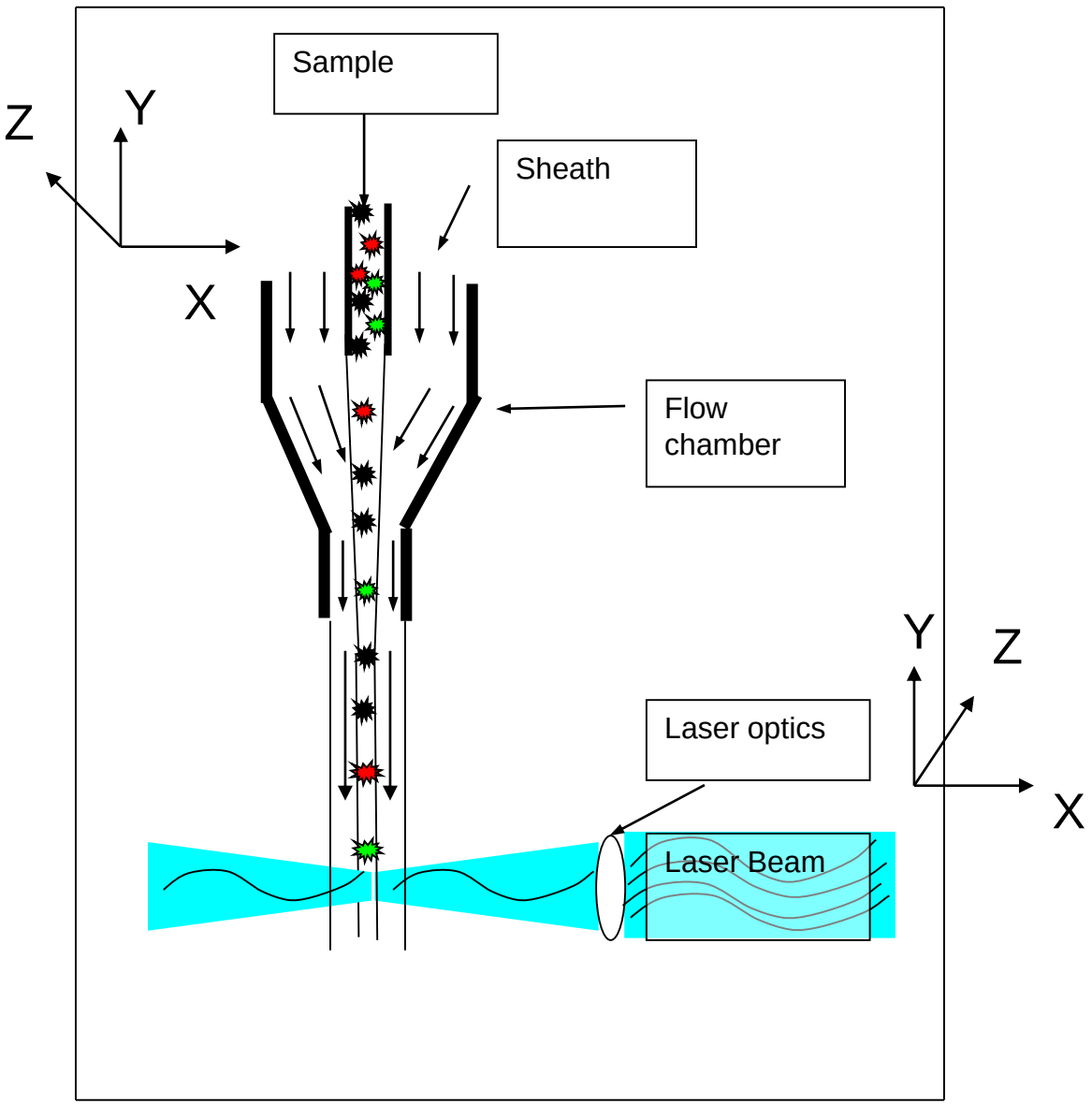
(Fluidics and Optics)

A flow cytometer has five main components

1. Lasers providing a monochromatic light
2. A flow cell with a liquid stream (sheath fluid), which carries and aligns the cells so that they pass in single file through the laser beam
3. Optical systems and filters regulating the light signals
4. Photomultiplier detectors (PMTs) that generate data on FSC (which provides an approximation of cell size) and SSC (which relates to cell complexity), as well as converting fluorescence signals from light into electrical signals that are processed by a computer
5. A computer for analysis of the signals.

Principles of Flow Cytometry

- For reliable analysis, the specimen must be in a **monodisperse suspension**.
- In a flow cytometer, isotonic fluid is forced under pressure into a tube that delivers it to the **flow cell**, where a fluid column with laminar flow and a high flow rate is generated (so called **sheath fluid**).
- The sample is introduced into the flow cell by a computer-driven syringe in the center of the sheath fluid, creating a coaxial stream within a stream (the so-called **sample core stream**).
- The pressure of the sheath stream hydrodynamically **aligns the cells** or particles so that they are presented to the light beam one at a time.
- Flow cytometers measure the amount of light emitted by fluorochromes associated with individual cells or particles.



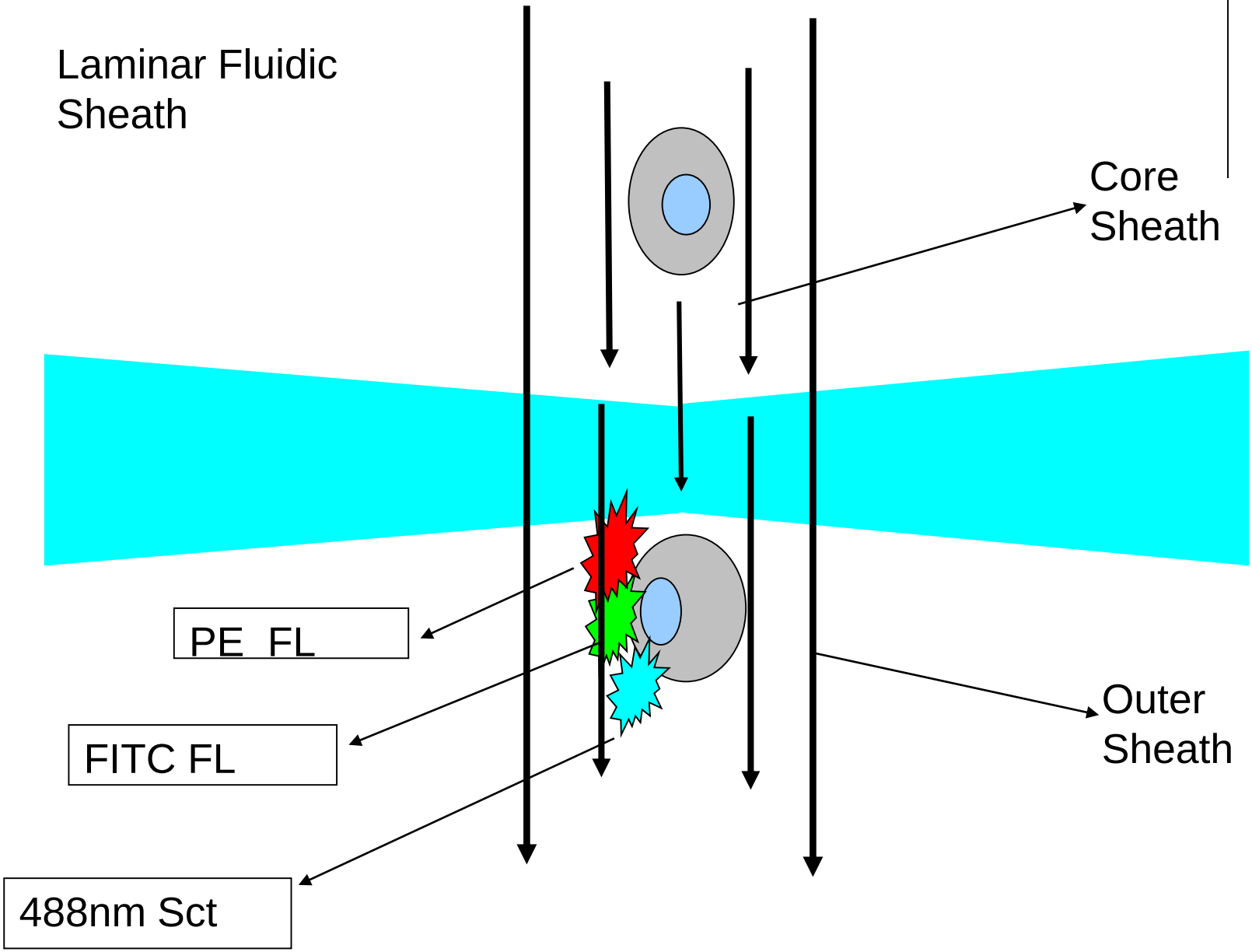
A single cell suspension is hydrodynamically focused with sheath fluid to intersect lasers

Cells are presented to the laser using principles of hydrodynamic focusing

Principles of Flow Cytometry

- A pair of light scatter channels provides an approximate measure of **cell size (FS) and granularity (SS).**

- FS and SS are used to set the **threshold** for separating debris, erythrocytes, and platelets from viable nucleated cells.
- **Live cells** scatter more light than dead and apoptotic cells and therefore have **higher FS**.
- SS is collected together with fluorescent light at **right angles** to the beam and is due to light reflected from internal structures of the cell.
- Cells with high granularity or vacuoles such as granulocytes or monocytes will have higher SS than ones with no granules such as lymphocytes or blast cells.


Laminar Fluidic Sheath
Core Sheath
PE FL
FITC FL
488nm Sct
Outer Sheath

Principles of Flow Cytometry

- For application in FCM, antibodies are conjugated with **fluorochromes**, dyes that absorb the light from the laser and emit light at longer wavelengths.
- The emitted light is focused by a lens onto fiberoptic cables and transmitted to octagonal **detectors**
- **Filters** in front of each of a series of detectors restrict the light that reaches the detector to only a small particular range of wavelengths (referred to as channels).
- The sensors **convert the photons to electrical impulses** that are proportional to the number of photons received and to the number of fluorochrome molecules bound to the cell.

TABLE OF FLUOROCHROMES COMMONLY USED IN CLINICAL FLOW CYTOMETRY

Probe	Ex (nm)	Em (nm)	MW	Acronym/Comments
Reactive and Conjugated Probes				
R-Phycoerythrin	480;565	578	240 k	PE
Red 613	480;565	613		PE-Texas Red
Fluorescein isothiocyanate	495	519	389	FITC
Rhodamine isothiocyanate	547	572	444	TRITC
X-Rhodamine	570	576	548	XRITC
Peridinin chlorophyll protein	490	675		PerCP
Texas Red	589	615	625	TR
Allophycocyanin	650	660	104 k	APC
TruRed	490,675	695		PerCP-Cy5.5
Alexa Fluor 647	650	668	1250	
Alexa Fluor 700	696	719		
Alexa Fluor 750	752	779		
Cyanine 5	(625);650	670	792	Cy5
Cyanine 5.5	675	694	1128	Cy5.5
Cyanine 7	743	767	818	Cy7
PE-TR-X	595	620	625	ECD
PE-Cy5 conjugates	480;565;650	670		Cychrome, Tri-Color, Quantum Red
PE-Cy7 conjugates	480;565;743	767		PE-Cy7
APC-Cy7 conjugates	650;755	767		APC-CY7
Nucleic Acid Probes				
4′,6-Diamidino-2-phenylindole	345	455		DAPI ,AT-selective
SYTOX Blue	431	480	~400	DNA
SYTOX Green	504	523	~600	DNA
Ethidium bromide	493	620	394	
7-Aminoactinomycin D	546	647		7-AAD, CG-selective
Acridine Orange	503	530/640		DNA/RNA
Thiazole Orange	510	530		TO (RNA)
Propidium iodide	536	617	668.4	PI

Em, peak emission wavelength (nm); Ex, peak excitation wavelength (nm); MW, molecular weight.

Nuclear Dyes* (a short List);

Dye	Lazer	Filters	Application:
Acridine Orange	Ar	G, R	DNA, RNA discrimination, Lysosome labeling
7-Aminoactinomycin D	Ar	R, LR	DNA content, Viability
DAPI	UV		
Ethidium Bromide	Ar	R, LR	dsDNA intercalator, Viability, Chromosome labeling
Ethidium Homodimer	Ar	R, LR	DNA content, Viability
Hoescht	UV		
LDS 751 (Styryl-8)	Ar	LR	DNA content, Viability, Leucocyte differentiation
Propidium Iodide	Ar, Ne	R, LR	DNA content, Viability, Chromosome labeling*
SYTO(R) 11, 12, 20, 22,16 (m)	Ar	G	DNA, RNA content of viable cells
SYTO(R) 17, 59, 61 (m)	Ne	LR	Cytoplasmic labeling in viable Bacteria and Eukaryotes
SYTOX(R) Green (m)	Ar	G	Chromosome labeling, Impermeant*
Thiazole Blue	HeNe	LR	Reticulocyte labeling
To-Pro?-3 (m)	HeNe	LR	Viability, photosensitive

Adapted from ComputCyte

Fluorochromes* (a short List);

Dye	Lazer	Filters	Application:
Alexa Fluor? 488 (m)	Ar	G	Substitute for FITC, better stability and intensity*
APC (Allophycocyanine)	HeNE	LR	Second most widely used Long Red dye
BODIPY(R) FL (m	Ar	G	Substitute for FITC, better stability and intensity*
BODIPY(R) 630/650 (m)	HeNE	LR	Substitute for LR dyes, better stability and intensity
CY5 (a)	HeNE	LR	Small molecule substitute for APC, TR*
CY5.5 (a)	HeNE	LR	Small molecule substitute for APC
FITC (Fluorescein)	Ar	G	Most widely used Green dye*
Oregon Green(R) 488 (m)	Ar	G	Substitute for FITC, better stability and intensity
PE (Phycoerythrin)	Ar	O	Most widely used Orange dye (R-form recommended)*
PE-APC tandem	Ar	LR	
PE-CY5 tandem	Ar	LR	
PerCP	Ar	LR	Long Red dye
PE-TR (Texas Red(R)) (m)	Ar, R	LR	Argon laser excited Texas Red dye
Rhodamine Green? (m)	Ar	G	Substitute for FITC, better stability and intensity
Rhodol Green? (m)	Ar	G	Substitute for FITC, better stability and intensity

Adapted from ComputCyte

Specialty Dyes* (a short List);

Dye	Lazer	Filters	Application:
BCECF	Ar	G	Cellular membrane potential
Calcium Green? (m)	Ar	G	Calcium measurements, ratioed with Fura Red
Carboxy-DCFDA (m)	Ar	G	Reactive oxygen intermediates*
Carboxy SNARF(R)-1 AM (m)	Ar	O/R ratio	Cellular pH measurements
DiIC(N)(5)	HeNE	LR ER	Mitochondrial membrane potential*
DiOC(N)(3)	Ar	G ER	Mitochondrial membrane potential*
Fluo-3 (m)	Ar	G	Calcium measurements
Fura Red? (m)	Ar	R	Calcium measurements

Flouresecnt Proteins and Markers (a short List);

Dye	Lazer	Filters	Application:
Green Fluorescent Protein	Ar	G	
JC-1	Ar	O, R	Mitochondrial membrane potential
NBD-C6-Ceramide	Ar	G	Golgi apparatus

Adapted from ComputCyte

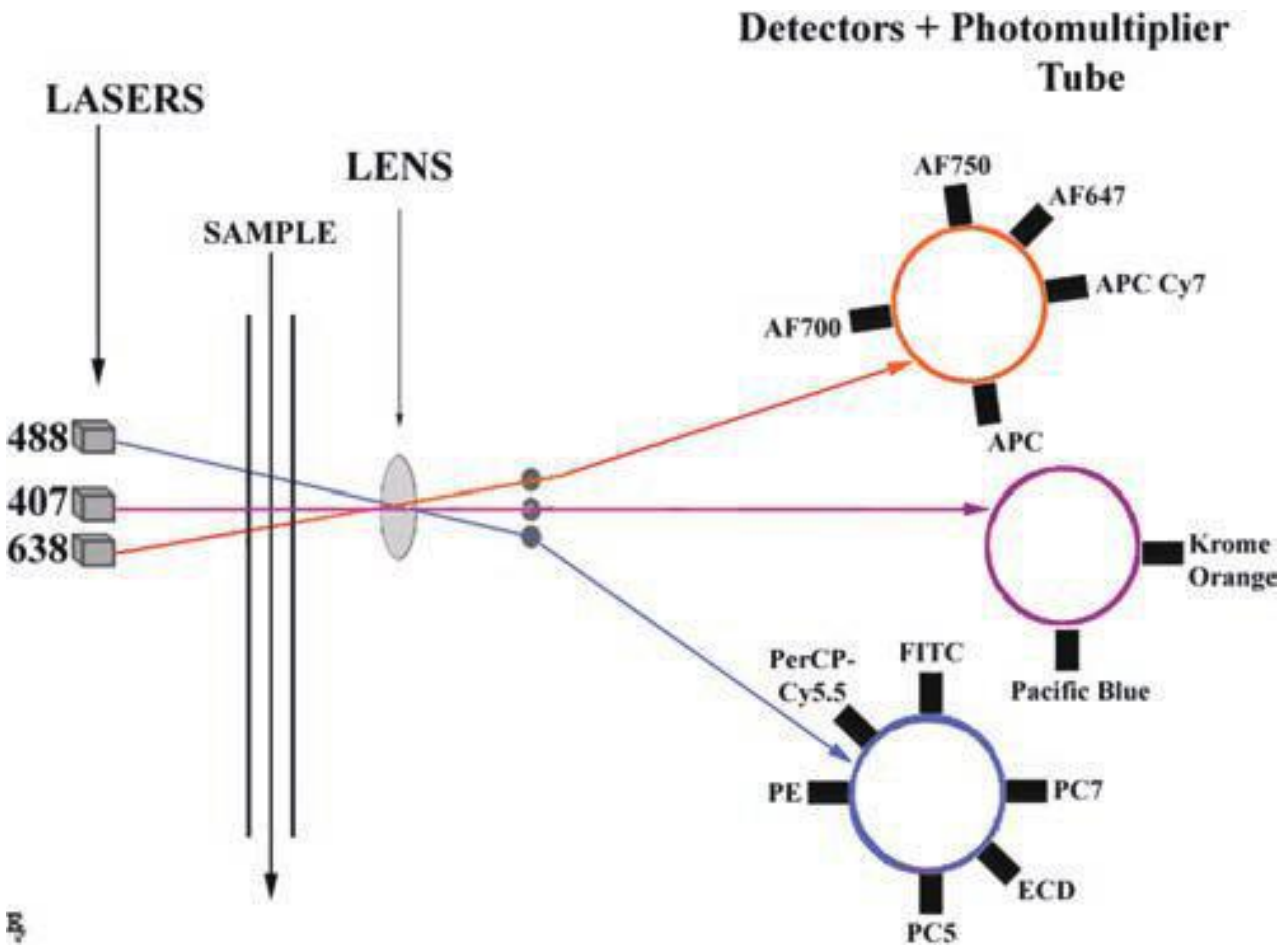
Principles of Flow Cytometry

- Most cells have low numbers of native fluorescent molecules that define their **background fluorescence**.
- Some of the light may come from **spillover** fluorescence emitted by a reagent measured in a different channel.
- The interference is corrected by applying fluorescence **compensation** based on data from single-stained samples.
  - This is usually done using cells or beads before or during the data acquisition phase.

Principles of Flow Cytometry

- Modern FCM data analysis software also allows **collection of uncompensated data and applying compensation during analysis**.

- Before data acquisition, standard reference particles (**fluorescent microspheres**) should be used to adjust the PMT voltage settings so that the beads fall in approximately the same location or the same “target channels,” predetermined for each fluorochrome.

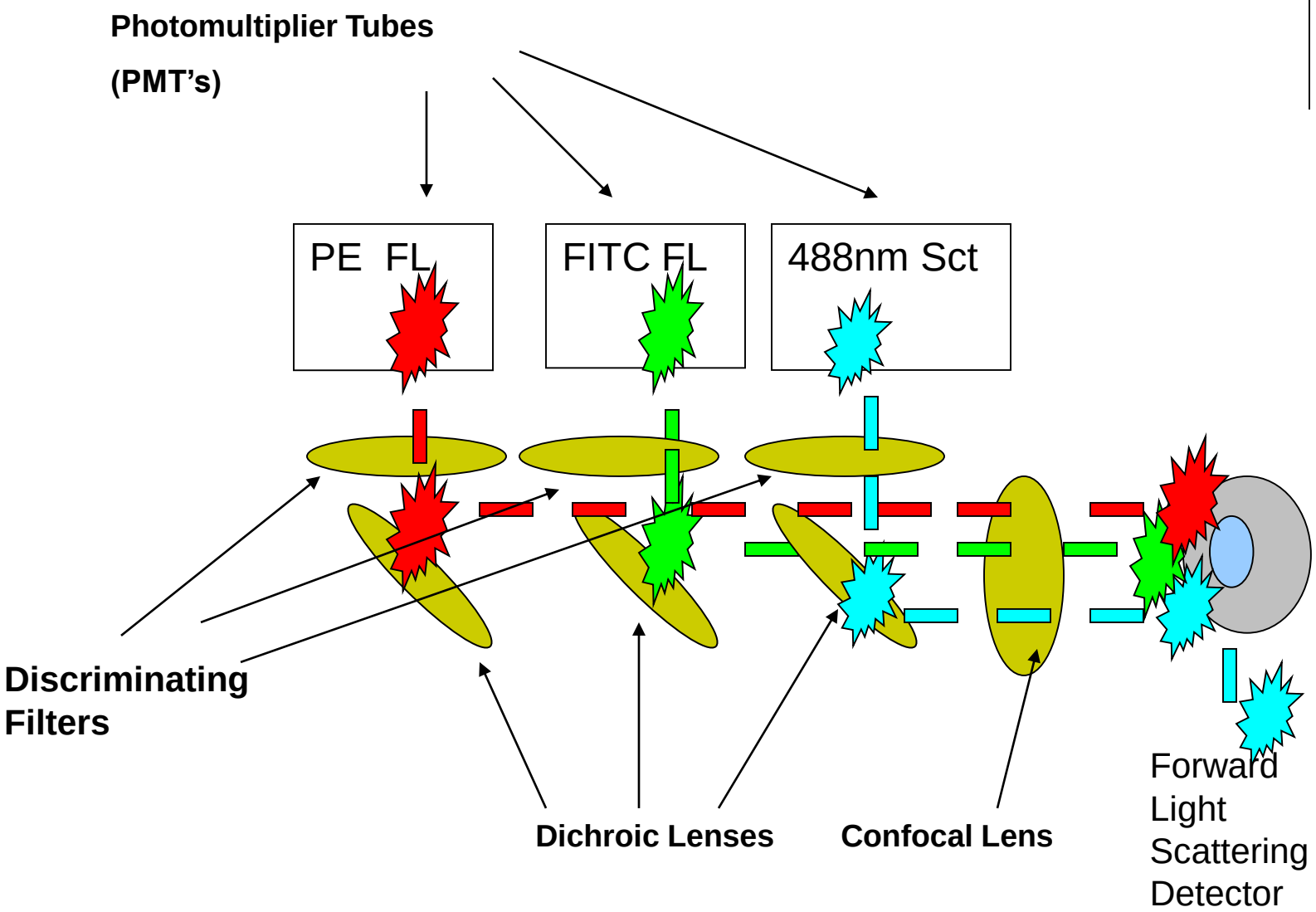


single cell suspension is hydrodynamically focused with sheath fluid to intersect lasers (three-laser system is shown).
Fluorescence signals are collected by multiple fluorescence emission detectors, separate for every laser.
Detected signals are amplified by photomultiplier tubes and converted to digital form for analysis

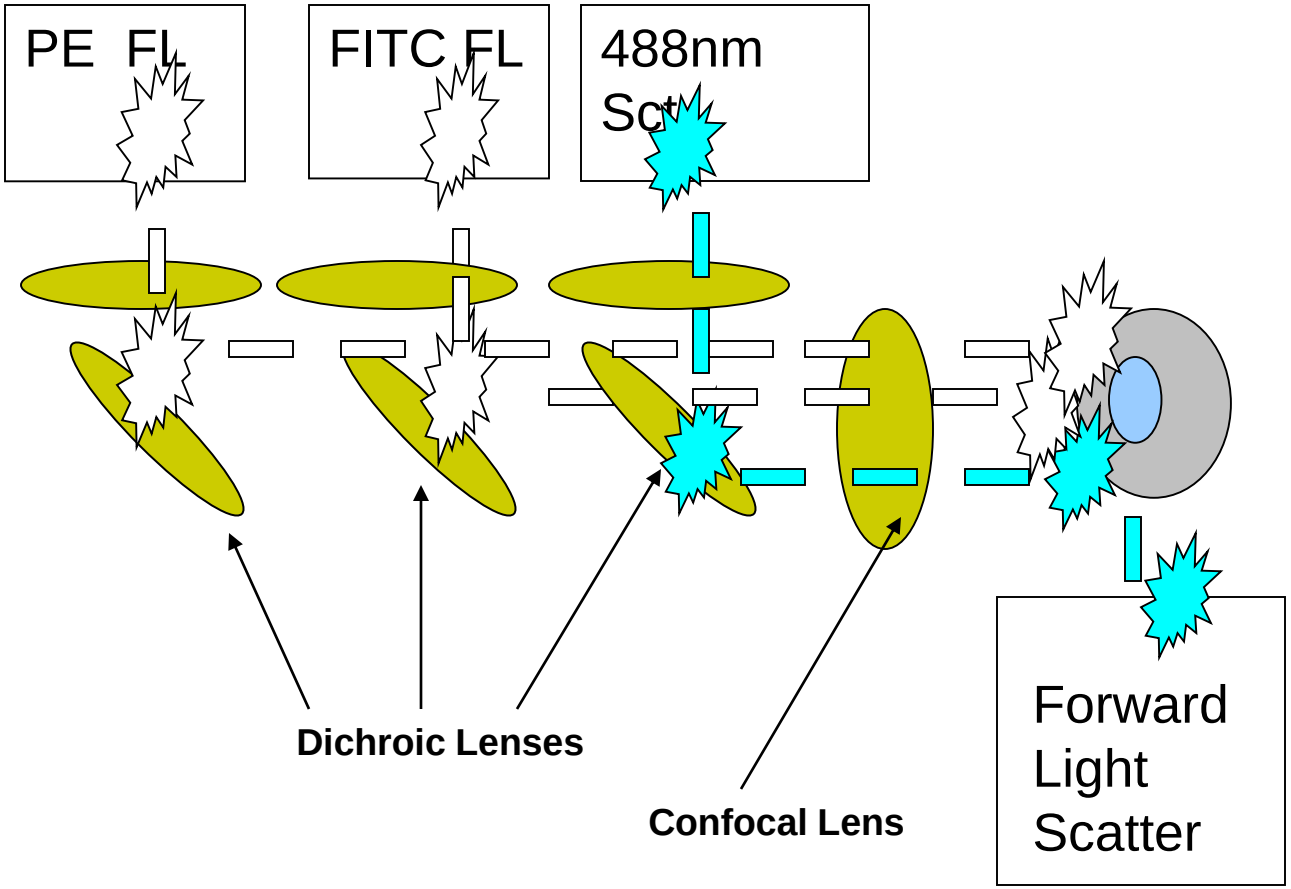
Principles of Flow Cytometry

- The fluorescent emissions are of low intensity and have to be amplified by **photomultiplier tubes** (PMT).
- PMTs count the specific photons and the remaining light is reflected to the next filter, where the process is repeated.
- Thus, most of the cell-associated fluorescence detected in a given channel is emitted by fluorochrome-coupled antibodies or other fluorescent reagents of interest.
- **Electrical impulses from photoelectrons collected by PMTs are converted to digital signals**.
- **Acquired FCM data are electronically stored in so-called list-mode files** that are a part of the medical record of the patient

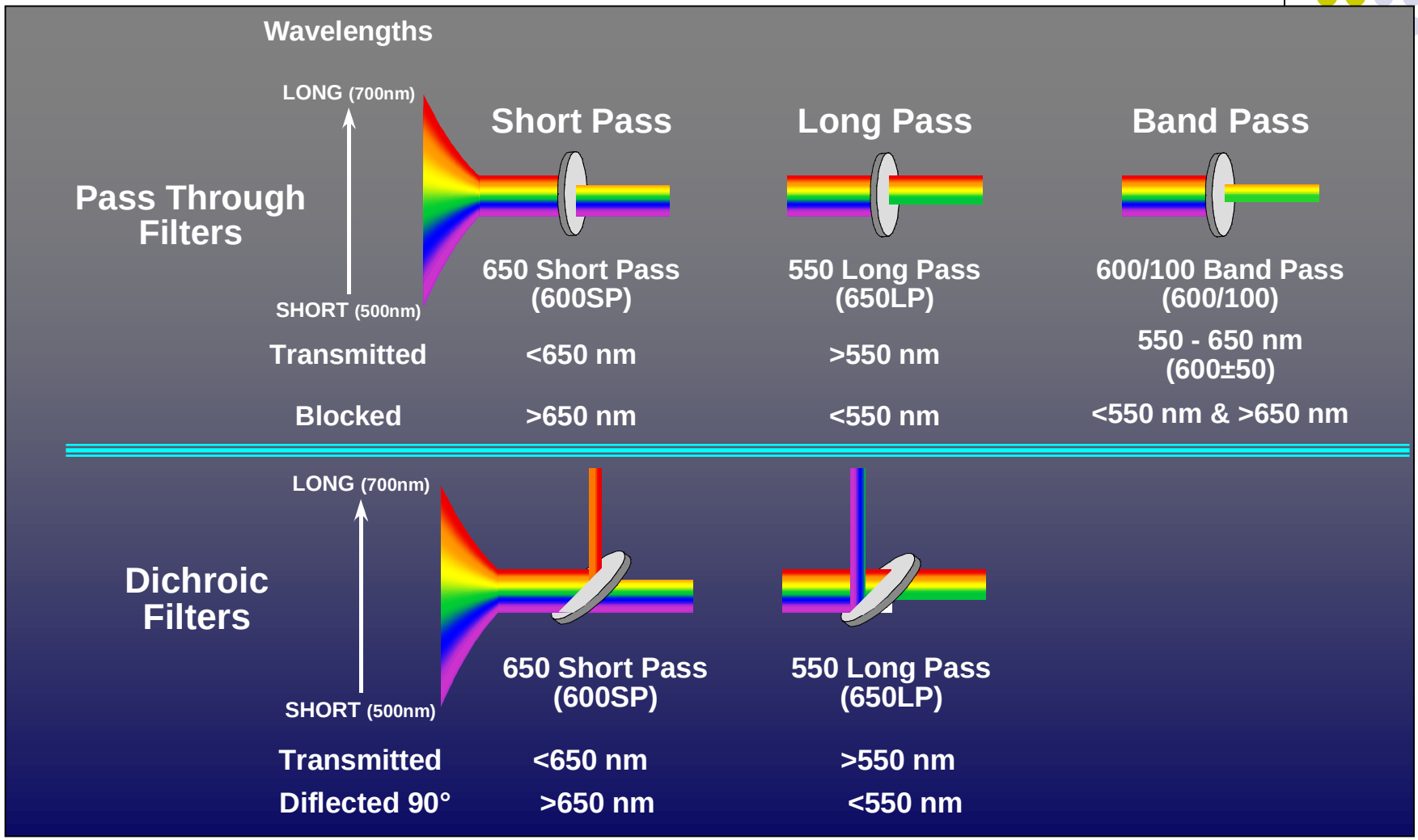
Each cell generates a quanta of fluorescence



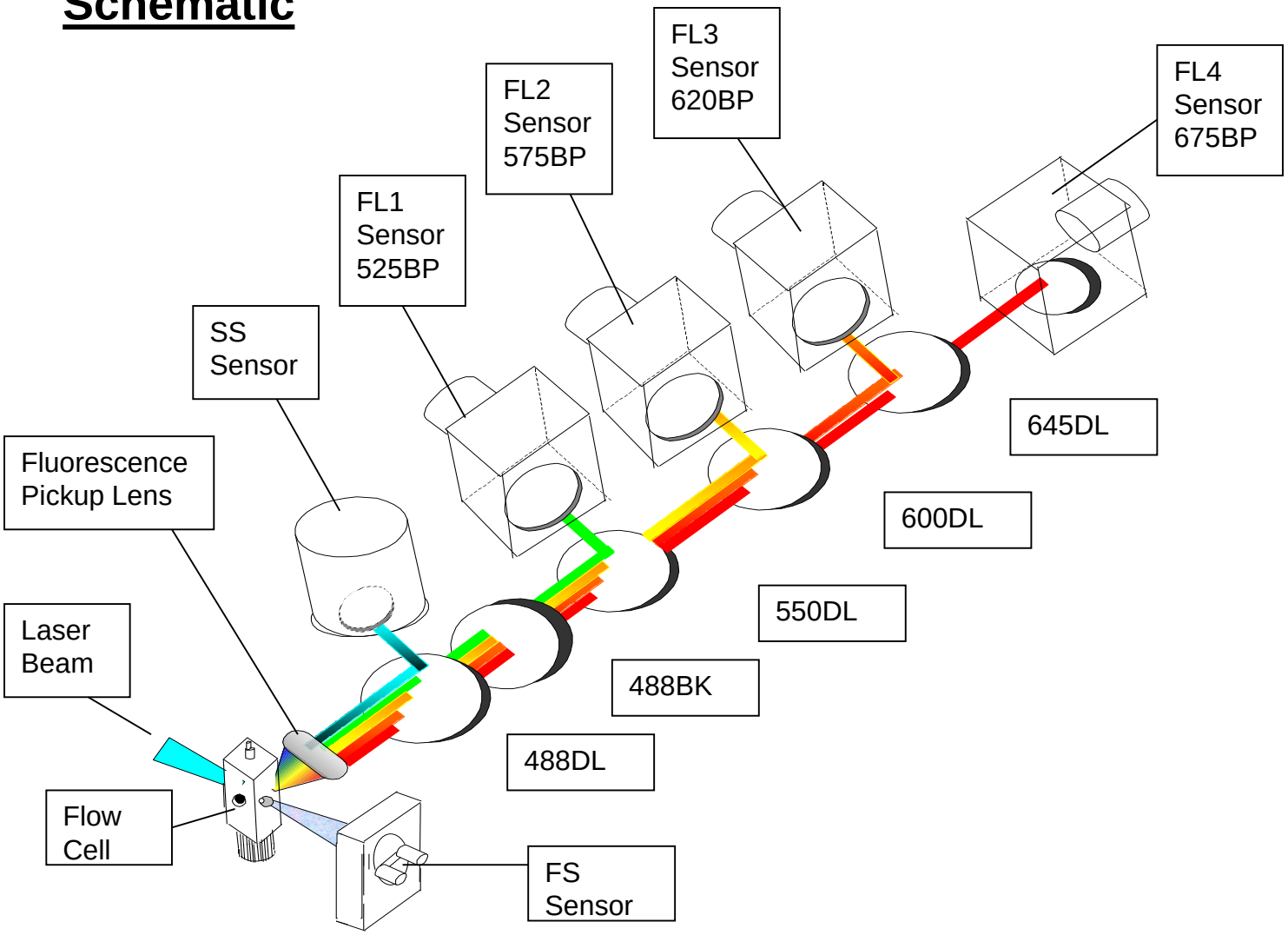
Negative cells are also detected



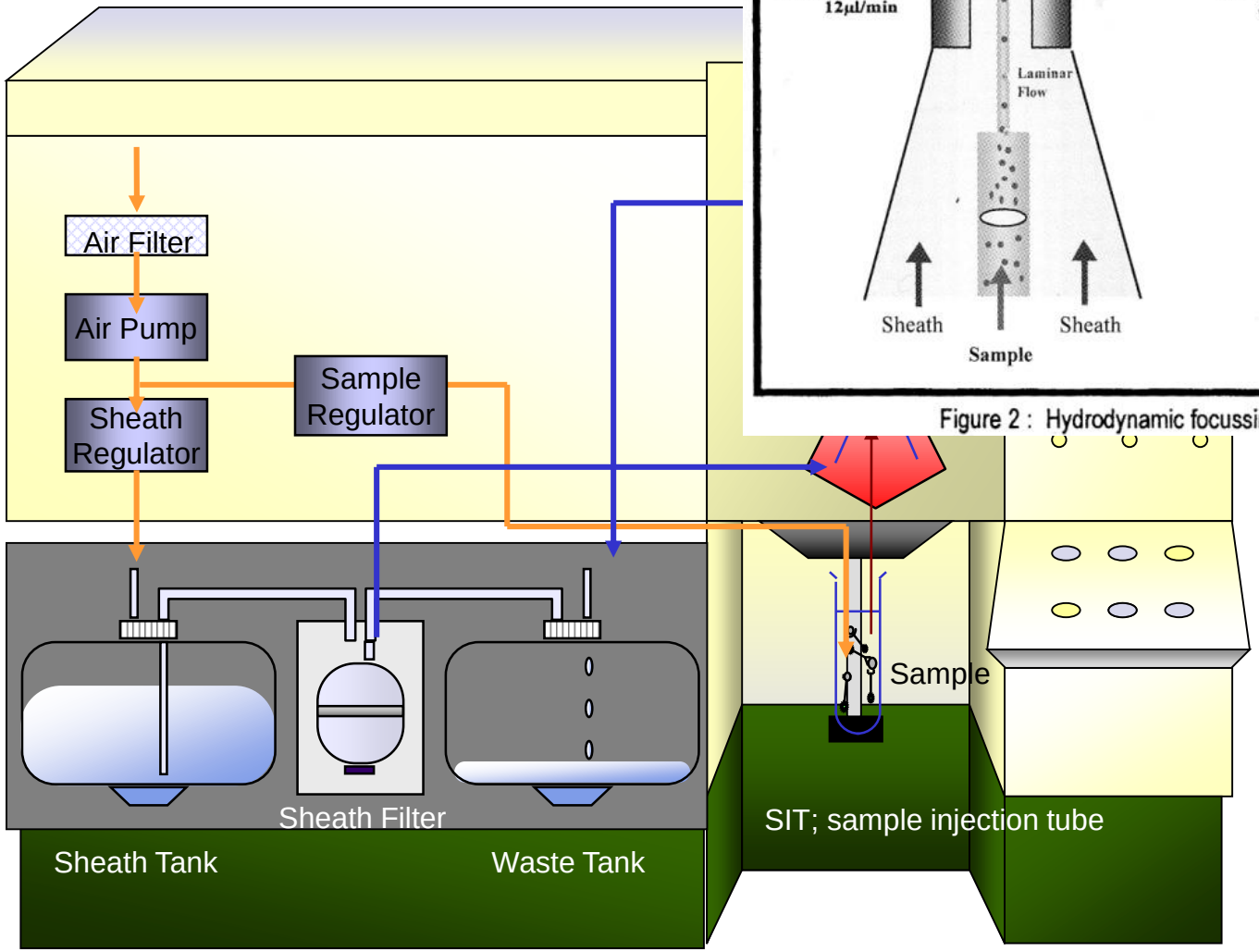
Types of Optical Filters



Optical Bench Schematic



Fluidic System



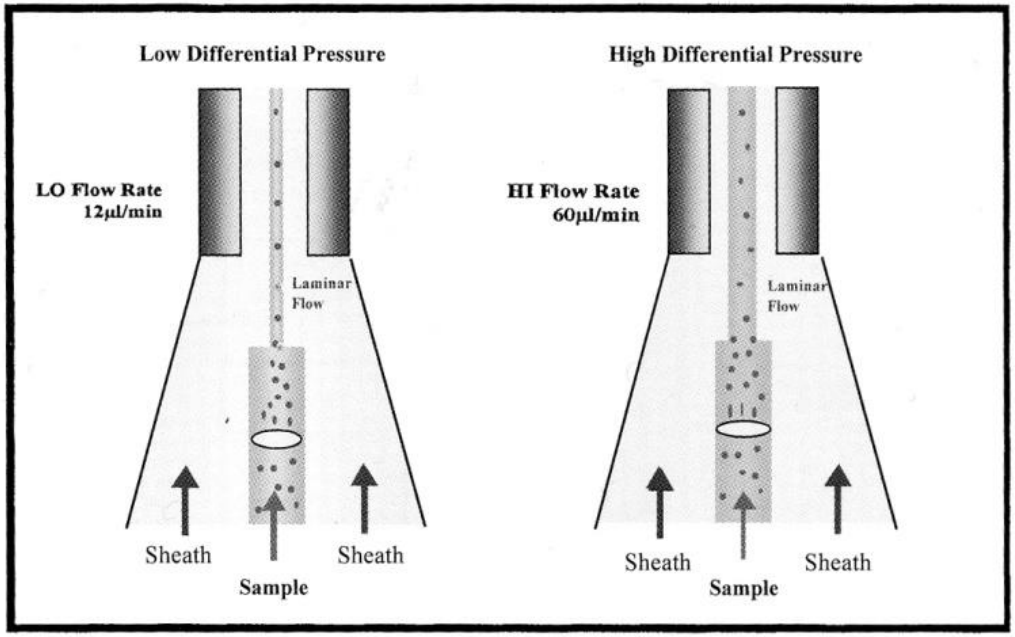
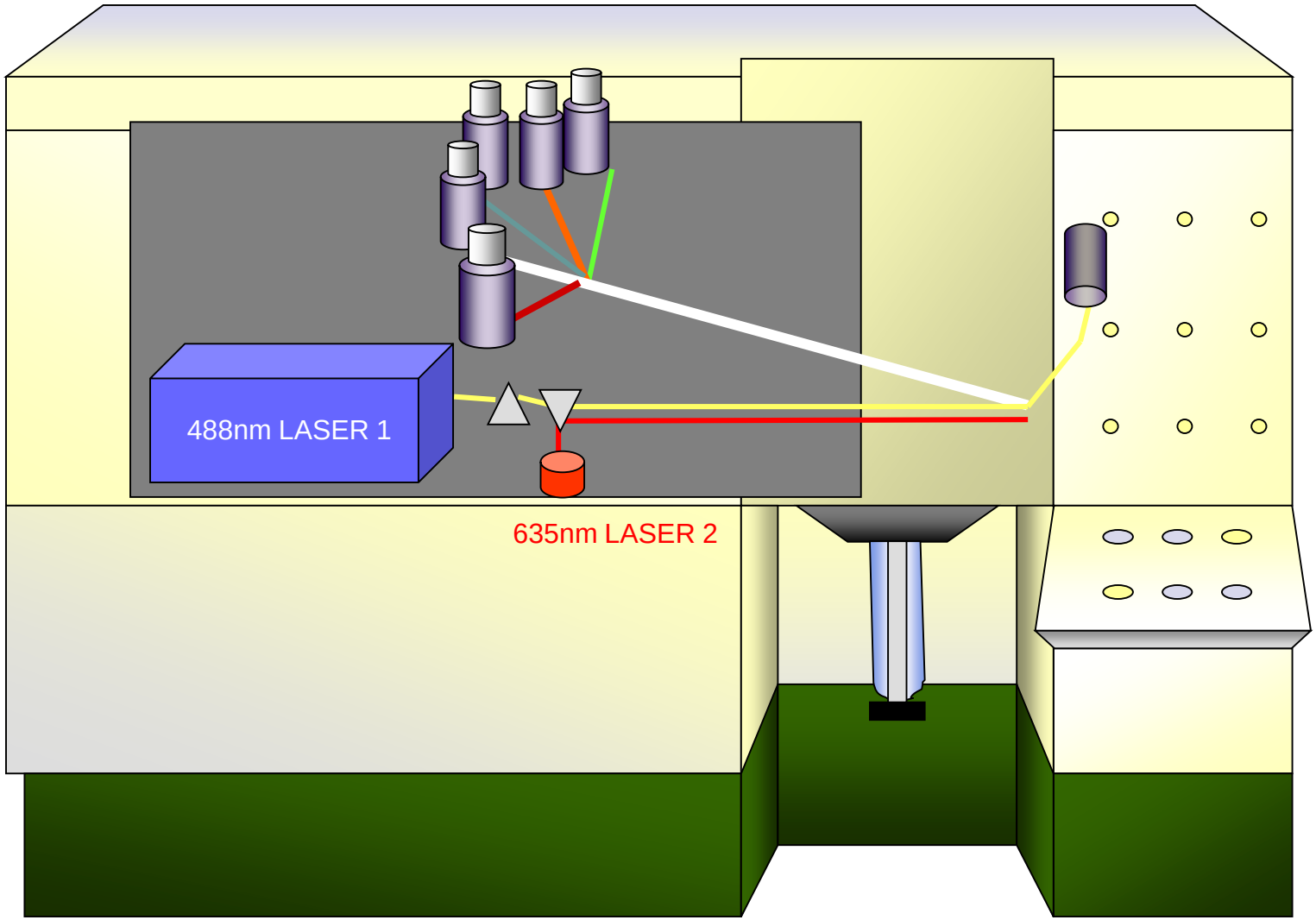


Figure 2 : Hydrodynamic focussing and FACSCalibur flow area

BD
BD Biosciences

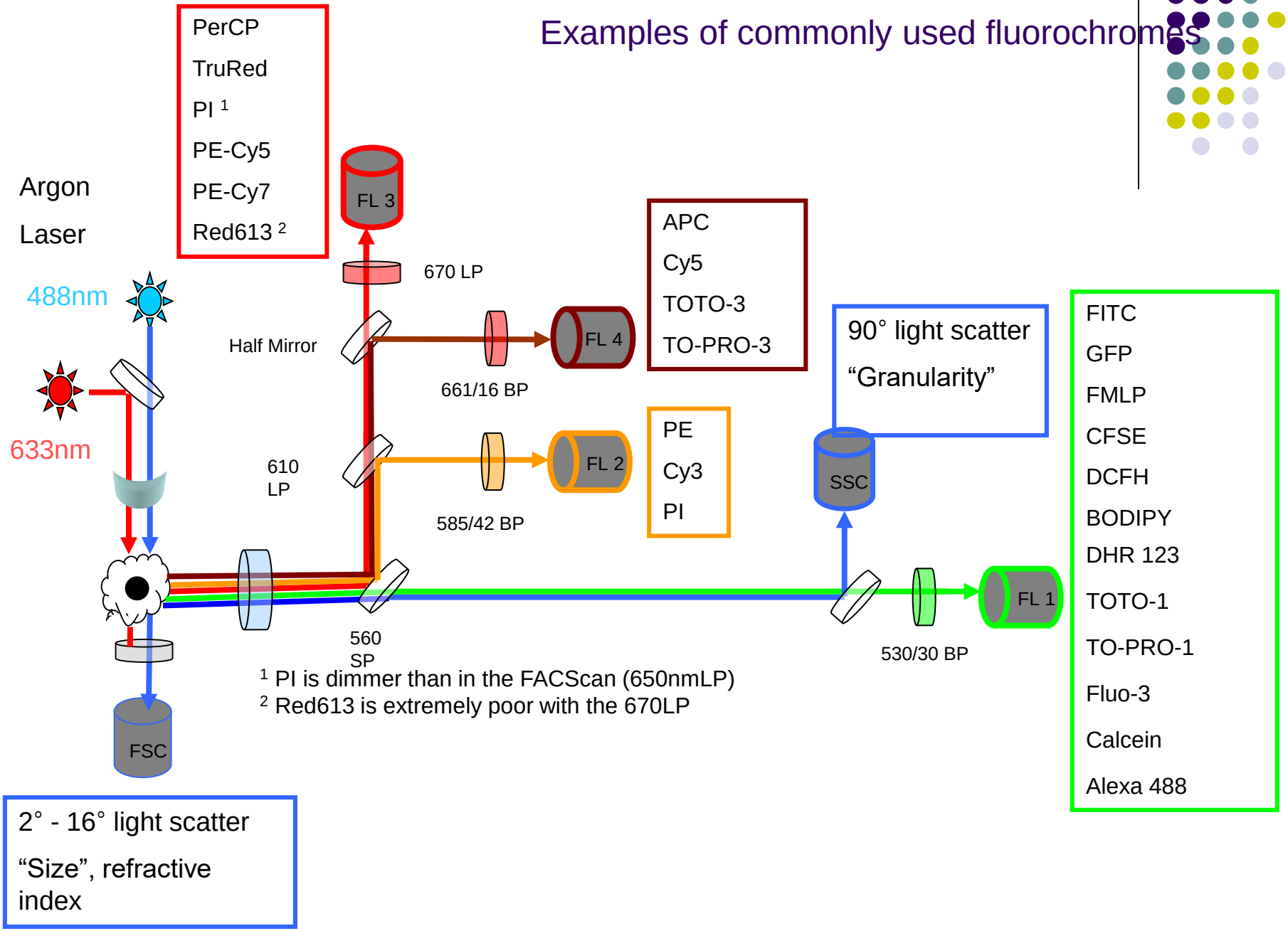
Optics System



BD
BD Biosciences

FACScan - FACS Calibur

Examples of commonly used fluorochromes



[1] PI is dimmer than in the FACScan (650nmLP)
[2] Red613 is extremely poor with the 670LP

Typical Instrument Configuration

Instrument	Laser	Exitation Laser Line (nm)	Fluorescence Channel	BD Biosciences Fluorochromes				
BD FACScan™	Argon (L1)	488	FL1 Green	FITC	Alexa Fluor® 488			
			FL2 Yellow	PE				
			FL3 Red	PE-Texas Red®	PE-Cy5*	PerCP	PerCP-Cy5.5	PE-Cy7
BD FACSCalibur™	Argon (L1)	488	FL1 Green	FITC	Alexa Fluor® 488			
			FL2 Yellow	PE				
			FL3 Red	PE-Texas Red®	PE-Cy5*	PerCP	PerCP-Cy5.5	PE-Cy7
	Red Diode (L2)	635	FL4 Red	APC*	Alexa 647			
BD FACStar™ Plus (typical setup)	Argon (L1)	488	FL1 Green	FITC	Alexa Fluor® 488			
			FL2 Yellow	PE				
			FL3 (1) Red	PE-Texas Red®	PE-Cy5*	PerCP-Cy5.5	PE-Cy7	
	HeNe (L2)	633	FL3 (2) Red	APC*	Alexa Fluor® 647			
			FL4 InfraRed	APC-Cy7				
BD FACSVantage™ SE (typical setup)	Argon (L1)	488	FL1 Green	FITC	Alexa Fluor® 488			
			FL2 Yellow	PE				
			FL6 Red	PE-Texas Red®	PE-Cy5*	PerCP-Cy5.5		
			FL3 InfraRed	PE-Cy7				
	Krypton (L2)	407	FL4 (1) Blue	Alexa Fluor® 405	Pacific Blue®			
	HeNe (L2 or L3)	633	FL4 (2) Red	APC*	Alexa Fluor® 647			
			FL5 InfraRed	APC-Cy7				
BD™ LSR (typical setup)	Argon (L1)	488	FL1 Green	FITC	Alexa Fluor® 488			
			FL2 Yellow	PE				
			FL3 Red	PE-Texas Red®	PE-Cy5*	PerCP	PerCP-Cy5.5	PE-Cy7
	HeCd (L2)	325	FL4, FL5 Blue					
	HeNe (L3)	633	FL6 Red	APC*	Alexa Fluor® 647	APC-Cy7		
BD™ LSR II (typical setup)	Argon (L1)	488	Green	FITC	Alexa Fluor® 488			
			Yellow	PE				
			Red	PE-Texas Red®	PE-Cy5*	PerCP	PerCP-Cy5.5	
			Infra Red	PE-Cy7				
	HeNe (L2)	633	Red	APC*	Alexa Fluor® 647			
			Infra Red	APC-Cy7				
	UV (L3)	355	Violet					
			Blue	Alexa Fluor® 405	Pacific Blue®			
	Violet (L4)	405	Blue	Alexa Fluor® 405	Pacific Blue®			
BD FACSAria™ (typical setup)	Argon (L1)	488	Green	FITC	Alexa Fluor® 488			
			Yellow	PE				
			Red	PE-Texas Red®	PE-Cy5*	PerCP		
			Far Red	PerCP-Cy5.5				
			InfraRed	PE-Cy7				
	HeNe (L2)	633	Red	APC*	Alexa Fluor® 647			
			InfraRed	APC-Cy7				
	Violet (L3)	407	Blue	Alexa Fluor® 405	Pacific Blue®			
BD FACSArray™ (typical setup)	Green Diode (L1)	532	Yellow	PE				
			Far Red	PerCP-Cy5.5	PE-Cy7			
	Red Diode (L2)	635	Red	APC*	Alexa Fluor® 647			
			Infra Red	APC-Cy7				

** APC and PE-Cy5 may be used together on instruments with cross-beam compensation.*

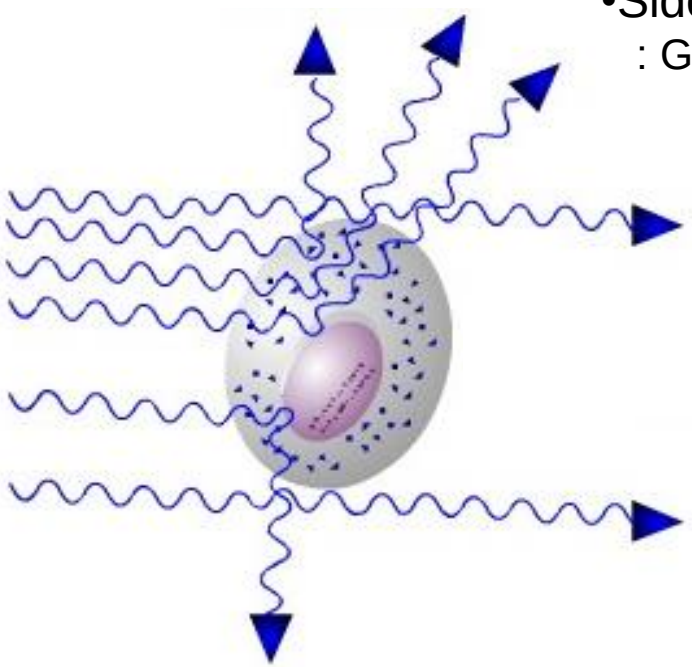
From Fluorescence to Computer Display

- Individual cell fluorescence quanta is picked up by the various detectors (PMT's).
- PMT's convert light into electrical pulses.
- These electrical signals are amplified and digitized using Analog to Digital Converters (ADC's).
- Each event is designated a channel number (based on the fluorescence intensity as originally detected by the PMT's) on a 1 Parameter Histogram or 2 Parameter Histogram.
- All events are individually correlated for all the parameters collected.

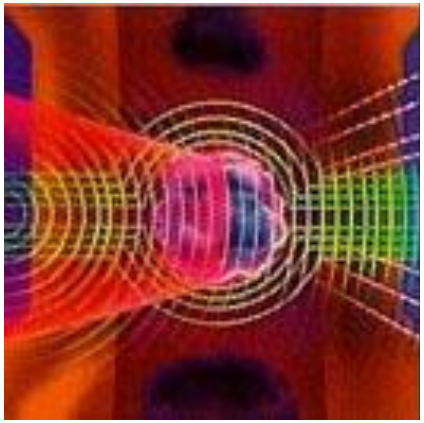
Properties of FSC and SSC

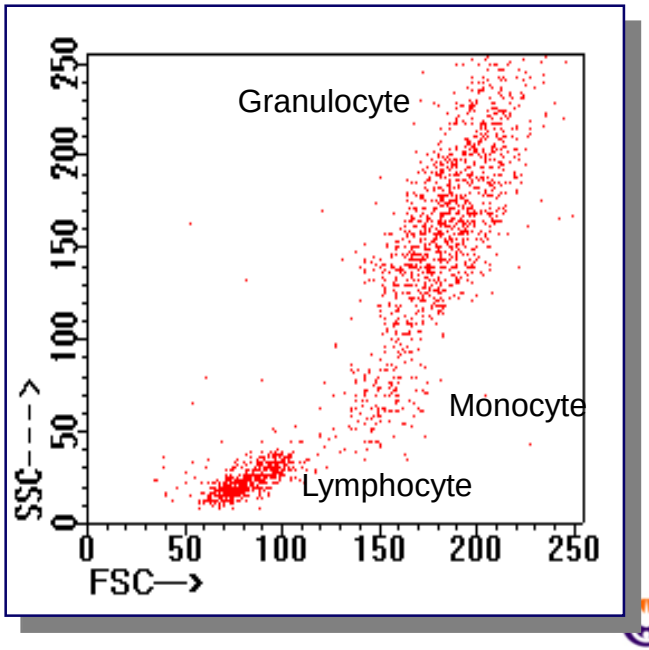
•Side Scatter (SSC)
: Granularity or Internal Complexity

LASER



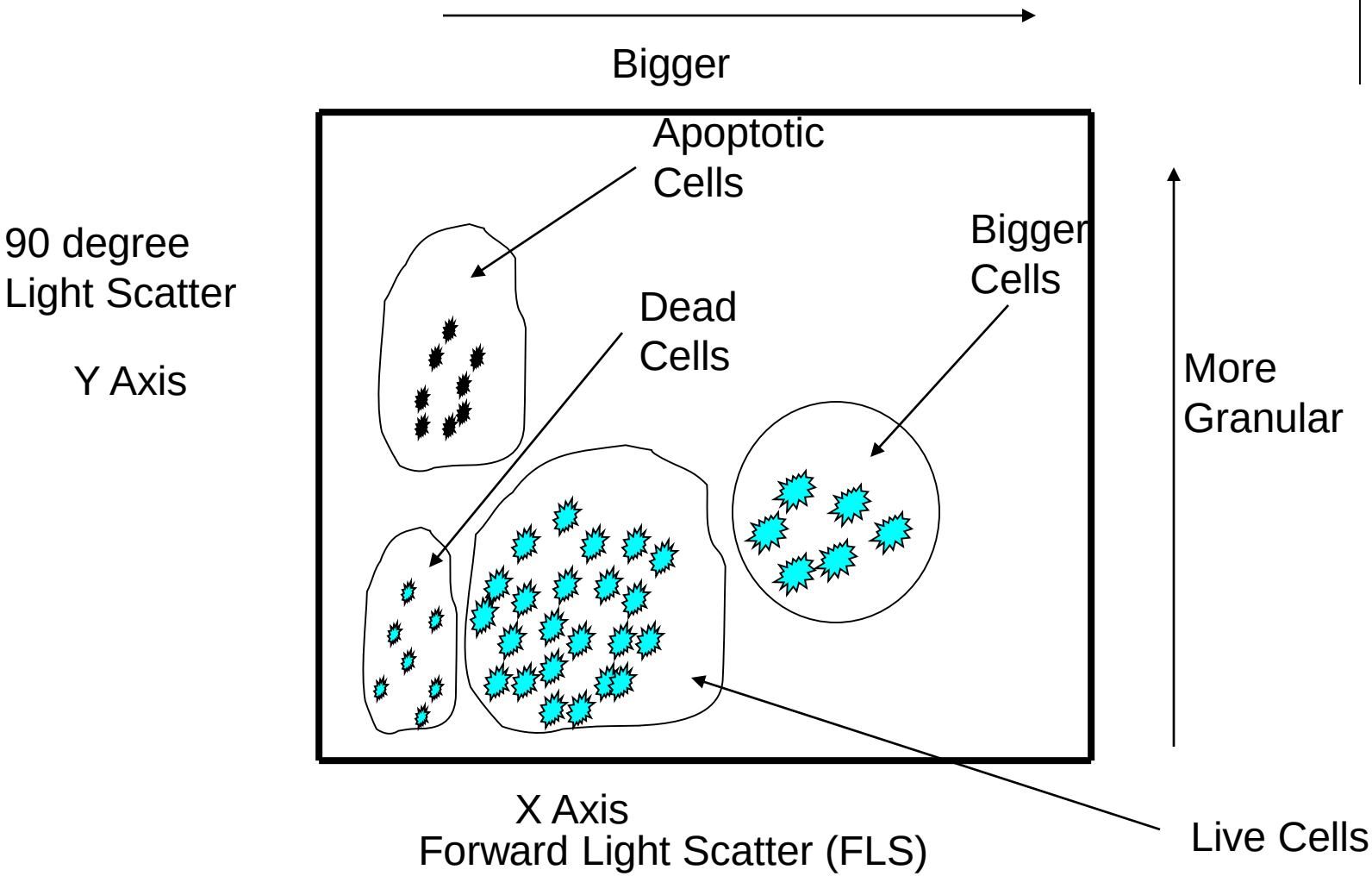
•Forward Scatter
(FSC) : Cell Size



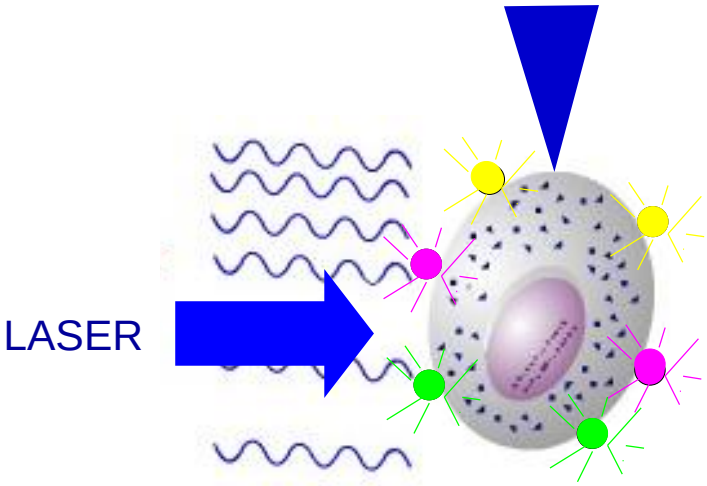


BD
BD Biosciences

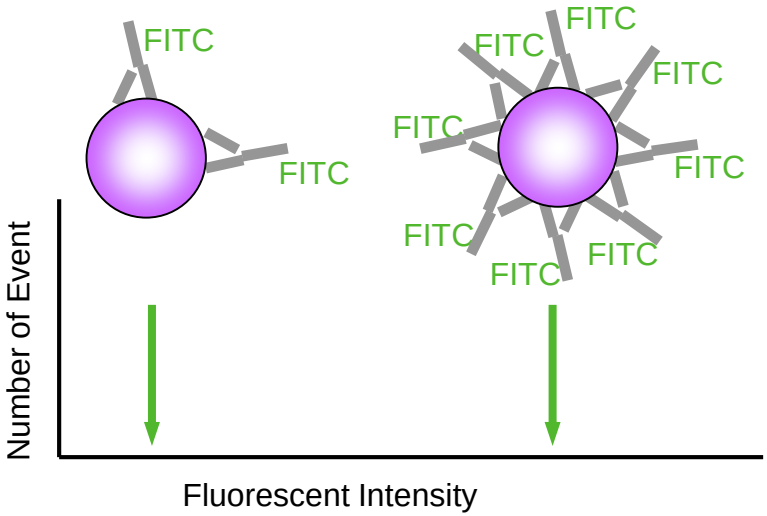
Light Scattering, 2 Parameter Histogram



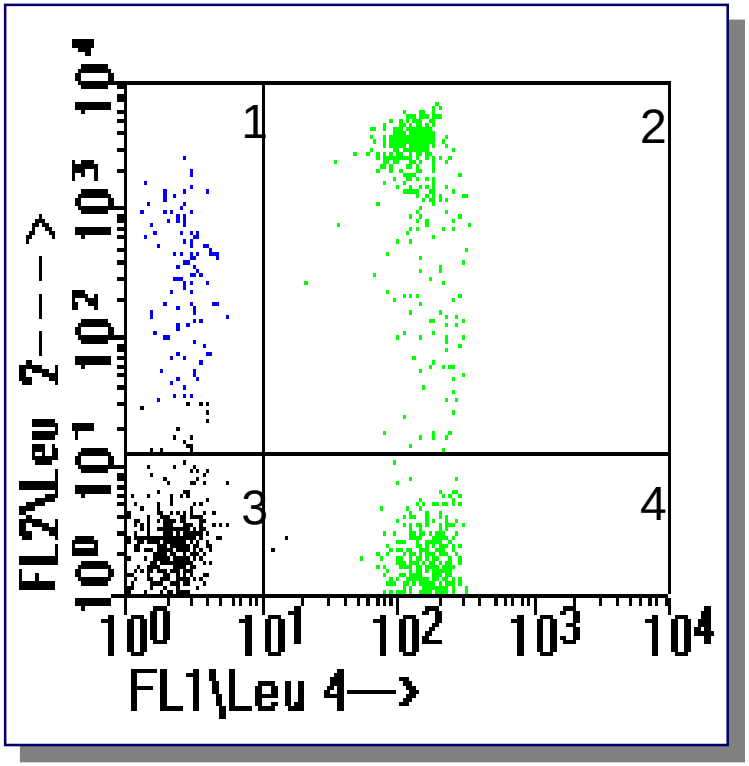
Fluorescence Signals



Emitted Fluorescence Intensity ∝ Binding Sites

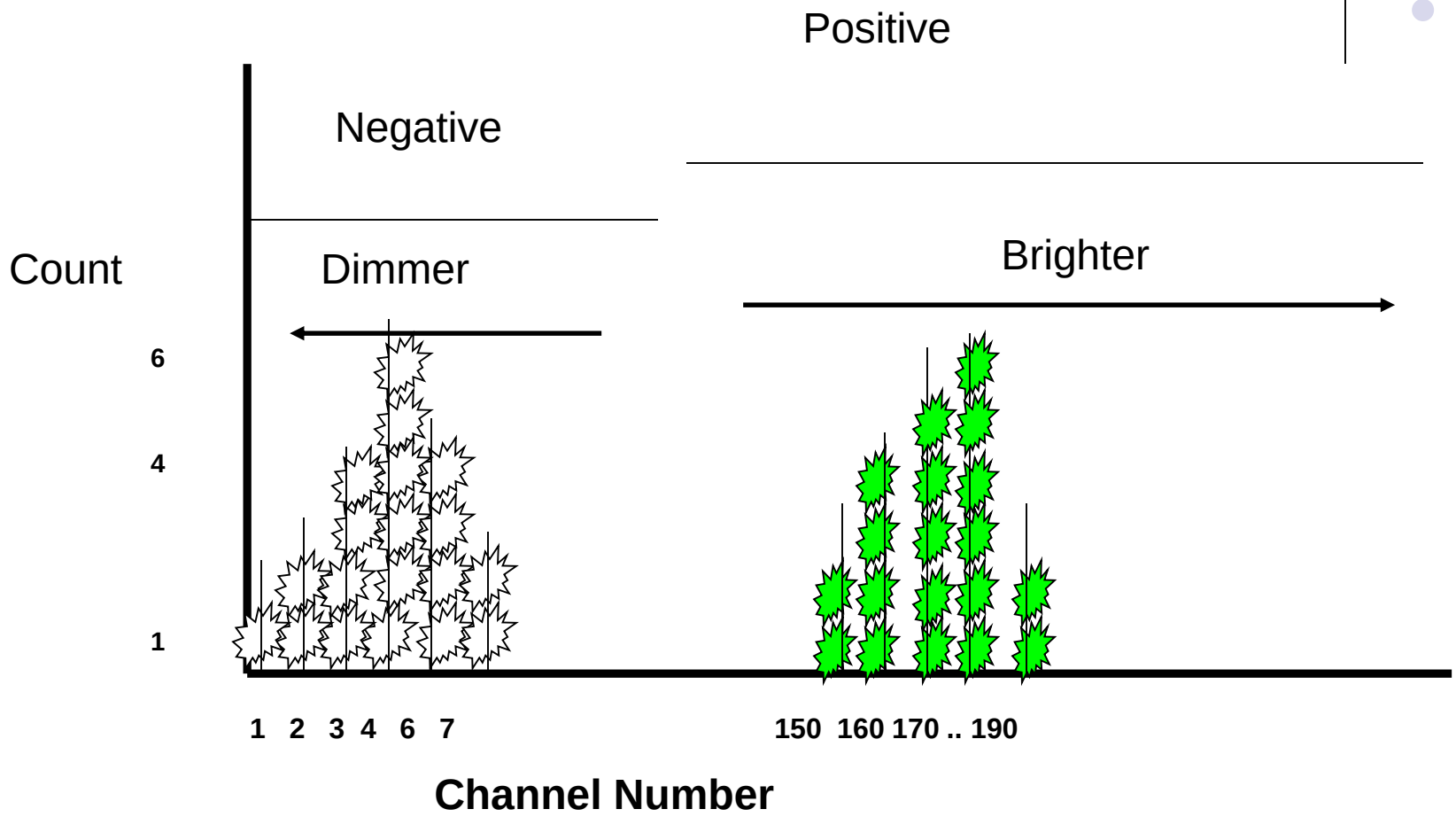


FL1/FL2



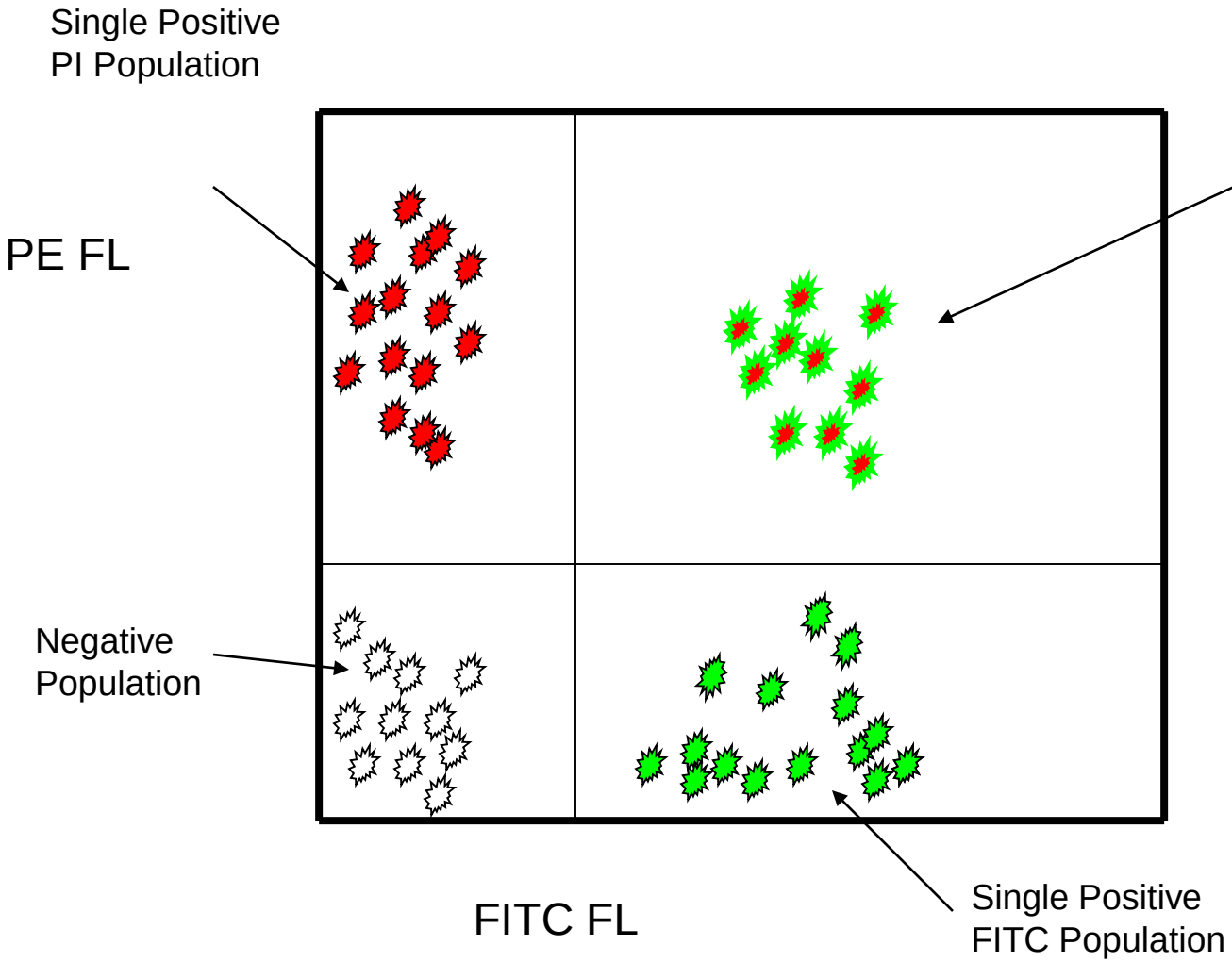
BD
BD Biosciences

1 Parameter Histogram



Fluorescence picked up from the FITC PMT

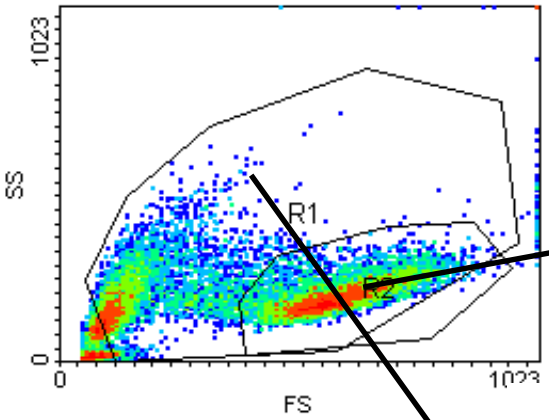
2 Parameter Histogram



Gating and Statistics

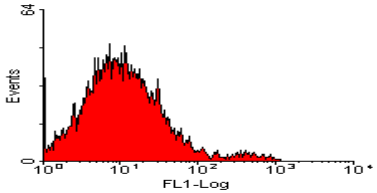
- Data generated in flow cytometry is displayed using **Multiparamater Acquisition and Display** software platforms.
- Histograms corresponding to each of the parameters of interest can be analyzed using statistical tools to calculate percentage of cells manifesting specific fluorescence, and fluorescence intensity.
- This information can be used to look at fluorescence expression within subpopulations of cells in a sample (gating).

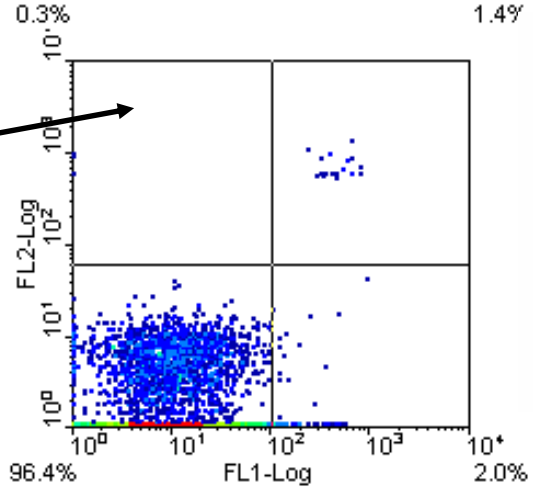
Flow Cytometry Data

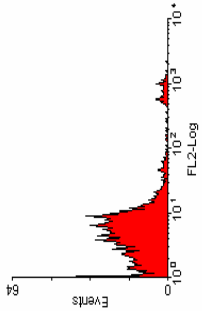


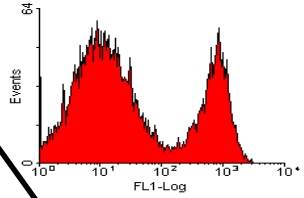
Smaller Region, Live cells mostly

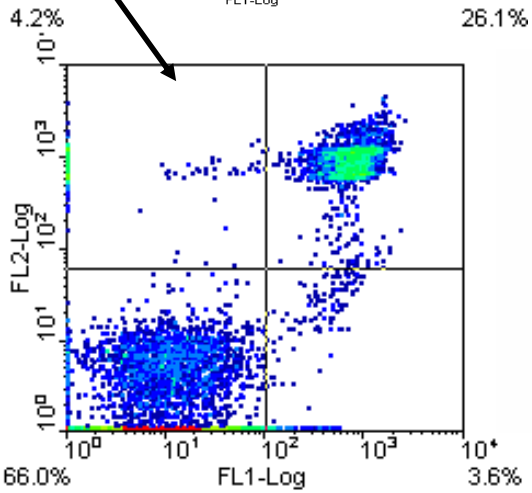
Larger Region includes all cells

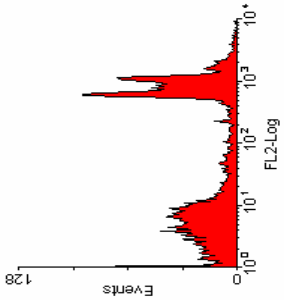












Monoclonal Antibodies

- Advances of FCM would not be possible without development of **monoclonal antibodies** (MAbs).
- **By the Nobel Prize winning hybridoma technology developed in 1975 by Köhler and Milstein**, lymphocytes from the spleen of an immunized mouse can be immortalized by fusion to myeloma cells that have lost the ability to make their own Immunoglobulins (Igs) but are capable of unlimited mitotic divisions.
- Through limited dilutions, individual cell lines (**hybridomas**) that produce an antibody of unique specificity, avidity, and isotype can be established.
- In the early days of the application of MAbs to immunology, many laboratories were immunizing mice with leukocytes.
- The obtained hybridomas produced many antibodies that reacted with leukocytes, but the identities of the molecular targets were not known.
- The reactivity spectrum of the antibody could be described by staining multiple different cell types, and in most cases the target antigen could be isolated by immunoprecipitation or Western blotting and its molecular weight and other structural characteristics determined.

Monoclonal Antibodies

- The first round of multilaboratory, blind, comparative analyses of antibodies was performed during the first Human Leukocyte Differentiation Antigen (HLDA) Workshop 1982 in Paris, France.

- Statistical analysis of data from several laboratories revealed "**clusters of differentiation (CD)**," named for the statistical procedure of cluster analysis and for the focus on leukocyte differentiation.

- Antibodies thought to be detecting the same molecule, and the molecule itself, were given a "CD" designation.

Monoclonal Antibodies

- An organization called the Human Leukocyte Differentiation Antigen Council has been established and nine subsequent HLDA workshops have characterized 350 CD antigens.
- The HLDA council reviewed and modified the objectives of HLDA in 2004, and changed the name of the organization to **Human Cell Differentiation Molecules** (HCDM).
- The reasoning behind the name change to HCDM was to break with tradition while retaining the letters “CD,” to maintain emphasis on molecules of human origin, to extend focus from leukocytes to other cell types interacting with leukocytes such as endothelial cell or stromal cell molecules, and to broaden the scope from cell-surface molecules to any molecule whose expression reflects differentiation, recognizing the growing values of intracellular molecules.
- The HCDM council keeps a comprehensive database of CD molecules (www.hcdm.org).

LIST OF CD ANTIGENS MOST COMMONLY USED IN FLOW CYTOMETRY IMMUNOPHENOTYPING OF HEMATOLOGIC SAMPLES

CD	Expression in Normal Hematopoietic Cell Types	MW (kD)	Function
CD1a	Cortical thymocytes, Langerhans cells, dendritic cells	49	Antigen presentation, w/β2m
CD2	Thymocytes, T-cells, NK cells	50	CD58 ligand, adhesion, T-cell activation
CD3	T-cells, thymocyte subset		w/TCR, TCR surface expression/signal transduction
CD4	Thymocyte subset, T-cell subset, monocytes, macrophages	55	MHC class II coreceptor, HIV receptor, T-cell differentiation/activation
CD5	Thymocytes, T-cells, B-cell subset	67	CD72 receptor, TCR or BCR signaling, T–B interaction
CD7	Thymocytes, T-cells, NK cells, small subset of hematopoietic progenitors	40	T costimulation
CD8	Thymocyte subset, T-cell subset, NK subset	32–34	MHC class I coreceptor, receptor for some mutated HIV-1, T-cell differentiation/activation
CD9	Eosinophils, basophils, platelets, activated T-cells	22–27	Cellular adhesion and migration
CD10	B-precursors, germinal center B-cells, thymocyte subset, neutrophils	100	Zinc-binding metalloproteinase, B-cell development
CD11a	Lymphocyte subsets, granulocytes, monocytes, macrophages	180	CD11a/CD18 receptor for ICAM-1, -2,-3, intercellular adhesion, T costimulation
CD11b	Granulopoietic cells, NK cells	170	Binds CD54, ECM, and iC3b
CD11c	Dendritic cells, granulopoietic cells, NK cells, and B-cell and T-cell subsets	150	Binds CD54, fibrinogen, and iC3b
CD13	Granulopoietic cells, monocytes	150–170	Zinc-binding metalloproteinase, antigen processing, receptor for corona virus strains
CD14	Monocytes, macrophages, Langerhans cells	53–55	Receptor for LPS/LBP, LPS recognition
CD15	Neutrophils, eosinophils, monocytes		Adhesion
CD16	Neutrophils, macrophages, NK cells	50–65	Component of low-affinity Fc receptor, phagocytosis, and ADCC
CD19	B-cells, plasma cells	95	Complex w/CD21and CD81, BCR coreceptor, B-cell activation/differentiation
CD20	B-cells	33–37	B-cell activation
CD21	B-cells and T-cells subsets	145, 110	Complement C3d and EBV receptor, complex w/CD19 and CD81, BCR coreceptor
CD22	B-cells	150	Adhesion, B-mono, B–T interactions
CD23	B-cells, eosinophils, platelets	45	CD19-CD21-CD81 receptor, IgE low-affinity receptor, signal transduction
CD24	Thymocytes, erythrocytes, lymphocytes, myeloid cells	35–45	Binds P-selectin
CD25	Activated B-cells and T-cells	55	IL-2Rα, w/IL-2Rβ, and γ to form high affinity complex
CD33	Granulopoietic cells, monocytes, dendritic cells	67	Adhesion
CD34	Hematopoietic precursors	105–120	Stem cell marker, adhesion, CD62L receptor
CD36	Platelets, monocytes, erythropoietic precursors	88	ECM receptor, adhesion, phagocytosis
CD38	High expression on B-cell precursors, plasma cells and activated T-cells, low on granulopoietic cells	45	Ecto-ADP-ribosyl cyclase, cell activation
CD41	Platelets, megakaryocytes	125/22	w/CD61 forms GPIIb, binds fibrinogen, fibronectin, vWF, thrombospondin, platelet activation and aggregation
CD42a	Platelets, megakaryocytes	22	Complex w/CD42b, c and d, receptor for vWF and thrombin, platelet adhesion to subendothelial matrices
CD45	Hematopoietic cells, multiple isoforms from alternative splicing	180–240	Tyrosine phosphatase, enhanced TCR and BCR signals
CD56	NK subset, T-cell subset	CD175–185	Neural cell adhesion molecule
CD57	NK subset, T-cell subset	110	HNK-1
CD59	Ubiquitous	18–20	Complement regulatory protein
CD61	Platelets, megakaryocytes	105	Integrin β3, adhesion, CD41/CD61 or CD51/CD61 mediate adhesion to ECM
CD62L	B-cells, T-cells subsets, monocytes, granulocytes, NK-cells, thymocytes	74, 95	CD34, GlyCAM, and MAdCAM-1 receptor, leukocyte homing, tethering, rolling
CD64	Monocytes, neutrophils	72	FCγRI, increases on neutrophils in sepsis
CD65	Granulopoietic cells		Phagocytosis
CD66	Neutrophils	90	Cell adhesion
CD68	Monocytes, neutrophils, basophils, mast cells,	110	Macrosialin

CD	Expression in Normal Hematopoietic Cell Types	MW (kD)	Function
CD71	Proliferating cells, erythroid precursors, reticulocytes	95	Transferrin receptor, iron uptake
CD79	B-cells, plasma cells	33–37	Component of BCR, BCR surface expression and signal transduction
CD103	B- and T-cell subsets	150, 25	w/integrin $\beta 7$, binds E-cadherin, lymph homing/retention
CD117	Hematopoietic progenitors, mast cells	145	Stem cell factor receptor, hematopoietic progenitor development/differentiation
CD123	Basophils, dendritic cell subset, hematopoietic progenitors	70	IL-3Rα, w/CDw131
CD133	Hematopoietic stem cells subset	120	
CD159c	NK	40	w/MHC class I HLA-E molecules, forms heterodimer with CD94
CD235a	Erythropoietic precursors	36	Glycophorin A

Sample Preparation

- Appropriate samples for clinical FCM include
  - peripheral blood (PB),
  - bone marrow (BM) aspirate,
  - disaggregated tissue including lymph node (LN) and other soft tissue biopsies
  - fine needle aspirations (FNA)
  - BM core biopsies,
  - cerebrospinal fluid (CSF),
  - other body fluids including effusions and lavage fluids, and
  - nuclei from paraffin-embedded tissue for DNA ploidy assays.

- With the exception of the latter, all other clinical FCM specimens should be considered biohazardous and labeled as such in accordance with national or regional safety standards.

Sample Preparation

- A test requisition form, whether printed or electronic, should accompany all specimens.
- This form should include unique patient identifiers, age, sex, diagnosis (if previously established) or suspect condition under consideration, name of the physician submitting the specimen, pertinent medication or recent treatment (including dates of chemotherapy or radiation), date and time of specimen collection, and source of the specimen (e.g., bone marrow aspirate, CSF, etc.).
- The requested test should appear on the specimen label or on the requisition accompanying the specimen.
- Complete blood count (CBC) should be provided for PB and BM samples.

Sample Preparation

- For PB, ethylene-diaminetetraacetic acid (EDTA), sodium heparin, or acid citrate dextrose (ACD) may be used.
- For BM aspirates, sodium heparin is the preferred anticoagulant, and is required if cytogenetic testing is to be performed on the same specimen.
- All tissue biopsies intended for FCM evaluation, including LN or other tissue biopsies should be transported in an adequate volume of an appropriate transport medium in a sterile container to optimize cell viability.
- CSF samples should be stabilized or analyzed immediately due to potential toxic effect on cell viability.

Sample Preparation

- All clinical samples should be analyzed as soon as possible.
  - As a general rule, 24 hours is preferred but 48 hours is considered the longest acceptable time frame for analysis.
  - If transport time is longer, a viability report is mandatory and the results should be interpreted cautiously.

- Room temperature (18°C to 25°C) is recommended for storage and transport.

Sample Preparation

- For specimens that are not highly degenerated, nonviable cells can be excluded from the analysis by meticulous FS versus SS gating.
  - Dead cells trap fluorochrome-conjugated antibodies and increase background fluorescence.
  - Fluorescent, DNA-binding dyes that are excluded from viable cells with intact plasma membranes and thus positive in nonviable cells, can also be applied.

Sample Preparation

- Whole PB/BM analysis **with erythrocyte lysis** is recommended for clinical immunophenotyping.
- Immunophenotyping of density gradient (Ficoll) separated mononuclear cells should not be used due to selective cell loss.
- For surface(s) staining, **the so-called "stain–lyse–wash"** method gives the best signal discrimination.
- Cells are first incubated with appropriate amounts of titrated MAb, then erythrocytes are lysed and cells finally are washed before acquisition.
- Several commercial lysis reagents, most of which also contain a fixative, are available.
- Samples to be **stained for sIg** should be thoroughly washed before incubation with MAb, in order to avoid false negative results due to the presence of serum Igs.

Sample Preparation
(cells and staining)

1. Whole blood
2. By density gradient: Ficoll-Hypaque, Percoll *etc*
3. By Ab-coated magnetic beads
4. By FACS (fluorescence-activated cell sorter
5. By Ab-based methods other than magnetic beads & FACS
6. *etc*

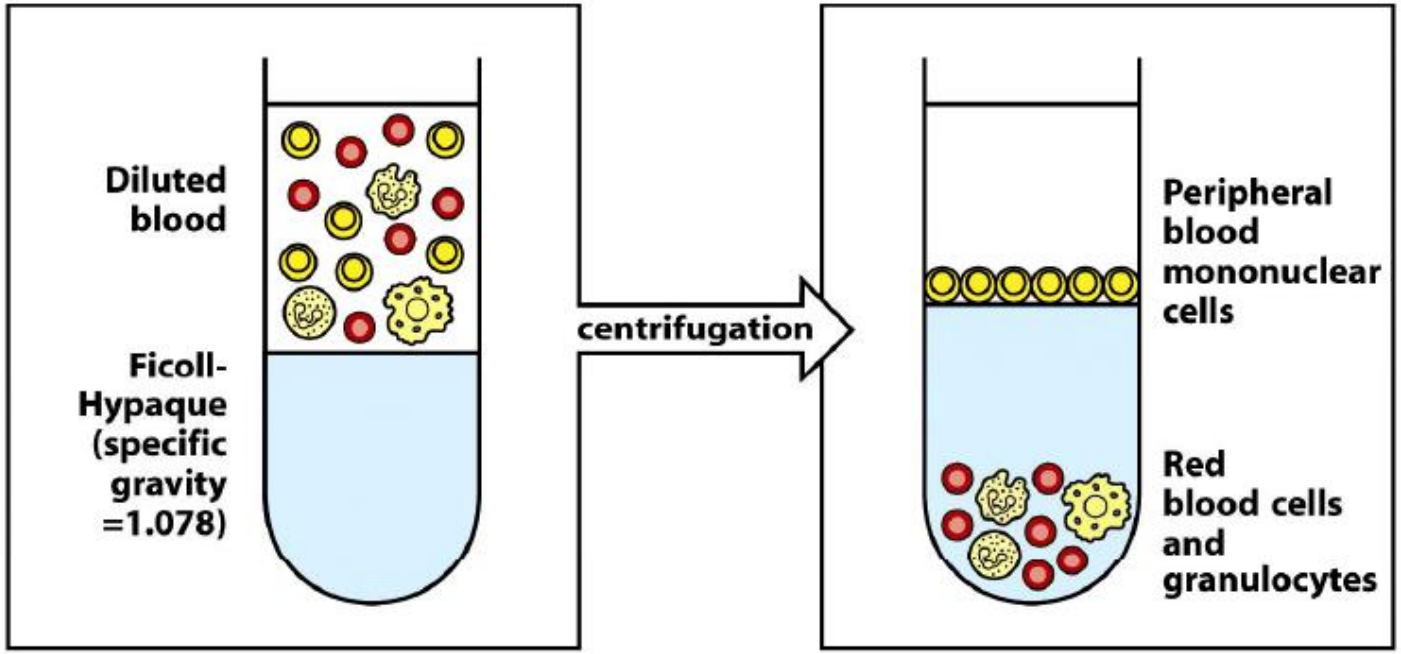
adapted from KAIST lecture

Isolation of lymphocytes by Ficoll-Hypaque

Principle; density gradient centrifugation

Ficoll; carbohydrate polymer, specific gravity=1.078

Usage; to isolate lymphocytes and monocytes from peripheral blood (human/mouse)



Adapted from KAIST (Shin, EC)

Ab-coated Magnetic Beads

Positive selection

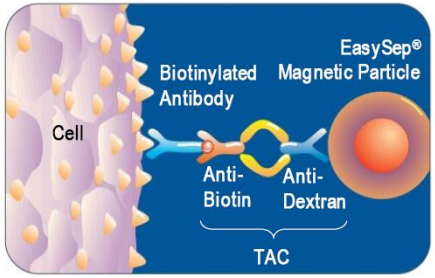
excellent purity (rare cell enrichment) and recovery

negative selection

removal of unwanted cells

if no specific Ab is available for target cells

if binding of the Abs to the target cells is not desired (activation, suppression)



TAC; bispecific tetrameric Ab complex

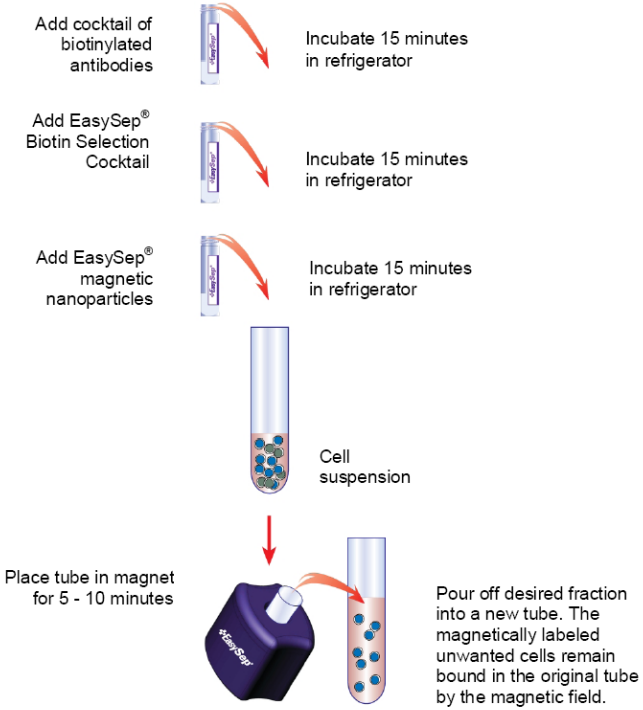
Commercial available sources for magnetic beads

Stemcell technologies, Miltnyi Biotec (MACS),

Dynal, Proimmune *etc*

Note for positive selection;

1. MACS magnetic beads are biodegradable and typically disappear after a few days in culture.
2. Because EasySep magnetic particles (~150 nm) are tiny, they do not interfere with downstream application.
3. In case of Dynal superparamagnetic beads (2.8 um), there is a step for separating magnetic beads.



Sample Preparation

- Evaluation of **intracellular** epitopes, including proteins, epigenetic protein modifications (e.g., protein phosphorylation, methylation, etc.), DNA, or RNA generally require that the target cell be **fixed and permeabilized** in order to allow MoAbs or target-binding dyes to cross the cytoplasmic and nuclear membranes.
  - Commercial fixation and permeabilization kits, with recommended protocols, are available from several manufacturers.
  - For newly developed tests, it is useful to check whether the obtained intracellular staining is associated with an expected localization, using fluorescence microscopy.
  - The specificity of the applied antibody should also be ensured.
- For cytoplasmic (cyt.) or nuclear (n) staining, it is important to use antibody conjugates that are free of unconjugated fluorochrome molecules that can stick to intracellular proteins nonspecifically.
- When simultaneous detection of surface and intracellular epitopes is necessary, the **surface staining is performed first**, then cells are **fixed and permeabilized**, and **finally intracellular epitopes** are stained.

Fluorochromes and Panels

- **Panel selection** should be based on specimen type with consideration of information provided by clinical history, medical indication, and morphology.
- Several guidelines and consensus papers giving lists of antigens proposed for diagnosis of hematologic malignancies have been published.
- Selecting which **antibody combinations** best delineate, distinguish, and measure key differences within the target populations of interest and the number of simultaneously measured antibodies is a critical step for FCM assays.
  - Serial dilution antibody titrations against both positive and negative cellular targets are necessary for antibody optimization.
- Choice of fluorochrome conjugate can affect background, specificity, and dynamic range of measurement.

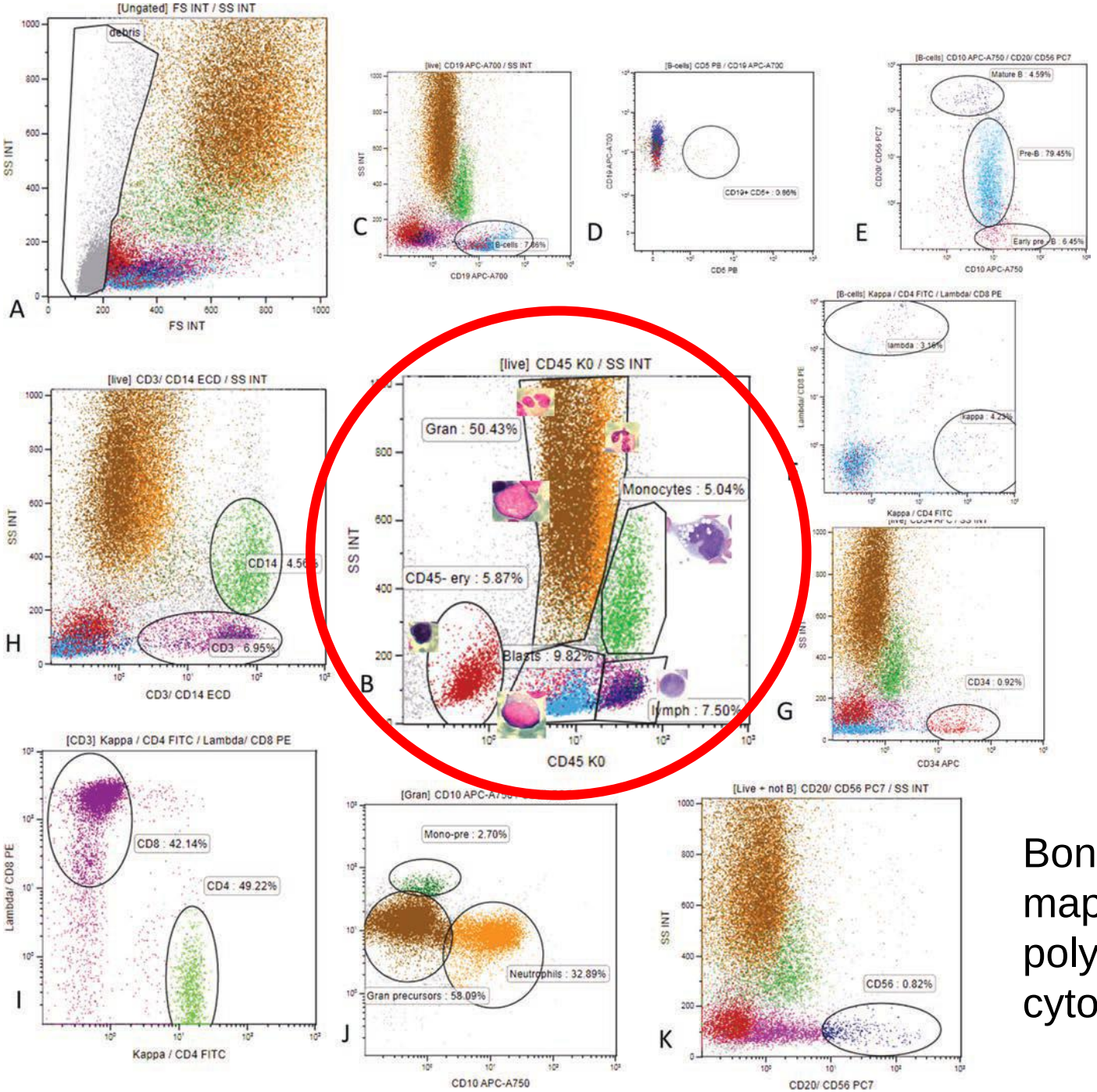
Fluorochromes and Panels

- Typically, one would choose a fluorochrome with **the best quantum efficiency/yield** as the antibody conjugate to identify the lowest antigen density so as to obtain the best possible signal-to-noise ratio possible.
- It is of high importance to reliably distinguish between antigen-positive and antigen-negative cell populations in order to accurately measure the population of positive cells.
- This can be a challenge in populations of cells weakly expressing antigens.
- Florescence-minus-one (FMO) controls give the maximum fluorescence expected for a given population in a given channel when the reagent used in that channel is omitted.
- These controls include both **autofluorescence** of the cells and the **spillover** that may be present even after compensation corrections and therefore such controls are best suited to determine boundaries between positive and negative cells for each subset.

Fluorochromes and Panels

- Often the **same anchor gating** antibodies are used in every tube thereby allowing consistent population gating strategies across all tubes of a panel.

- In immunophenotyping of lymphocyte subsets and in the diagnosis of leukemia/lymphoma, **CD45 anchor gating** has been shown to provide differential population identification correlated to morphologic microscopic differentials:
  - Mature lymphocytes are characterized by low side scatter and strong CD45 expression.
  - Monocytes have higher SS and strong CD45 expression.
  - Erythropoietic precursors are CD45 negative and have low SS.
  - Granulopoietic precursors and granulocytes are weakly CD45 positive and have high SS.
  - Early hematopoietic precursors of various lineages, including CD34+ stem cells, are characterized by low CD45 expression and low SS.



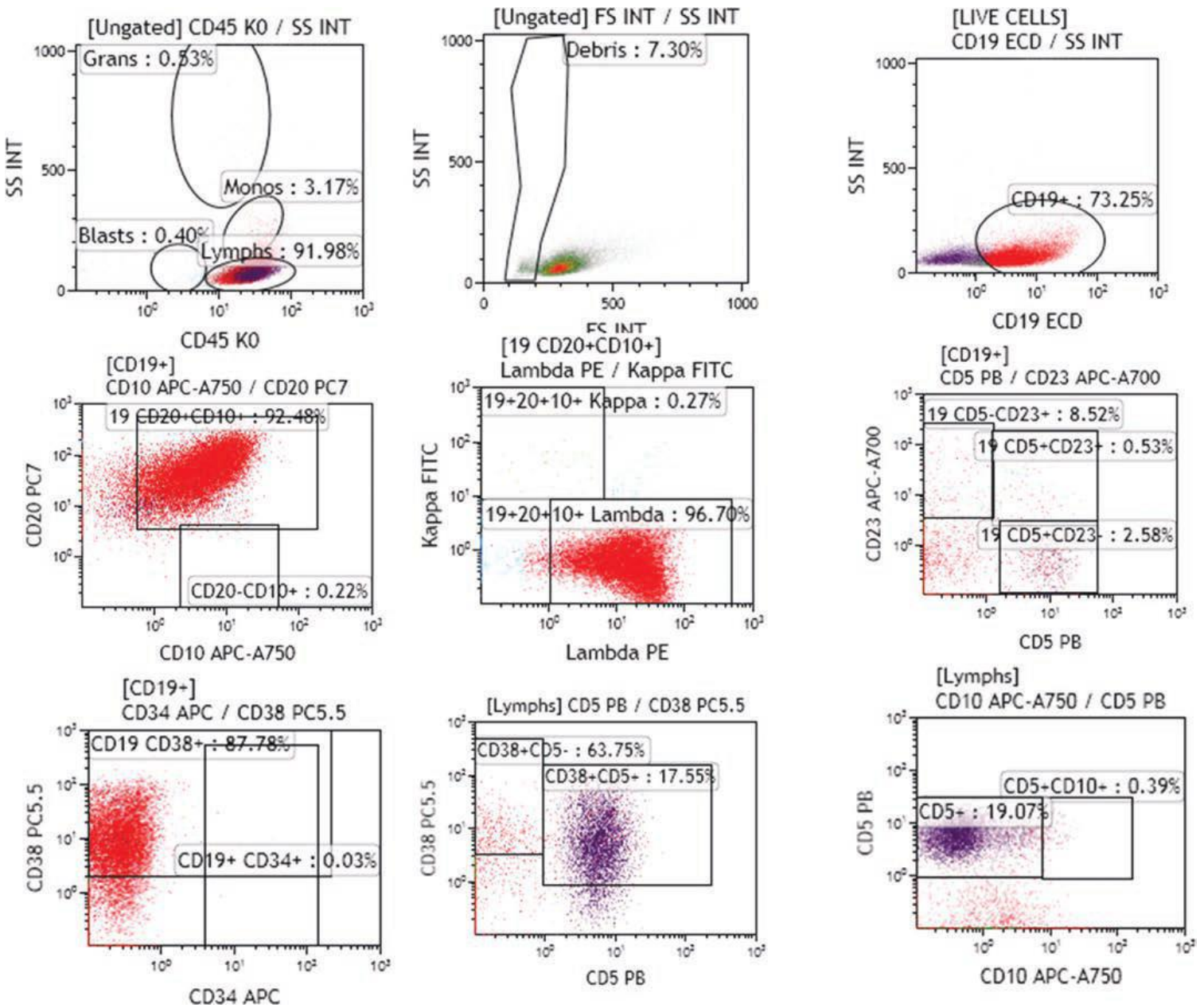
Bone marrow mapping with polychromatic flow cytometry.

Fluorochromes and Panels

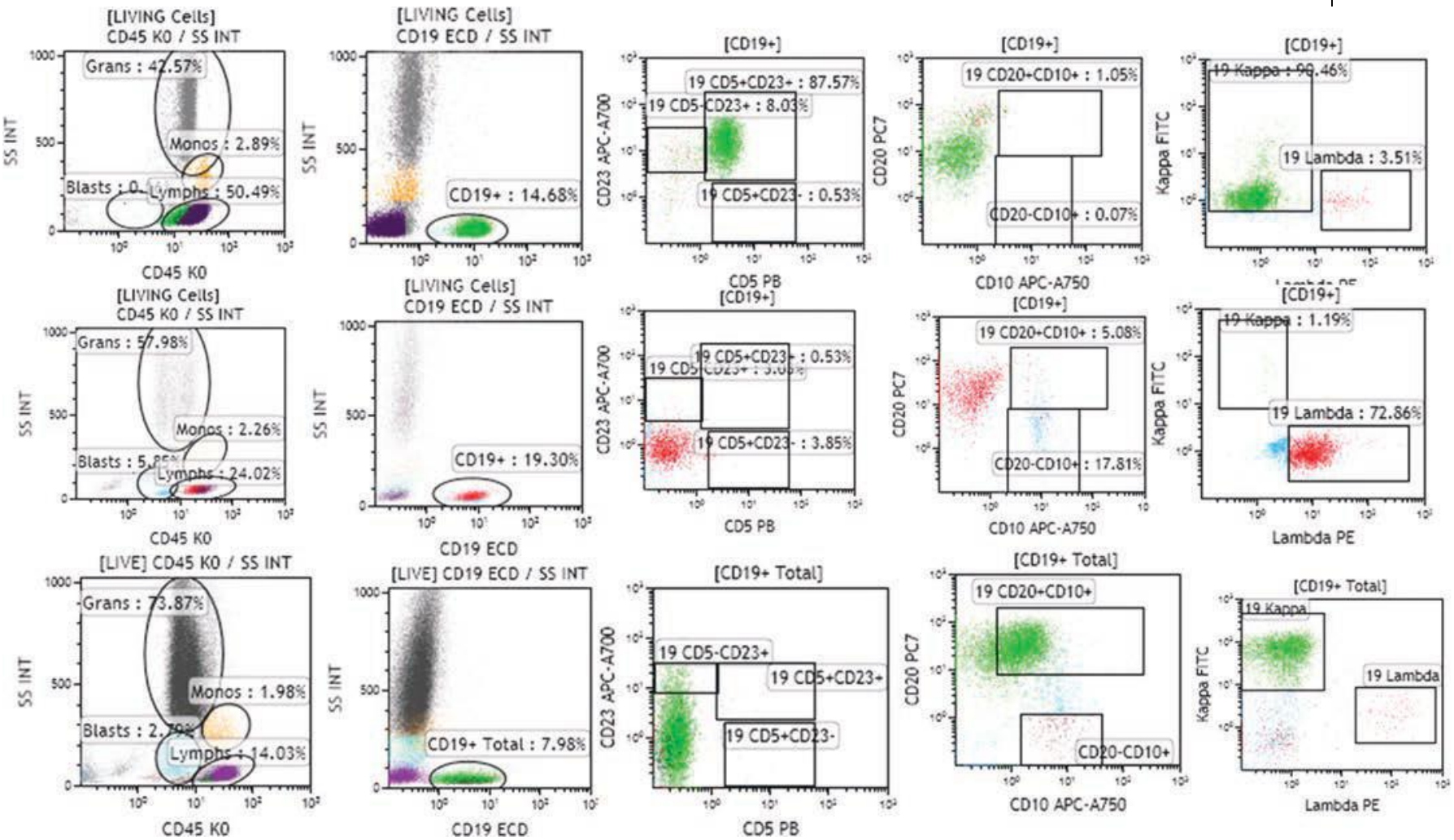
- The localization of these subpopulations on the CD45/SS plot can be confirmed by multicolor staining of various lineage-associated antigens together with CD45 (plots C–K) and visualization of cell clusters positive for given antigen combinations on the CD45/SS plot B) by so-called back-gating using color-coding.

- In multicolor FCM, lineage-associated antigens that are broadly expressed through maturation of investigated cell lineage can be used for gating in conjunction with SS and CD45 (e.g., CD19 for B-cells, CD3 for T-cells).

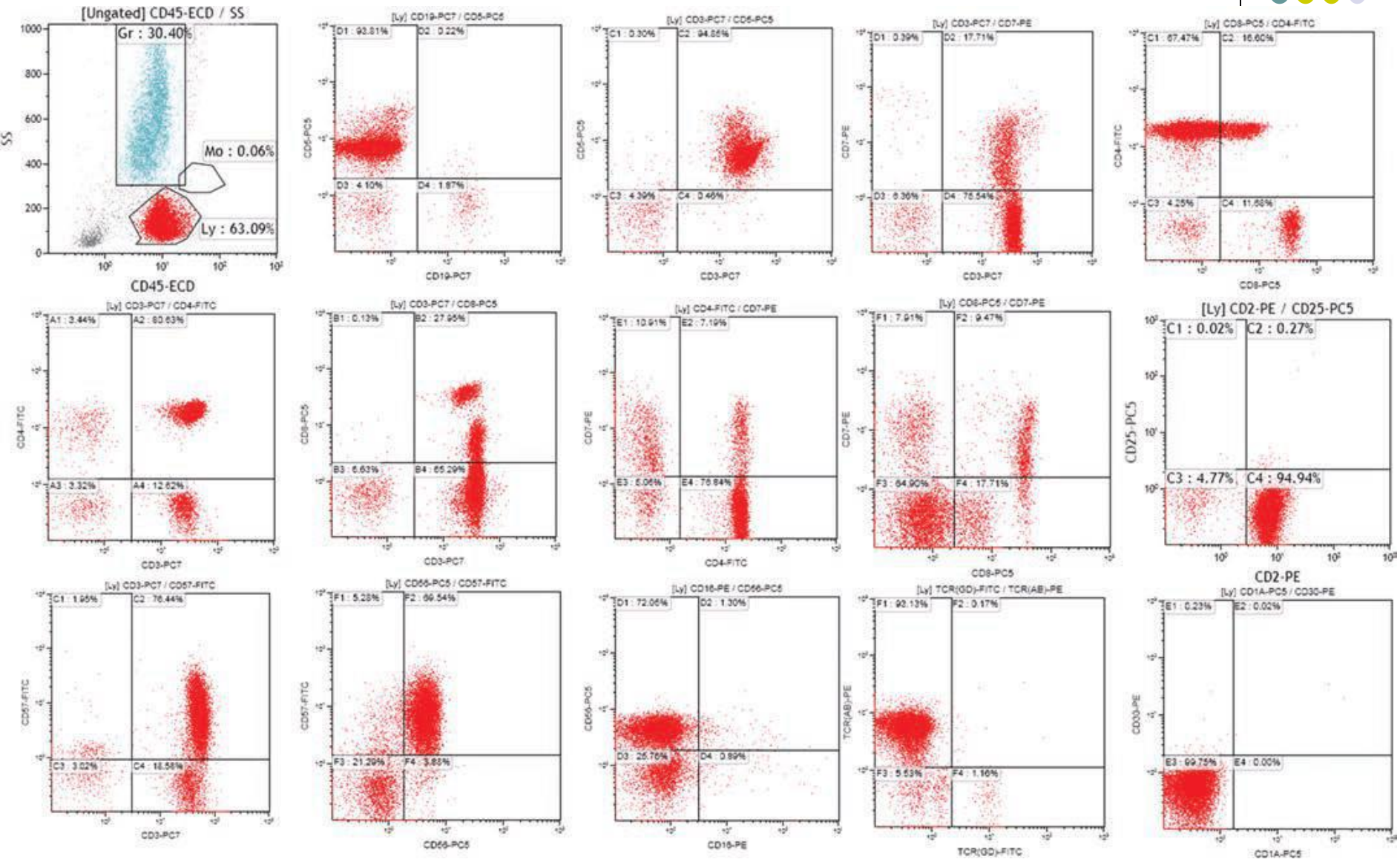
Examples of analysis of B-cell compartment in bone marrow samples.



Examples of analysis of B-cell compartment in bone marrow samples



Example of aberrant T-cell population detected in peripheral blood of a patient with lymphocytosis



Flow cytometry for the diagnosis and monitoring of hematologic neoplasms 1/4

- FC evaluates individual cells in suspension for the presence and absence of specific antigens (phenotype).

- Several steps are taken in the application and interpretation of the immunophenotype:

  1. identification of cells from different lineages and determination of whether they are mature or immature;

  2. detection of abnormal cells through identification of antigen expression that differs significantly from normal;

Blood. 2008; 111:3941-3967

Flow cytometry for the diagnosis and monitoring of hematologic neoplasms 2/4

3. **detailed documentation of**

- **the phenotype of abnormal cell populations (ie, the presence or absence of antigens)**
- **increased or decreased intensity of staining by fluorochrome labeled antibodies, in comparison to their normal cell counterpart**

Blood. 2008; 111:3941-3967

Flow cytometry for the diagnosis and monitoring of hematologic neoplasms 3/4

4. **evaluation of whether the information available is diagnostic of a distinct disease entity and,**

- **if not, development of a list of possible entities with suggestion of additional studies that might be of diagnostic value such as**
  - **immunohistochemistry,**
  - **conventional cytogenetic,**
  - **FISH,**
  - **molecular diagnostic studies;**

Blood. 2008; 111:3941-3967

Flow cytometry for the diagnosis and monitoring of hematologic neoplasms 4/4

5. **provision of immunophenotypic information that**
   - **might be of additional prognostic value,**
   - **include the identification of targets for potential directed therapy.**

Blood. 2008; 111:3941-3967

Flow Cytometry testing

- **When a specimen is received for FC testing, a decision is made regarding the cell lineages and antigens to be evaluated that is based on the type of specimen and other available information, such as**

1. **the medical indication for testing listed on the requisition,**
2. **clinical history,**
3. **morphologic findings,**
4. **history of prior flow cytometric testing,**
5. **results of other laboratory testing, and**
6. **possibly results of any preliminary screening testing performed in the flow cytometric laboratory.**

Blood. 2008; 111:3941-3967

Flow cytometry testing

- For the medical indications identified by the 2006 Bethesda group, consensus was reached on the cell lineages that should be evaluated and the antigens to include in a primary evaluation of each lineage.

- In addition, general recommendations were made on the approach used to evaluate these antigens by flow cytometry.

- Using this approach, flow cytometric immunophenotyping of clinical specimens can provide a rapid screen for hematologic neoplasms and play a key role in diagnosis and classification.

Blood. 2008; 111:3941-3967

Cell Lineages to be Evaluated for Each Medical Indication

Medical indication	Lineage to be evaluated
Anemia	B, T, M, P
Leukopenia	B, T, M, P
Thrombocytopenia	B, T, M, P
Pancytopenia	B, T, M, P
Neutrophilia	M (limited)
Monocytosis	M
Lymphocytosis	B, T
Eosinophilia	T, M
Erythrocytosis	M (limited)
Thrombocytosis	M (limited)
Blasts in blood or marrow	B, T, M
Lymphadenopathy	B, T
Extranodal masses	B, T
Splenomegaly	B, T, M (limited)
Transformation of chronic leukemia—B cell	B
Transformation of chronic leukemia—T or NK cell	T
Staging for non-Hodgkin lymphoma—B cell	B
Staging for non-Hodgkin lymphoma—T/NK cell	T
Skin rash	B, T
Atypical cells in body fluids (CSF, serous, ocular, etc.)	B, T, M (limited)
Monoclonal gammopathy	B, P
Unexplained Plasmacytosis of bone marrow	B, P
Monitoring of Rx response (unknown diagnostic immunophenotype)	
Mature B cell neoplasm	B
Mature T or NK cell neoplasm	T
Acute lymphoid leukemia—B cell	B
Acute lymphoid leukemia—T cell	T
Acute myeloid leukemia	M
MDS/MPD/Overlap Syndrome	M
Plasma cell neoplasm	P

B, B cell; T, T cell; M, myeloid; P, plasma cell.

Cytometry Part B (Clinical Cytometry) 72B:S14–S22 (2007)

Consensus reagents

Table 3
Consensus Reagents for Initial Evaluation for Hematopoietic Neoplasia

Lineage	Primary reagents
B cells	CD5, CD10, CD19, CD20, CD45, Kappa, Lambda
T cells and NK cells	CD2, CD3, CD4, CD5, CD7, CD8, CD45, CD56
Myelomonocytic cells	CD7, CD11b, CD13, CD14, CD15, CD16, CD33, CD34, CD45, CD56, CD117, HLA-DR
Myelomonocytic cells (limited)	CD13, CD33, CD34, CD45
Plasma cells	CD19, CD38, CD45, CD56

Cytometry Part B (Clinical Cytometry) 72B:S14–S22 (2007)

EXAMPLES OF 10-COLOR FLOW CYTOMETRY PANELS[a] IN IMMUNOPHENOTYPING OF LEUKEMIA AND LYMPHOMA

Panel	FITC[b]	PE	ECD	PC5.5	PC7	APC	APC-AF700	APC-AF750	PB	KO
B-cell	kappa	lambda	CD19	CD38	CD20	CD34	CD23	CD10	CD5	CD45
T-cell	CD57	CD11c	CD8	CD3	CD2	CD56	CD7	CD4	CD5	CD45
AML-granulo	CD65	CD13	CD14	CD33	CD34	CD117	CD7	CD11b	CD16	CD45
AML-mono	CD36	CD64	CD56	CD33	CD34	CD123	CD19	CD38	HLA-DR	CD45
AML-ery-ly	CD71	CD11c	CD4	CD33	CD34	CD2	CD10	CD235a	CD15	CD45
ALL-B	CD58	CD22	CD38	CD33	CD34	CD123	CD10	CD19	CD20	CD45
ALL-T	CD7	CD1a	CD8	CD33	CD34	CD2	CD10	CD4	CD5	CD45
AL-cytoplasmic	TdT	MPO	CD14	CD33	CD34	cytCD79	cytCD22	CD19	cytCD3	CD45

[a]These panels are in current clinical use at the Flow Cytometry Lab., Department of Laboratory Medicine, University Health Network, Toronto General Hospital, Toronto, Ontario, Canada.
[b]Characteristics of fluorochromes are given in Table 2.1.

Data Analysis and Reporting

- Fluorescence data may be presented using either **linear** or **logarithmic** amplification.
- In linear amplification, fluorescence differences are directly proportional to differences of fluorochrome concentration between cells.
- Logarithmic amplification compresses a wide input range, which may cause difficulties in resolving populations with similar fluorescence intensities.
- “Logicle” (or “biexponential”) displays have recently been designed for the display of FCM data so that they incorporate the useful features of logarithmic displays but also provide accurate visualization of populations with low or background fluorescence.

Data Analysis and Reporting

- During analysis, data is presented in form of:
  - **Histograms (for one parameter),**
    - where relative fluorescence or scatter is on the x-axis and the number of events with given characteristics on the y-axis
  - **Two-parameter dot plots**,
    - where each signal is visualized by one dot and given a parameter on the x- and y-axes; various cell populations can be then “painted” with different colors
  - **Density plots,**
    - where hotspots indicate large numbers of events resulting from discreet population of cells and colors can give the graph a three-dimensional feel
  - **Contour diagrams**,
    - where joined lines represent similar numbers of cells

Data Analysis and Reporting

- Analysis is usually focused on identifying and quantifying subsets of cells.
- Successful analysis will depend on correct marker selection and panel design. Cell counts and percentages are typically reported.
- The choice of gating strategy depends on the panel used and specific populations of interest.

Data Analysis and Reporting

- In immunophenotyping of PB and BM, the analysis can be focused on lymphocytes (CD45 bright gate), B-lymphocytes, blasts (CD45 dim gate), T-lymphocytes and natural killer (NK) cells, on monocytes, or include all living cells in the sample (debris excluded).

- In tissue samples (lymph nodes, FNA, body fluids) a broad lymphocyte gate is usually applied.

- The parent population should be clearly identified when percentages are reported: a fraction may represent a percentage of all living cells in the sample (debris excluded), a percentage of lymphocytes, a percentage of B-cells, a percentage of T-cells, or a percentage of blasts.

Data Analysis and Reporting

- In hematology, assays are usually designed to characterize abnormal cell populations or stages of cell development.
- In these tests, marker intensities are used to identify the immunophenotype of the cells at various stages of differentiation.
- Markers with good dynamic range and proper spillover compensation are critical.
- Intensity results are typically reported as medians or geometric means.
- A comparison to **control** populations either external such as beads or internal such as normal mature cells is often used.
- If fluorescence intensity is comparable to normal mature cells, it is reported as “normal”: positive if it corresponds to normal cells, “dim” if it is weaker than in normal cell population, or “bright” if it is stronger than in normal cells.

Data Analysis and Reporting

- Most currently used analysis software allows cross-platform application for analysis and makes it possible to create analysis **templates** that are a useful tool for assuring that the analysis is always performed in the same way.
  - Templates help to include all critical elements, and they can serve as an example of how the analysis should be performed.

- Due to the highly complex nature of multiparameter analysis, it is recommended that experienced interpreters with knowledge of instrumentation, software, and data analysis produce the templates and supervise the reporting.

Data Analysis and Reporting

The final report should contain:

1. Demographic identification of patient
2. Identification of the hospital or division sending the sample
3. Type of specimen (bone marrow aspirate, peripheral blood, other biologic fluids)
4. Timing of observation (first diagnosis or follow-up)
5. Diagnostic hypothesis made by the sender
6. List of antigens and type of immunofluorescence analysis carried out
7. Absolute number of cells in the sample
8. Quality of the sample, in terms of viability
9. General description of the gating procedure
10. Immunophenotype of abnormal cells present in the sample
11. Description of other (normal) cells
12. Diagnostic conclusions
13. Comments and/or recommendations for further testing.

Dye & Single Color Compensation

Running Samples

- Prepare samples.
- One sample should be completely negative.
- This sample should be analyzed first. This sample is used for adjusting the PMT's **amplification** voltage.
- Adjust the PMT Voltage until you can see a population peak in the first decade of your 1 parameter and or your two parameter plot.These samples are used for adjusting **Spectral** **Overlap**.
- Once the instrument settings are optimized, run samples and collect data.

Tip 4.

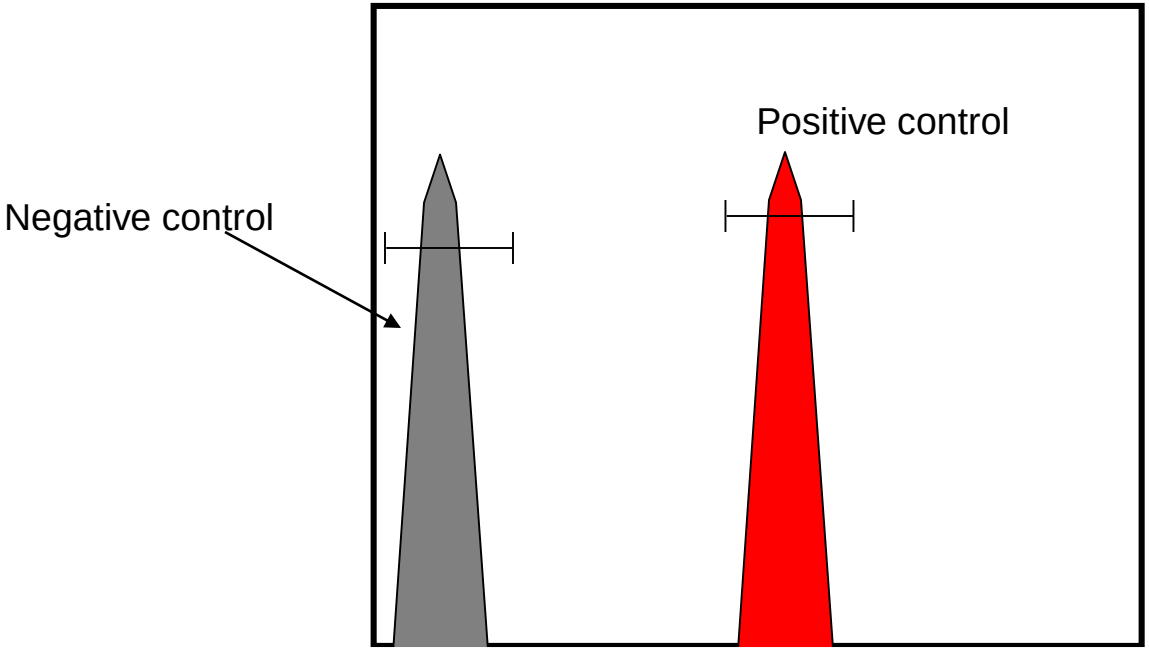
Optimizing PMT voltage: the rules

1. Establish Instrument Baseline: A sensible PMTV value for each detector as a default starting point
   - Digital: Increase PMT voltage until no further improvement (decrease) in CV is observed on (dim) setup particles
   - Analog: Increase PMT voltage until no further improvement in S/N is observed on positive and negative setup particles
   - PMT voltage should not be lower than baseline to begin characterizing stained cells
2. Run stained sample: If several photoelectrons/cell in stained background (high signal), then PMT voltage *may* be lowered until lower part of background approaches noise (can be good information if left alone)
   - Difficult to determine on analog instruments
3. In samples with very bright fluorescence, PMT voltage should be lowered to keep brightest on scale regardless of where background ultimately resides

Single-stained BD™ CompBeads ;

Anti-mouse ig, κ
Anti-rat Ig, κ
Anti-rat/hamster Ig, κ

plus negative control (FBS)



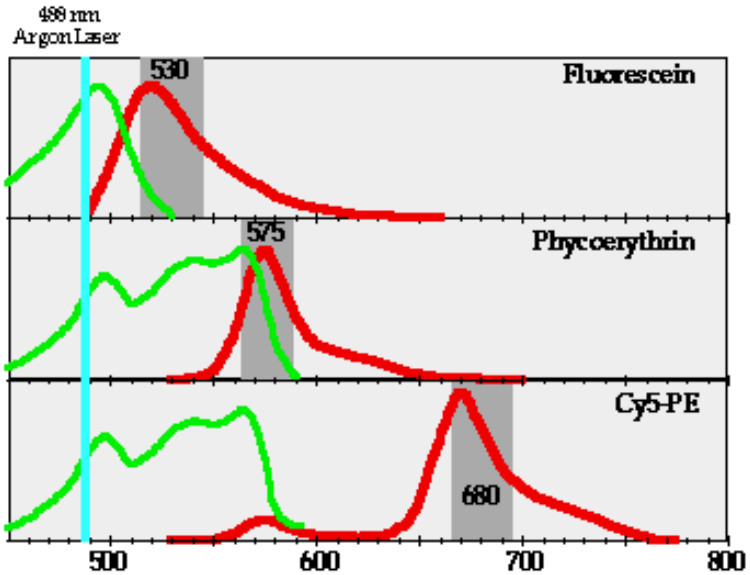
2. Single Color Compensation

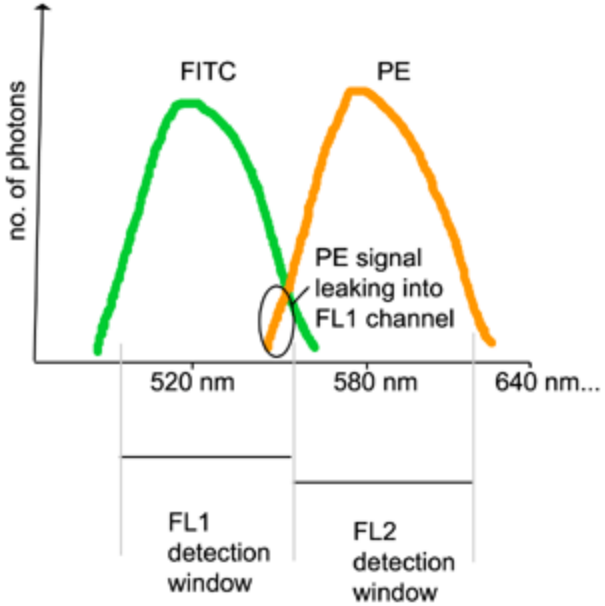
The single most common source of data error in multi-color flow cytometry experiments

What is the Single Color Compensation?

is the process by which we correct for fluorescence “spillover”.

*video training @ http://www.flowjo.com/





How important is proper compensation?

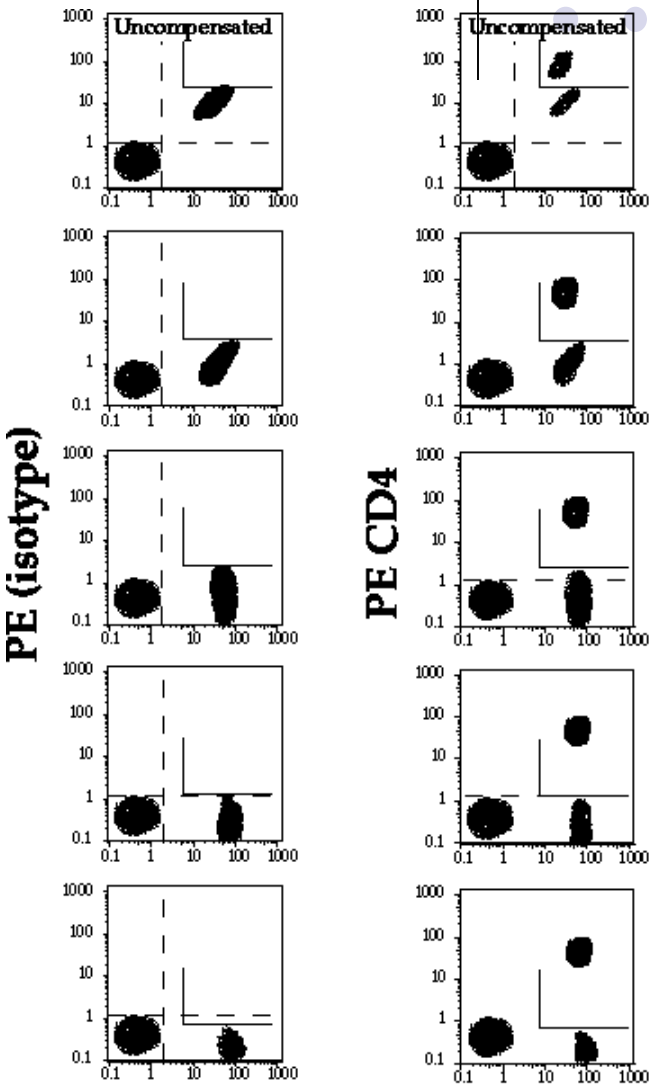
The simple answer is that most of what we have been using flow to do for the past decade has *not* *required absolutely correct compensation*.

For just determining the frequencies of populations, exact compensation is not necessary. This is because, for the most part, the subpopulations that we are interested in enumerating are easily distinguished on the basis of a bright reagent.

However, proper compensation is absolutely necessary for

1) proper antigen density measurements

2) distinguishment dim populations from negative populations: under-compensation will result in overestimating the frequency of the dim cells; over-compensation will result in underestimating the frequency.



Adapted from www.drmr.com/compensation

How does compensation work?

1. *Determine the spectral overlap values by a single color*
2. *Calculate the compensation value*

As an example for correction,

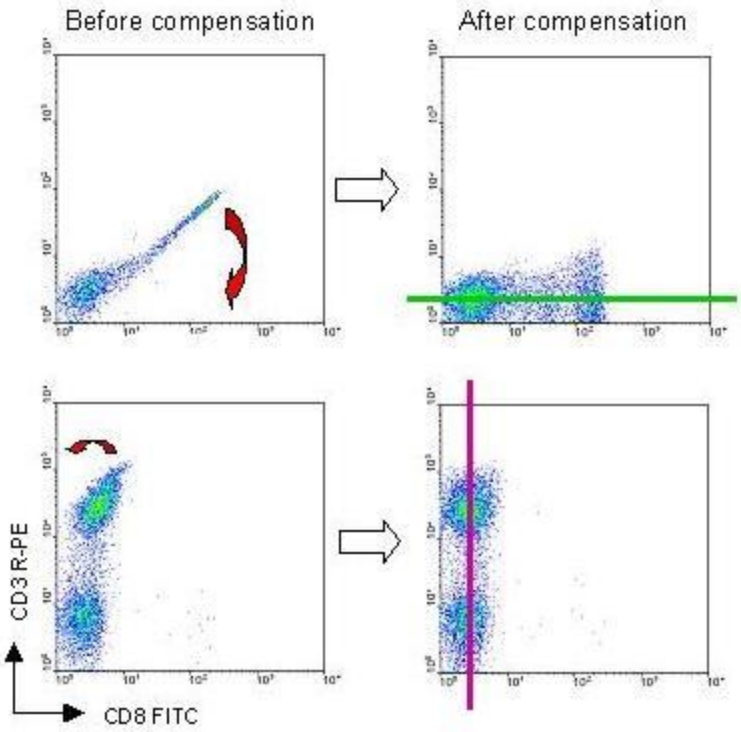
Correcting for FITC fluorescence appearing in the PE channel.

if the amount of yellow fluorescein signal in the FL2 channel is 15% of the green fluorescein signal in the FL1 channel (i.e., "15% compensation"), then we can exactly determine the "true" PE fluorescence of a cell, even in the presence of FITC fluorescence, as:

$$PE_{true} = PE_{measured} - (0.15) \times FITC_{measured}$$

Correcting for PE fluorescence appearing in the FITC channel. For instance, if the amount of (green) PE signal in the fluorescein channel is 2% of the (yellow) signal in the PE channel (i.e., "2% compensation"), then we can exactly determine the true FITC fluorescence of a cell as:

$$FITC_{true} = FITC_{measured} - (0.02) \times PE_{measured}$$

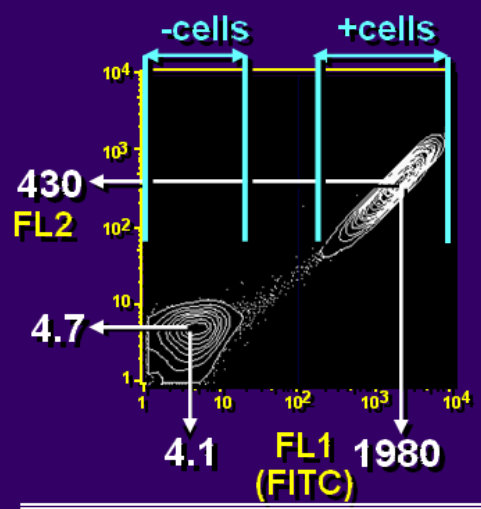


Adapted from www.proimmune.com

Adapted from www.drmr.com/compensation

Calculate compensation value

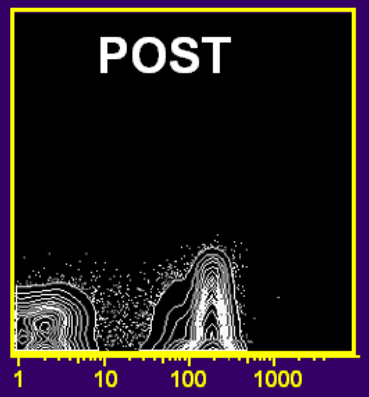
Principles of Compensation - Calculations



Calculation of FL1 Spillover into FL2 (FL2 - %FL1)

$$\% = \frac{\text{FL2 Median}^{+\text{cells}} - \text{FL2 Median}^{-\text{cells}}}{\text{FL1 Median}^{+\text{cells}} - \text{FL1 Median}^{-\text{cells}}} \times 100$$

$$\% = \frac{430 - 4.7}{1980 - 4.1} \times 100 = 20.3$$



Proper compensation for FL2 is achieved when

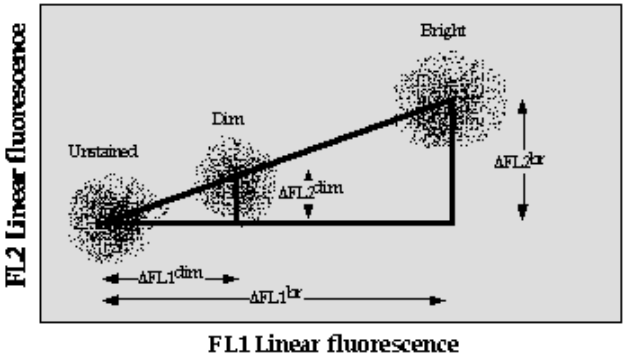
$$\text{FL2 Median}^{+\text{cells}} = \text{FL2 Median}^{-\text{cells}}$$

Myths about setting compensation

1) You should set your compensation on the same tissue you are going to analyze

2) Compensation controls should be the same intensity as the reagent to be use

Bright reagents require more compensation than dim (dull) reagents



3) Compensation can be set by eye

4) Compensation settings can be saved and used from day-to-day

5) Improper compensation doesn't affect the data very much

Requirements for Proper Compensation

1. *The fluorescence spectrum (% spillover) of the compensation control reagent should be identical to the reagent used in the experiment*
   Critical for tandem reagents
   even similar fluorochromes like FITC/Alexa488 or APC/C5

2. *The negative and positive populations must have the same autofluorescence*
   don't use CD3+ lymphocytes and CD3- monocytes

3. *The positive population should be a bright as possible*

4. *Take enough events to get statically accurate numbers*

- *Type of Single color control*

  - *BD CaliBRITE™ beads,*
  - *Single-stained cellular controls; popular*
  - *Single-stained BD™ CompBeads ;*

  > provide a convenient method of presenting single color control
  > Best match of spectra

Practical Issue

Analog compensation

CellQuest of FACS Calibur™

Correct for dye spillover to align stained populations in dye space w/o bias from spectral overlap.

all adjustments are made pair-wise FL1-%FL2, etc and not available FL2-%FL4

> What to suggest? finish compensation by using software (FlowJo etc)

Software (digital) compensation

FACSDiva™ of BD FACS Canto II, LSRII, LSRII Green and FACS aria

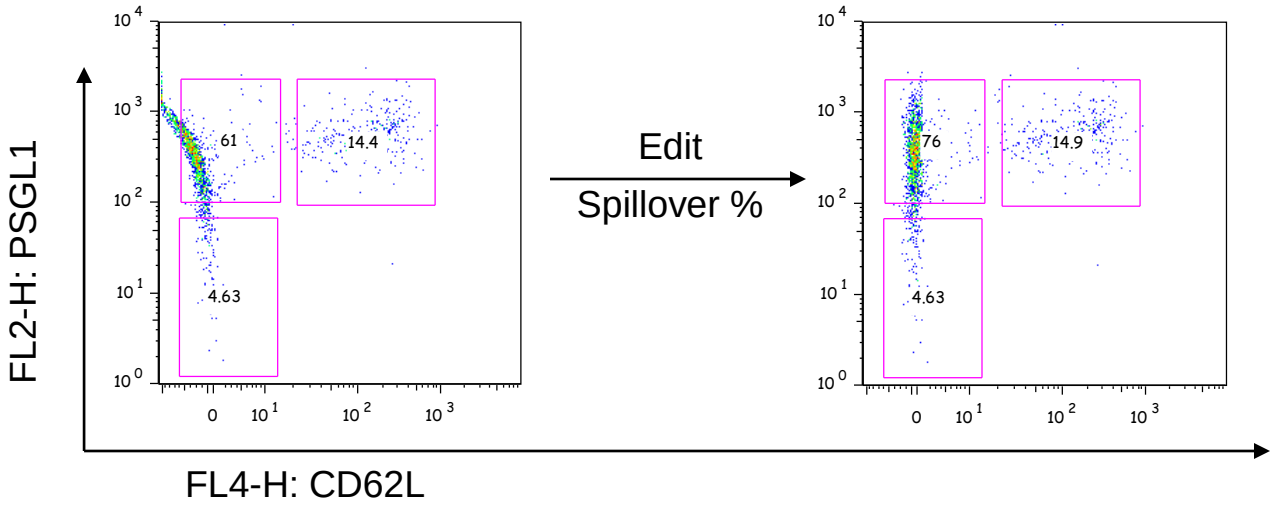
automatically calculate spectral overlap for compensation value

Not a substraction, use matrix algebra and compensation coefficients

	Analog	Digital
Algorithm	Subtractive: pulse matching	Corrective: matrix algebra
PMTV	Critical – narrow ranges for pulse matching	Above noise
Comp Error	Yes	No
Linearity	Up to 6 – 10% error	Linear across dynamic range

Tip 1.

Make and Edit compensation matrix by FlowJo



	FL1-H	FL2-H	FL3-H	FL4-H
FL1-H		0	0	0.946
FL2-H	0		0	**0.813**
FL3-H	0.177	0		0
FL4-H	0	0	0.741	

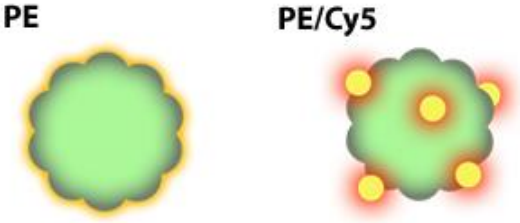
	FL1-H	FL2-H	FL3-H	FL4-H
FL1-H		0	0	0.946
FL2-H	0		0	**0**
FL3-H	0.177	0		0
FL4-H	0	0	0.741	

[Compensation matrix]

Row; primary fluorochrome
Column; detector
Each entry; the amount of spillover (%)

Tandem Dyes

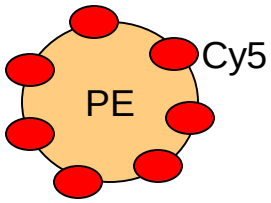
- A tandem is composed of two covalently attached fluorescent molecules (one of which serves as the donor and the other as acceptor) that behaves as a unique fluorophore with the excitation properties of the donor and the emission properties of the acceptor.
- This is possible through the phenomenon of Förster resonance energy transfer (FRET), also known as fluorescence resonance energy transfer. This allows one fluorophore to pass its excitation energy to a neighboring fluorophore, which then emits the photon of light.
- This transfer of energy is dependent on the proximity and orientation of the donor and acceptor molecules.



Comparison of PE and PE/Cy5. Both molecules excite by the blue or green/yellow lasers, based on the excitation properties of PE. While PE emits maximally at 578 nm, PE/Cy5 emits at 667 nm.

Use of tandem fluorophores

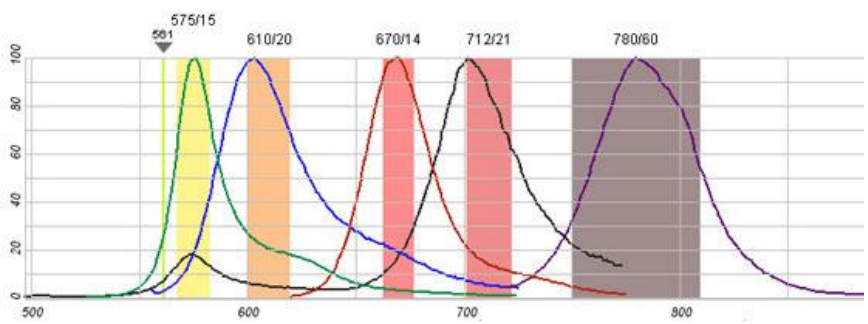
PE-Cy5, PE-Cy5.5, PE-Texas Red®, PE-Cy7*,
APC-Cy5.5, APC-Cy7*



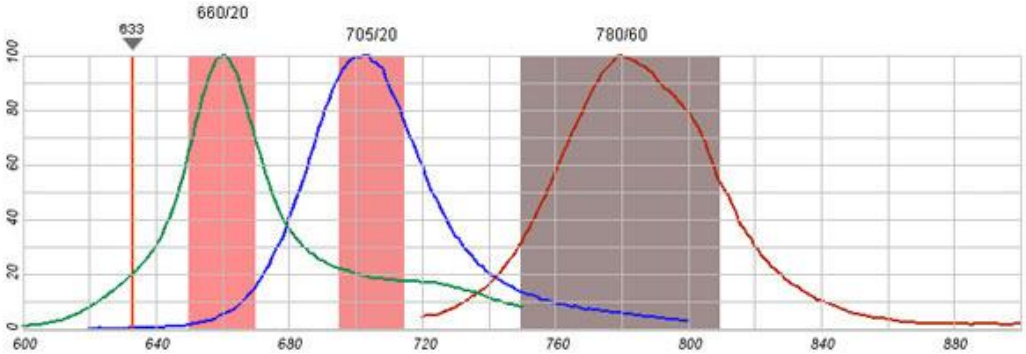
Fluorescence resonance energy transfer (FRET) technology (<10nm)
Spectral overlaps vary from lot to lot;
FRET efficient in tandem pairs tends to be different each time the conjugation chemistry is performed.

APC; Allophycocyanin, a protein from the light-harvesting phycobiliprotein family
PE ; Phycoerythrin, a protein from light-harvesting phycobiliprotein family,
present in cyanobacteria, red algae and cryptomonads

** They can degrade in the presence of light, heat, and fixation*



PE, PE-TxRed, PE-Cy5, PE-Cy5.5, PE-Cy7



APC, APC-Cy5.5, APC-Cy7

Validation of Assays and Quality Assurance

- In clinical settings, the results obtained in FCM **must be interpreted in relation to clinical information and to the results of other techniques** (morphology, cytogenetics, molecular genetics, fluorescence in situ hybridization [FISH]), which are used as a validation of the information provided by FCM.

- Newly established panels have to be validated by comparison to reference methodology, interlaboratory comparison, or verification with specimens obtained from patients with a confirmed diagnosis.

  - A minimum of 10 to 20 samples (10 normal, 10 abnormal) is recommended for accuracy assessment.

  - The acceptance criteria will also be variable depending on the required degree of accuracy for the intended use, nevertheless should be clearly defined for each assay.

- Ninety percent, or greater, agreement between methods is generally required for accuracy.

Validation of Assays and Quality Assurance

- All instruments have to follow daily quality checks according to manufacturers' recommendations.
- Participation in a suitable **external quality assurance (EQA)** program should be undertaken.
- Many proficiency testing programs are in existence operating at local, national, or international levels.

Validation of Assays and Quality Assurance

- The more common uses of FCM should be subjected to EQA and many of the larger international programs such as those operated by **UK NEQAS** for Leukocyte Immunophenotyping and the College of American Pathologists offer FCM EQA programs for leukemia and lymphoma diagnosis, lymphocyte subset monitoring, paroxysmal nocturnal hemoglobinuria (PNH), and CD34+ stem cell enumeration.

- Many of these programs use stabilized material enabling samples to be transported long distances such that data from large international cohorts can be examined to search for any instrument or reagent bias.

- The frequency of the samples issued by such programs is recommended to be at least four times per year to ensure continued performance monitoring.

Cell Sorting

- Some flow cytometers are capable of physically separating the cells (fluorescence activated cell sorter, FACS) based on differences in any measurable parameters.

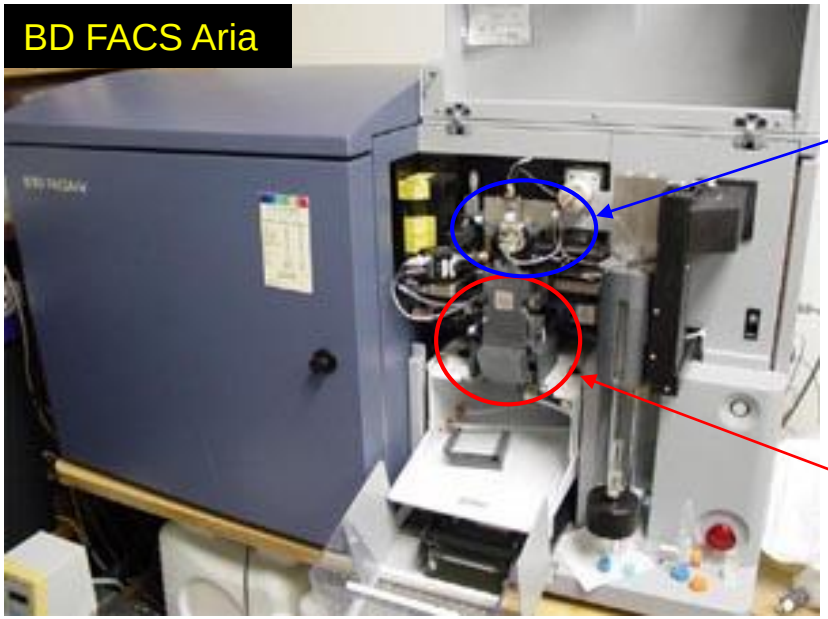
- Sorting is achieved by droplet formation.

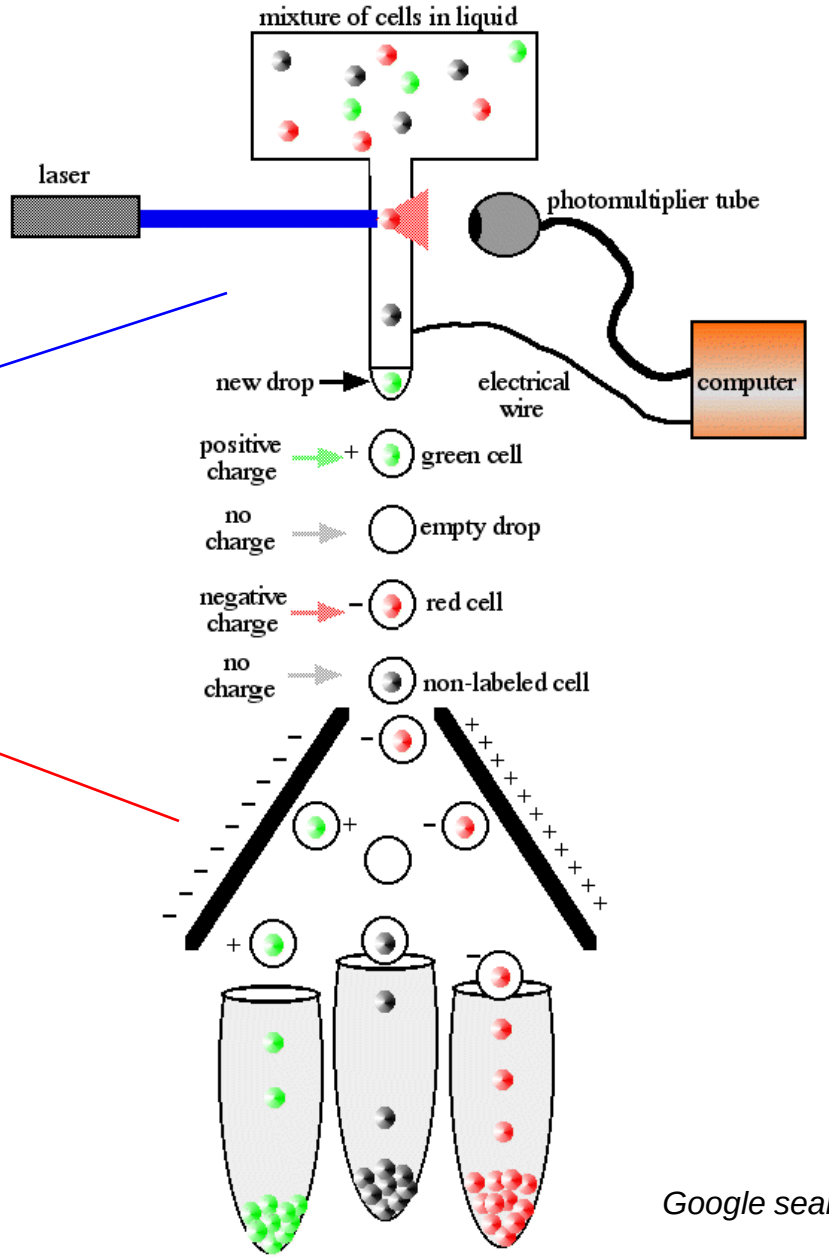
- The basic components of any sorter are:
  1. A droplet generator
  2. A droplet charging and deflecting system
  3. A collection component
  4. The electronic circuitry for coordinating the timing and generation of droplet-charging pulses

Cell Sorting

- The flow chamber is attached to a piezoelectric crystal, which vibrates at a certain frequency so that when the fluid carrying the cells passes through the nozzle, forming a jet in air with a velocity of 15 m/s, the vibration causes the jet to break up in precisely uniform droplets, approximately 30,000 to 40,000/s.
- Each droplet, when separated from the jet, can be charged and deflected by a steady electric field and is collected in a receptacle.
- Almost every cell is isolated in a separate droplet.
- When the cell is analyzed a sorting decision is made, and until the proper electrical charge pulse is applied to the droplet containing the cell, there is a transit time determined by several factors, such as flow velocity, droplet separation, and the cell preparation. If two cells cannot be separated the sorting is aborted.

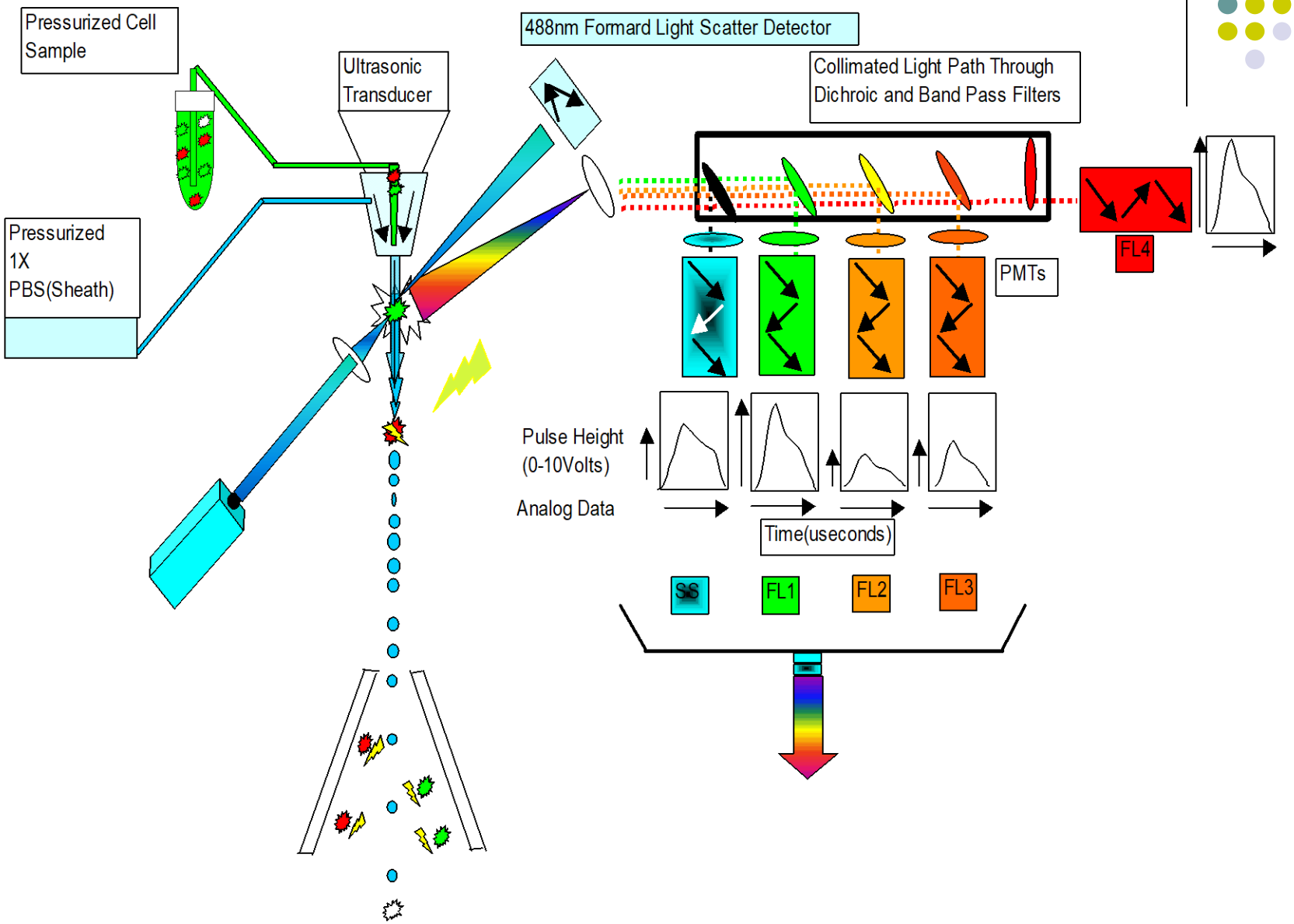
FACS sorting





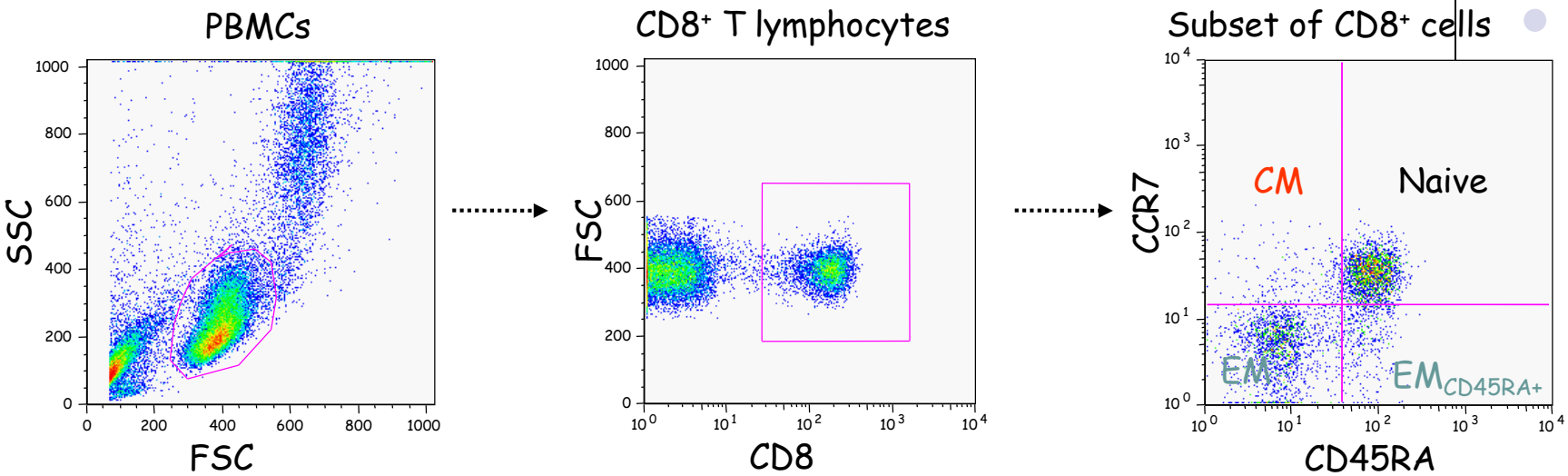
Google search

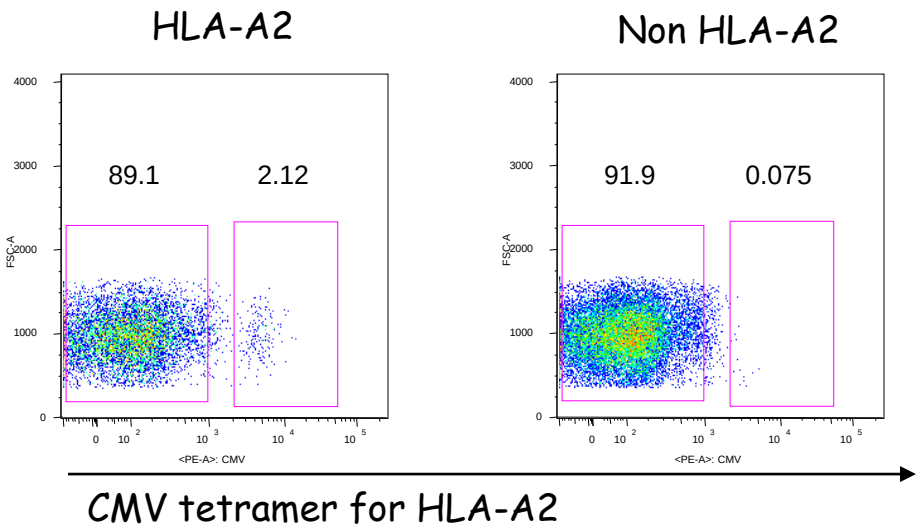
Flow Cytometry and sorting



Applications

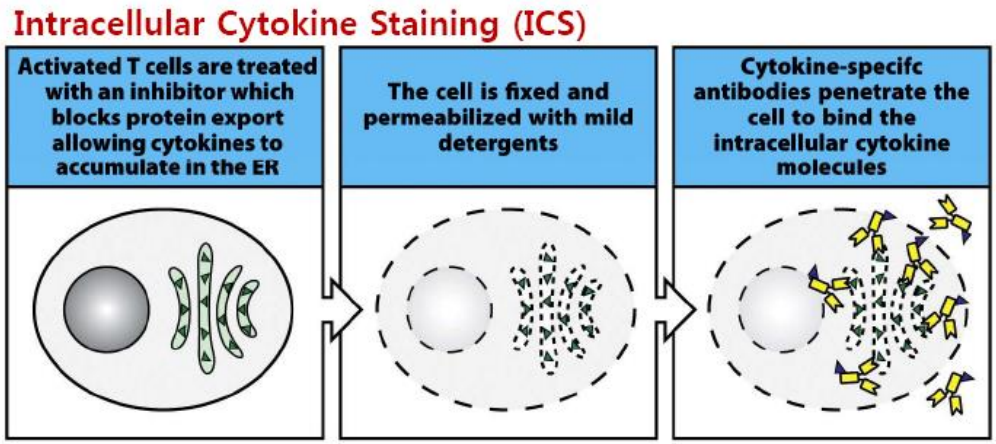
1. Surface phenotype, Ag-specific T cells

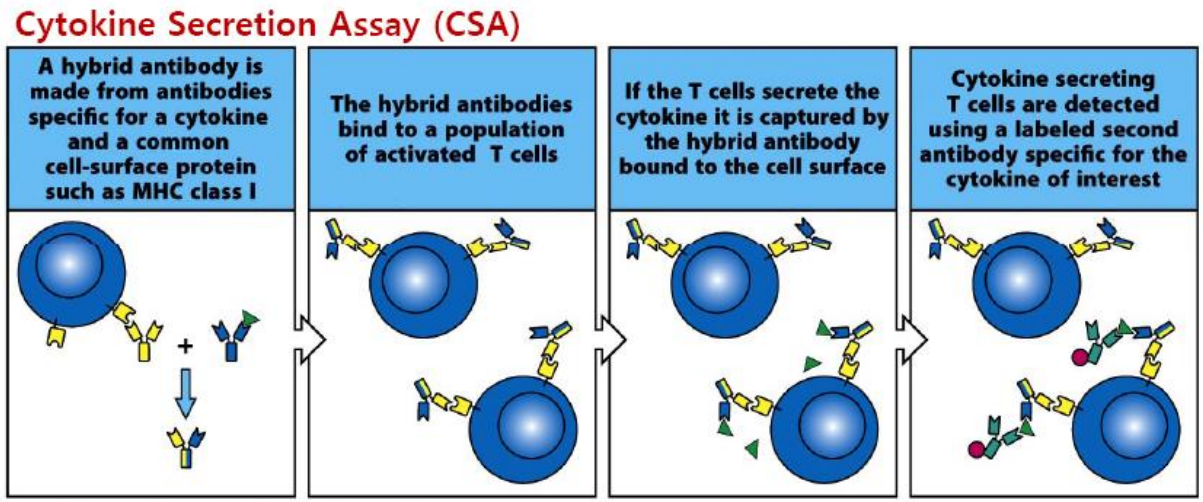




CMV-specific CD8+ T cells

2. Cytokine productions

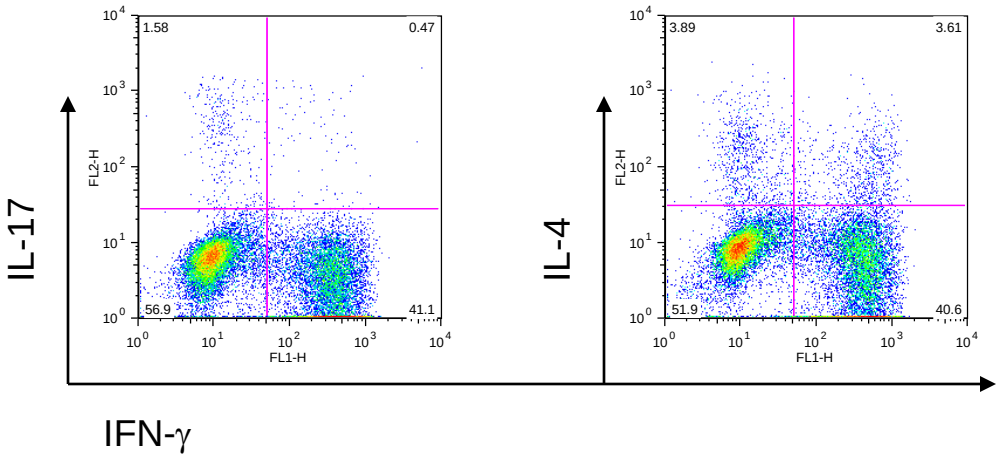




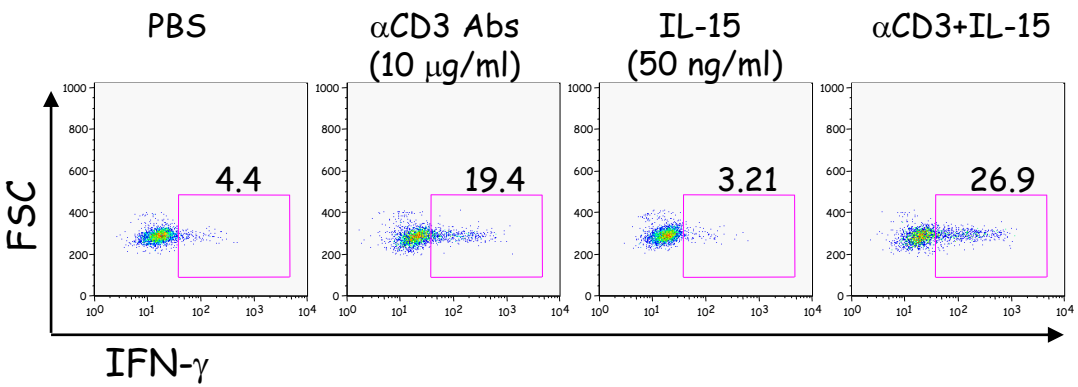
Fixative; PFA
Perm; Sapoinin, PEG (BD Perm II solution for human)

Adapted from KAIST (Shin, EC)

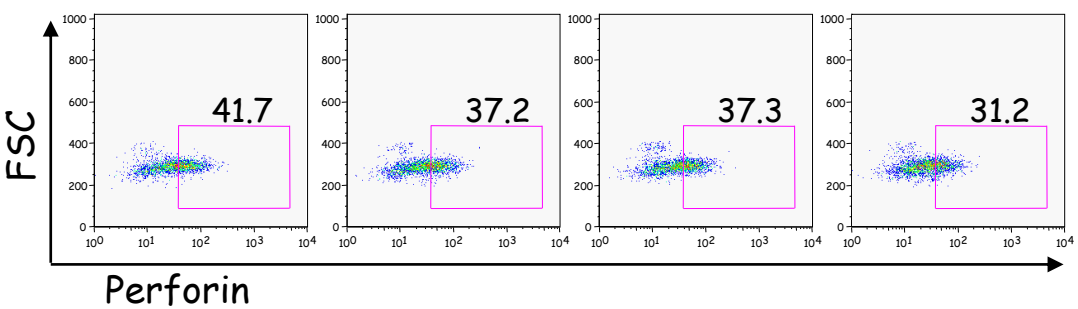
Representative cytokine staining



Sort CD4⁺ cells from PBMC
Stimulate cells with PMA/ION
in the presence of GolgiStop®
Fix and Perm with BD buffer
Stain cells with Abs against IFN-γ, IL-17 and IL-4



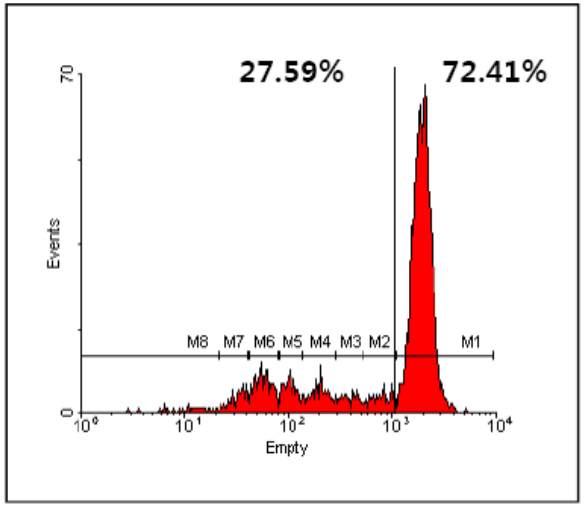
+ aCD28 (2 μg/ml)
+ aCD49d (2 μg/ml)



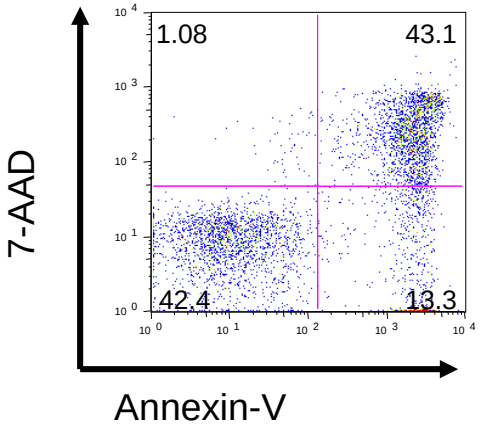
Stain cells with Abs for surface Ags
Stimulate cells with indicated cytokine and/or Abs
in the presence of Golgiplug®
Fix and Perm with BD buffer
Stain cells with Abs against IFN-γ and perforin

3. Cell proliferation, Cell cycle, Apoptosis

CFSE; cell proliferation



Apoptosis



Cell Cycle

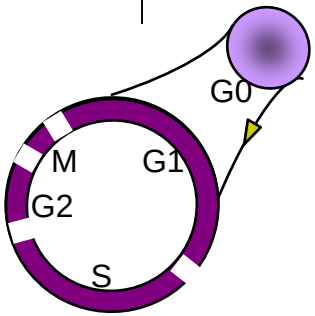
Go : 2n
(Gap0) resting state

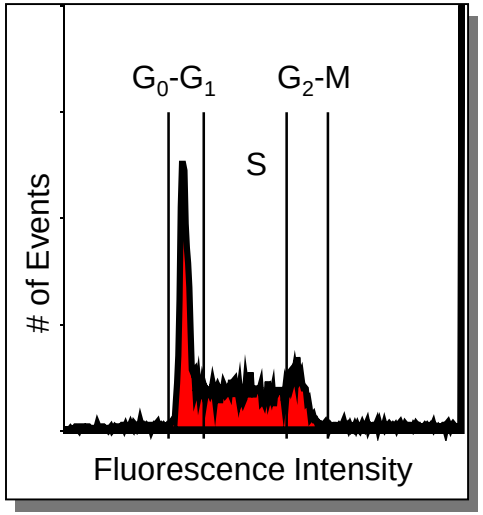
G1 : 2n
(Gap1) RNA & protein synthesis to prepare for S phase

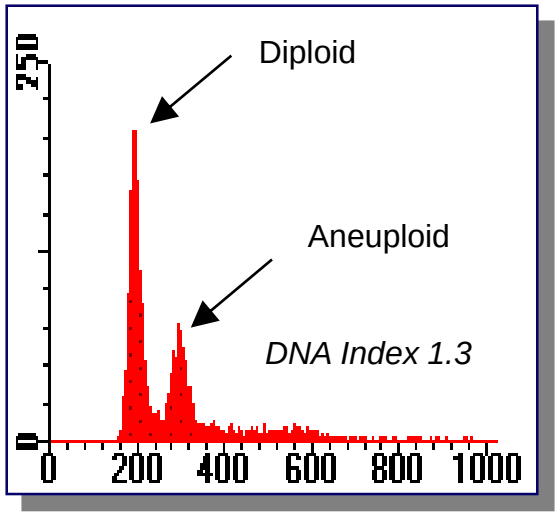
S : 2n~4n
(Synthesis) DNA Synthesis

G2 : 4n
(Gap2) RNA & protein synthesis before cell division

M : 4n
(Mitosis) preparation for daughter cell production







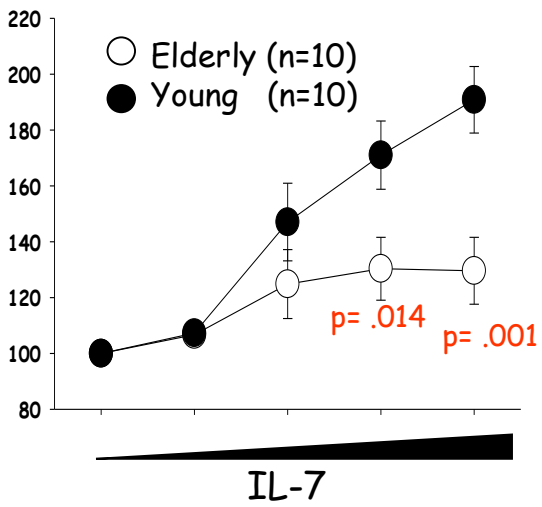
Adapted from BD biosciences

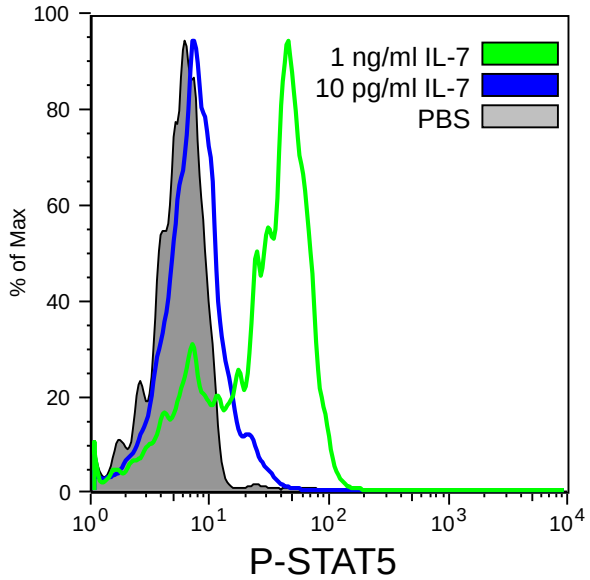
4. Intracellular protein

Phospho protein;

p-STAT1, p-STAT5
KINASES (p38 MAPK,P44/42 MAPK, JNK/SAP).
Members of cell survival pathways (AKT/PKB)
T cell activation pathway (TYK2)
p-ERK

Granzyme, Perforin

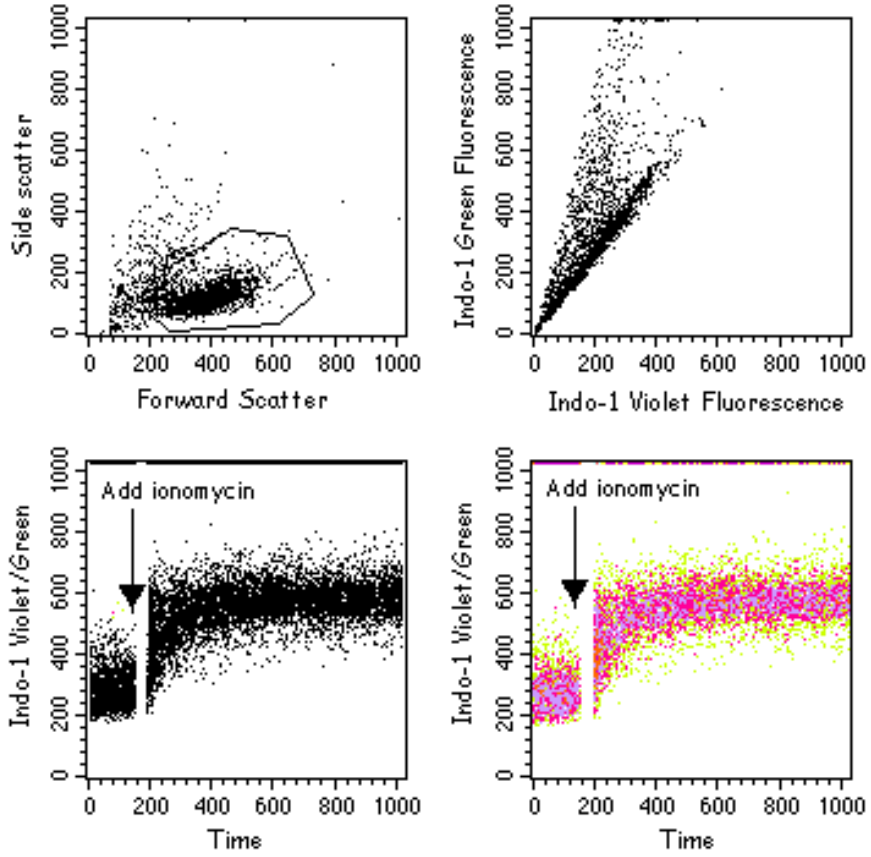


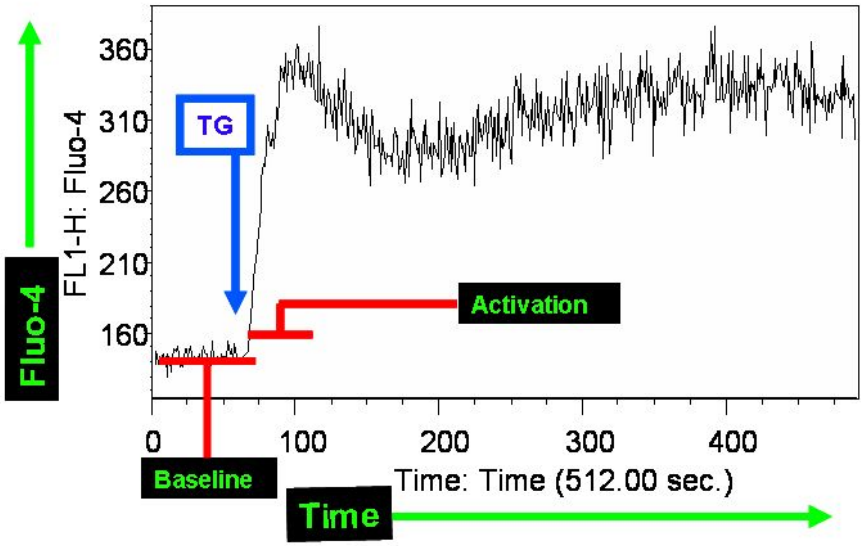


Stain cells with Abs for surface Ags
Stimulate cells in the presence
or absence of IL-7
Fix with 2% formalin
Permeabilize with 90% methanol
Stain cells with Abs for p-STAT5

Fixative; PFA
Perm; Methanol

5. Intracellular Calcium





Legend. Jurkat T-cells were loaded with 1 μM Fluo-4 for 45 min at 37°C and adjusted to 1 x 10^6/ml in calcium free PBS. After a 30 second baseline was collected, thapsigargin (Tg) (5 μg/ml) an endoplasmic reticulum (ER) ATPase inhibitor was added. The subsequent release of internal stores of calcium from the ER into the cytoplasm was detected by Fluo-4 (activation phase) before moving to miotchondria.

UV (em 390_violet & 500_green)
Indo-1

488 (blue laser)
Fluo-4

Google search

Tip 1.

Which fluorochromes and when …

ANTIGEN DENSITY	FLUOROCHROME
low	Phycoerythrin (PE), APC
low-intermediate	CY5
high	FITC, PerCP

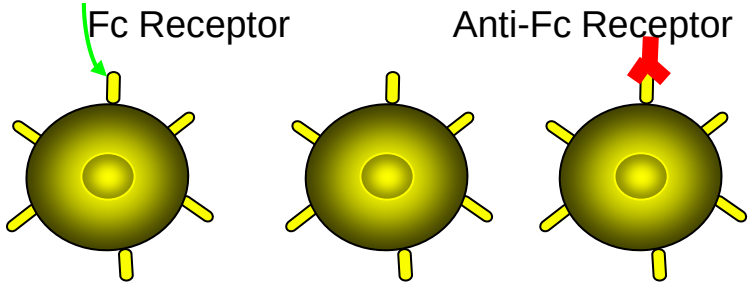
Tip 2.

Non-specific binding

Fc receptor blocking,

Mouse systems, FcγII/III CD16/CD32

Human, human Ig or 10% autologous serum in PBS



Immunoglobulin Isotype Controls

Same fluorochrome-conjugated antibody of irrelevant specificity which has the same Ig isotype

Ligand blocking control

Pre-block with anti-cytokine antibody

Tip 3.

Advantages/ Disadvantages of Using More Colors

Advantages

Save Time, Reagents, Samples

(1) 6-color stain = (15) 2-color stains

Exponential increase in information

Data from (1) 6-color stain » (15) 2-color stains

identify new/rare population (<0.05%)

internal controls

Disadvantages

Must carefully choose combinations of fluorochrome conjugates

All reagents not available in all colors

Greater potential for errors in compensation

Proper controls required

Normal Hematopoiesis

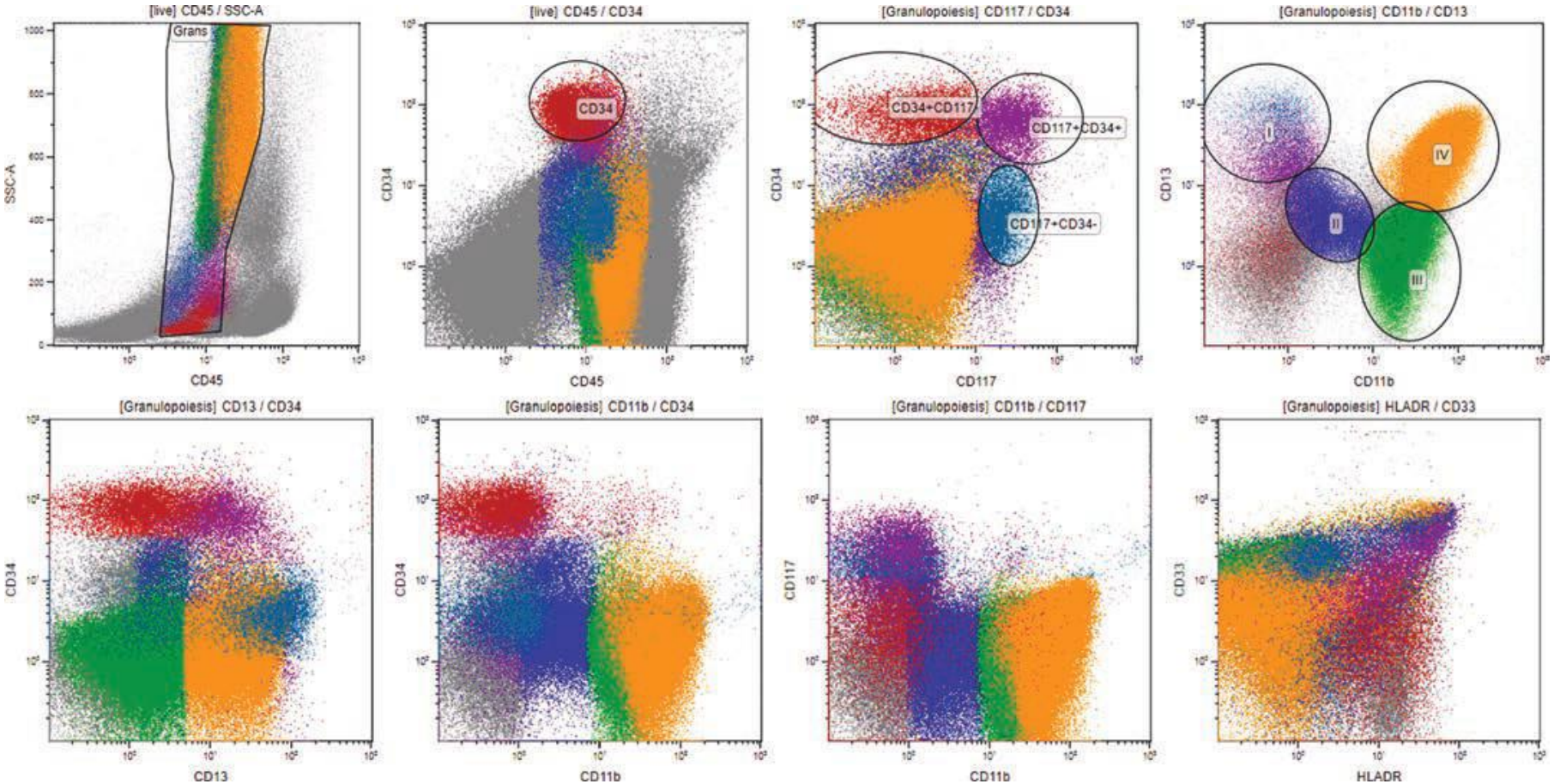
- Knowledge of levels and expression patterns of various antigens in normal hematopoietic cells at different stages of development provides a frame of reference for recognition of abnormal differentiation patterns.

SURFACE MARKER EXPRESSION DURING MATURATION OF GRANULOPOIETIC PRECURSORS IN THE BONE MARROW

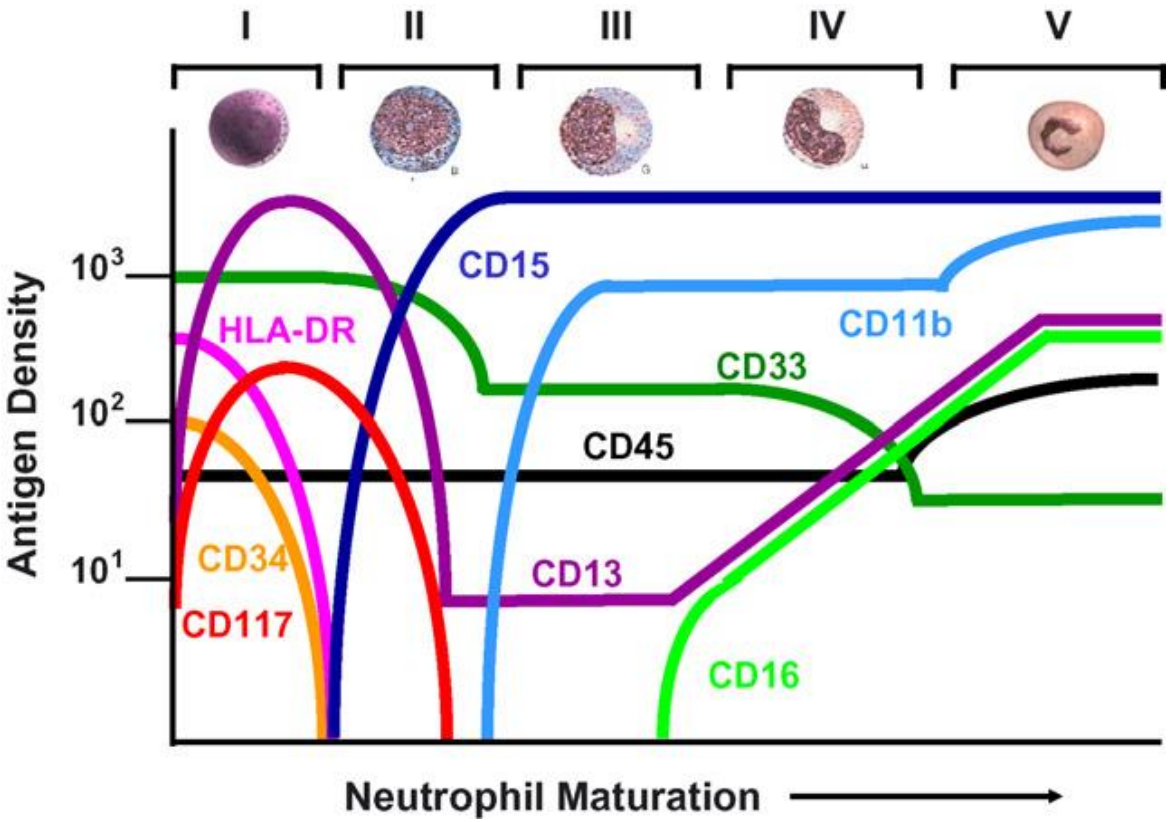
Antigen	Blasts	Promyelocytes	Myelocytes	Metamyelocytes	Bands	Segmented Neutrophils
CD10	–	–	–	–	–	+
CD11a	d	d	d	+	+	+
CD11b	–	–	d	+	+	b
CD11c	–	–	d	d	d	d
CD13	d	+	+	d	d/+	b
CD15	–/+	d/+	+	+	+	+
CD16	–	–	–	d	+	b
CD18	+	+	b	+	+	+
CD24	–	–	+	+	+	+
CD33	–/d/+	b	+	d	d	d
CD34	d/+	–	–	–	–	–
CD35	–	–	–	–	d	d
CD44	b	+	d	d	+	b
CD45RA	d	d	–	–	–	–
CD45RO	–	–	–	d	+	b
CD54	+	+	–/d	–/d	–/d	–/d
CD55	b	+	+	b	b	b
CD59	b	b	b	b	b	b
CD62L	+	+	+	+	+	+
CD64	d	d	+	+	–	–
CD65	–/+	d	+	+	b	b
CD66a	–	–	+	+	+	+
CD66b	–	b	b	+	+	+
CD66c	–	b	b	+	+	+
CD117	d	+	–	–	–	–
CD133	d	–	–	–	–	–

–, Negative; –/+ or (d), partially positive (or dim); d, dim, weakly positive; +, positive; b, bright, strongly positive.

Flow cytometry analysis of maturation in granulopoiesis.



Antigens and myeloid maturation



I myeloblasts
II promyelocytes,
III myelocytes
IV metamyelocytes /bands
V neutrophils.

Leukemia Research 32 (2008) 5–17

Immature Cells of Normal Bone Marrow

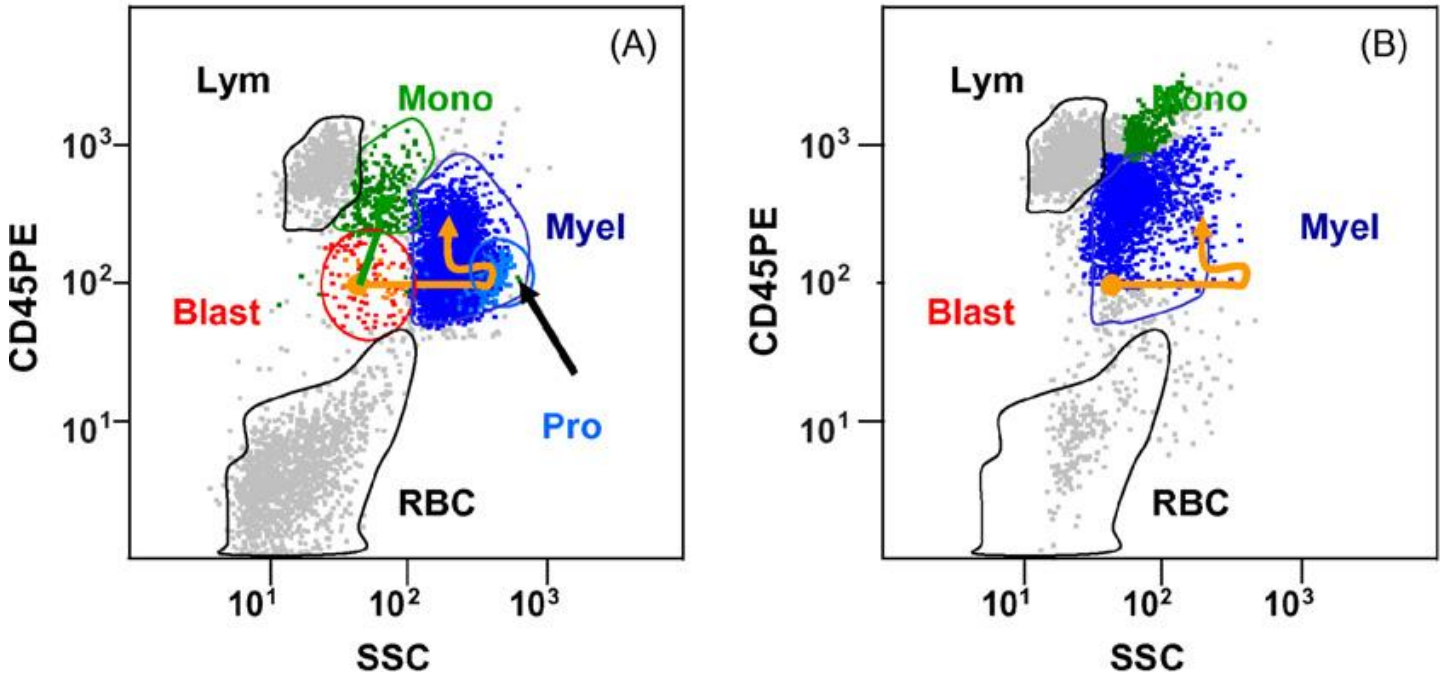
- CD34+ hematopoietic progenitor and precursor cells (HPC) that constitute most cells of the CD45/dim (blast) region are a heterogeneous cell population.
  - A small fraction of pluripotent stem cells with long-term repopulating cell activity have been associated with the CD34/CD38− phenotype.
  - These cells are very rare in normal BM (usually <0.1%), but may increase in regenerating BM and in myelodysplastic syndromes (MDS).

- CD34/CD45dim cells also include a major fraction of HPC already committed to different hematopoietic lineages (erythroid, neutrophil, monocytic, dendritic cell (DC), basophil, mast cell (MC), eosinophil, and megakaryocytic) and variable numbers of CD34+ B-cell precursors (BCP).

Immature Cells of Normal Bone Marrow

- Human stem cells are defined by expression of CD90 and CD49f and are CD45RA negative.
- Early myeloid progenitors were isolated based on the expression of IL-3 receptor, a chain (CD123) or FLT3 (CD135), and CD45RA.
- Myeloid, but not erythroid, progenitors express CD123 and CD135, and the transition from common myeloid to granulocyte-macrophage progenitor is marked by acquisition of CD45RA.

FC CD45 gating

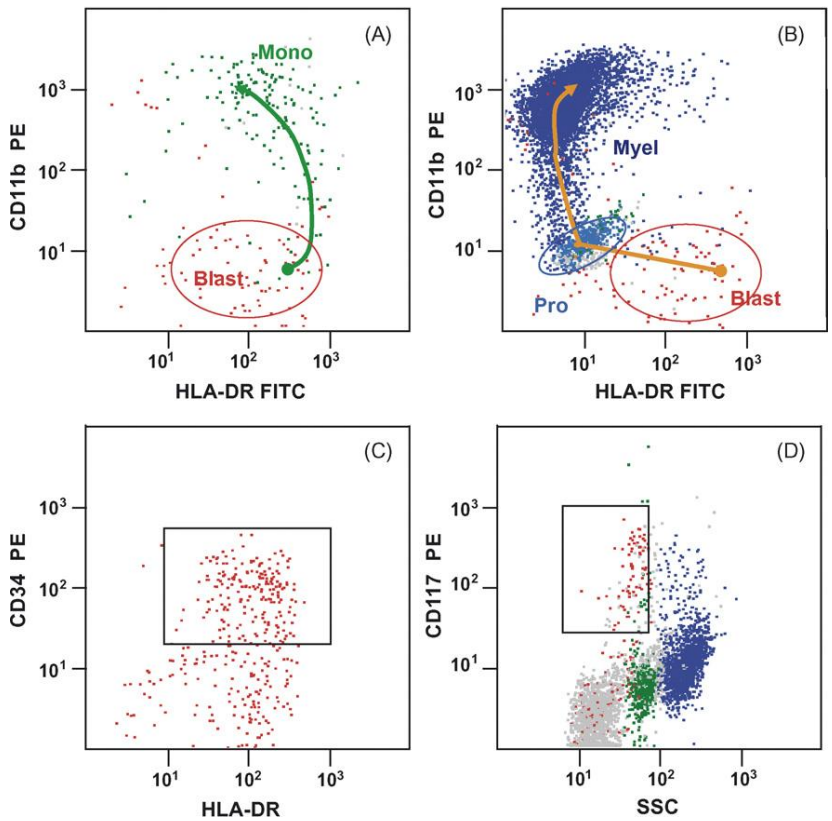


normal

mds

Leukemia Research 32 (2008) 5–17

Normal BM stained with HLA-DR-FITC, CD11b-PE and CD45 PerCP, HLA-DR-FITC, CD34-PE, CD45 PerCP, CD117



immature myeloblasts are red, monocytoid cells are green while maturing myeloid cells are blue.

(A) Cells in the blast and monocyte regions, illustrate the maturation from blast to monocyte noted by the green arrow.

(B) Cells within the blast and myeloid regions illustrate the maturation to neutrophils depicted by the gold arrow.

(C) CD34+, HLA-DR positive cells are only found in the blast region.

(D) CD117 positive cells are identified in both the blast and myeloid region. However, the expression of CD117 on blast cells can be identified based on low SSC.

Leukemia Research 32 (2008) 5–17

Granulocytic Differentiation

- Several antigens change their expression intensity during maturation of granulopoiesis.
- Characteristic normal patterns for various antigen combinations have been identified using multicolor analysis.
- Continuous variation in the expression of CD13, CD11b, and CD16 that occurs as the blasts/promyelocytes mature to neutrophils makes the combinations of these antigens very useful in delineating granulocyte maturation.

Granulocytic Differentiation

- CD13 is expressed at high levels on CD34+ HPCs and CD117+ precursors (promyelocytes).
- CD13 is then down-regulated and dimly expressed on intermediate precursors (myelocytes)
- it is gradually up-regulated again as the granulocytic cells develop into segmented neutrophils.

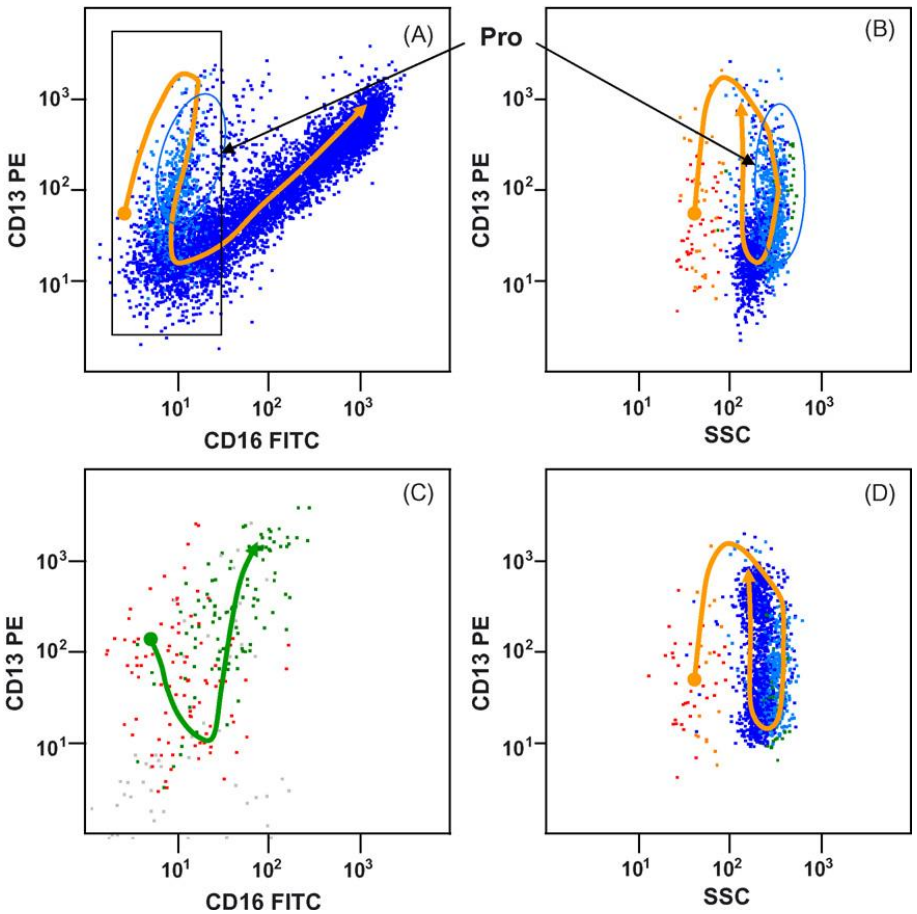
Granulocytic Differentiation

- CD11b and CD16 are initially expressed at low levels, but their expression increases during maturation.
- Expression of CD33 is particularly useful if followed together with expression of HLA-DR.
- CD34+ cells are HLA-DR positive and become weakly positive for CD33.
- With maturation, CD34 disappears and CD33 expression is up-regulated, followed by down-regulation of HLA-DR and slight down-regulation of CD33 in most mature forms.
- .

Granulocytic Differentiation

- CD15 and CD65 appear when cells are restricted to neutrophil differentiation.
- CD66, CD16, and CD10 are the markers of mature, band, and segmented neutrophil granulocytes and can be applied to evaluate blood contamination of aspirate.

Normal CD13, CD16, and SSC patterns.



Leukemia Research 32 (2008) 5–17

(A) Gold arrows follow the intensities of CD13 and CD16 paired antigens from myeloblasts to neutrophils in the classic sickle shape.

(B) The relationship between CD13 and SSC on early myeloid maturation is shown by gating on CD16 negative maturing myeloid cells in the CD13/CD16 histogram (A). Myeloblasts gain CD13 as they mature to promyelocytes, then precipitously lose CD13 as they become myelocytes.

(C) Myeloblasts lose CD13 to become monocytes (both gated by CD45/SSC, then re-express this molecule.

(D) The relationship between CD13 and SSC on all myeloid cells (including both CD16 negative and positive maturing myeloid cells) illustrates the limitation of displaying the data in only 2 dimensions.

Monocytic Differentiation

- CD14, CD36, and CD64 are considered as monocyte-associated markers, CD14 being the most specific.
- During maturation toward promonocytes, progenitors down-regulate CD34 and CD117 and gain the expression of CD64, CD33, HLA-DR, CD36, and CD15, with an initial mild decrease in CD13 and an increase in CD45.
- Maturation toward mature monocytes leads to a progressive increase in CD14, CD11b, CD13, CD36, and CD45, with a mild decrease in HLA-DR and CD15.
- Mature monocytes show expression of bright CD14, bright CD33, variably bright CD13, bright CD36 and CD64, and low CD15.

Erythropoietic Differentiation

- Early erythropoietic precursors are found in the blast area and can be identified by very bright CD44, bright CD71, intermediate CD36, positivity for HLA-DR, and expression of CD117 with “dim” CD45.

- Glycophorin A (CD235a) is expressed at a low level at this stage.

- Maturation to the basophilic erythroblast is accompanied by a decrease in CD44, disappearance of CD45 and acquisition of bright CD235a expression.

- At transition to the polychromatophilic/ orthochromatophilic stage, erythroblasts show loss of HLA-DR, further decrease in CD44, and a mild decrease in CD36.

Lymphocyte Differentiation

AVERAGE RELATIVE FREQUENCY OF MAJOR LYMPHOID CELL SUBSETS IN NORMAL TISSUES

Subset	Peripheral Blood[a] Children (%)		Peripheral Blood[a] Adults (%)	Bone marrow[b] (%)		Lymph Nodes[a] (%)	Tonsils[a] (%)	Spleen[a] (%)
	2–5 Years	5–15 Years		Children	Adults			
CD19+ B-cells	24	17	12	10	3	41	51	55
CD3+ T-cells	64	68	72	6	12	56	49	31
CD4+ CD3+ T-helper	37	38	44	3.2	6.5	48	42	17
CD4+CD8+ T-cytotoxic	24	26	24	2.6	4.2	10	6	14
Natural killer (all NK subsets)	10	13	13	2	4	1	<1	15

[a]Percentage of cells in the lymphocyte region (CD45 bright).
[b]Percentage of total bone marrow cells.

Lymphocyte Differentiation

- B-cell differentiation in the normal human bone marrow:
  1. common lymphoid progenitor (CLP): early B (E-B) stage.
     - CD34+ CD10+ TdT+ CD79a+ CD19neg
  2. pro-B-cell stage
     - CD34+ CD19+ CD10+ TdT+ CD20- cytIgM-.
  3. pre-B
     - CD34– CD19+ CD10+ CD20.
  4. immature (IM)-B-cells.
     - CD34–CD19+CD20+CD10dim/− IgM+
  5. mature B-cells
     - CD10-CD19+ CD20+ IgM+ IgD+.

Lymphocyte Differentiation

- Pre-B and IM B-cells constitute the majority of B-cells in BM of children, whereas mature B-cells are most frequent in adult BM.
  - In children with BM regeneration after infection or chemotherapy and in transient hyperplasia of B-cell progenitors, subpopulations of IM and mature B-cells co-expressing CD5 have been identified.
  - CD5+ B-cells are the major population of B-cells in fetal life, and their percentage decreases with age.
- Knowledge of antigen expression patterns of B-cell subsets in normal BM is essential for follow-up studies of minimal residual disease (MRD) in patients treated for B-precursor acute lymphoblastic leukemia (ALL).

IMMUNOPHENOTYPIC CHANGES DETECTED BY FLOW CYTOMETRY DURING B-CELL DEVELOPMENT IN NORMAL BONE MARROW

	CLP	Early B	Pro-B	Pre-BI	Large Pre-BII	Small Pre-BII	Immature-B	Mature B	Plasma cells
CD34	+	+	+	–	–	–	–	–	–
CD10	+	+	+	+	+	+	+/dim	–	–
CD19	–	–	+	+	+	+	+	+	+
Cyt.CD79a	–	+	+	+	+	+	+	+	+
Cyt.CD22	–	+	+	+	+	+	+	+	+
TdT	–	–	+	–	–	–	–	–	–
mCD22	–	dim	dim	+	+	+	+	+	–
CD20	–	–	–	+	+	+	+	+	–
sIgM	–	–	–	–	–	–	+	+	–
sIgD	–	–	–	–	–	–	–	+	–
sIg κ or λ	–	–	–	–	–	–	–	+	–
cyt.Ig κ or λ	–	–	–	–	–	–	–	+	+

CLP, common lymphatic precursor; cyt., cytoplasmic; s, surface; TdT, terminal deoxynucleotidyl transferase.

Lymphocyte Differentiation

- T-cells
- T-cell production is maintained throughout life by thymic seeding of BM-derived progenitors.
- Rare (<0.1%) T-cell–restricted precursors, which express pre-Ta protein on the cell surface and are CD34+CD7+CD45RA+, were identified in human BM.
- No TdT-positive T-cells expressing cytoplasmic CD3 are found in normal BM.
- Most mature T-cells in the BM co-express CD7, CD5, CD2, and membrane CD3 and are either CD4 or CD8 positive.
- Minor subsets of CD7+ cells lacking other “pan-T” antigens, small subsets with co-expression of CD4 and CD8, and a subset lacking CD4 and CD8 have been identified.
- A small population of CD7– T-cells (<10% of T-cells) can also be seen in normal and reactive conditions.

Minor Bone Marrow Cell Subsets

- In healthy donors, **eosinophils** represent 2% to 3% of blood leukocytes.
- Numbers of eosinophilic precursors may vary considerablynin reactive BM.
- Eosinophilic myelocytes can be identified by
  - high side scatter,
  - intermediate CD45 (at a level slightly higher than neutrophilic mylocytes),
  - low to intermediate CD11b,
  - intermediate CD13, and low CD33 with bright CD66b and no CD16 expression.
- Mature eosinophils show
  - increased levels of CD45 and CD11b with a decrease in CD33 and are negative for CD16.

Minor Bone Marrow Cell Subsets

- **Basophils** are the least common granulocyte subset (0.5% of total blood leukocytes and about 0.3% of nucleated BM cells in healthy individuals).

- Basophils are
  - positive for CD9, CD13, CD22 (dimmer than mature B-lymphocytes), CD25 (dim), CD33, CD38 (bright), CD45 (dimmer than lymphocytes and brighter than myeloblasts), and CD123 (bright), and are
  - negative for CD3, CD4, CD19, CD34, CD15, CD64, CD117, and HLA-DR.

- In some individuals, basophils are positive for CD11b.

Minor Bone Marrow Cell Subsets

- **Bone marrow mast cells** (BMMCs) are present in normal BM at a very low frequency 0.021% +/− 0.0025% of the nucleated cells.
- BMMCs are clearly identifiable on the basis of their light scatter properties and strong CD117 expression.
- Normal BMMCs are virtually always positive for the CD9, CD11c, CD29, CD33, CD43, CD44, CD45, CD49d, CD49e, CD51, CD54, CD71, and FcgRI antigens.
- Other markers such as CD11b, CD13, CD18, CD22, CD35, CD40, and CD61 display a variable expression in normal individuals.
- BMMC are negative for the CD34, CD38, and CD138 antigens.

Minor Bone Marrow Cell Subsets

- **Dendritic cells** (DCs) comprise two main subpopulations:
- conventional DCs (cDCs) and interferon-producing plasmacytoid (p) DCs.
  - Human cDCs are Lineage (Lin) negative HLA-DR+ cells that express high levels of CD11c and consist of a major blood dendritic cell antigen (BDCA)3− and a minor BDCA3+ population.
  - Human Lin-HLA-DR+ pDCs are defined by absence of CD11c expression and by high levels of CD123 (the IL-3Ra chain) and BDCA2.
  - The CD11c+HLA-DR+BDCA3− population can be further subdivided into CD16+ and CD16− populations.
- cDCs in lymphoid tissues arise from a distinct population of committed cDC precursors (pre-cDCs) that originate in bone marrow and migrate via blood.
- Spleen cDCs arise from a distinct population of Lin neg CD11c+ major histocompatibility complex (MHC) class II neg immediate cDC precursors (pre-cDCs).
- Pre-cDCs originate from bone marrow Lin neg CD117int FLT3+ CD115+ common DC progenitors.
  - The direct progenitor of pDCs is contained within the CD34 low compartment of cord blood, fetal liver, and bone marrow.
  - These progenitors (pro-pDCs) co-express CD45RA, CD4, and high levels of CD123.

Minor Bone Marrow Cell Subsets

- **NK cells** are positive for CD2 & CD7 but negative for CD3 and CD5.
- In humans, there are two major subsets of NK cells:
  - one expressing high levels of CD56 and low or no CD16 (CD56hiCD16+/−),
  - the second that is CD56+CD16hi
  - CD56hiCD16+/− cells display relatively lower cytolytic activity and produce more cytokines than the CD56+CD16hi cells.
- The immature NK cells developing from committed NK-cell precursors are defined by expression of CD161 (NKR-P1).
- These cells do not express CD56 or CD16.
  - Immature NK cells can be induced to express these markers as well as the activating and inhibitory receptors, CD94 (NKG2A) and killer inhibitory receptors (KIR), upon culture with stromal cells and cytokines such as IL-15 or Flt3-L.
- A total of 30% to 60% of CD56dim CD16bright NK cells in healthy adults express CD57, which is not expressed on immature CD56bright NK cells.
  - CD57+ NK cells express a repertoire of NK-cell receptors, suggestive of a more mature phenotype, and proliferate less when stimulated with target cells and/or cytokines.