Introduction to Flow Cytometry

-- BD FACSCanto II™

Daisy Kuo
Assistant Product Manager
E-mail: daisy_kuo@bd.com
BDBiosciences

Outline

- Basic Concept of Flow Cytometry
- FACSCanto II System Introduction
- Application Examples

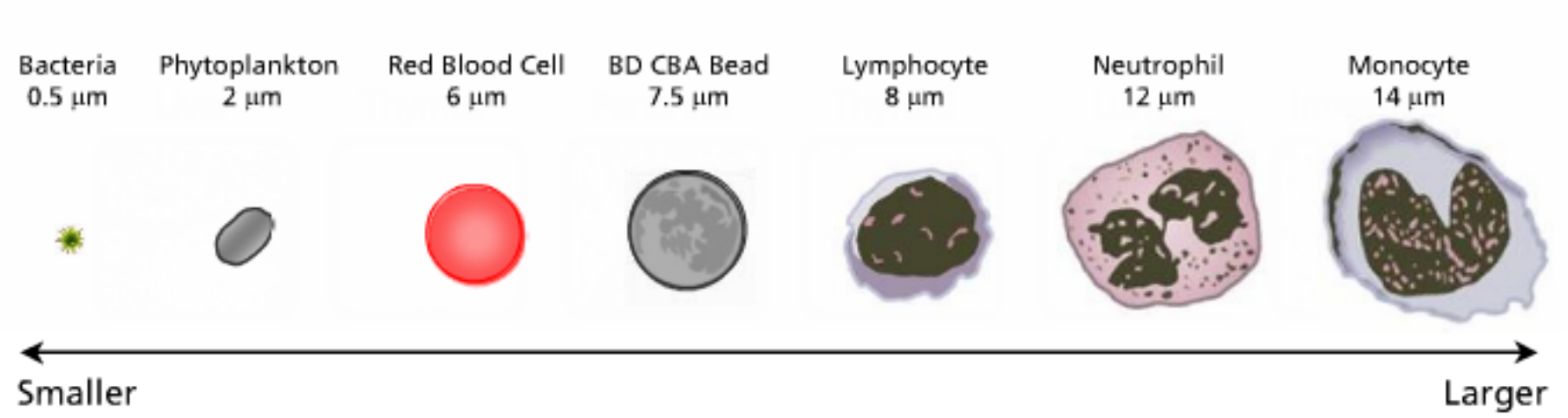
What is Flow Cytometry?

- Flow = Fluid
- Cyto = Cell
- Metry = Measurement

- A variety of measurements are made on cells, cell organelles, and other objects **suspended in a liquid** and flowing at rates of **several thousands per second** through a flow chamber.

Particle Size

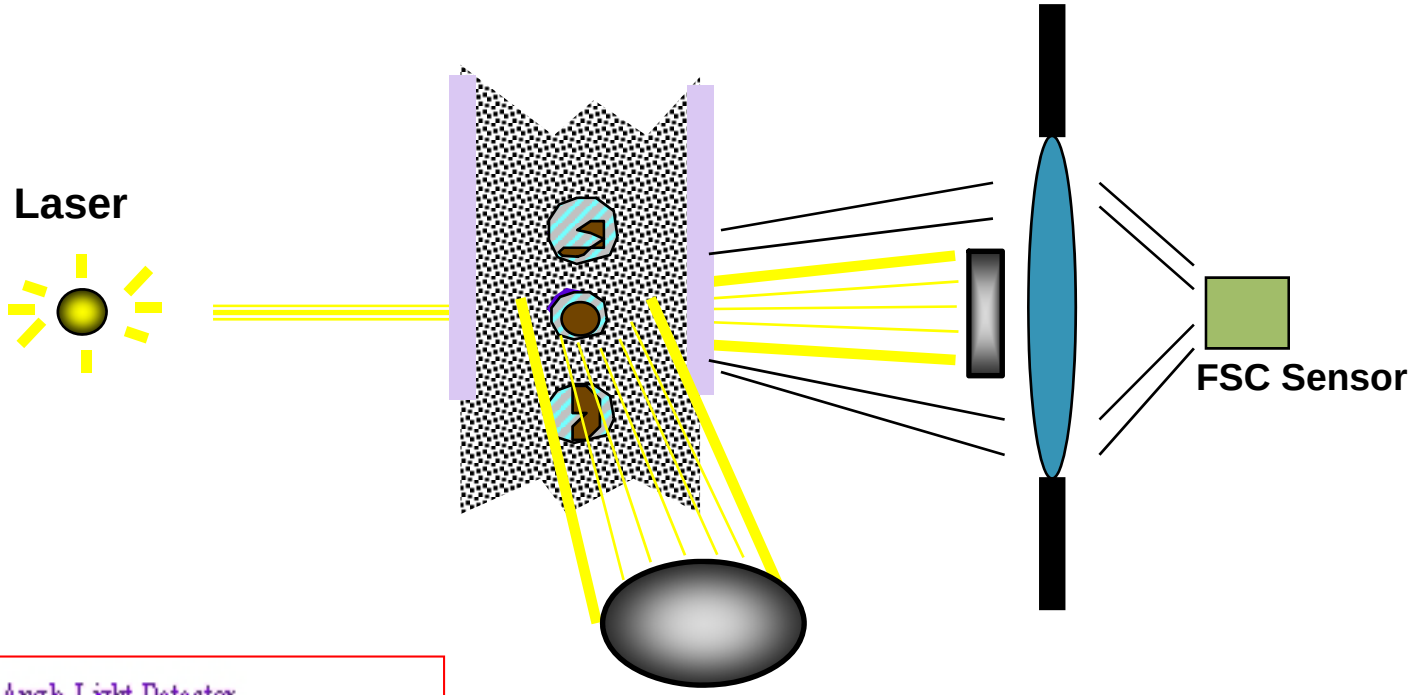
- Detection range: 0.5~50um

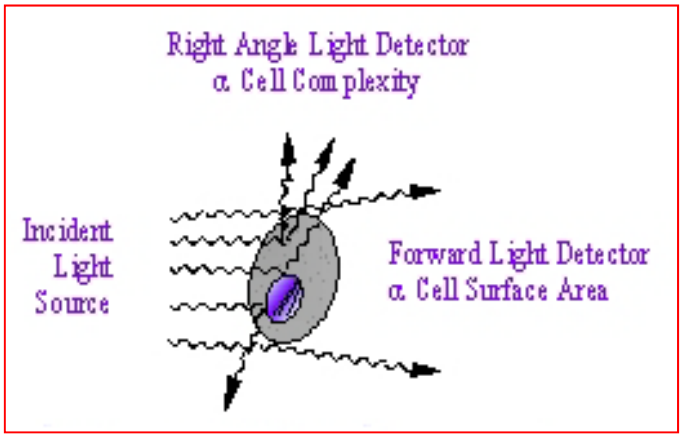


What Can a Flow Cytometer Tell Us About a Cell?

- Its relative size (Forward Scatter—FSC)
- Its relative granularity or internal complexity (Side Scatter—SSC)
- Its relative fluorescence intensity

Scatter Light





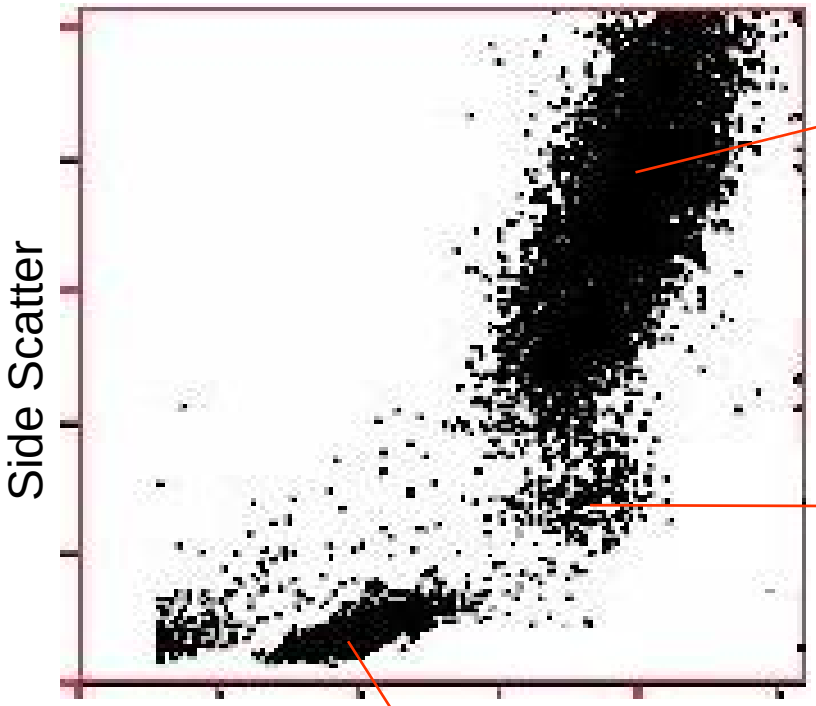
Forward Scatter—diffracted light

- **Related to cell surface area**
- **Detected along axis of incident light in the forward direction**

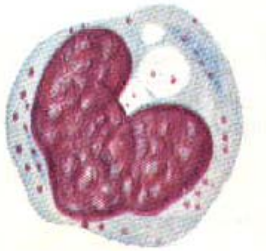
Side Scatter—reflected and refracted light

- **Related to cell granularity and complexity**
- **Detected at 90° to the laser beam**

Lysed Whole Blood



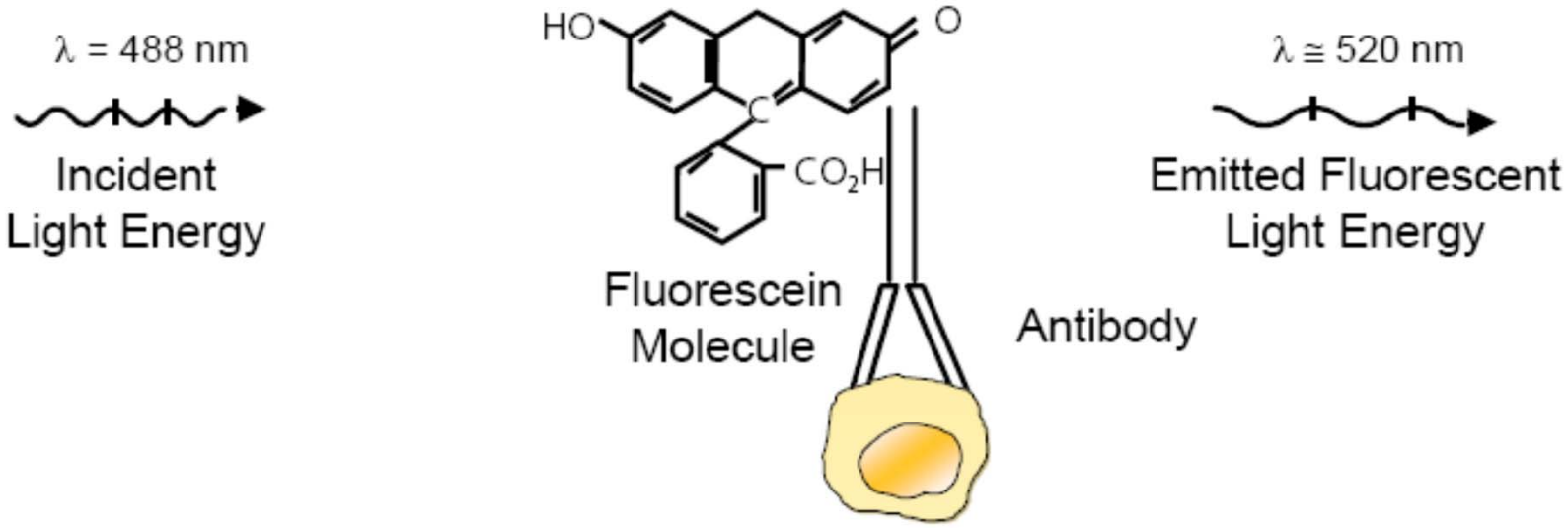
Neutrophils



Monocytes

Lymphocytes

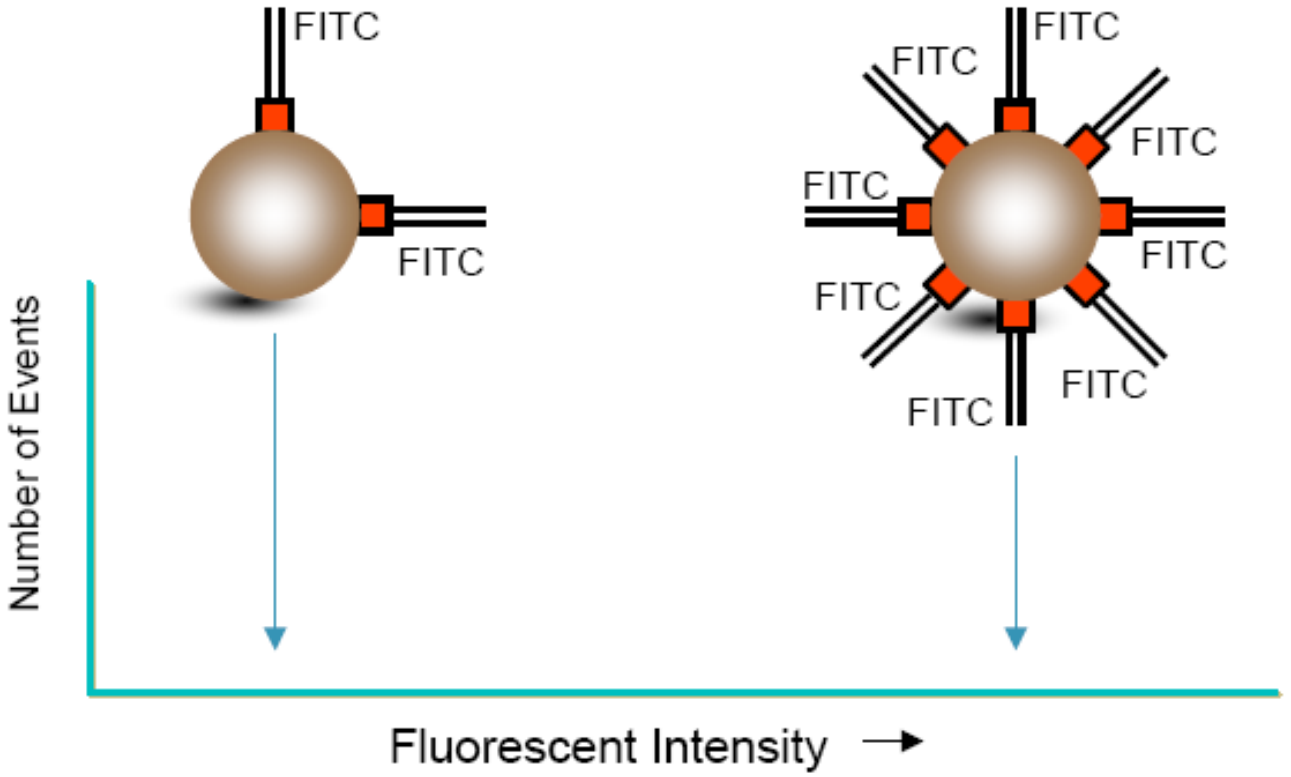
Fluorescence Light



- The fluorochrome absorbs energy from the laser.
- The fluorochrome releases the absorbed energy by:
  - vibration and heat dissipation.
  - emission of photons of a longer wavelength.

Fluorescence

Emitted fluorescence intensity proportional to binding sites



BD FACSCanto II™

Subsystems

Fluidics

To introduce and focus the cells for interrogation.

Optics

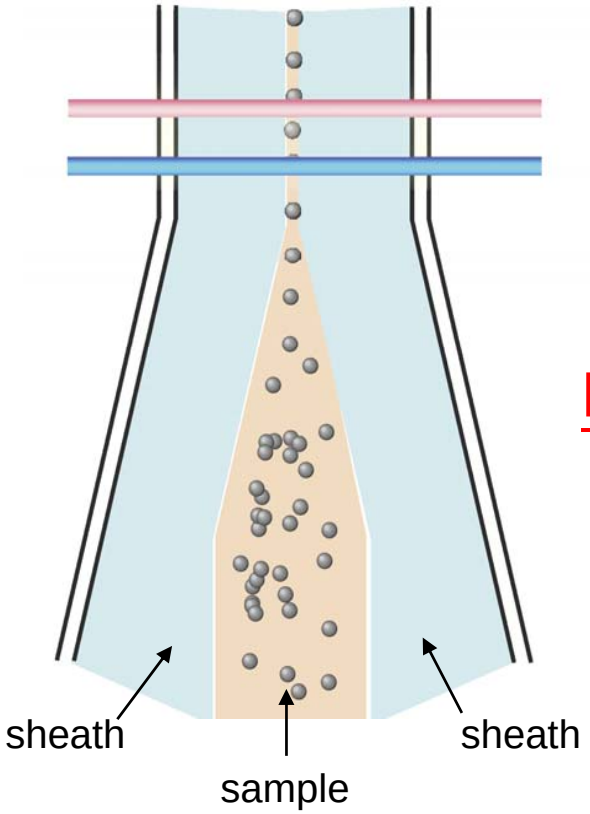
To generate and collect the light signals.

Electronics

To convert the optical signals to proportional digital signals, process the signals, and communicate with the computer.

Sample Flow

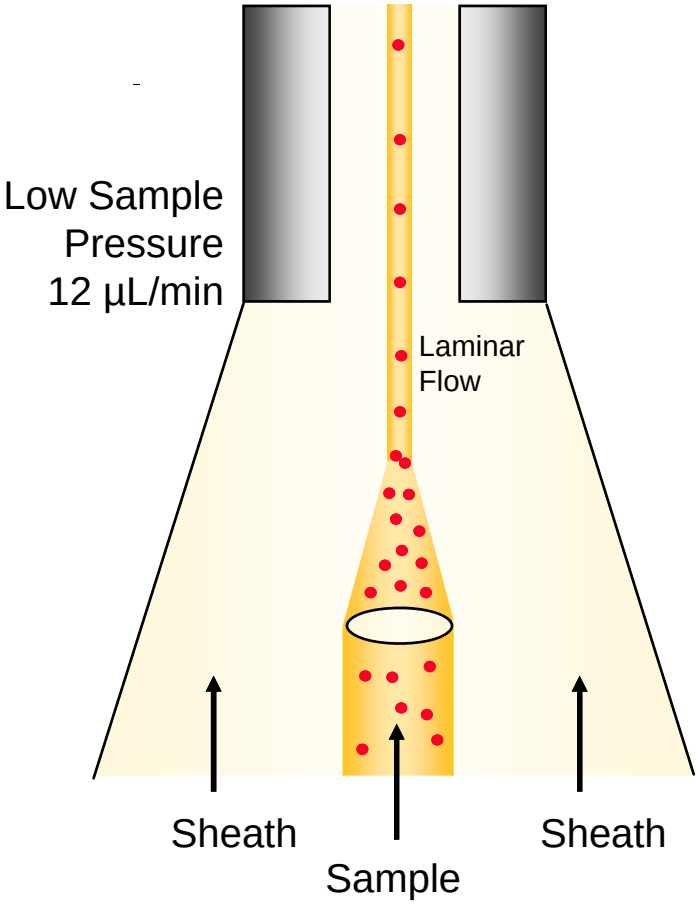
Excitation
Lasers



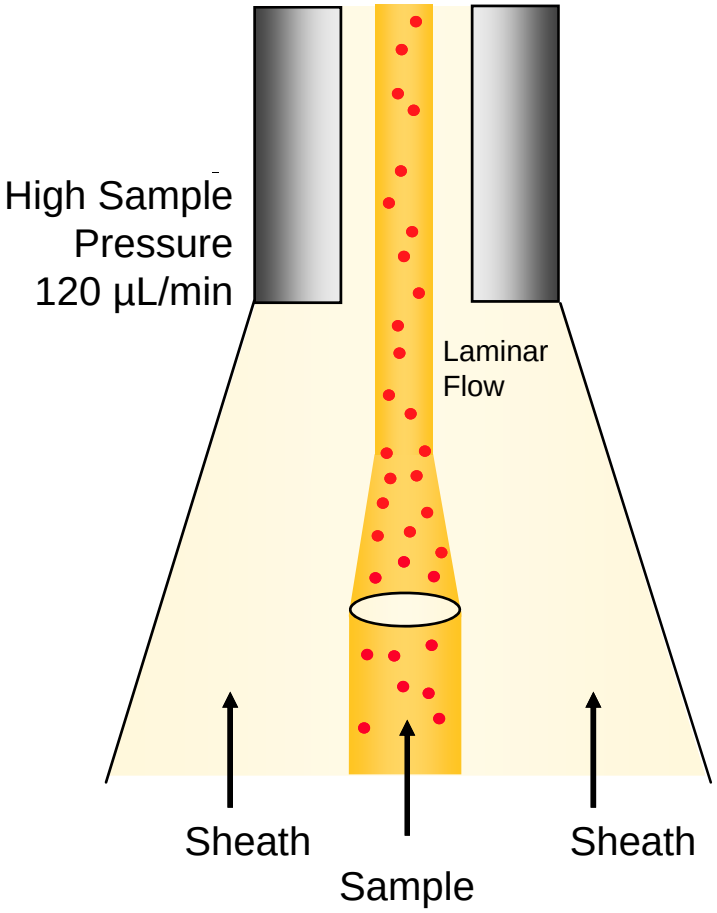
Hydrodynamic Focusing

Sample Differential

Low Differential Pressure



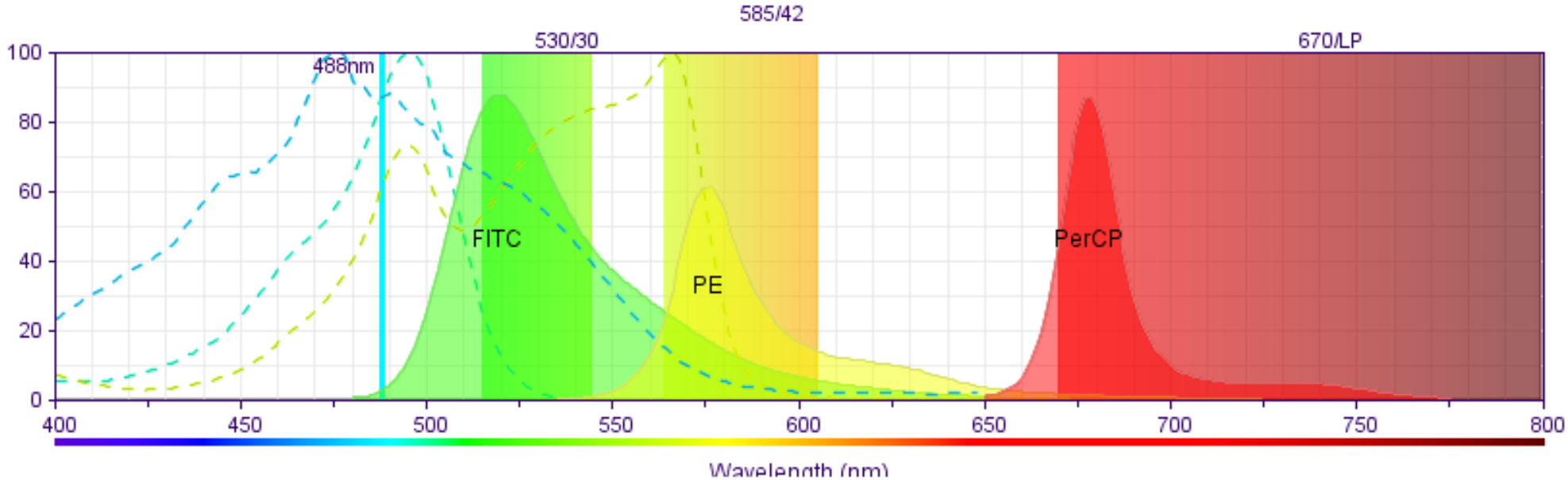
High Differential Pressure



Optics

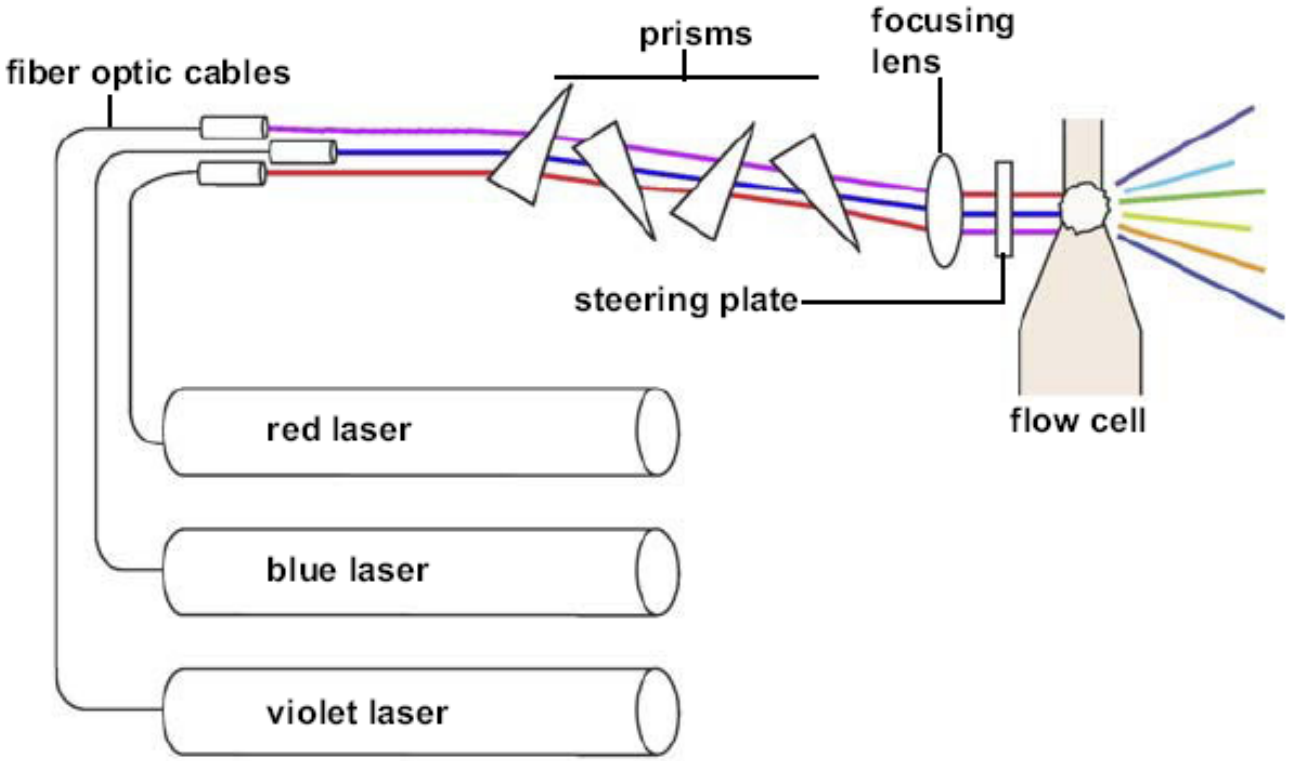
- Excitation optics
  - Lasers
  - Lenses to shape and focus the laser beam
- Collection optics
  - A collection lens to collect light emitted from the article-laser beam interaction
  - A system of optical mirrors and filters to route specified wavelengths of emitted light to designated optical detectors

Fluorochrome Spectra

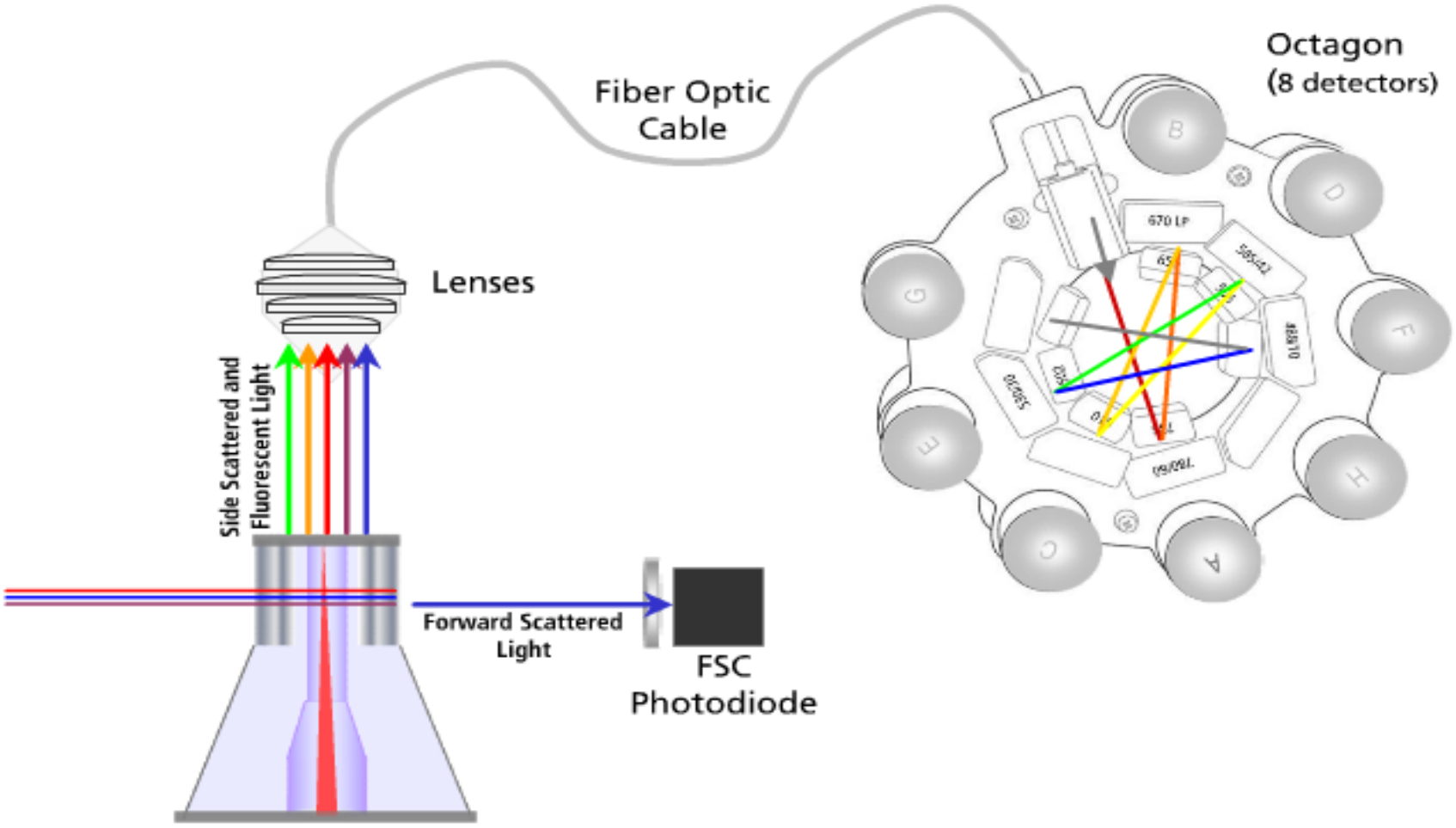


Excitation Optics

- Spatially separated laser beams lower the possibility of fluorescence spillover



Collection Optics



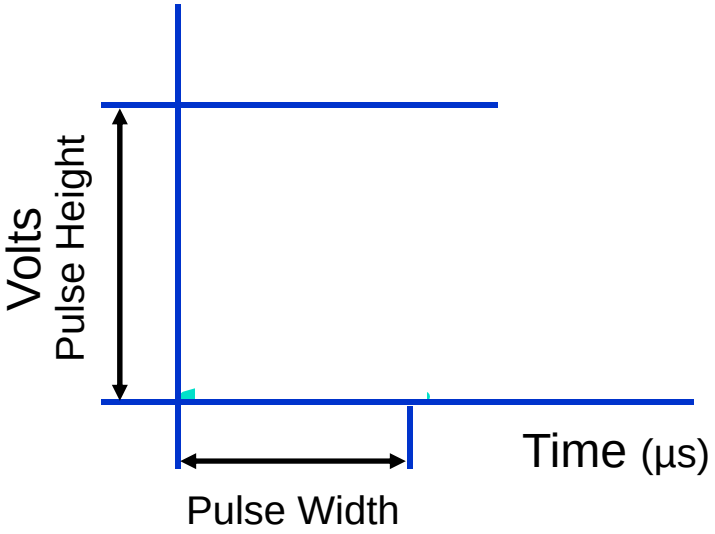
Optics-- Configuration

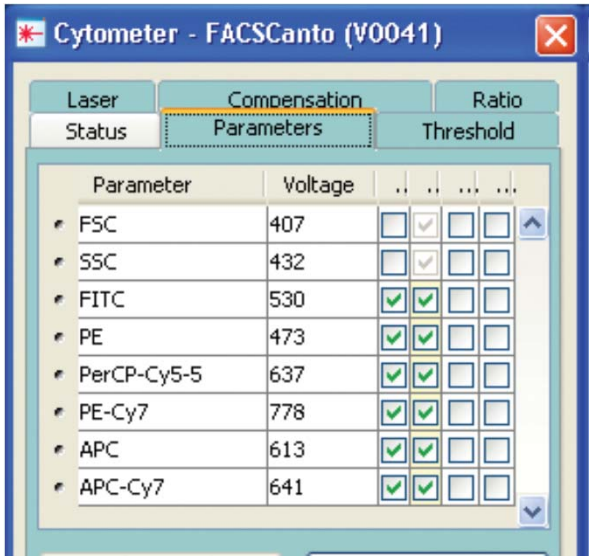
Laser	Primary Fluorochrome	PMT	Dichroic Mirror	Bandpass Filter	Other Fluorochrome
488 nm (blue)	Side Scatter	F	none	488/10	
	FITC	E	502LP	530/30	GFP
	PE	D	556LP	585/42	PI
	—	C	610LP	blank optical holder	
	PerCP or PerCP-Cy5.5	B	655LP	670LP	PI, PE-Cy5.5, 7-AAD
	PE-Cy7	A	735LP	780/60	
633 nm (red)	APC	C	none	660/20	Alexa Fluor® 633
	—	B	685LP	blank optical holder	
	APC-Cy7	A	735LP	780/60	
407 nm (violet)	Pacific Blue™	B	none	450/50	DAPI, Hoechst Dye
	AmCyan	A	502 LP	510/50	Cascade Blue®

Electronics

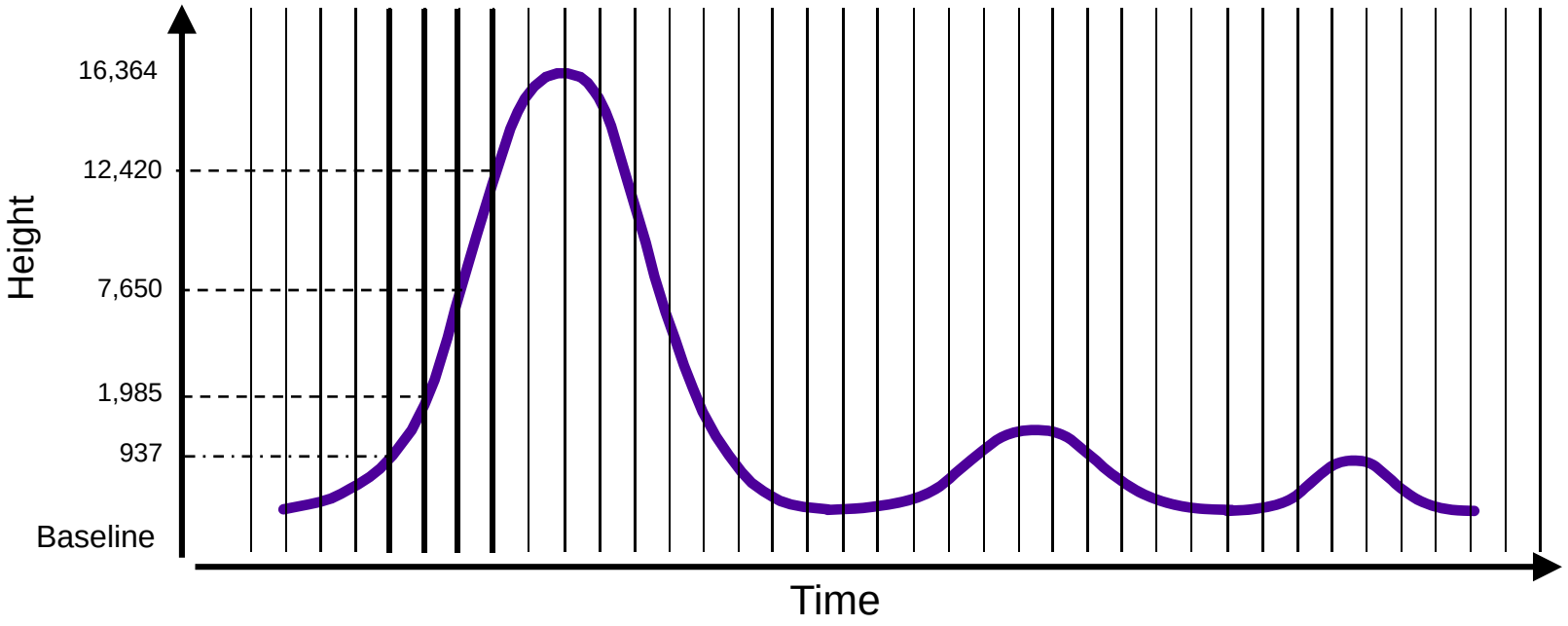
- PMTs and preamps convert photons to voltage pulses.
- Analog-to-digital converters translate analog signals to proportional digital signals.
- Compute area and height for each pulse.
- Perform compensation and calculate ratios and width.
- An embedded computer interfaces with the computer workstation for data transfer.

Creation of a Voltage Pulse





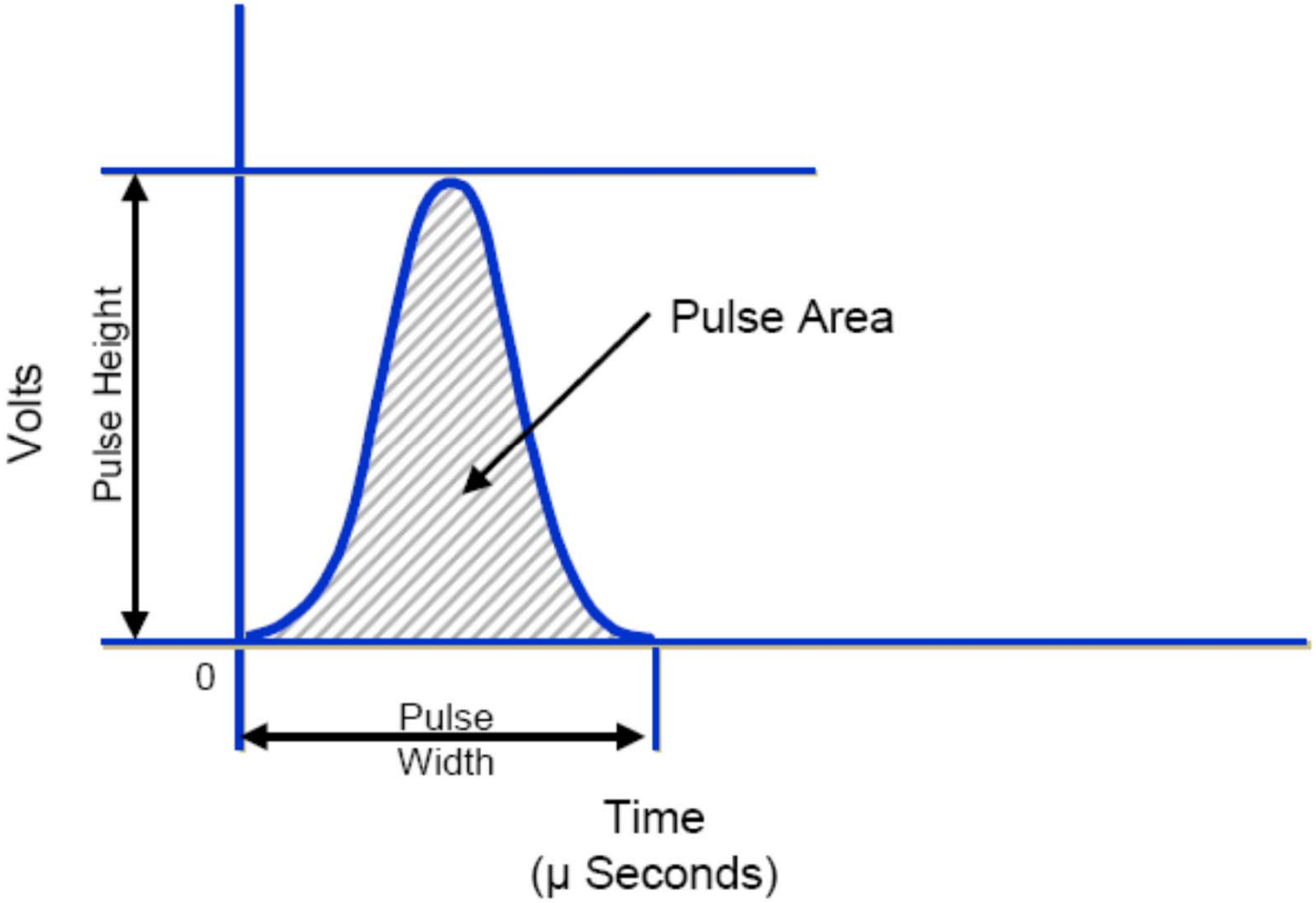
Analog-to-Digital Converter



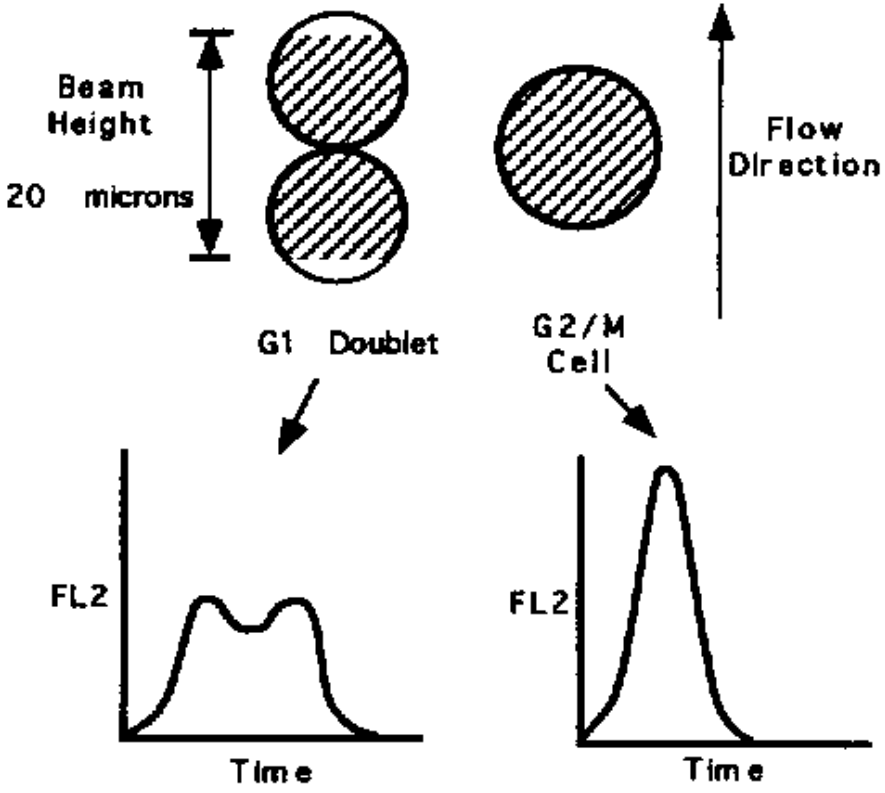
318	367	319	375	423	432	937	1985	7650	12420	15300	13256	5791	2471	842	433	331	311	308	376	349	414	823	1373	903	514	338	418	307	317	353	313	703	403	378	308	406	405	303

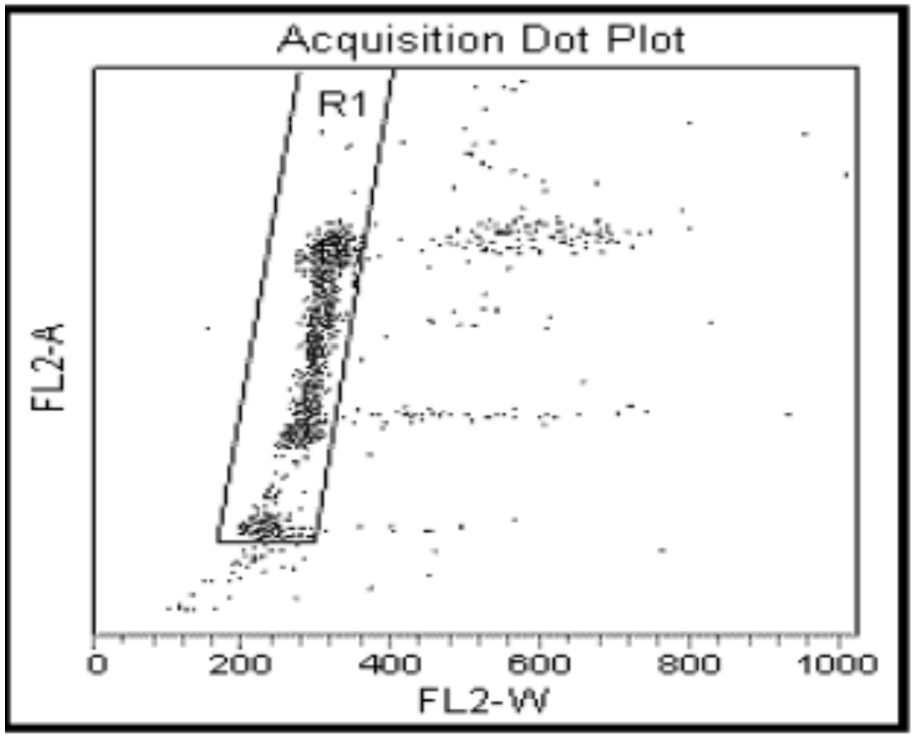
Digitized values

Quantification of a Voltage Pulse



Doublet Discrimination

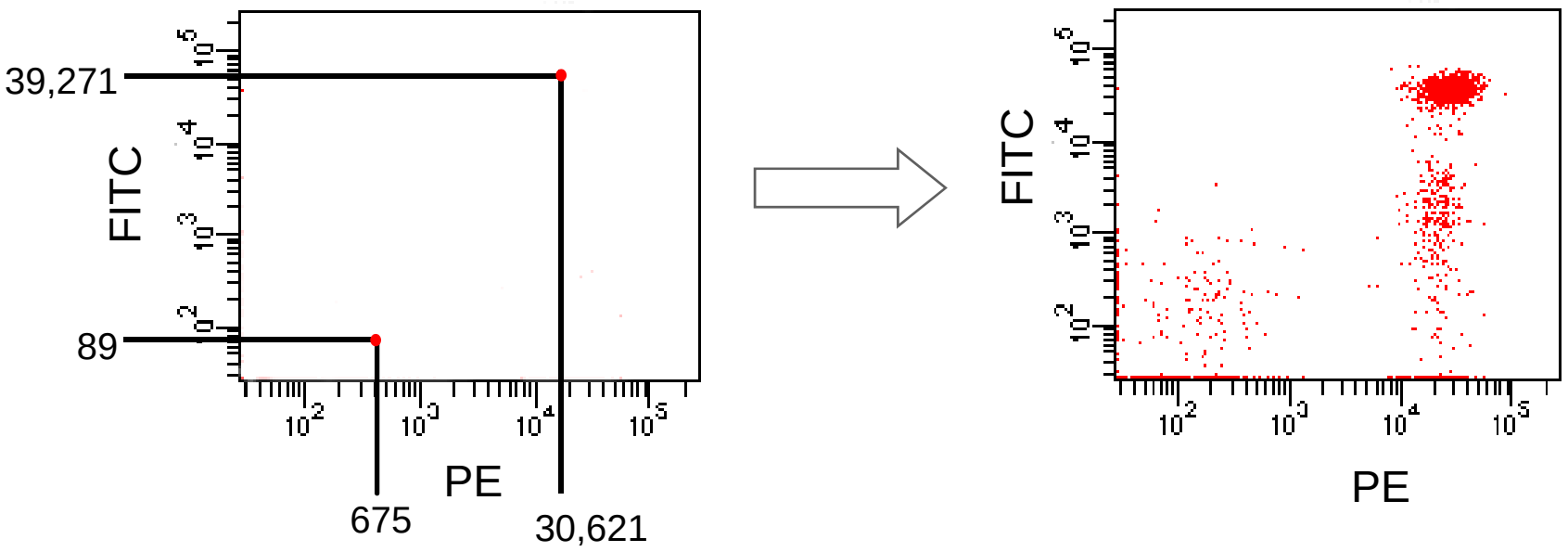




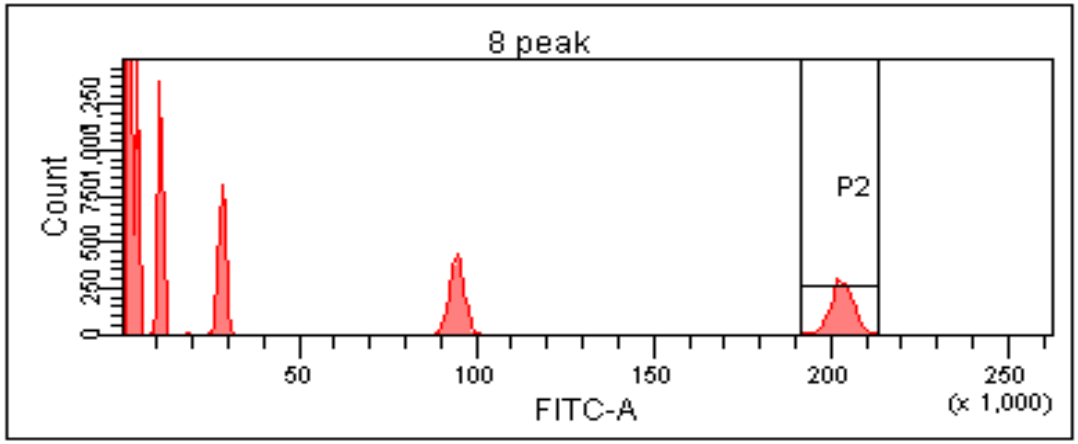
Data Storage

List-Mode Data

	Time	FSC	SSC	FITC	PE
Event 1	0	60	120	89	675
Event 2	10	160	65	39,271	30,621
Event 3	30	650	160	22,688	6,189

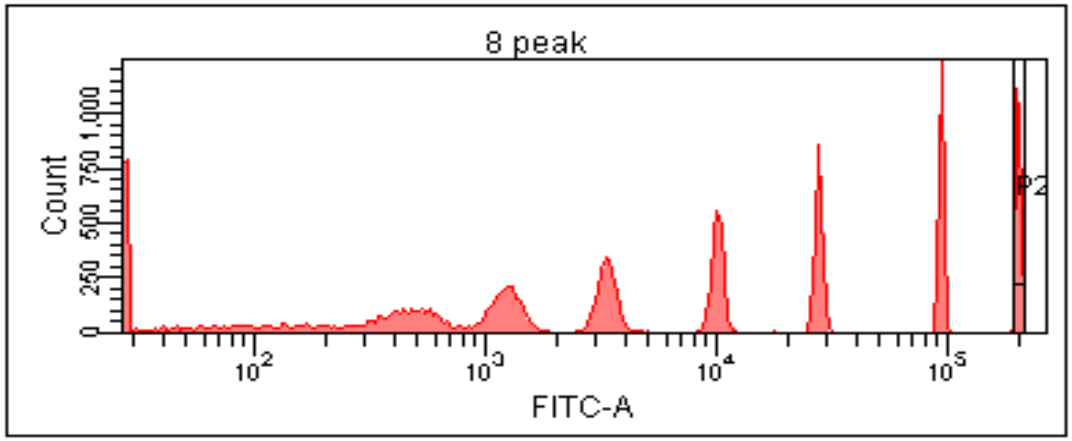


Data Display: Linear vs Log



Tube Name: 8 peak

Population	#Events	FITC-A Mean
P1	16,589	42,948
P2	2,124	203,334



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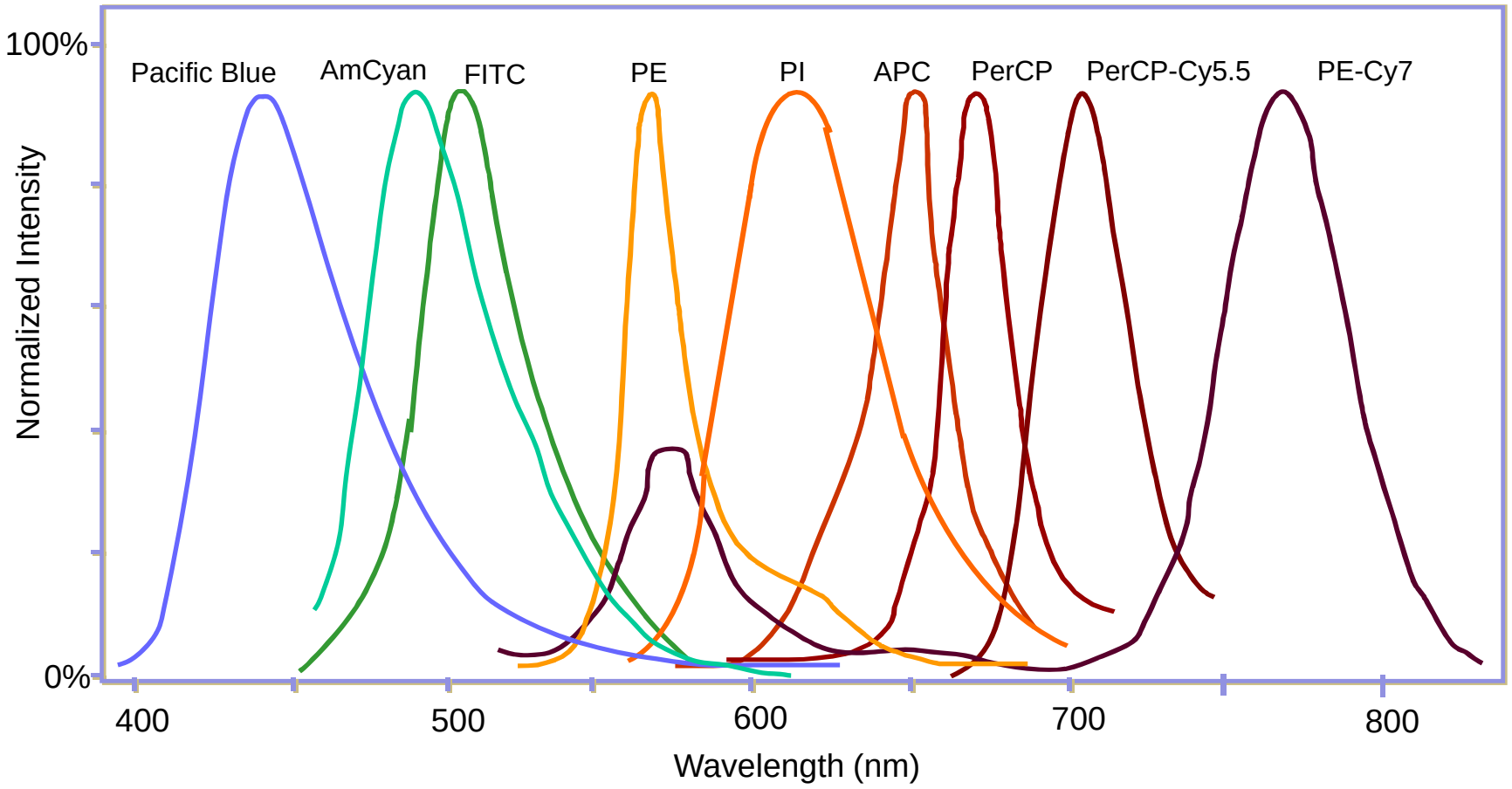
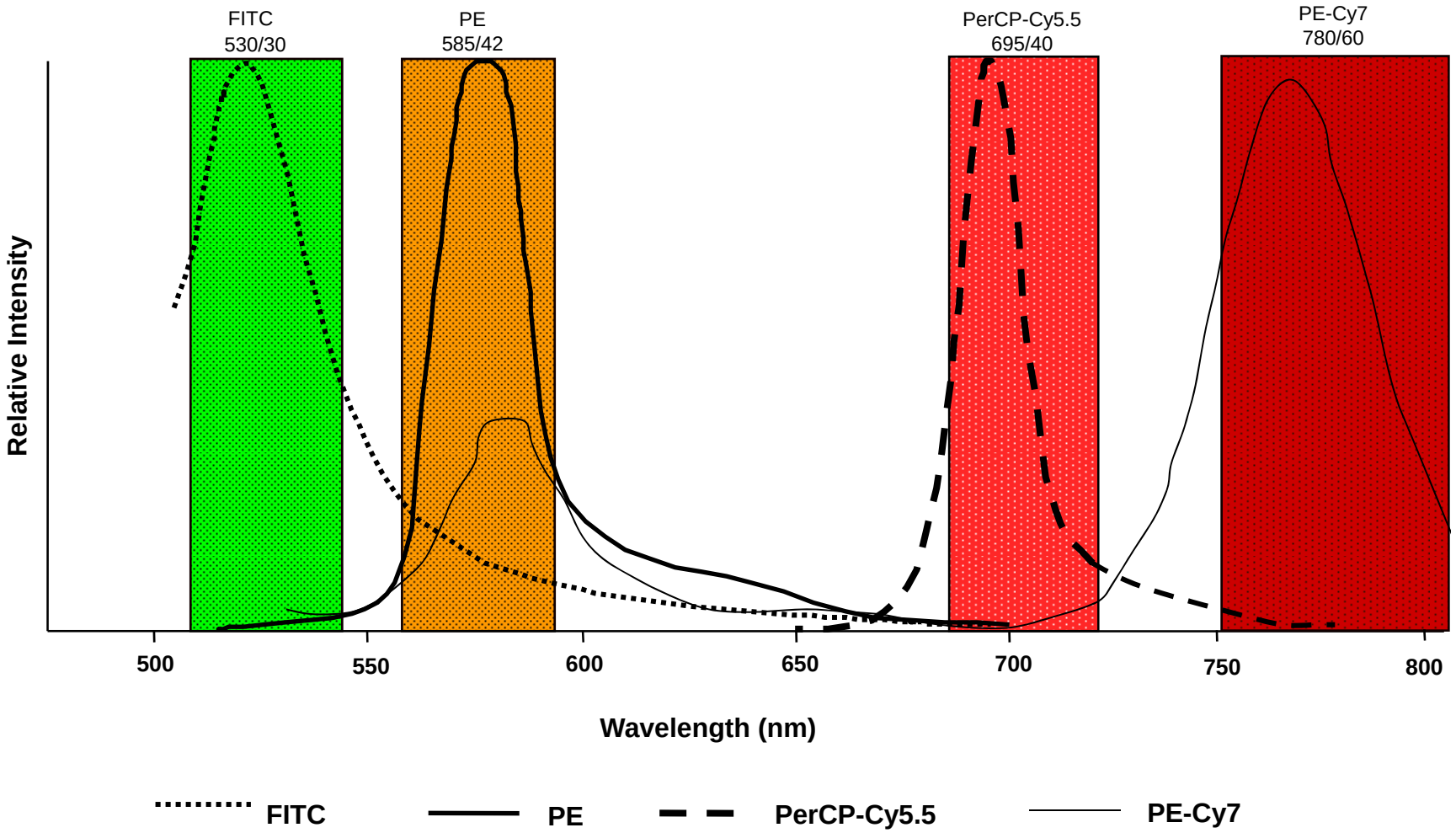
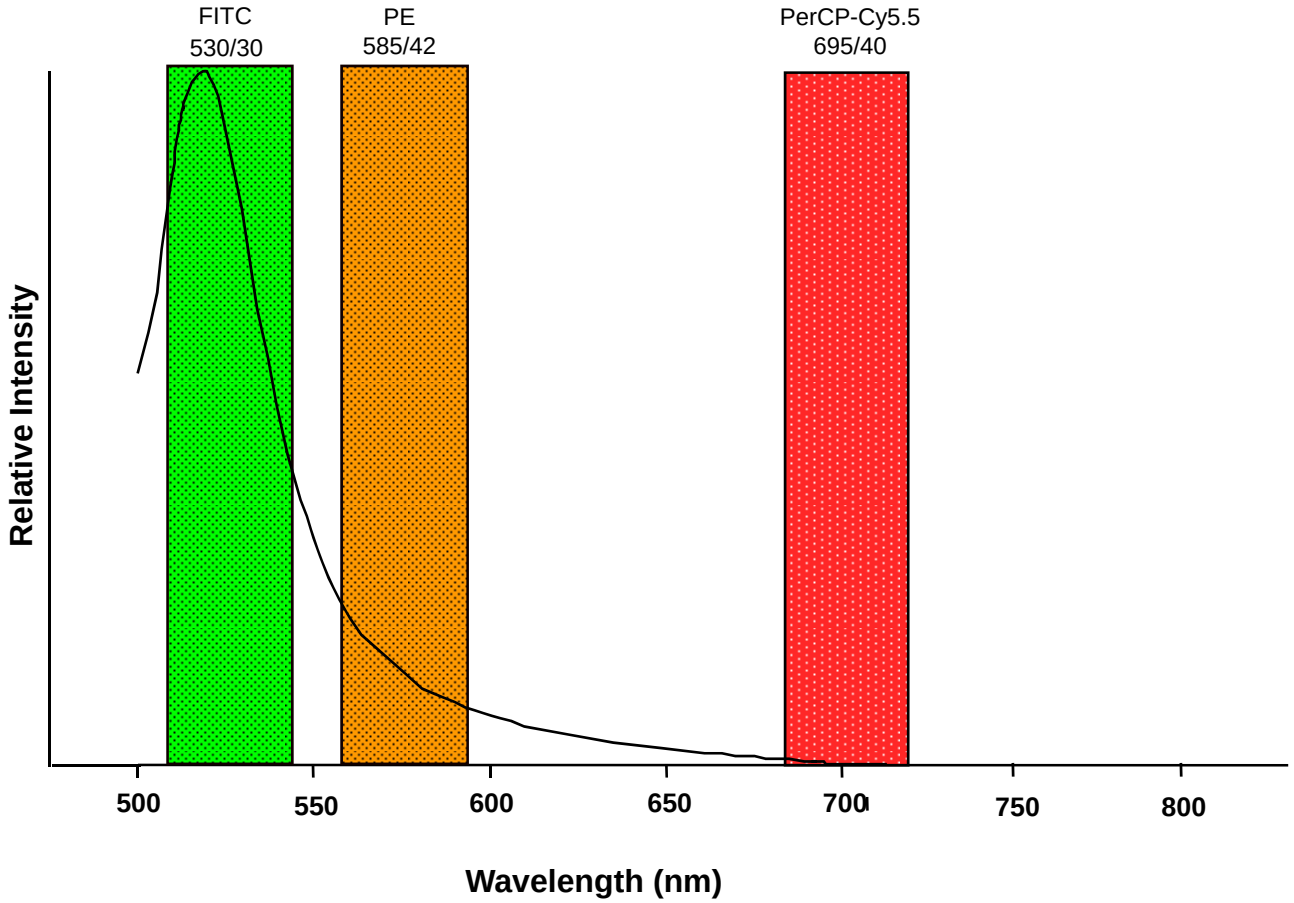
Spectral Overlap– Compensation Theory

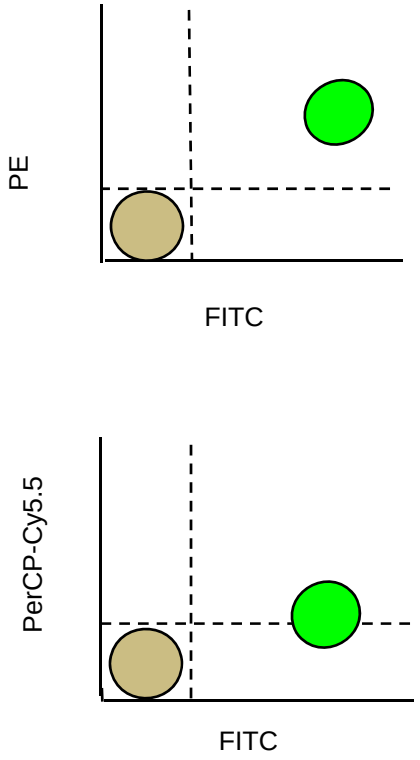


Spillover


FITC
530/30
PE
585/42
PerCP-Cy5.5
695/40
PE-Cy7
780/60
Relative Intensity
500
550
600
650
700
750
800
Wavelength (nm)
FITC
PE
PerCP-Cy5.5
PE-Cy7

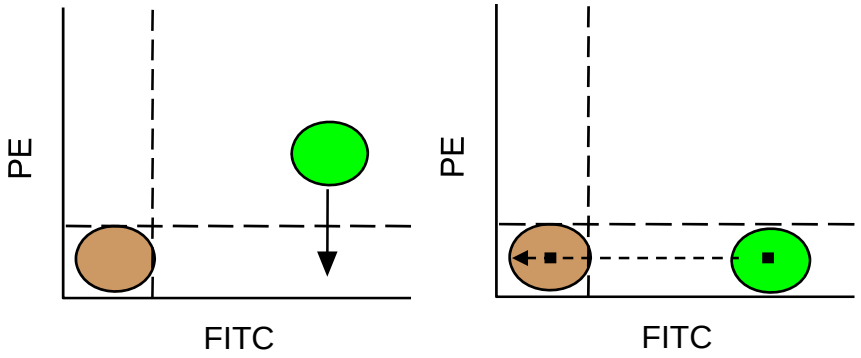
FITC Spillover



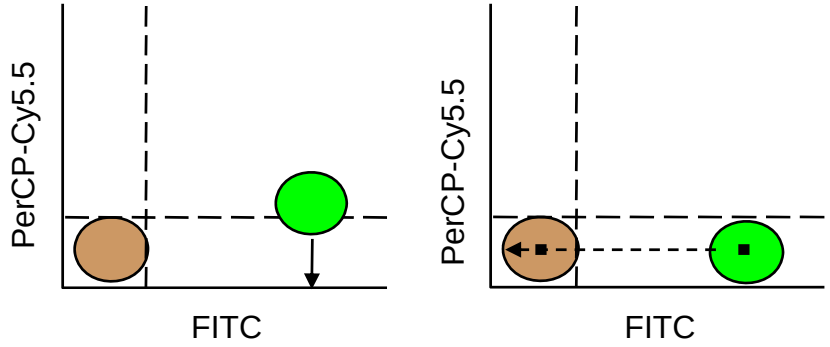


FITC Compensation

PE-%FITC

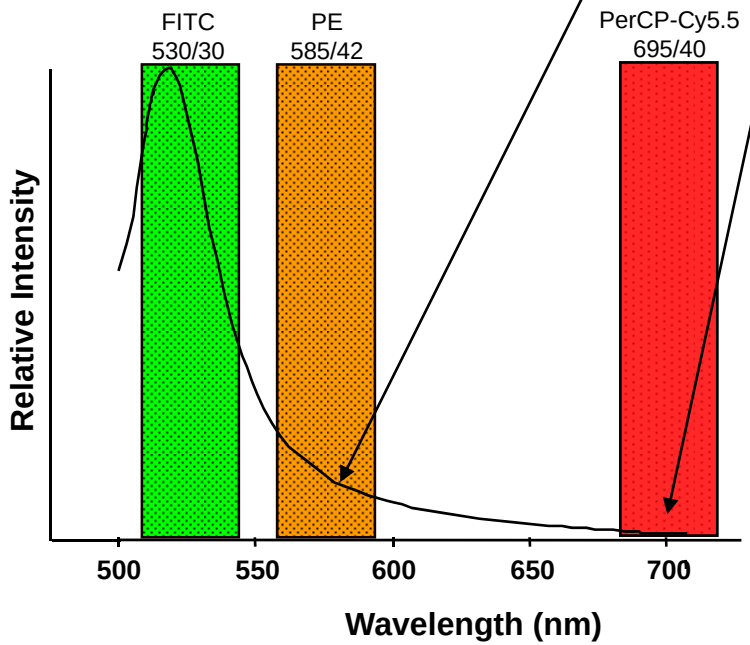


PerCP-Cy5.5-%FITC



To lower cluster, increase value.

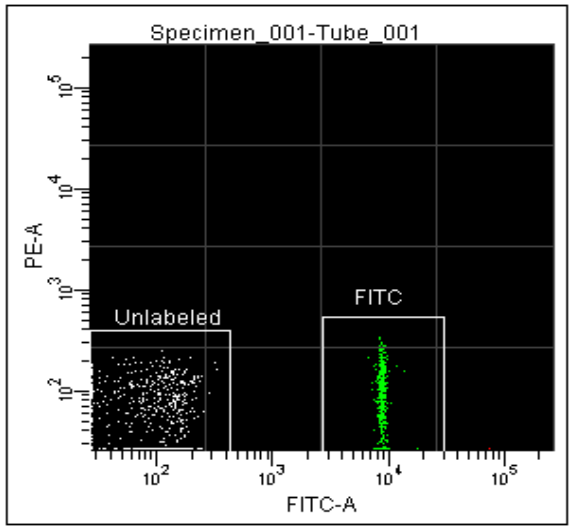
	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	20.10
•	PerCP-Cy5-5	FITC	0.90



Compensation Examples

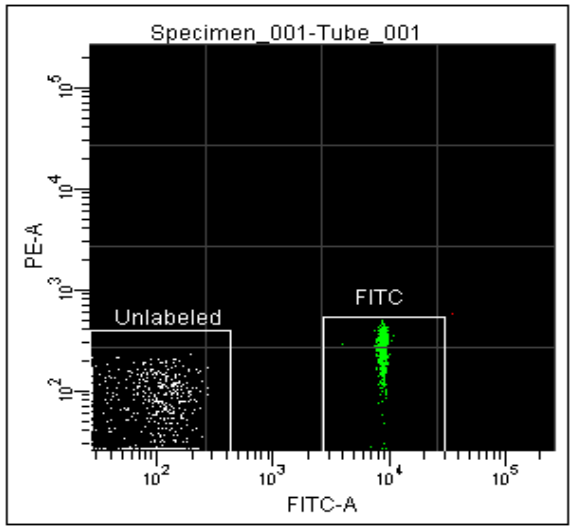
Incorrect Compensation

Correct Compensation



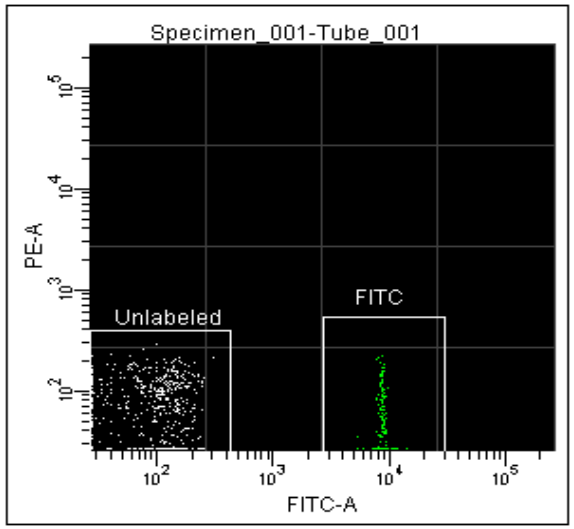
Population	PE-A Mean
Unlabeled	77
FITC	73

Undercompensation



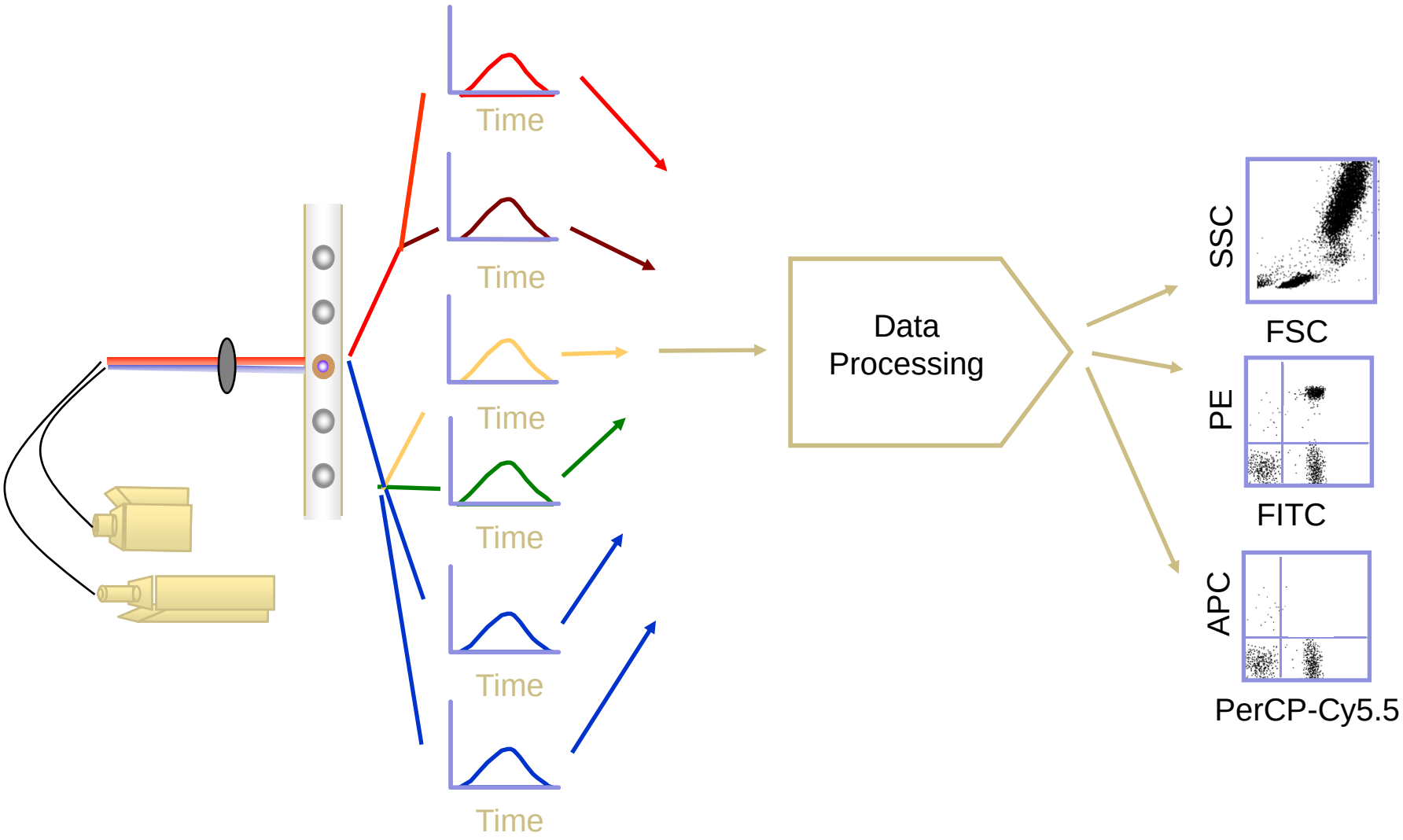
Population	PE-A Mean
Unlabeled	72
FITC	245

Overcompensation



Population	PE-A Mean
Unlabeled	70
FITC	-54

Review

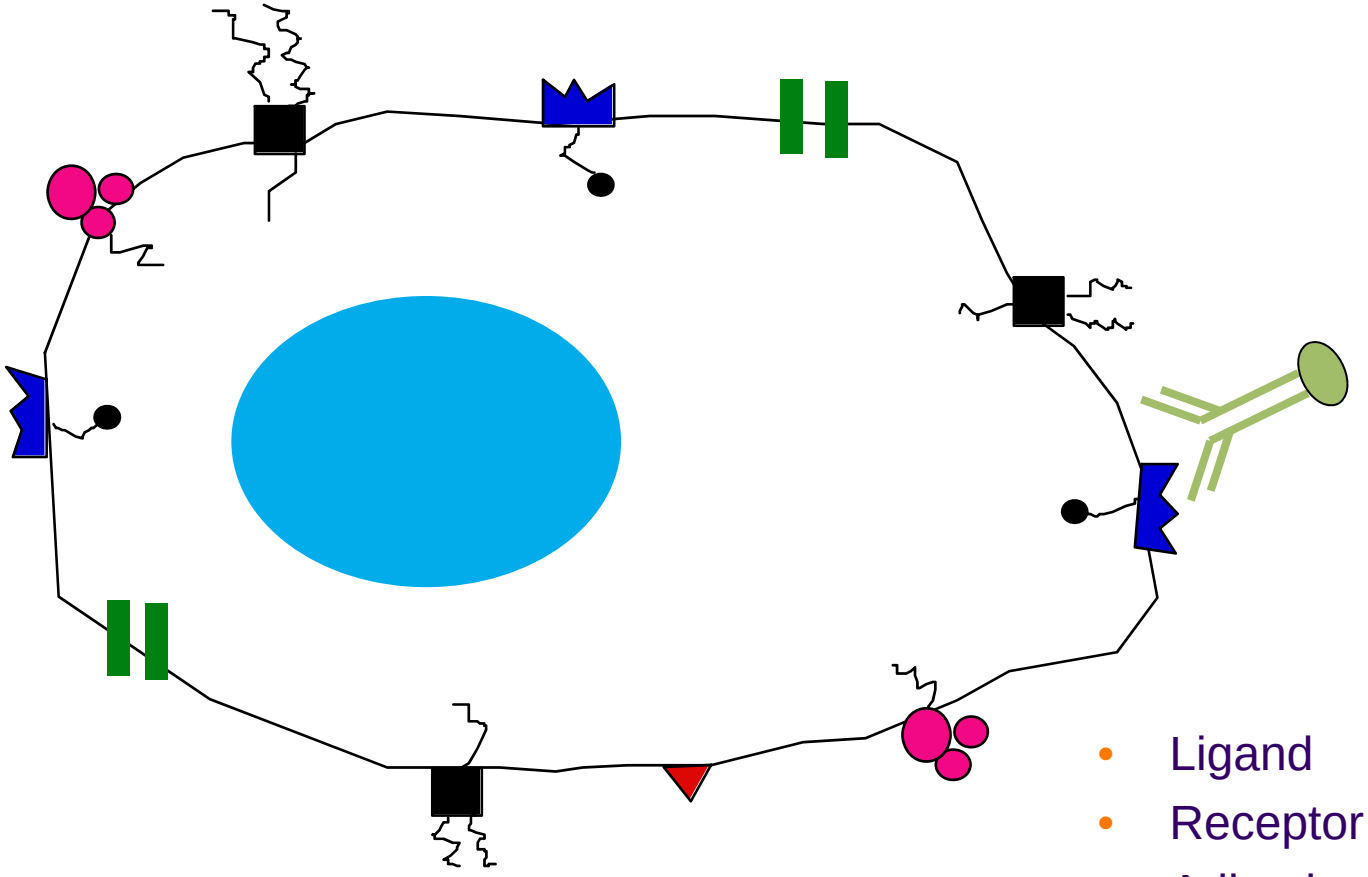

Time
Time
Time
Time
Time
Time
Data
Processing
SSC
FSC
PE
FITC
APC
PerCP-Cy5.5

Application Examples

Applications

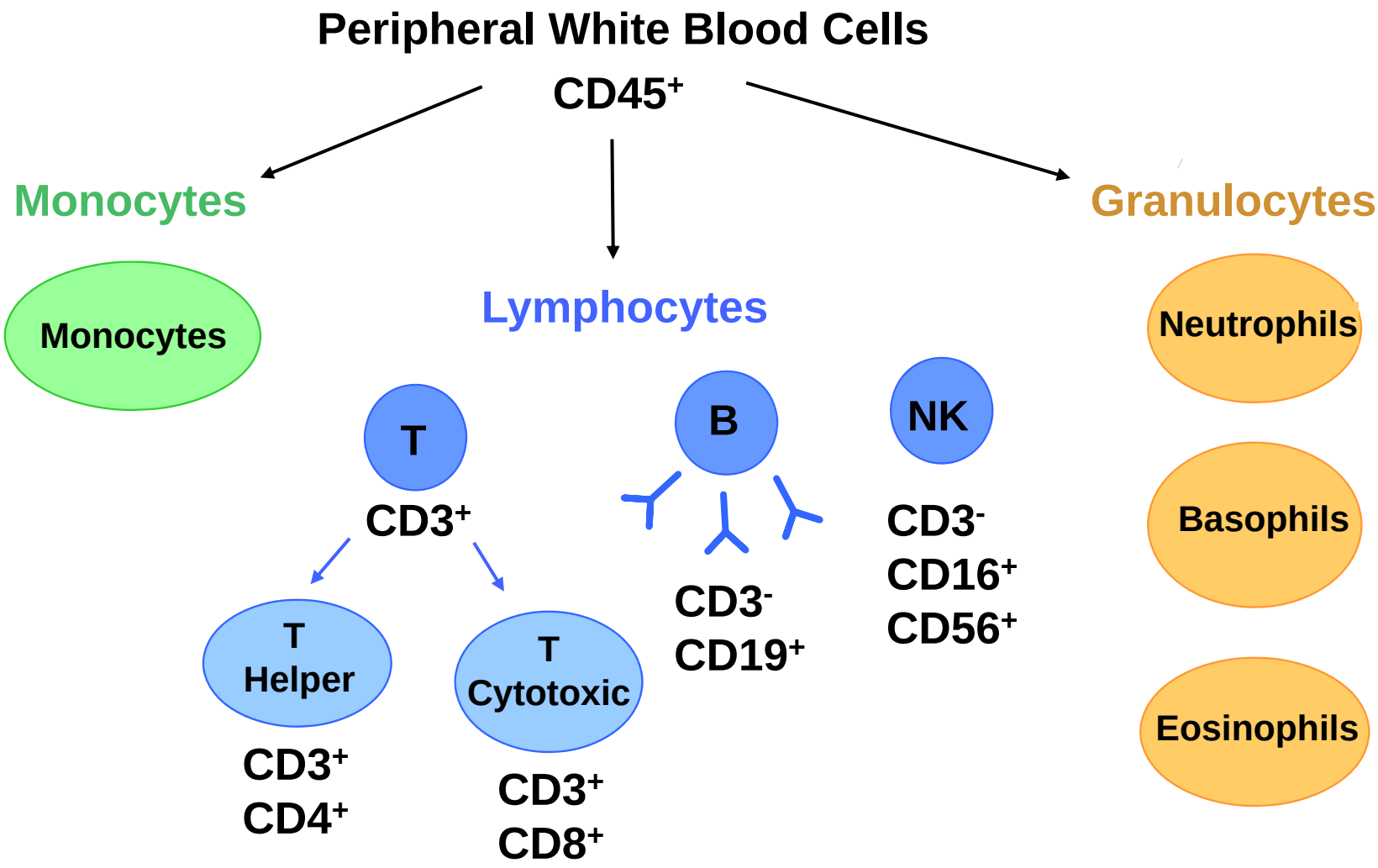
- Phenotype Analysis (Cell Surface Antigens/Markers)
- Intracellular Analysis
  -- Eg. Cytokines, Signal Transduction molecules…etc.
- DNA Analysis
  -- Eg. Viability, Cell cycle, Apoptosis…etc.
- Cell Fuction Analysis
  -- Eg. Free radicals, Ca^{2+}, Reporter genes…etc.
- CBA (Cytometric Bead Array)
- Others

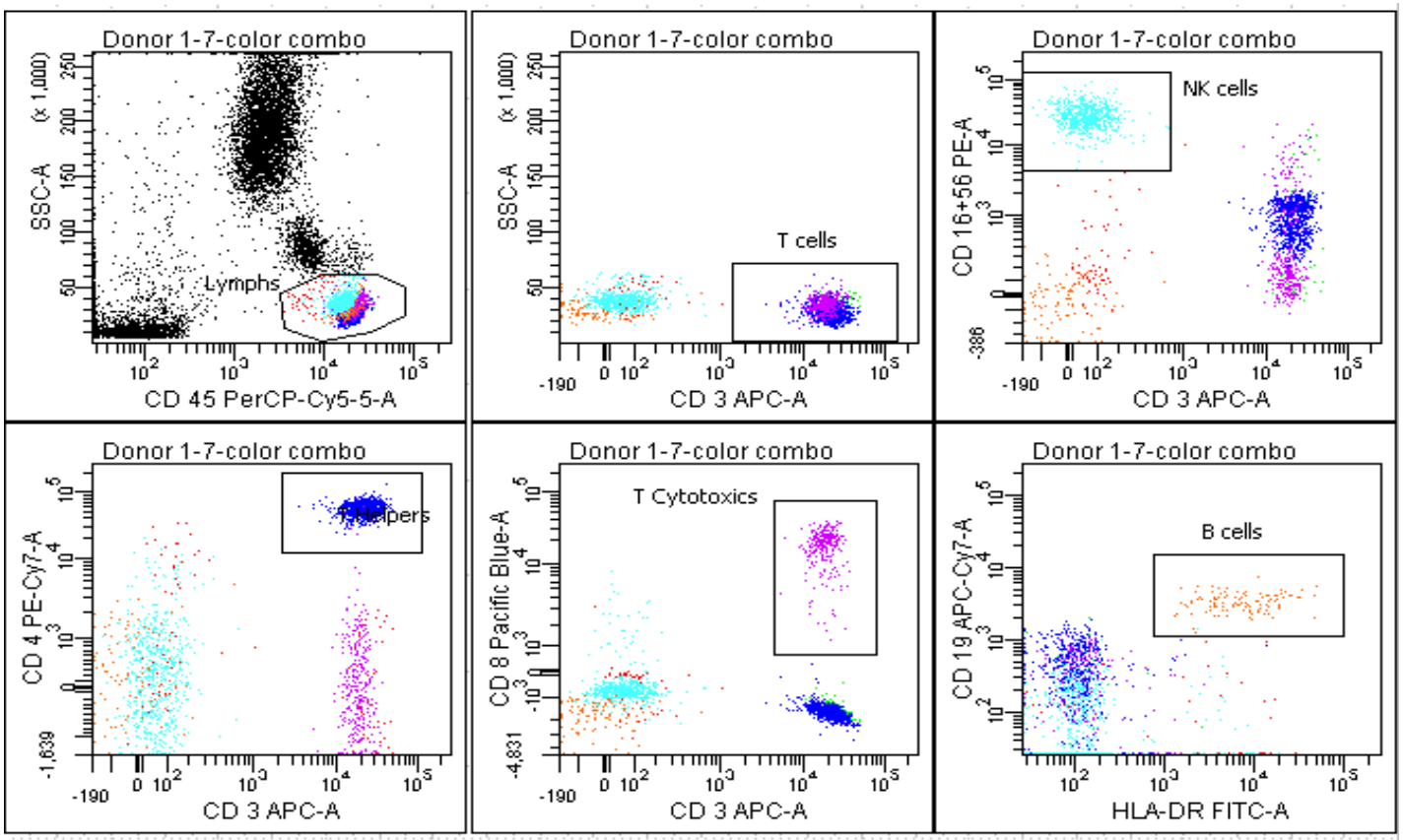
Phenotype Analysis



- Ligand
- Receptor
- Adhesion molecule
- …etc

Lymphocyte Immunophenotyping



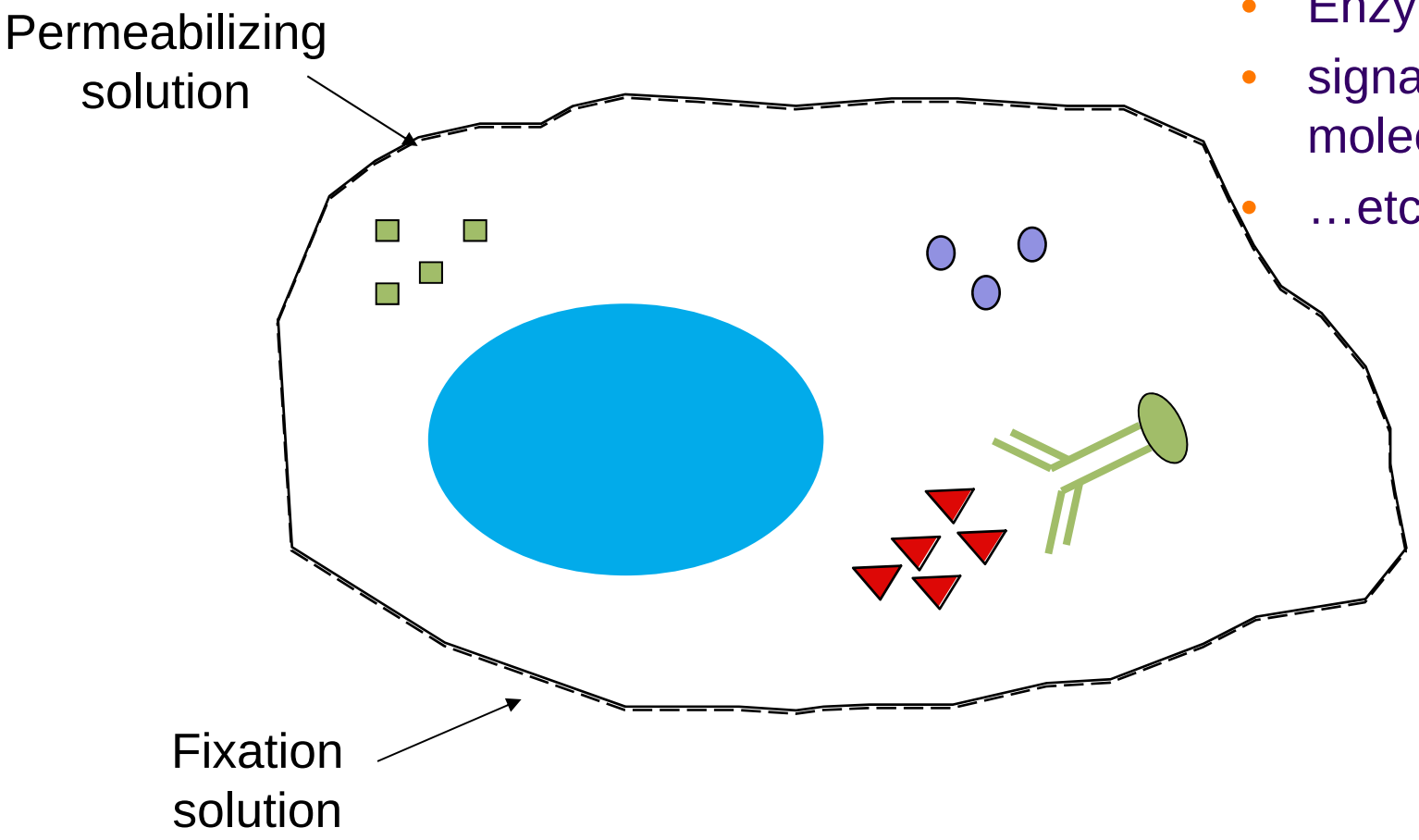


Tube: 7-color combo

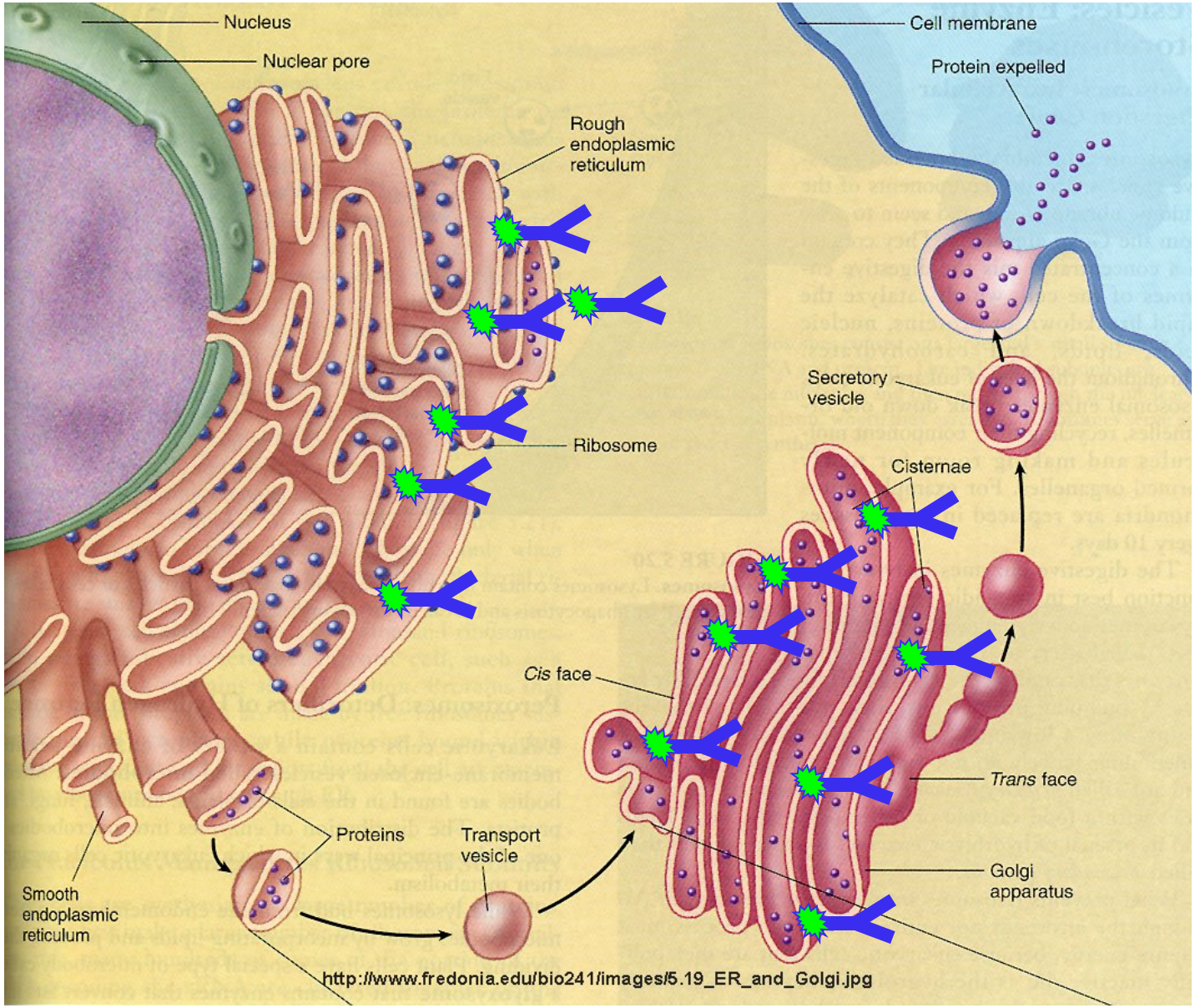
Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
Lymphs	1,948	19.5	19.5
T cells	1,195	61.3	12.0
T Helpers	860	72.0	8.6
T Cytotoxics	300	25.1	3.0
B cells	111	5.7	1.1
NK cells	586	30.1	5.9

Intracellular Analysis

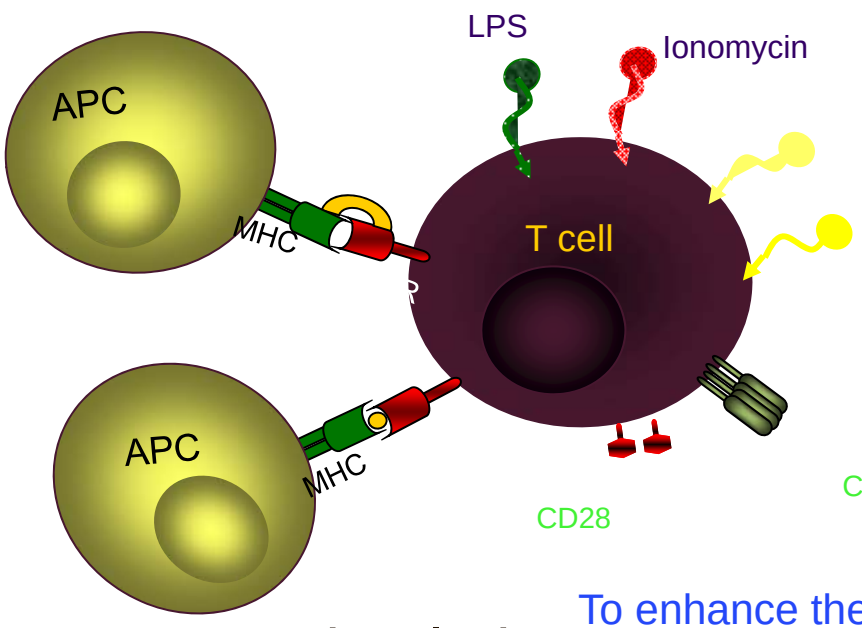
- Cytokine
- Enzyme
- signal transduction molecule
- …etc.



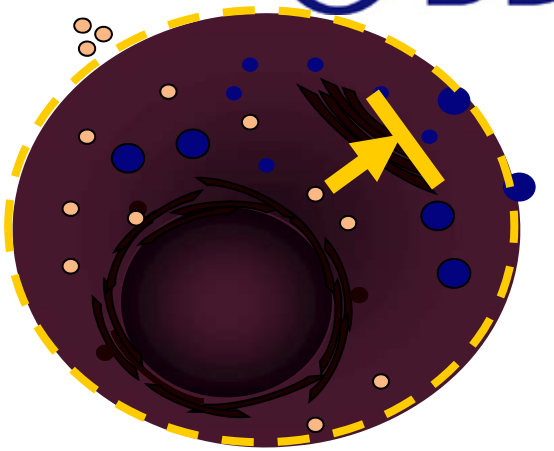
Cytokine Detection



Picture From www.fredonia.edu



Con A



Stimulation

To enhance the accumulation of intracellular cytokines.

Secretion stop

(Brefeldin A or Monensin)

Only *in vitro*

Monensin: Cytokines accumulate in the ER
Brefeldin A: in Golgi complex.

To maintain structural integrity.
Formaldehyde or glutaraldehyde
Keep the protein structure and doesn't change the (accessibility of the) epitopes too much

Fixation

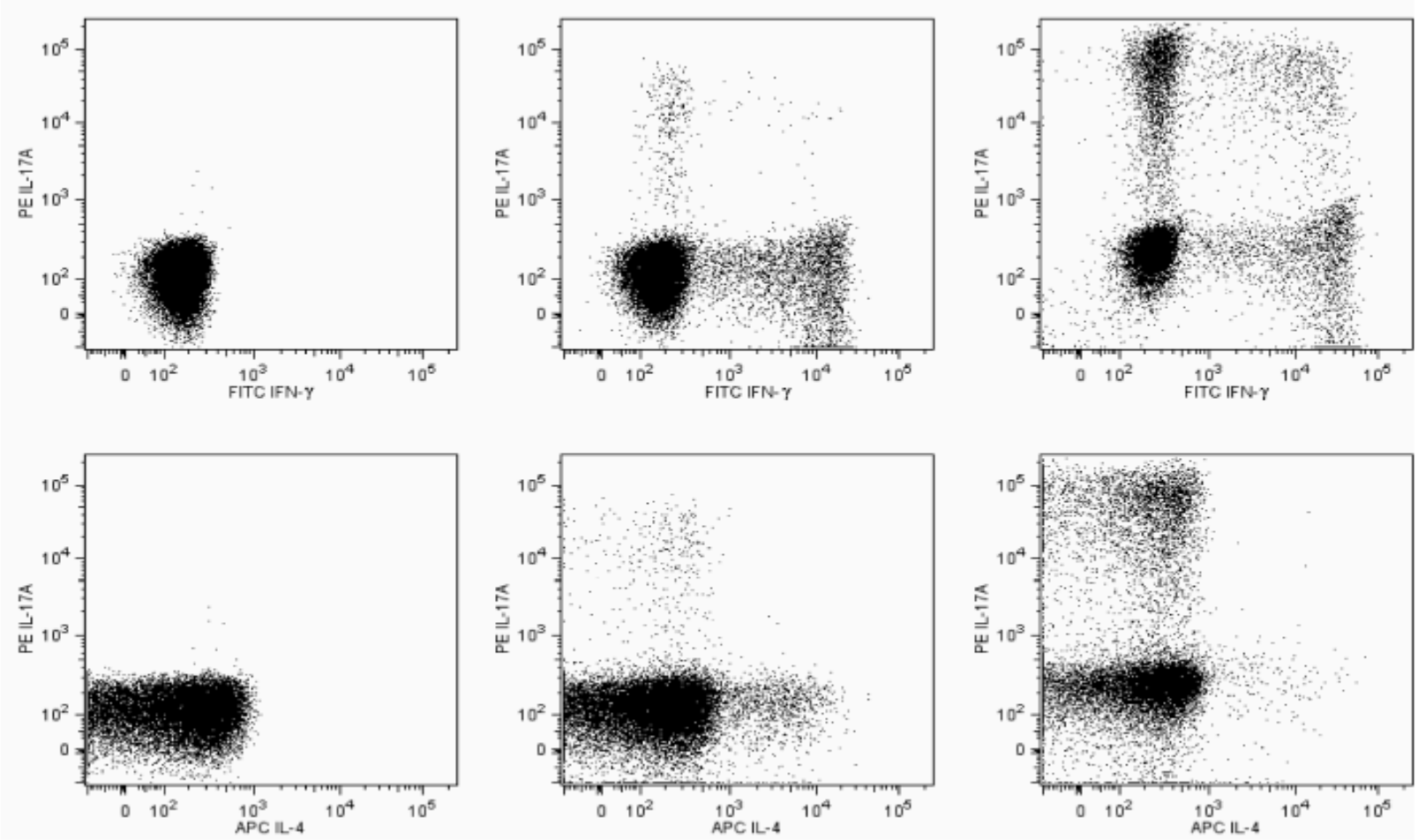
Permeabilisation

Saponin (permeablisation buffer).

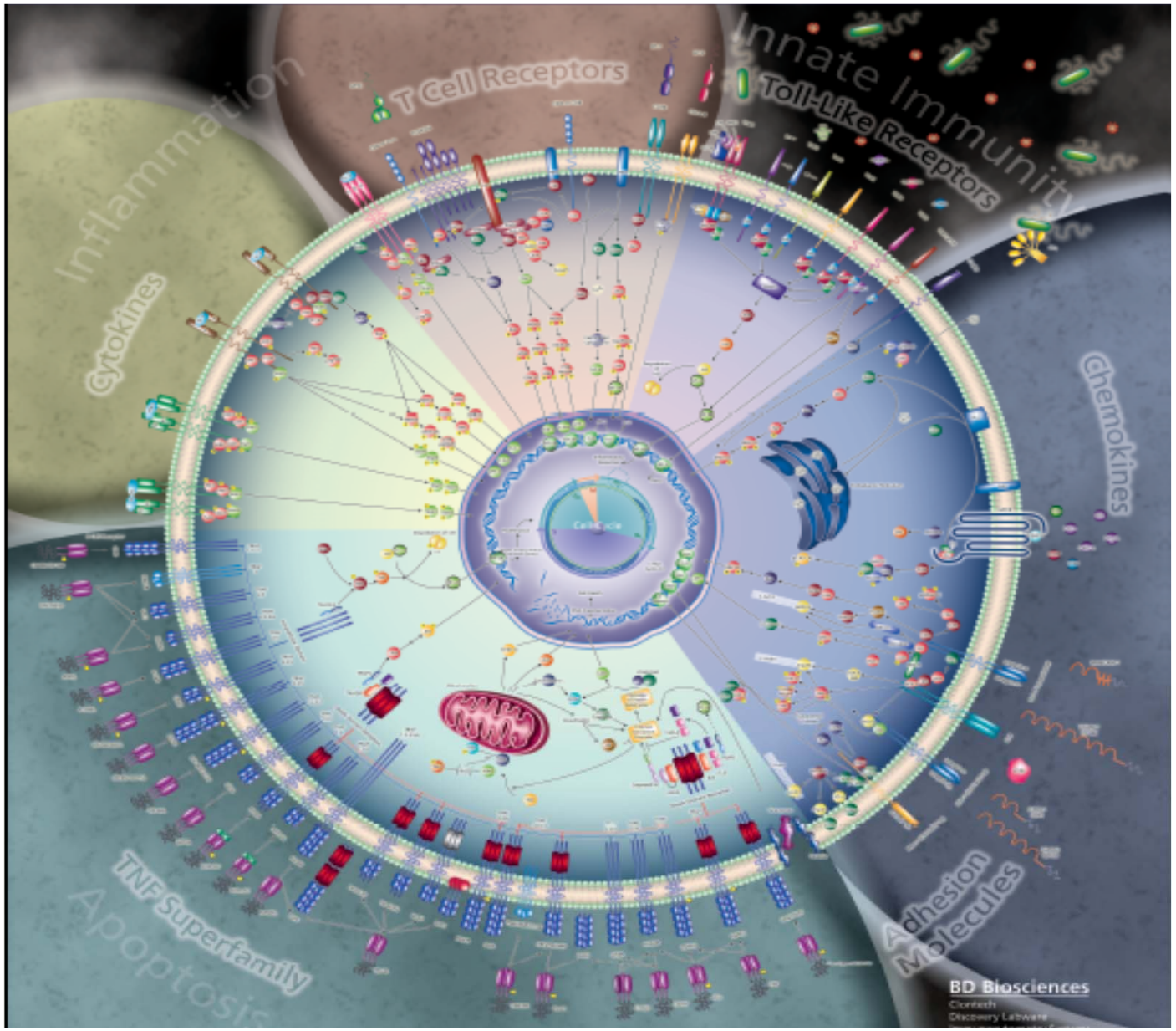
Intracellular Staining

Combination of Cell Surface and Cytoplasmic Staining

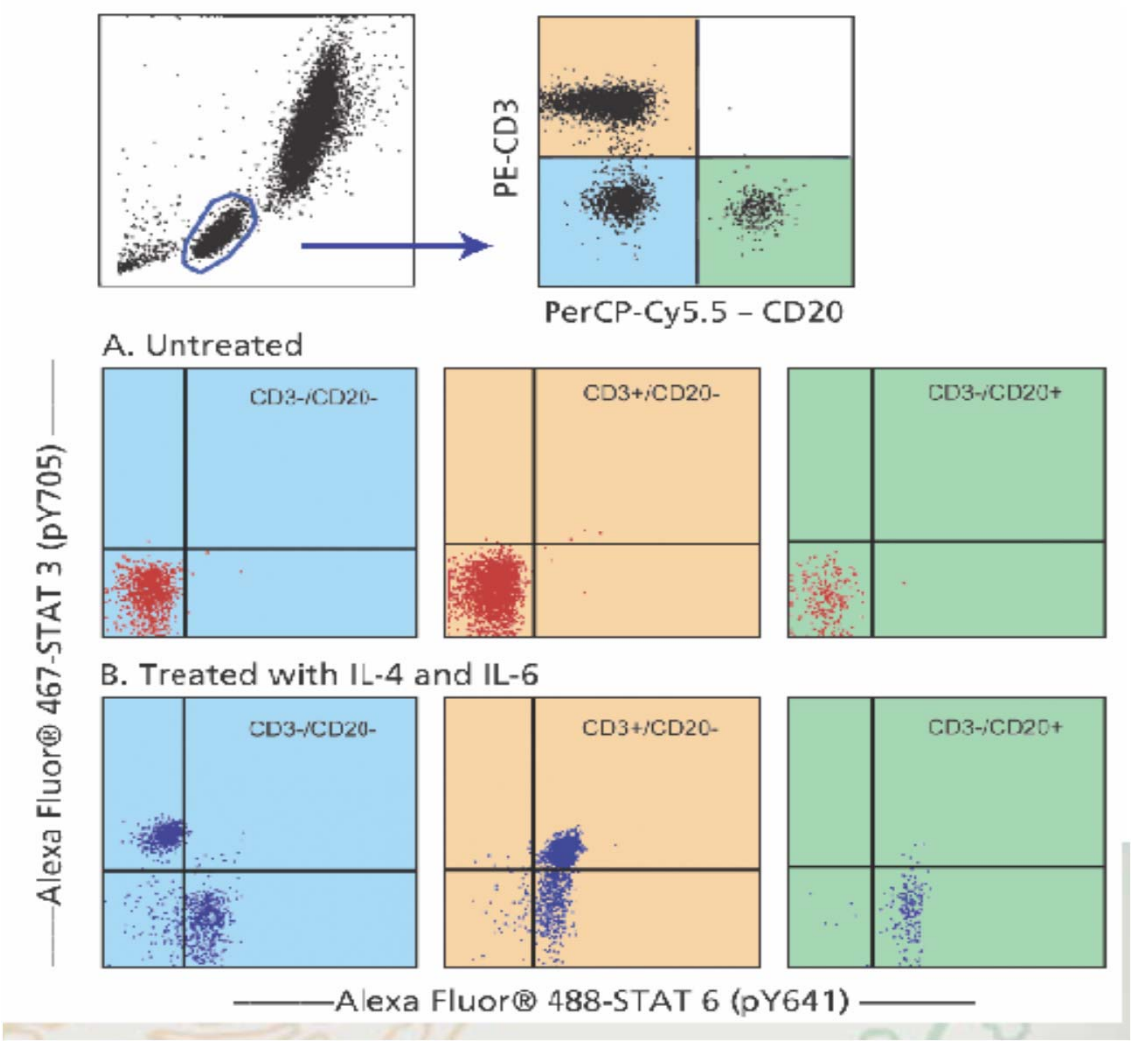
Th1/Th2/Th17 Phenotyping Kit



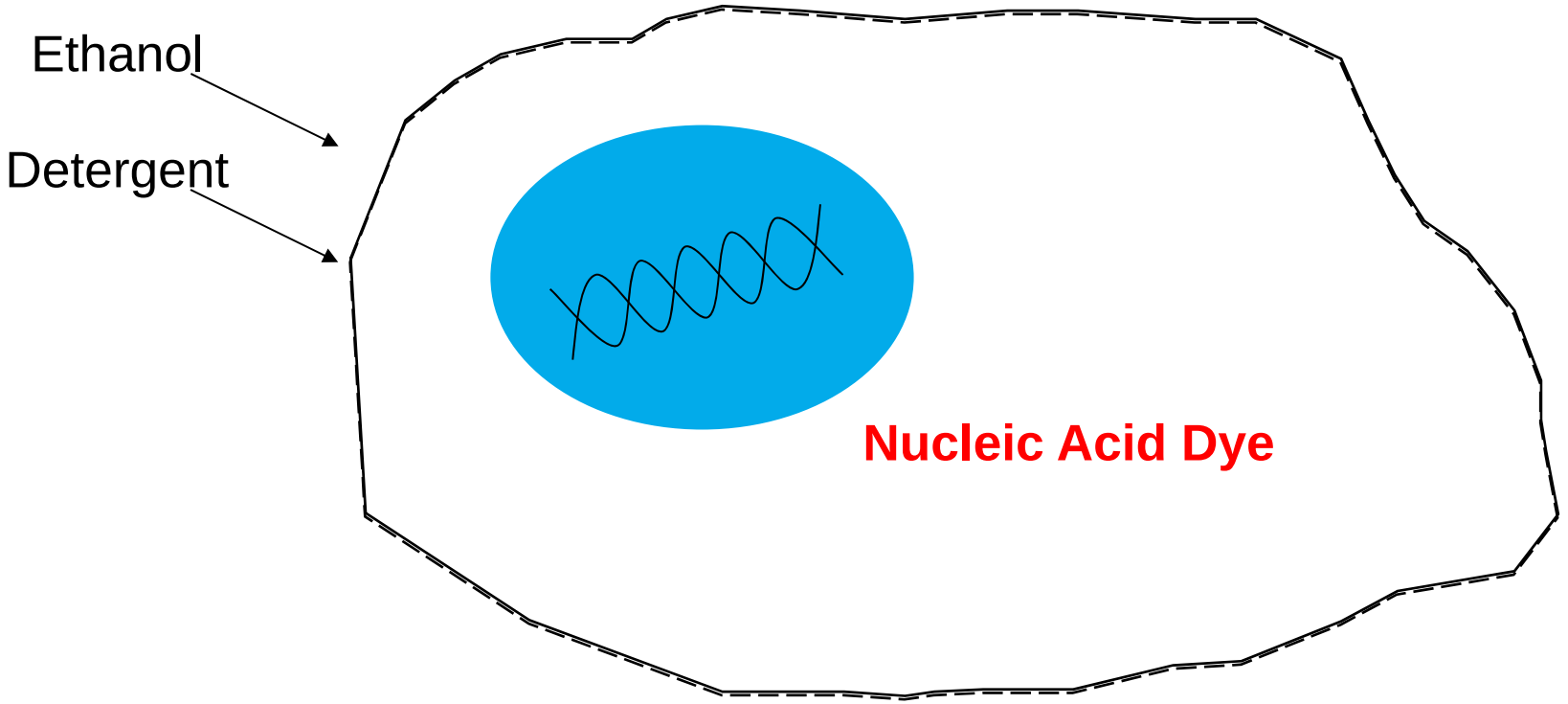
Signal Transduction



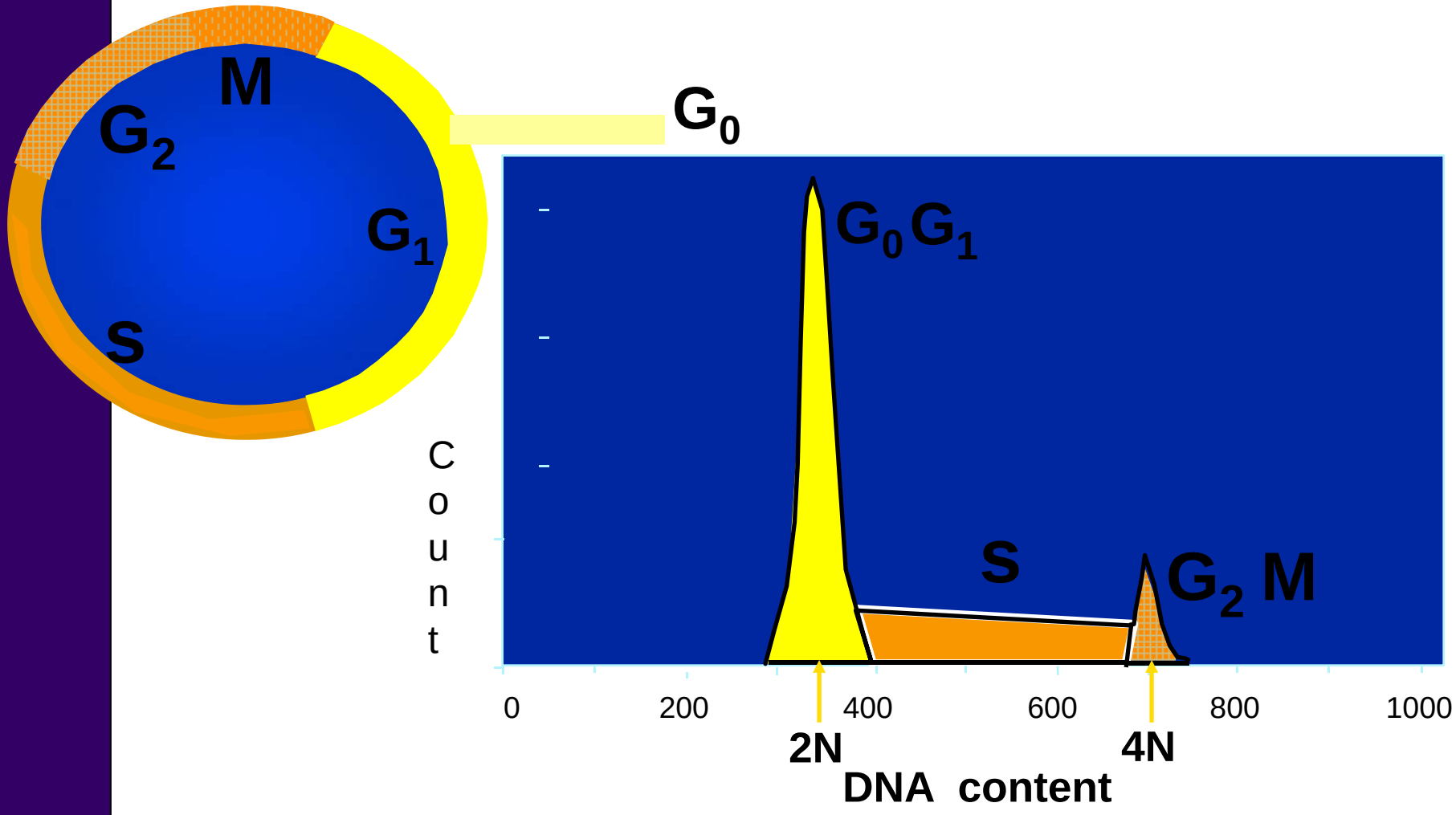
Intracellular Staining in Activated Lysed Whole Blood



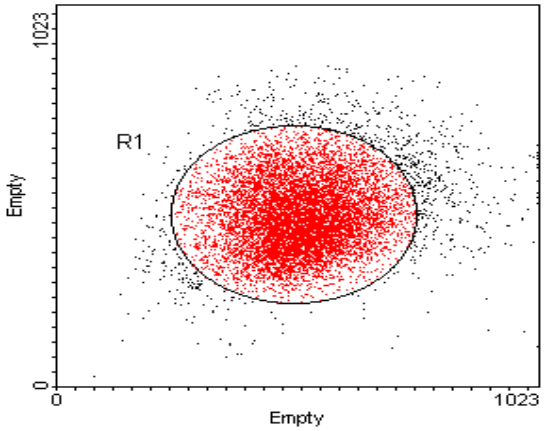
DNA Analysis

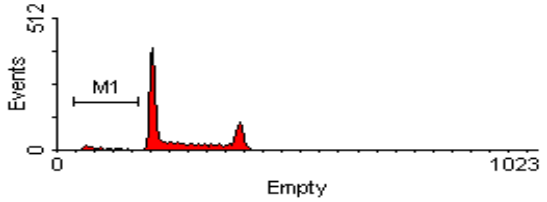


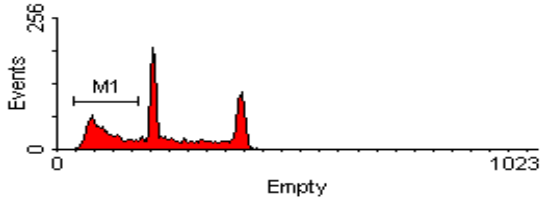
Cell Cycle Analysis

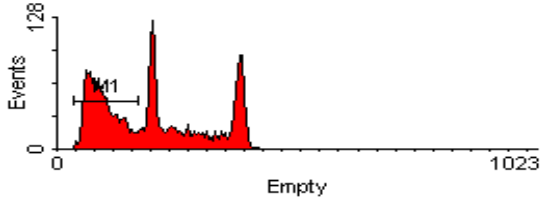


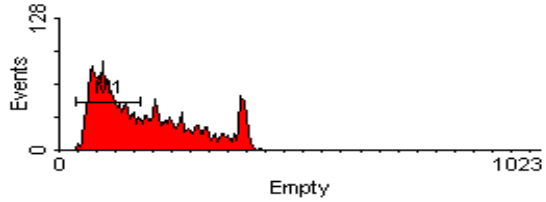
Apoptosis (Sub G1)







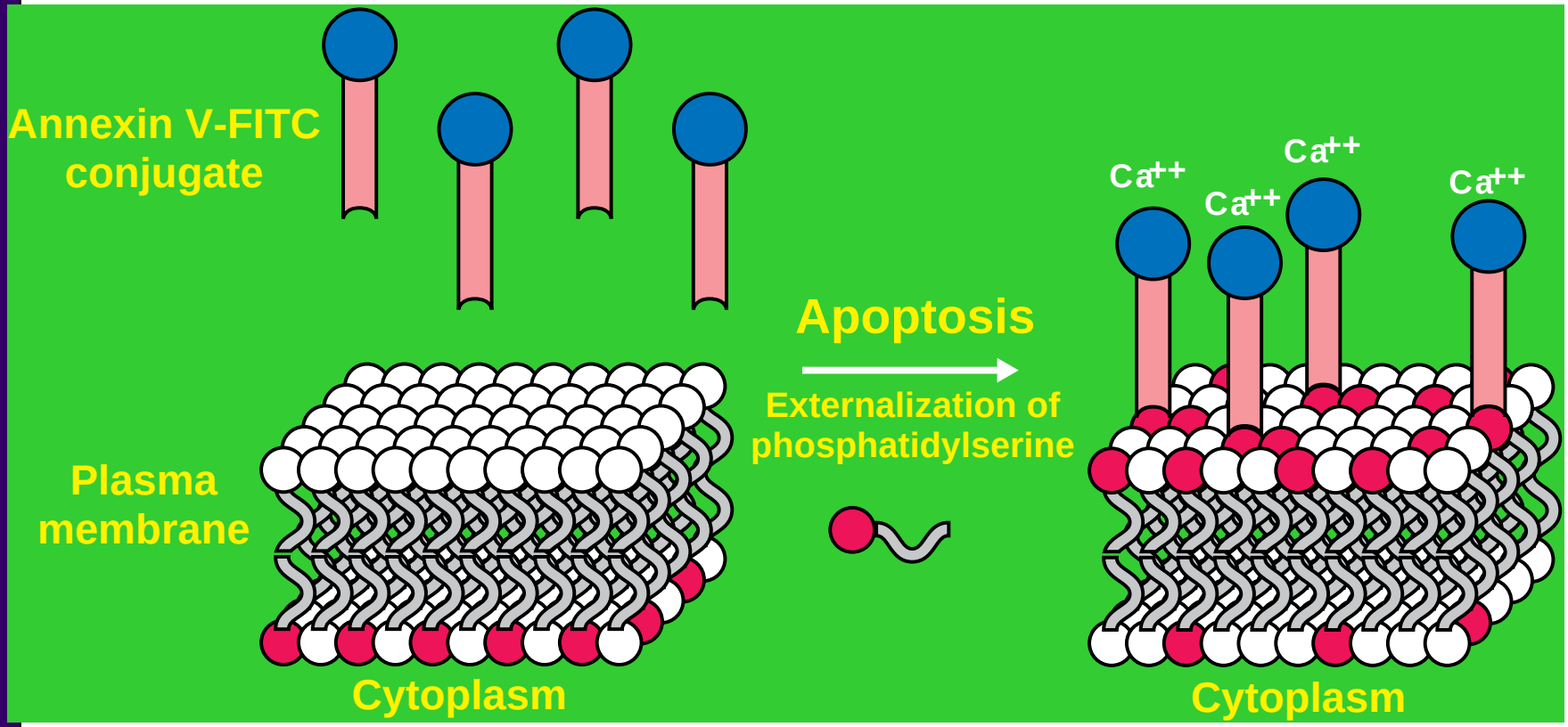




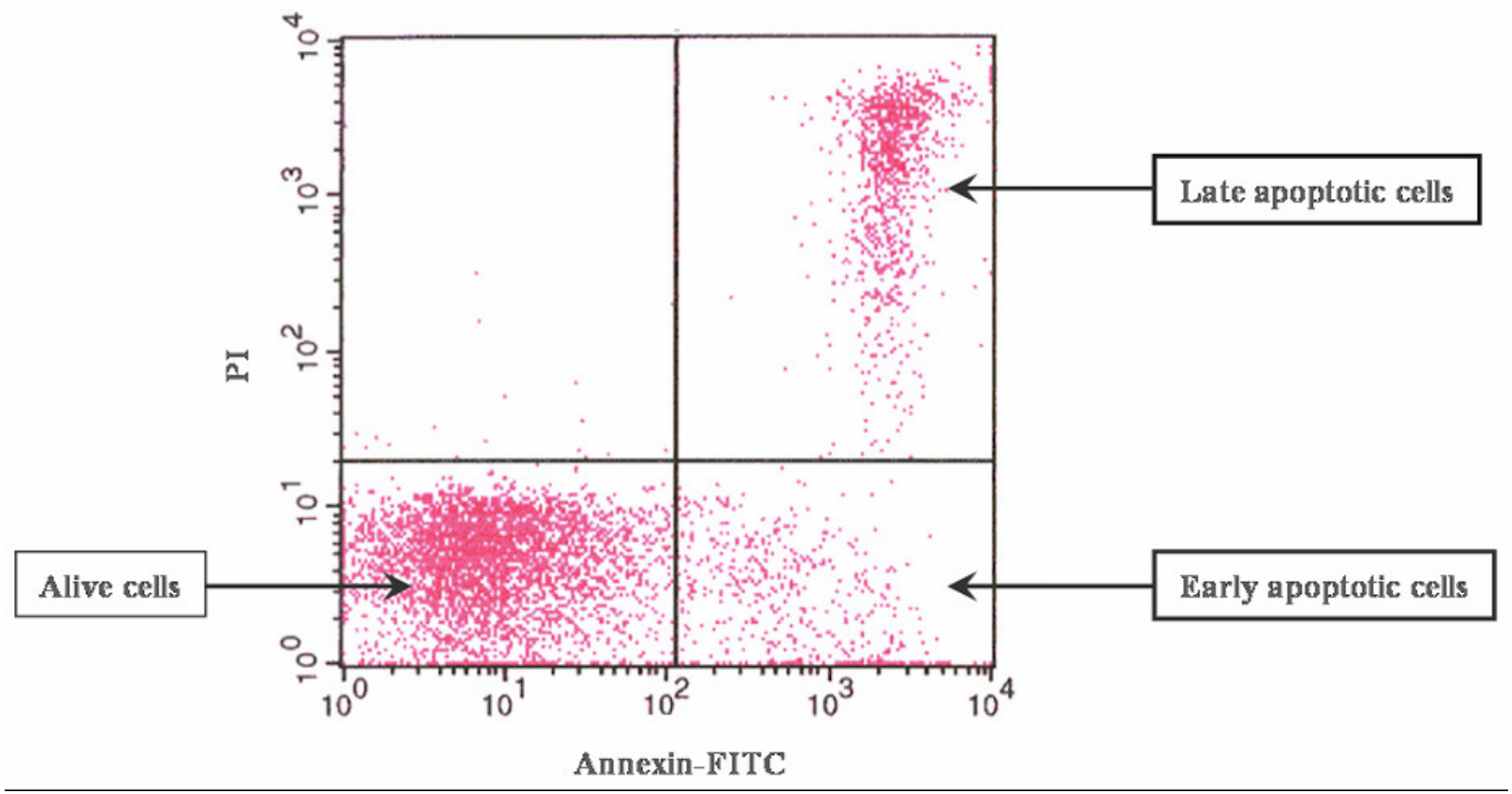
Cell Function Analysis

- Membrane Potential (DiOC6, JC-1)
- Oxidative Metabolism (Free Radicals)
- Intracellular PH Value (Snarf-1)
- Ca++ Influx (Fluo-4/Fura Red, Indo-1)
- Phagocytosis
- Cell Proliferation (PI, BrdU, Intracellular Cyclins)
- Apoptosis (Annexin V, active Caspase-3)

Annexin V Assay



Annexin V/PI Double Staining



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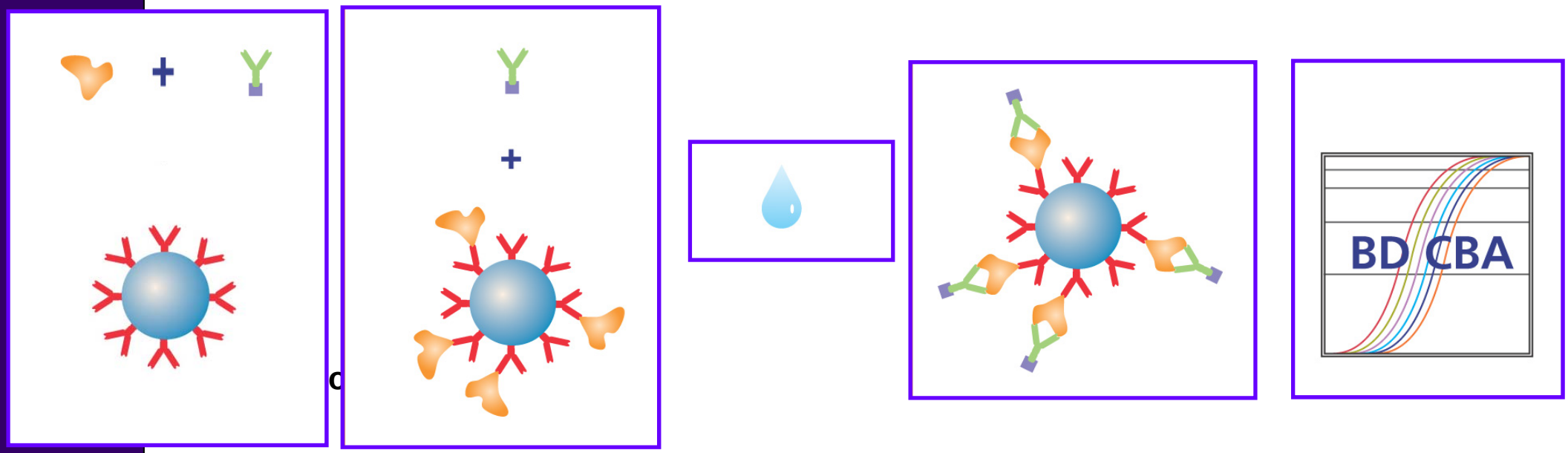
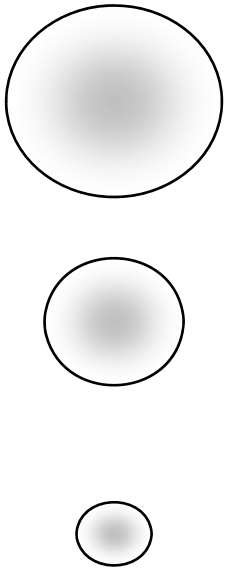
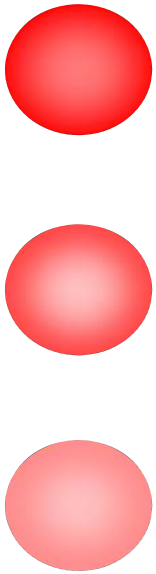
Cytometric Beads Array (CBA)



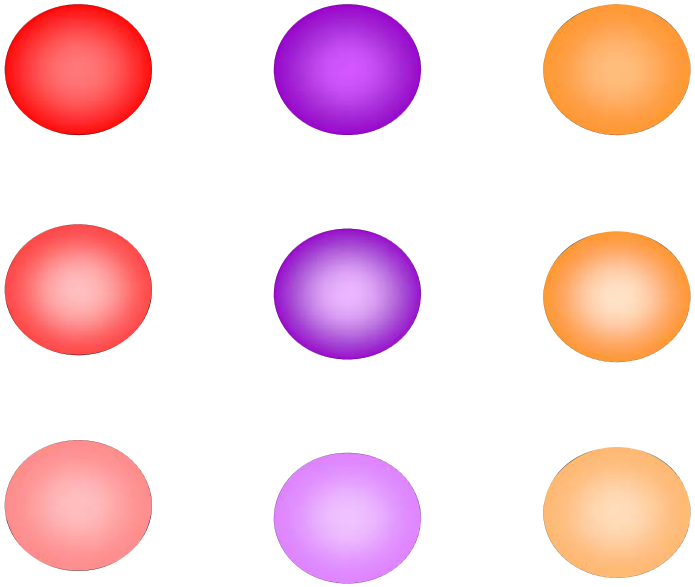
Beads Provide a Flexible Platform



Multiple sizes



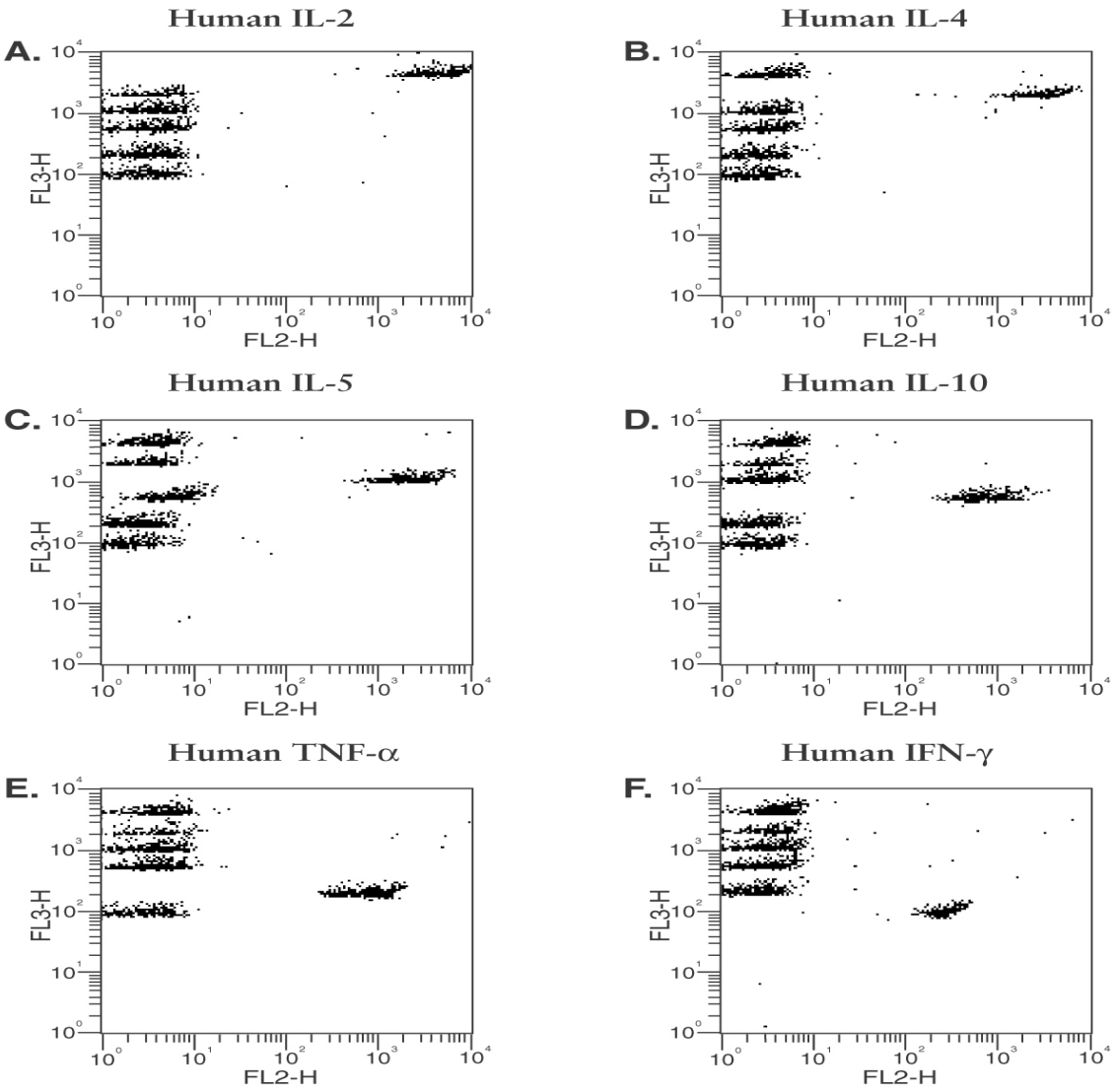
Different fluorescence intensities



Different colors with different intensities

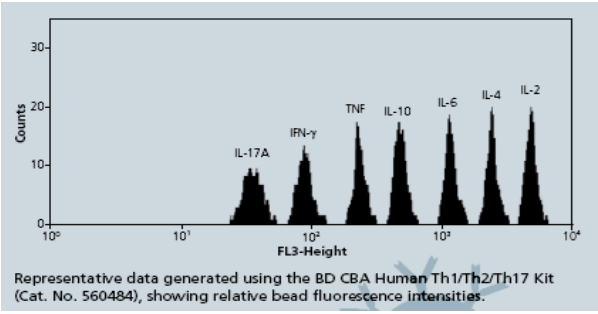
Advantages of Bead-Based Immunoassays

- Analyze multiple analytes simultaneously
- Reduced sample volume requirements
- Reduced hands-on time by parallel analysis of samples
- Wide dynamic range of fluorescence detection (requires fewer sample dilutions)



Proteins Measured

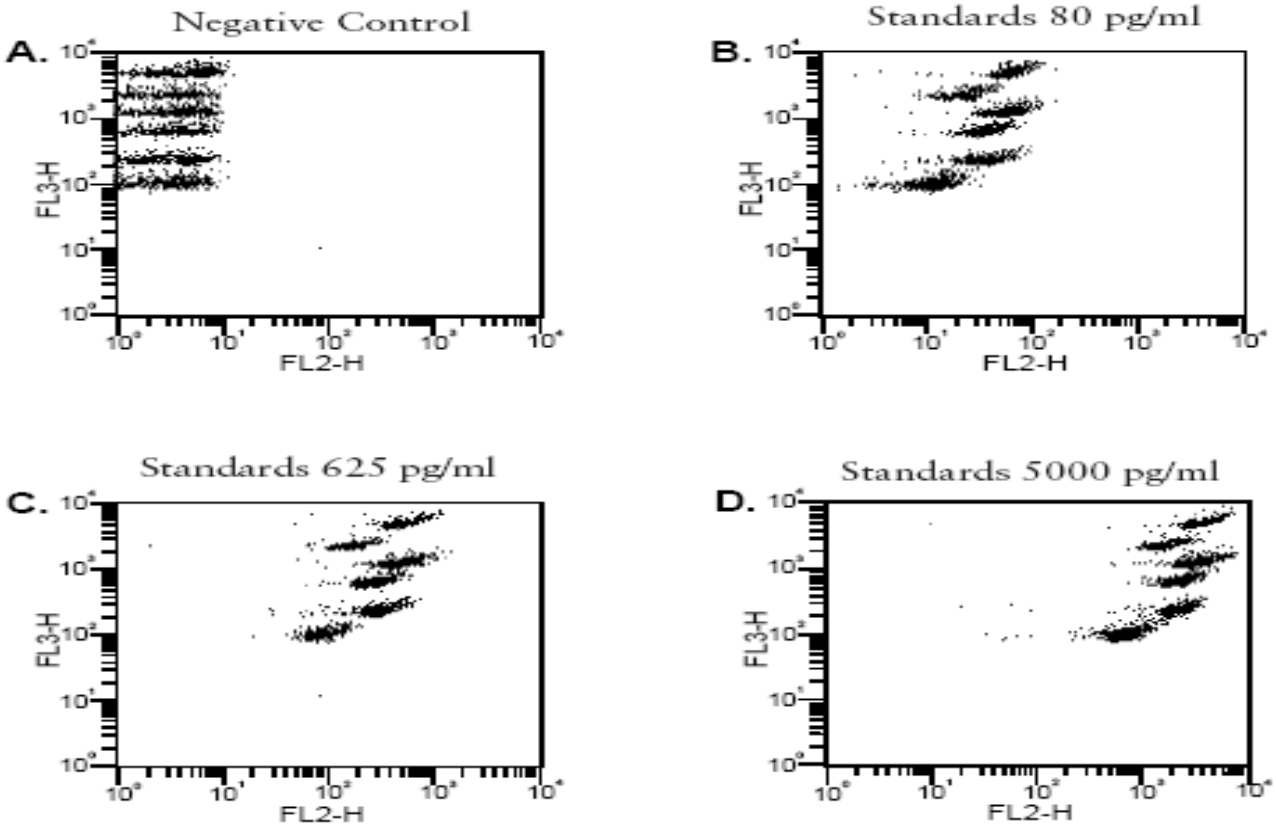
A. Interleukin (IL)-2
B. IL-4
C. IL-5
D. IL-10
E. Tumor Necrosis Factor-α
F. Interferon-γ



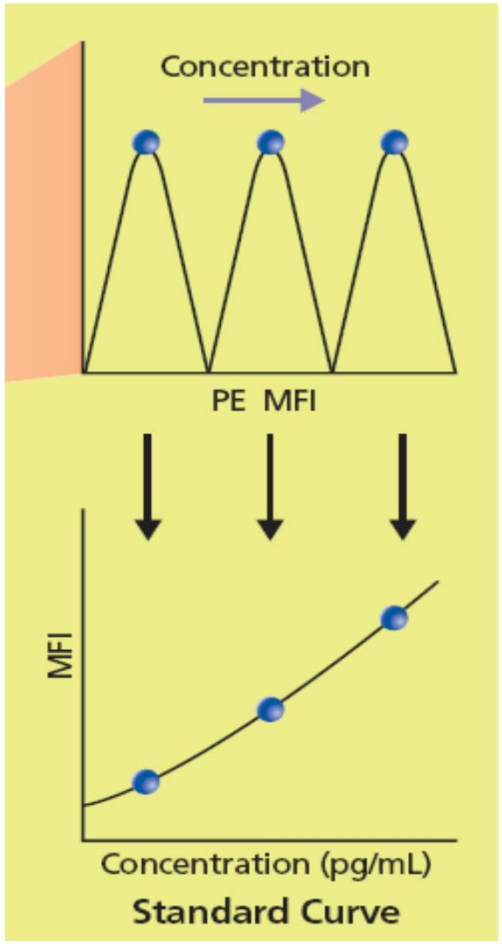
Representative data generated using the BD CBA Human Th1/Th2/Th17 Kit (Cat. No. 560484), showing relative bead fluorescence intensities.

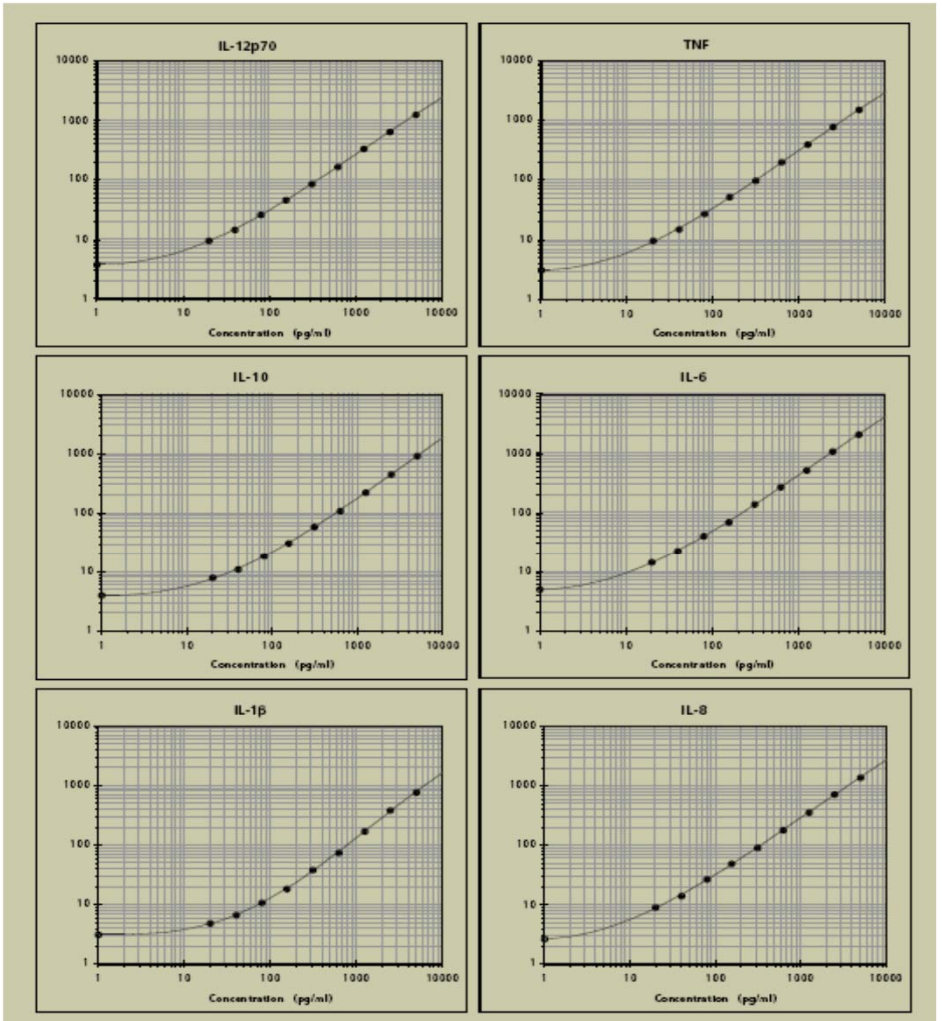
Cytometry Beads Array (CBA)

Typical Data



Standard Curves

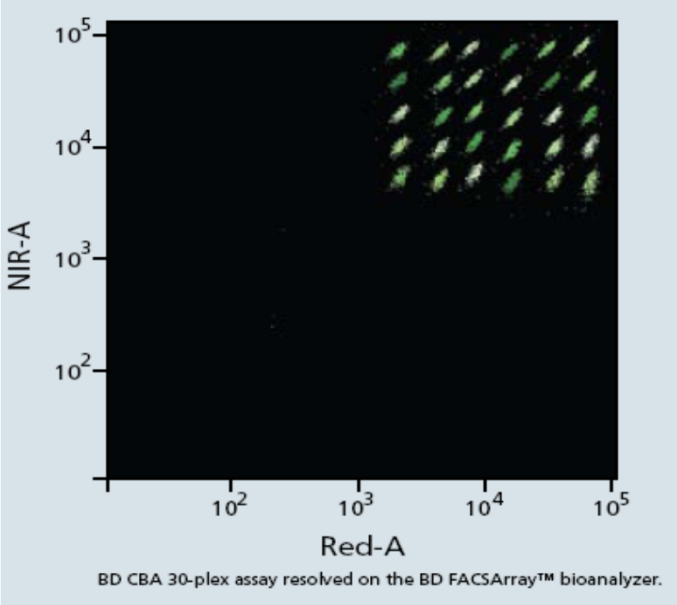




Representative standard curves generated using the BD CBA Human Inflammatory Cytokines Kit.

CBA Flex Sets

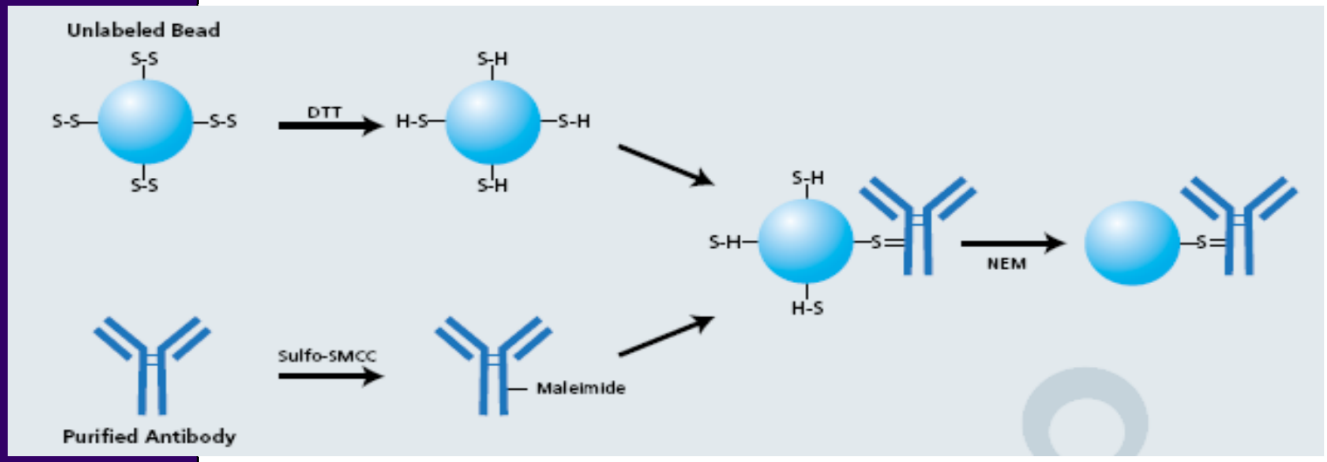
- Open configuration (Up to 30 plex)
- Clustering based on Red and NIR fluorescence intensity
- Need to be used at dual-laser(488nm blue v.s 633nm red) instrument

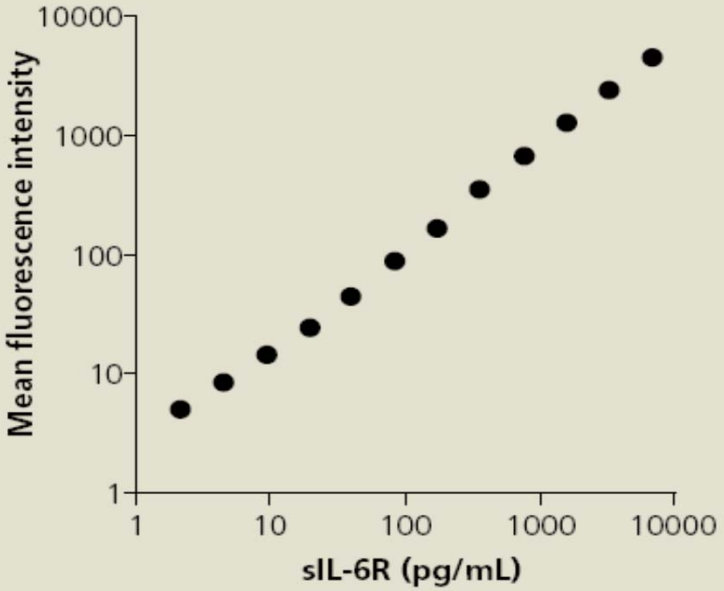


BD CBA 30-plex assay resolved on the BD FACSArray™ bioanalyzer.

CBA Functional Beads

- Can be conjugated with any Ab





Standard curve for a soluble IL-6 receptor assay generated using BD CBA Functional Bead E4 following the conjugation procedure in the BD CBA Functional Bead Conjugation Buffer Set manual.

Data courtesy of Joseph Cannon and Gloria Sloan, Medical College of Georgia.