Flow Cytometry

Basic Introduction and Applications

BD Biosciences
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Outline

- Introduction to Flow Cytometry
- Application Examples

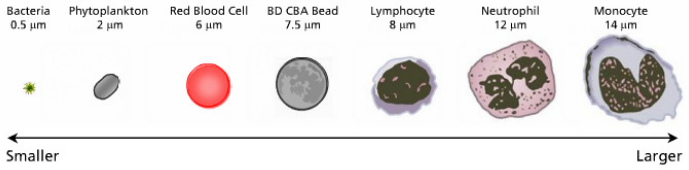
What is Flow Cytometry?

- 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: 0.5~50µm



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

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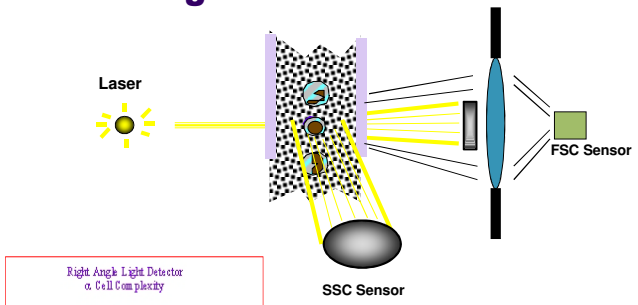
Scatter Light



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

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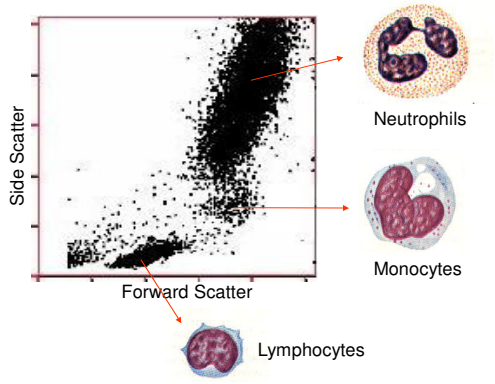
Lysed Whole Blood



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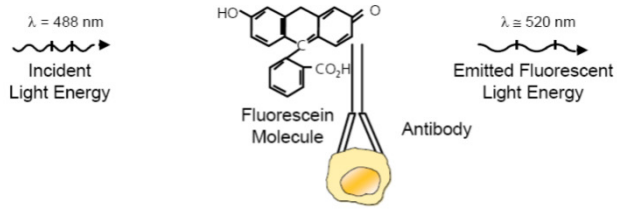
Fluorescence Light



- 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.

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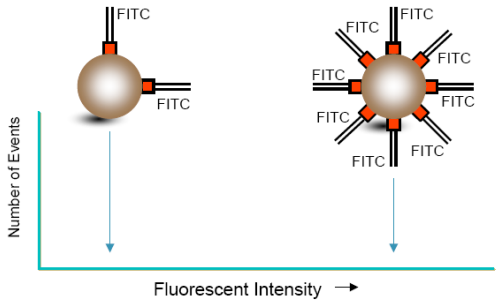
Fluorescence

Emitted fluorescence intensity proportional to binding sites



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BD Flow Cytometers

FACSCantoII

- *細胞分析儀主體*
- *系統液流車*
- *BD FACStation 電腦工作站*

2 Lasers, 6 Colors

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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.

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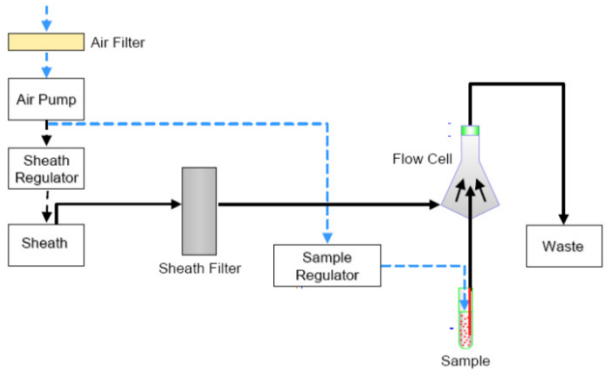
Fluidics



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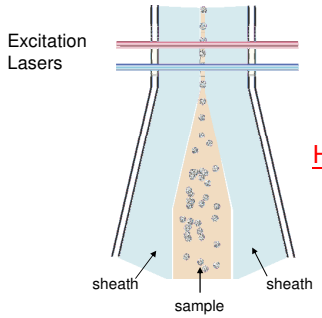
Sample Flow



Hydrodynamic Focusing

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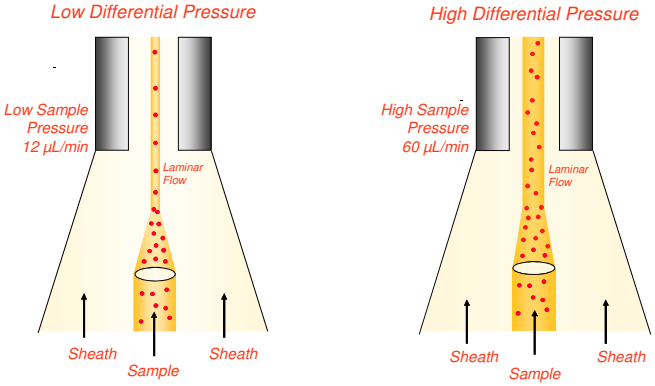
Sample Differential



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Fluorochrome Spectra

Fluorochrome	Fluorescence Emission Color	Ex-Max (nm)	Excitation Laser Line (nm)*	Em-Max (nm)
Hoechst 33342	Blue	350	355, 375	461
BD Horizon™ V450	Blue	404	405	448
Pacific Blue™	Blue	401	405	452
BD Horizon™ V500	Green	415	405	500
AmCyan	Green	457	405	491
Alexa Fluor® 488	Green	495	488	519
FITC	Green	494	488	519
PE	Yellow	496, 564	488, 532, 561	578
BD Horizon™ PE-CF594	Orange	496, 564	488, 532, 561	612
PI	Orange	351	488, 532, 561	617
7-AAD	Red	543	488, 532, 561	647
APC†	Red	650	633, 635, 640	660
Alexa Fluor® 647	Red	650	633, 635, 640	668
PE-Cy™5†	Red	496, 564	488, 532, 561	667
PerCP	Red	482	488, 532	678
PerCP-Cy™5.5	Far Red	482	488, 532	695
Alexa Fluor® 700	Far Red	696	633, 635, 640	719
PE-Cy™7	Infrared	496, 564	488, 532, 561	785
APC-Cy7	Infrared	650	633, 635, 640	785
BD APC-H7	Infrared	650	633, 635, 640	785

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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

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Excitation Optics

- *FACSCantoII*
  --488nm Blue Laser
  --633nm Red Laser

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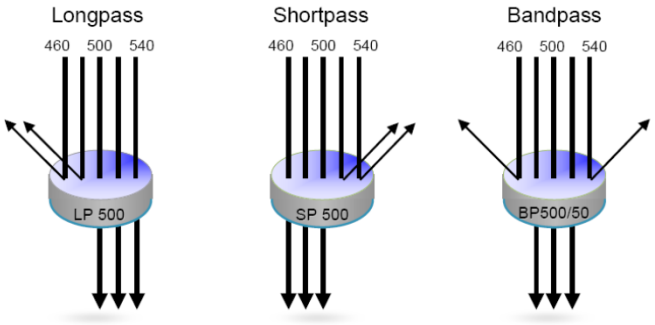
Optical Filters



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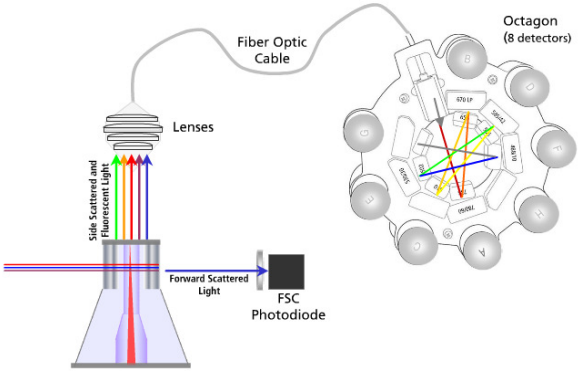
FACSCanto Collection Optics



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FACSCanto—Octagon and Trigons

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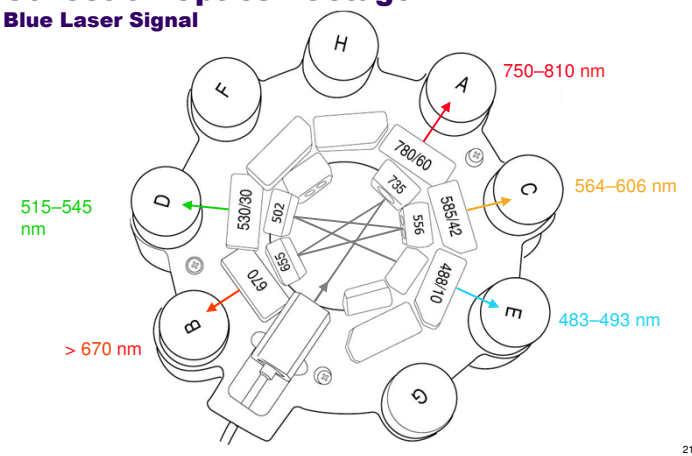
Collection Optics—Octagon

Blue Laser Signal



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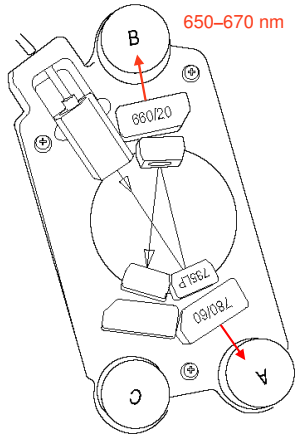
Collection Optics—Trigon

Red Laser Signal



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FACSCanto Configuration

Wavelength (nm)	PMT Position (nm)	Intended Dyes	Other Dyes
488 (blue)	A	PE-Cy7	
	B	PerCP, PerCP-Cy5.5	PI, PE-Cy5.5, DSRed
	C	PE	PI
	D	FITC	GFP
	E	Side Scatter (SSC)	
633 (red)	A	APC-Cy7	
	B	APC	Alexa Fluor® 633

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Electronics

- Convert analog signals to proportional digital signals.
- Compute area, height and width for each pulse.
- Interface with the computer for data transfer.

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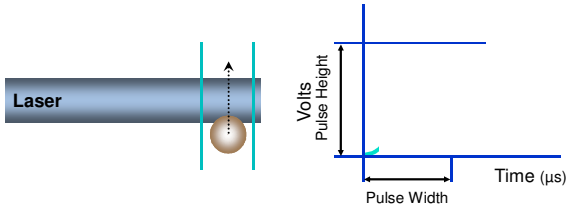
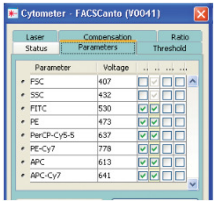
Creation of a Voltage Pulse





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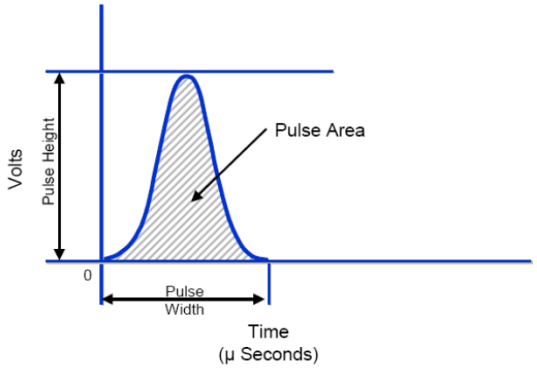
Quantification of a Voltage Pulse



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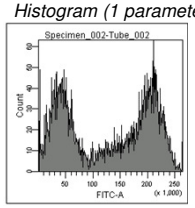
Data Storage

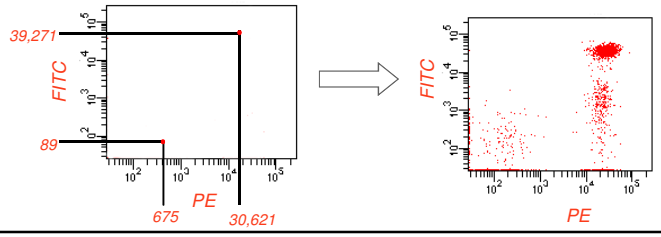
List-Mode Data

	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	16	22,688	6,189

Histogram (1 parameter)



Dot Plot (2 parameters)



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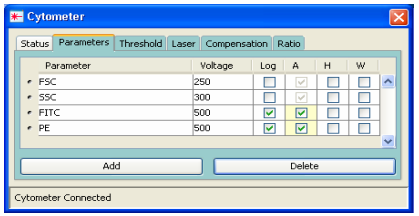
Data Display

- Linear Scaling
- Log Scaling



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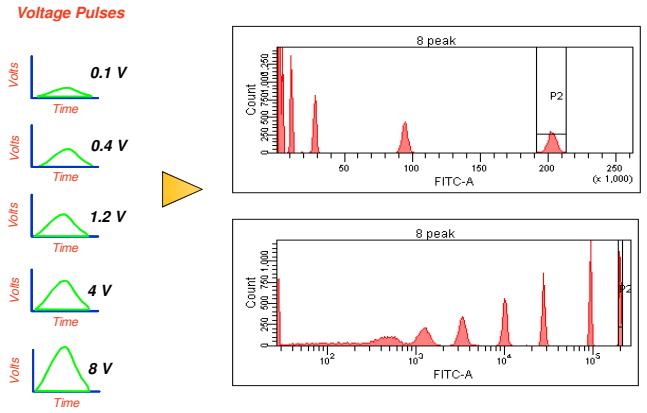
Linear v.s Log



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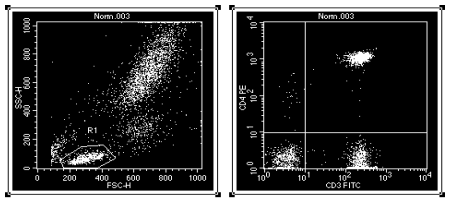
Linear v. Log Amplification



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

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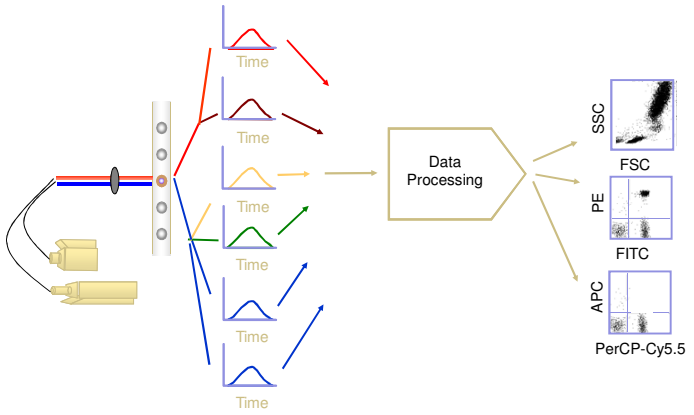
Review



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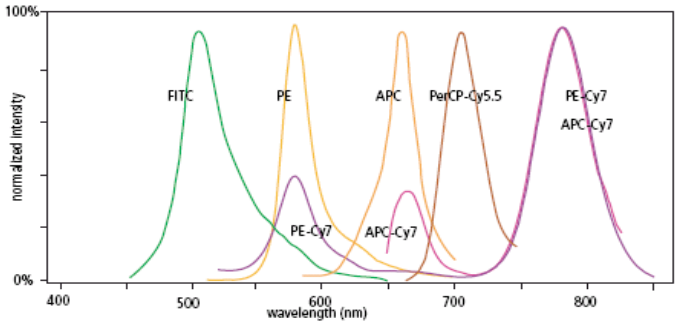
Compensation Theory

Emission Optics—Spectral Overlap



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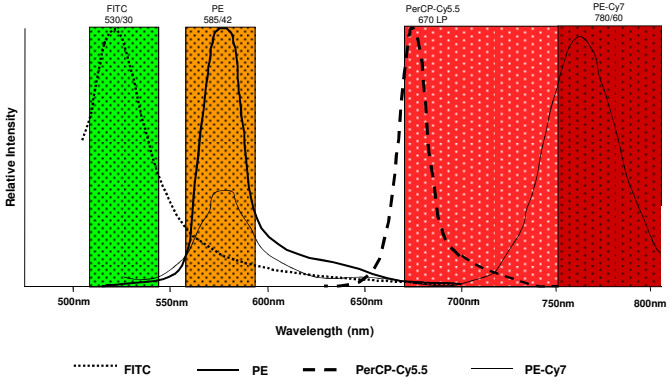
Spillover



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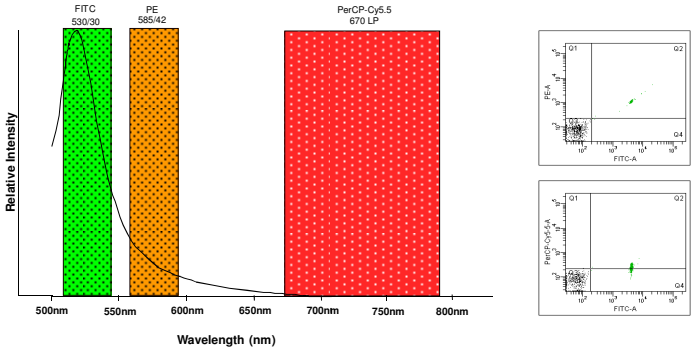
FITC Spillover



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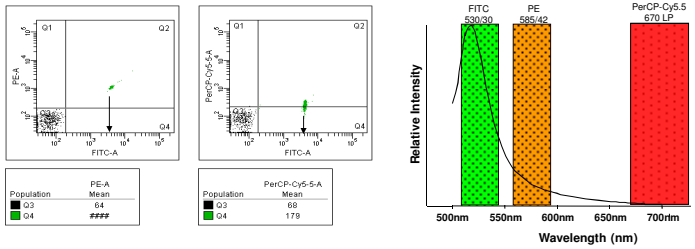
FITC Compensation



Fluorochrome	- % Fluorochrome	Spectral Overlap
PE	FITC	0.00
PerCP-Cy5-5	FITC	0.00

increase values

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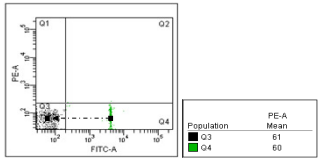
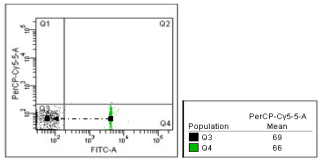
FITC Compensation



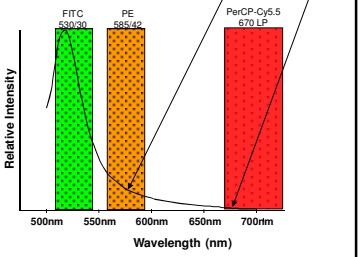
Population	PE-A Mean
Q3	61
Q4	60



Population	PerCP-Cy5-5-A Mean
Q3	69
Q4	66

To lower cluster, increase value.

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



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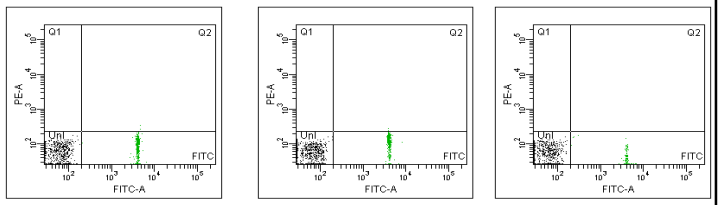
Compensation Examples

Incorrect Compensation

Correct Compensation | Undercompensation | Overcompensation



Population	PE-A Mean
Unl	64
FITC	69

Population	PE-A Mean
Unl	61
FITC	96

Population	PE-A Mean
Unl	62
FITC	-1

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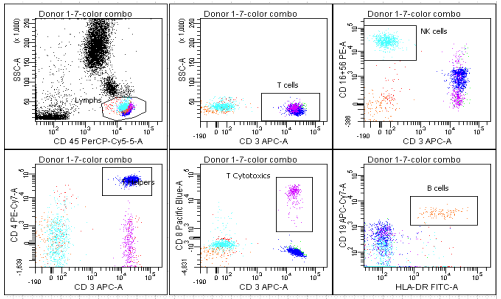
Multicolor Analysis



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

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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)*

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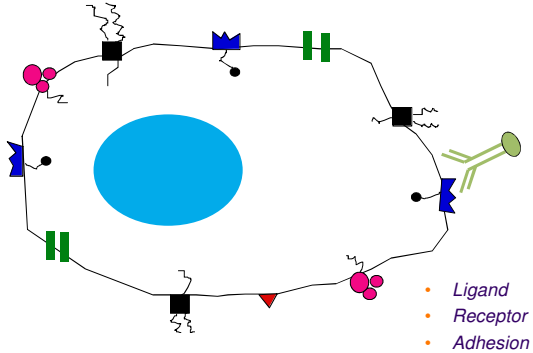
Phenotype Analysis



- *Ligand*
- *Receptor*
- *Adhesion molecule*
- *…etc*

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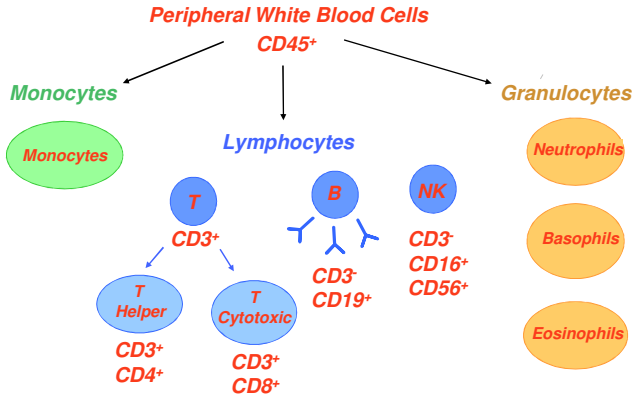
Lymphocyte Immunophenotyping



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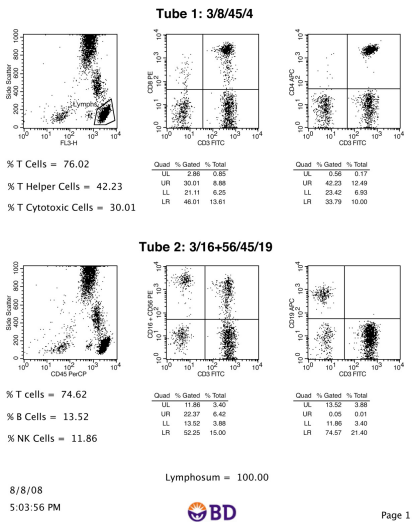

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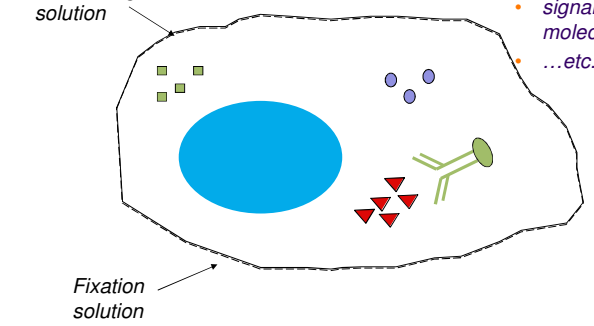
Intracellular Analysis

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



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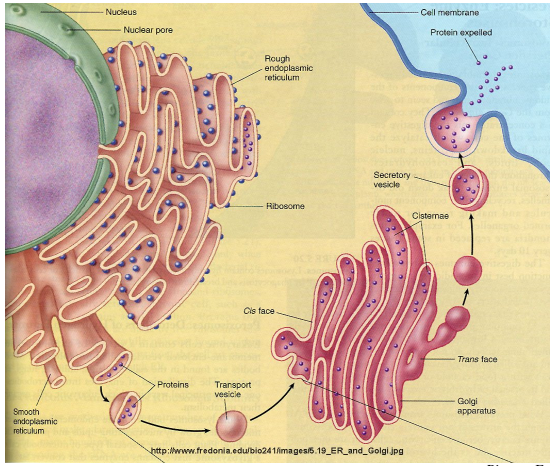
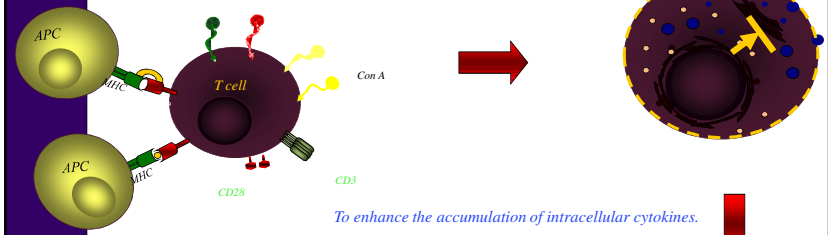
Cytokine Detection



Picture From www.fredonia.edu



Stimulation

To enhance the accumulation of intracellular cytokines.

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

Secretion stop

(Brefeldin A or Monensin)

Only in vitro

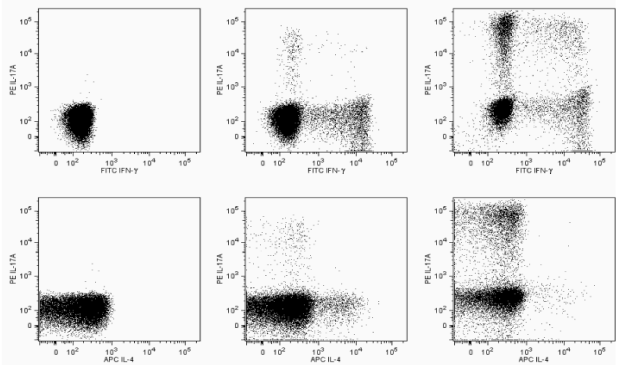
To maintain structural integrity.
Formaldehyde or glutaraldehyde
Keep the protein structure and doesn't change the (accessibility of the) epitopes too much

Intracellular Staining ← Permeabilisation ← Fixation

Saponin (permeablisation buffer).

Combination of Cell Surface and Cytoplasmic Staining

Th1/Th2/Th17 Phenotyping Kit



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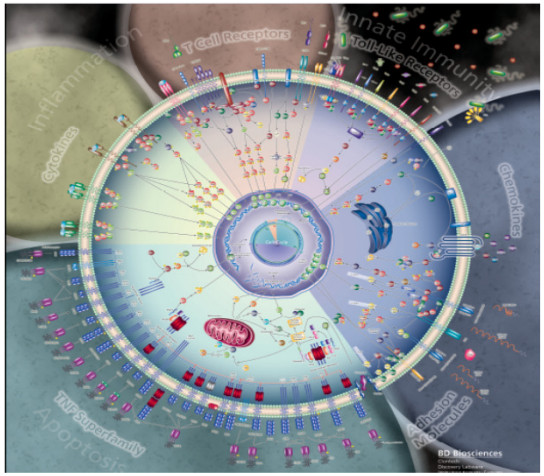
Signal Transduction



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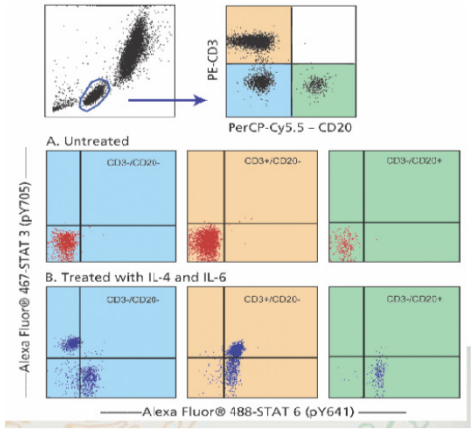
Intracellular Staining in Activated Lysed Whole Blood



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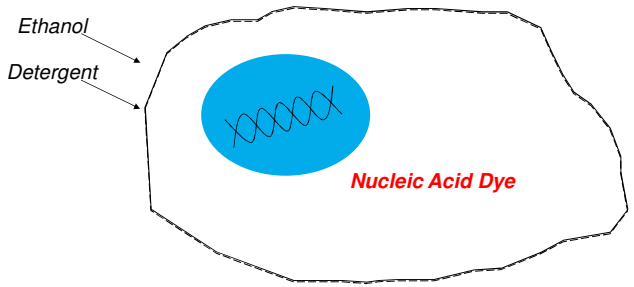
DNA Analysis



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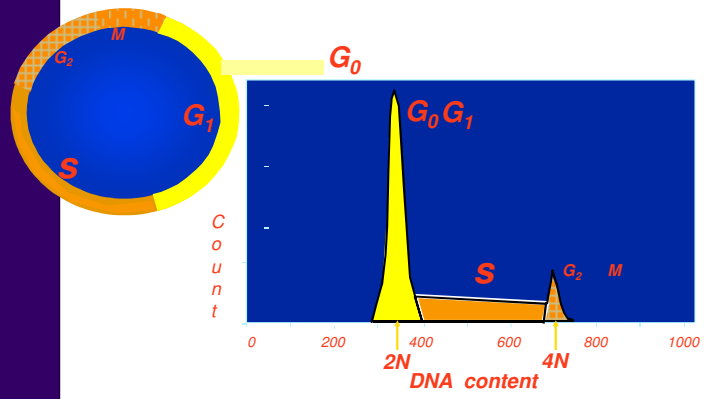
Cell Cycle Analysis



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Sample Preparation

- Ethanol fixation
  -- Long term storage
- Detergent/Hypotonic cell permeabilization
  -- Better resolution
  -- Compatible with multicolor analysis if mild detergent is used
- Analysis in live cells
  -- For cell sorting

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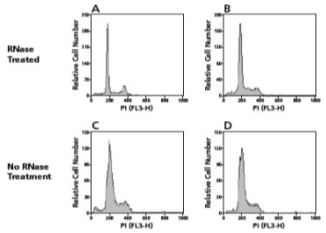
DNA Dye Selection

- **PI:** The most popular DNA dye with high specificity; excited by 488nm blue laser
- **7-AAD:** Alternative blue-excited dye for multicolor staining; less specificity
- **DAPI:** UV excitation with high specificity; for both fixed and un-fixed cells
- **Hoechst:** UV excitation with high specificity; for un-fixed cells

Other Considerations

1. RNase Activity



2. Cells v.s PI Concentration Consistency
3. Sample Filtration (35um Filter)

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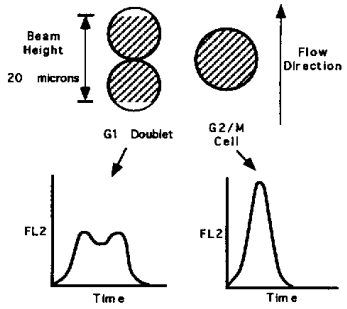
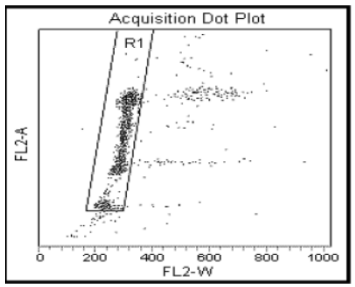
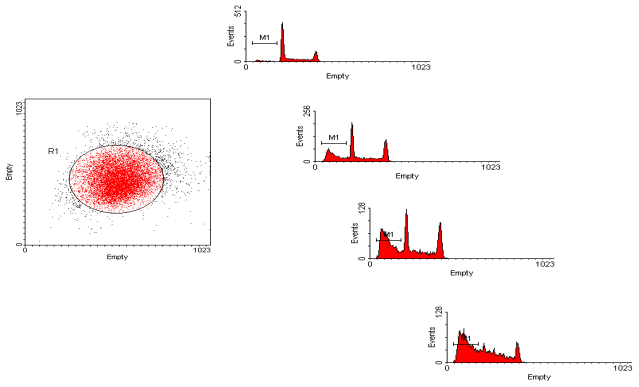
DDM (Doublet Discrimination)





Apoptosis (Sub G1)



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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)*

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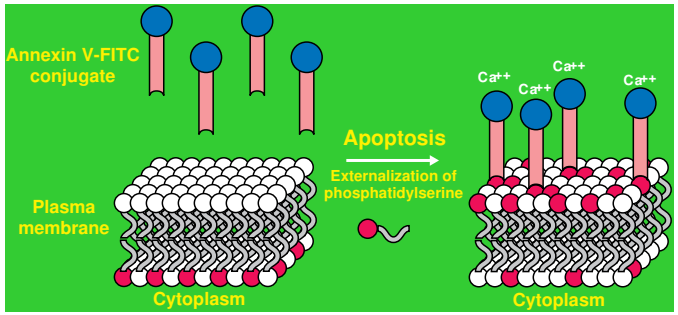
Annexin V Assay



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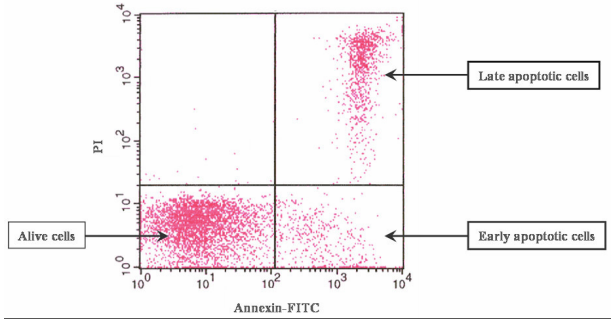
Annexin V/PI Double Staining



Bordón et al. Radiation Oncology 2009 4:58

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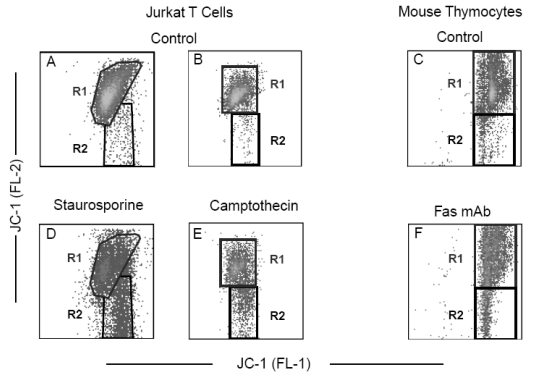
Mitochondria Membrane Potential (JC-1)



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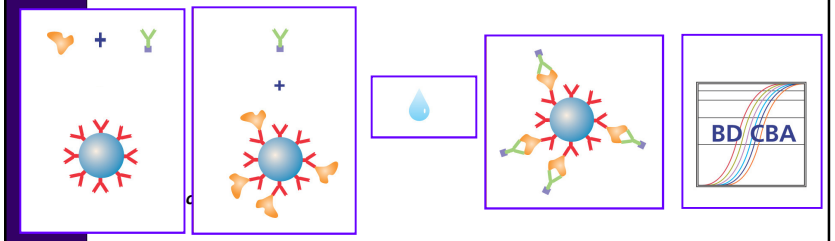
Cytometric Beads Array (CBA)



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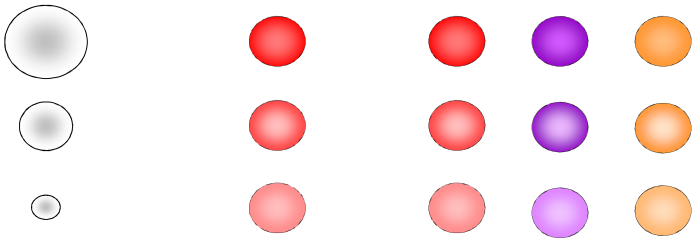
Beads Provide a Flexible Platform



Multiple sizes

Different fluorescence intensities

Different colors with different intensities

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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)

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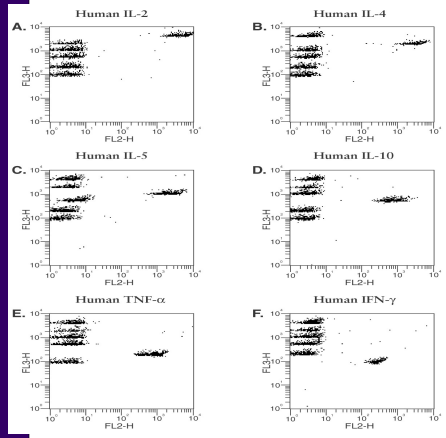
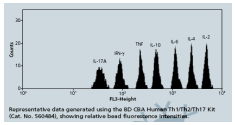


Proteins Measured

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.

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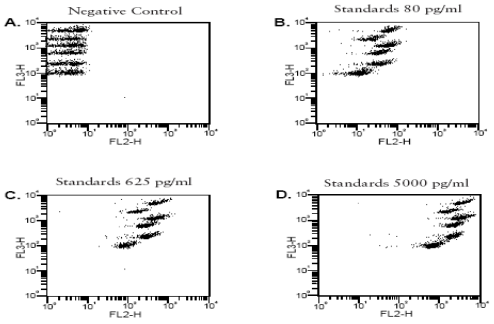
Cytometry Beads Array (CBA)

Typical Data



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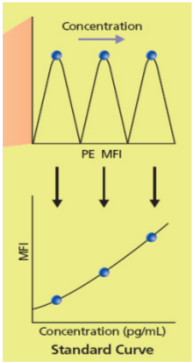
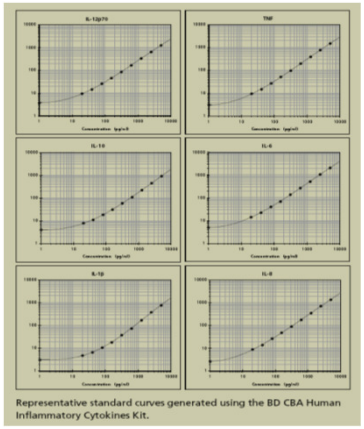
Standard Curves





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

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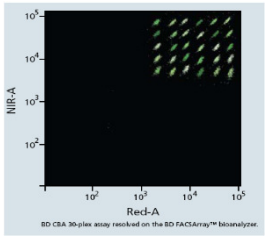
CBA Flex Sets

- *Open configuration (Up to 30 plex)*
- *Clustering based on Red and NIR fluorescence intensity*



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

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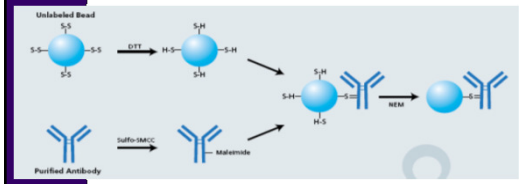
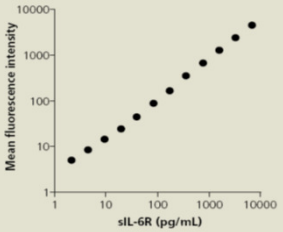
CBA Functional Beads

- *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.

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Thank You!