Introduction to Flow Cytometry

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Outline

- Instrumentation
  - Fluidics
  - Lasers, light detectors and optics
- Fluorescence
- Spectra
- Multi-parameter flow cytometry
- Flow data display

Learning points

- What are the parts of a flow cytometer?
- What is a flow cytometer used for?
- How are fluorescent dyes and monoclonal antibodies used in flow cytometry?
- What limits the number of colors or parameters measured?
- How are flow cytometric data displayed?

Flow cytometry

- Microscopy – “static” examination
- “Flow” – cells flow past the “microscope” or the point of interrogation
- Advantage over microscopy is that large numbers of cells can be examined quickly
- Disadvantage is that cells cannot be examined *in situ* (must be in suspension) and morphology of cells is not examined

Examples of applications of flow cytometry

- "Phenotypic" identification of cell type; e.g., the percentage of T cells in blood that express CD4
  - Cannot determine absolute count (unless using counting beads); typically determine count in conjunction with CBC data
- Functional examination of cells – cytokines, proliferation
- Multiple other applications

Instrumentation

- Analyzers
- Vs.
- Cell sorters (also analyze)

Len Herzenberg

Len and Lee Herzenberg

1973

The first FACS publication in Science, 1969

Cell Sorting: Automated Separation of Mammalian Cells as a Function of Intracellular Fluorescence

Abstract. *A system for high-speed sorting of fluorescent cells was able to sort mouse spleen cells from Chinese hamster ovarian cells after development of fluorochromasia. Highly fluorescent fractions separated after similar treatment from mouse spleen cells immunized to sheep erythrocytes were enriched in antibody-producing cells by factors of 4 to 10.*

H. R. HULETT, W. A. BONNER
JANET BARRETT
LEONARD A. HERZENBERG
Department of Genetics, Stanford University School of Medicine, Stanford, California 94305

Reprinted from SCIENCE.
7 November 1969, volume 166, pages 747-749

First instrument used an arc lamp rather than a laser

B and T cell hybridomas

From: Handbook of Experimental Immunology, 3rd Edition, D. M. Weir, Editor
Blackwell Scientific Publications Ltd., Edinburgh, Scotland, 1978

CHAPTER 25

Cell hybrids of myelomas with antibody forming cells and T-lymphomas with T cells:

A new means of making large amounts of monoclonal antibodies and a potential means of making large numbers of cells carrying out specific T cell functions

Monoclonal antibodies key for FACS

L. A. HERZENBERG, LEONORE A. HERZENBERG & C. MILSTEIN

Herzenberg & Becton-Dickinson

Herzenberg -1972 - Argon laser flow sorter - placed an argon laser onto their sorter and successfully did high speed sorting - Coined the term Fluorescence Activated Cell Sorting (FACS). This instrument could detect weak fluorescence with rhodamine and fluorescein tagged antibodies. A commercial version was distributed by BD in 1974 and could collect forward scatter and fluorescence above 530 nm.

Herzenberg was the recipient of the very Prestigious 2006 Kyoto Prize for his work in development of fluorescence based flow cytometry. Many people well known in the field were trained in his lab.

(Photo from the official Kyoto Prize website)

Components of a flow cytometer

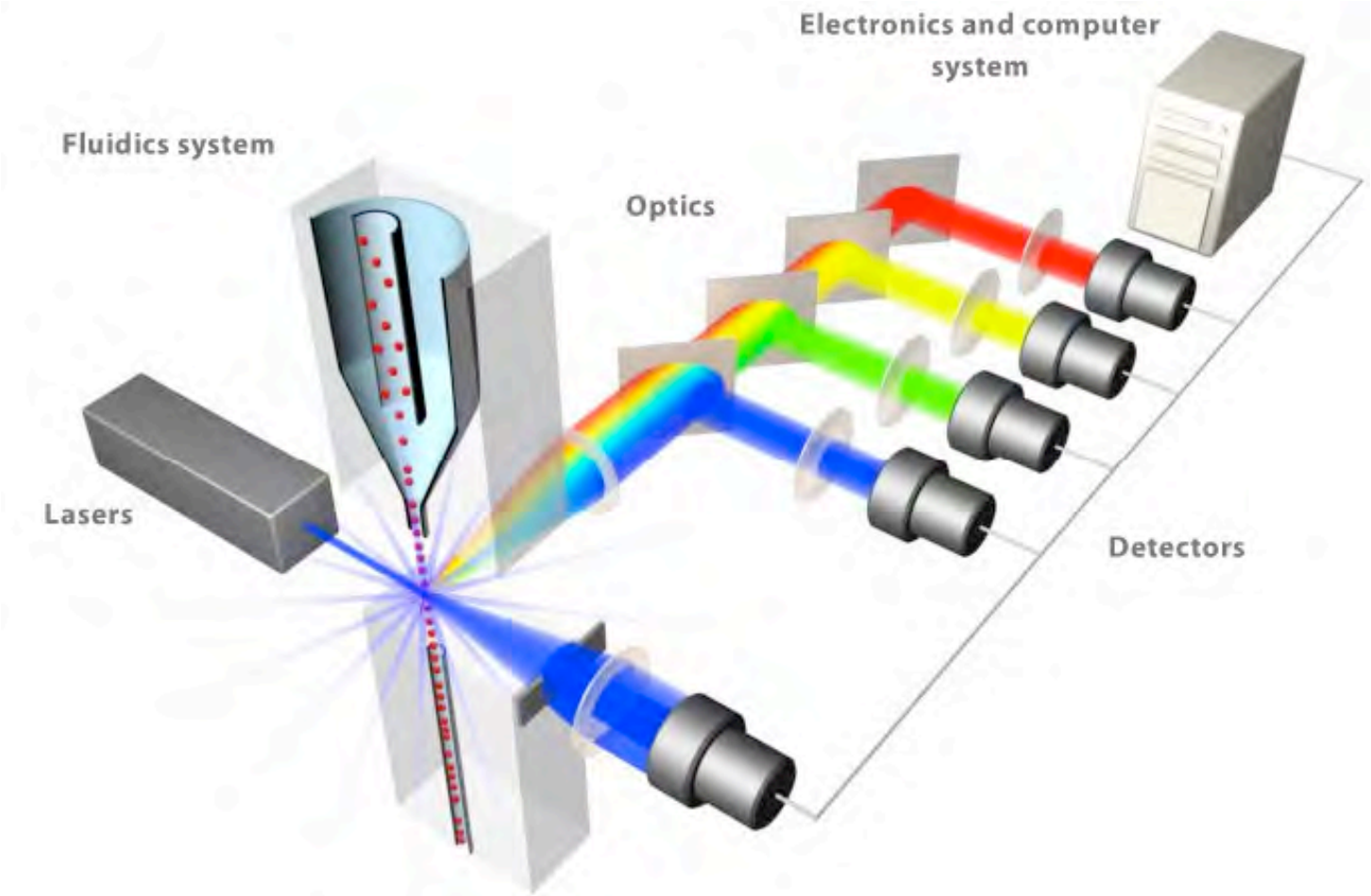
- Fluidics system
- Laser(s)
- Light detectors
- Optical path
- Electronics

- (Cell sorting components)

Fluidics

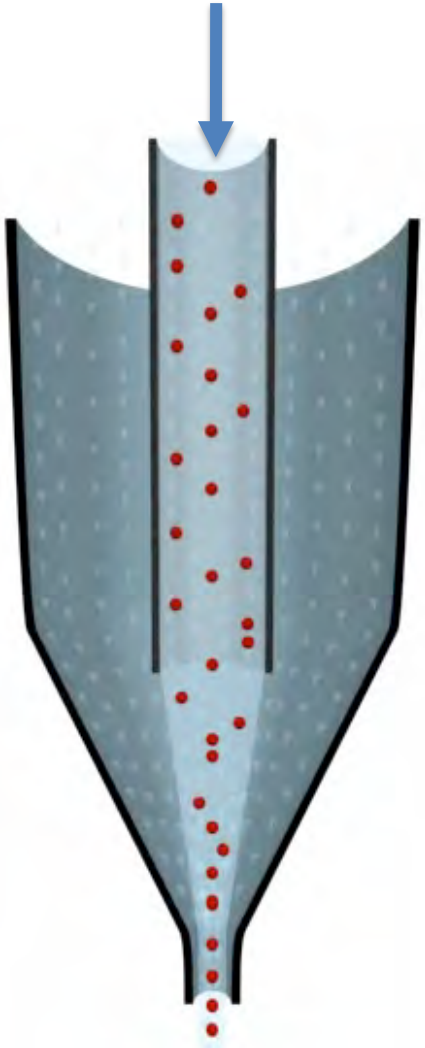
- Fluidics are critical to placing the cell in the correct position to be “viewed” at the point of interrogation
- “Alignment” refers to the focus of the laser beam on the cell in the fluid stream
- Alignment can change either due to laser beam moving or fluid stream moving relative to the laser
  - The latter is more common and can often be corrected by cleaning or priming nozzle

Flow cytometer
5-parameter, 3-color example



http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

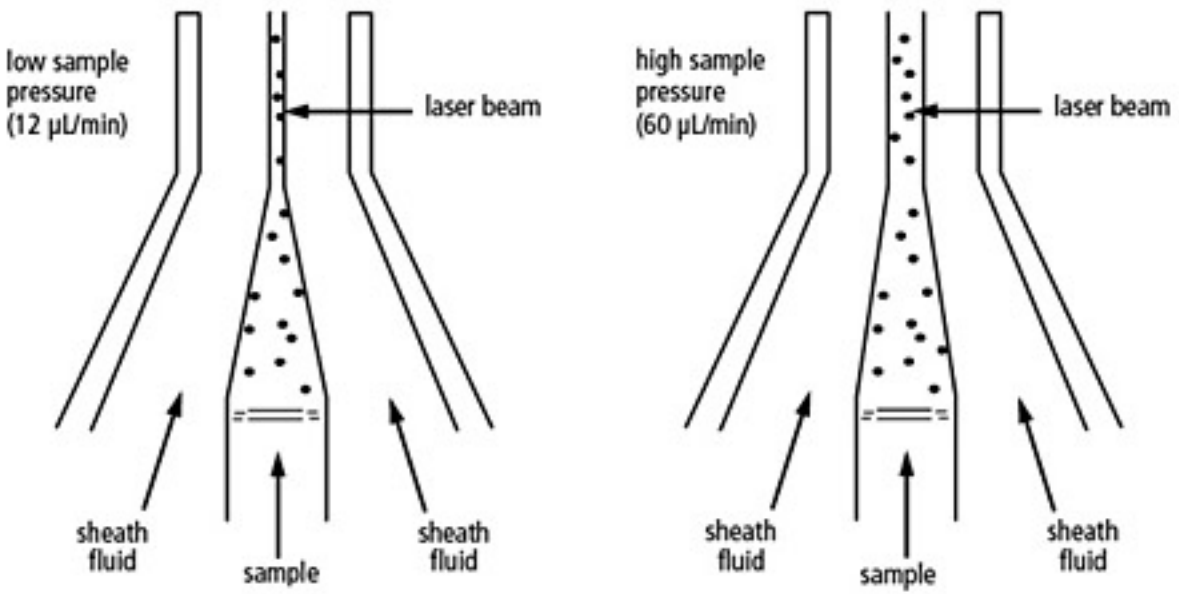
Hydrodynamic focusing



- Nozzle or flow cell
- Cell stream in center
- Sheath fluid stream surrounds cells and focuses cells
- Single cell per cross section
- Cells pass through laser one cell at a time

http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

In some cytometers, cells flow up



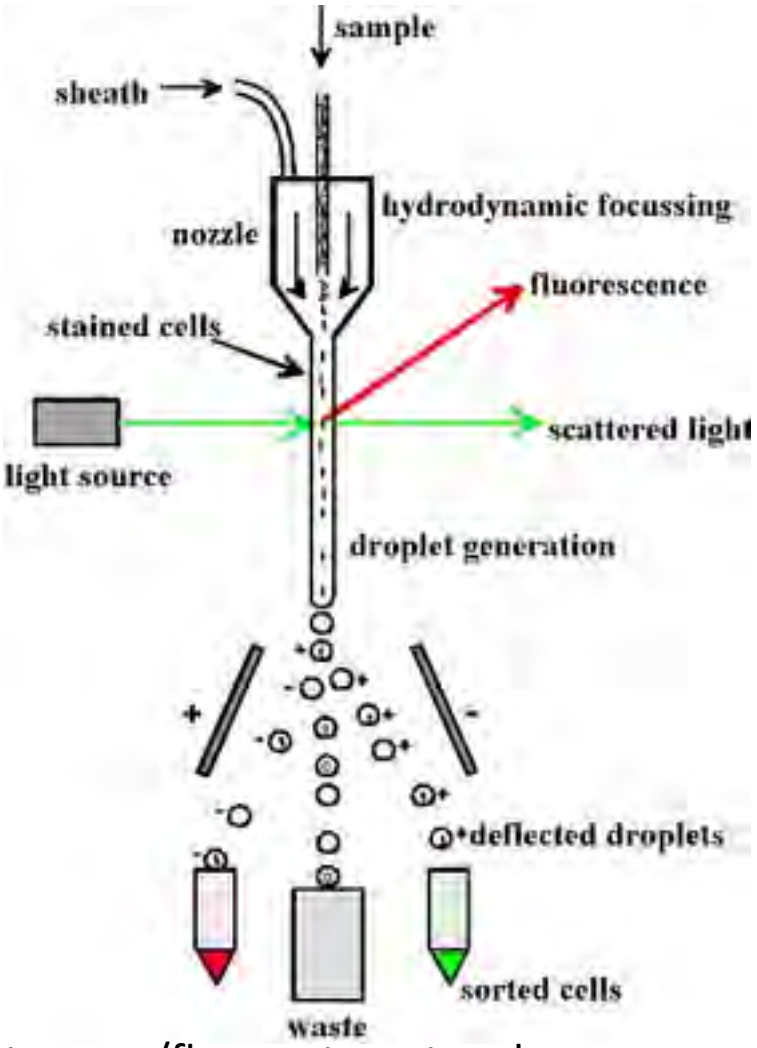
Hydrodynamic focusing of the sample core through the flow cell

http://www.bdbiosciences.com/instruments/lsr/features/index.jsp

Cell analysis vs. cell sorting

- Some flow cytometers can physically separate or sort cells of interest in addition to analyzing cells
- FACS refers to Fluorescence-Activated Cell Sorting
- Each cell to be sorted receives a positive or negative charge and is then deflected from the fluid stream by charged plates
- Same technology as inkjet printers

Cell sorting

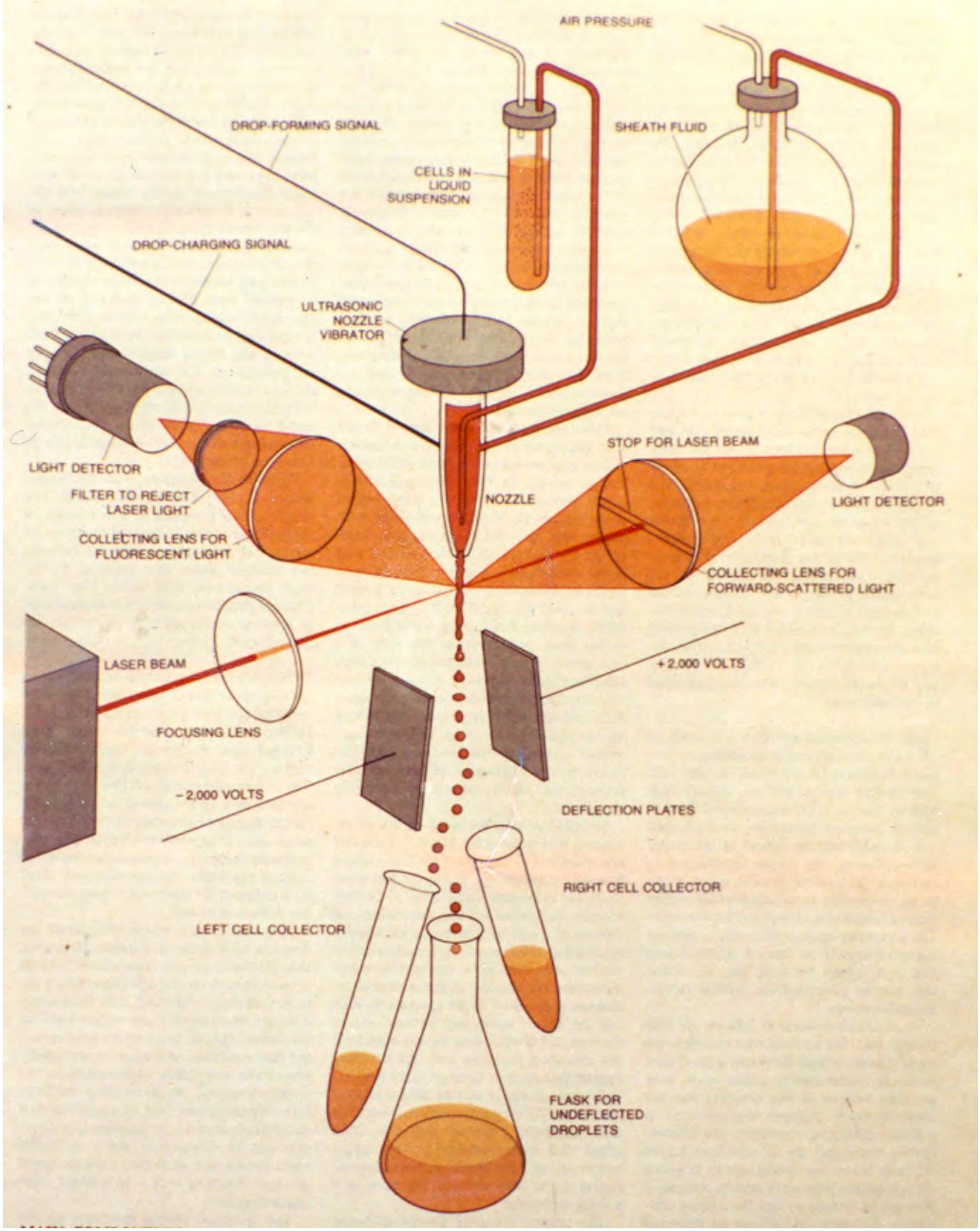


http://www.appliedcytometry.com/flow_cytometry.php

Leonard A. Herzenberg,
Richard G. Sweet
and
Leonore A. Herzenberg

Scientific American

*March **1976***



Laser light

- Light Amplification by Stimulated Emission of Radiation
- A non-laser light source could be used, but for multi-color flow, light of specific wavelengths is needed
- Also, lasers provide light of sufficient intensity to excite fluorescent dyes to the level necessary to be detectable

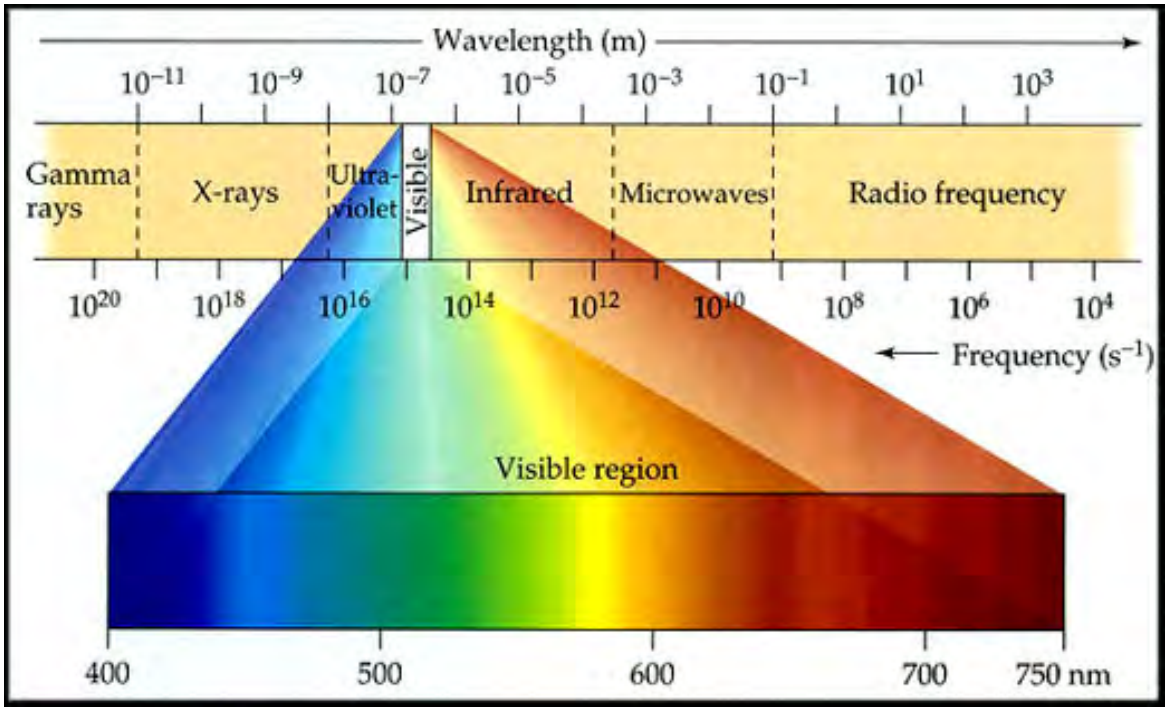
Light collector

- Photomultiplier tubes (PMTs)
- Convert light to an electrical signal
- Can be very sensitive, as little as one photon
- Amplifies signal

- Voltage applied to the PMT can be adjusted and determines the signal intensity

Brief note on light

- Wavelength determines color
- Higher energy light has shorter wavelengths (blue) and lower energy longer wavelengths (red)



http://www.earthtym.net/image.htg/em-spectrum.jpg

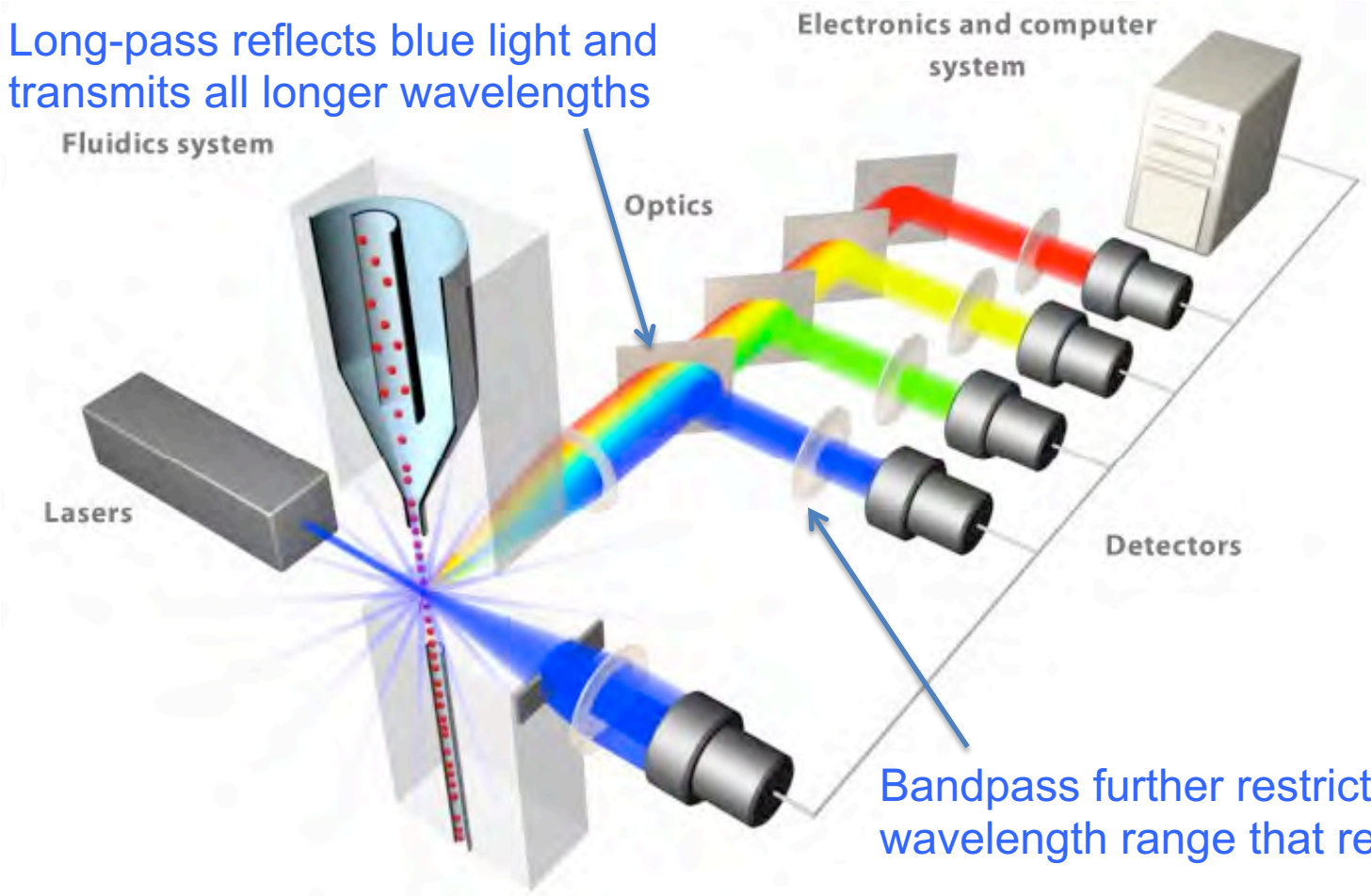
Optical path

- Path of laser light directed to the fluid stream
- Path of scattered light from the cell to each PMT
  - Can be quite complex for 20-parameter instruments
- Either can be “direct” and positioned using mirrors or transmitted through optical fibers
- Alignment is critical

Light filters

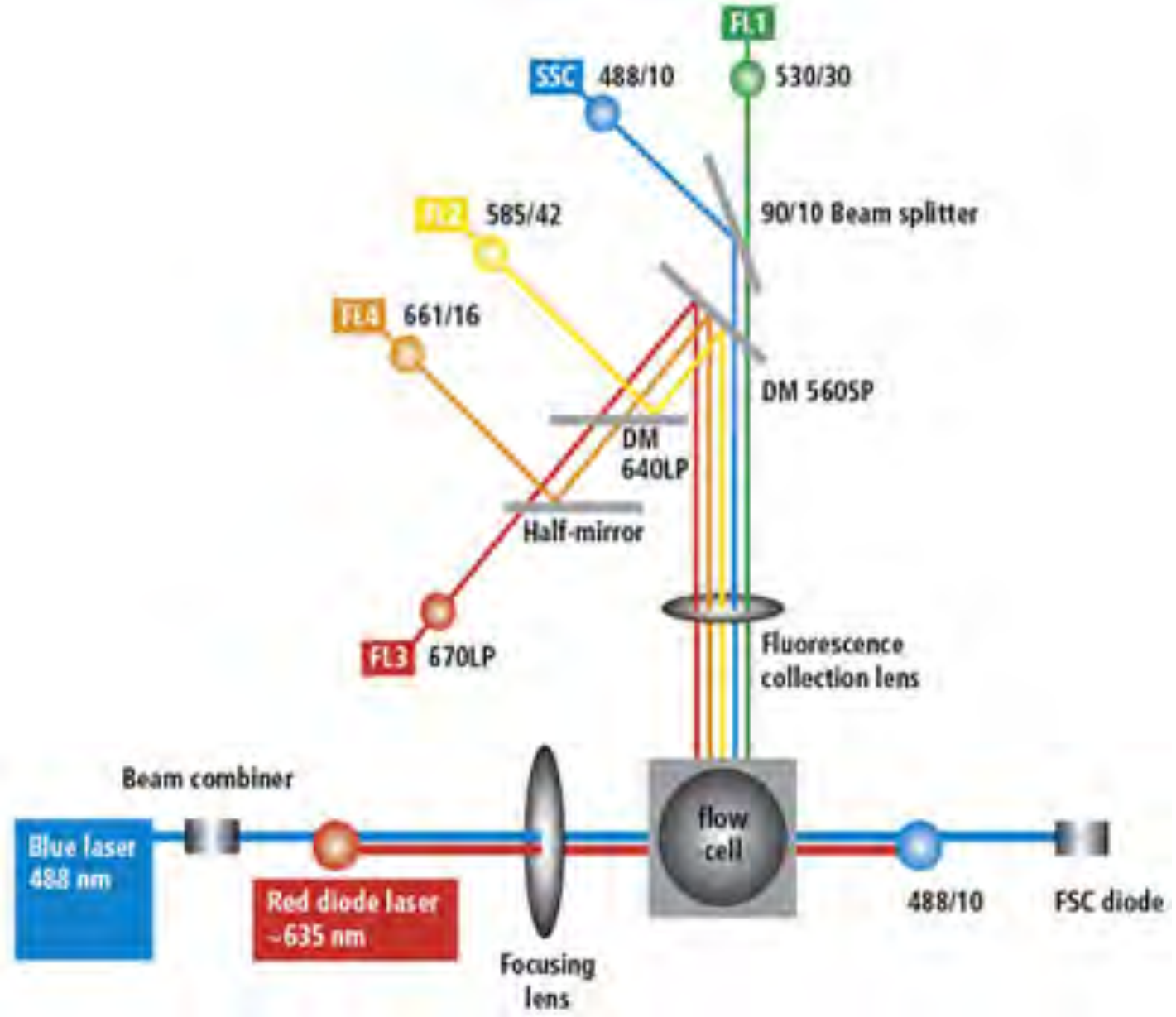
- Restrict light to selected wavelengths
- Each PMT has a bandpass filter so that only light of a selected wavelength range reaches the PMT
- Naming: center/range, e.g., **530/30** passes light of **515 to 545 nm**
- Other filters reflect all light above or below a wavelength (long- or short-pass filters)

Flow cytometer
5-parameter, 3-color example



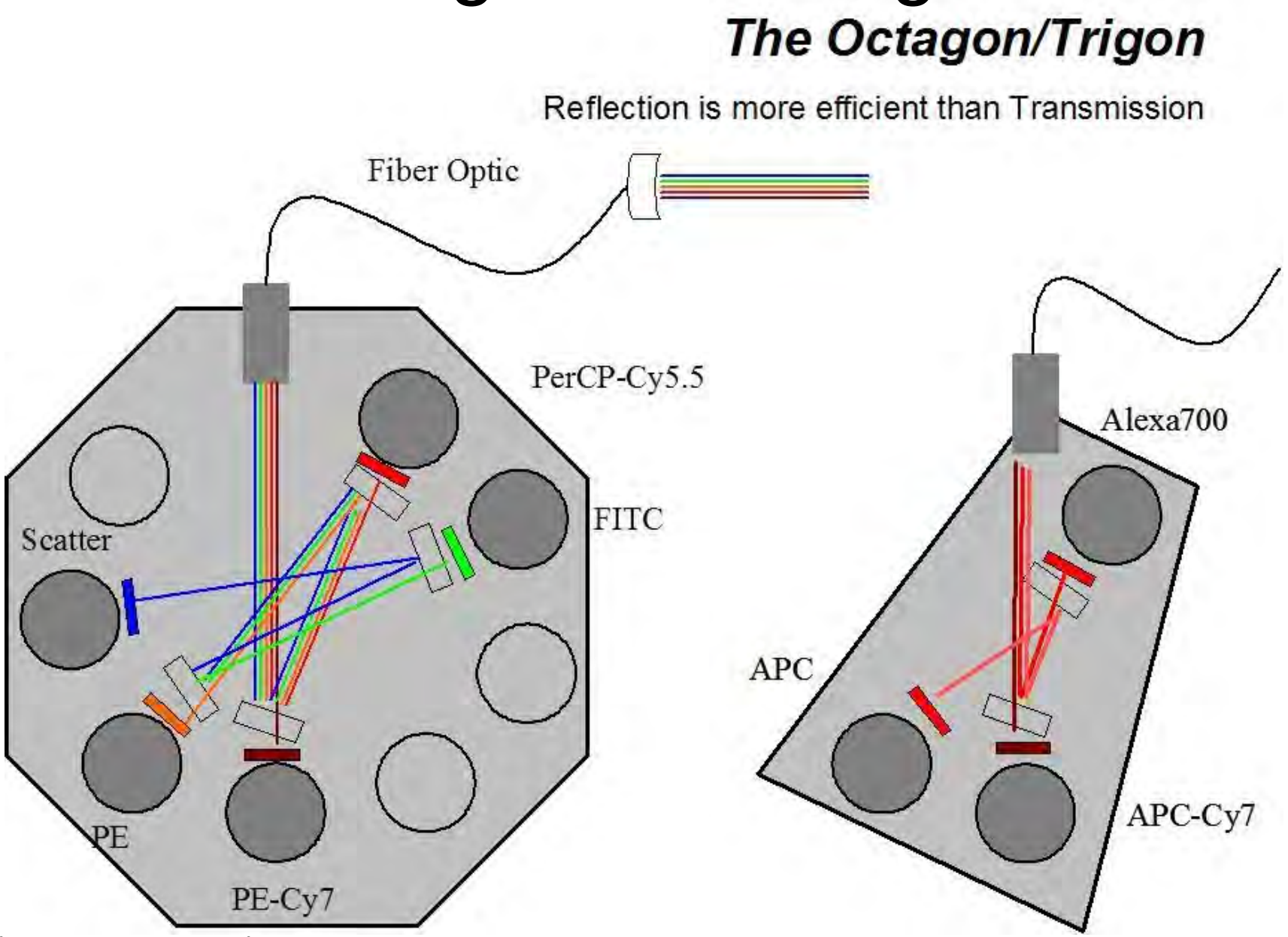
http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

BD FACSCalibur Optical Path Configuration



http://www.bdbiosciences.com/instruments/facscalibur/features/index.jsp

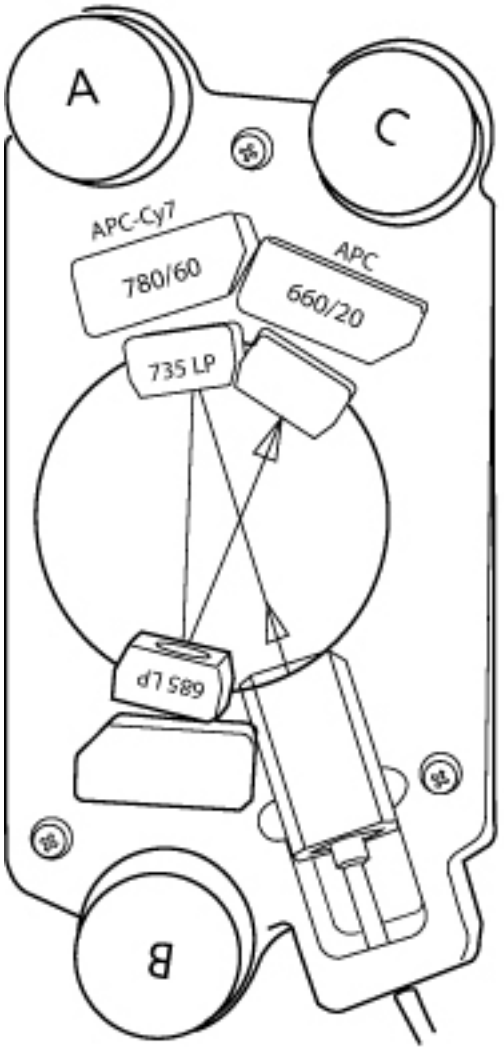
BD Octagons and Trigons



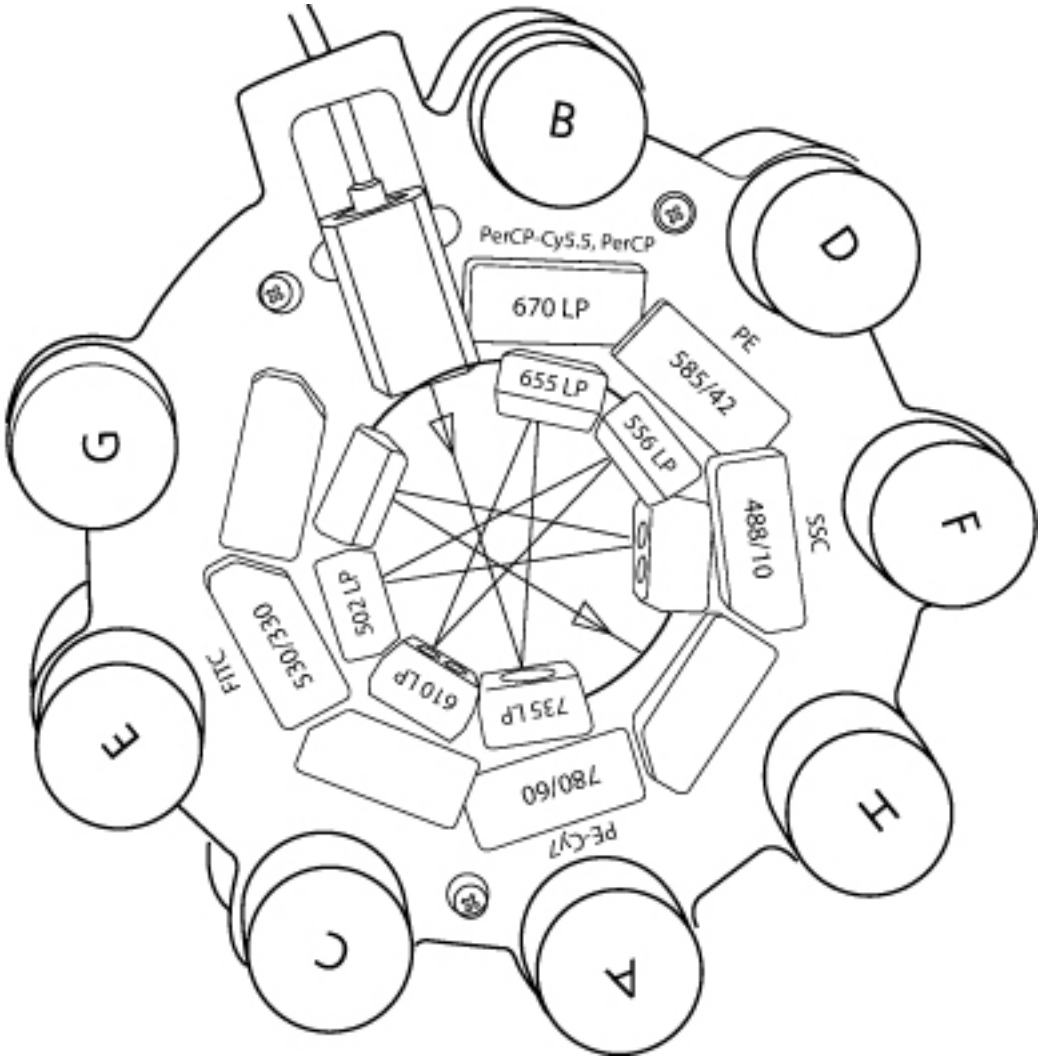
http://facs.scripps.edu/FACSAria.html

Examples of Trigon and Octagon

Red Laser



Blue Laser



www.bdbiosciences.com/
wcmimages/octagon_canto.jpg

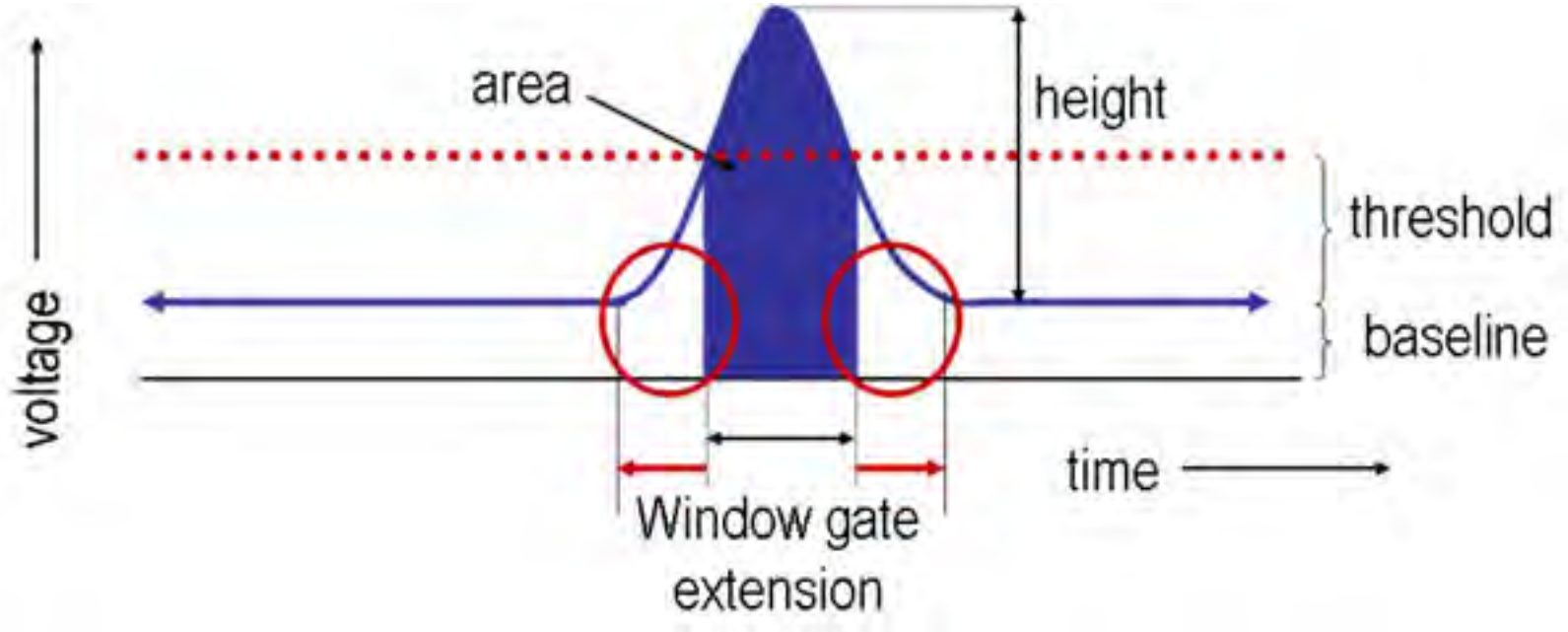
Laser	Laser Power	Channel	Instrument	Bandwidth	Long Pass	Old Detector Name
Blue	488/100mW	B710	L	710/50	685	
			M	710/50	680	PerCP Cy5.5
			Y	710/50	685	
		B515	L	515/20	505	
			M	510/30	505	FITC
			Y	515/20	505	
		SSC	L	488/10	None	
			M	488/10	None	SSC
			Y	488/10	None	
Green	532/150mW	G780	L	780/40	740	
			M	780/60	735	PE Cy7
			Y	780/40	740	
		G710	L	None	None	
			M	None	None	PE Cy5.5
			Y	None	None	
		G660	L	660/40	635	
			M	660/40	635	PE Cy5
			Y	660/20	640	
		G610	L	610/20	595	
			M	610/20	595	PE Texas Red
			Y	610/20	600	
		G575	L	575/26	None	
			M	575/26	None	PE
			Y	575/26	None	
Red	628/200mW	R780	L	780/60	735	
			M	780/60	755	APC Cy7
			Y	780/60	755	
		R710	L	710/50	680	
			M	710/50	690	Alx700
			Y	710/50	685	
		R660	L	660/20	None	
			M	660/20	None	APC
			Y	660/20	None	
Violet	405/100mW	V780	L	780/60	770	
			M	780/60	770	Qdot800
			Y	780/60	770	
		V750	L	None	None	
			M	None	None	Not Used
			Y	None	None	
		V710	L	710/40	685	
			M	710/40	685	Qdot705
			Y	710/40	685	
		V655	L	660/40	635	
			M	660/40	635	Qdot655
			Y	660/40	635	
		V610	L	610/20	585	
			M	610/20	585	Qdot605
			Y	610/20	585	
		V570	L	575/25	550	
			M	575/25	557	Qdot585
			Y	575/25	557	
		V510	L	510/20	495	
			M	510/20	495	Am Cyan
			Y	510/20	495	
		V450	L	450/50	None	
			M	450/50	None	Pacific Blue
			Y	450/50	None	
Ultra Violet*	355/20mW	UV730	L	730/45	685	
			M	730/45	685	
			Y	730/45	685	
		UV395	L	379/28	None	
			M	379/28	None	
			Y	379/28	None	

Example configurations for LSR instruments (L, M, Y)

What is measured?

Shape of electronic signal as cell passes through laser

- Typical shape of signal
- Height and/or area is used

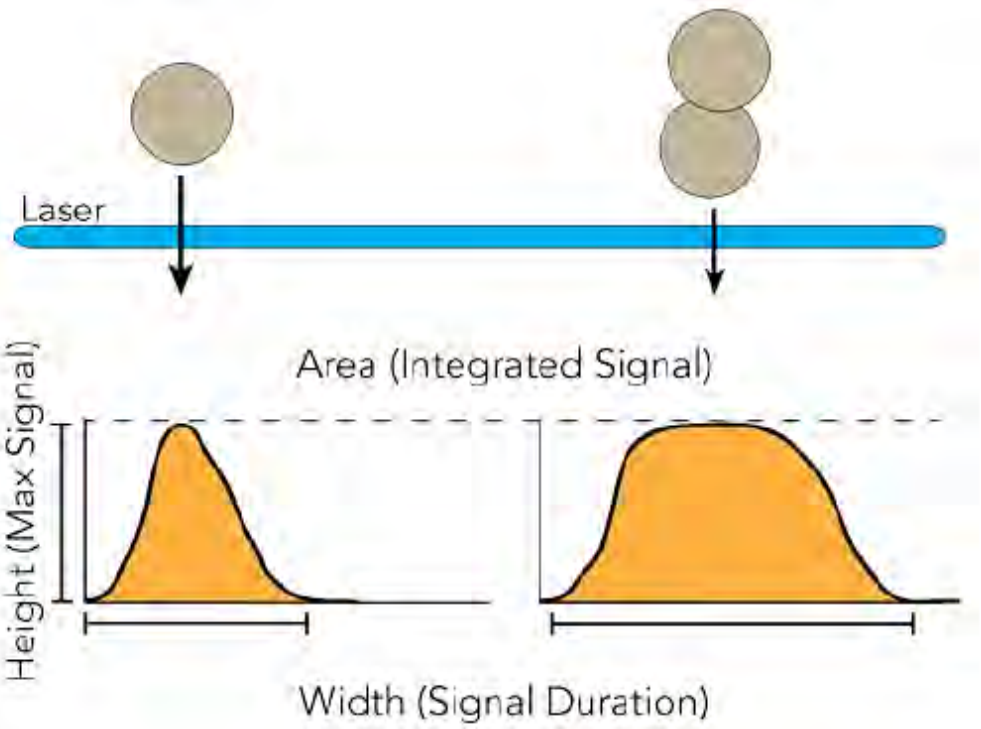


http://mi.img.cas.cz/FACS/LSRII/System%20description.aspx?id=root_7_1_7

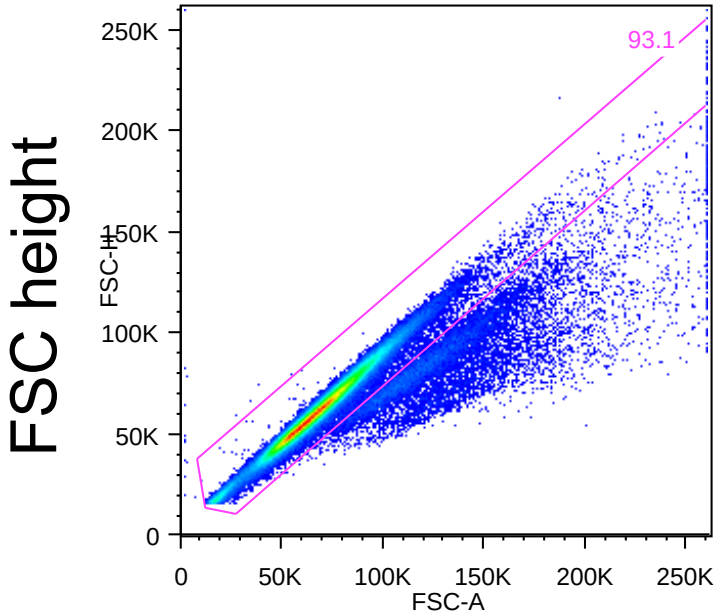
Doublets

- In flow cytometry (and CyTOF), a major concern and potential for artifact is two cells counted together, either physically associated or simply side-by-side in the fluid stream
- These are referred to as doublets
- Examining area vs. height (or width) allows exclusion of doublets
  - The doublet orients in the direction of the fluid stream and thus the height is not increased, but the width and area are increased

Example of doublets exclusion via FS area vs. height



Doublets are higher for area as compared to height



Electronics

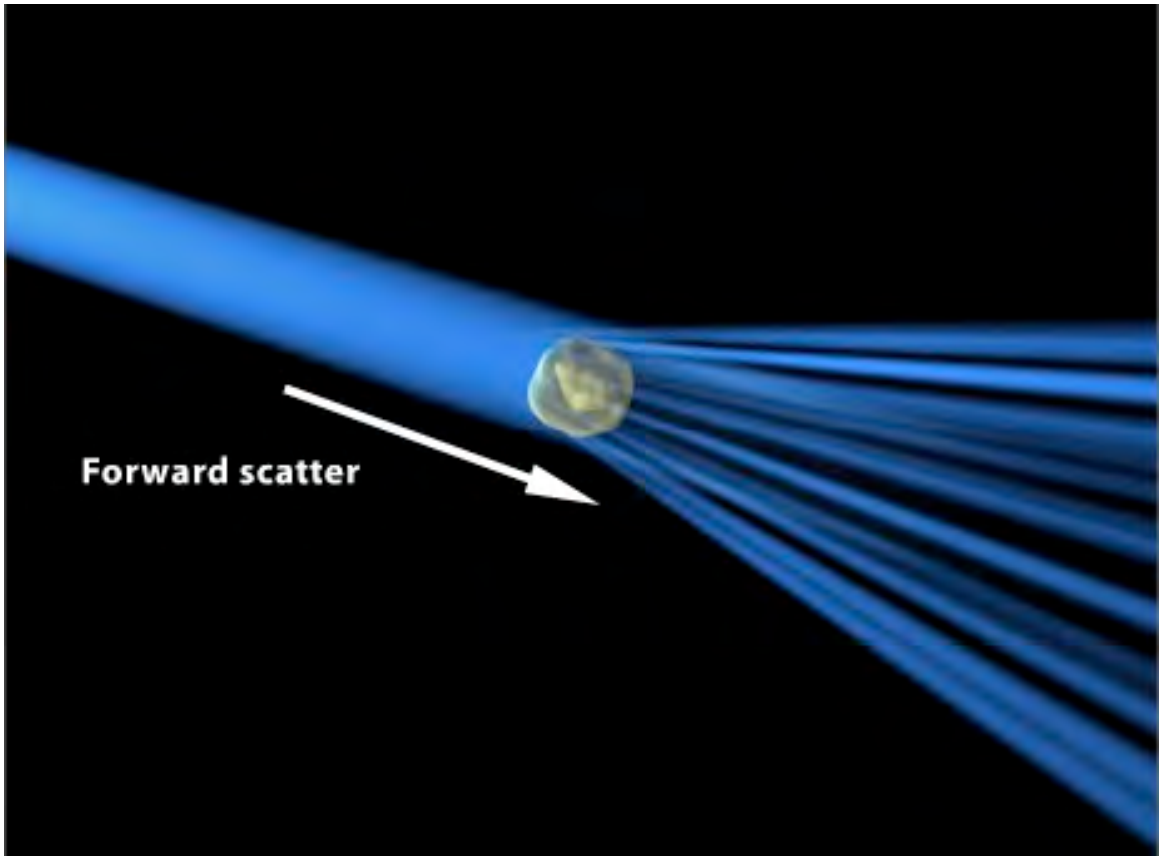
- Older instruments:
  Due to large dynamic range required, logarithmic amplifiers are used on “analog” instruments (data is collected after log transformation)
- Newer “digital” instruments collect data before log transformation

What is detected: scattered light

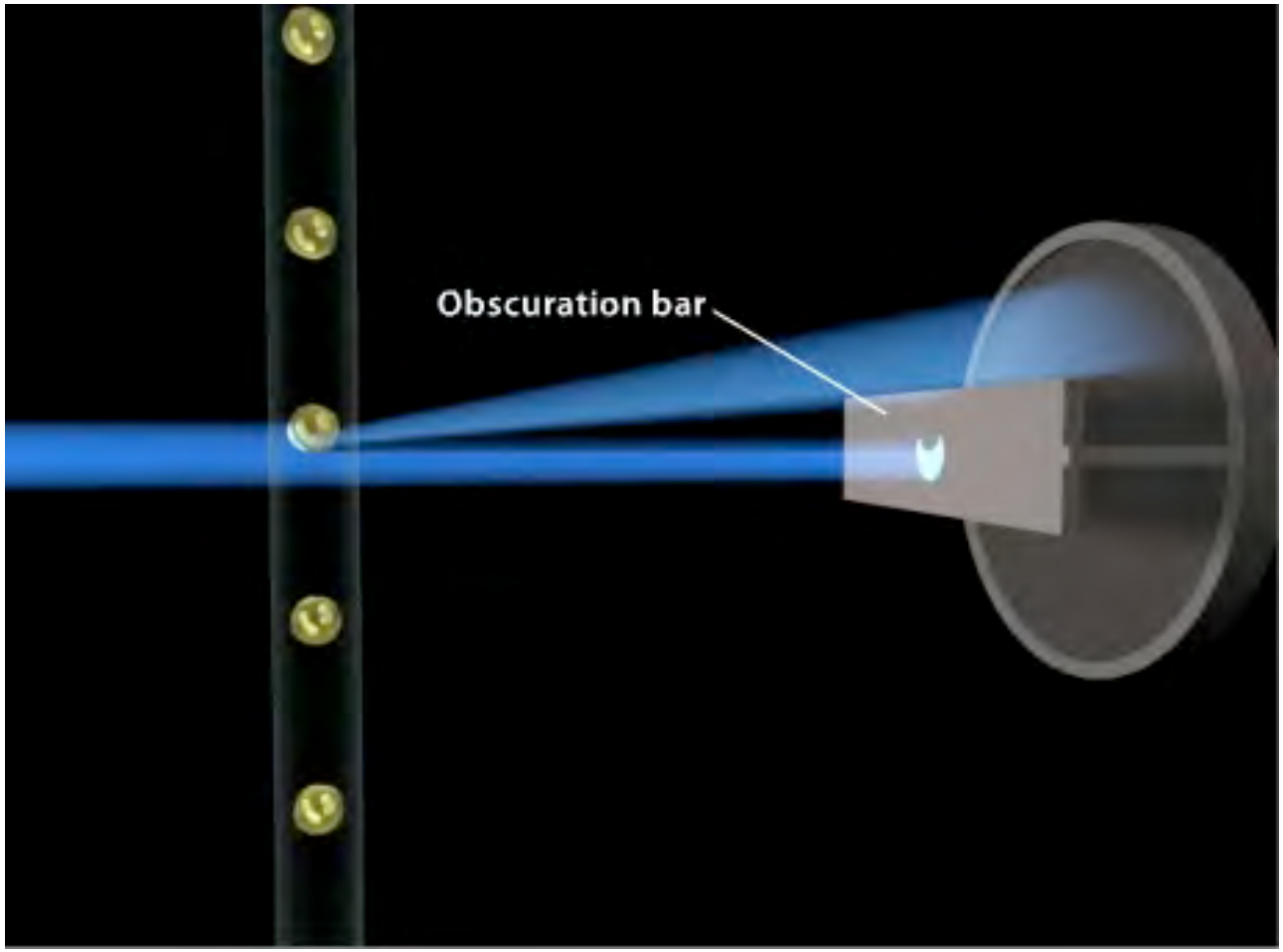
- As cells pass through laser, multiple signals are collected:
  1. Scattered light
  2. Fluorescence
- Light is scattered by the cell and detectors collect the scattered light
  1. Forward scatter
  2. Side scatter

Forward scatter (FS)

- Often associated with the size of a cell
- E.g., helps to distinguish smaller lymphocytes from larger monocytes



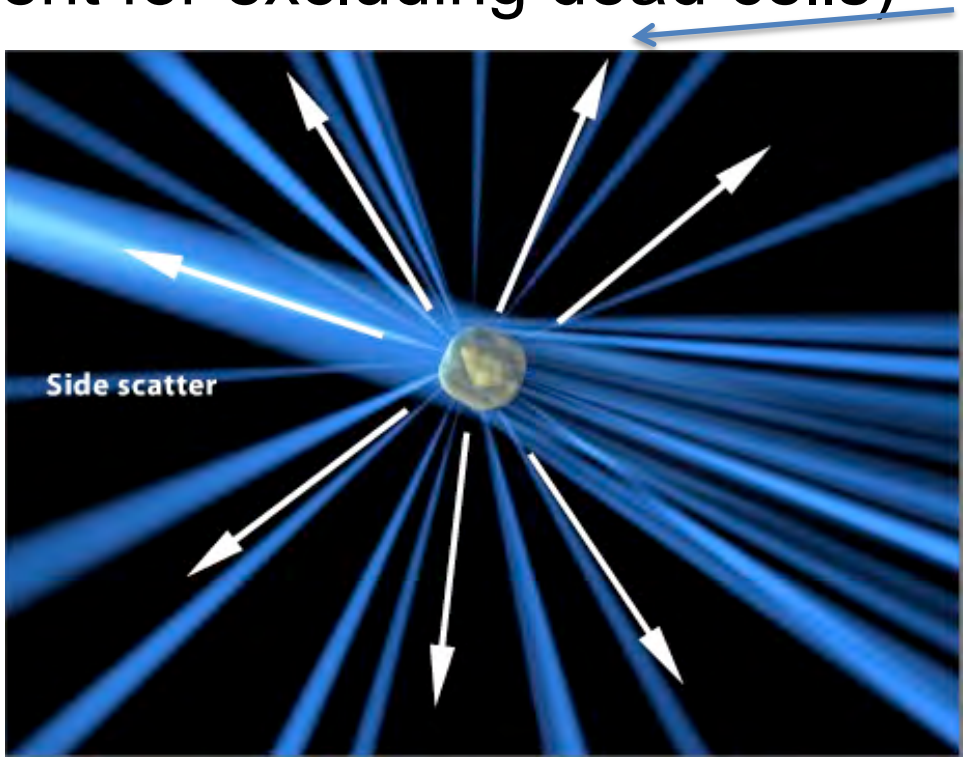
Direct laser light must be removed from forward scatter



http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

Side scatter (SS)

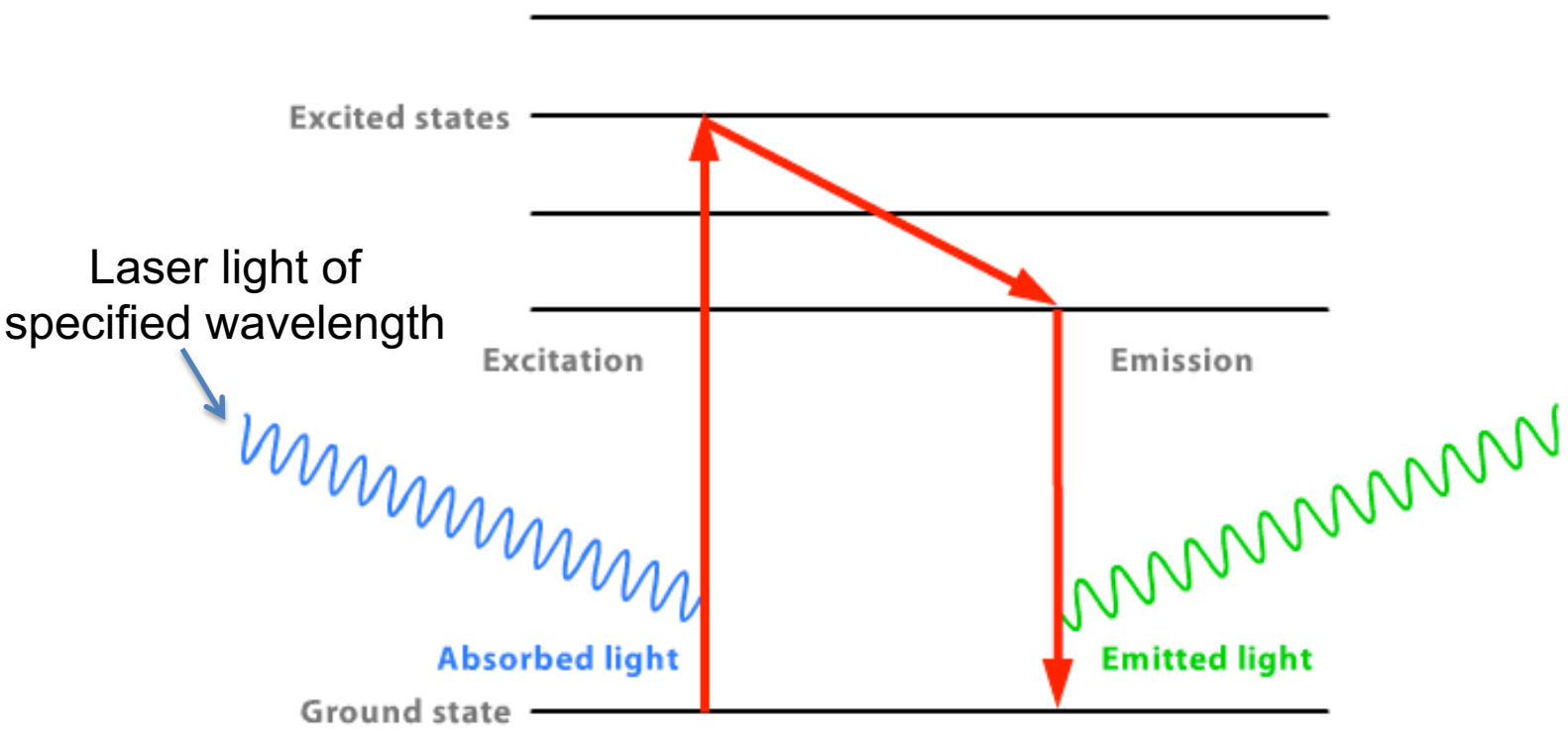
- Can be measured at multiple angles, but typically is 90 degrees
- Associated with granularity of cells, e.g., granulocytes are high for SS
- In combination with FS, useful in identifying lymphocytes from monocytes and granulocytes (not sufficient for excluding dead cells)



What is detected: fluorescence

- As cells pass through laser, fluorescence light from each cell is detected at the appropriate PMT
- Fluorescence from the fluorescent dyes used as tags or markers for cell antigens (but also includes auto-fluorescence)
- The fluorescent light is emitted in all directions, but is only collected and examined from one angle (typically 90 deg)

Fluorescence: emitted light is of longer wavelength (lower energy)



Higher energy: shorter wavelength
Lower energy: longer wavelength

http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

$$E = \frac{hc}{\lambda}$$

h=Planck's constant
c=speed of light

Fluorescent molecules

- Each has excitation and emission characteristics and these can be displayed as spectra
- Commonly used fluorescent molecules have been derived from living organisms or have been created artificially
- E.g., phycobiliproteins are from algae
  - Phycoerythrin, or PE
  - Allophycocyanin, or APC

Commonly-used fluorescent dyes

- PE and APC
- Fluorescein or FITC
- Texas red, often as tandem, PE-TR
- Cyanine dyes, often as tandems, PE-Cy7
- Alexa dyes in many colors, e.g., Alx488, Alx655
- Quantum dots
- Pacific Blue
- Propidium iodide (PI)
- “Brilliant” dyes

Brilliant fluorophores

- Conductive organic polymers
  - Stems from discovery that earned the 2000 Nobel prize in chemistry
- Synthetically tunable network of π-orbitals
  - Many repeat units in a polymer chain
- Extraordinarily high molecular extinction coefficients, similar to PE and APC
- Series of tandem dyes
- Initial series was violet excited, now UV and blue excited

Chattopadhyay et al, Cytometry Part A, 81A: 456-466, 2012

Fluorescent compound intrinsic properties

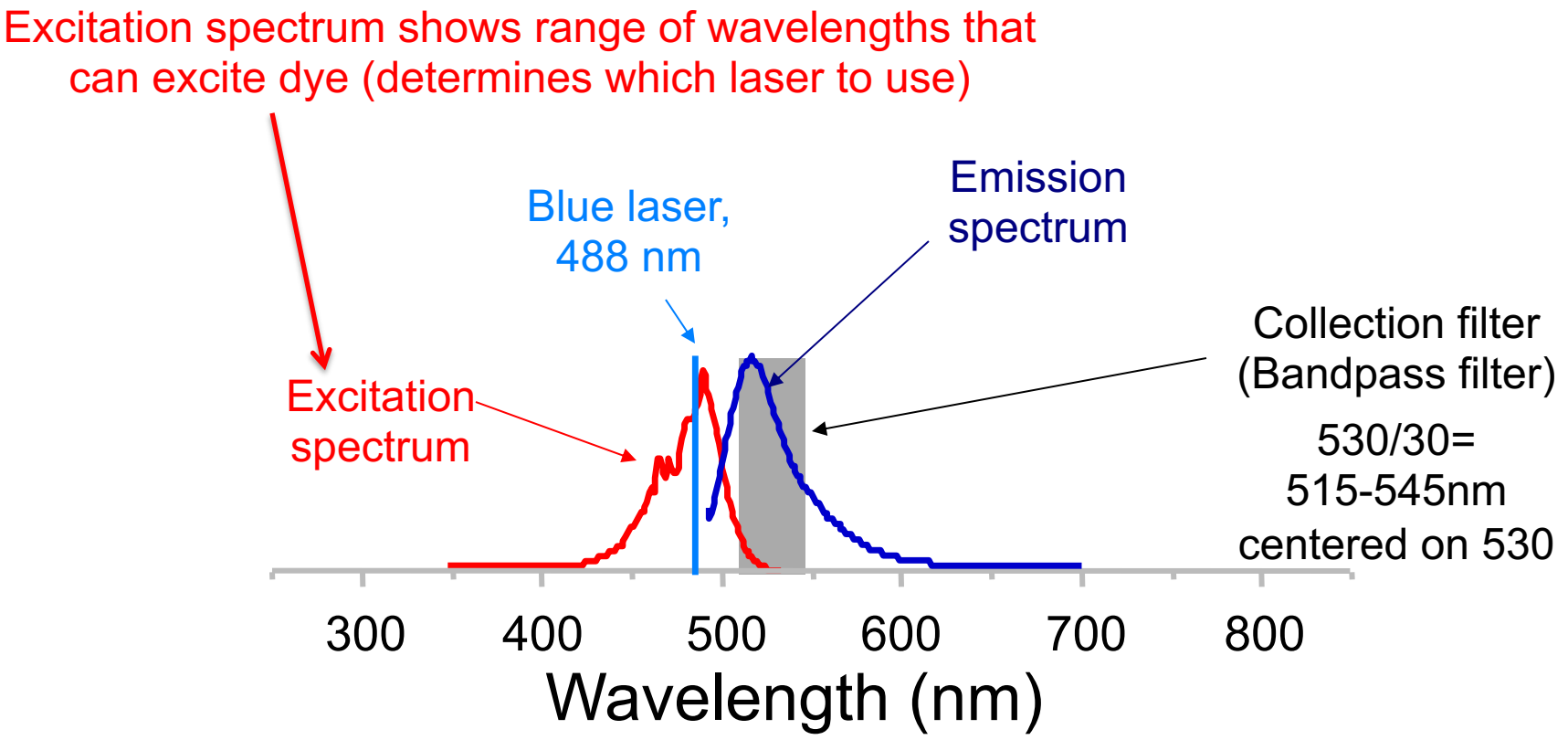
- Quantum yield – probability of emitting a photon once a photon of light is absorbed (efficiency of energy conversion)
- Molar extinction coefficient – probability of absorbing a photon of light at the wavelength of excitation

Brilliant violet characteristics

Fluorochrome	Quantum Yield	Molar Extinction Coefficient[a]	Excitation/Absorbance
Brilliant Violet	0.69	2,500,000	Violet (405 nm)
R-PE	0.82	1,960,000	Blue (496 nm)
APC	0.68	700,000	Red (650 nm)
Quantum Dot 655	≈0.3[b]	5,700,000	Violet (405 nm)
Quantum Dot 585	≈0.7[b]	2,200,000	Violet (405 nm)
AlexaFluor 660	0.37	132,000	Red (663 nm)
Fluorescein	0.5	86,000	Blue (488 nm)
AlexaFluor 488	0.92	71,000	Blue (495 nm)
Pacific Blue	0.78	46,000	Violet (405 nm)

[a]Measured at the indicated excitation/absorbance wavelength, $cm^{-1}M^{-1}$
[b]Quantum yields generally increase for larger Quantum Dots

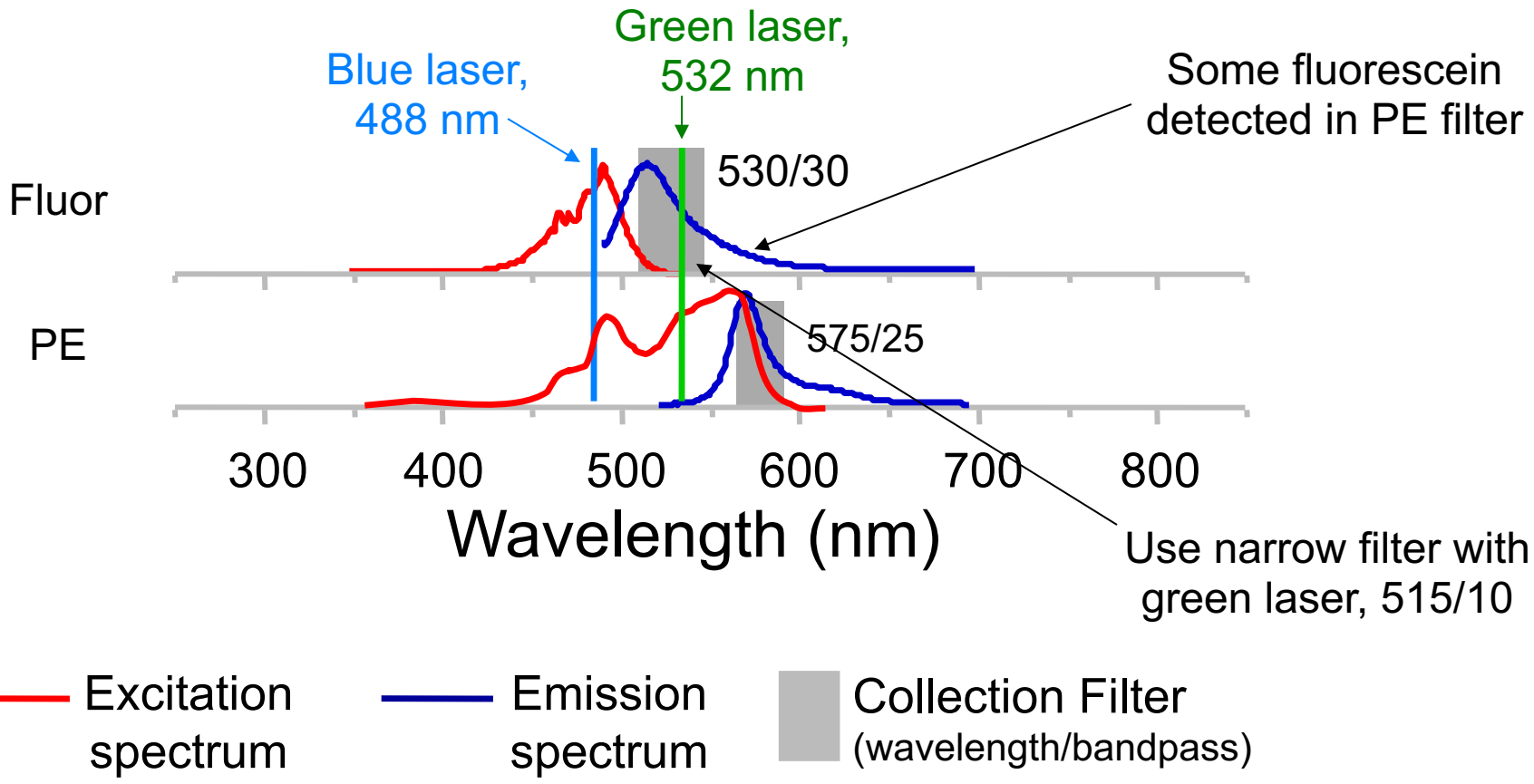
Spectra of Fluorescein



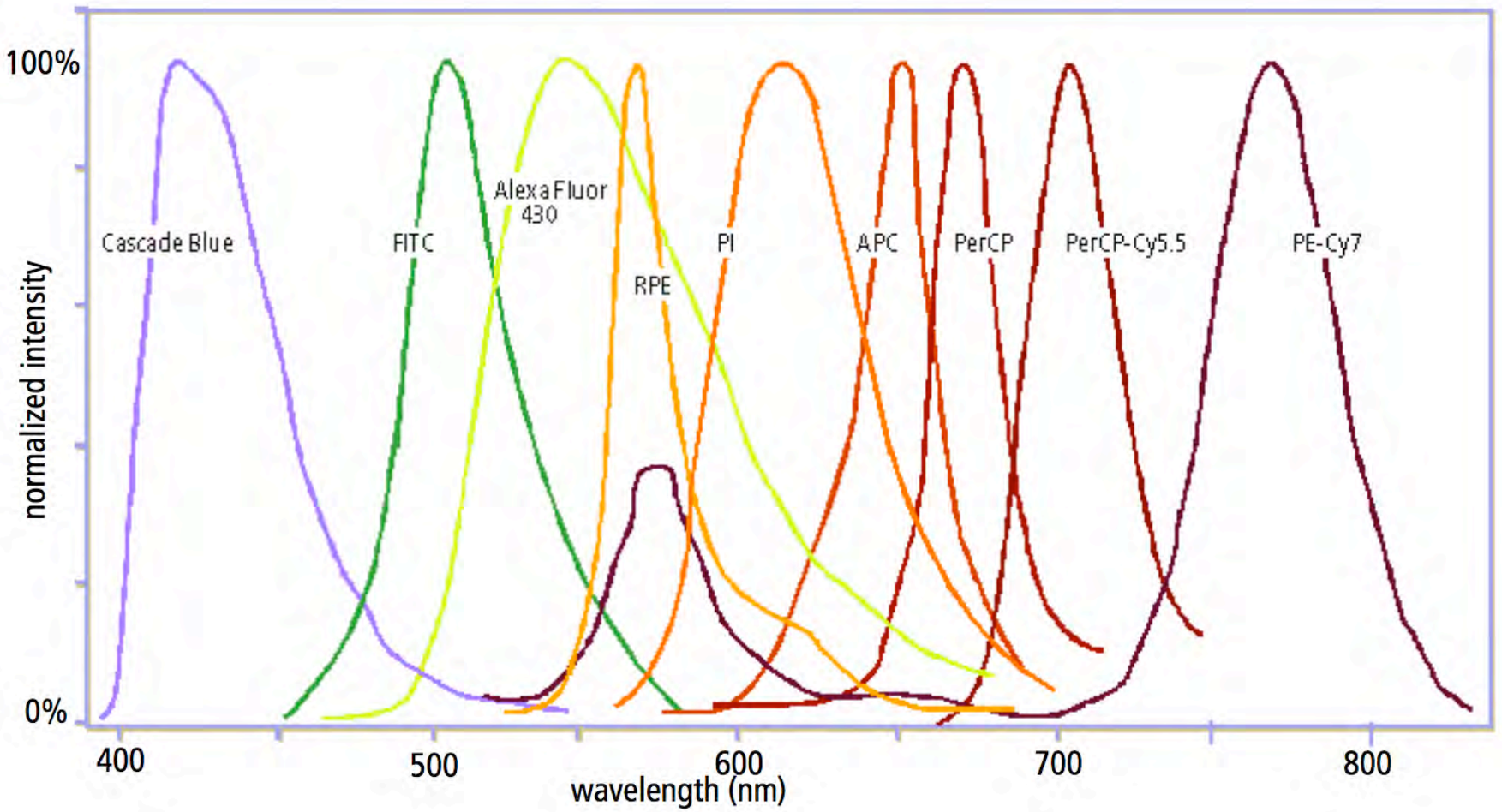
Useful resources = http://www.bdbiosciences.com/spectra/
http://probes.invitrogen.com/resources/spectraviewer/

Spectra of dyes: 2-color example

Different emission spectra allow signals to be distinguished



Emission spectra of common dyes



http://www.rockefeller.edu/fcrc/pdf/642221_BD_LSRII_Users_Guide.pdf

Cell identification

Specific cell labeling

- Flow cytometry was made feasible because of two technologies: lasers and monoclonal antibodies
- Monoclonal antibodies are “specific”, i.e., bind to only a single antigenic determinant
- Antibodies are an important component of the immune system, but for flow, they are simply used as a tool
- For human studies, antibodies to human antigens are produced in other species (mouse, rat)

Cell antigens

- Antigens refer to “determinants” that can induce an antibody response
- Different cell types often express different antigens
- For the purpose of flow these antigens are simply used as “markers” even though they likely have important functions for that particular cell type

Fluorochrome-antibody reagents

- Monoclonal antibodies to the marker of interest provide specificity
- To allow visualization in flow, the antibody must be labeled with a fluorescent dye
- Many different methods for attaching the fluorescent dye to the antibody have been developed (typically through covalent chemical bonds)
- These are often referred to as antibody-dye conjugates or reagents

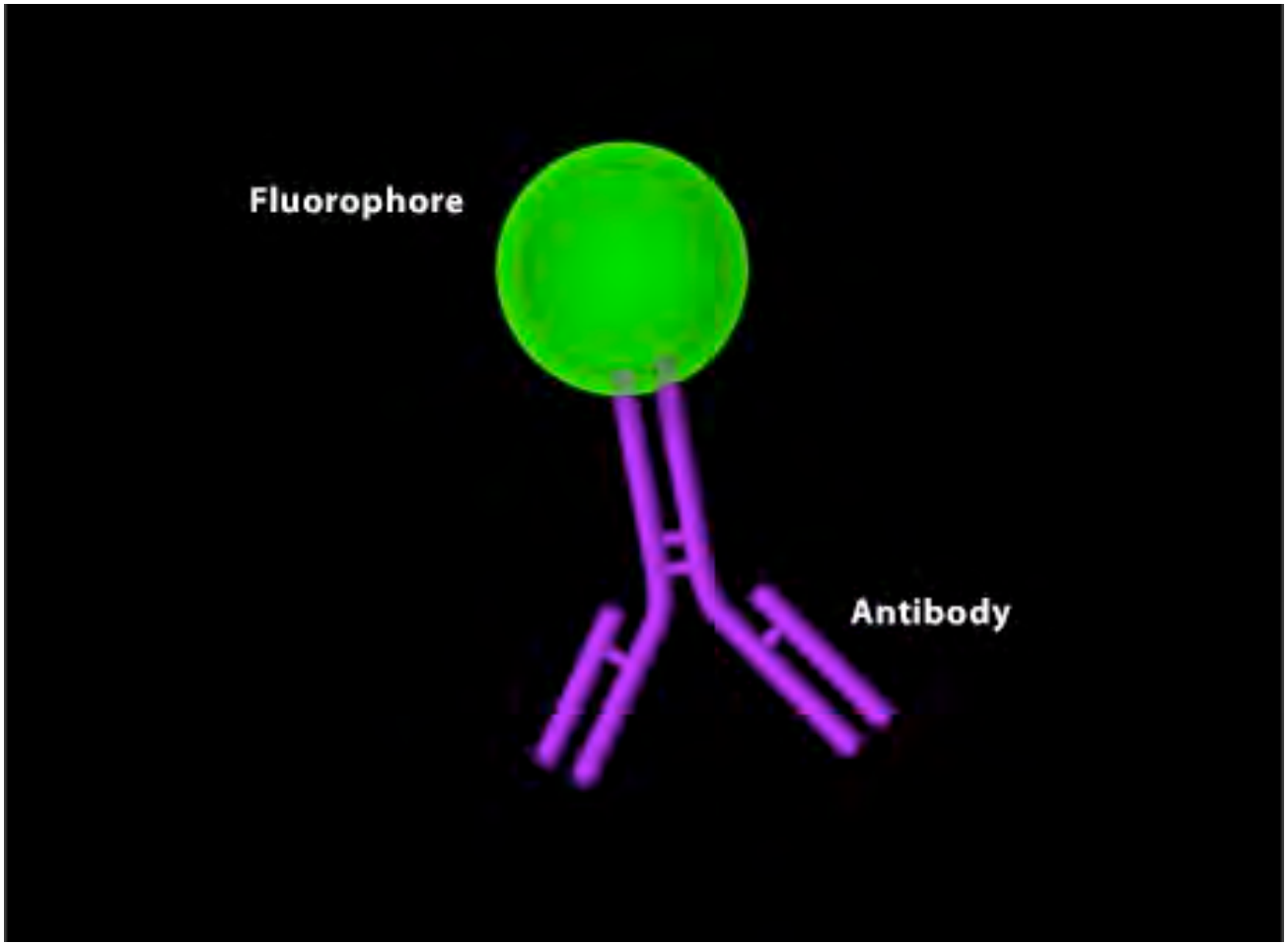
Fluorescent labeling of cells

- Specificity is typically provided by monoclonal antibodies specific for selected cell antigens (such as CD4, CD8…)
- Antibodies of different specificities are tagged or labeled with fluorescent molecules of different “colors”
- Cells are labeled by reaction with the antibody-dye reagent (“staining”). Any reagent that does not stick to the cell is washed off.

Note on nomenclature

- Antigens of interest on cells often have been assigned “CD” numbers (cluster of differentiation)
- These antigens may have been chosen as the marker of interest because of a function they perform, or simply because they have been shown to label a cell of interest

Example of larger fluorophore attached to antibody



E.g., PE or APC

http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

Multi-parametric flow cytometry

- Multiple measurements on each cell (in addition to forward and side scatter)
- A single marker is often insufficient to identify cell of interest
- A common example is measurement of T cells
  - Three markers typically used
  - CD3 is one component of the T cell receptor and therefore marks all T cells
  - CD4 and CD8 mark the two major types of T cells
  - (CD8 also expressed on CD3-negative NK cells and thus the need to use CD3 for specific identification of CD8+ T cells)

Multi-parametric flow cytometry requirements

- Dyes of different “colors” used in combination
- Must be able to distinguish the signals from each of these dyes
- Choose dyes that emit light of different colors
- Use different detectors for each dye and only collect light of the appropriate color in each detector

Nomenclature

- Each different dye in a multi-parameter flow assay is often referred to as a “color”, such as an “8-color” experiment
- On the flow cytometer, each color requires a light path and light detector and this is often referred to as a channel
- The color and/or channel/detector may be named using the name of the fluorescent dye such as the FITC or PE channel or simply numbered (FL1)
  - Generic naming by laser color and center of bandpass filter, e.g., B515, G575

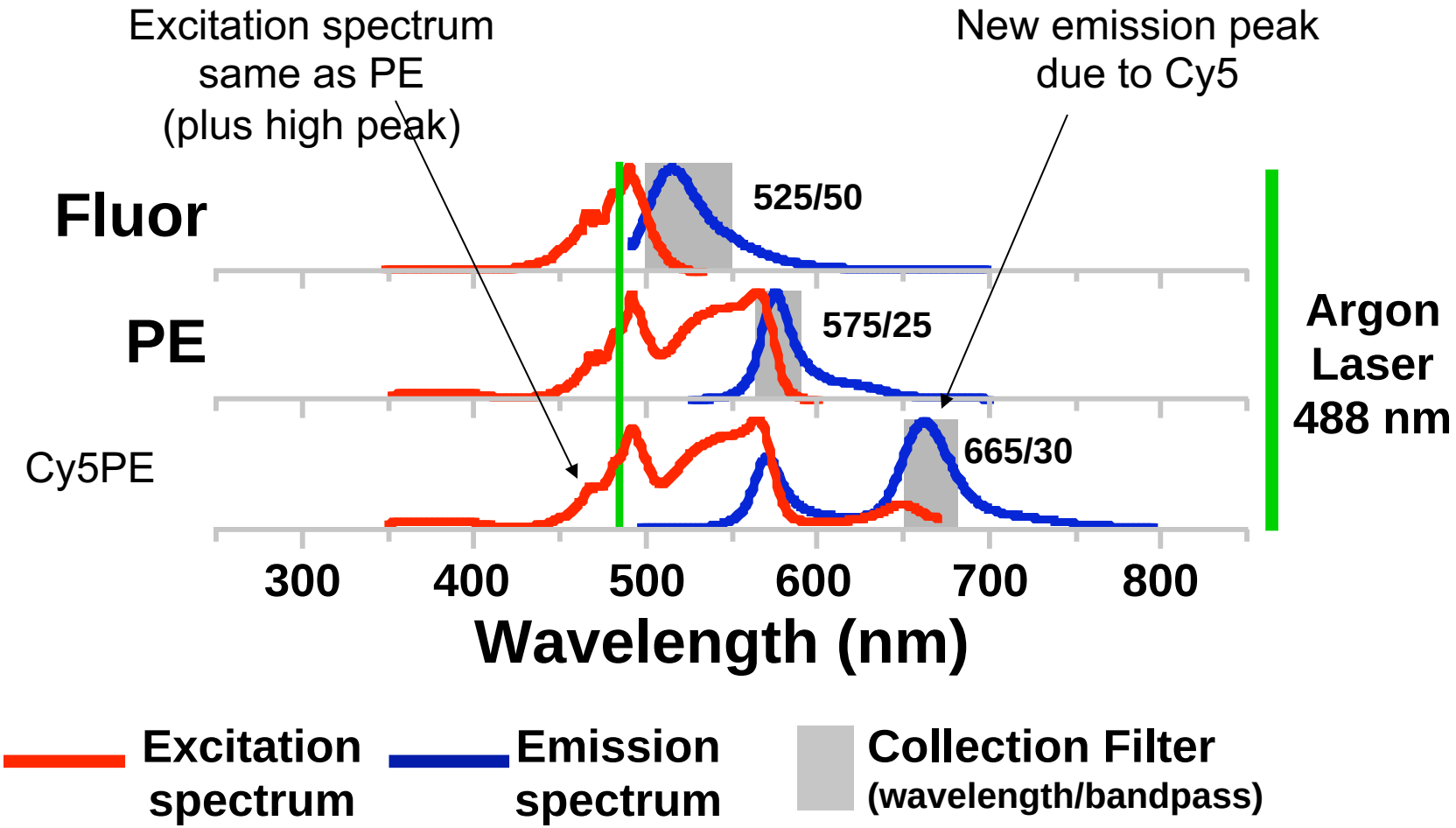
Why multiple lasers

- Each laser type emits light of a specific wavelength(s)
- Multiple lasers allow an increase in the total number of colors or markers that can be examined in each assay (because there is greater choice in dyes)
- Dyes that are excited at different wavelengths can be used in combination (even if they emit at similar wavelengths)

Tandem dyes

- Dyes covalently linked so that energy from one dye is transferred to the other (resonance energy transfer)
- Ideally, when the dye is excited, it emits mainly at the wavelength characteristic of the second dye (with little emission from the first dye)
- Examples: PE-Cy5, PE-TR (ECD), PerCP-Cy5.5, PE-Cy7, APC-Cy7
- Note that most of the newer brilliant dyes are tandems although not obvious from the name

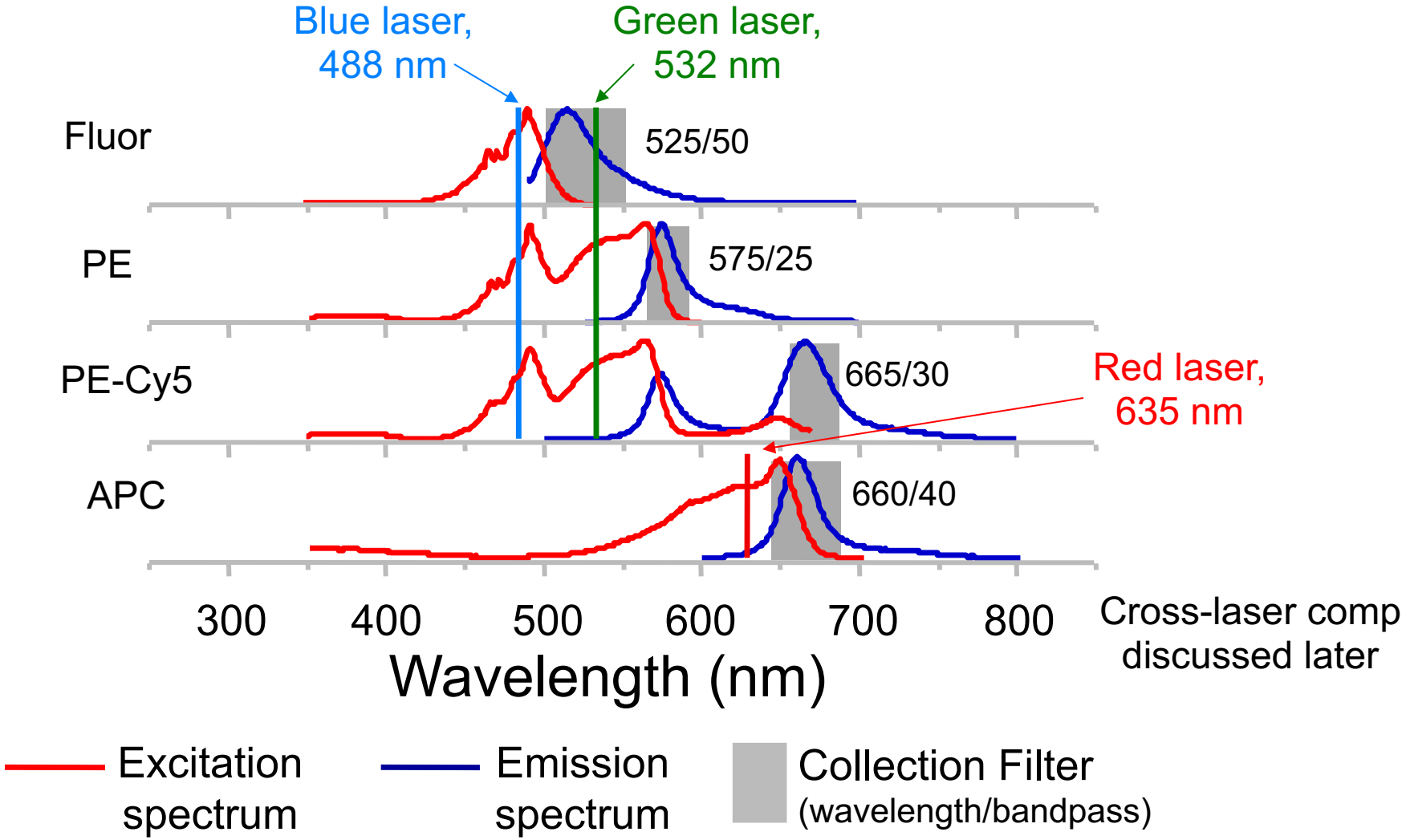
Spectra: 3-color example (one laser)



Additional lasers

- The addition of a second (or third or fourth) laser allows for use of dyes excited at another wavelength(s)
- There are time delays for different lasers, so even if dyes have similar emission spectra, the signals can be distinguished by time
  - But, if a dye is excited by more than one laser, then “cross-laser” compensation is required

Spectra: 4-colors (2 lasers)

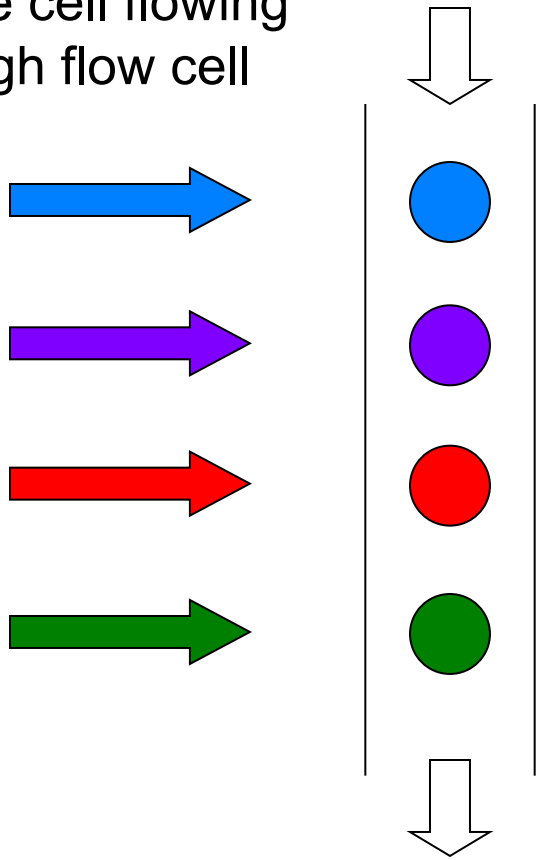


Cross-Laser Excitation

- Cells are exposed to light from each laser at different times
- Electronic signals due to the excitation of dyes by each laser are only collected during the time that the cells are exposed to that laser
- If a dye is excited by only one laser, then even if the emission spectrum for this dye overlaps with another, the signals are distinguished in time
- This is not the case when a dye is excited by more than one laser

Laser Delay

A single cell flowing through flow cell



Signal collected at:

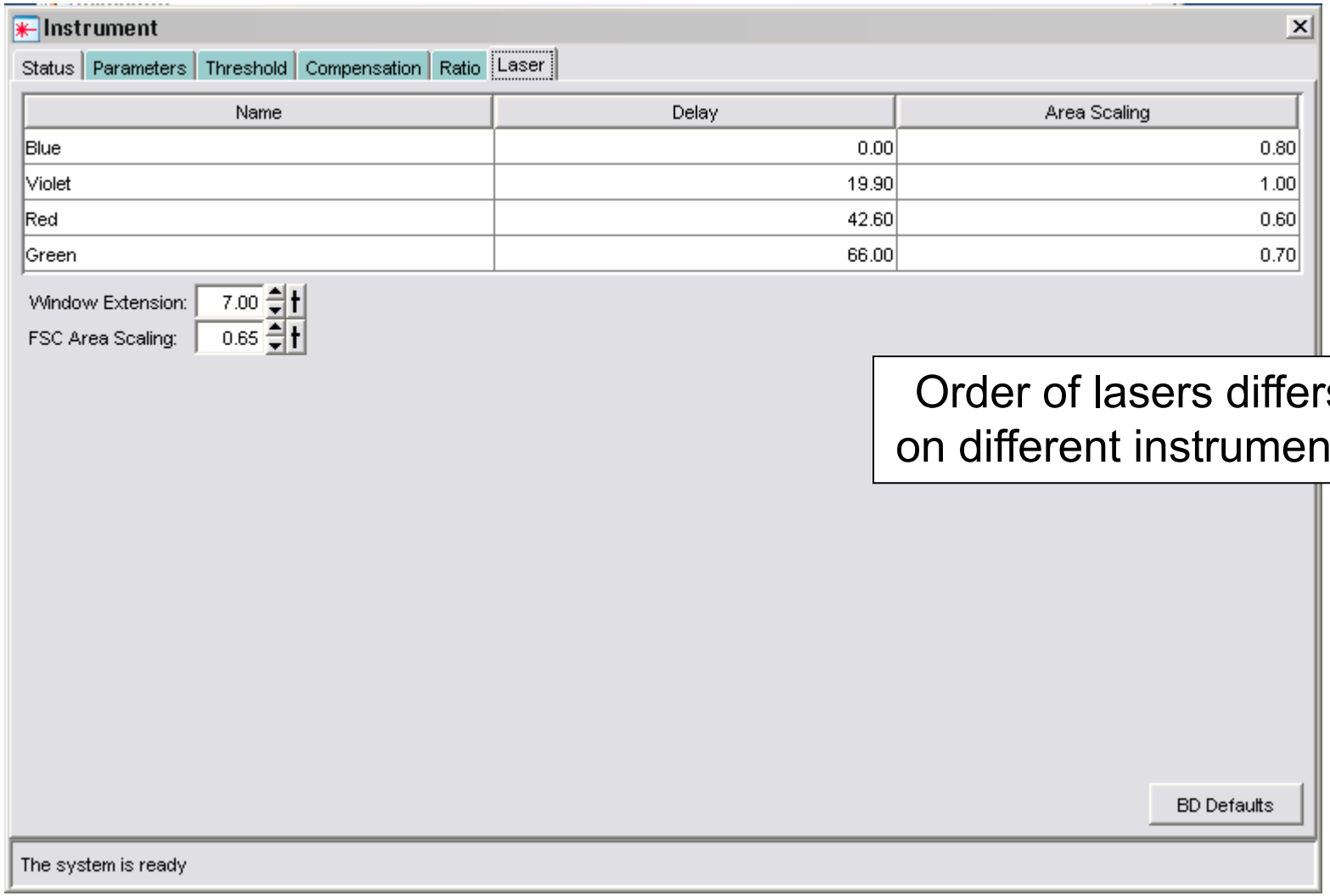
time T_{blue}

time T_{blue}+violet delay

time T_{blue}+red delay

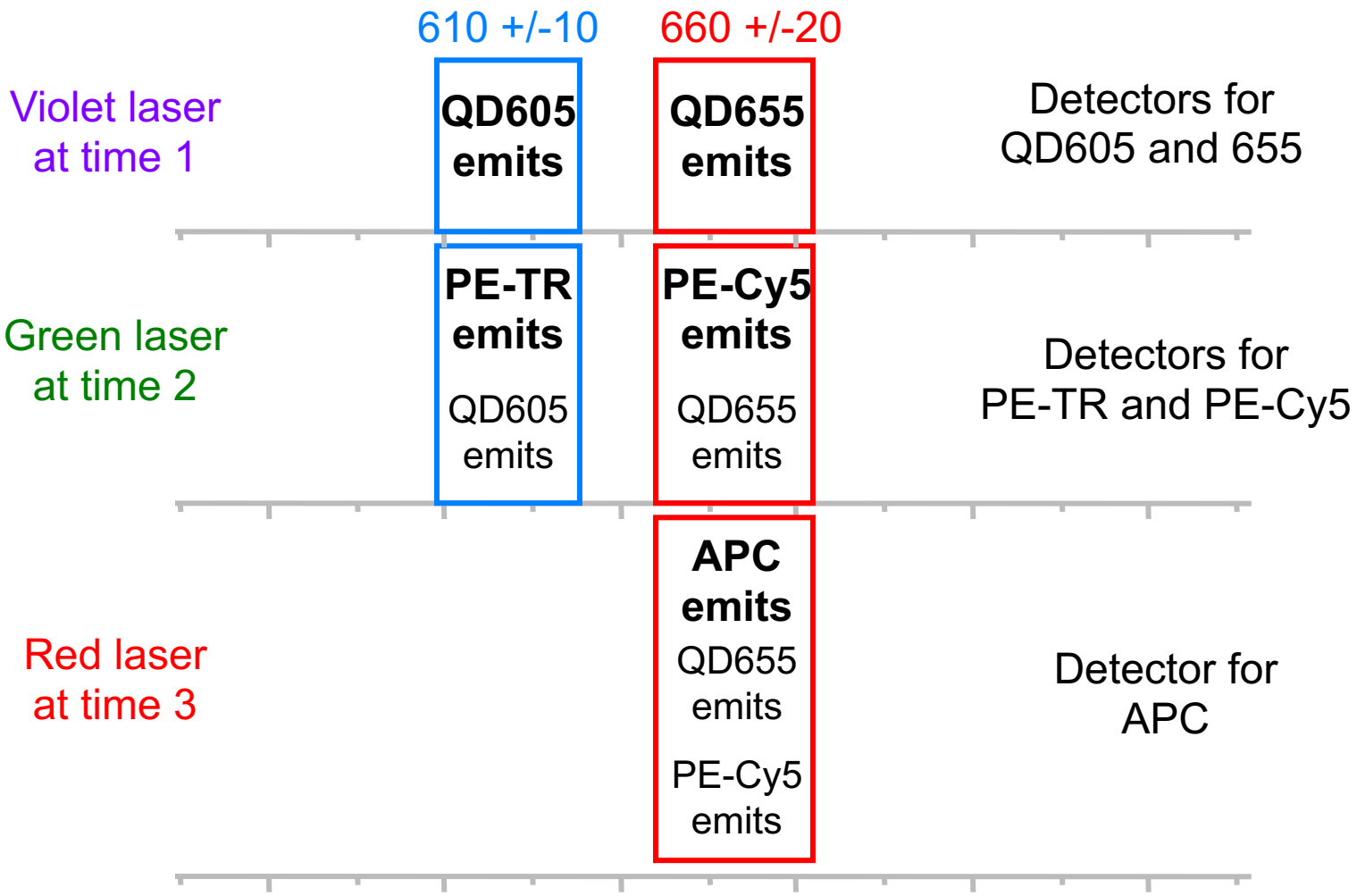
time T_{blue}+green delay

Laser Delay Settings



Order of lasers differs on different instruments

Cross-laser compensation

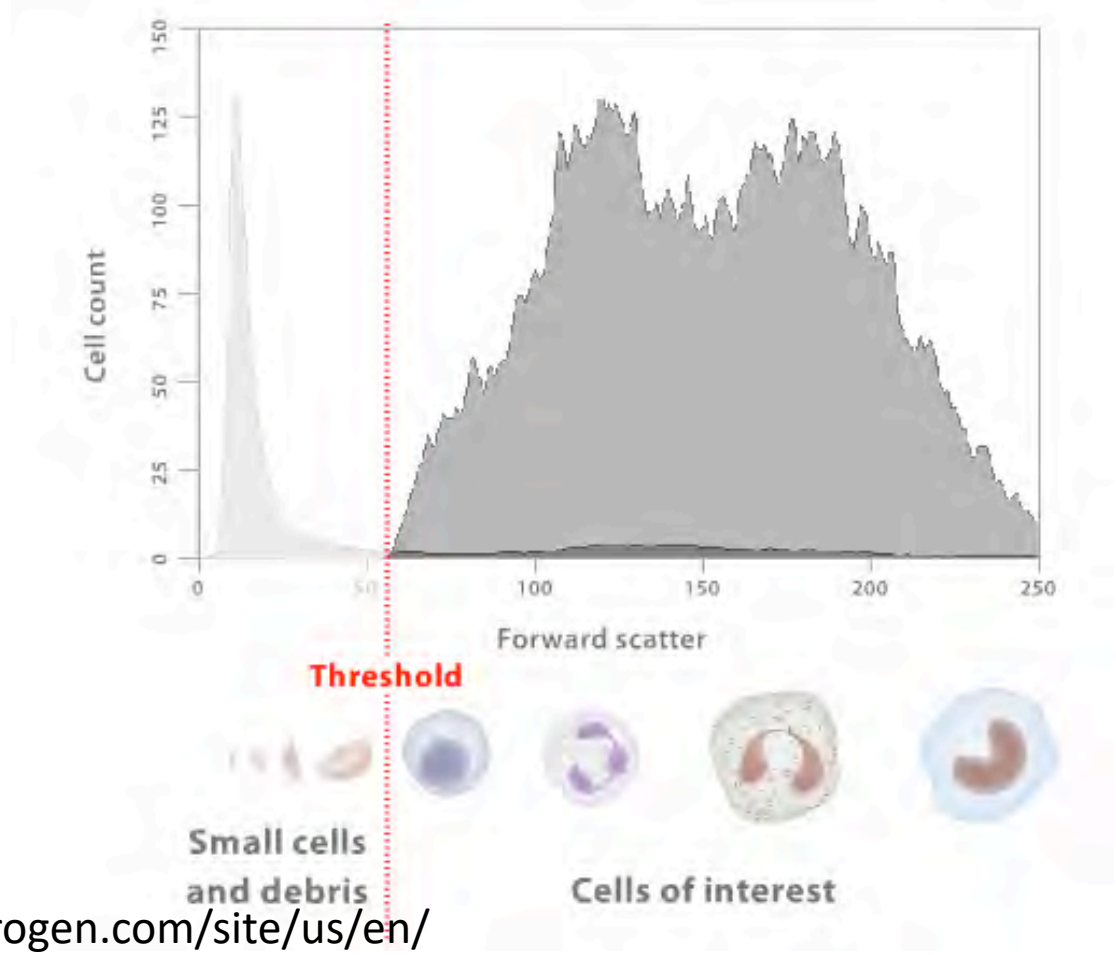


Flow data display

Flow data display

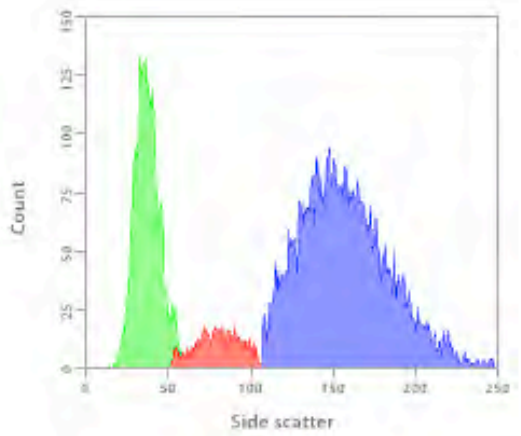
- Single dimensional histogram
- Two-dimensional
  - Dot plot
  - Contour plot
  - Pseudo-color plot

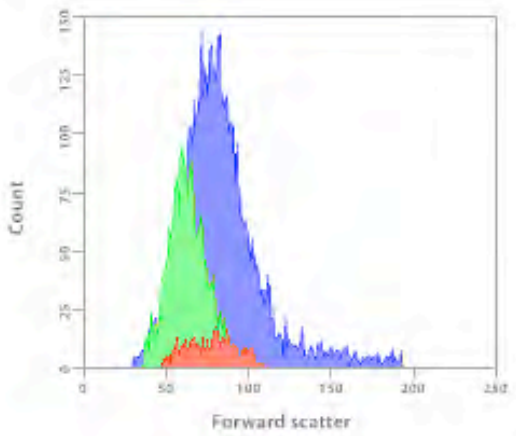
Histogram display: distribution of number of cells (y-axis) vs. signal intensity (x-axis) (forward scatter example)

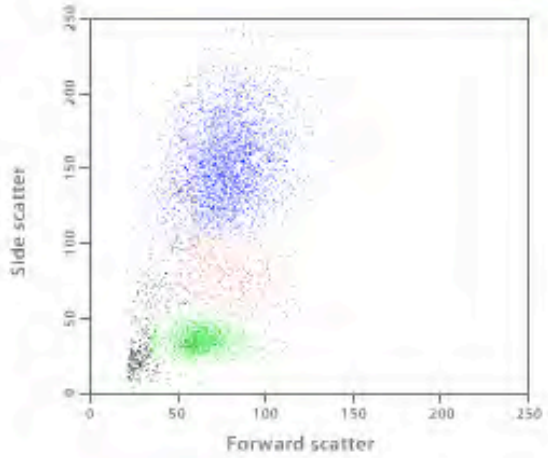


http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

Two-dimensional plots vs. histograms

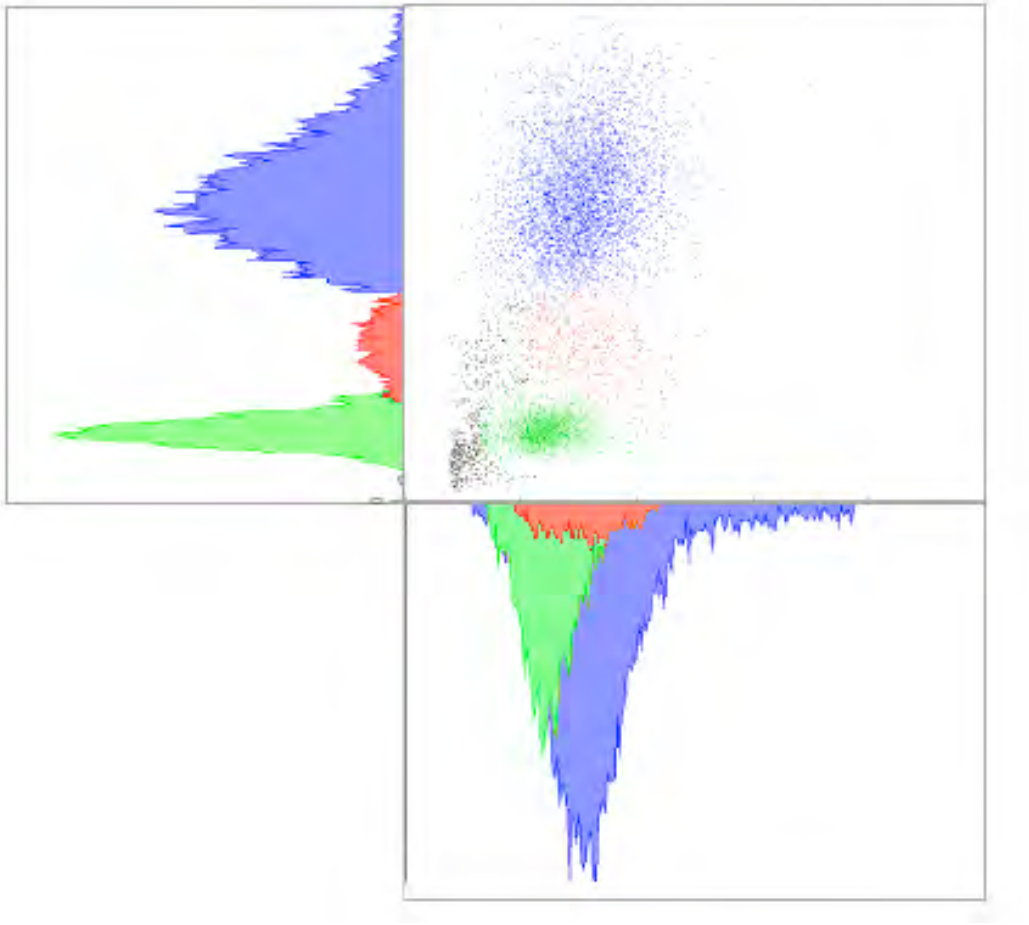






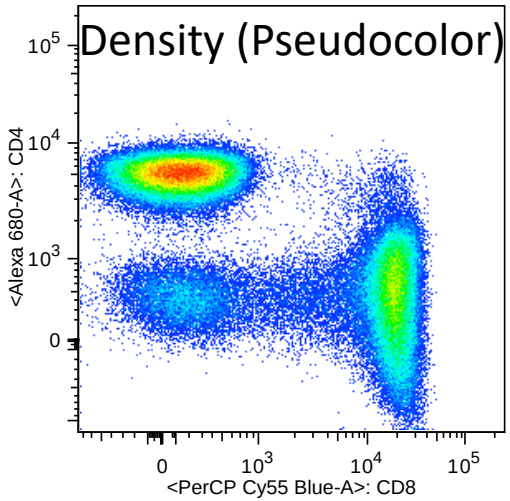
http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

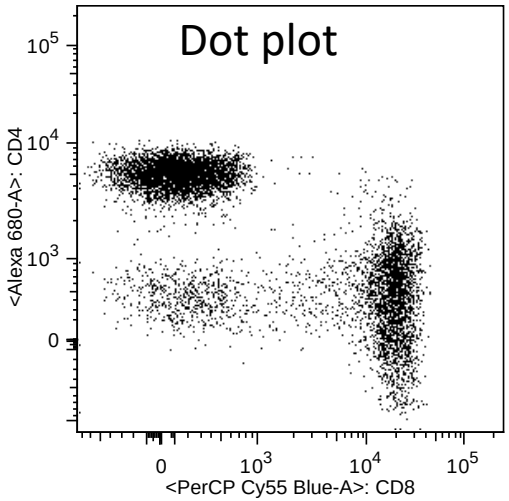
Visualizing two dimensions

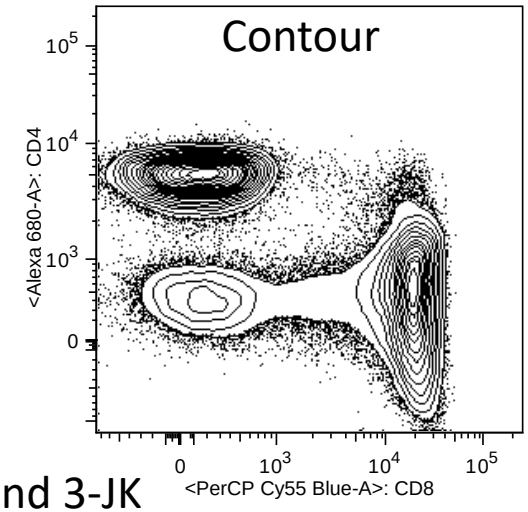


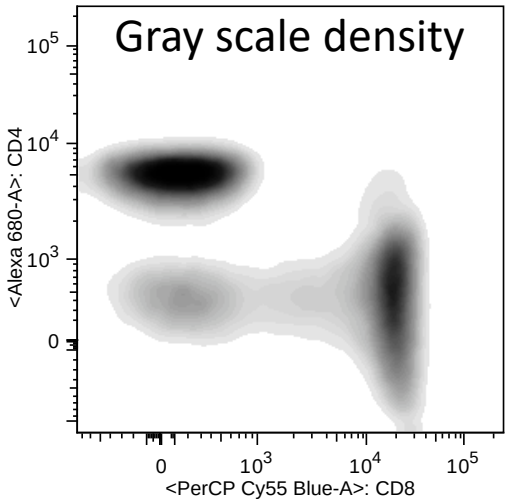
http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

Display options



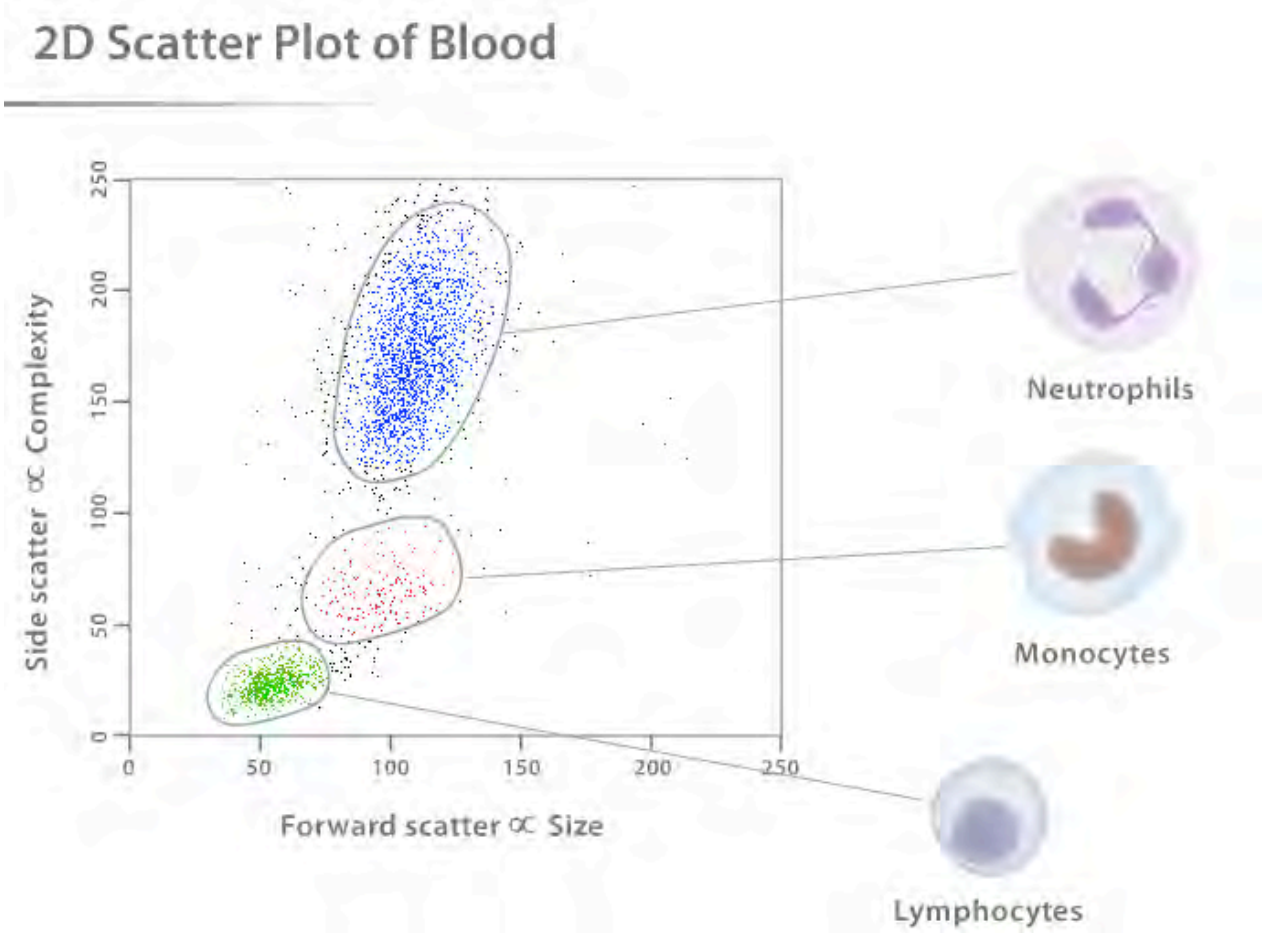






091014-NK Cell Round 3-JK

Even scatter alone (without fluorescence signal) can identify cell types



http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

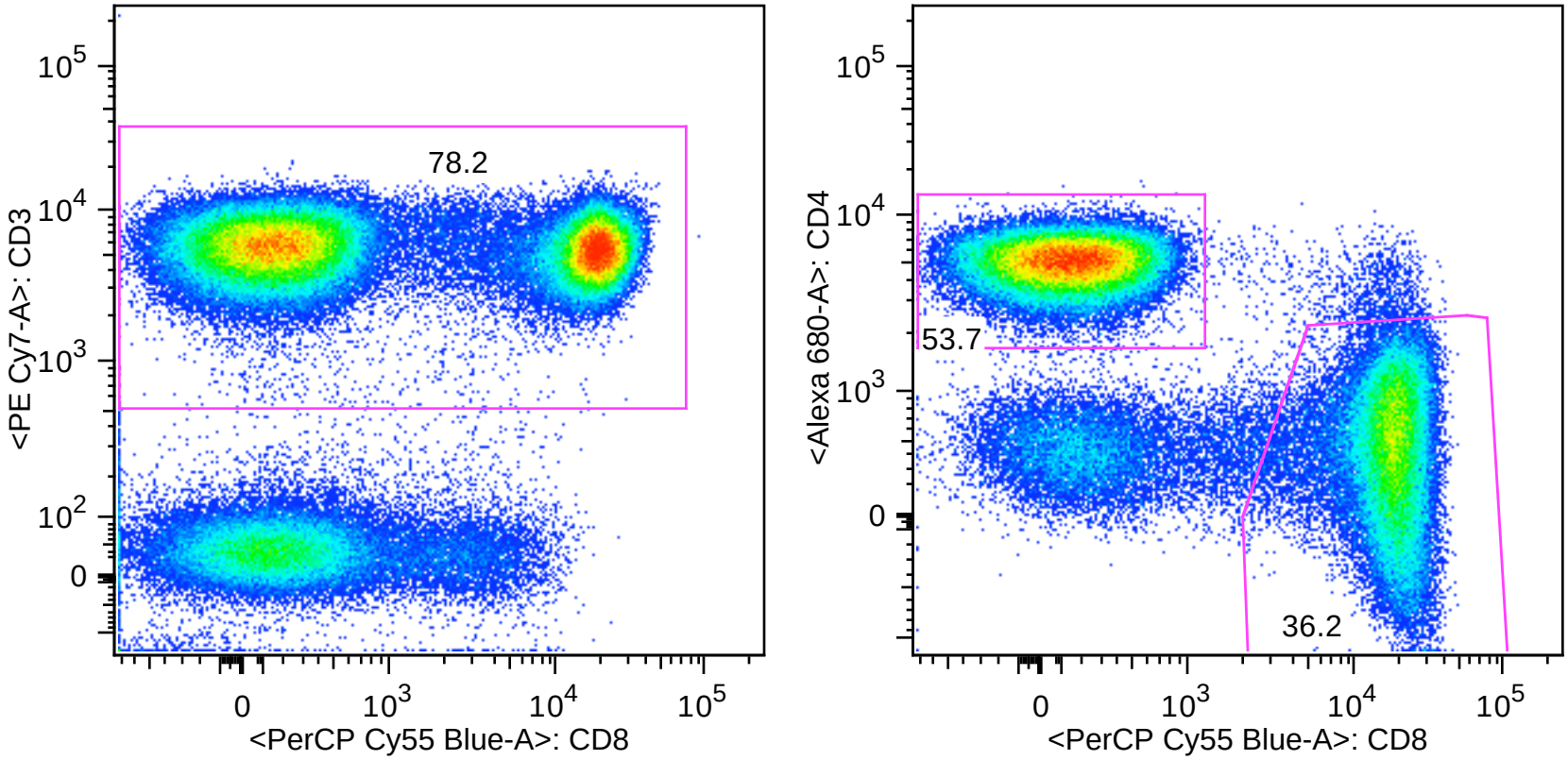
Flow data analysis

- A “gate” is used to select a group of cells of interest
- It is typically a rectangle or polygon
- Typically, multiple gates are used and often in a hierarchical format (a gating tree) in which each new gate is a subset of cells in the prior gate

Gating tree example in Flow Jo

374079.fcs		218184	NonComp	SAC 214404...	DMSO
S	96.4	210294			
Live	99.6	209549			
L	70.4	147586			
3+	77.6	114523			
4+	30.6	35088			
107...	0.023	8			
IFNg+	0.046	16			
IL2+	0.054	19			
TNF+	0.063	22			
8+	56.1	64267			
107...	0.15	98			
IFNg+	0.025	16			
IL2+	0.011	7			
TNF+	0.067	43			
374094.fcs		212640	NonComp	SAC 200087...	ENV
S	94.6	201253			
Live	91.2	183454			
L	65	119265			
3+	76.2	90916			
4+	51.3	46623			
107...	0.015	7			
IFNg+	0.015	7			
IL2+	0.056	26			
TNF+	0.06	28			
8+	37.5	34096			
107...	0.047	16			
IFNg+	0.018	6			
IL2+	0.026	9			
TNF+	0.047	16			

Gating example: T cells identified as $CD3^+$, then $CD4^+$ and $CD8^+$ cells gated



091014-NK Cell Round 3-JK

Next generation fluorescence-based cytometry

- Although mass cytometry (CyTOF) allows for high dimensional single-cell analysis, introduction of new fluorescence dyes and new instrumentation is now allowing fluorescence-based cytometry to achieve similar capabilities

CyTOF vs. fluorescence cytometry

	CyTOF	Fluorescence cytometry
Labels	Metal isotopes (minimal overlap)	Fluorophores (must compensate)
Multiplexing	30+	Currently 20-26; 30+ under development
Signal intensity	Moderate to poor	Moderate to excellent
Background signal	Minimal	Autofluorescence high in some colors in large cells
Efficiency	At best 30% of input cells	30% minimum; generally higher
Throughput	Low (10 minutes per sample)	High (<1 minute per sample)
Panel design	Panel design not limited by compensation, but some reagents poor	Panel design requires expertise, but potential for excellent separation

Beyond 18 colors in fluorescence cytometry

- Most current commercial flow cytometry instruments limited to 20 parameters/18 colors
- 18 color-capability enabled by use of multiple lasers and multiple fluorescent labels with varied excitation and emission spectra
- But existing fluorescent labels made it difficult to design staining panels near 18 colors
- Introduction of “brilliant” fluorophores as series of dyes is rapidly enabling 18 colors and beyond
- Previously limited by instrumentation (optics can be expanded, but electronics limited to 20 parameters)

New instrumentation

- New BD X50 (Symphony) instrument with capability up to 50 parameters
- New electronics decrease background and thus increase sensitivity
- Same fluidics as the LSR/Fortessa, allows for high throughput capability
- Current configuration for 30 parameters/28 colors using 5 lasers, but expandable
  - Current Diva software limited to 30 parameters

Prototype X50 instrument at the Vaccine Research Center/NIH

New BD X50 Symphony instrument (“Rooibos”) before shipping to Cape Town HVTN laboratory

Summary

- New instruments with >18 color capability allow for greater flexibility in panel design for ≤18-color panels
- Panel design for >18 colors will require iteration, but due to brightness of new dyes, there is less need to carefully assign selected markers to a few bright fluorochromes
- New technology retains all advantages of traditional flow cytometry – speed, high cell yield, potential for cell sorting in the future