Introduction to Flow Cytometry

-- BD LSR II

Daisy Kuo 郭正佼
Assistant Product Manager
BD Biosciences
Daisy_kuo@bd.com

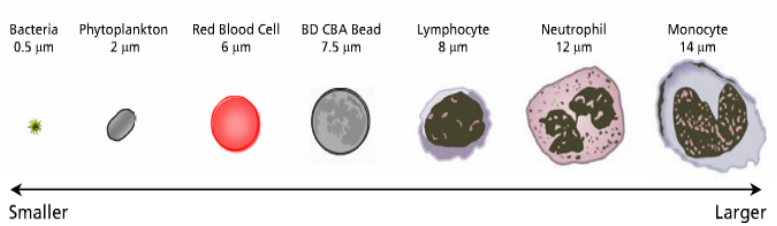
What is Flow Cytometry?

- 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

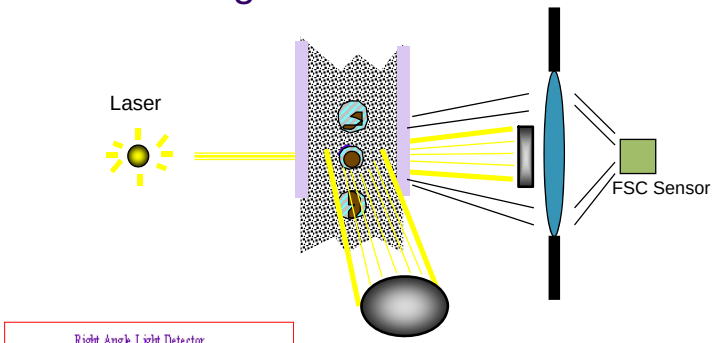
- Detection range: 0.5~50um

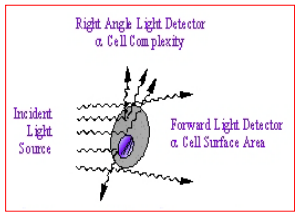


What Can a Flow Cytometer Tell Us About a Cell?

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

Scatter Light

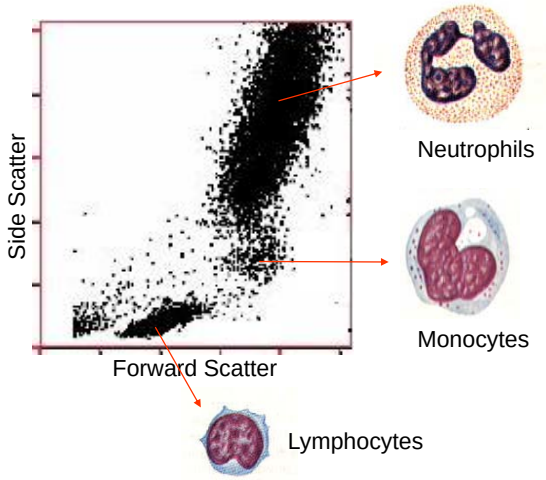




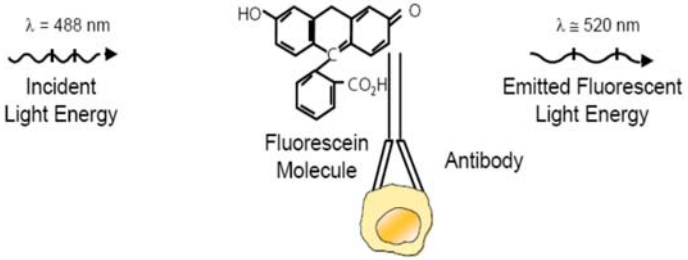
Forward Scatter—diffracted light
- Related to cell surface area
- Detected along axis of incident light in the forward direction

Side Scatter—reflected and refracted light
- Related to cell granularity and complexity
- Detected at 90° to the laser beam

Lysed Whole Blood



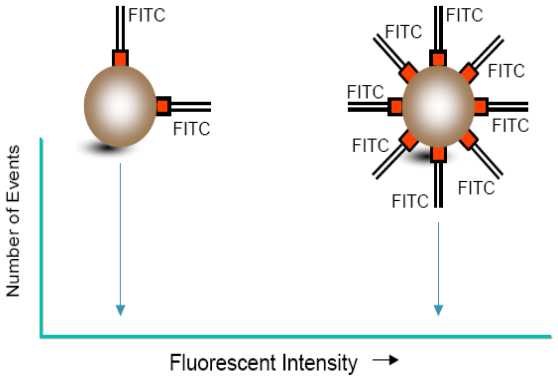
Fluorescence Light



- 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 LSR II

- Up to 4 lasers, 18 colors

Subsystems

Fluidics

To introduce and focus the cells for interrogation.

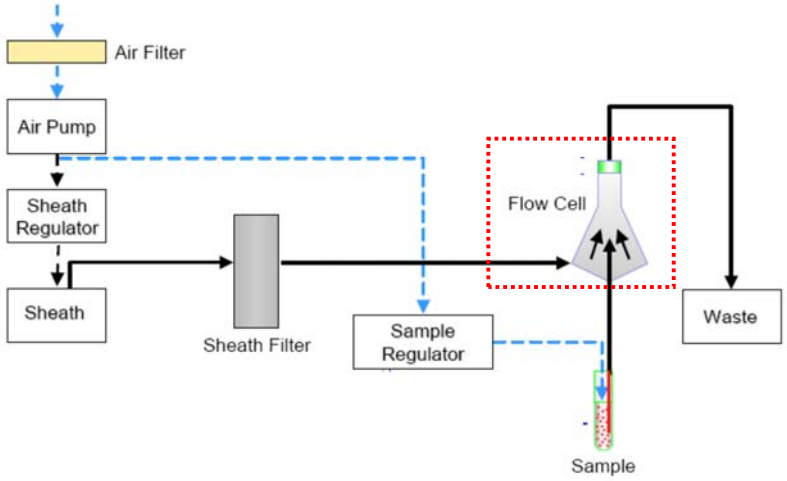
Optics

To generate and collect the light signals.

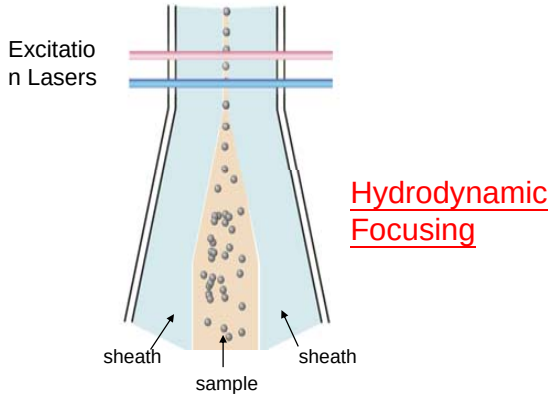
Electronics

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

Fluidics – BD LSR II™

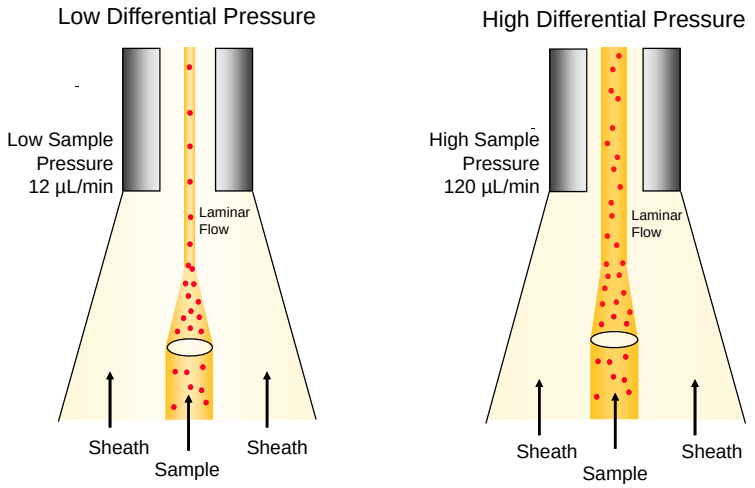


Sample Flow



Hydrodynamic Focusing

Sample Differential

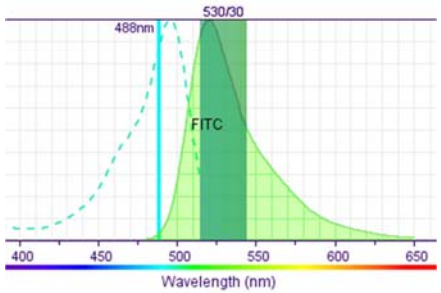


BD LSR II Optical System

- **Excitation optics**:
  - Lasers
  - Filters and mirrors that route the laser light to the fluid stream
- **Collection optic**:
  - Fiber optic cables that direct the emitted light to the appropriate emission block
  - Filters that direct the signals in the emission block to the appropriate photomultiplier tube (PMT)

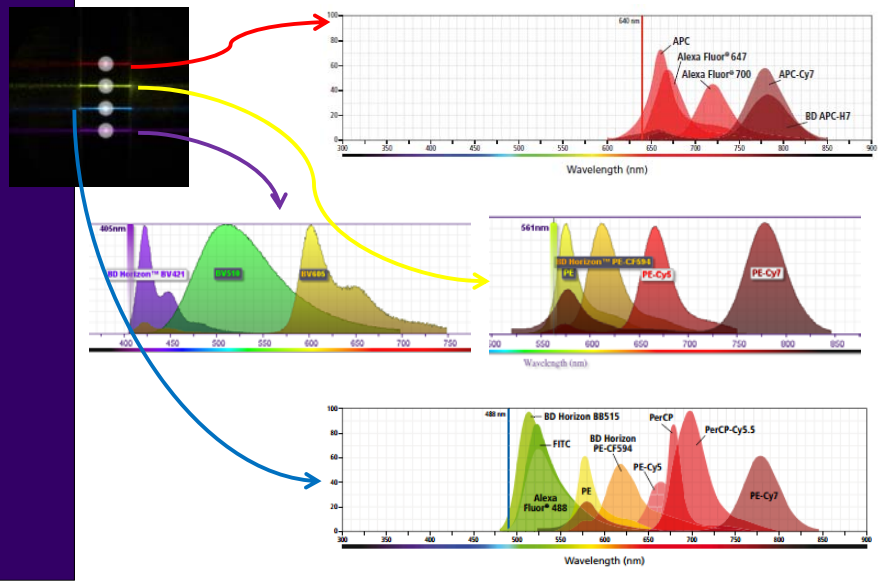
Excitation and Emission

- Use the maximum excitation wavelengths to determine lasers that can be used to excite the fluorochrome.
- Use the maximum emission wavelengths to determine filters and PMTs that can be used to measure the signal.

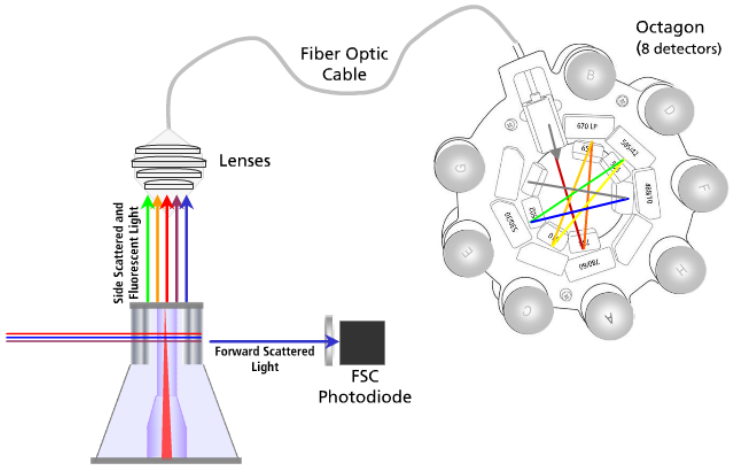


- **www.bdbiosciences.com/spectra**

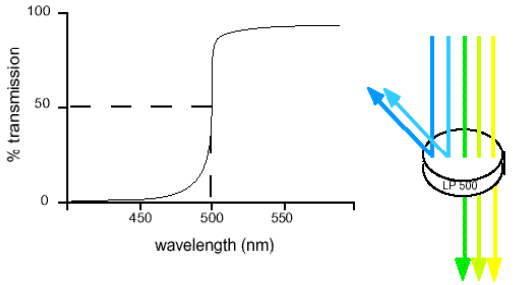
LSR II Optics (4 Lasers, 17 Colors)



Collection Optics

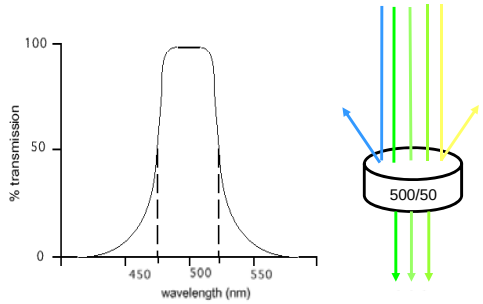


Optical Filters



Longpass Filter (LP)

Optical Filters



Bandpass Filter (BP)

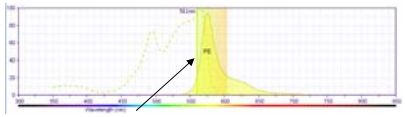
488nm Blue Laser (6 Colors)

- Basic laser which is equipped in almost all Flow Cytometers
- Generates FSC/SSC
- Example fluorochromes excited:
  - FITC, Alexa488, GFP, CFSE
  - PI
  - PerCP and PerCP tandems, 7-AAD
  - PE and PE tandems

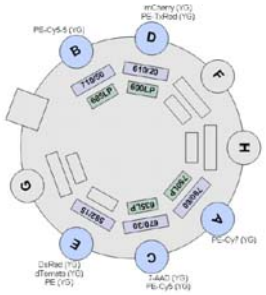
PMT	Longpass Dichroic Mirror	Bandpass Filter	Fluorochrome				
H	N/A	N/A					
G	NONE	488/10	SSC				
F	505LP	530/20 (520~540)	FITC	Alexa488	GFP		
E	550LP	575/26 (562~588)	Alexa532				
D	600LP	610/20 (600~630)			PI		
C	655LP	660/20 (650~670)	Alexa 547		PI	7AAD	
B	685LP	695/40 (675~715)	PerCP	PerCP-Cy5.5	PI	7AAD	
A	755LP	780/60 (750~810)					

561nm Yellow-Green Laser (5 Colors)

- Closest wavelength to PE excitation peak, provides better sensitivity to PE and PE tandems
- No spillover from FITC
- Optimal excitation for DsRed, mCherry, RFP, and other fruit proteins



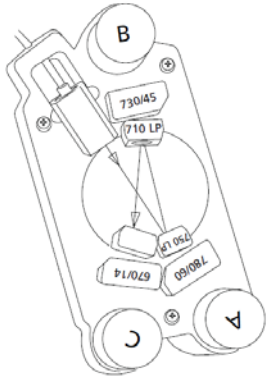
561nm is close to the PE max. excitation peak



PMT	Longpass Dichroic Mirror	Bandpass Filter	Fluorochrome		
H	N/A	N/A			
G	N/A	N/A			
F	N/A	N/A			
E	550LP	586/15	PE		PI
D	600LP	610/20	PE-Texas Red	mCherry	PI
C	635LP	670/30	(PE-Cy5)	(7AAD)	PI
B	685LP	695/40	PE-Cy5.5	(7AAD)	PI
A	750LP	780/60	PE-Cy7		

640nm Red Laser (3 Colors)

- Optimal to use for populations that are highly autofluorescent when excited by the blue laser
- Example fluorochromes excited:
  - APC, Alexa647
  - APC-Cy7, APC-H7
  - Alexa700



PMT	LP mirror	BP filter	Fluorochromes
A	750	780/60	APC-Cy7, APC-H7
B	710	730/45	Alexa Fluor® 700
C	—	670/14	APC

405nm Violet Laser (3 Colors)

- Ideal for Brilliant Violet (BV) dyes
  - BV421
  - BV510
  - BV605
  - BV650
- Can be used for Qdots

PMT	Longpass Dichroic Mirror	Bandpass Filter	Fluorochrome					
C	N/A	450/50	Pacific blue	BV421	Alexa405	DAPI	Hoechest	ViviD
B	505LP	525/50	Alexa 430	BV510	Qdot525	AmCyan		
A	556LP	585/42	Qdot585		Pacific Orange			

Fluorochrome/Antigen Combination

Antigen Density

low → medium → high

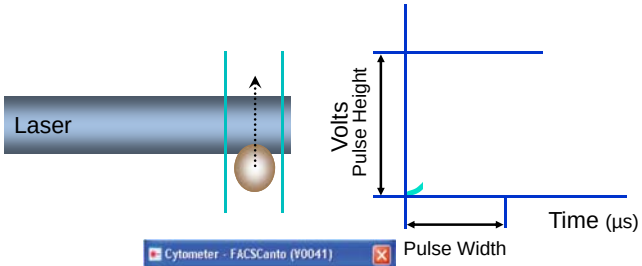
Fluorochrome

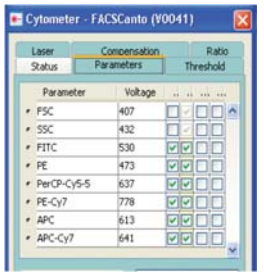
Laser	Very Bright	Bright	Moderate	Dim
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	BD Horizon™ BV605 BD Horizon™ BV786	BD Horizon™ BV510	BD Horizon™ V450 BD Horizon™ V500
Blue (488 nm)	BD Horizon™ BB515 BD Horizon™ PE-CF594 PE-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon™ PE-CF594 PE-Cy™5 PE-Cy™7			
Red (640 nm)		APC Alexa Fluor® 647		Alexa Fluor® 700 APC-H7 APC-Cy7

Electronics

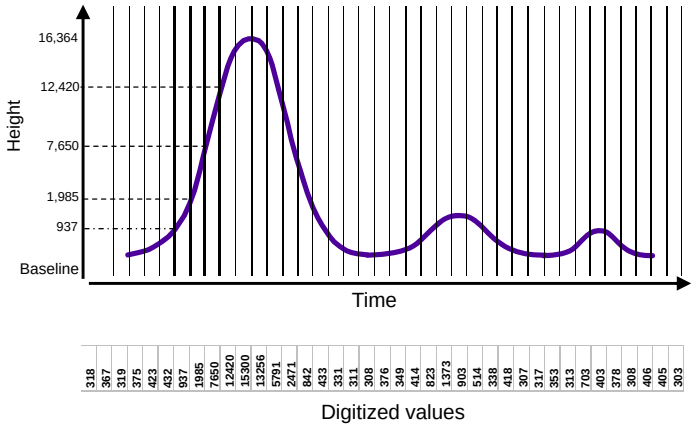
- Optical signals are converted to electronic signals.
- Voltage pulse height, area, and width are analyzed.
- Analog signals are converted to digital signals.
- Data is transferred to the computer.

Creation of a Voltage Pulse

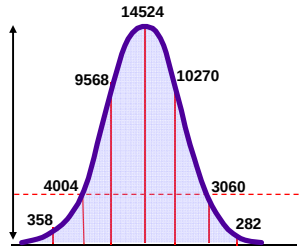




Analog-to-Digital Converter



Parameters



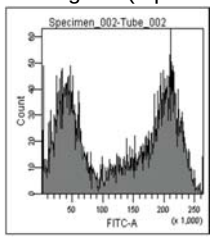
- **A**rea: Sum of all height values
- **H**eight: Maximum digitized value
- **W**idth: Time

Data Storage

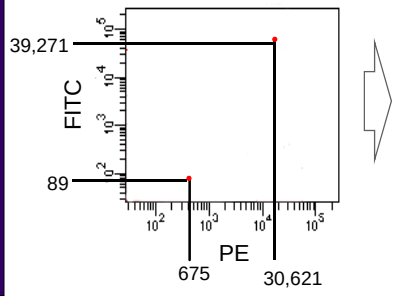
List-Mode Data

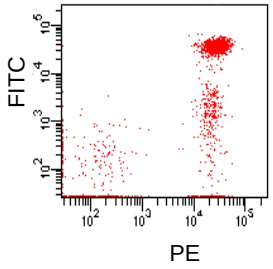
	Time	FSC	SSC	FITC	PE
Event 1	0	60	120	89	675
Event 2	10	160	65	39,271	30,621
Event 3	30	650	160	22,688	6,189

Histogram (1 parameter)



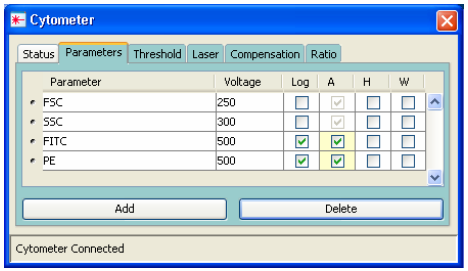
Dot Plot (2 parameters)





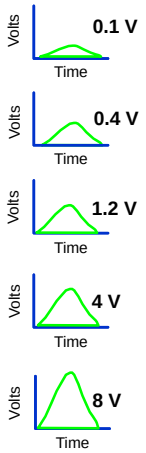
Data Display

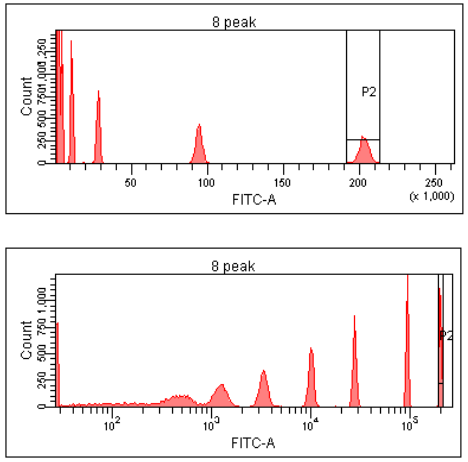
- Linear Scaling
- Log Scaling



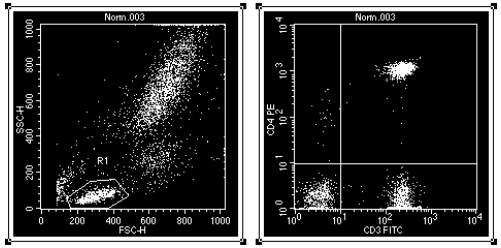
Linear v.s Log

Voltage Pulses



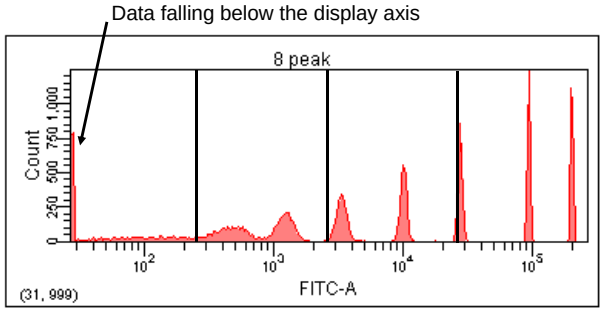


Linear v. Log Amplification

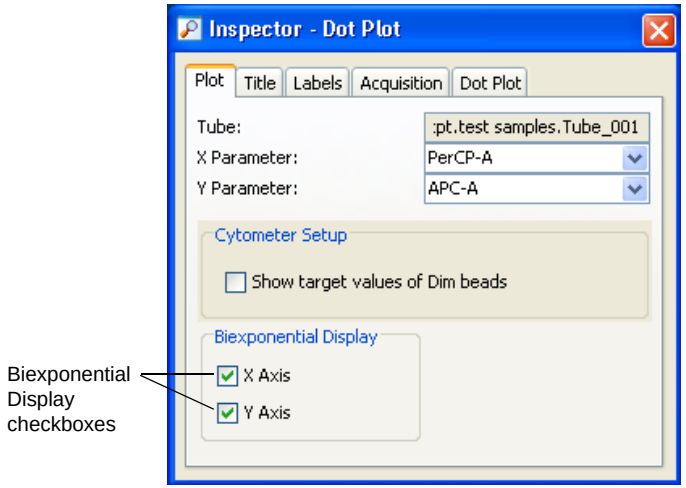


- **Linear** amplification is usually used for light scatter parameters and DNA analysis.
- **Log** amplification is used for fluorescence signals with a large dynamic range, or small particle detection.

Negative Values

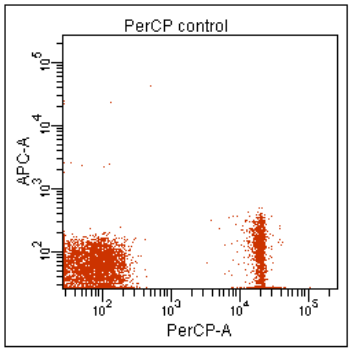


Biexponential Display

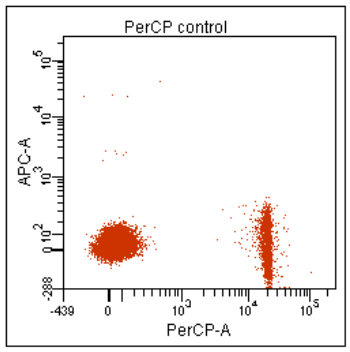


Log vs Biexponential Scaling

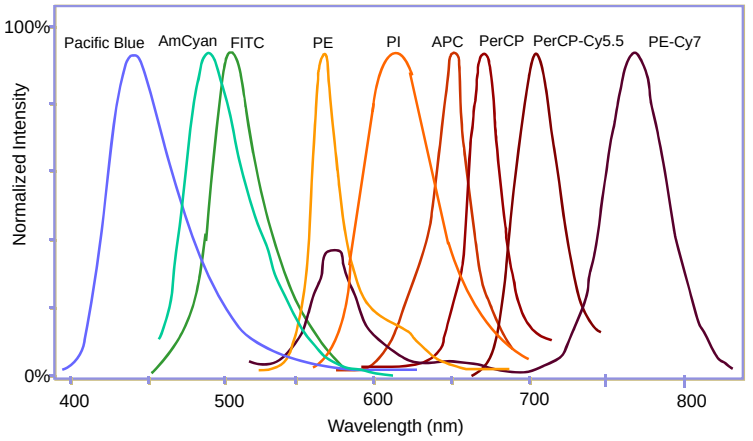
log scaling



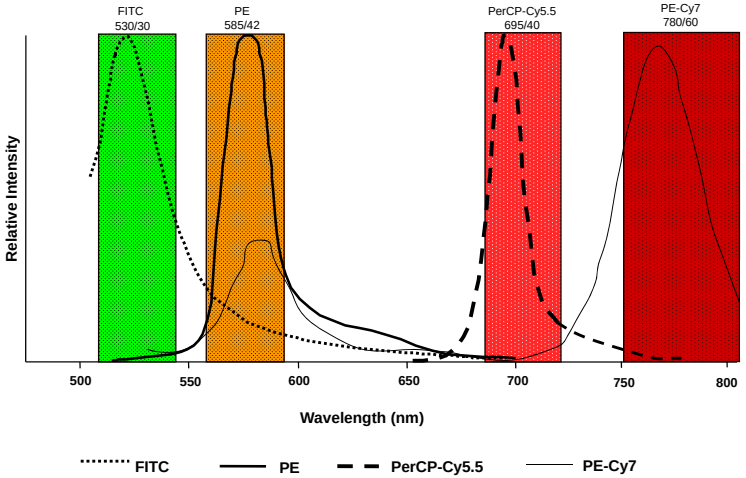
biexponential scaling



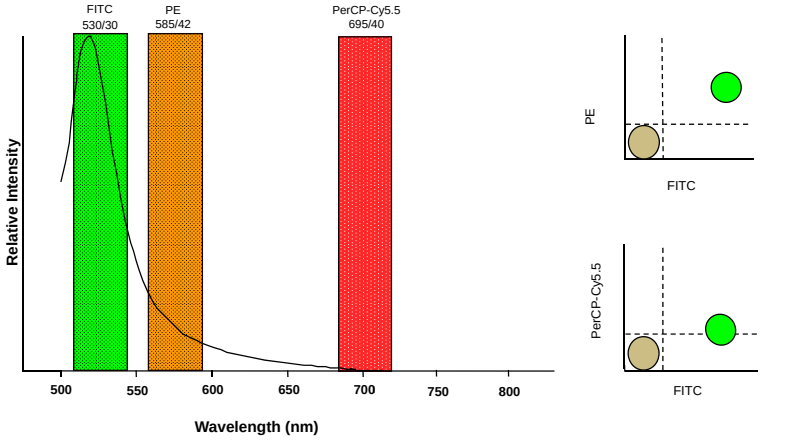
Spectral Overlap– Compensation Theory



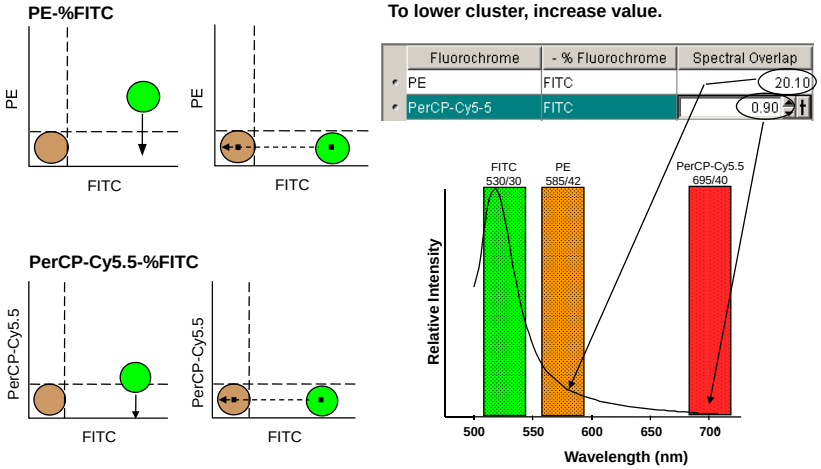
Spillover



FITC Spillover

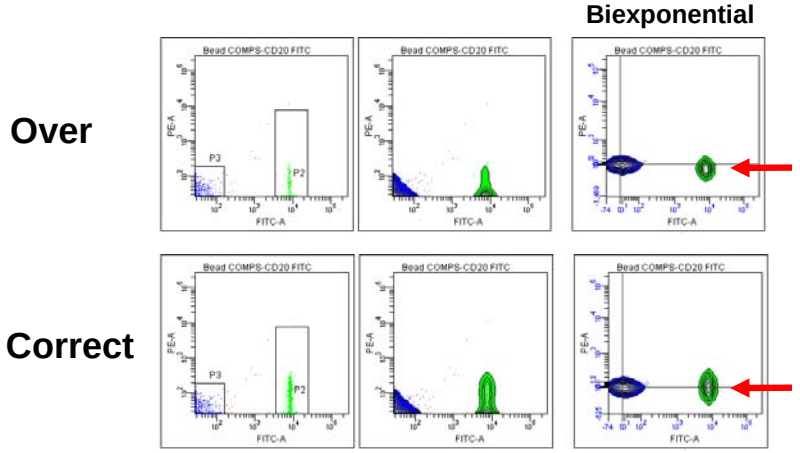


FITC Compensation

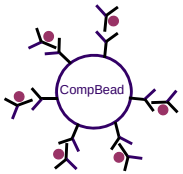


Compensation QC

Biexponential display reveals compensation problems.



BD CompBeads/CompBeads Plus



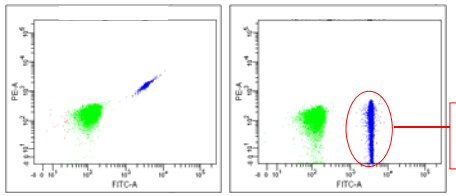
A **convenient** way to create **effective** single-color compensation controls

- Use the same antibodies as in the experimental samples.
- Create bright and uniform positive fluorescence peaks.
- Avoid using limited sample.
- Mimic autofluorescence background of lymphocytes/cell lines
- Beads are coated with anti-mouse, anti-rat, and anti-hamster kappa.

Data Spread Due to Spillover

⇧ spillover = ⇩ resolution

Uncompensated Compensated



Maecker HT, Frey T, Nomura LE, Trotter J. **Selecting fluorochrome conjugates for maximum sensitivity**. *Cytometry A* 2004; **62**:169-173.

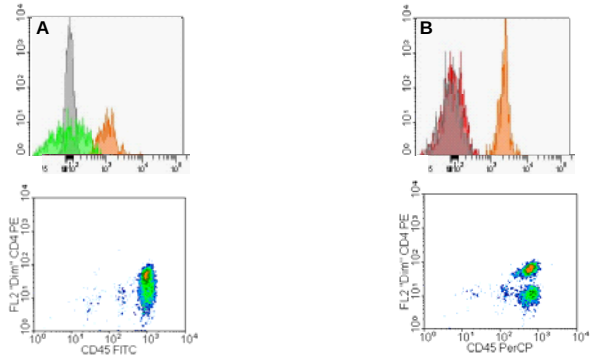
Population Lost Due to Spillover

Unstained lymphocytes

Dim PE CD4+ lymphocytes

CD4- lymphocytes stained with FITC anti-CD45, compensated

CD4- lymphocytes stained with PerCP anti-CD45, compensated

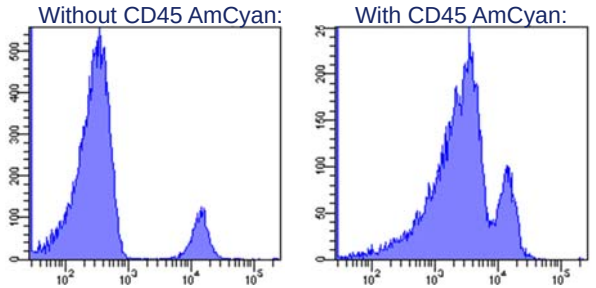


Dual Excitation Reduces Resolution

Fluorochromes that are excited by more than one laser cause high spillover.

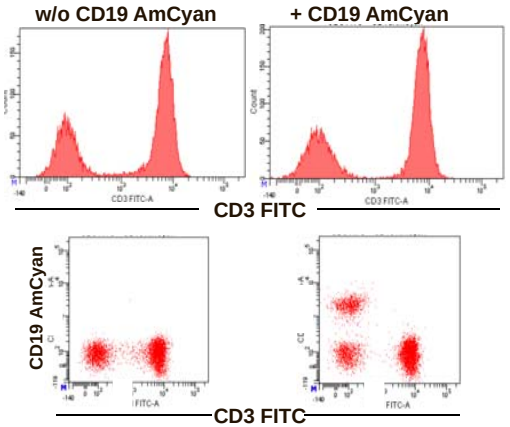
- AmCyan excited by the violet and blue lasers spills into the FITC detector.
- PE-Cy5 excited by the blue and red lasers spills into APC detector.

Without CD45 AmCyan: With CD45 AmCyan:



BUT Spillover Happened...

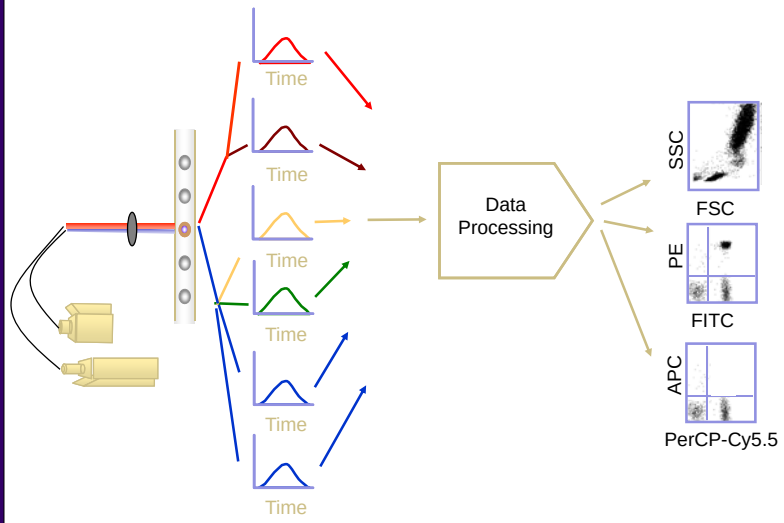
- **ONLY if they are co-expressed on the same cell!**
  Bound to different cells: No spill over effects!



Multicolor Panel Design Principles

1. Consider antigen co-expression based on biology
2. Group antigens by high, medium and low antigen densities
3. Pair brightest fluorochromes on specificities with the lowest antigen densities
4. Minimize spillover by spread antigens across different lasers

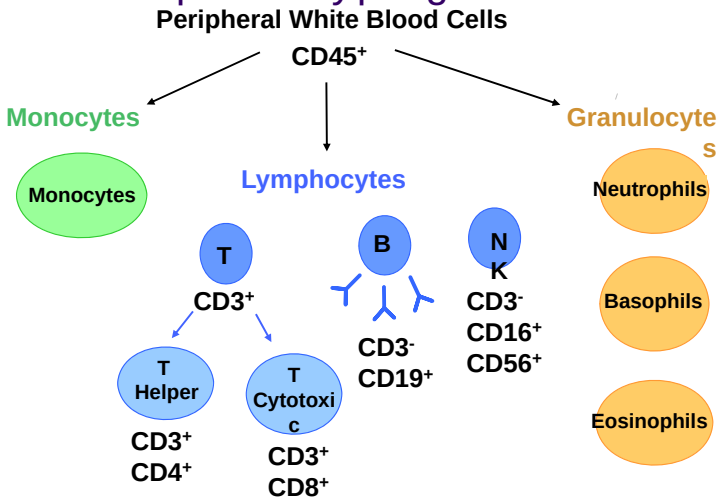
Review

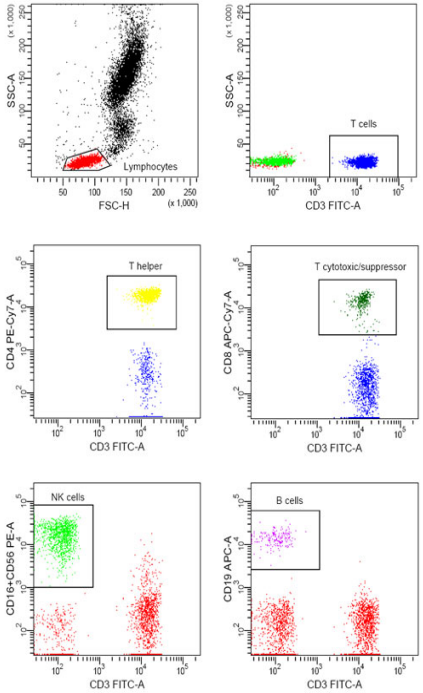


Applications

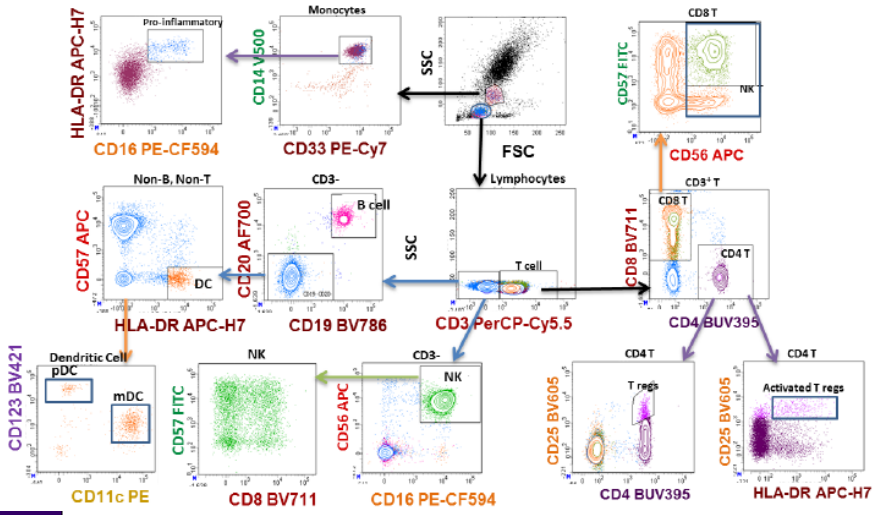
- Phenotype Analysis (Cell Surface Antigens/Markers)
- Intracellular Analysis
  -- Eg. Cytokines, Signal Transduction molecules…etc.
- DNA Analysis
  -- Eg. Viability, Cell cycle, Apoptosis…etc.
- Cell Function Analysis
  -- Eg. Free radicals, Ca^{2+}, Reporter genes…etc.
- CBA (Cytometric Bead Array)

Lymphocyte Immunophenotyping

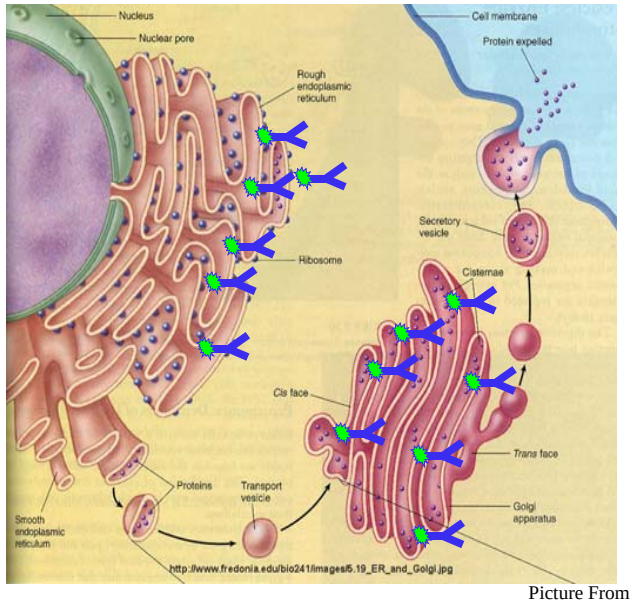




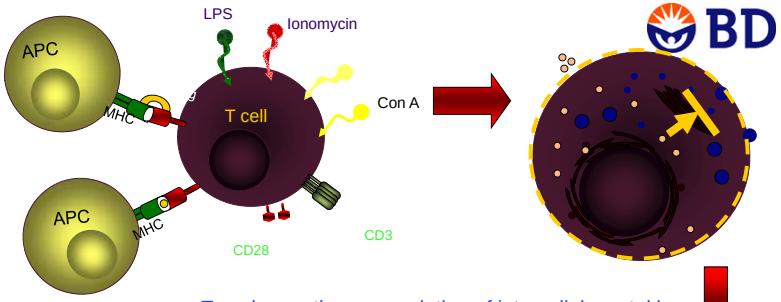
14-color analysis: T/B/NK/NK-T/Mono/Dendritic cell subsets



Cytokine Detection



Picture From www.fredonia.edu



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.

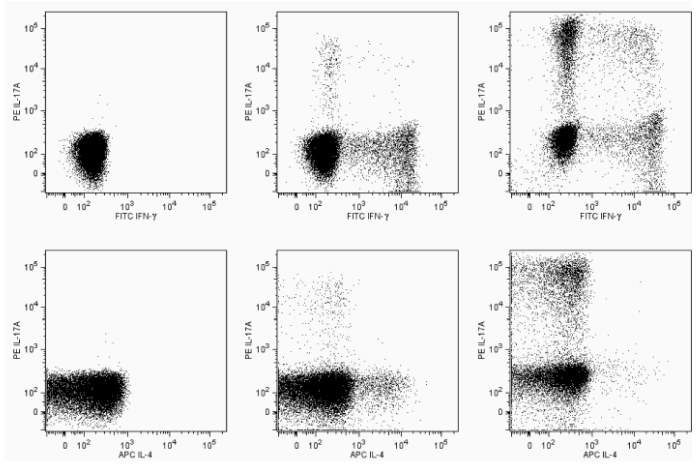
To maintain structural integrity.
Formaldehyde or glutaraldehyde
Keep the protein structure and doesn't change the (accessibility of the) epitopes too much

Fixation → Permeabilisation → Intracellular Staining

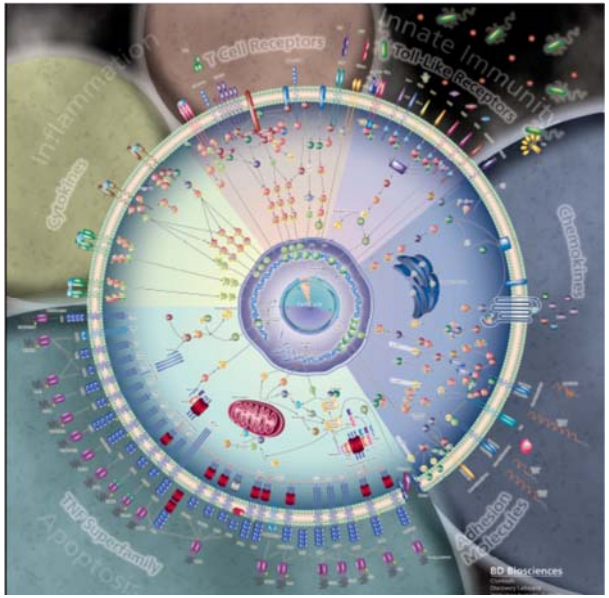
Saponin (permeablisation buffer).

Combination of Cell Surface and Cytoplasmic Staining

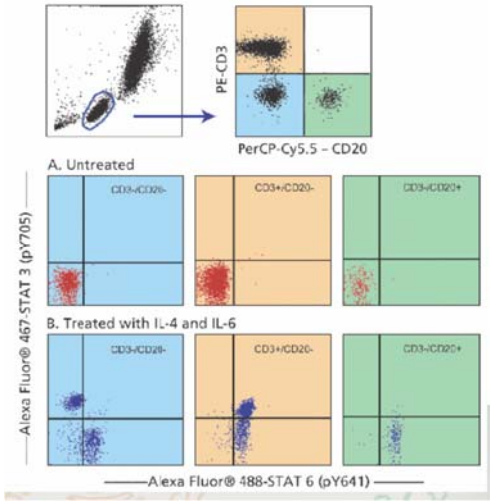
Th1/Th2/Th17 Phenotyping Kit



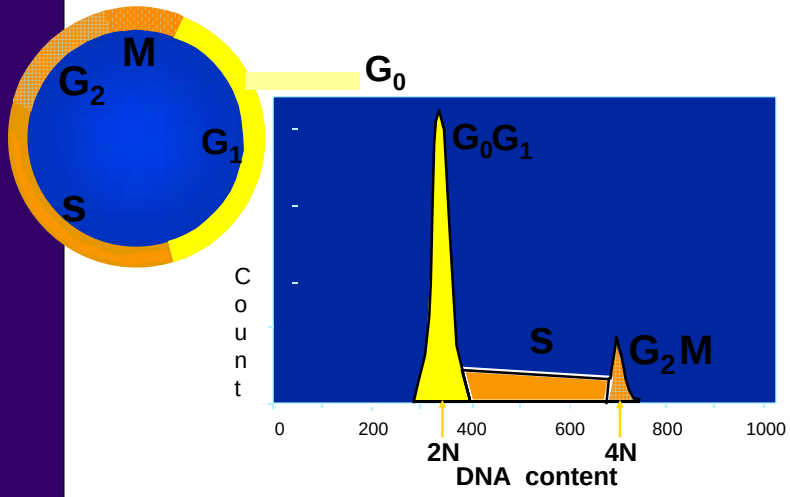
Signal Transduction



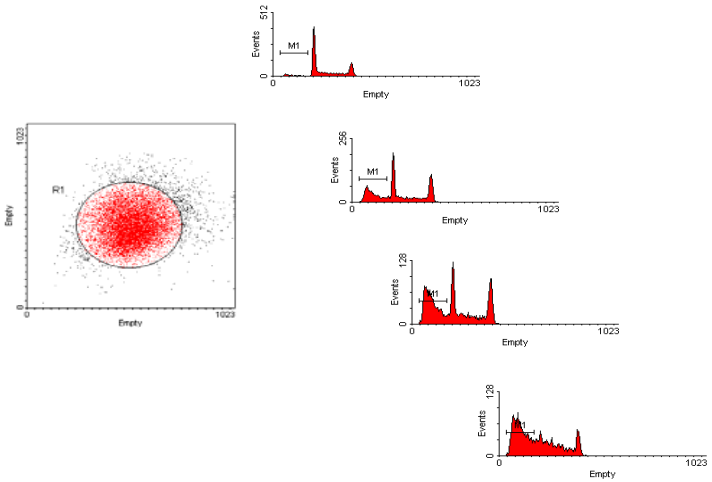
Intracellular Staining in Activated Lysed Whole Blood



Cell Cycle Analysis



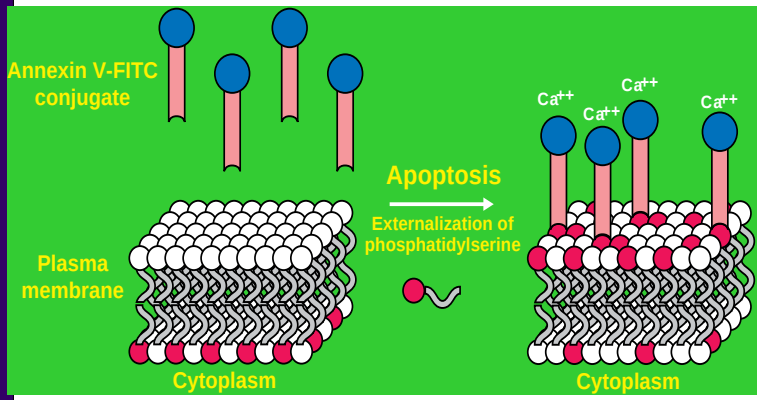
Apoptosis (Sub G1)



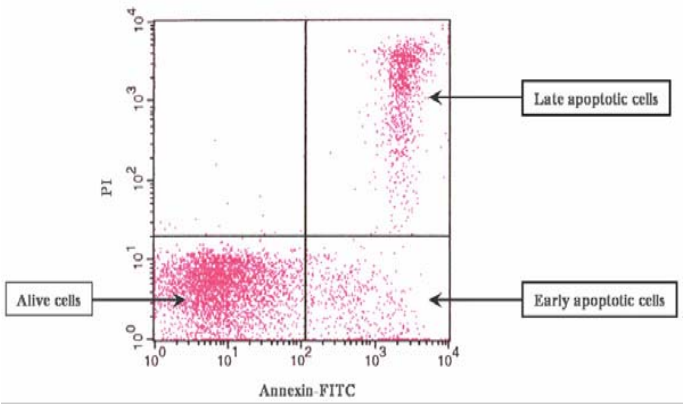
Cell Function Analysis

- 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

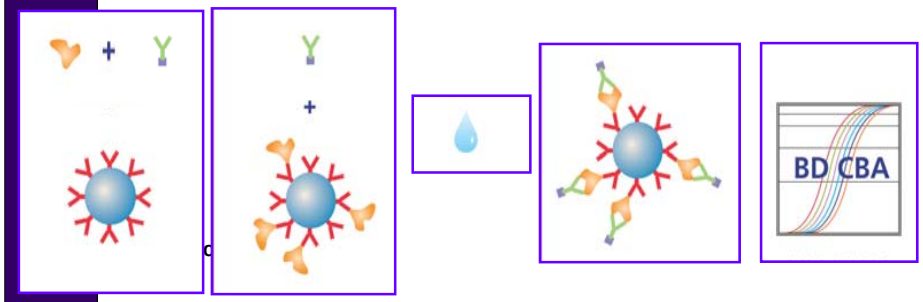


Annexin V/PI Double Staining

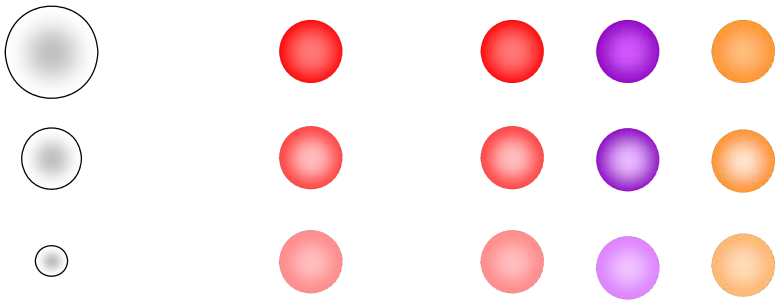


Bordón *et al. Radiation Oncology* 2009 4:58

Cytometric Beads Array (CBA)



Beads Provide a Flexible Platform



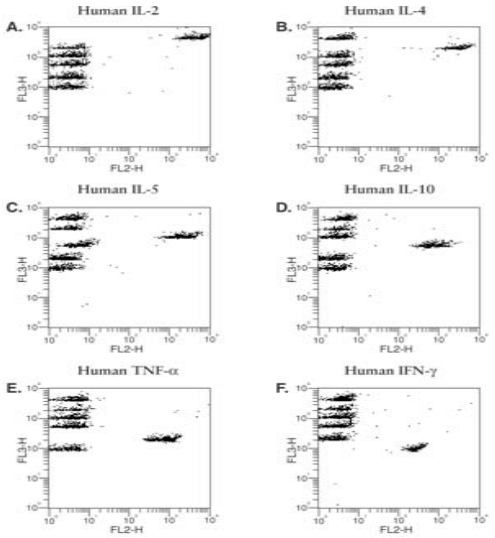
Multiple sizes

Different fluorescence intensities

Different colors with different intensities

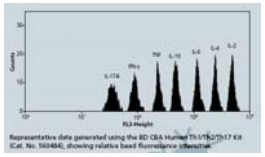
Advantages of Bead-Based Immunoassays

- 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)



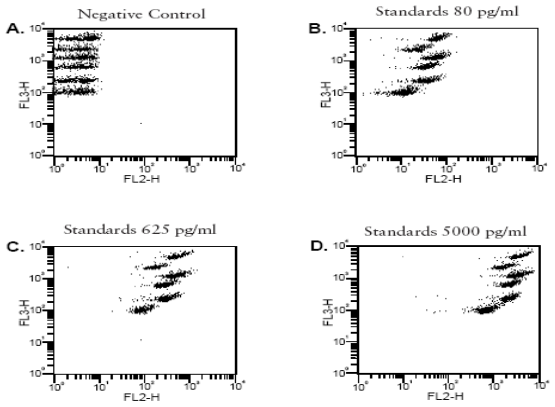
Proteins Measured

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

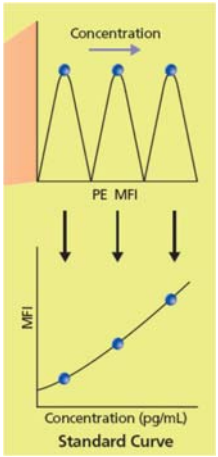


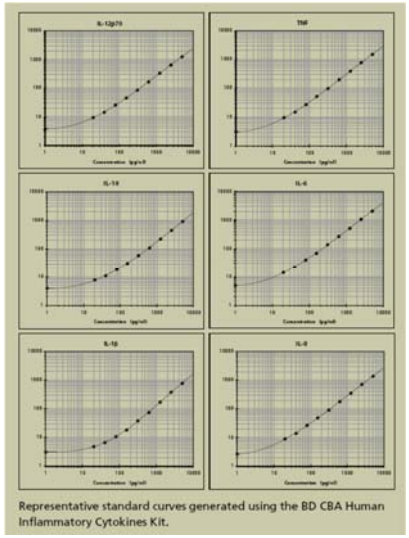
Cytometry Beads Array (CBA)

Typical Data



Standard Curves

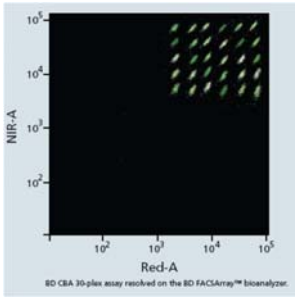




Representative standard curves generated using the BD CBA Human Inflammatory Cytokines Kit.

CBA Flex Sets

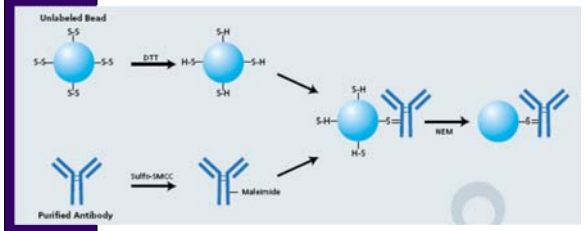
- Open configuration (Up to 30 plex)
- Clustering based on Red and NIR fluorescence intensity
- Need to be used at dual-laser(488nm blue v.s 633nm red) instrument

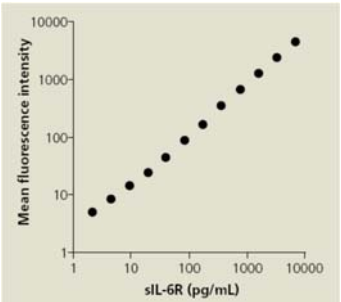


BD CBA 30-plex assay resolved on the BD FACSArray™ bioanalyzer.

CBA Functional Beads

- Can be conjugated with any Ab or Ag





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.

Thanks for your attention~

Questions?