

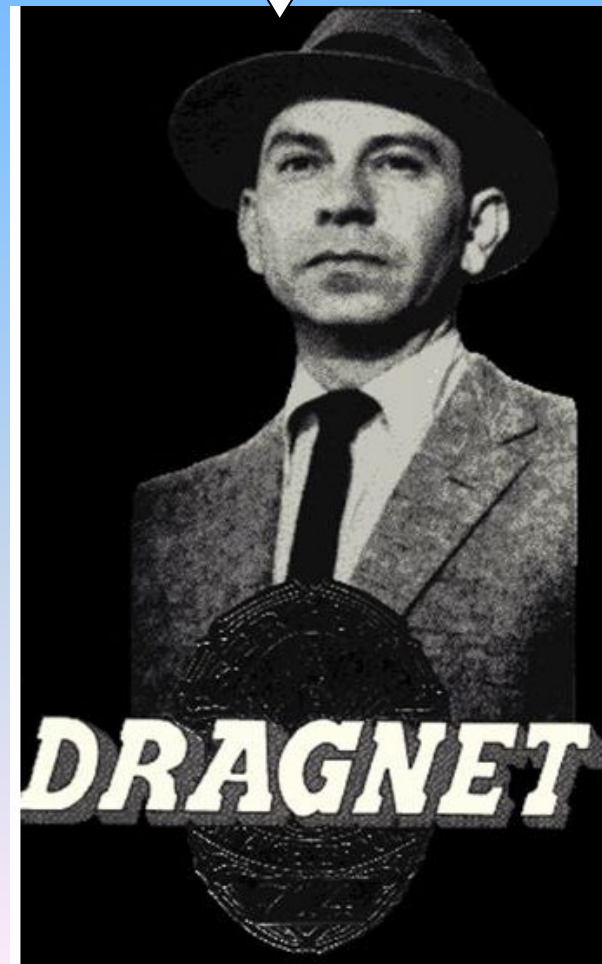


MASS GENERAL | MIT | HARVARD

Michael Waring

Director, Ragon Institute Imaging Core Facility
Cambridge, Massachusetts, USA

Just the
FACS,
ma'am.



Ragon Institute Imaging Core

- Maintain and oversee operation of the Institute's flow cytometry equipment (Cytometers and cell sorters, as well as Luminex and Imaging Cytometry technologies)
- Provide training and guidance in experimental design in flow cytometry.
- ragoncytometry@mgh.harvard.edu will send your message to all core staff!
- Core resources also include confocal and fluorescent microscopy and slide scanners

Ragon Institute Imaging Core

Flow Cytometry training syllabus

- “Just the FACS” class
 - Review online tutorials and resources beforehand:
 - www.newenglandcytometry.com>cytometry training
- Instrument Orientation—90 minutes, limit 3 per session
 - changing tanks, using software—one session covers all LSR cytometers.
 - Watching another operator recommended
- Proficiency test—10 minutes
 - demonstrate ability to change fluidics tanks without help
 - “cleared” to run independently

We are happy to help set up new protocols or experiments at any time!

Instrument Orientations

All sessions are held in room 333 (core lab) and announced over email.

If none of the times work for you, we can schedule additional sessions as necessary.

“Request Training” for the Diva instruments is just to “request access” to booking and having an account on the workstation.

Flow Cytometry

- Measuring physical and chemical characteristics of particles in a fluid stream as they pass through one or more lasers.
- FACS=Fluorescence Activated Cell Sorting—not all flow cytometry is FACS! (sometimes you are “just looking”)
- Uses fluorescence to measure expression levels of multiple markers on individual cells in a population
- Allows the researcher to characterize and count mixed populations of cells at rates over 10,000 events per second

Information We Get From a Flow Cytometer

- Physical properties—based on physical interaction of the particle with laser light, measured in the same wavelength as laser.
 - Relative size
 - Relative granularity or internal complexity
 - Often a characteristic profile for a given particle
- Chemical Properties—based on signals from reagents that interact with the laser light.
 - Relative Fluorescence intensity in different colors.
 - Allows us to determine positive expression/presence of a fluorescent marker of interest.

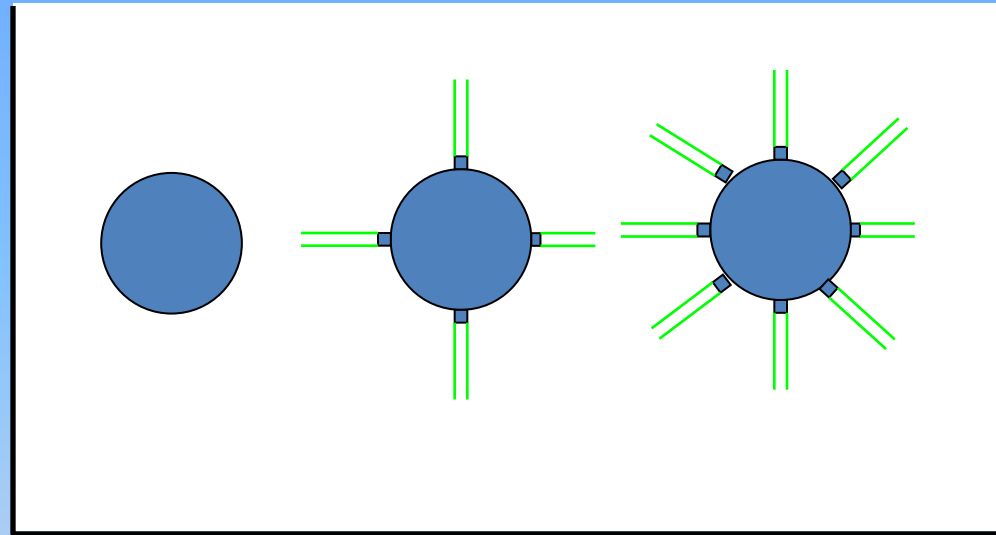
Fluorescence Intensity

- Measuring fluorescent proteins (GFP), fluorescent labels that directly bind to the particle, or fluorescently labeled antibodies that bind to the particle.
- Provides a measure of the protein or DNA content.

Emitted Fluorescence is proportional to the number of binding sites

Identify cells that express high levels of a given protein or marker.

CAUTION: “Negative” does NOT mean “zero expression”! Cytometry cannot identify cells that only express a few molecules of a protein.



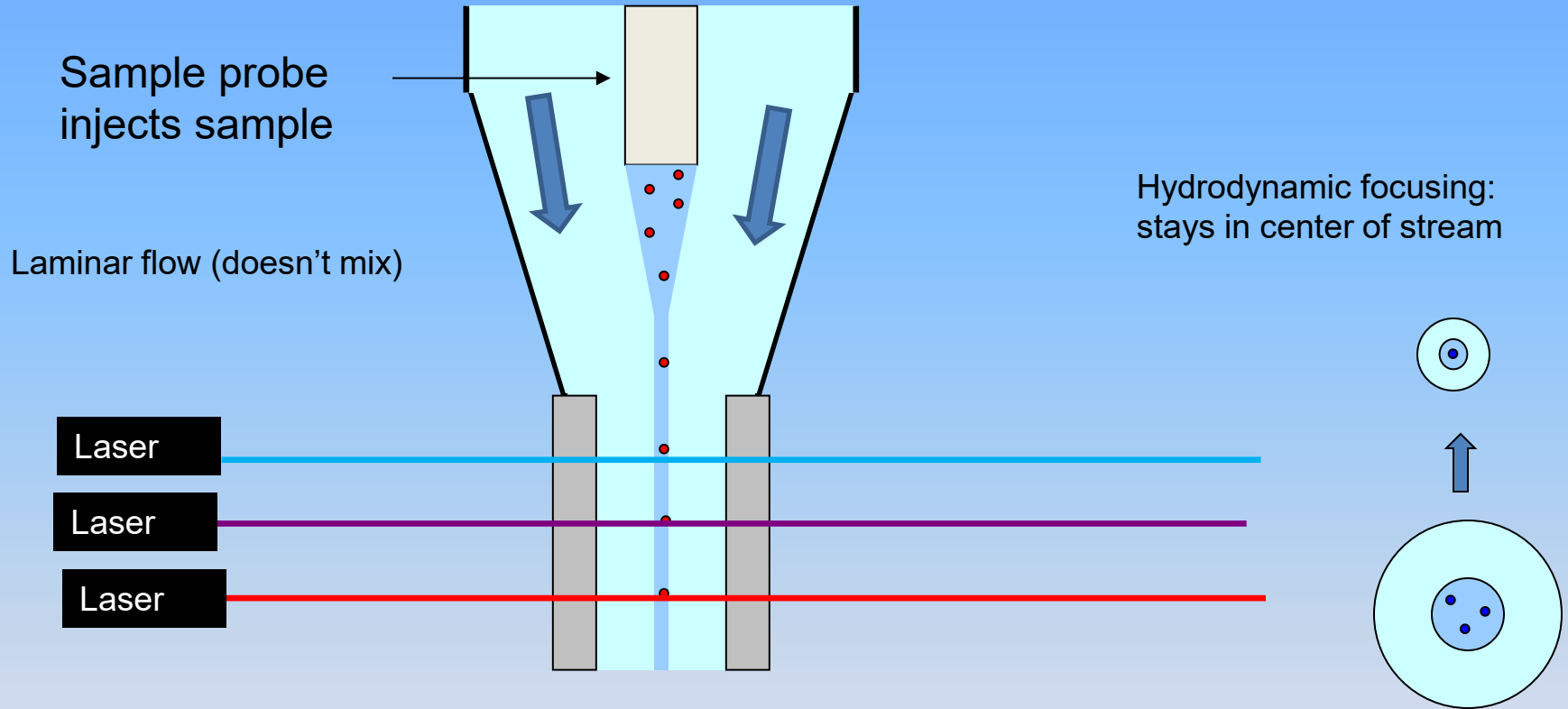
Fluorescence Intensity

"CCR5-negative" memory CD4 T cells do express CCR5 -- not enough to be positive by flow cytometry-- but enough to be infected by HIV and killed! (See Mattapallil et al., Nature, 434, 1093-1097, 2005)



Key Component: Flow Cell

Quartz cuvette, tapers towards outlet. Continuous flow of saline (sheath fluid) carries and focuses sample to pass it through the laser(s).



Lasers produce light signals from dyes on the cells to identify what each one is.

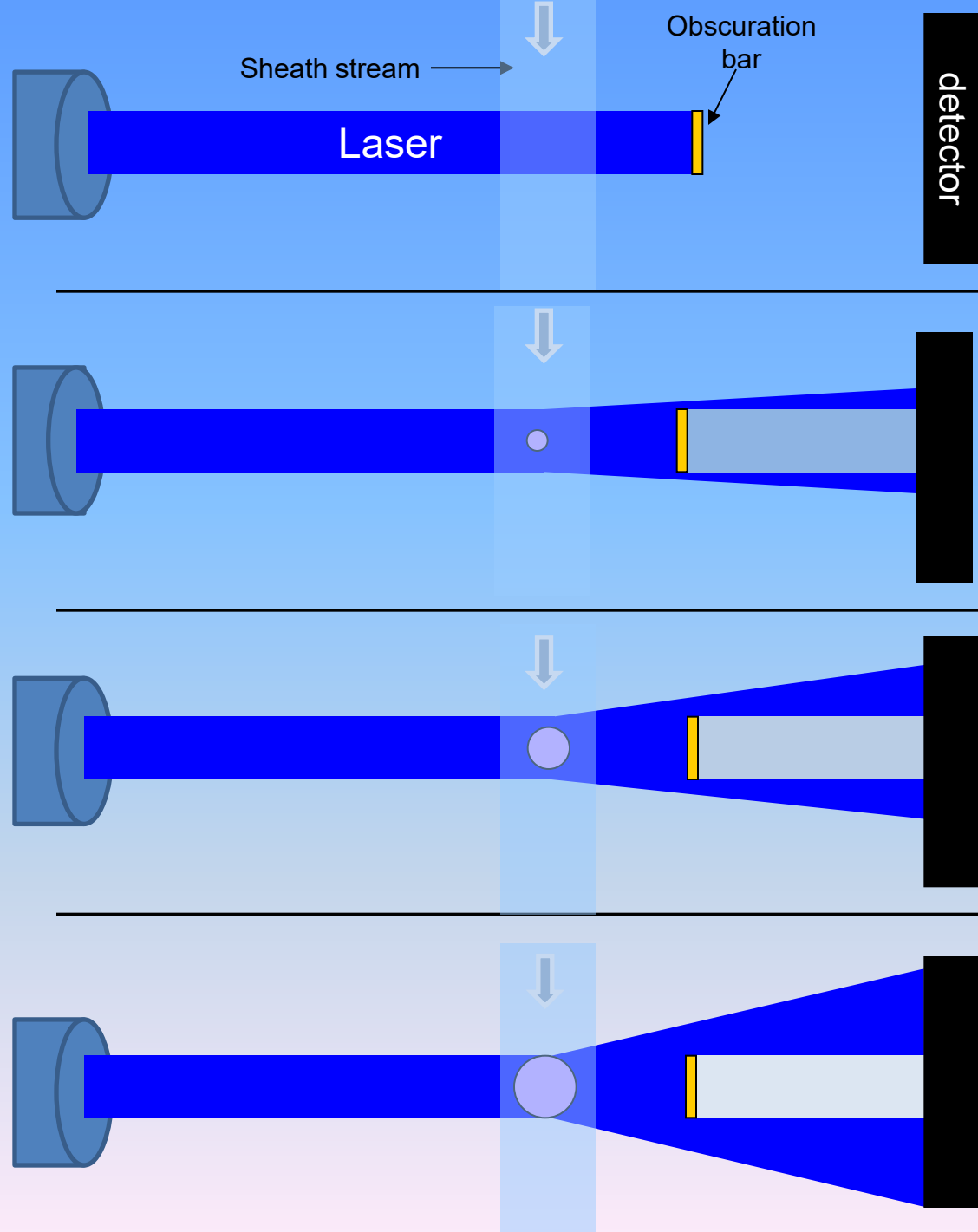
Forward scatter, small angle scatter, FSC

Diffraction light

Related to particle's surface
area and refractive index

Detected along axis of
incident light in forward
direction.

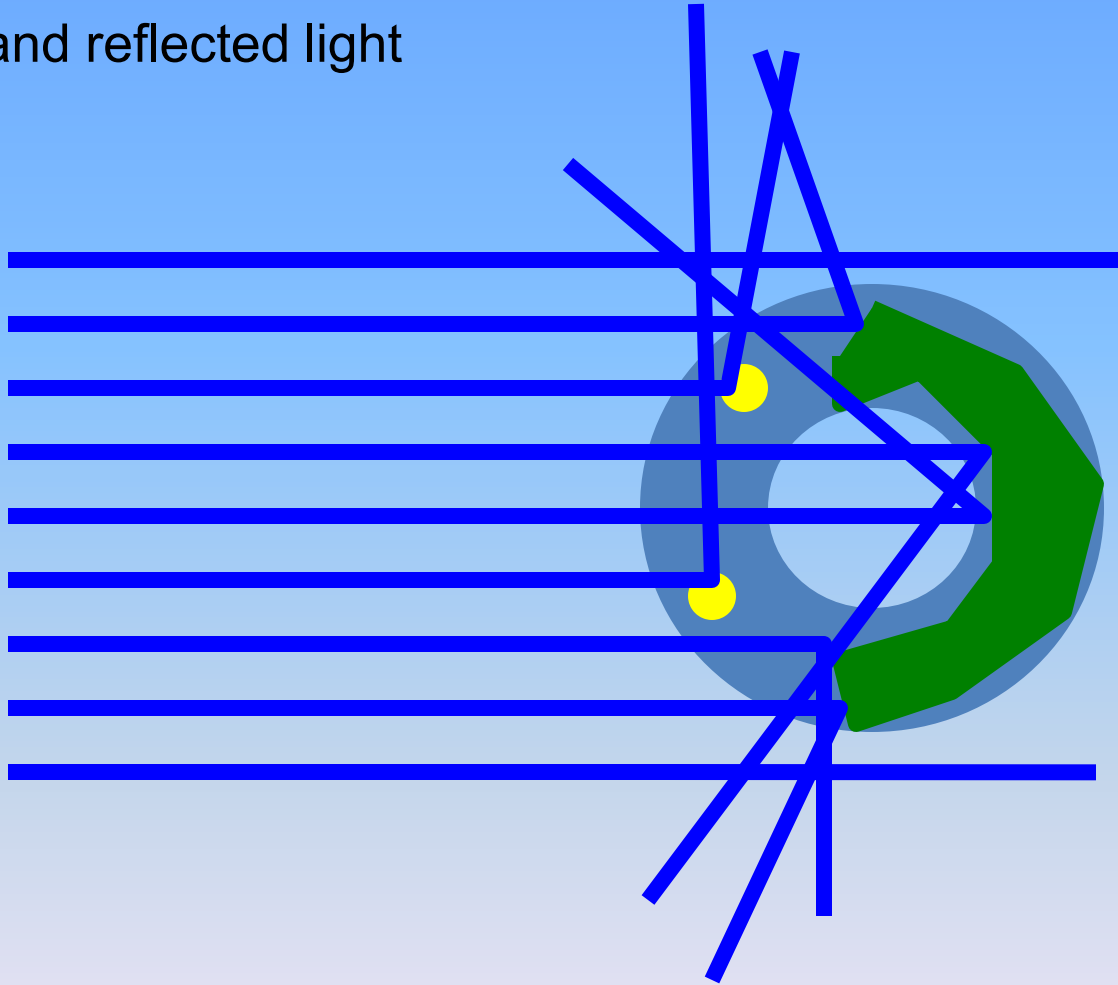
5um bead $\neq$ 5um cell



Side Scatter, Wide angle scatter, SSC

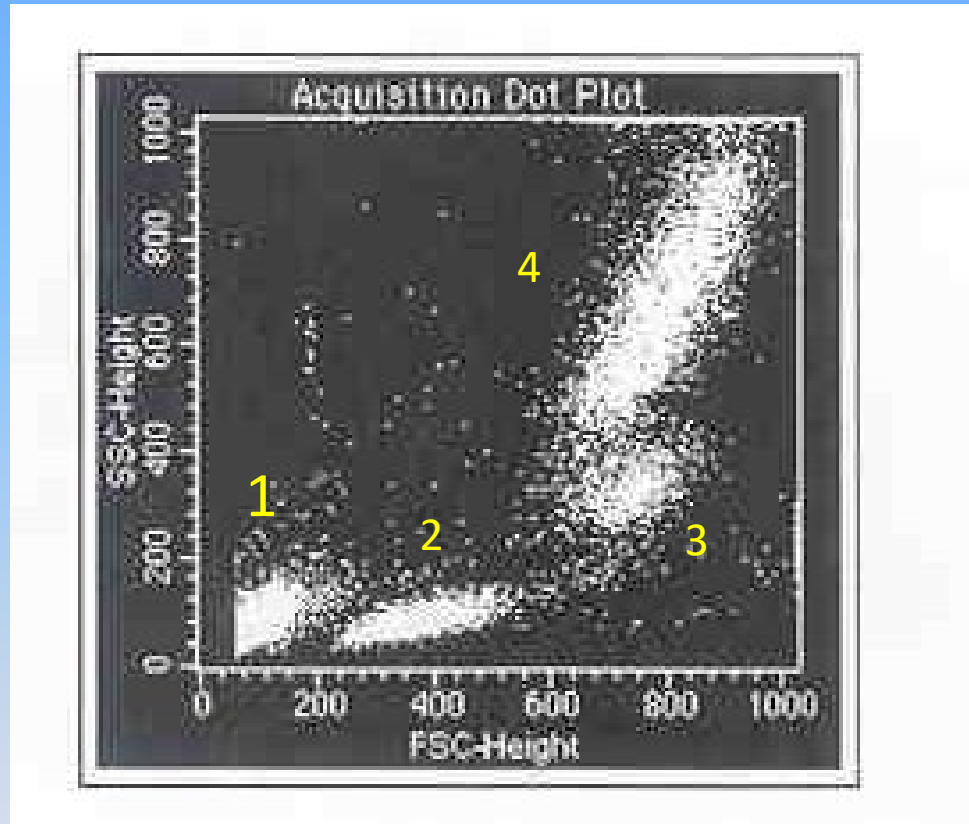
Measure of cell granularity and complexity

Refracted and reflected light



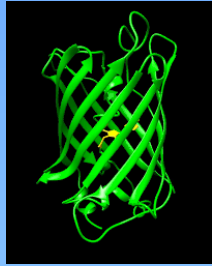
Light scatters in all directions, but SSC usually measured 90 degrees from incident light

Cells usually have a characteristic “scatter” profile

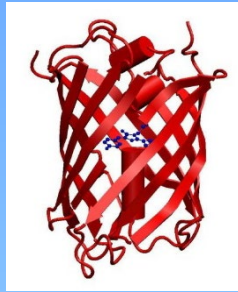


(Guess which cluster are “GRANULOcytes”?)

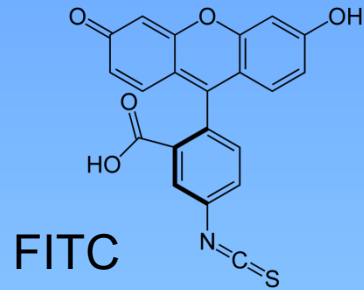
Multiple types of fluorescent markers



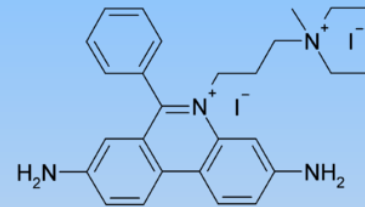
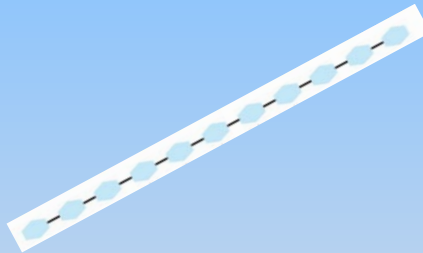
GFP



RFP



Qdot



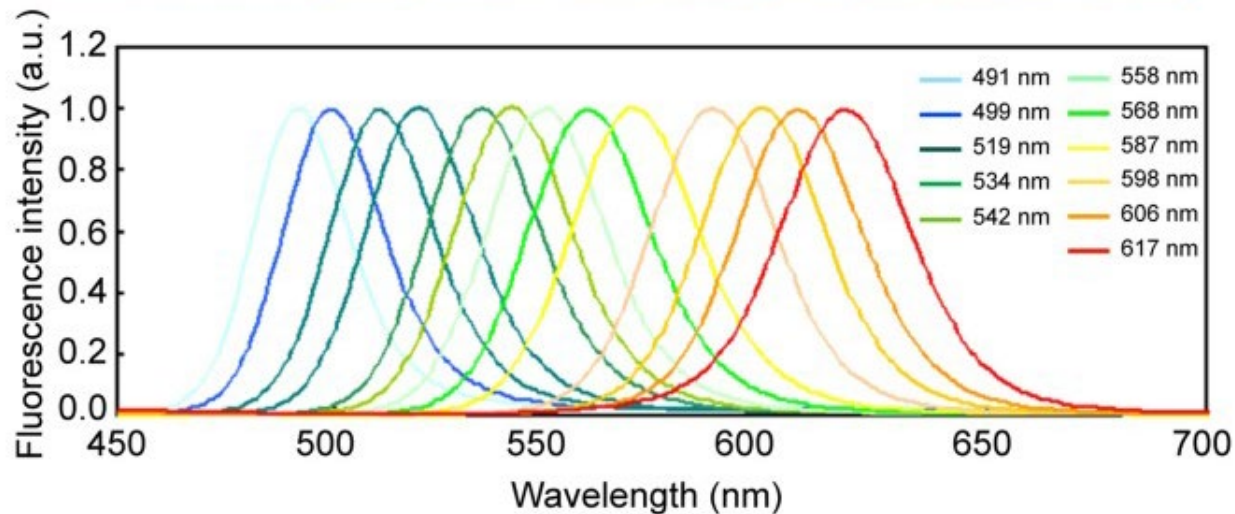
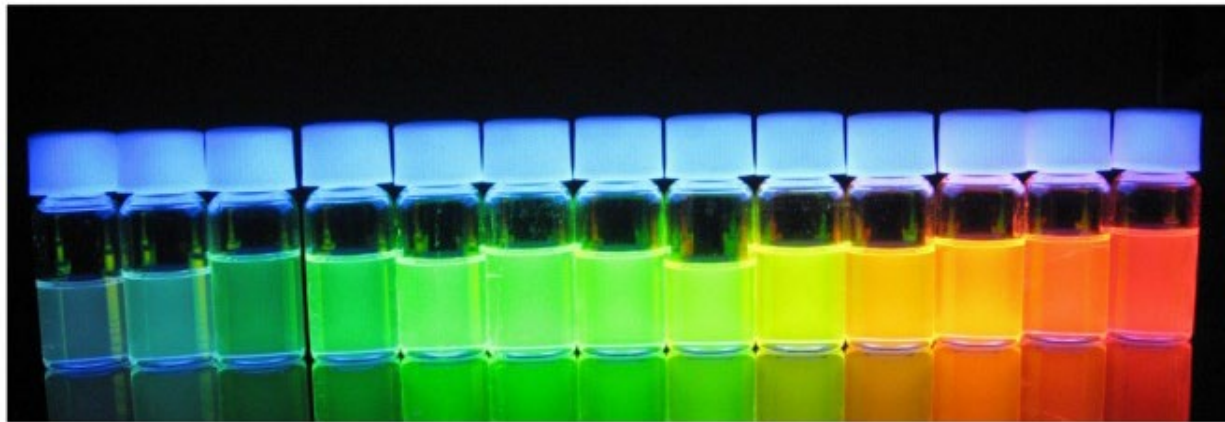
Propidium Iodide
(binds DNA)

Fluorescent proteins (GFP, RFP) first isolated from jellyfish.

A fluorochrome is an organic molecule, Qdots are nanocrystals of semiconductor coated in polymers. Brilliant dyes are polymer strings of chemically tunable optical units to provide higher signals.

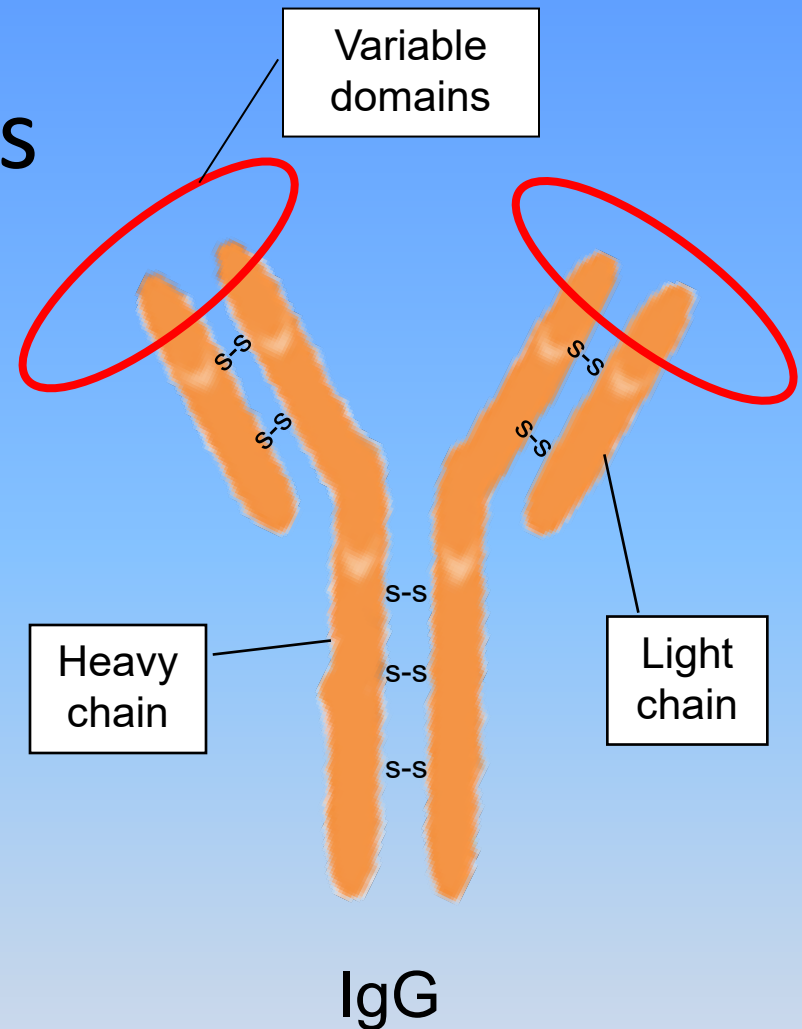
All will absorb light energy (i.e. from a laser) of certain wavelengths and emit at a longer wavelength. "Stokes shift"

Different color for each target



Antibodies

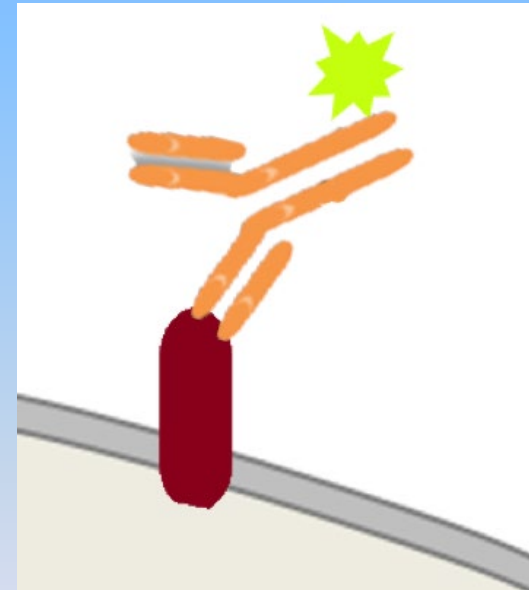
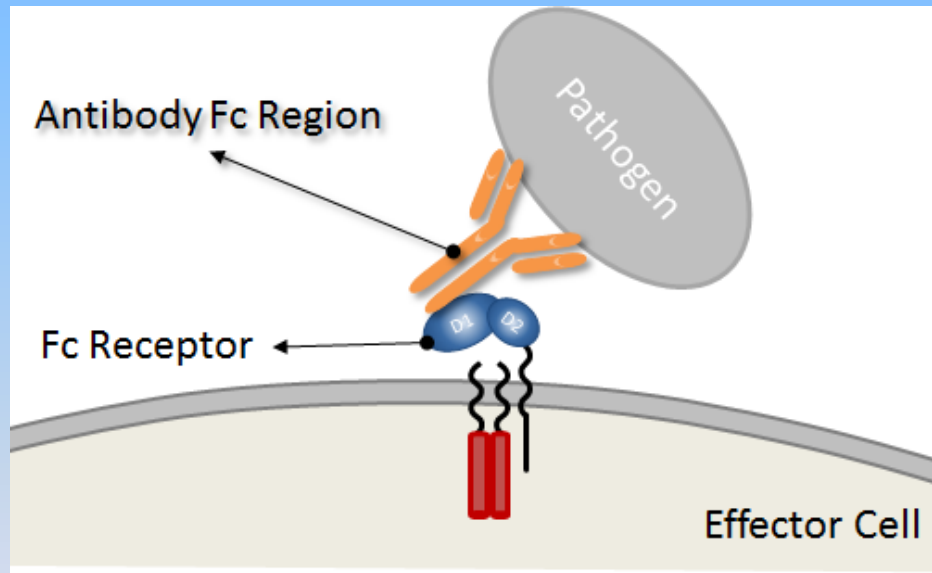
- Bind to specific proteins or epitopes
- Immunoglobulins come in 5 classes
 - IgG, IgA, IgM, IgD, IgE
- IgG-most often used in cytometry.
 - Heavy chain determines class and subclass (1, 2a, 2b, 3, 4)
 - Light chain either Kappa or Lambda
 - Constant domain gives the structure, variable domain gives specificity



Antibodies to markers of interest

What its really for...

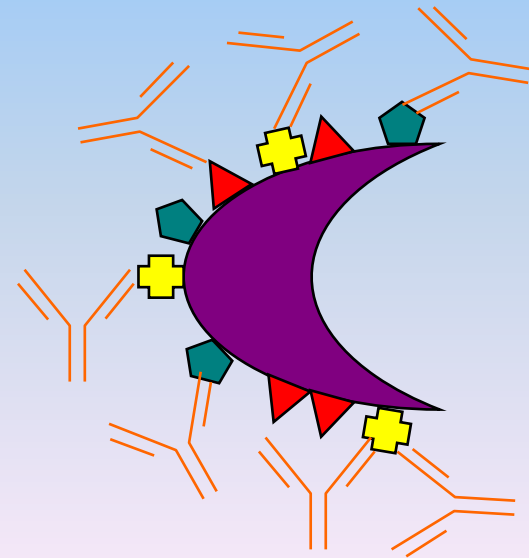
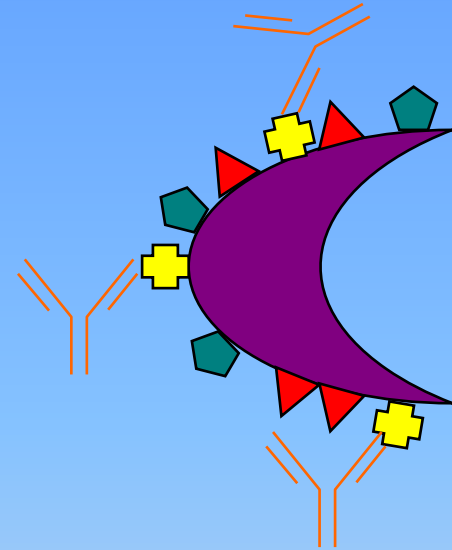
How we use it in flow...



Antibodies to a particular protein of interest will allow us to label cells expressing that protein

Antibodies 2

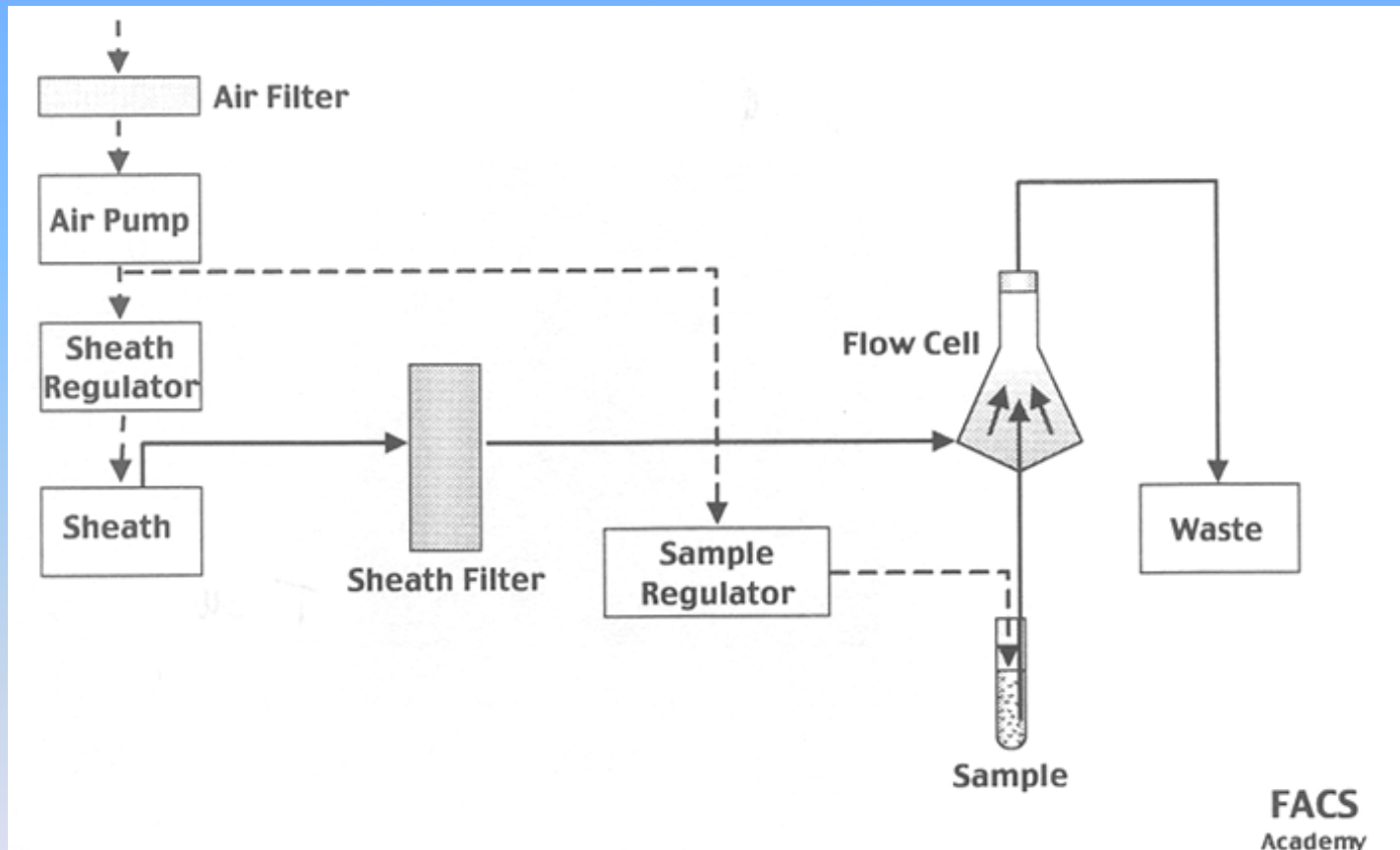
- Monoclonal
 - all antibody molecules are immunochemically identical and recognize the same epitope on an antigen
 - High specificity, no lot to lot variability in affinity
 - May not recognize fixed samples.
- Polyclonal
 - Each molecule will recognize different epitopes on the antigen.
 - Can “saturate” an antigen giving higher signal
 - Can be more nonspecific binding, can have lot-to-lot variation in affinity.



A cytometer needs to combine:

- Fluidics system
 - Introduces and focuses the cells for “interrogation”
- Optical system
 - Generate and collect light signals
- Electronics system
 - Change optical signals to electronic signals and digitize for computer analysis

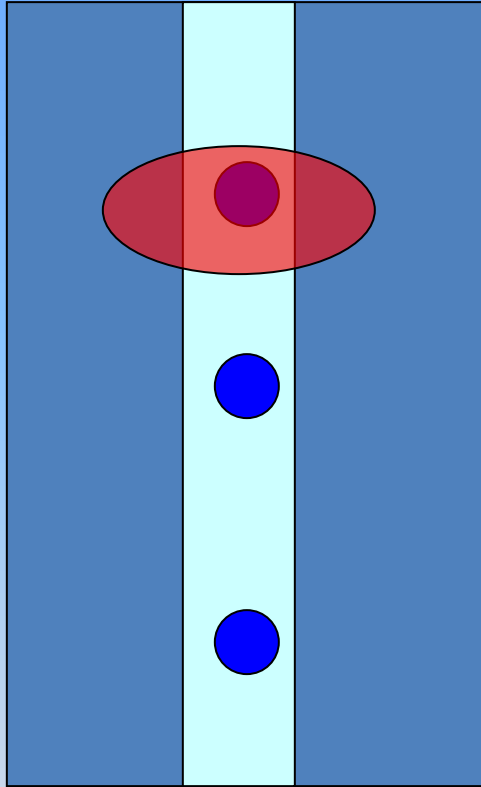
Fluidics



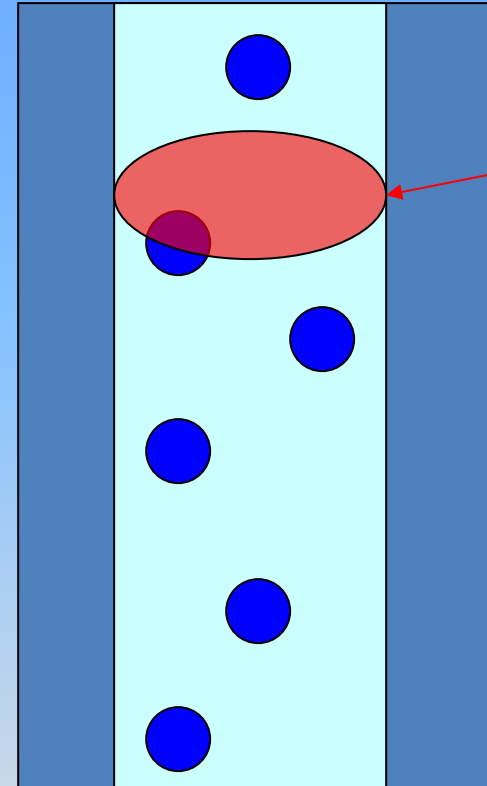
Most cytometers set up same way—sheath fluid supply, inject sample into flowing stream, quartz cuvette for interrogation.

Effect of flow rate on particle placement

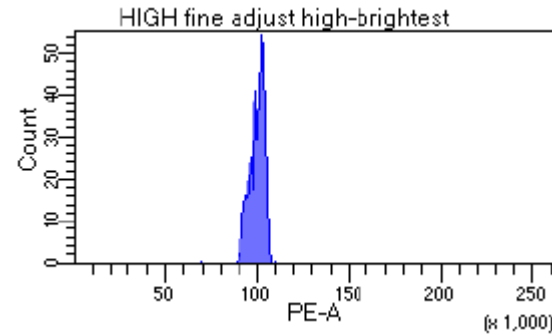
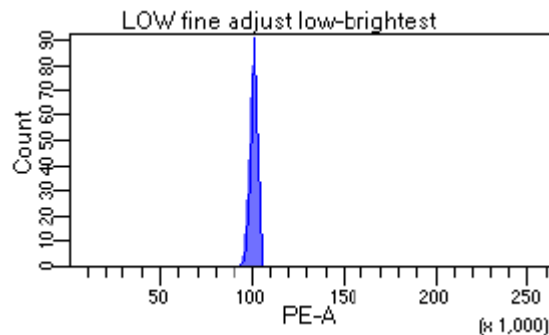
“Low” flow rate



“High” flow rate



Laser beam spot



Flow Rate vs. Event Rate

Instrument controls determine the sample difference (“flow rate”, volume of sample per second), but the “event rate” also depends on sample concentration.

A concentrated sample on low producing 5000 eps will have more accurate measurements than a dilute sample on high at 500 eps

A cytometer needs to combine:

- Fluidics system
 - Introduces and focuses the cells for “interrogation”
- Optical system
 - Generate and collect light signals
- Electronics system
 - Change optical signals to electronic signals and digitize for computer analysis

There are 2 parts to the Optics

-Excitation Optics generate and focus the light.

-Collection Optics gather the emitted light and separate the different wavelengths.

Lasers—EXCITING!

Cytometers will have one or more lasers to excite the fluorochromes:

- Unlike a microscope that uses white light with an “excitation filter” to choose the optimum wavelength, the laser color determines what fluorochromes can be used on that instrument.
- Common excitation wavelengths:
 - 488 (blue)
 - 635 (red)
 - 405 (violet)
 - 532 (green)
 - 350 (UV)
 - 561 (yellow-green)

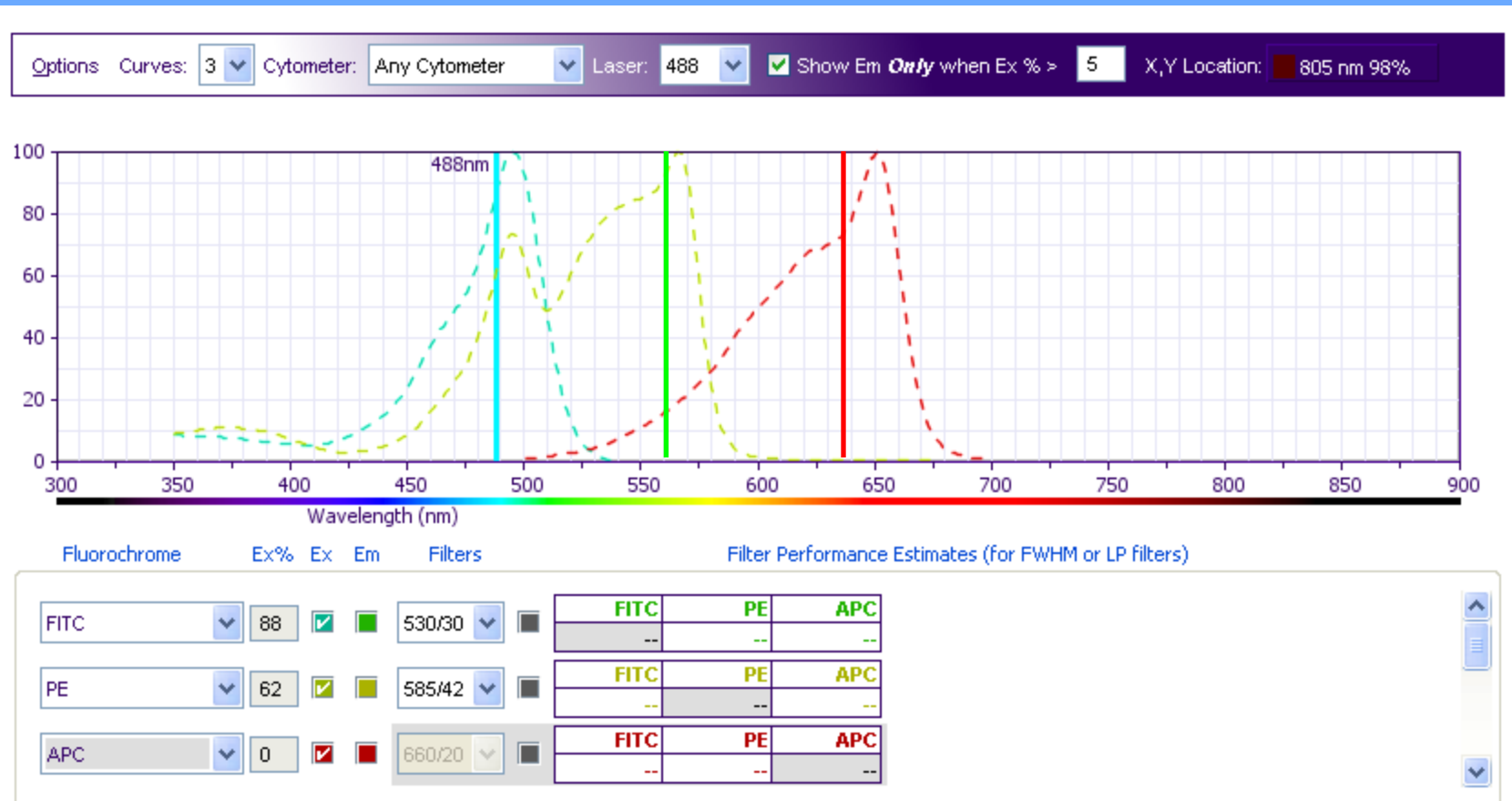
488, 489, whatever it takes...



“Mr Mom”, 1983

EXCITATION SPECTRA www.bdbiosciences.com/spectra

What fluorochromes can be used on a given instrument?



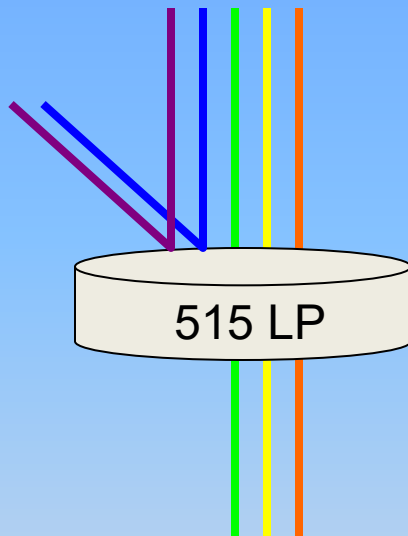
- FITC almost maximally excited by 488
- PE sufficiently excited by 488 for good signal
- PE near max with 561 (yellow-green).
- APC requires 635 excitation
- Multiple laser lines add to the # of parameters that can be measured simultaneously

Collection Optics consist of:

- A collection lens to collect light emitted from the particle-beam interaction
- A system of optical mirrors and filters to route specified wavelengths of the collected light to designated optical detectors

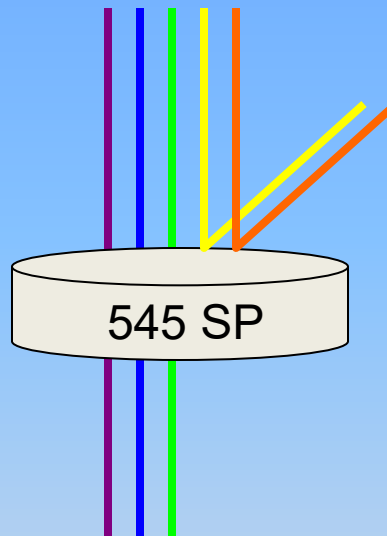
Collection Optics: Separating the Spectrum

Optical filters--3 main types



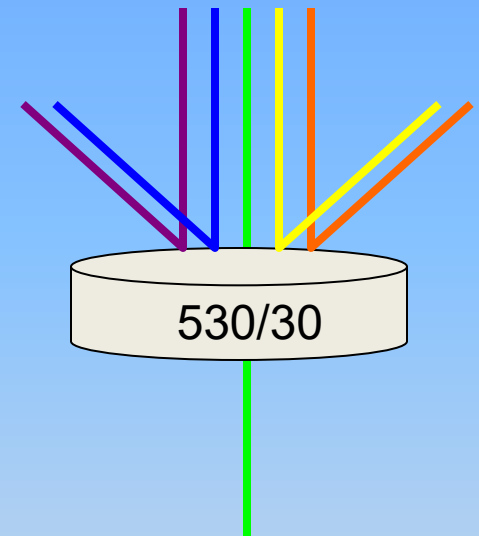
Long pass

Longer than cutoff
passes through,
shorter is reflected



Short pass

Shorter than cutoff
passes through,
longer is reflected



Band pass

Allows a band of light to
pass through. First number
indicates midpoint, second
number indicates width (in
this case 515-545)

Combinations of filters determine the wavelength of light that reaches your detector

LSR II and LSR Fortessa: Detector array

- Optical fibers carry light from flow cell to detector array-- less loss of light.
- Inner ring of longpass filters
- Middle ring of bandpass filters
- Outer ring of PMTs
- Filters are interchangeable



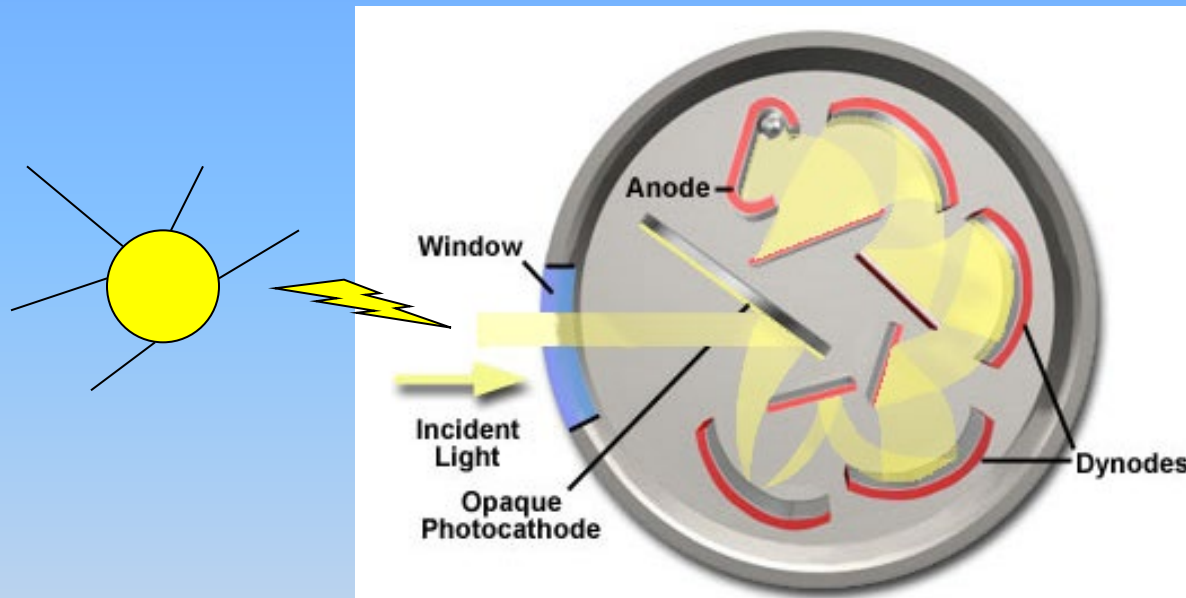
A cytometer needs to combine:

- Fluidics system
 - Introduces and focuses the cells for “interrogation”
- Optical system
 - Generate and collect light signals
- Electronics system
 - Quantify signals

Electronics

- Converts optical signals to proportional electronic signals (voltage pulses)
- Analyze voltage pulse height, area, or width
- Interfaces with computer for data transfer.

Conversion of Optical Signals to Proportional Electronic Signals



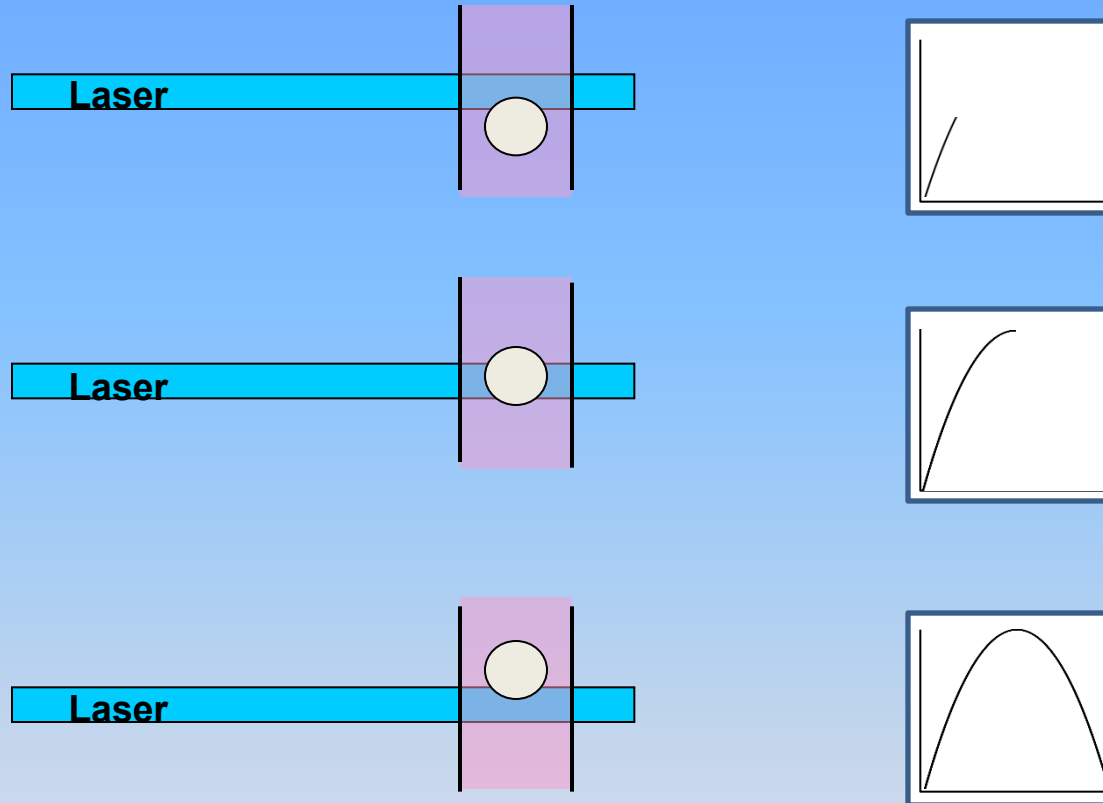
PhotoMultiplier Tube (PMT), photodiode (PD), and Avalanche Photodiode (APD)—generate voltage when struck by photons.

PD used for FSC which produces more light.

PMT amplifies the signal depending on voltage supplied.

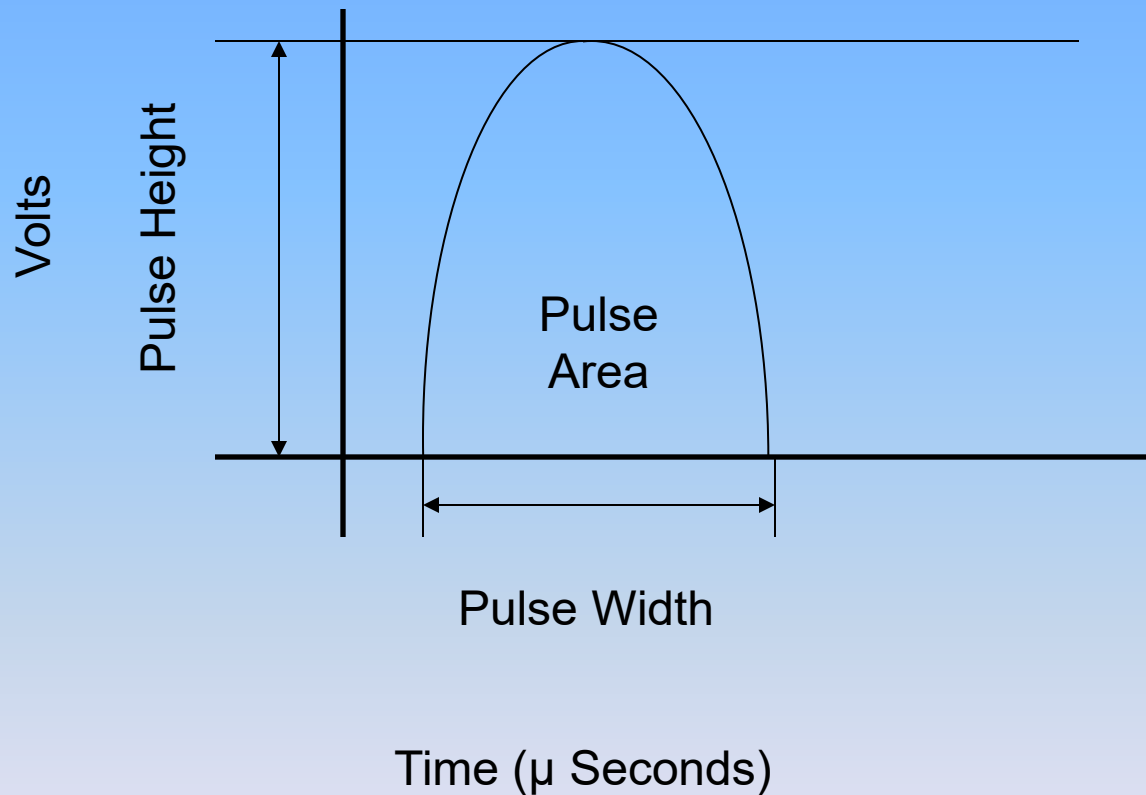
Detectors are color blind, so it is important that filters separate different colors of light.

Creation of a Voltage Pulse



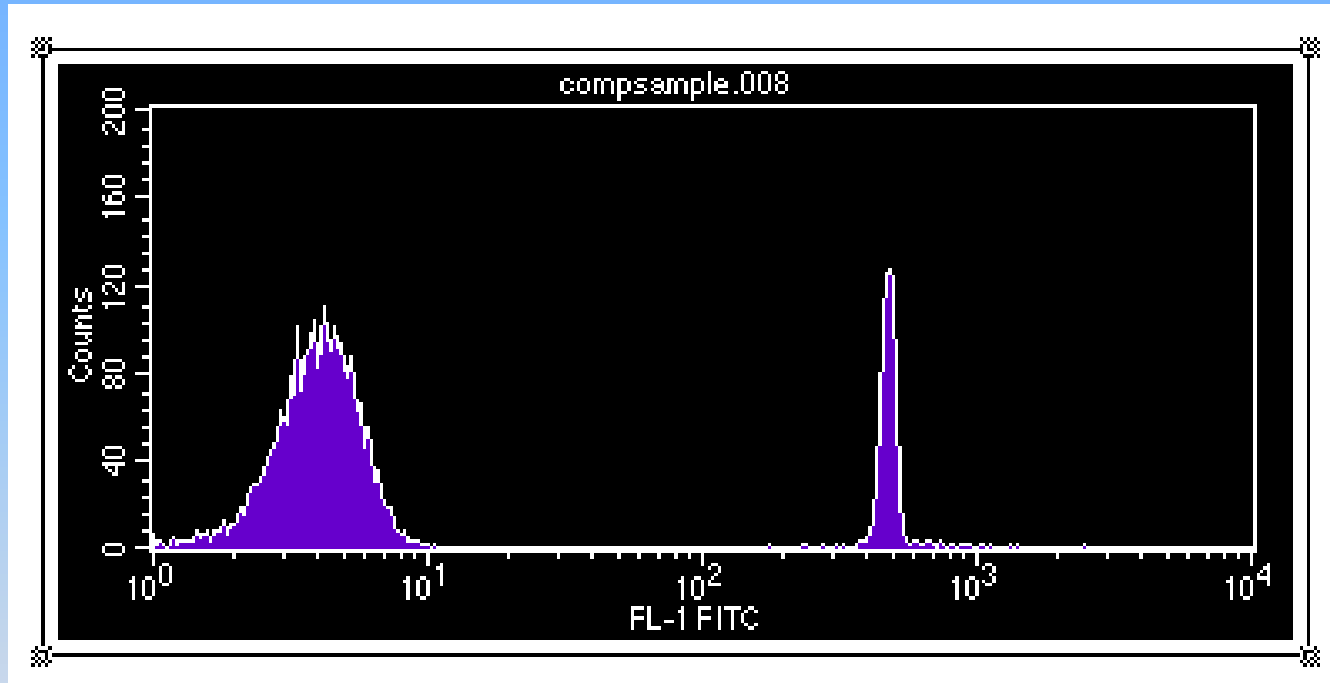
- Maximal signal when particle is entirely within the laser beam.
- Pulse generated for each parameter being measured, for each particle.
- Total signal is collected, not just what is bound to particle!
(e.g. unbound dye, Phenol Red in culture media)
- Light signal turned into voltage signal, which can be quantified.

Quantification of a Voltage Pulse



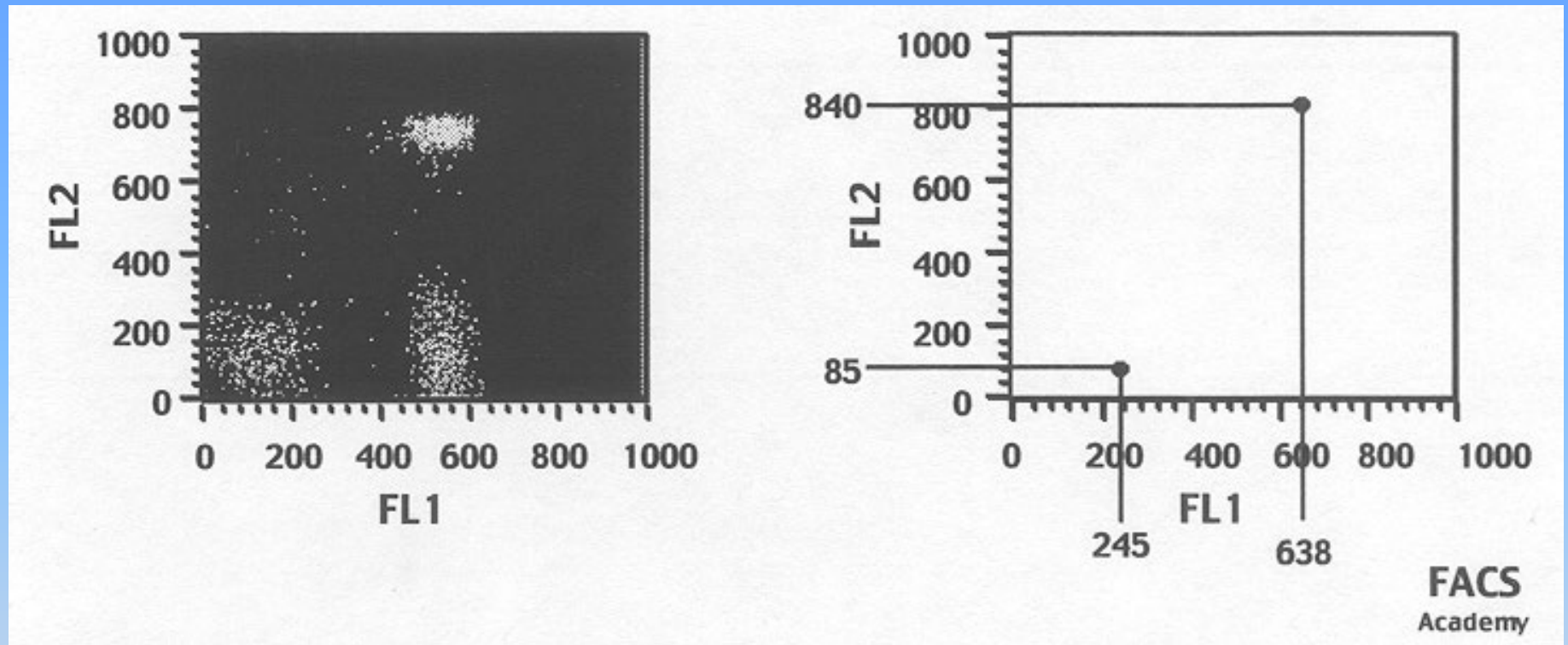
Histogram

Single Parameter



Histogram displays the number of events with a given signal intensity

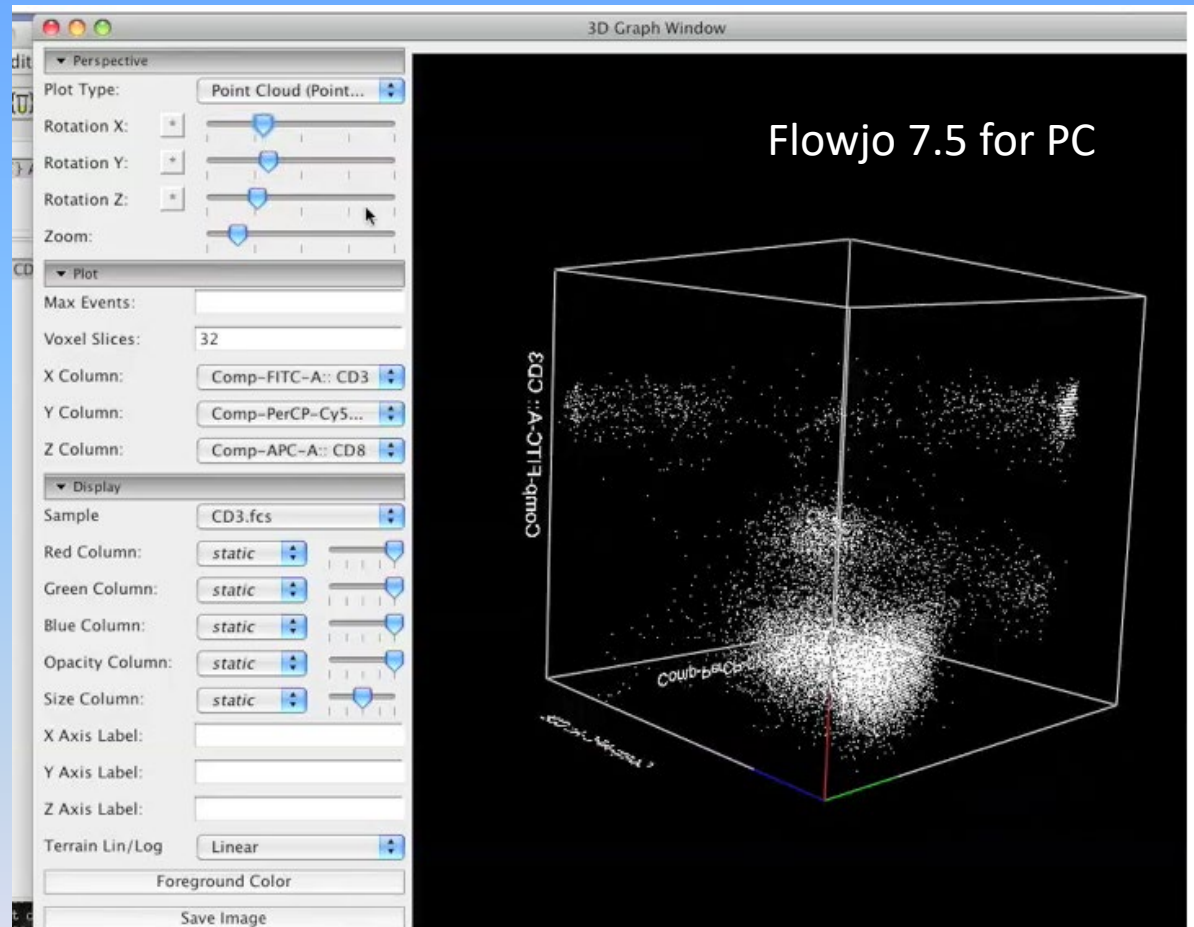
Dot Plot



Bivariate plot or bivariate histogram, events map to regions of the graph. Allows you to visualize different populations among particles— look at 1 parameter would see only + or -, looking at 2 you can identify ++.

Can then select particular populations and use other parameters to further classify them.
Identify protein markers, fluorescent proteins, DNA content for cell cycle, etc...

3D plots in Flowjo

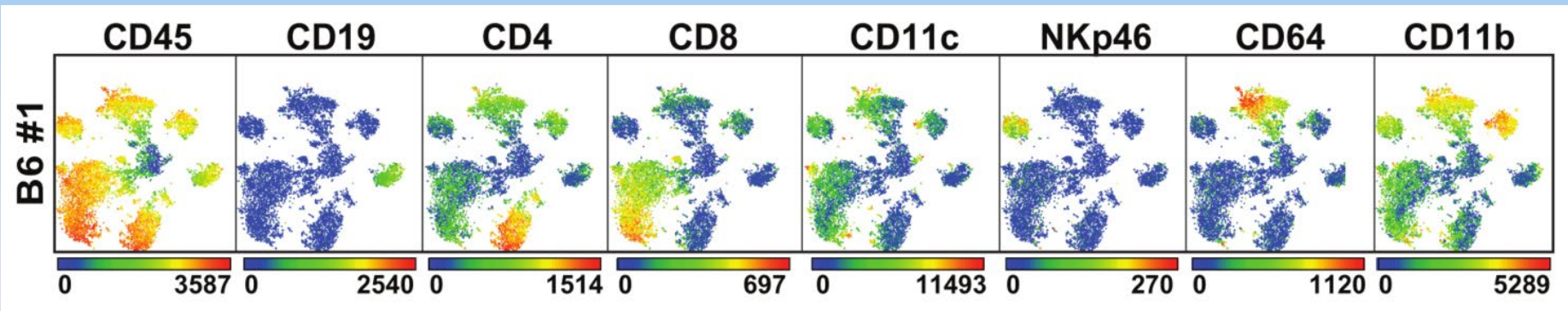


<http://docs.flowjo.com/d2/workspaces-and-samples/ribbons-and-tabs/ws-ribbon-band-visualizations/>

https://www.youtube.com/watch?v=_kBUoSj0C6k

Automated Analysis

- Clustering algorithms to identify populations (t-SNE, other methods)
- Heat maps show what markers expressed by each cluster

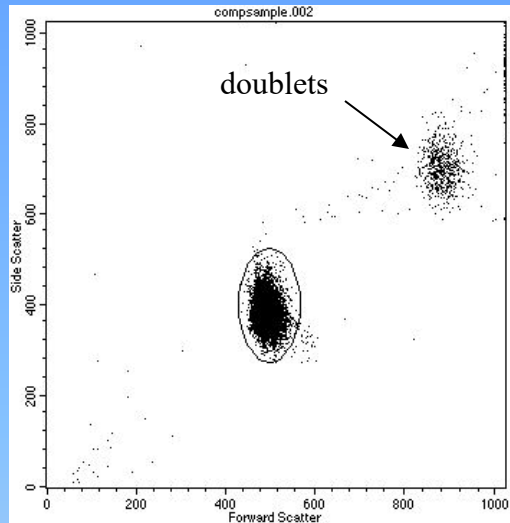


Instrument Setup

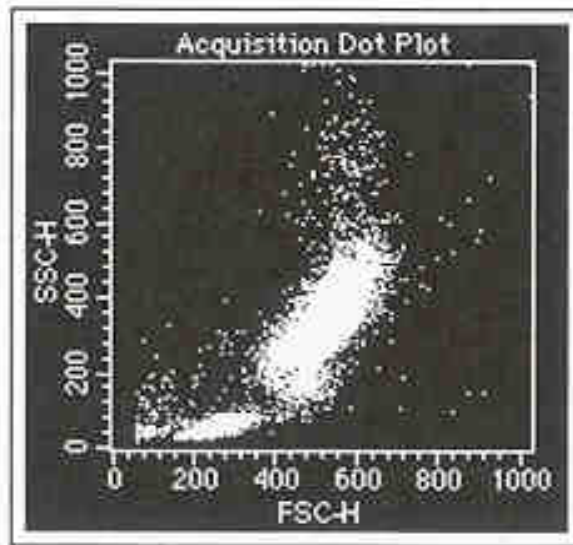
- User adjusts sensitivity of detectors so that:
 - Events of interest are on scale
 - “Negative” fluorescence on the left/bottom, providing maximum dynamic range for positive signals

Setting FSC and SSC

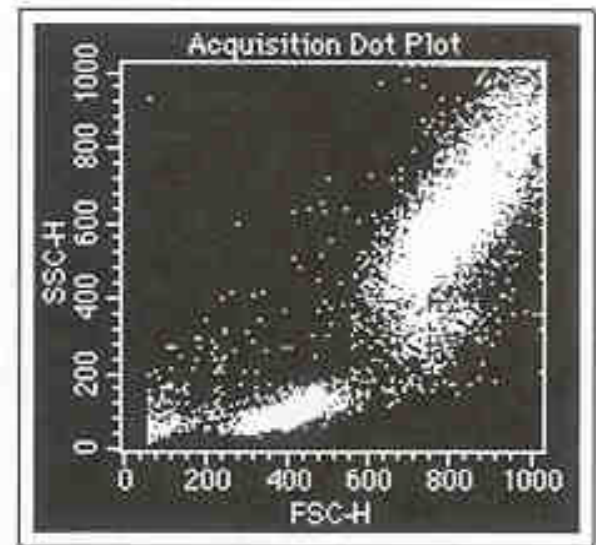
Beads



**Lysed
Whole
Blood**

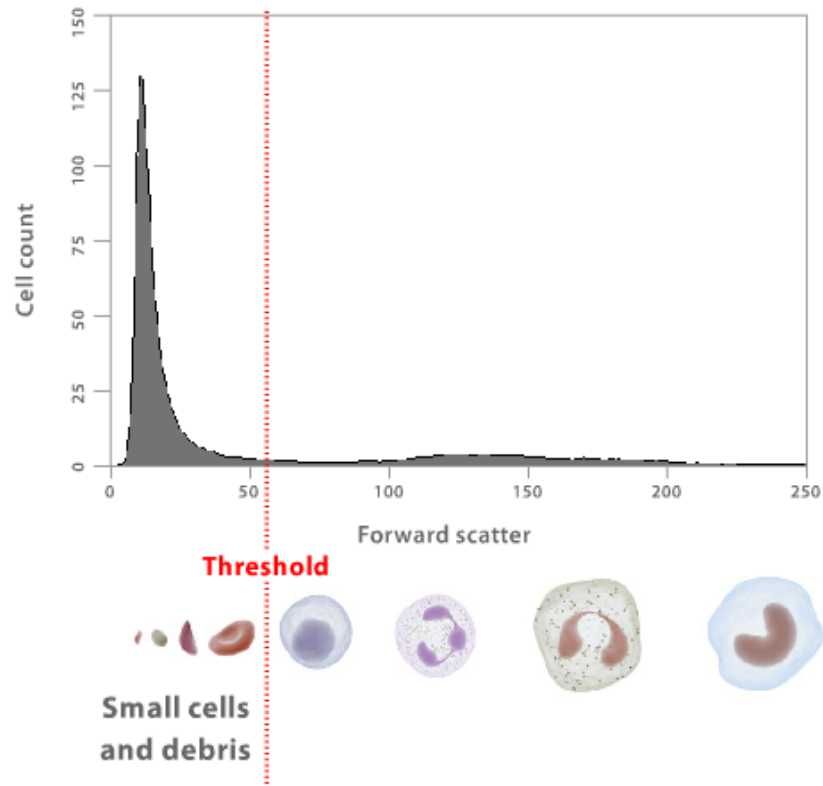


Before optimizing



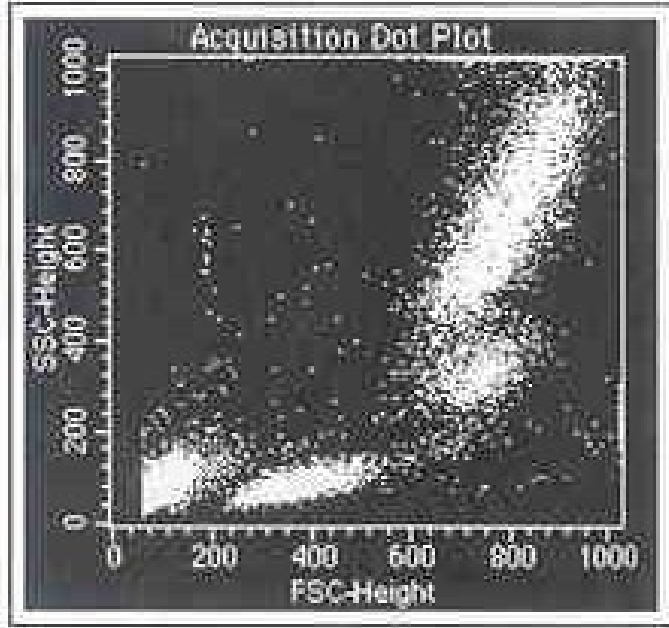
Optimized

Forward Scatter Threshold

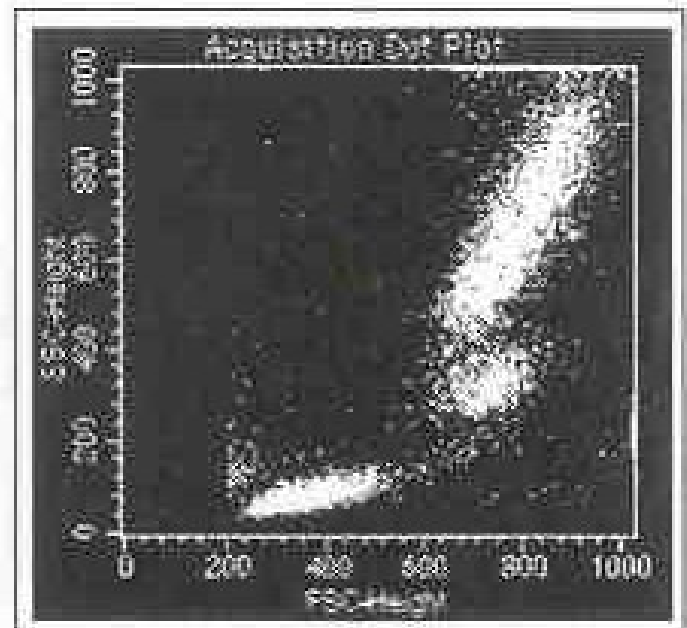


Forward scatter used as trigger signal. Events below cutoff are ignored.

Adjust FSC Threshold



Before



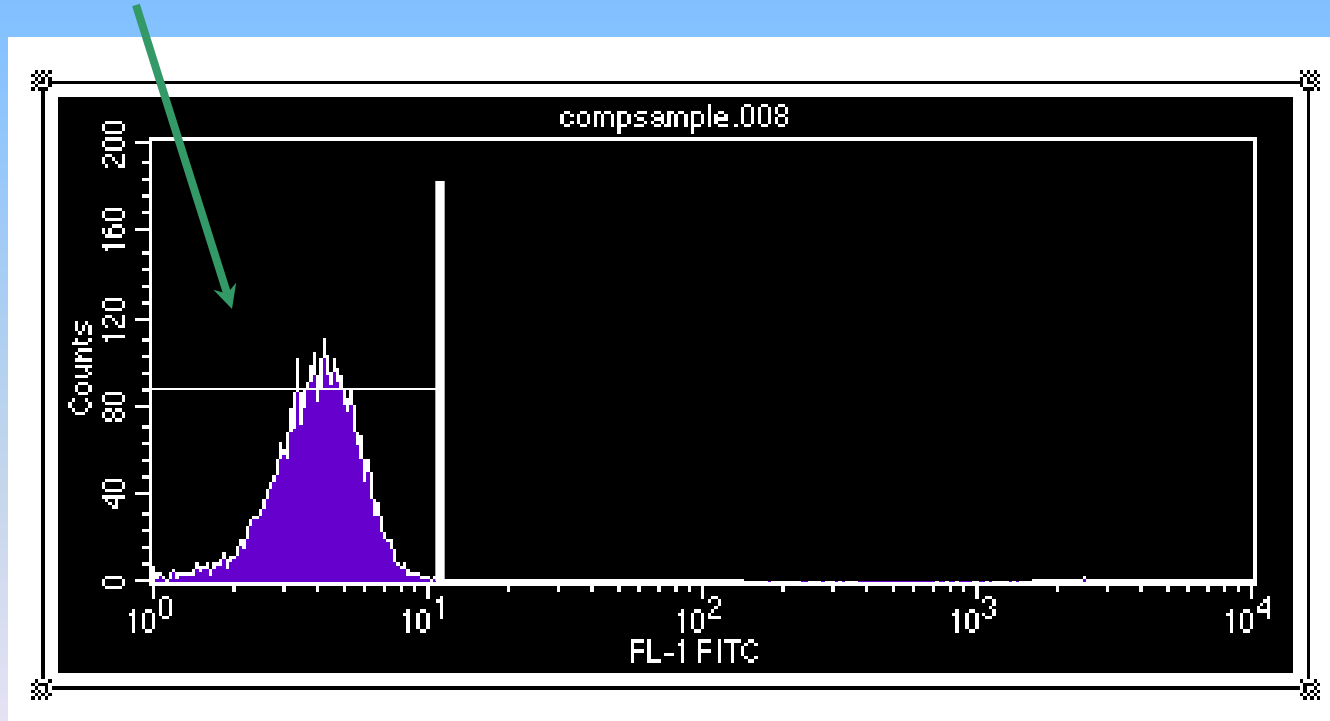
After

Eliminates debris, RBC's, platelets, instrument noise.

Set appropriately for your assay! Apoptotic cells have lower FSC than healthy cells, so make sure they don't fall below threshold.

Setting Starting Voltages for Fluorescent Parameters

For each color, adjust voltage so that the negative population is in the first decade



Autofluorescence

“Negative” signal on cells or beads is autofluorescence due to flavins, porphyrins and other molecules (e.g. chlorophyll in plants) or properties of the material (plastics fluoresce in certain excitation wavelengths).

Different particles will have different levels of autofluorescence (e.g. lymphs vs. monos, different cell lines, beads of different plastics) affecting sensitivity in certain parameters with high base signals.

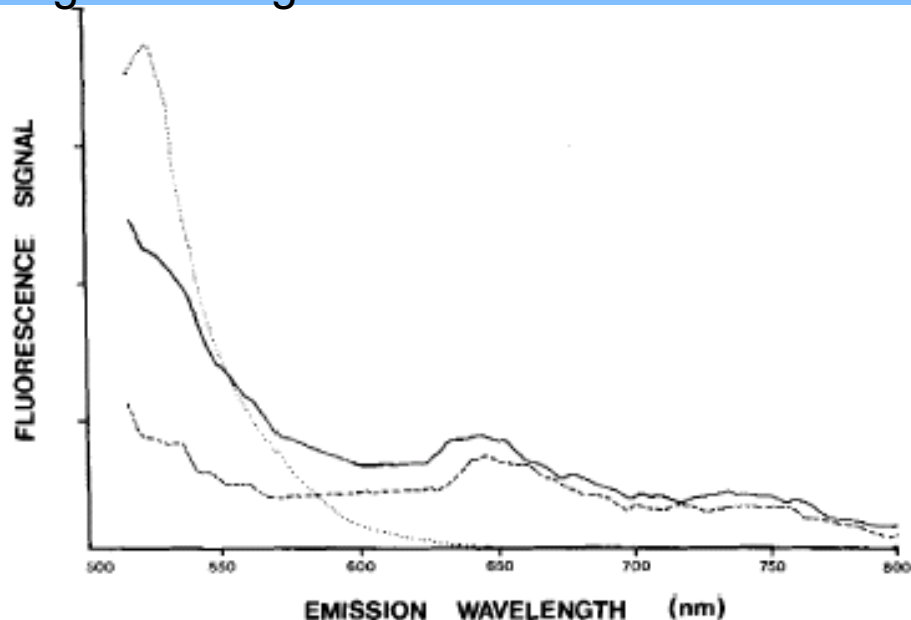
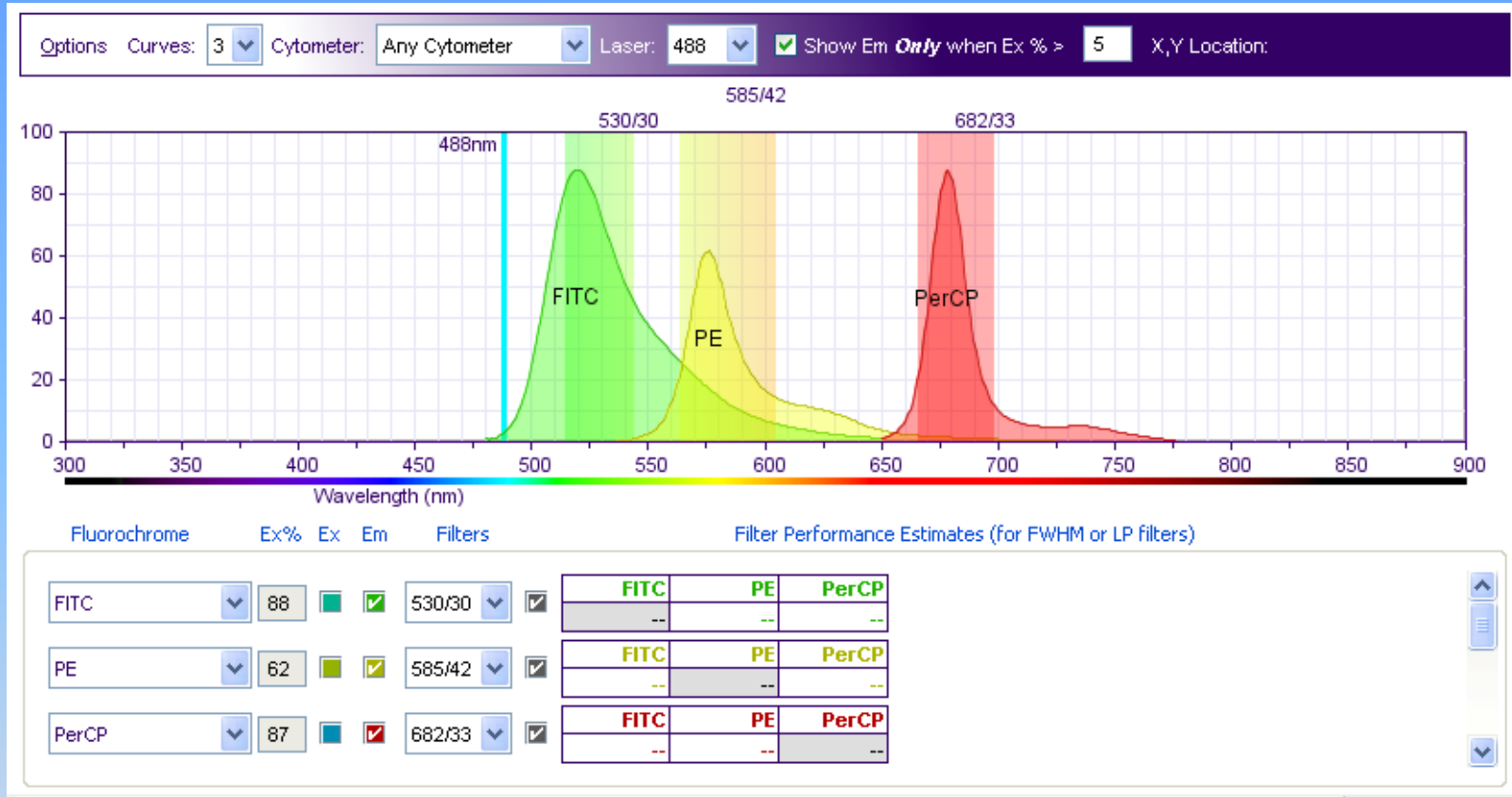


FIG. 1. Fluorescence emission spectra of L cells (solid line), 143 cells (broken line), and FITC-conjugated antibody to TROP-1 (dotted line) excited at 488 nm. Excitation window = 8 nm. Measurements were taken in 5-nm steps. The Raman scatter peak (570-600 nm) has been removed from the spectra. The spectra are uncorrected for photomultiplier spectral sensitivity to approximate what would be expected with the flow cytometer photomultipliers.

Emission Spectra



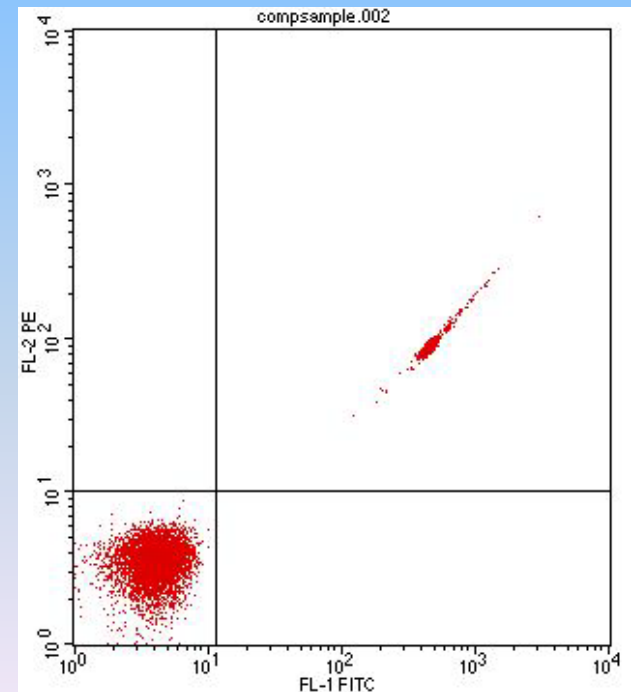
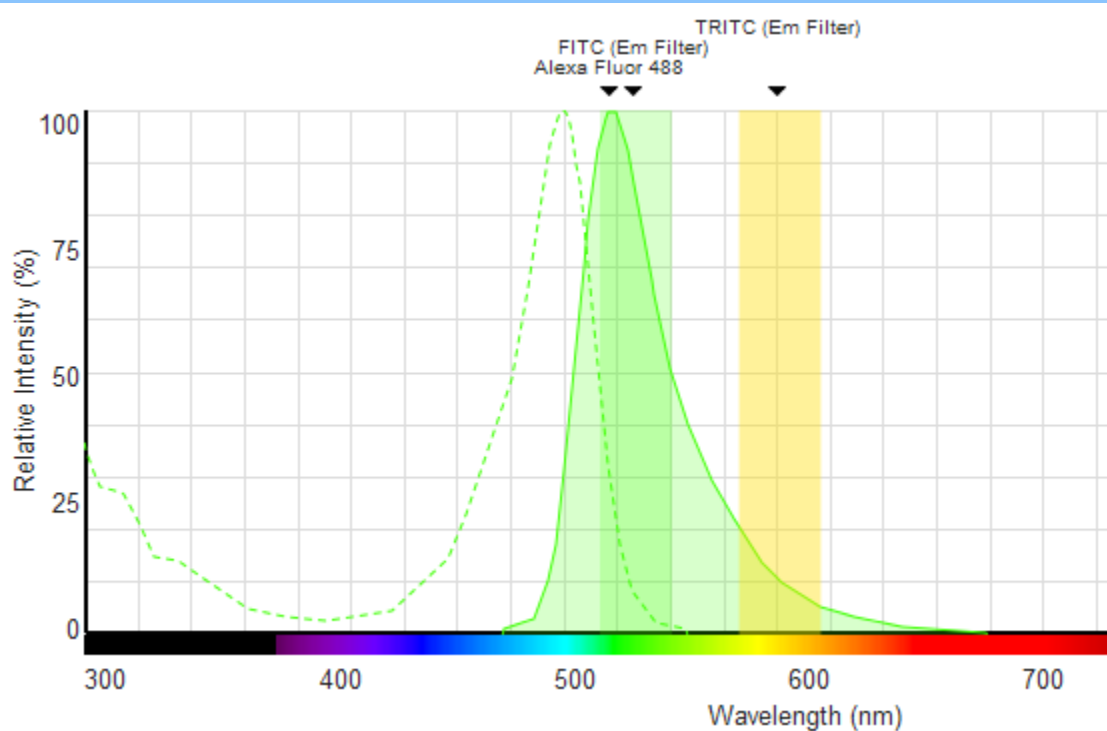
Excitation and Emission spectra of fluorochromes can be viewed using Spectraviewers linked from newenglandcytometry.com, "Helpful Links>Spectraviewers"

Emission occurs over a range of wavelengths, with measurements usually made at the peak of signal.

Spectral overlap or spillover necessitates "compensation" to remove background signal in other channels.

Compensation

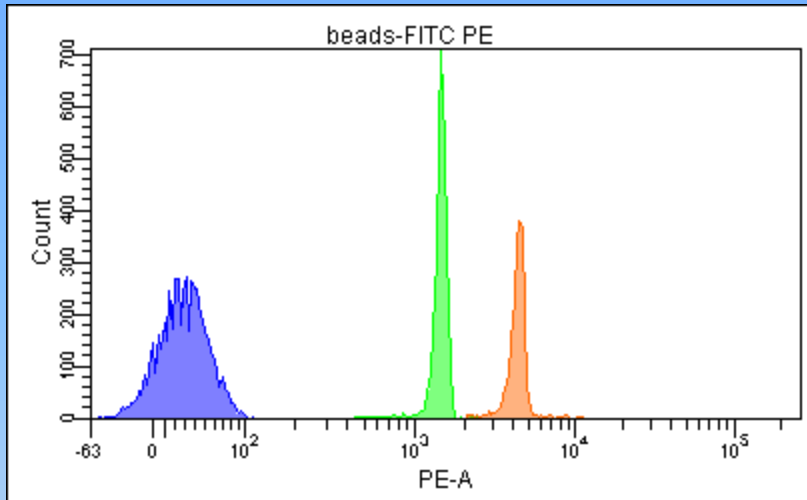
- Corrects for the emission of one fluor into the detector used to measure another.



Compensation—FITC and PE

Compensation removes background signal that is a normal property of the dyes used to detect labeling. FITC emits in the range of the PE detector

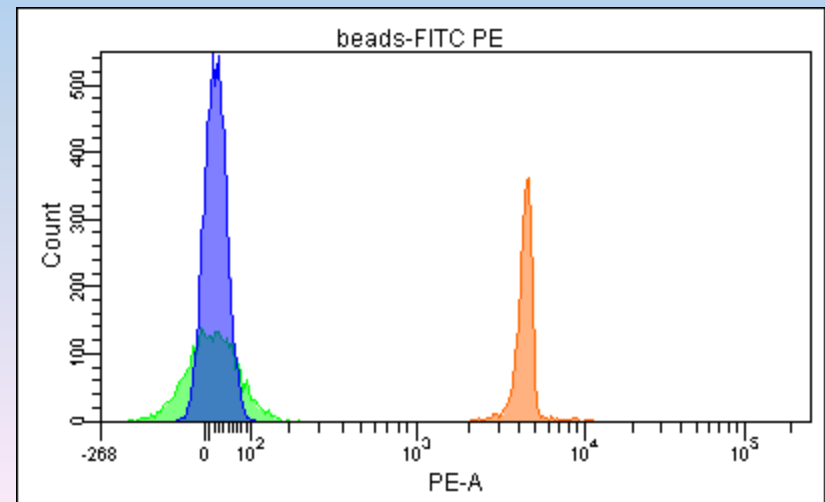
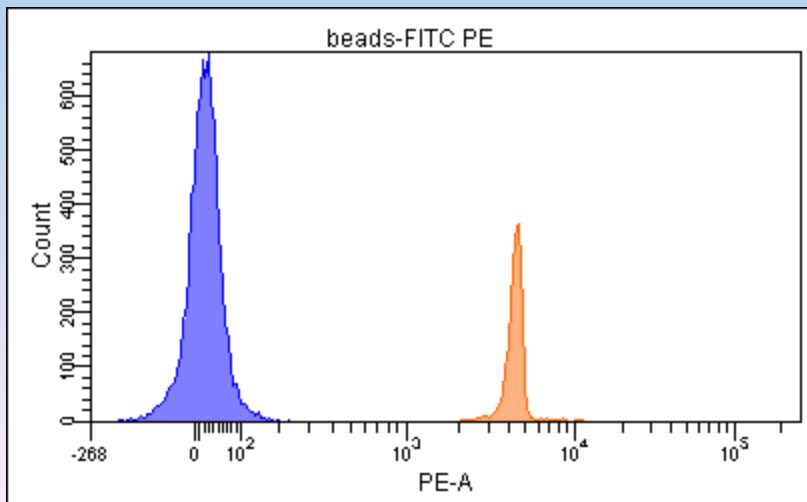
Uncompensated



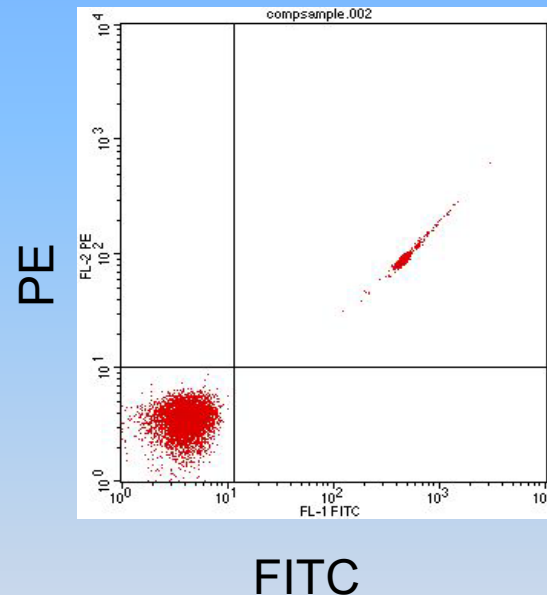
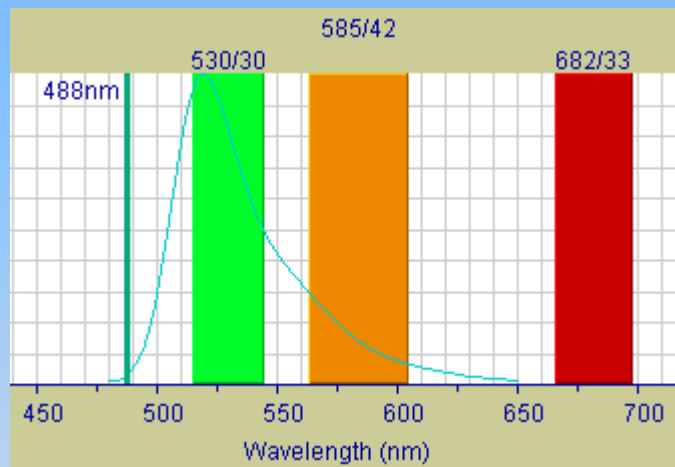
- negative
- PE labeled
- FITC labeled

When compensation is properly applied, a single-stained particle will have the same mean signal in all other channels as an unstained particle.

Compensated



FITC fluorescence Overlap



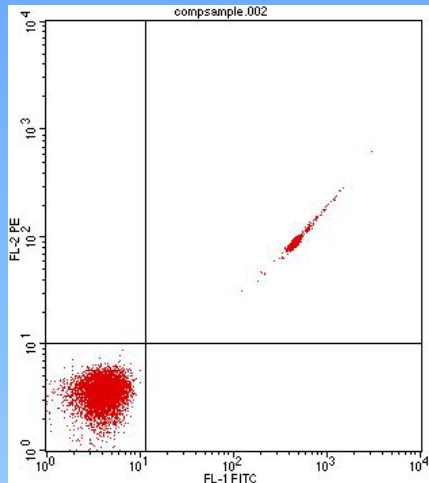
At given voltages, the amount of signal in the spillover detector is proportional to the signal in the primary detector:

100 FITC $\rightarrow$ 20 PE, then 50 FITC $\rightarrow$ 10 PE

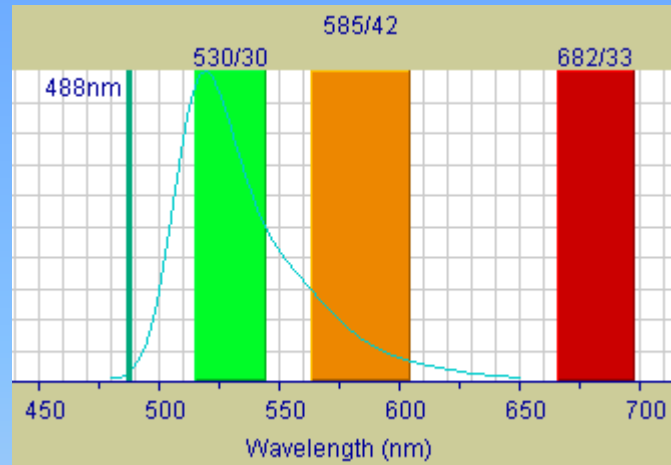
Set up a ratio between the signals

FITC Compensation

FL2 (PE)



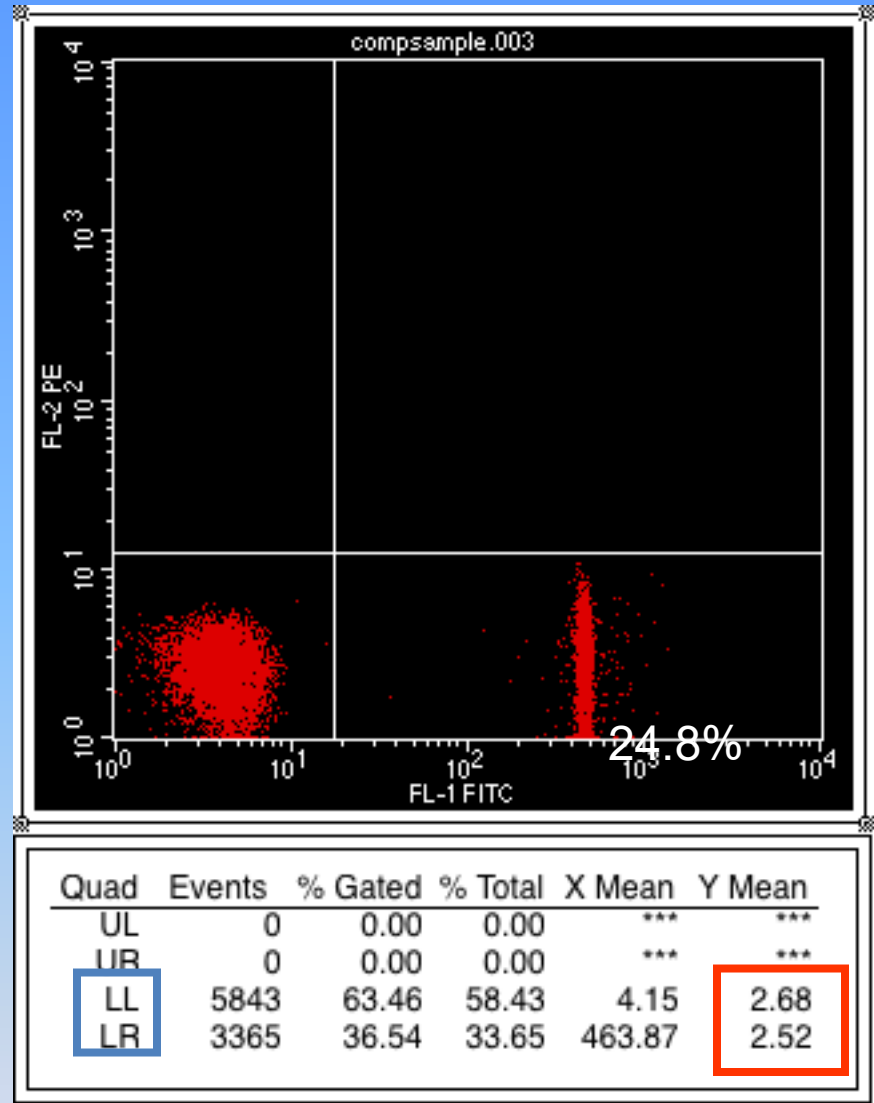
FL1 (FITC)



Compensation			
FL1 -	0.0		%FL2
FL2 -	0.0		%FL1
FL2 -	0.0		%FL3
FL3 -	0.0		%FL2

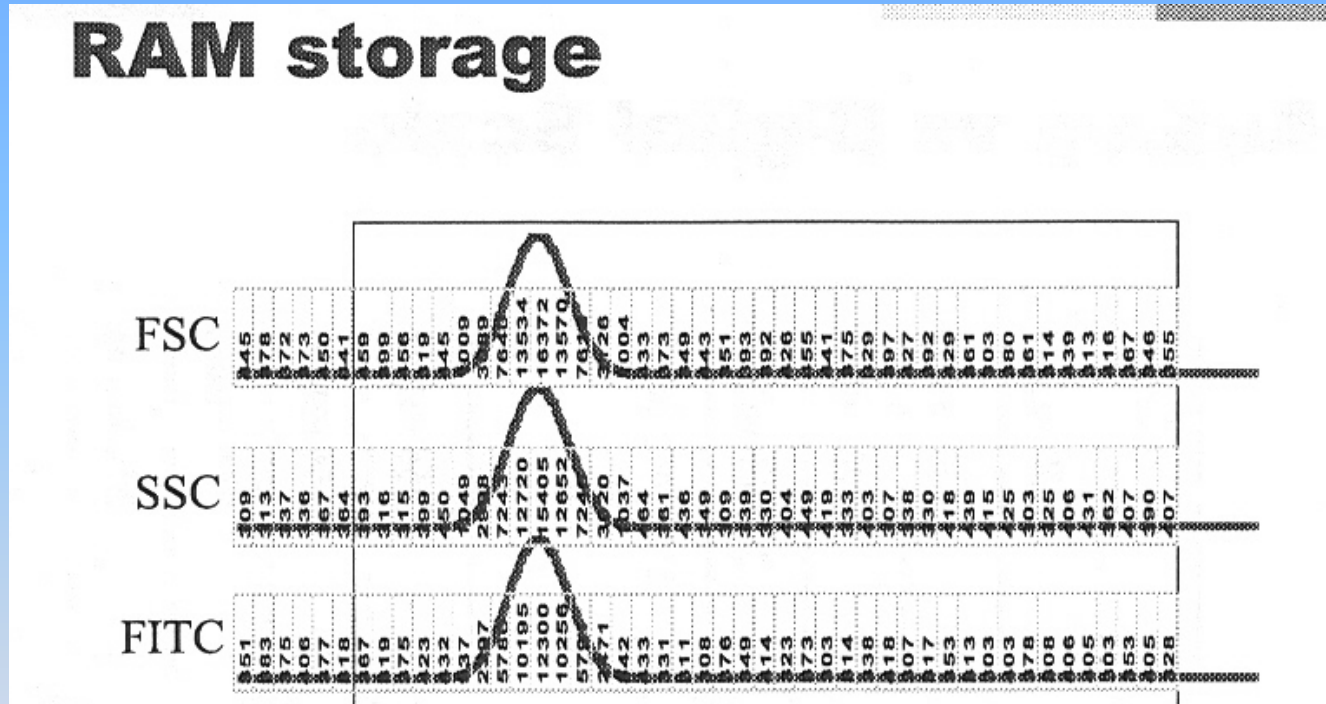
Subtract % of signal in primary channel **from** signal detected in the overlap channel to remove background.

Goal: mean signal
of + and –
populations in
overlap channel is
equal



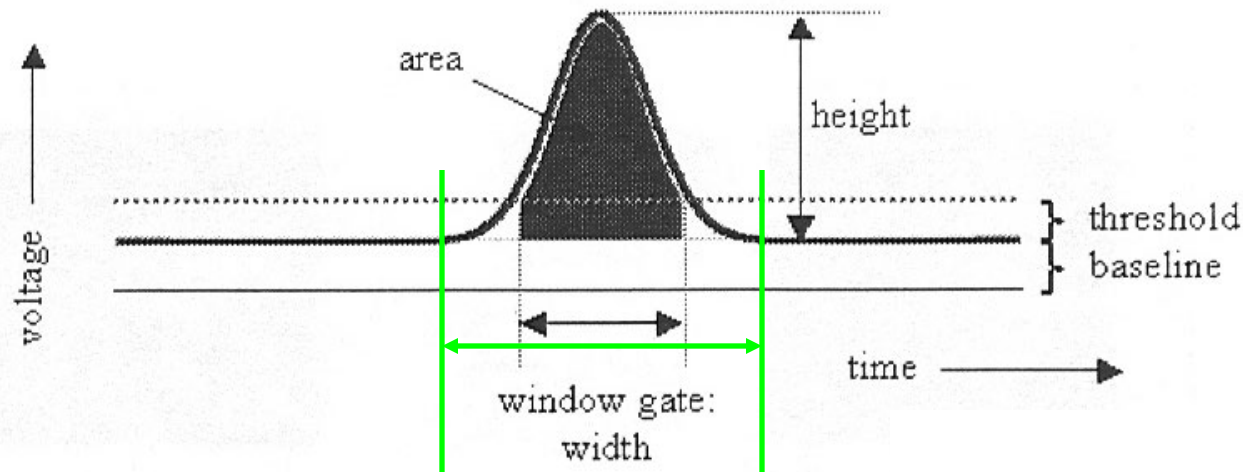
Comparisons should be made between each pair of parameters in the experiment,
but compensation will not be necessary for all pairings
(4 color experiment=12 pairs, 10 color experiment=90!)
25 color= 600 (n^2-n)

Signal pulses in Digital acquisition



Voltage pulse assigned a value up to 16k, sampled 10 million times per second, and number stream stored in RAM while events are identified.

Convert Numbers to Parameters



Window extension

- For height: maximum digitized value
- For Area: sum of all height values
- For width: $\text{area}/\text{height} \times 64k$ (spread values out more)
- Digital data is displayed on 262,144 scale

Computer looks in threshold parameter (usually FSC) and when an event is present, it calculates the signal levels in that time window for each of the parameters.

Window extension—overlap leads to aborts.

From detection to display

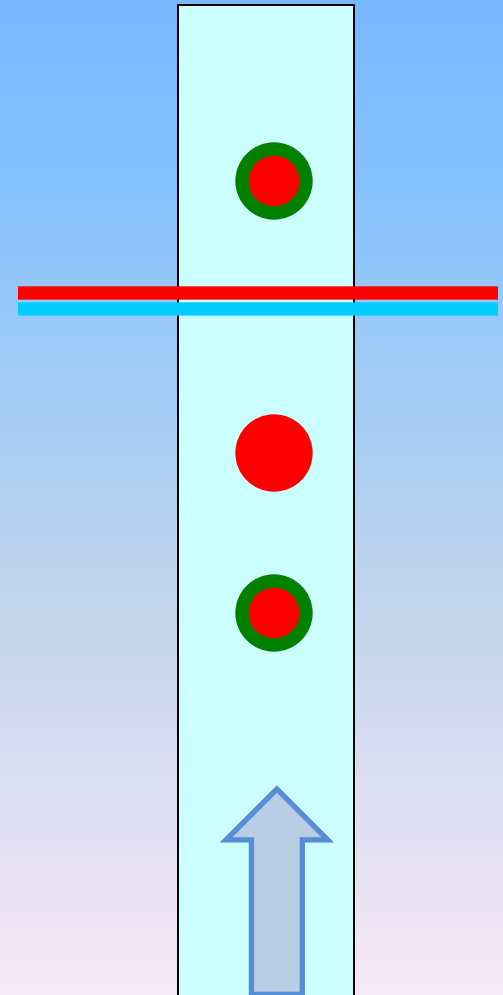
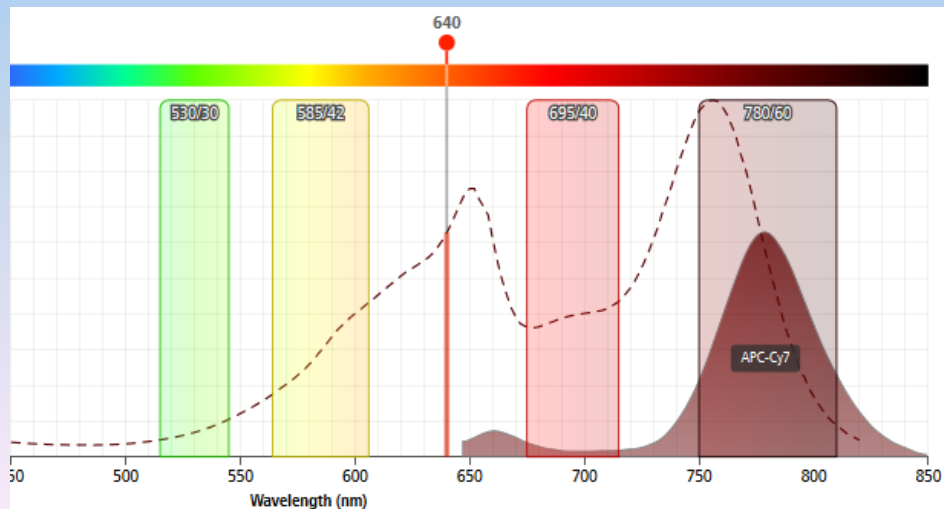
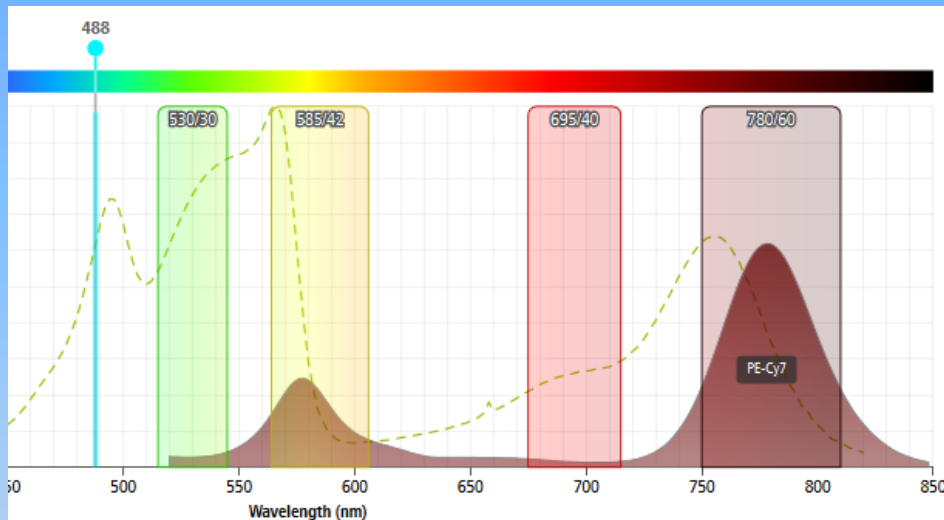
- FACSDiva (digital)
 - Light signal converted to voltage pulse
 - Analog-to-Digital converter assigns value from 0-16,384
 - “Digitized” signals sent to computer, values converted to parameters (A, H, W)
 - *”List-mode” data file generated—
 - Compensation applied mathematically
 - Files are .fcs “flow cytometry standard”
 - User defines display mode
 - (log, lin, biexponential)

List-Mode Data				
	FSC	SSC	FL1	FL2
Event 1	30	60	638	840
Event 2	100	160	245	85
Event 3	300	650	160	720

Can't change voltage after acquisition, signal has already been generated!

Multiple Lasers

When cell passes through lasers at the same time, it is not possible to distinguish which laser caused the excitation, so only a single dye in each region of the spectrum can be used in the panel.
e.g. PE-Cy7 and APC-Cy7



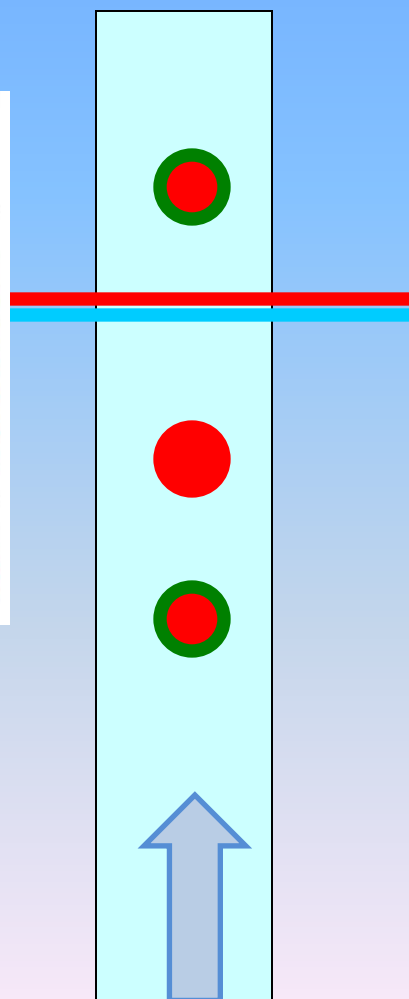
Multiple Lasers

When cell passes through lasers at the same time, it is not possible to distinguish which laser caused the excitation, so only a single dye in each region of the spectrum can be used in the panel.
e.g. PE-Cy7 and APC-Cy7

SONY SH800 BL2 Sorter configuration (4 lasers):

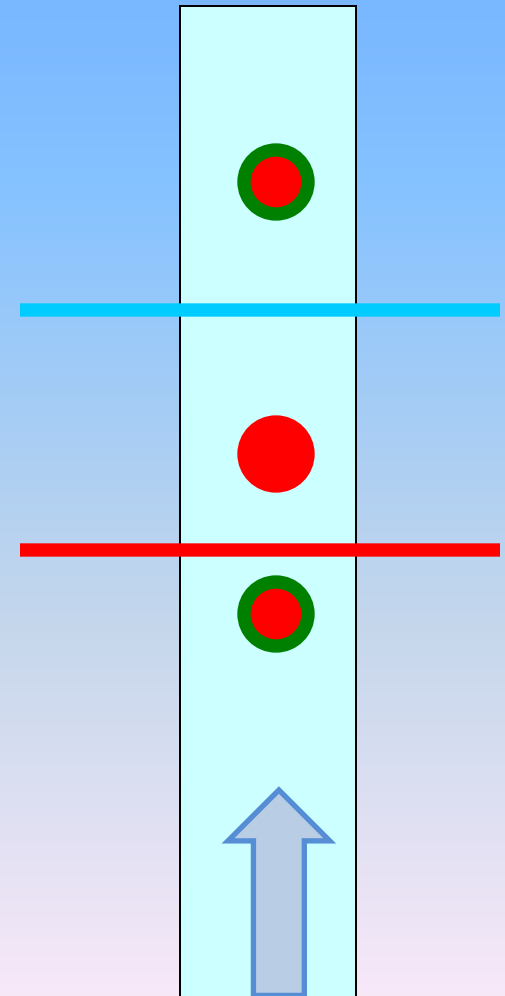
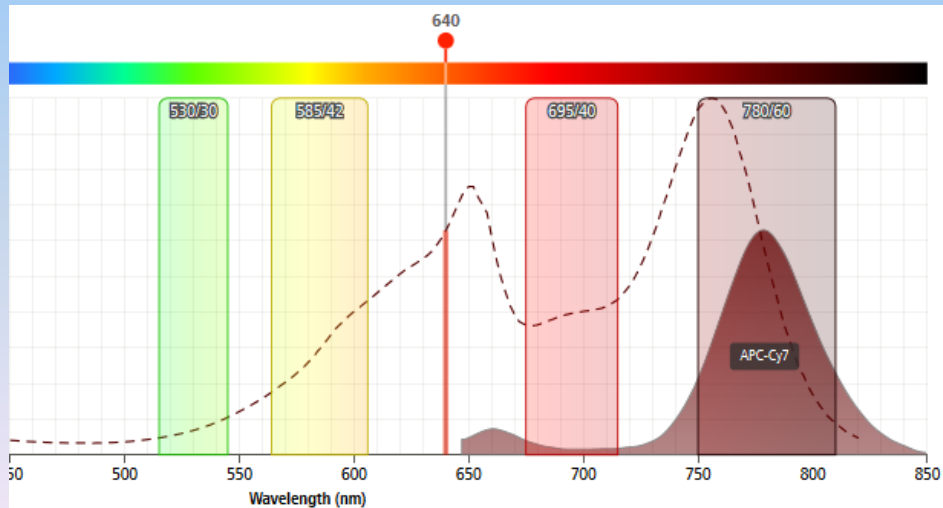
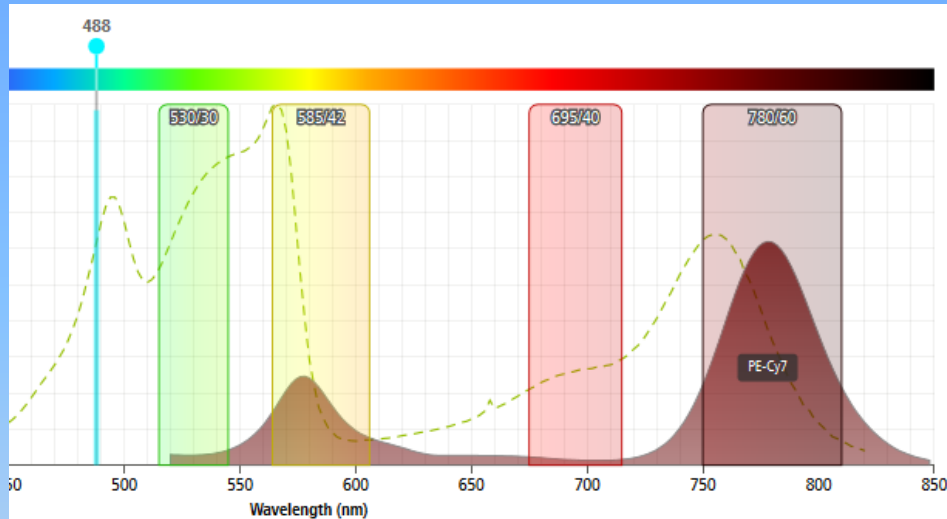
Colinear lasers 405, 488, 561, 640 (choose one marker per column)

FL1 (450/50)	FL2 (525/50)	FL3 (600/60)	FL4 (665/30)	FL5 (720/60)	FL6 (785/60)
BV421	AmCyan	BV570	BV650	BV711	BV786
AF405	Pacific Orange	BV605	7AAD	PE-Cy5.5	PE-Cy7
DAPI	eGFP	PE	PE-Cy5	PerCP-Cy5.5	BB700
Pacific Blue	CFSE	PE-Texas Red	PerCP	APC-Cy5.5	APC-Cy7
mCFP	FITC	PI	APC	AF700	APC-H7
	eYFP	DsRed	Cy5		APC-eFluor 780
	AF488	tdTomato	AF647		
		mCherry			
Zombie Violet					
LiveDead Violet	LiveDead Green	LiveDeadRed	LiveDead Far Red		LiveDead Near IR



Multiple Lasers are spatially separated

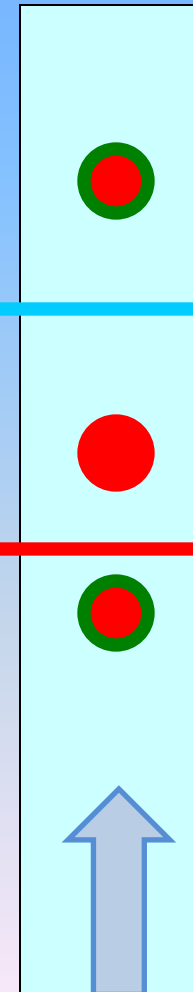
Allows measurement of signals in the same region of the spectrum for each laser, allowing for more parameters



Multiple Lasers are spatially separated

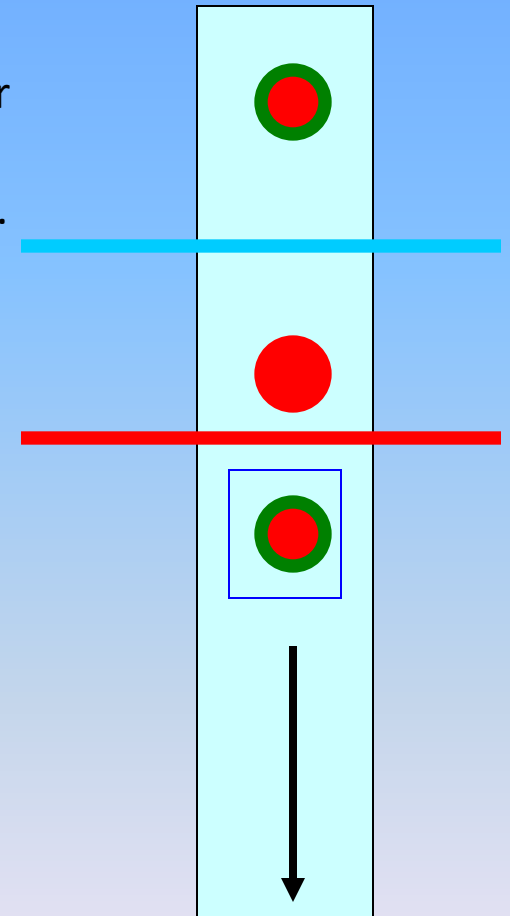
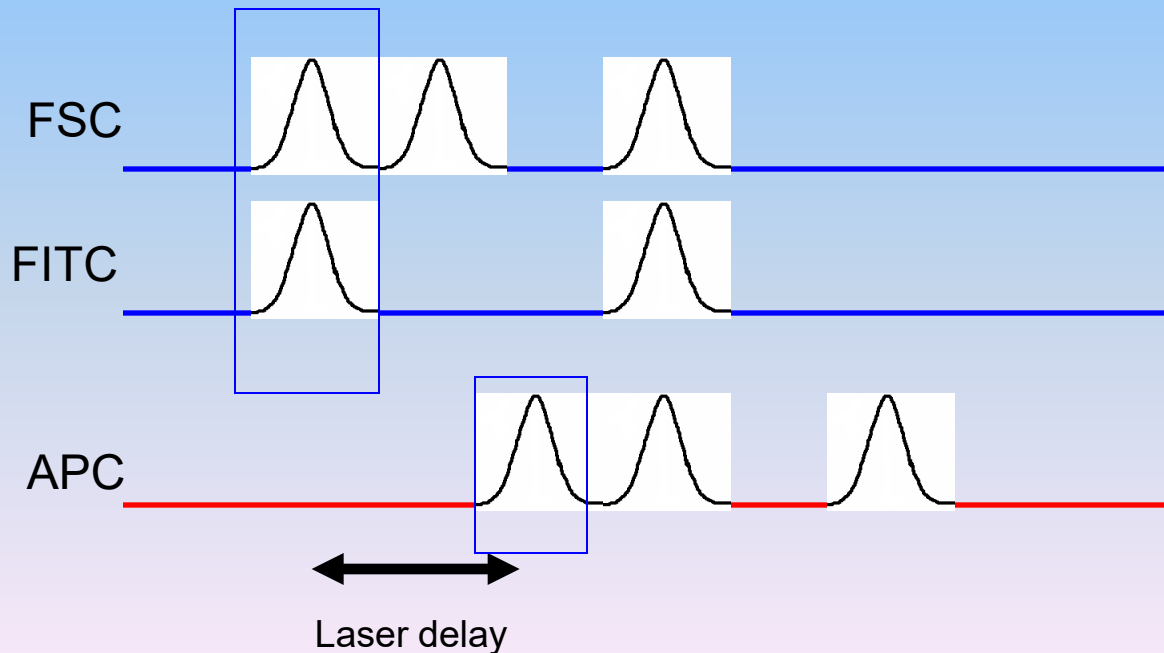
Allows measurement of signals in the same region of the spectrum for each laser, allowing for more parameters

	Fluorochrome	Detector	LP	BP
488 nm 200 mW	BB790	Blue A	750	780/60
	BB755	Blue B	730	750/30
	BB700, PerCP-Cy5.5, PerCP-eFluor710	Blue C	690	710/50
	BB660, PerCP, AO(RNA),	Blue D	635	670/30
	BB630, JC-1 Orange	Blue E	600	610/20
	BB 515, FITC, Alexa Fluor 488 , GFP, YFP, CFSE, Chameleon Yellow, LiveDead Green, AO(DNA), VioBright FITC, JC-1 Green	Blue F	505	530/30
	SSC	Blue G		488/10
561 nm 150 mW	PE-Cy7, PE-Vio770	YG A	750	780/60
	PE-Cy5.5, PE-Alexa 700	YG B	690	710/50
	PE-Cy5, 7-AAD, CyChrome	YG C	635	670/30
	PE-Texas Red, mCherry, PI, PE-CF594, LiveDeadRed, Alexa 568, Alexa 594, PE Alexa594	YG D	600	610/20
	PE, Dil, DsRed	YG E		586/15
637 nm 140 mW	APC-Cy7, APC-Alexa 750, LiveDeadNearIR, APC-H7, Alexa 750, APC-eFluor780	Red A	740	780/60
	Alexa 700, APC-R700, APC-Cy5.5, VT680, Cy5.5	Red B	690	710/50
	APC, Alexa 647, Cy5, LiveDeadFarRed, Sytox Red	Red C		670/30
405 nm 100 mW	BV786, BV785, Qdot 800	Violet A	750	780/60
	BV750, Qdot 750	Violet B	710	740/35
	Qdot 705, BV711, Q700	Violet C	690	710/50
	Qdot 655, BV650	Violet D	635	670/30
	Qdot 605, BV605, eFluor605NC	Violet E	600	610/20
	BV570, Pacific Orange, Alexa 430, Cascade Yellow, LiveDead Yellow, Qdot 585	Violet F	550	586/15
	BV510, AmCyan, CFP, V500, Qdot 525, LiveDeadAqua, eFluor 506, FixVia-eFluor506	Violet G	450	515/30
	BV421, V450, Pacific Blue, Alexa 405, Marina Blue, eFluor450, Cerulean, Cascade Blue, LiveDeadViolet, Cell Tracer Violet	Violet H		431/28



When Lasers are Spatially Separated

Signals from different lasers for the same particle will occur at different times, so a correction must be applied. This “Laser Delay” is dependent upon stable flow of the stream.



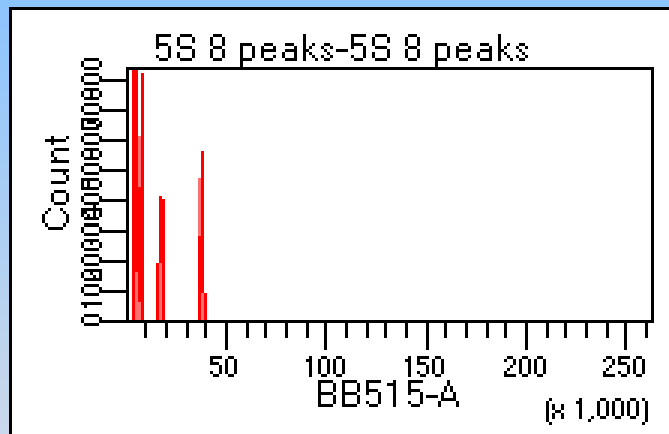
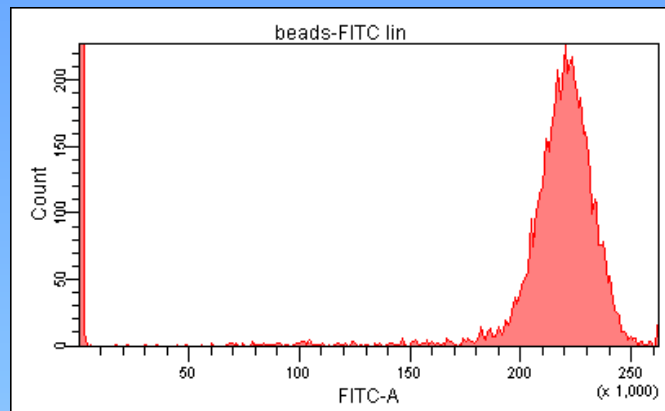
Basic Setup

- In addition to the experimental samples, you will need an unstained sample and single stained samples for each color in the experiment.
- Use the unstained sample to set voltages so that the negative population is in the lower quarter of the plot
- Sequentially run and record each single stained sample, software has automated compensation that does the comparison for you after controls are recorded.
- *Changing voltage after setting compensation will invalidate the settings!* (you've changed the ratio)
- You can now run your experiment samples.

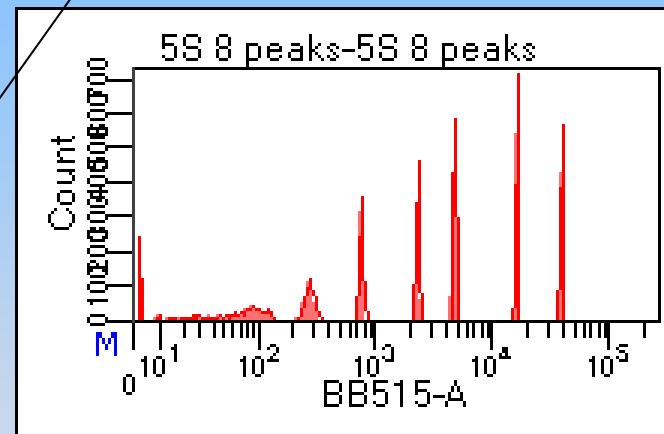
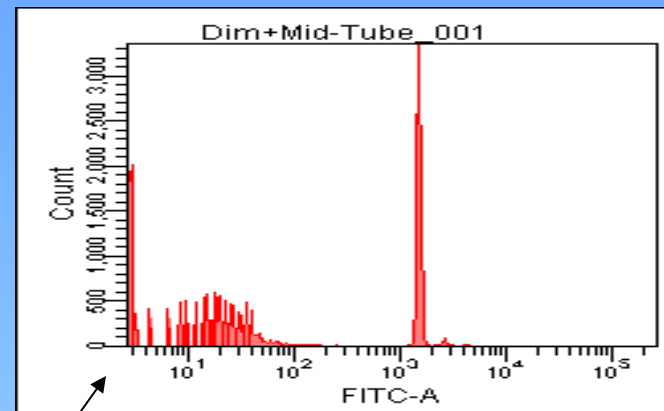
Linear

Display modes

Log



Compresses low values, high values are spread out



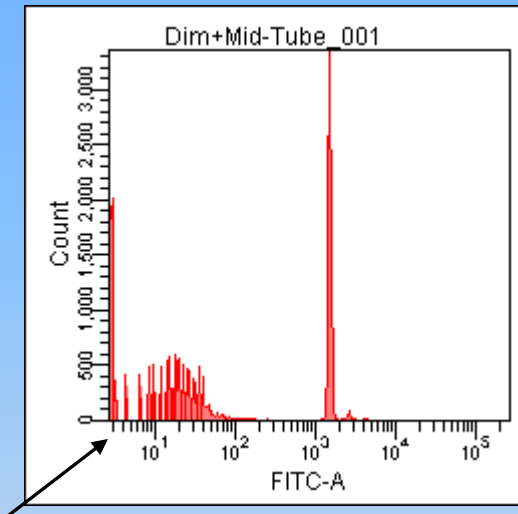
Compresses high values, low values are spread out

Events on the axis

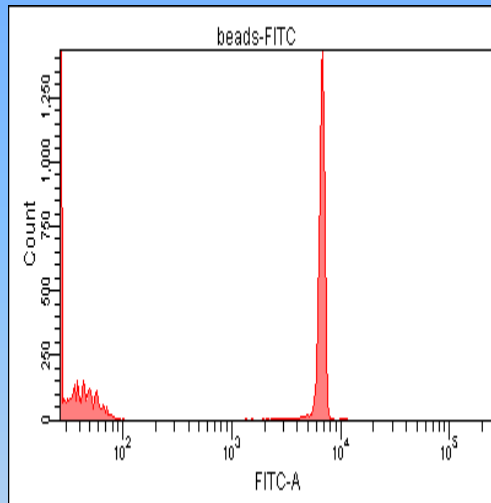
Can switch between linear and log (but signals won't be optimized for both!) Anything below the first displayed channel would be displayed on the axis but retain its value.

Data Display

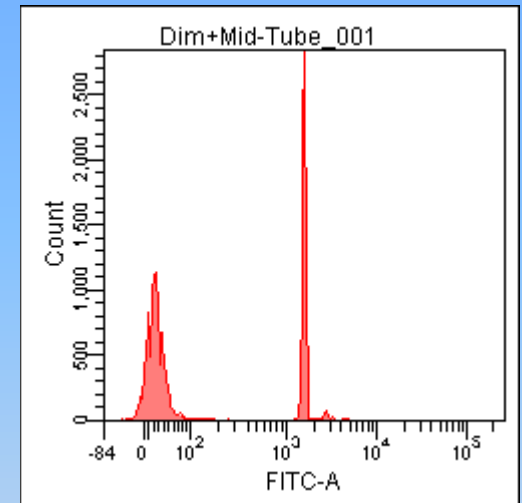
Log scale 5 decades



Log scale 4 decades



Logicle/ Biexponential



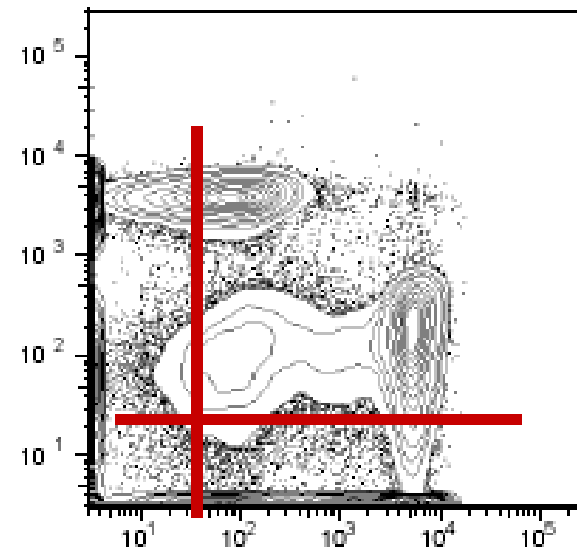
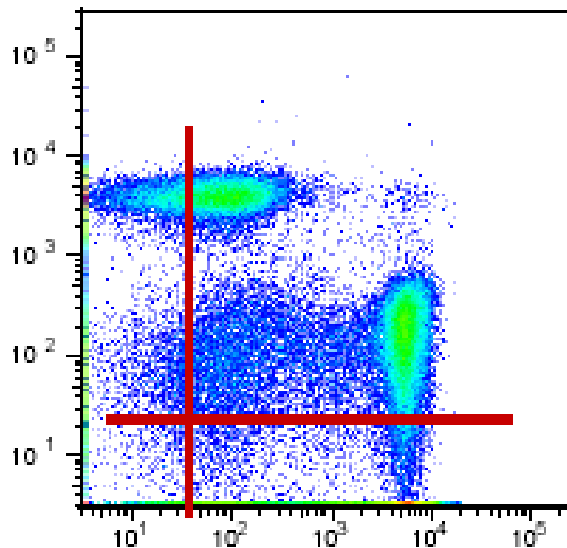
The system uses 18-bit precalculated log lookup tables, which explains the discrete nature of the histogram at the lower end. In the lowest decade of the log plot, only ten possible values are available for display.

BD Biosciences

David Parks and Wayne Moore at Stanford with Mario Roederer at the VRC-NIH developed the “Logicle” hybrid scale for data display, published in Cytometry in 2006.

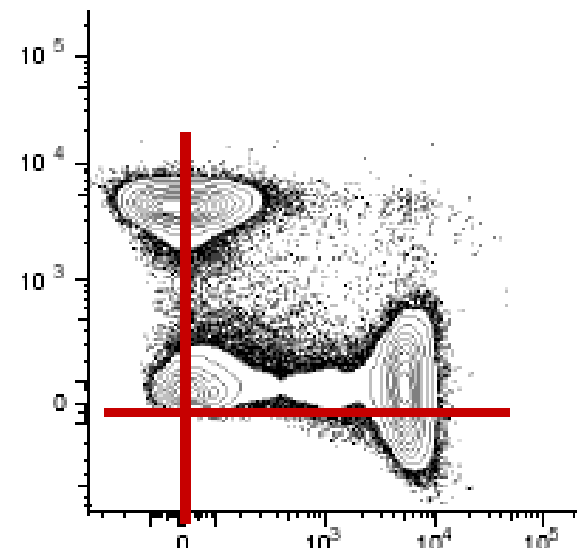
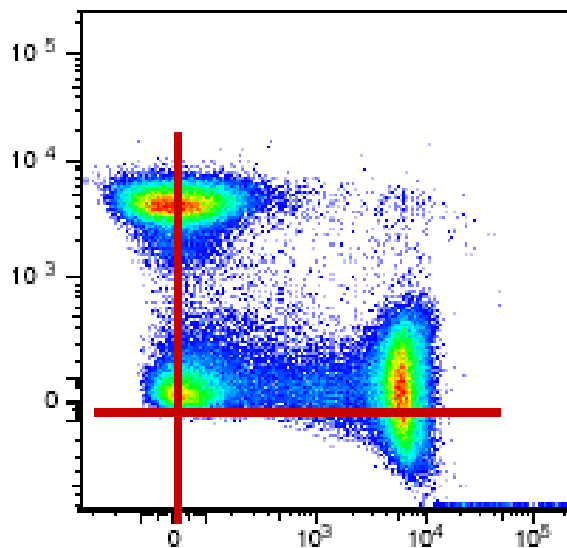


CD4



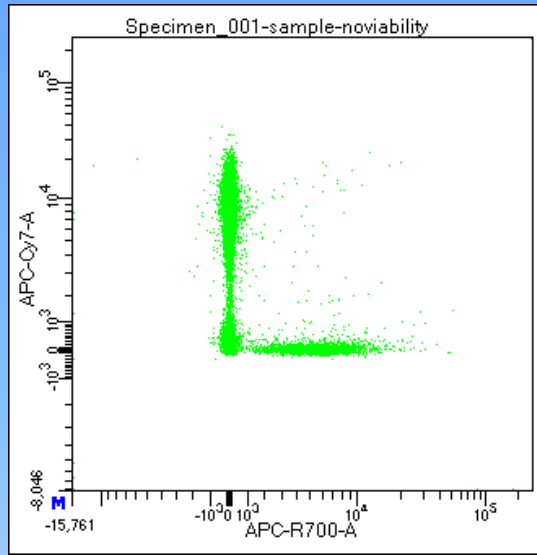
Median

Transformed

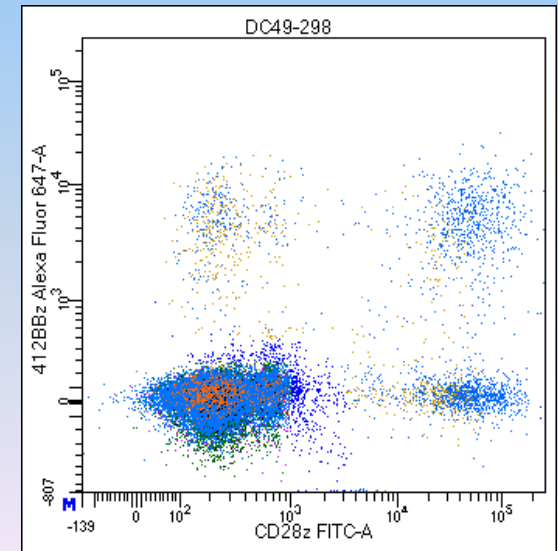
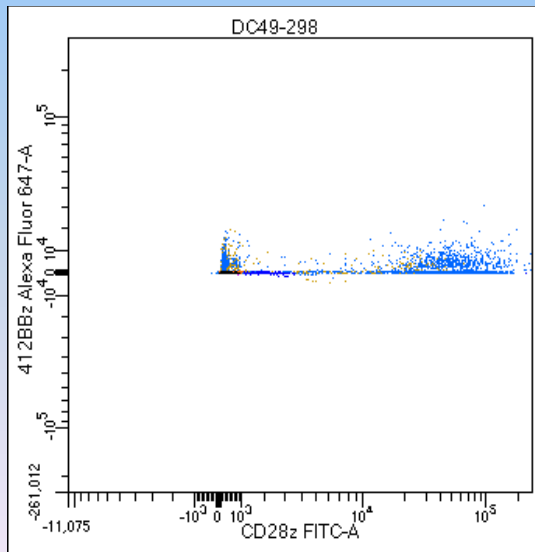
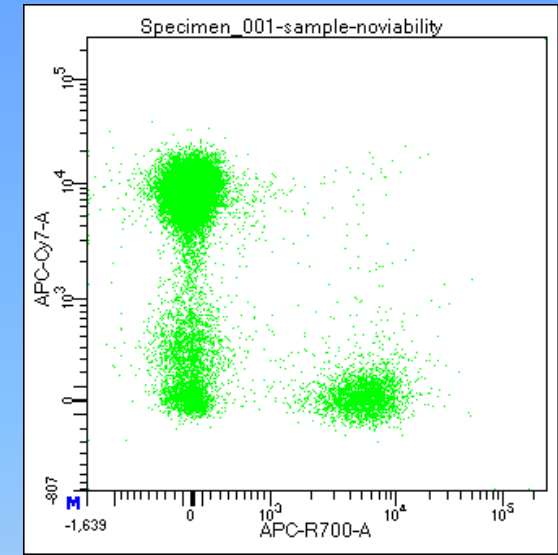


CD8

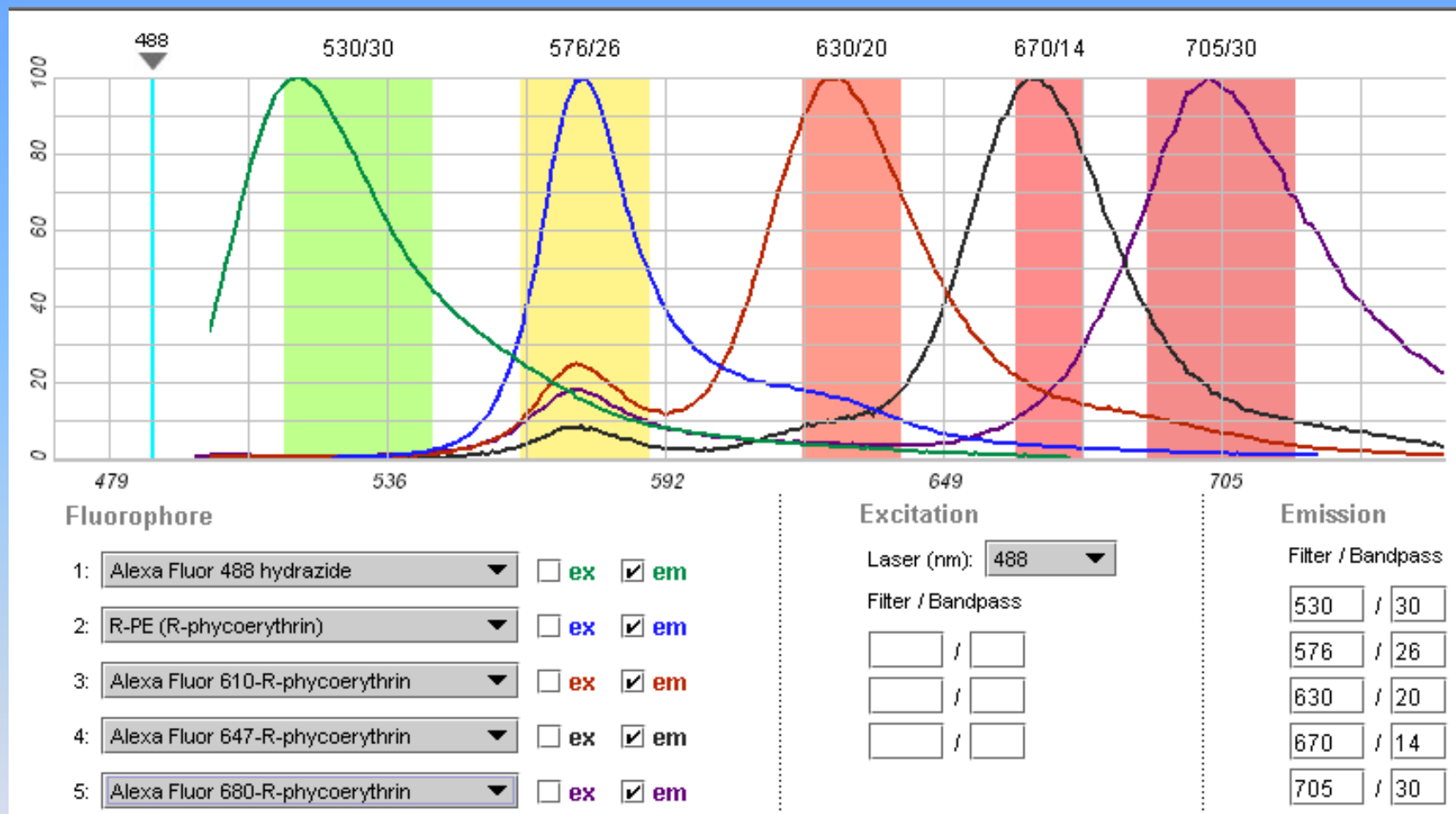
Biexponential Scaling can be adjusted/optimized



Scaling can be adjusted to optimize how much “negative space” is displayed, and how much of the area around 0 is compressed.



Invitrogen Spectraviewer -- Emission curves



- Overlap of fluorochromes, get PE in FITC, etc...
- Tandem dyes—provide more parameters off of a single laser. Notice overlap into PE channel—not 100% transfer of energy.

Special considerations with tandem dyes

- For single color fluorochromes (FITC, PE, APC, etc), a surrogate marker can be used when target antigen is expressed in low levels or on a small population of cells.
- Because efficiency of transfer can differ between tandem molecules, compensation tube MUST be same as reagent in sample.
- Spillover can change for tandems from exposure to light, extended incubations in fixative, or when left overnight.

Three types of staining controls

1) Instrument setup and validation

Compensation

2) Biological

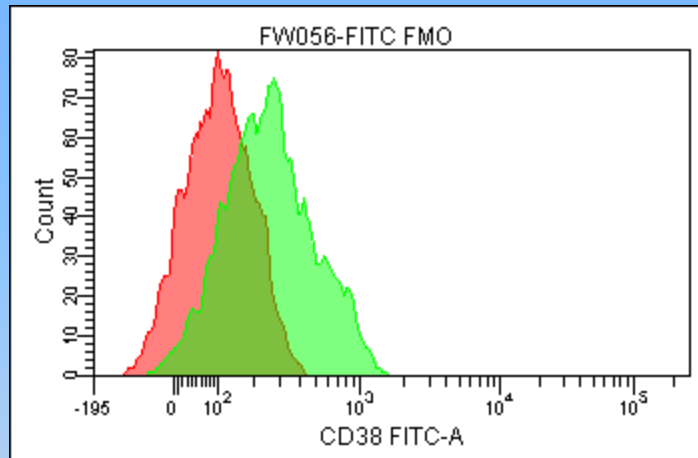
3) Staining/gating

FMO, Nonspecific binding

1. Compensation Controls

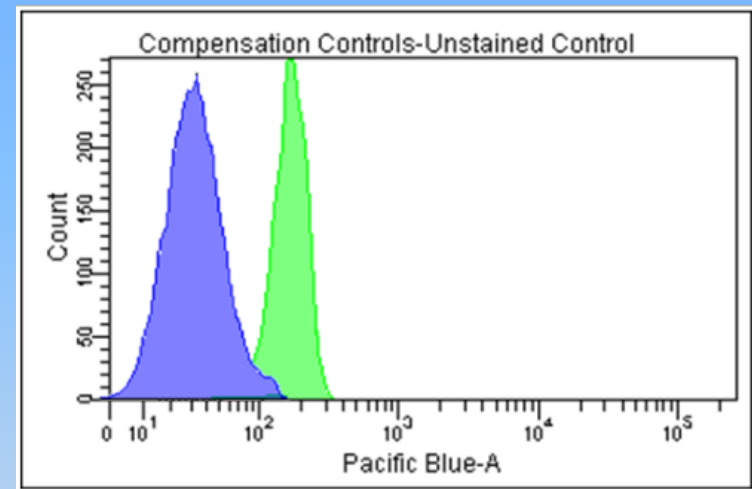
- Single color samples at least as bright as the reagent in your experiment (GFP counts!)
- One sample for every color, and one for each unique lot of tandem label (PE Cy5, APC Cy7, etc)
 - For tandems, amount of escaped light will vary from lot to lot, antibody to antibody, and possibly experiment to experiment. Comp must be done with every run.
- Any “carrier” particle (bead or cell) as long as + and – populations have same unstained fluorescence
 - Important to match the positive and negative particles for compensation comparisons. Only want to correct for contribution of FLUOROCHROME. Lymphs and monos, or lymphs and beads, will have different autofluorescence.

Different particles have different amounts of autofluorescence

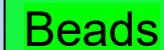


 Lymphocytes

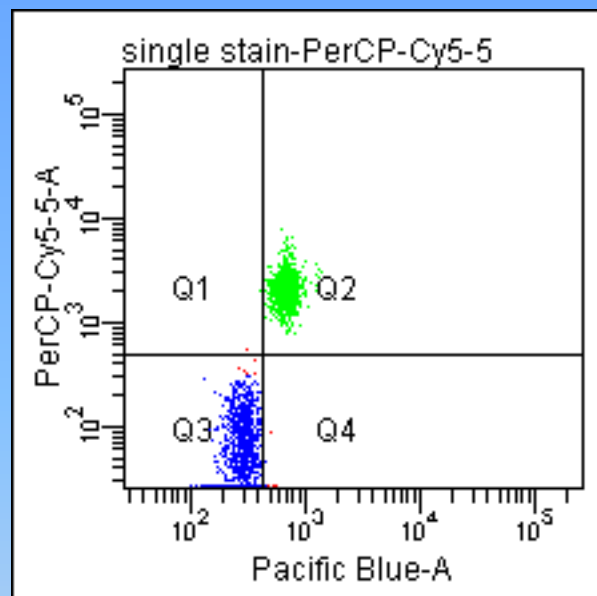
 Monocytes



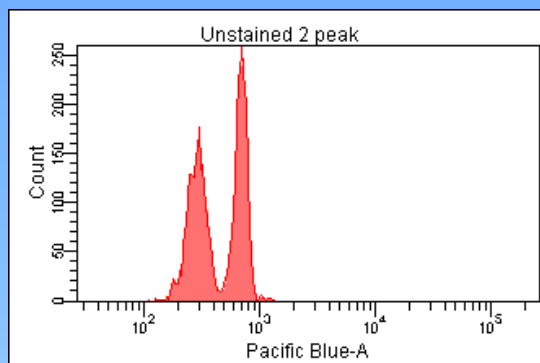
 Cells

 Beads

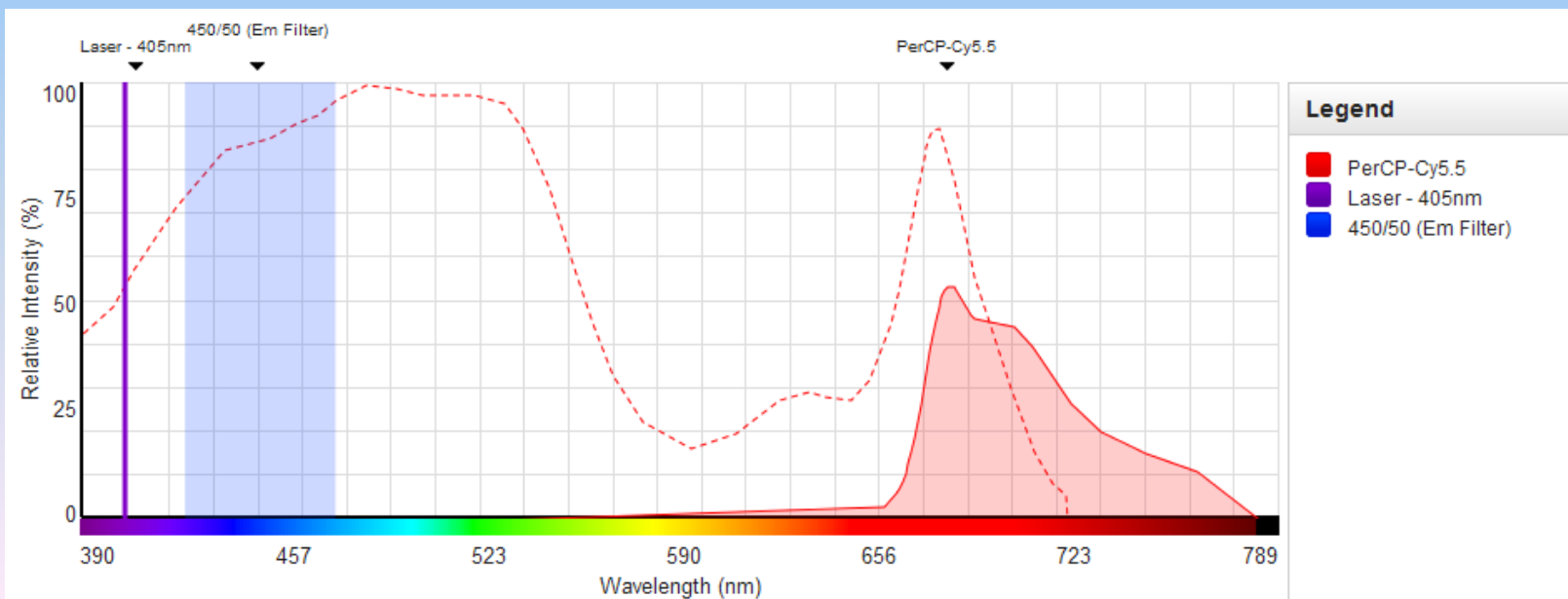
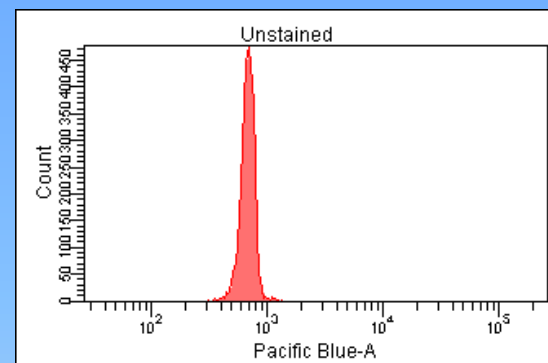
Mis-matched positive and negative compensation controls



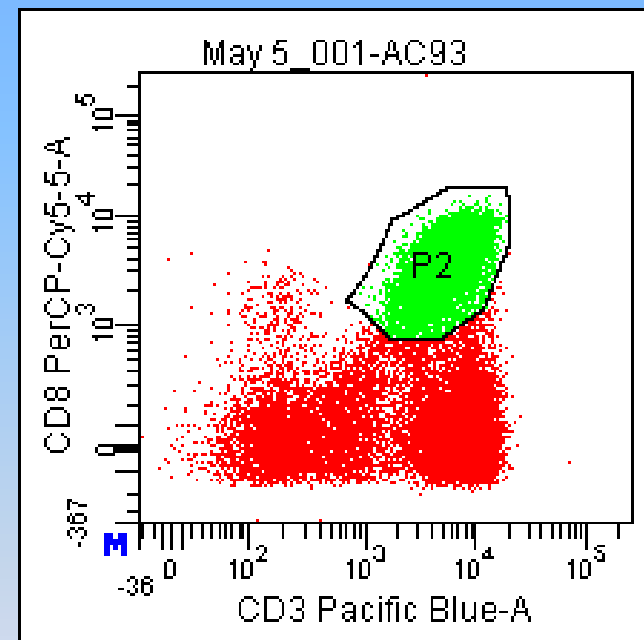
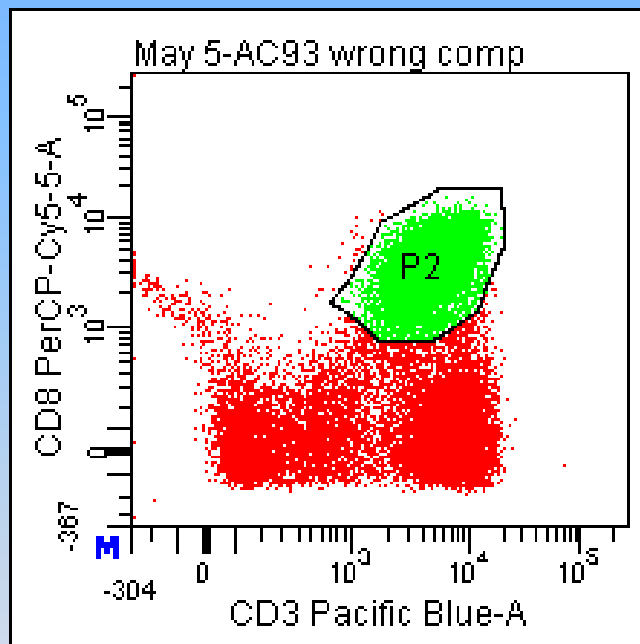
Mixed unstained



Unstained "binding beads"



Mismatched controls lead to improper compensation



Pac Blue -% PerCP-Cy5.5
19.2 vs 0.17



2. Biological Controls

- “Normal” sample to verify proper detection of populations
- Run identical sample with each experiment for daily variation
 - If “biological control” from different days shows 5% variation and your experimental samples 3%, not significant.
 - If biological control is 1%, and experimental is 3%, then it IS more likely to be significant.

3. Staining Controls

- Unstained cells are not a proper controls for determining positive vs. negative expression in multicolor experiments.
 - unstained—changing more than 1 variable (leaving out multiple reagents), no longer a control!
- The best control is to stain cells with all reagents except the one of interest.

FMO

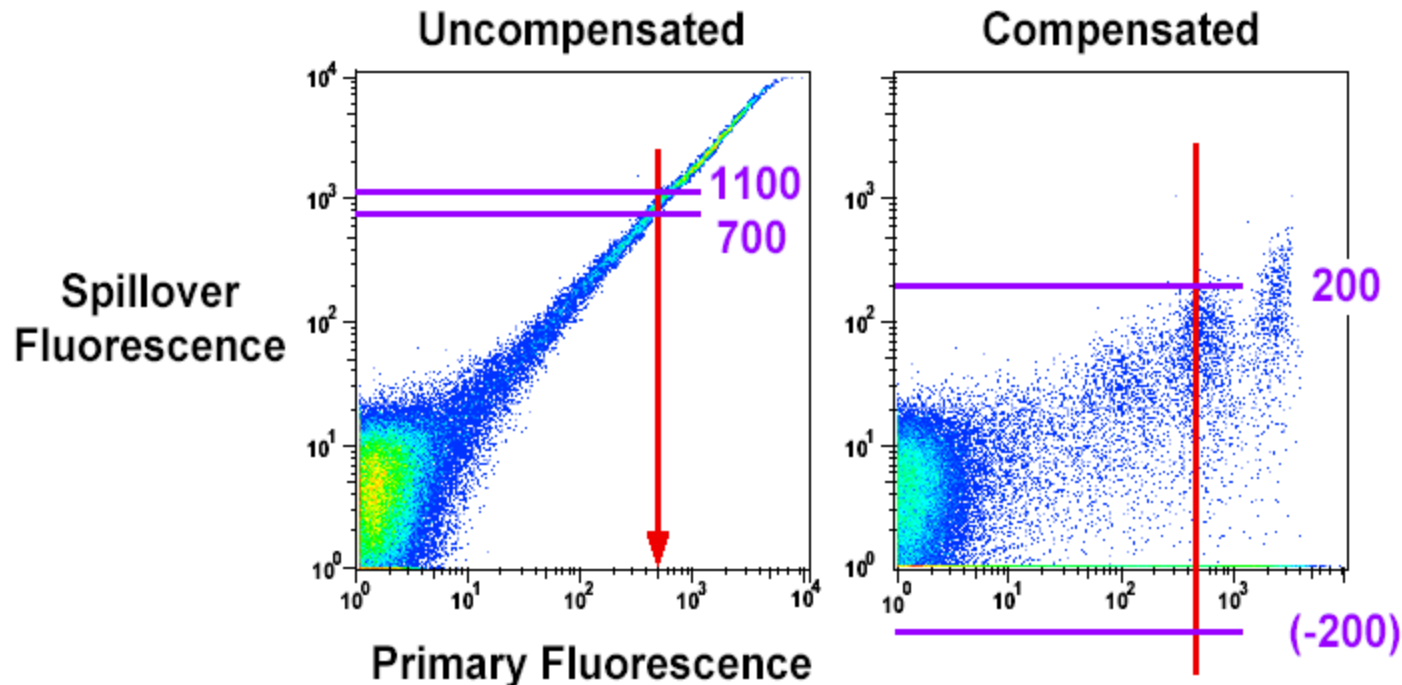
“Fluorescence Minus One”

Why do we need FMO?

Spread of Compensated Data

- +/- boundary for data that is correctly compensated may not match boundary for unstained sample.
- Difference is due to measurement errors and cannot be corrected.
- You must be sure to distinguish between incorrect compensation and measurement error

Imperfect Measurement Leads to Apparent Spread in Compensation



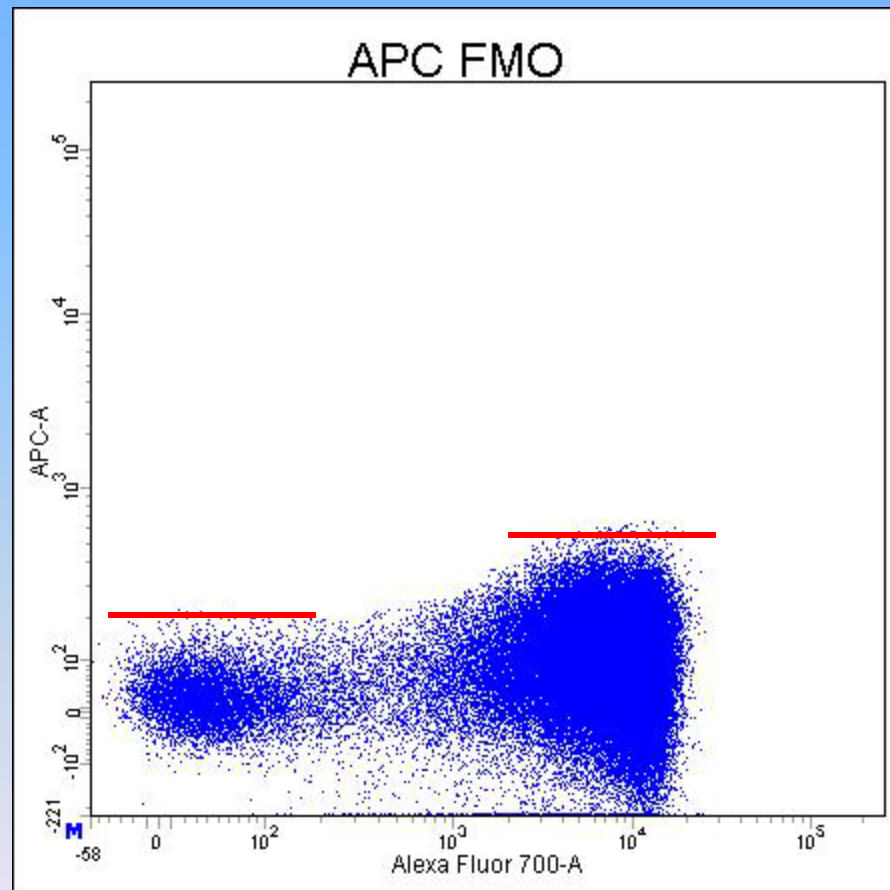
Why is there a 400-unit spread? Photon counting statistics.

400 units of separation will remain, but viewing in lower section of log scale will cause it to spread out. In log scale, cannot see the negative (value) population.

Fluorescence Minus One (FMO)

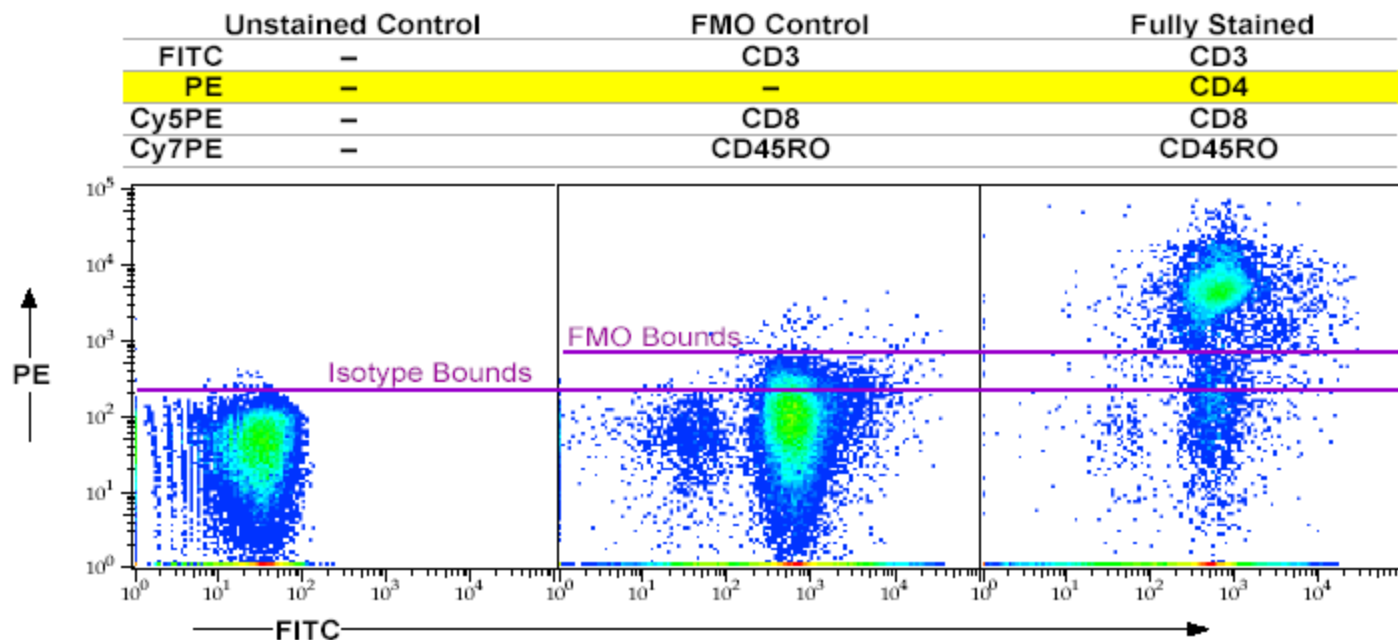
- Needed to identify the cutoff for +/-
- Threshold for + can depend on the fluorescence levels in other channels.

All the other colors may contribute small amounts of signal to the measured color, thus increasing the cutoff for “negative”.



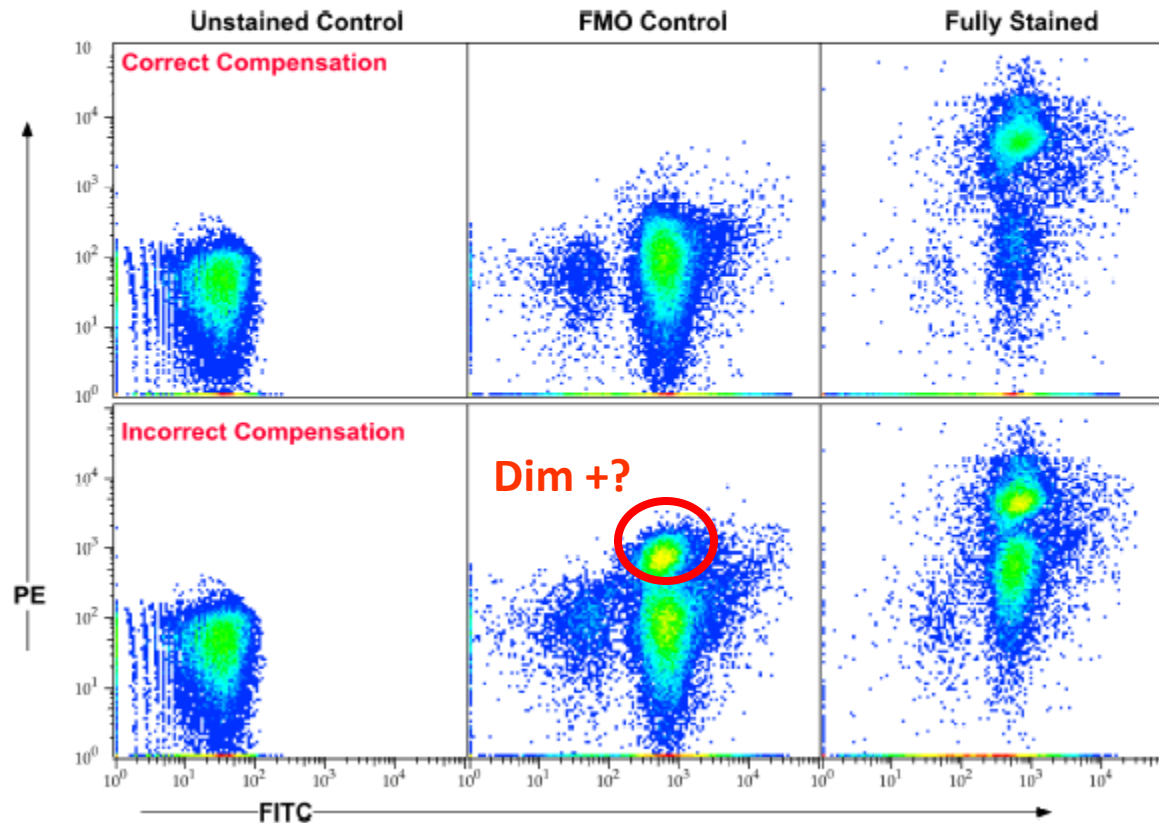
Identifying CD4 cells with 4 colors

PBMC were stained as shown in a 4-color experiment.
Compensation was properly set for all spillovers



Complex Interactions in Compensation

The same data is shown with correct or wrong Cy5PE->Cy7PE comp setting. Note that neither of these channels is shown here!



Why/How?

Compensation

$$F_1 = f_1 + k_{21}f_2 + k_{31}f_3 + k_{41}f_4 \quad (1)$$

Observed **uncompensated signal** is the sum of the **primary fluorescence** plus the spillover of all **other colors** in the sample.

Compensation: The Calculation

$$F_1 = f_1 \quad + k_{21}f_2 \quad + k_{31}f_3 \quad + k_{41}f_4 \quad (1)$$

$$F_2 = k_{12}f_1 \quad + f_2 \quad + k_{32}f_3 \quad + k_{42}f_4 \quad (2)$$

$$F_3 = k_{13}f_1 \quad + k_{23}f_2 \quad + f_3 \quad + k_{43}f_4 \quad (3)$$

$$F_4 = k_{14}f_1 \quad + k_{24}f_2 \quad + k_{34}f_3 \quad + f_4 \quad (4)$$

Using a single stained control allows you to determine the contribution of each color to all of the other channels—any signal above background in the other channels is known to be from that one fluorochrome and “k” for that pair can be determined.

growing frustrated in their pursuit of compensation

The diagram illustrates the relationship between four fluorescence channels (F₁, F₂, F₃, F₄) and their respective measured values (f₁, f₂, f₃, f₄). The equations are as follows:

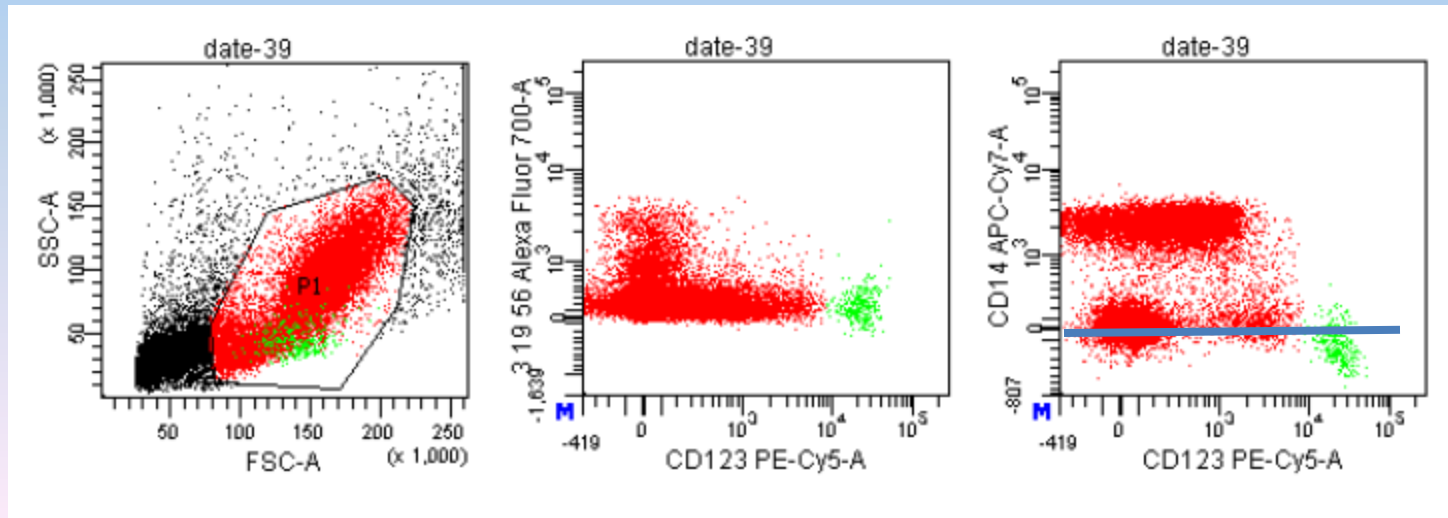
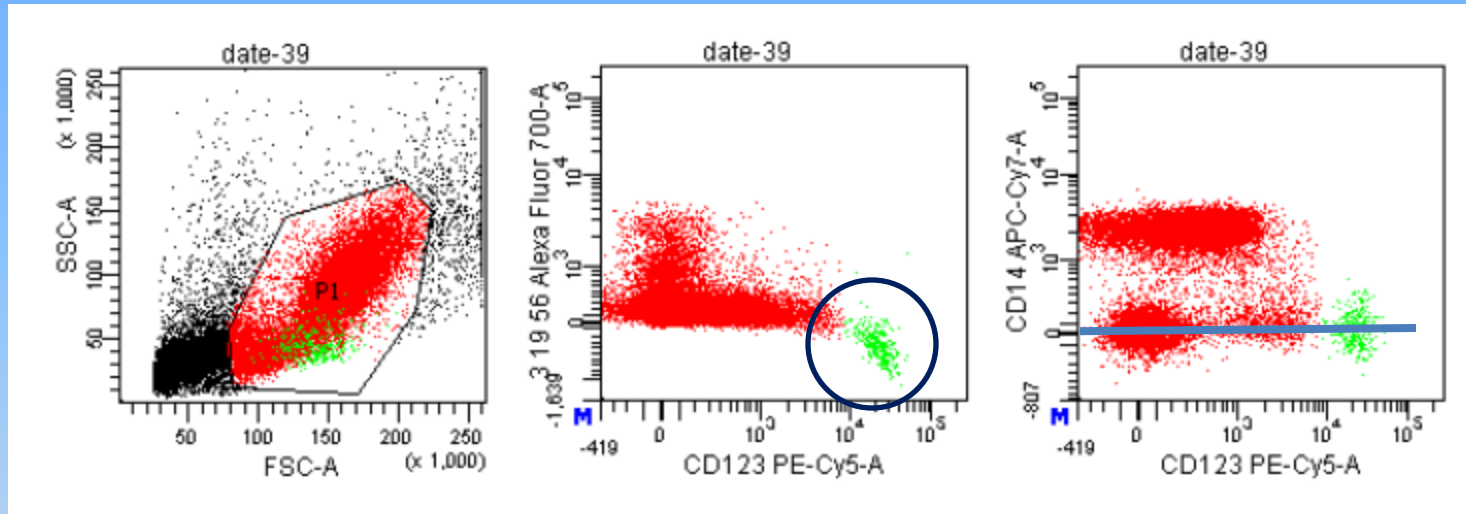
$$\begin{aligned}
 F_1 &= f_1 + k_{21}f_2 + k_{31}f_3 + k_{41}f_4 & (1) \\
 F_2 &= k_{12}f_1 + f_2 + k_{32}f_3 + k_{42}f_4 & (2) \\
 F_3 &= k_{13}f_1 + k_{23}f_2 + f_3 + k_{43}f_4 & (3) \\
 F_4 &= k_{14}f_1 + k_{24}f_2 + k_{34}f_3 + f_4 & (4)
 \end{aligned}$$

Key features of the diagram:

- The term f_1 in equation (1) is highlighted with a green box.
- The entire left side of equation (1) ($F_1 =$) is enclosed in a blue box.
- The term $+ f_2$ in equation (2) is highlighted with an orange box.
- The term $+ k_{23}f_2$ in equation (3) is highlighted with a purple box.
- The term $+ f_3$ in equation (3) is highlighted with a red box.
- The term $+ f_4$ in equation (4) is highlighted with a dark red box.
- Arrows indicate dependencies: a purple arrow points from the $+ k_{21}f_2$ term in (1) to the $+ f_2$ term in (2); another purple arrow points from the $+ k_{31}f_3$ term in (1) to the $+ f_3$ term in (3); a third purple arrow points from the $+ k_{23}f_2$ term in (3) to the $+ f_3$ term in (3).

Values for fluorescence are solved simultaneously based on the calculated spillover coefficients and the total measured fluorescence in each channel, so when compensation is applied, an error in one constant will impact the values for other channels.

Don't try to "correct" compensation manually on samples stained with multiple colors, as you sometimes can't visually tell where the error is and can change the display of OTHER parameters



Gating: FMO Controls

FMO controls are the proper way to identify positive vs. negative cells

FMO controls can help identify problems in compensation that are not immediately visible

FMO controls should be used whenever accurate discrimination is essential or when antigen expression is relatively low

**If you were to do FMOs for all colors in a 10 color panel, that would be 10 single-stained controls (10 tests of antibody) and 10 FMOs (9 tests of antibody each, or 90)=100 tests of antibody before a single sample tube is stained.

Compensation of Multicolor Data

It is impossible to set proper compensation using visual guides (dot plots, histograms)

- Biexponential is useful for identifying gross overcompensation, but should not be used for setting compensation visually.
- Use statistics (medians of gated cells)
- Use automated compensation tools

Compensation Control options

- Antibody capture beads are an excellent way to set compensation. Don't need to use precious cells, usually give bright, uniform signal. Must make sure compatible with your reagents.

- BD's CompBeads and CompBeads Plus only recognize IgG k, limited by species--2 separate vials, keep together as a set!

- Beckman's VersaComp--Reacts with all mouse and rat, most hamster, and rabbit polyclonal IgG.

- *Some BD reagents conjugated to PE do not bind well.

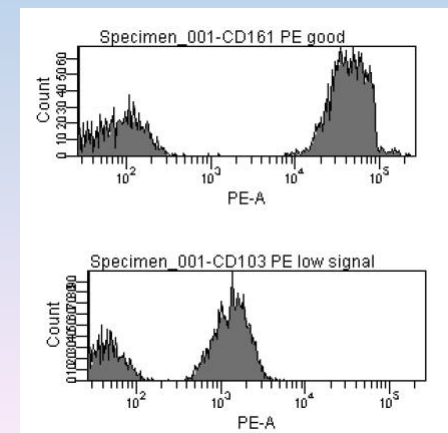
- OneComp

- Spherotech COMPTrol (varied intensity) bind antibodies raised in mouse.

- Spherotech EasyComp pre-stained particles

- Amine coated beads for the fixable live/dead amine reactive dyes. ARC beads from ThermoFisher, Spherotech and other companies have their own.

CD103 PE lot # 6175690 cat # 550260 On VersaComp beads



POP QUIZ!!

Which EasyComp beads should NOT be used??

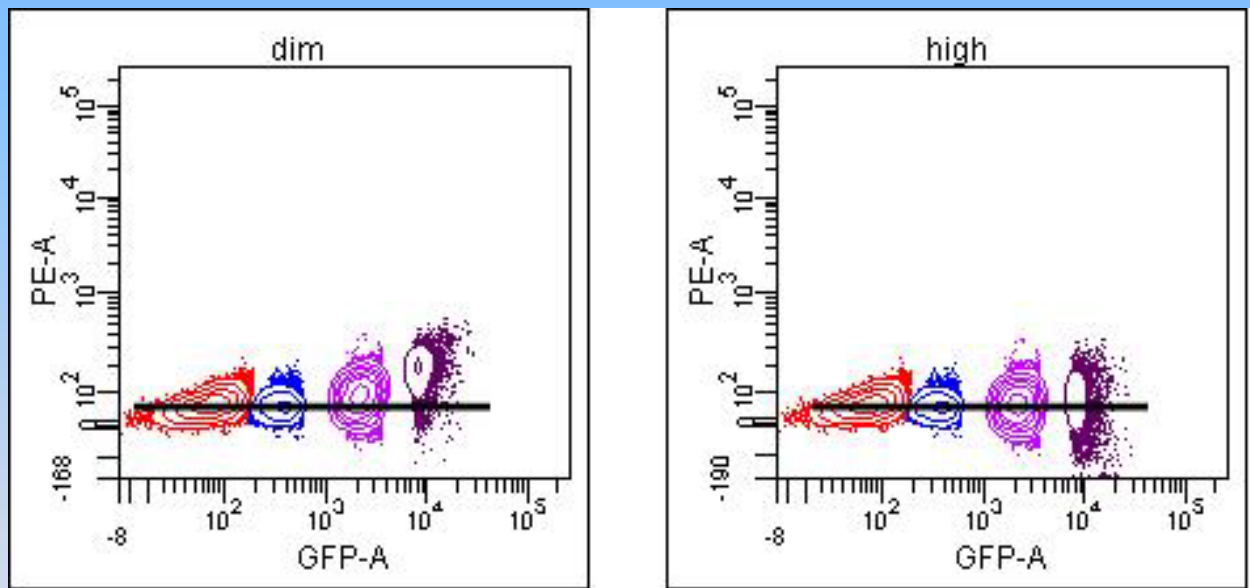
SPHERO™ EasyComp: Spectral Matching Particles

- Surface labeled polystyrene particles with commonly used fluorophores
- Used for setting compensation in a wide variety of channels for any flow cytometer
- Supplied as individual products or as kits for use in different models of flow cytometers

Catalog No.	Description	Concentration
ECFP-B	EasyComp Fluorescent Particles, Blank	10 ⁷ /mL
ECFP-F1	EasyComp Fluorescent Particles, FITC	10 ⁷ /mL
ECFP-F2	EasyComp Fluorescent Particles, PE	10 ⁷ /mL
ECFP-F3	EasyComp Fluorescent Particles, PE-TR	10 ⁷ /mL
ECFP-F4	EasyComp Fluorescent Particles, PE-Cy5	10 ⁷ /mL
ECFP-F5	EasyComp Fluorescent Particles, PE-Cy7	10 ⁷ /mL
ECFP-F6	EasyComp Fluorescent Particles, APC	10 ⁷ /mL
ECFP-F7	EasyComp Fluorescent Particles, APC-Cy7	10 ⁷ /mL
ECFP-F8	EasyComp Fluorescent Particles, PerCP	10 ⁷ /mL
ECFP-F9	EasyComp Fluorescent Particles, GFP	10 ⁷ /mL
ECFP-K1	EasyComp Fluorescent Particle Kit (Blank, FITC, PE & PE-Cy5), 4 vials	10 ⁷ /mL
ECFP-K2	EasyComp Fluorescent Particle Kit (Blank, FITC, PE, PE-Cy5 & APC), 5 vials	10 ⁷ /mL
ECFP-K3	EasyComp Fluorescent Particle Kit (Blank, FITC, PE, PE-TR & PE-Cy5), 5 vials	10 ⁷ /mL
ECFP-K4	EasyComp Fluorescent Particle Kit (Blank, FITC, PE, PE-TR, PE-Cy5, PE-Cy7, APC & APC-Cy7), 8 vials	10 ⁷ /mL

Any of the TANDEMS will not properly match the tandem dye on your antibody!
Each lot is different!

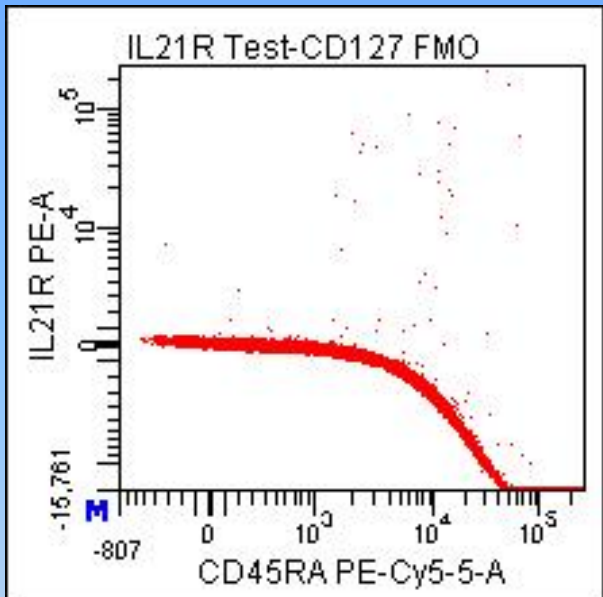
Compensation controls must be
matched to your experiment and
AS BRIGHT OR BRIGHTER
than your stained samples.



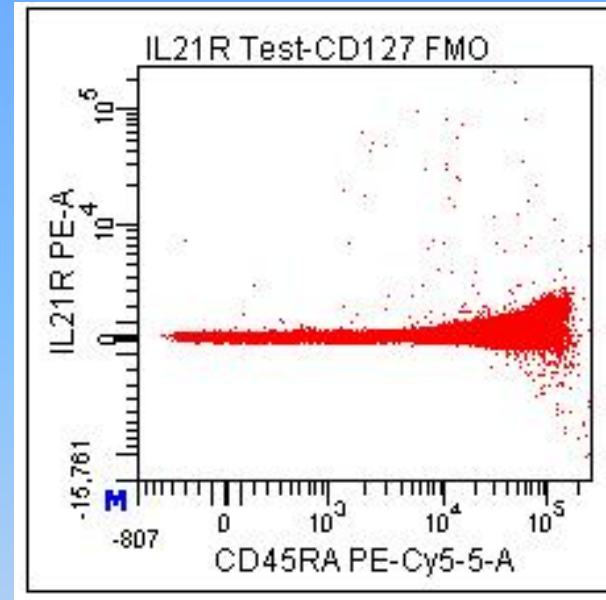
Dim signals may not give the necessary precision in calculating the spillover (4.00% vs 4.12% makes a difference with signals of 10,000!)

<http://newenglandcytometry.com/2013/01/15/as-bright-or-brighter/>

Compensating with the wrong tandem dye



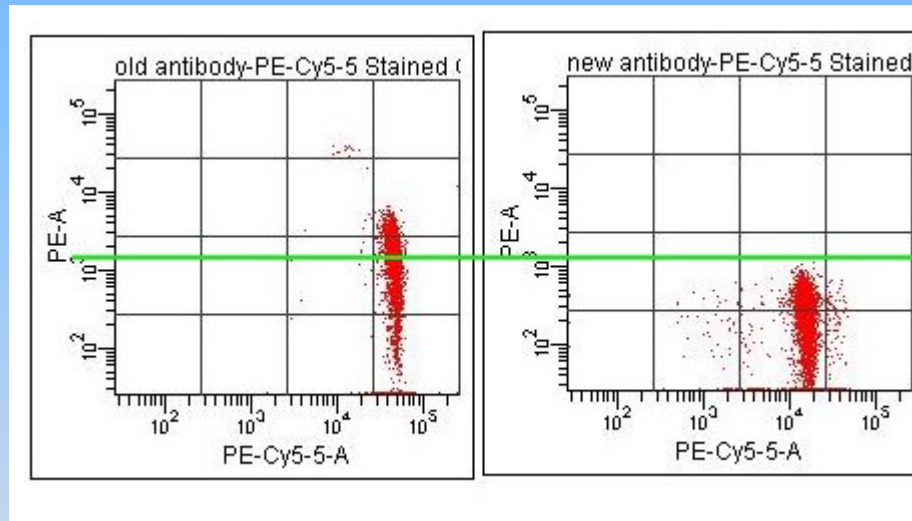
42.6%



7.59%

Must use identical tandem dye to ensure proper compensation.
(example is compensated with an old vial of same lot/order of label)

Impact of higher spread between channels

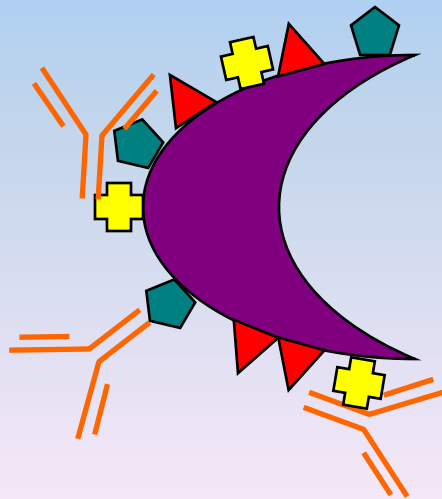


Left is “old tandem” that has broken down so more spillover into PE, more spread, reduced resolution!

Increasing compensation indicates the tandem is breaking down, so you will have more spillover into other channels and less sensitivity--staining has to be 10x brighter for your PE marker to identify it when using the “old” vial here.

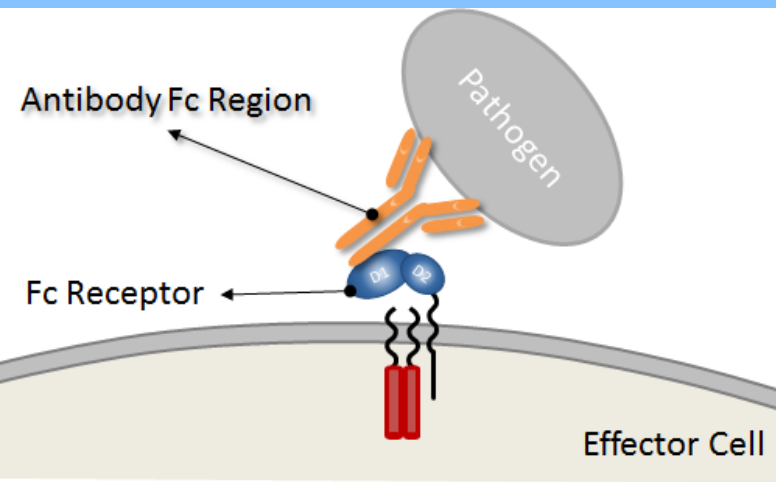
Nonspecific binding

- Other regions of the antibody could stick to epitopes contained in your sample.
- Perhaps “Unwanted binding” is a better term, as some is very specific (Fc receptors)
- “Isotype controls” can be used to determine levels of this nonspecific binding.

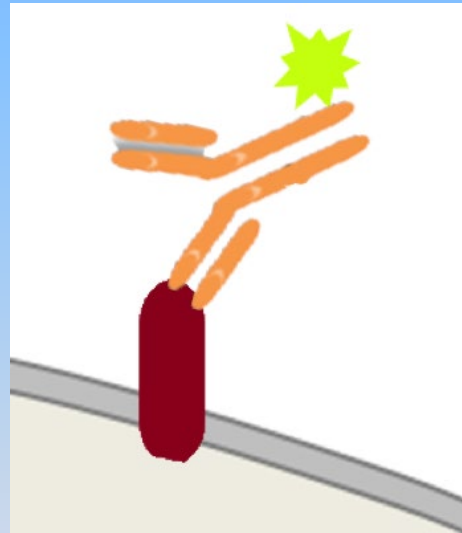


Antibodies

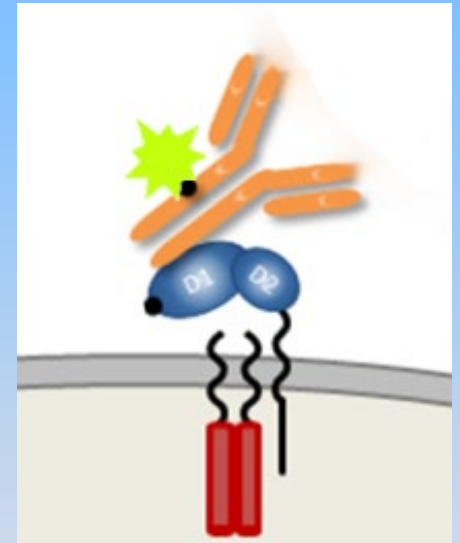
What its really for...



How we use it in flow...



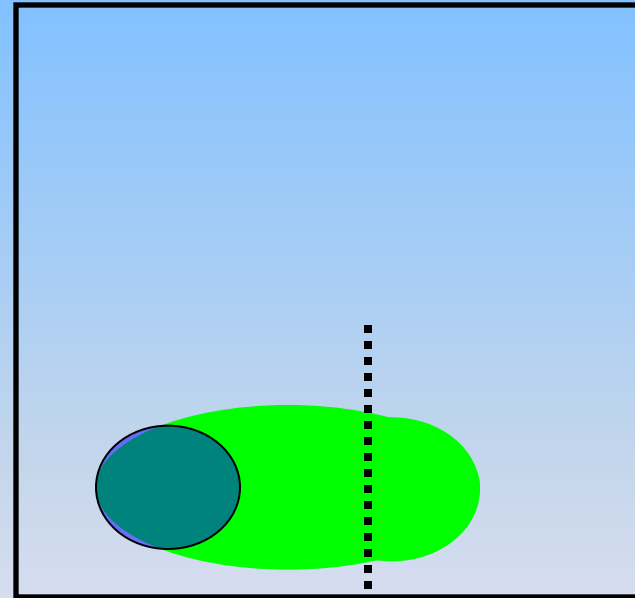
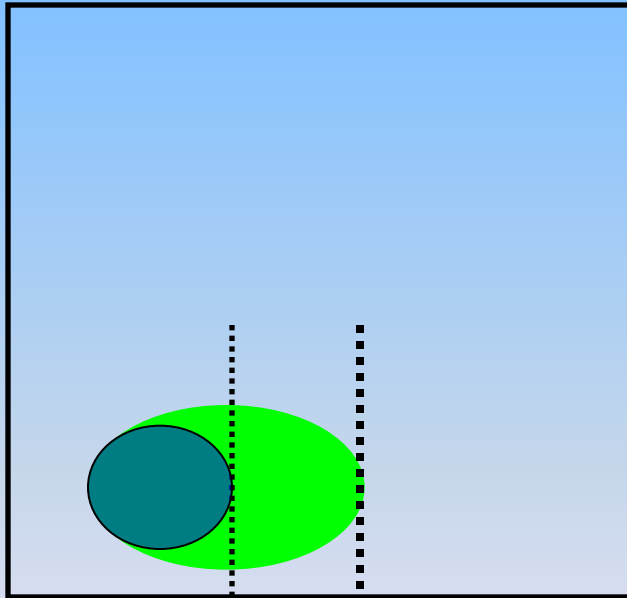
How it can go “wrong”...



Monocytes and macrophages—Fc receptors! Wrong part of the antibody might bind

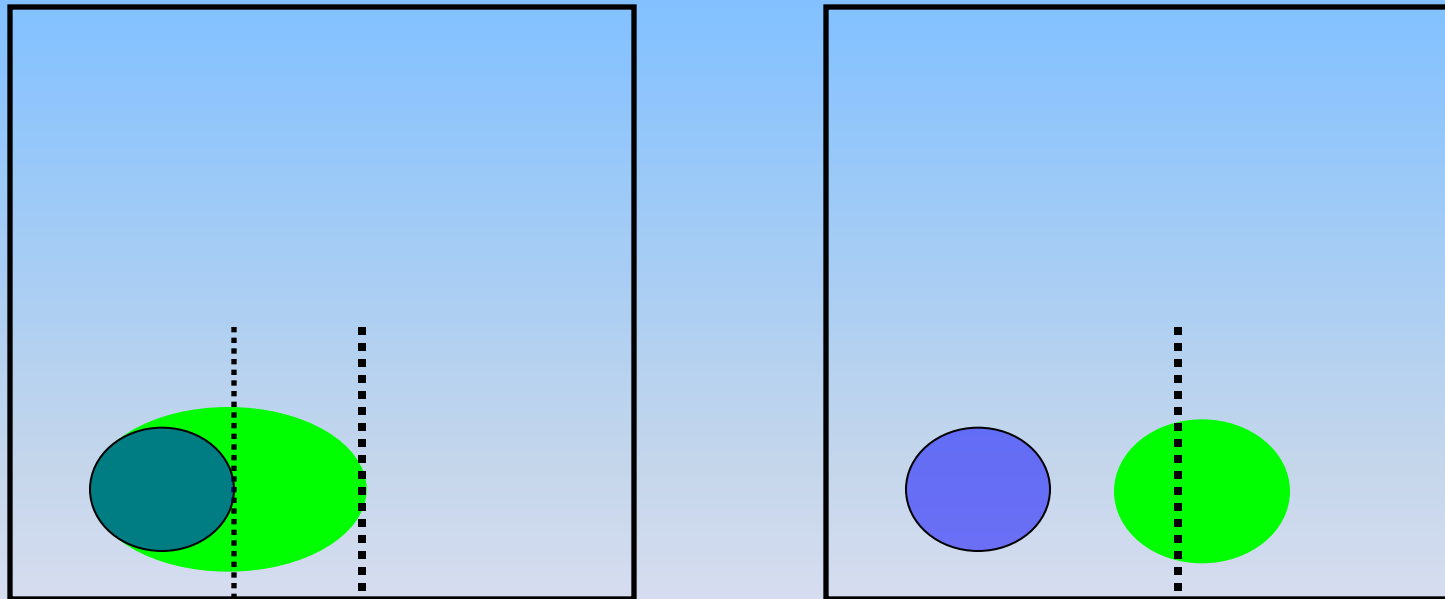
Isotype controls

- Antibody of same species and subtype (“Mouse IgG 2a kappa”), but directed against something not found in the sample so that any binding that occurs is due to interaction of the constant domain of that subtype.
- Can be used to show if there is background binding of the antibody type
- Use to verify that there is no background, NOT to set gates



Isotype controls

- Antibody of same species and subtype (“Mouse IgG 2a kappa”), but directed against something not found in the sample so that any binding that occurs is due to interaction of the constant domain of that subtype.
- Can be used to show if there is background binding of the antibody type
- Use to verify that there is no background, NOT to set gates



Use blocking agents or other treatments to prevent the background staining (Fc block to prevent Fc receptor binding, etc). Verify with isotype.

Isotype controls

Can tell you IF there is an issue with background binding, but not for setting boundaries of populations.

Control changes only ONE variable.

Other variables that must be accounted for:

1. Concentration (different for each antibody)
2. Fluor:Protein ratio
3. “Nonspecific” binding of variable region in Isotype

One case where isotype QC'd for surface labelling was used for intracellular staining, and generated artificial background signal (variable region recognized intracellular epitope)

Isotype controls discussion from Purdue Cytometry

<http://www.cyto.purdue.edu/hmarchiv/2005/1777.htm>

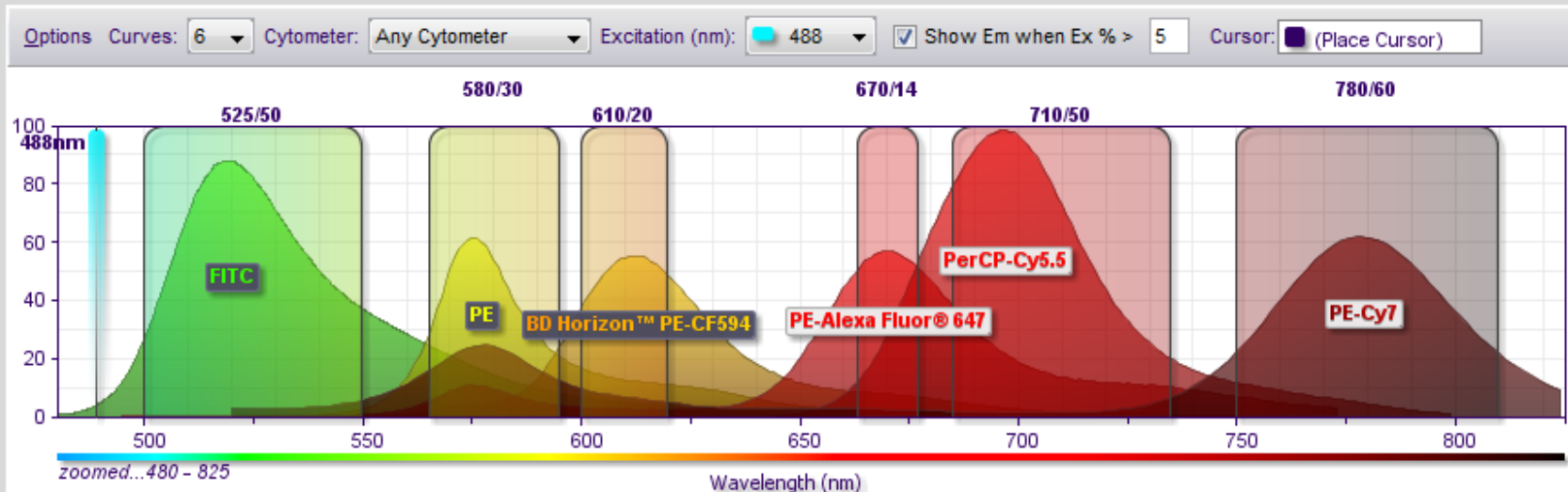
<http://www.cyto.purdue.edu/hmarchiv/2005/1650.htm>

O'Gorman MRG, Thomas JA: Isotype Controls-Time to Let Go?; Cytometry 38:78-80, 1999

Keeney M et al.: Isotype controls in the analysis of lymphocytes and CD34+ stem and progenitor stem cells by flow cytometry--time to let go!, Cytometry 34:280-283, 1998.

Challenges in flow: Spectral overlap

BD FLUORESCENCE SPECTRUM VIEWER A MULTICOLOR TOOL



Fluorochrome					% Max	☐ Ex	☒ Em	Filters	☒	FITC	PE	PerCP-...	PE-Cy7	BD Hori...	PE-Ale...
1	FITC	88.0			525/50	☒				71.3 %	1.5 %	--	4.0 %	0.3 %	1.1 %
2	PE	61.6			580/30	☒				14.5 %	61.0 %	--	21.9 %	22.5 %	28.7 %
3	PerCP-Cy5.5	98.4			710/50	☒				--	2.4 %	69.4 %	4.0 %	9.6 %	107.3 %
4	PE-Cy7	61.8			780/60	☒				--	--	8.0 %	60.5 %	0.3 %	13.8 %
5	BD Horizon™ PE...	55.2			610/20	☒				3.2 %	20.7 %	--	5.5 %	40.9 %	6.9 %
6	PE-Alexa Fluor® ...	56.8			670/14	☒				--	2.1 %	13.5 %	1.2 %	10.9 %	25.5 %

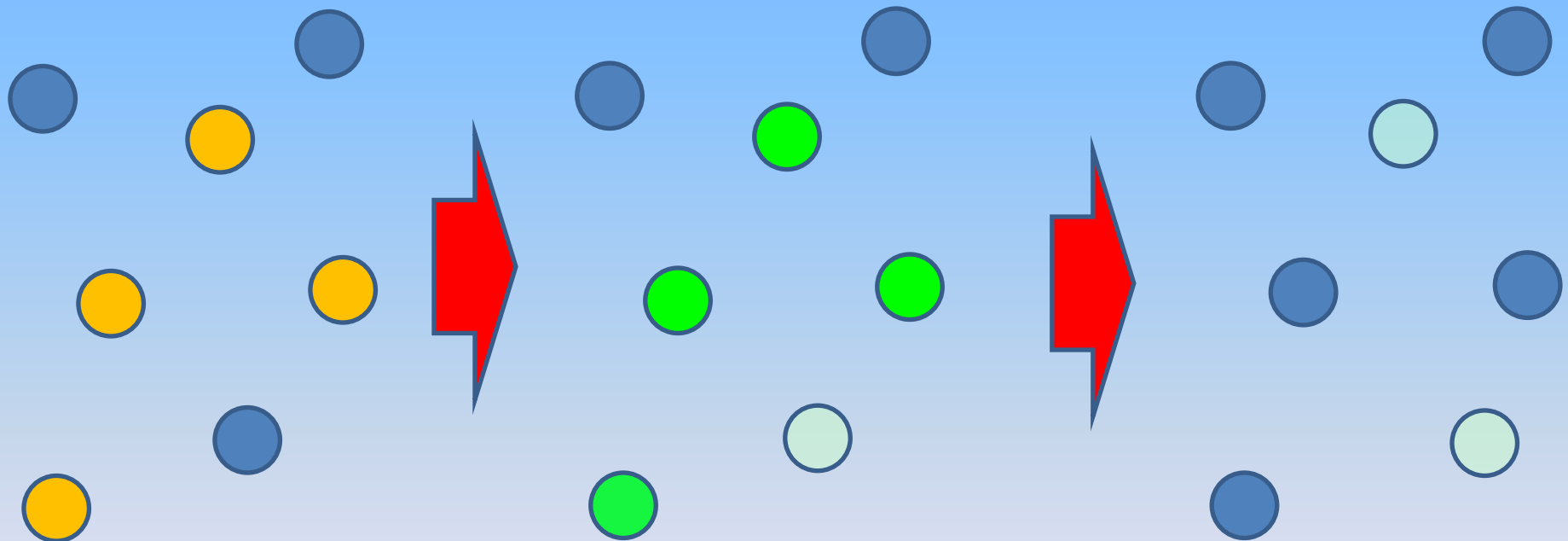


It is more difficult to detect signal where there is a lot of background light...

Orange color marker has high spillover into the green channel. Before compensation, significant signal is present.

Which “green” cells are double positive?

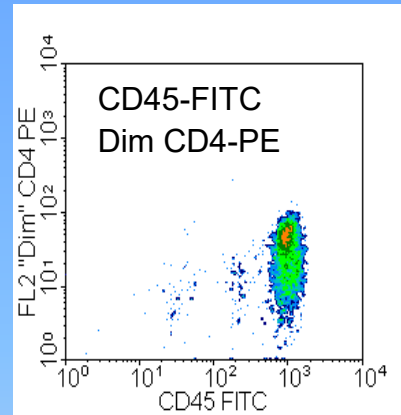
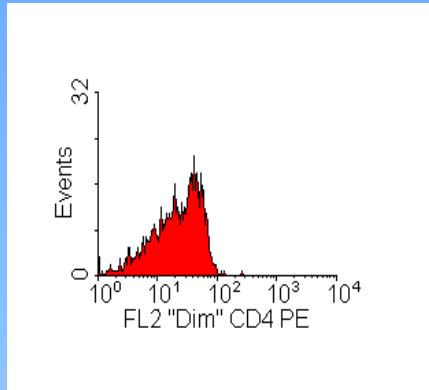
Can't tell because the spillover of orange masks the presence of the dim green signal.
Like looking for stars on a sunny day—they are there, but too much background light!



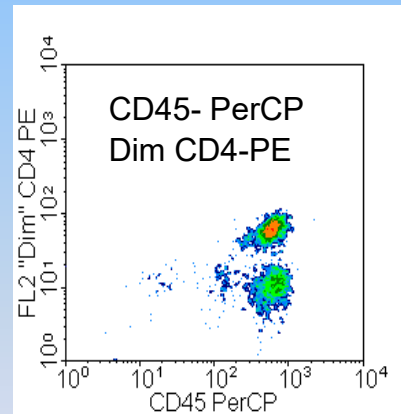
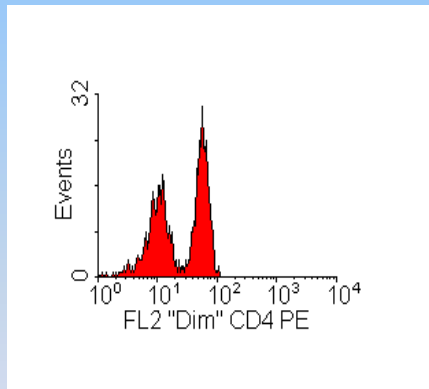
High Spillover from other channels—less sensitivity

What does this do to your data?

Effect of Spillover on Double Stained Cells



CD45 FITC makes dim CD4 difficult to measure due to FITC spillover into PE and resultant "spread"



CD45 PerCP allows same dim CD4 cells to be separated from bkg. – little spillover into PE

The spread in the compensated FITC labeled CD45 single +’s contributes to background in the PE channel, making it impossible to separate PE labeled CD4 dim from CD4 negative cells also + for CD45. By using PerCP CD45 instead, no spread so CD4 dim easily distinguished from negative.

Selecting a Panel

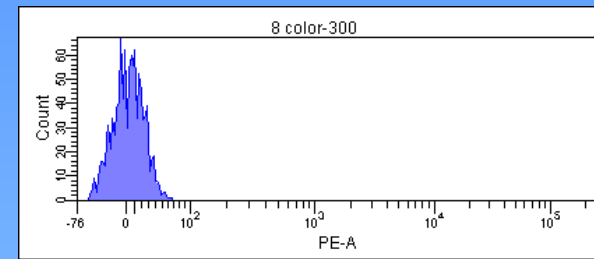
- Avoid using a dim marker in a channel adjacent to a bright marker with high spillover
- Separate reagents as much as possible (3 colors, use 3 different laser lines)
- Titrate your antibodies! Optimizes signal and saves reagent.
- Do NOT run a full experiment without validating the panel first!

Optimizing settings

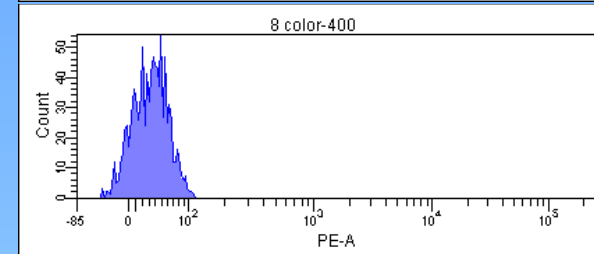
Same sample of 2 beads of similar fluorescence, recorded at different voltages.

If voltage is set too low, dim particles will not resolve themselves from negative particles. The lowest voltage that allows separation should be used.

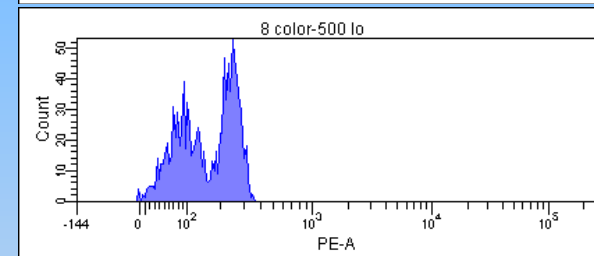
Higher voltage will not improve resolution but WILL increase overlap % from other channels.



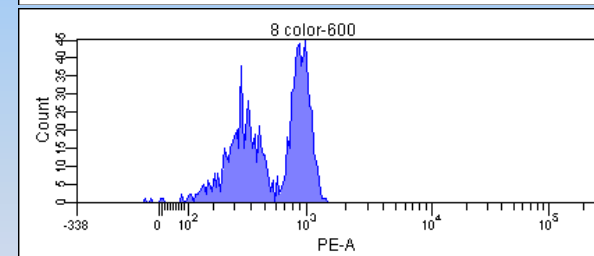
300V



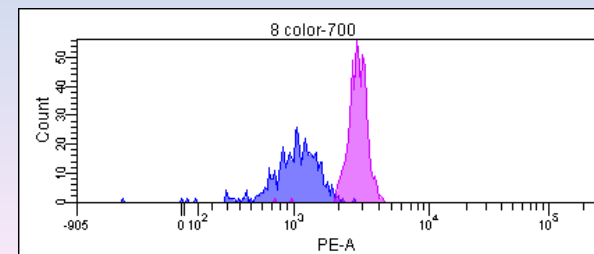
400V



500V



600V

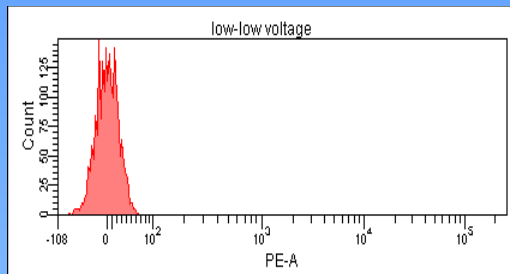


700V

The correct voltage to use will depend on brightness of target population

Only Noise

Light to PMT is blocked



Dim

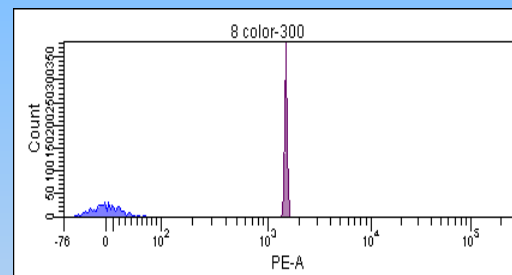
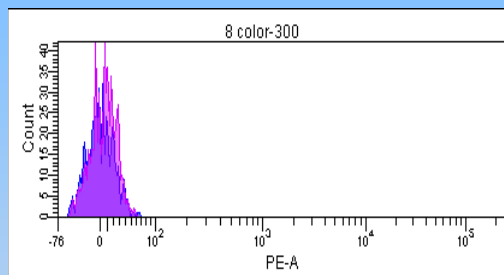
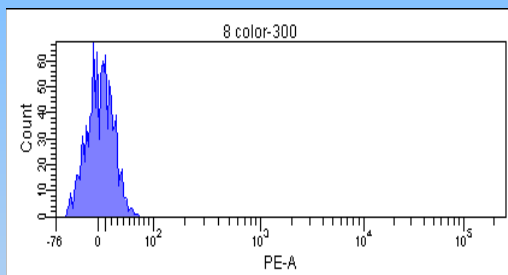
Dim (overlay)

Bright

300V

No separation for dim

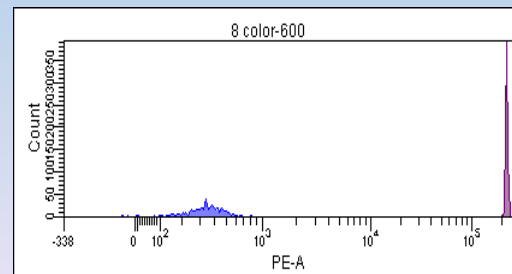
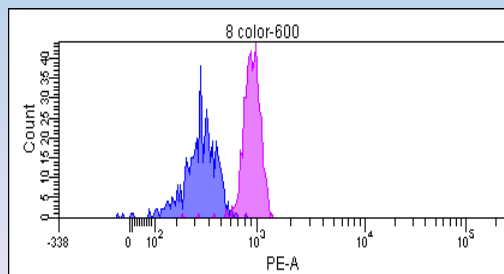
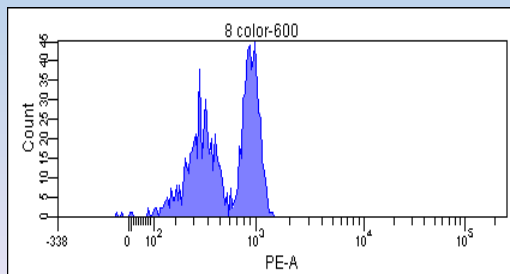
Good separation for bright

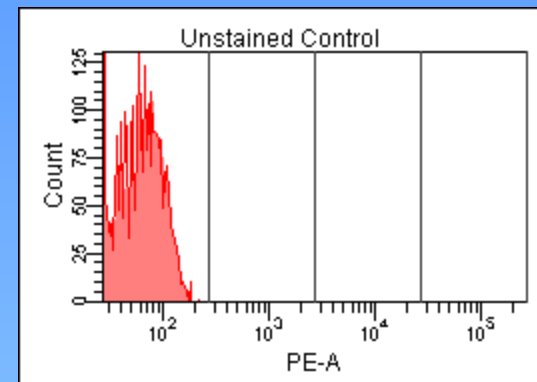
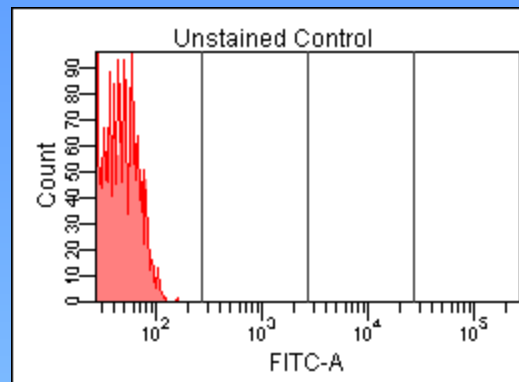
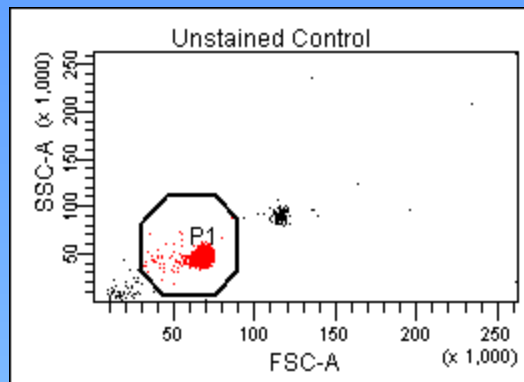


600V

Maximum separation for dim

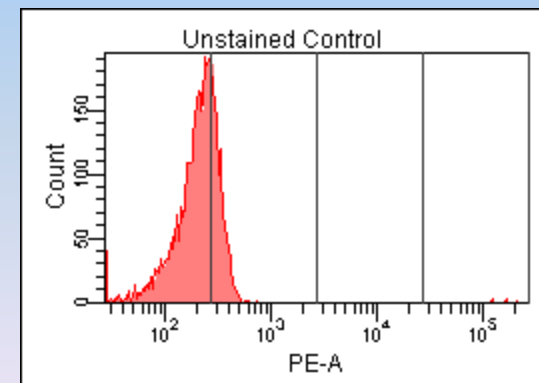
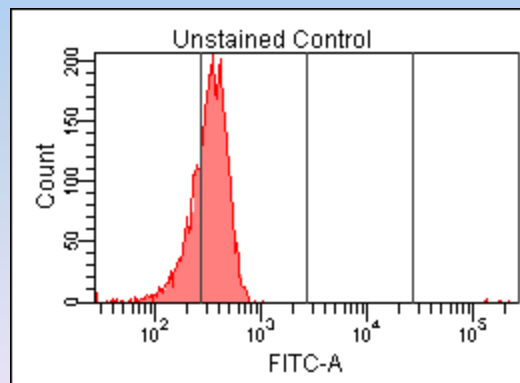
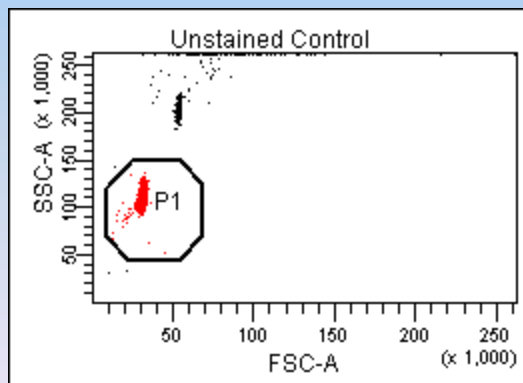
Brights off scale





Population	#Events	%Parent	FITC-A Median	PE-A Median
■ P1	4,839	96.8	18	42

For optimal resolution of dim signals, the target value for the negative population in your sample is 8-10 times the standard deviation of the noise, which minimizes the effect of the noise on your measurements.



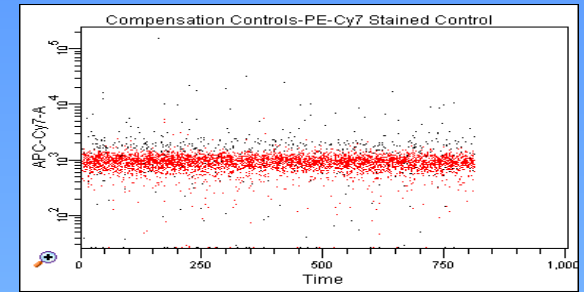
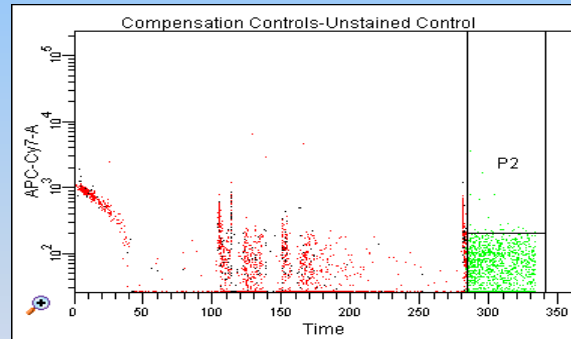
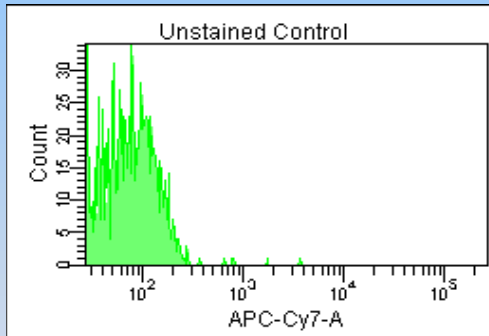
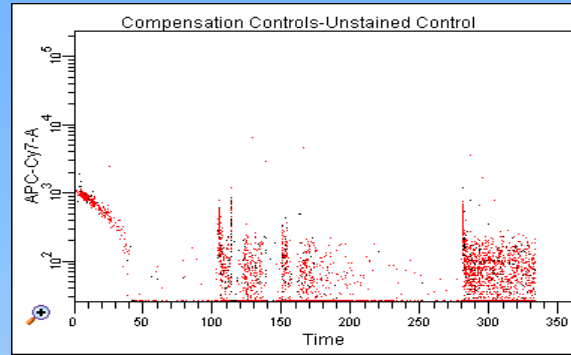
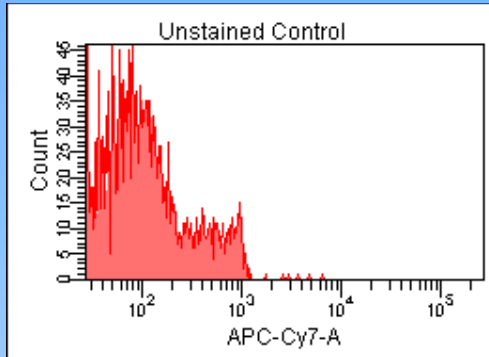
Population	#Events	%Parent	FITC-A Median	PE-A Median
■ P1	4,621	92.4	331	216

Settings may be adjusted for a specific sample

- Not all samples will require “optimal settings”
- Bright or off scale will not need optimal separation.
- May be better to titer antibody instead of changing voltage (saves reagent, and compensation considerations—very bright antibody will have more overlap into other channels, and reducing voltage in primary channel will NOT reduce signal in overlap channels-- but using less antibody WILL)

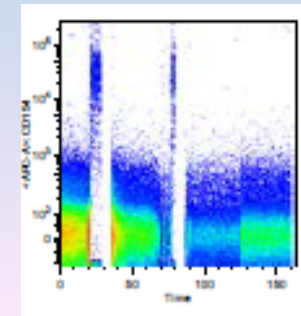
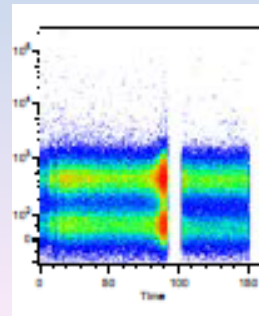
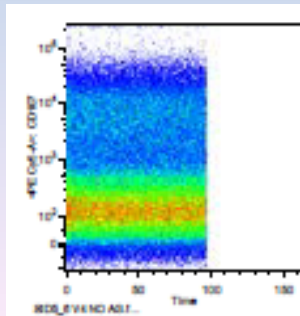
“Time Gating”

- View dot plot of time (x axis) vs parameter
- Signals should be consistent for duration of data file.
- Disturbance in the stream can cause fluctuations.

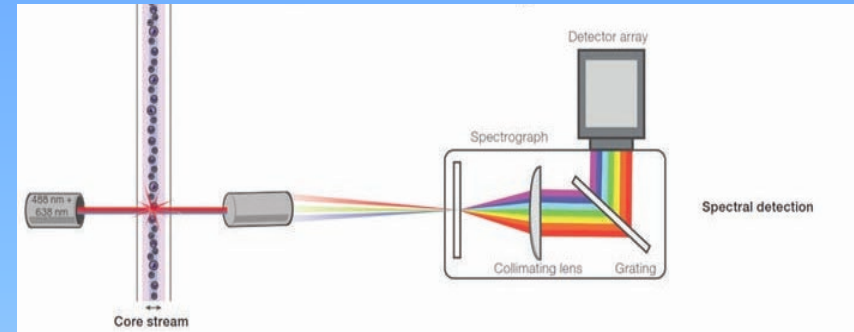
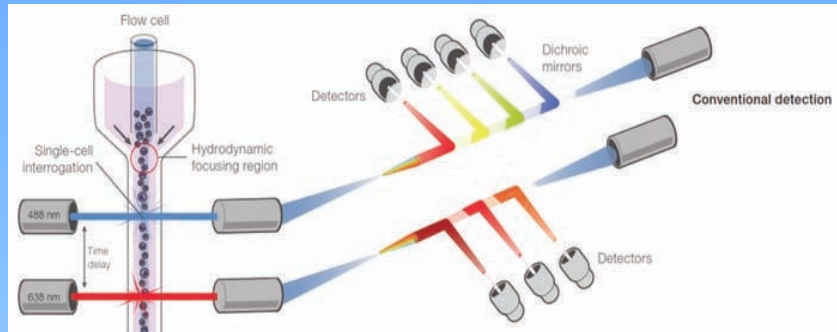


If it does fluctuate, you can draw an interval gate against “time” to exclude the initial events.

When running tubes, good idea to click “acquire”, wait a few seconds to see signals stabilize, then click “record”



2 Cytometer Designs



- Conventional

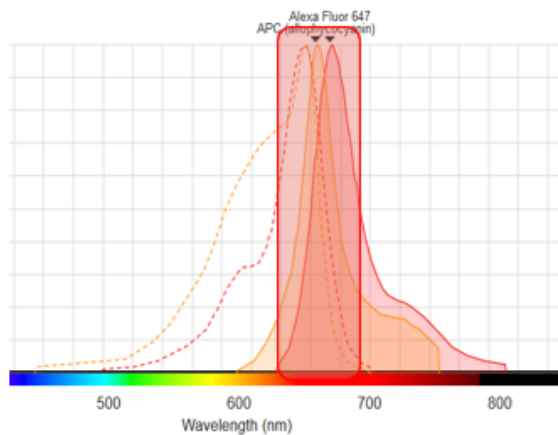
- Each detector is assigned to a particular fluorochrome.
- Only one marker per channel can be measured
- “compensation” is used to correct for spillover
- “spillover” becomes noise/error in the OTHER detectors

- Spectral

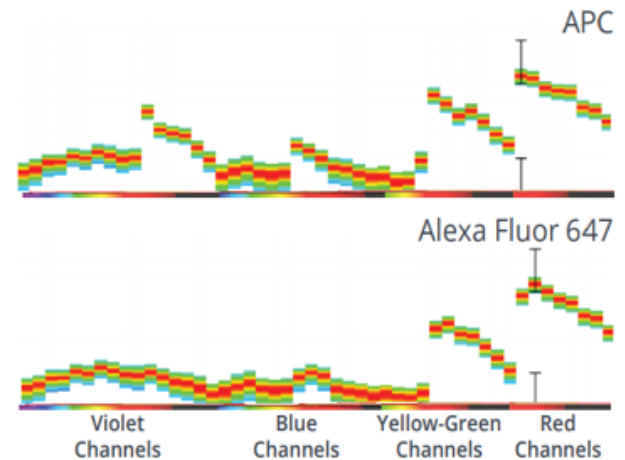
- Array of detectors generate a profile for each fluorochrome
- Colors need a unique profile, high overlap can be used.
- “unmixing” is used to determine total signals for each fluorescent marker
- “Error” is spread over multiple detectors, so less data spread

Distinguishing Fluorochromes

Conventional: must pick one



Spectral: can use both!



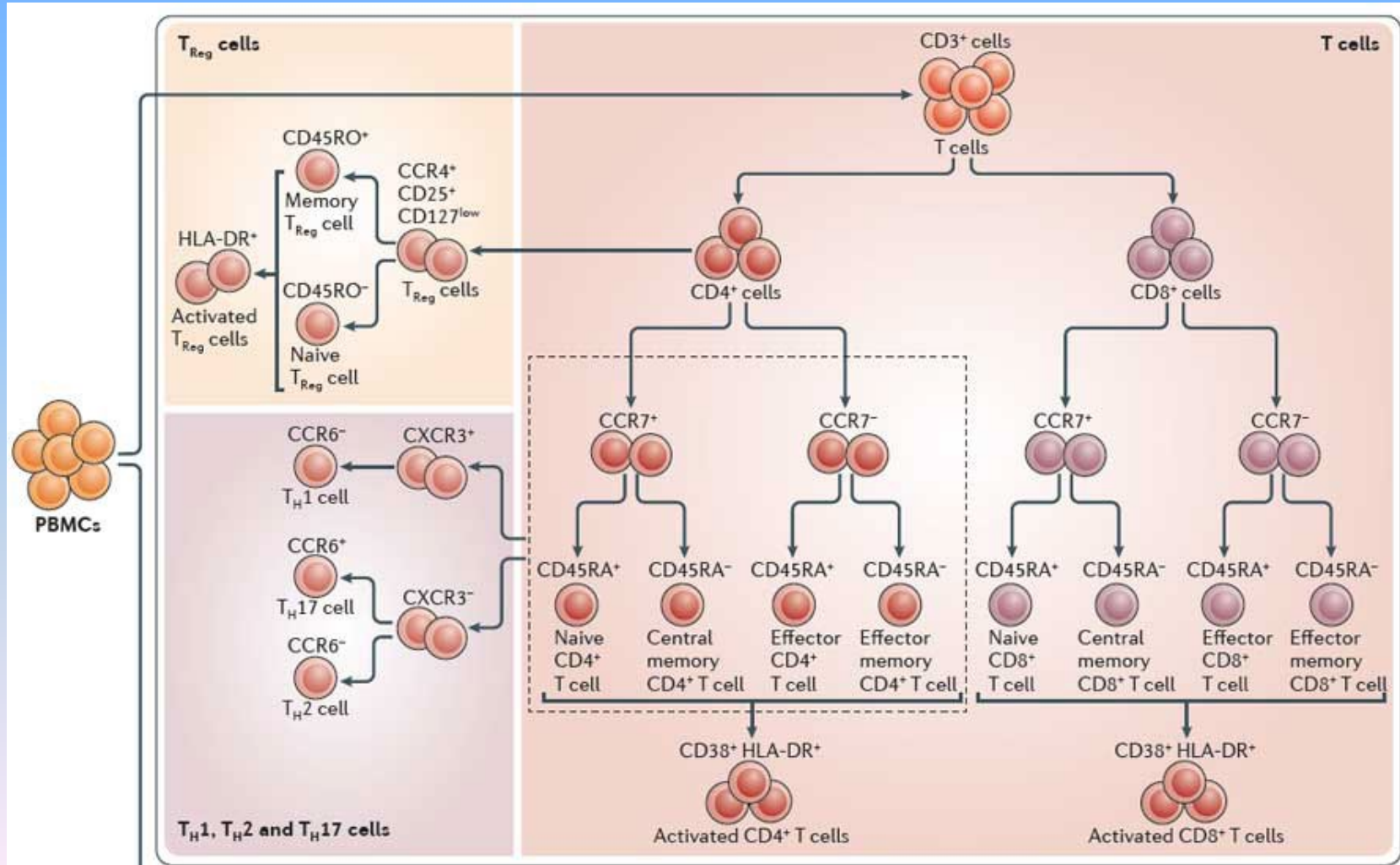
Applications

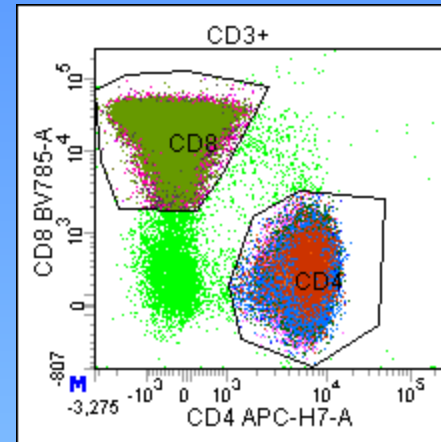
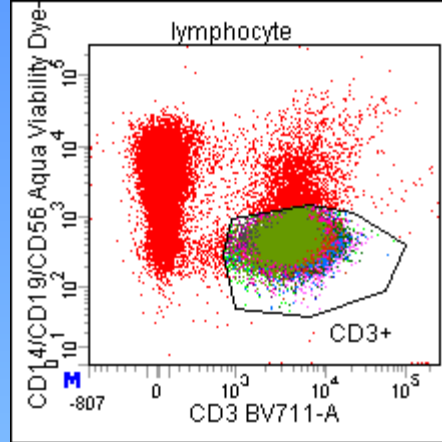
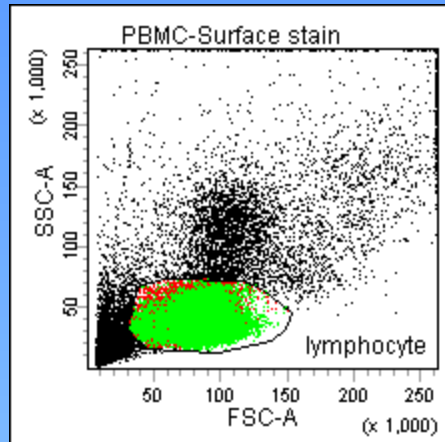
- Immunophenotyping
- ICS
- Proliferation
- Protein phosphorylation
- Cell Cycle
- Apoptosis
- Stem Cells

Standardizing immunophenotyping for the Human Immunology Project

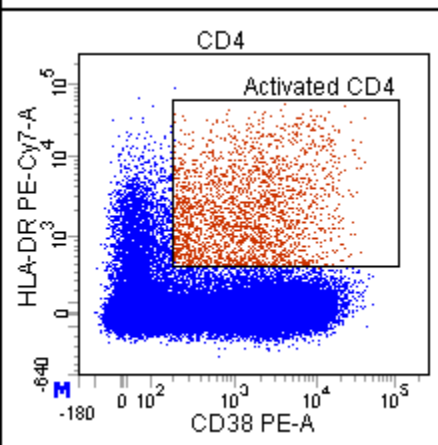
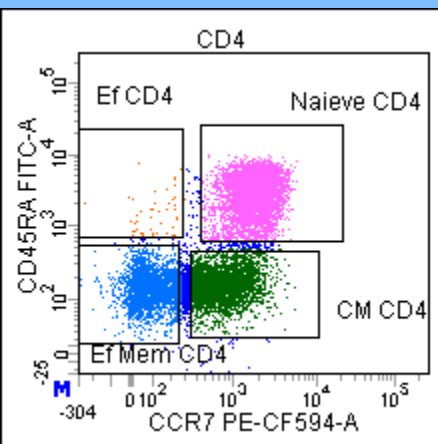
Holden T. Maecker, J. Philip McCoy & Robert Nussenblatt

Nature Reviews Immunology 12, 191-200 (March 2012)



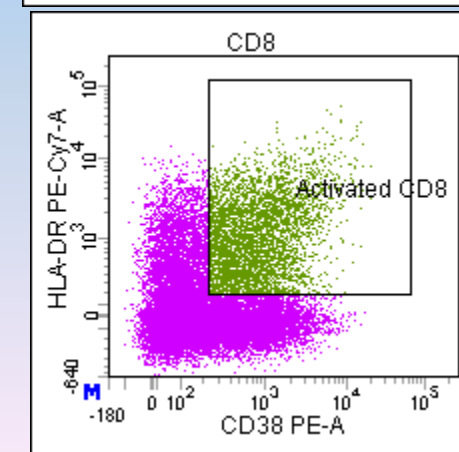
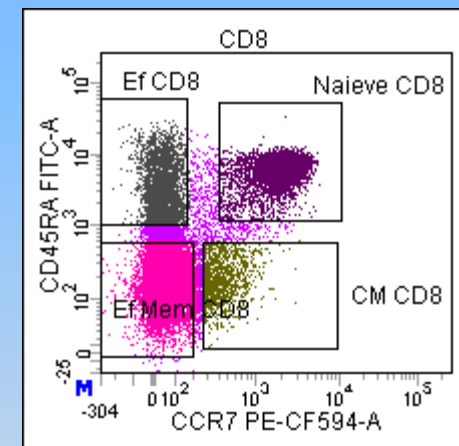


Phenotyping

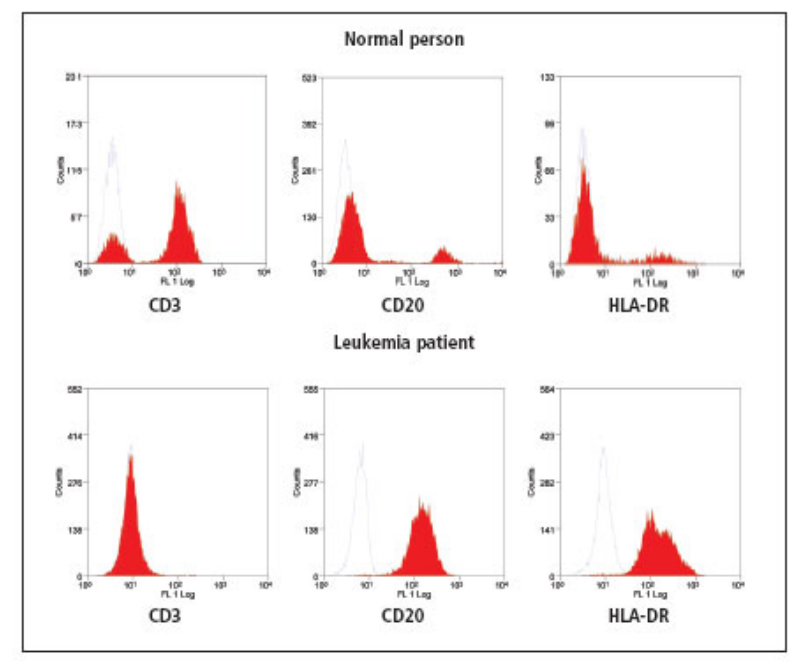


Tube: Surface stain

Population	#Events	%Parent	%Total
All Events	587,518	####	100.0
lymphocyte	275,561	46.9	46.9
CD3+	172,497	62.6	29.4
CD4	62,036	36.0	10.6
Naive CD4	26,171	42.2	4.5
CM CD4	16,787	27.1	2.9
Ef Mem CD4	14,762	23.8	2.5
Ef CD4	264	0.4	0.0
Activated CD4	2,358	3.8	0.4
CD8	104,988	60.9	17.9
Naive CD8	20,787	19.8	3.5
CM CD8	4,894	4.7	0.8
Ef Mem CD8	57,569	54.8	9.8
Ef CD8	13,063	12.4	2.2
Activated CD8	21,228	20.2	3.6



Immunophenotyping: Identifying Cancers



CD3-A normal person has a significant proportion of CD3-positive lymphocytes. In the patient with leukemia, staining for CD3 is absent.

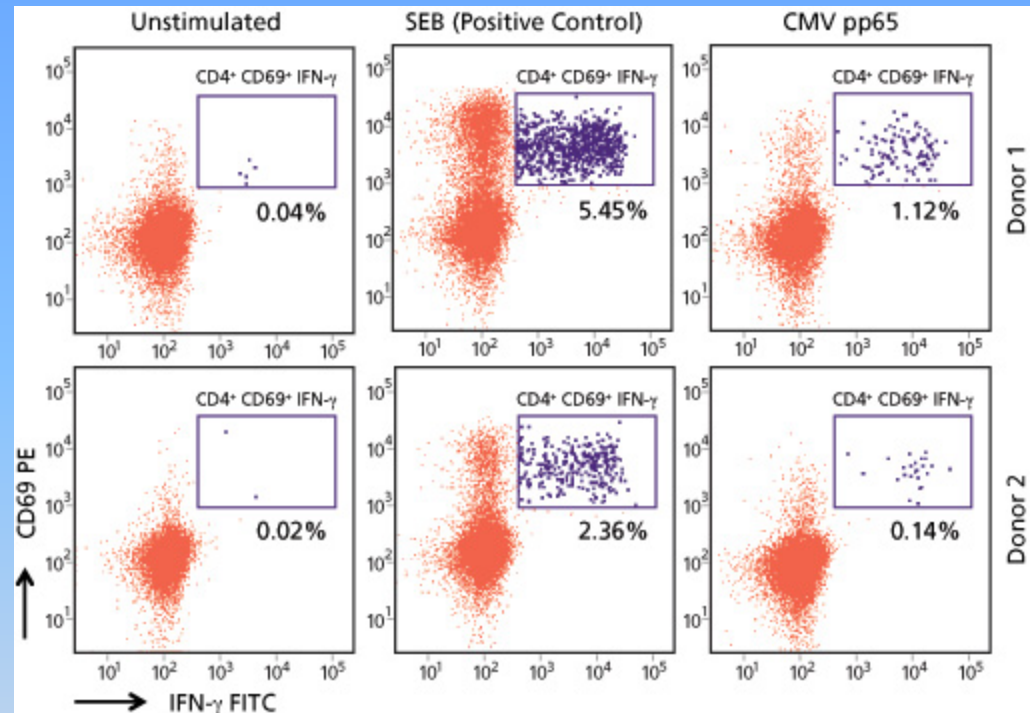
CD20-In the leukemia patient there are a large number of cells staining positive for CD20. In the healthy person only a few stain positive.

HLA-DR-The leukemia patient is HLA-DR-positive. In the normal person only a small number of cells stain positive.

Being CD3-negative, CD20-positive and HLA-DR-positive, a clinician could diagnose with certainty that this patient is suffering from a B cell lineage leukemia or lymphoma. The precise classification of disease may be determined using further antibodies.

Intracellular Cytokine Staining; ICS

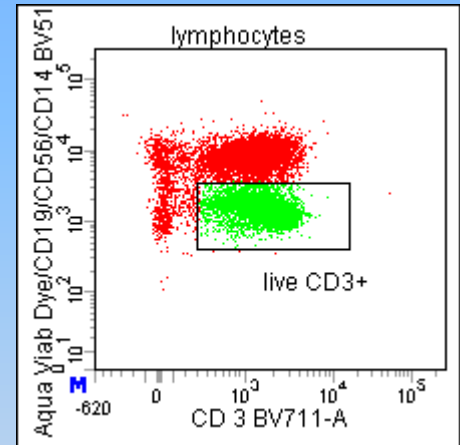
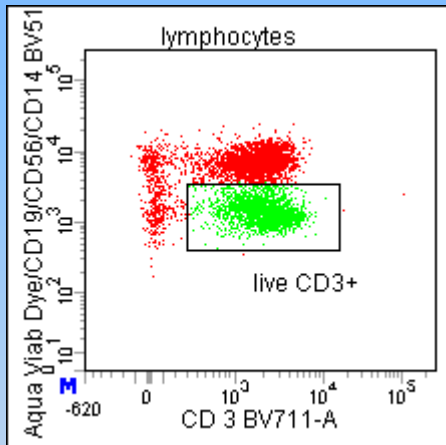
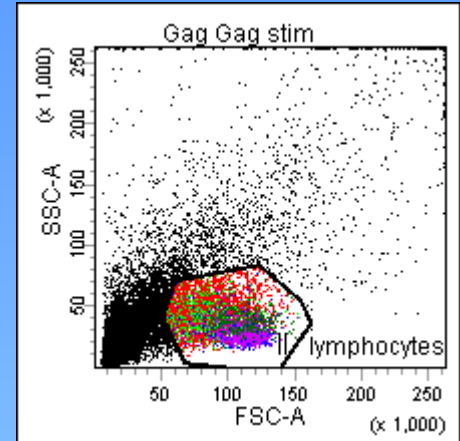
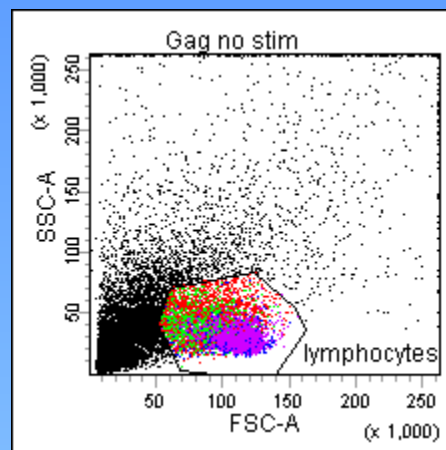
**Antigen-specific IFN- γ
production by cytomegalovirus
(CMV) pp65- stimulated
CD4⁺CD69⁺ T lymphocytes**



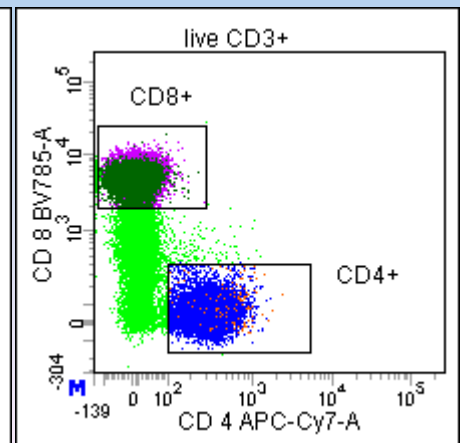
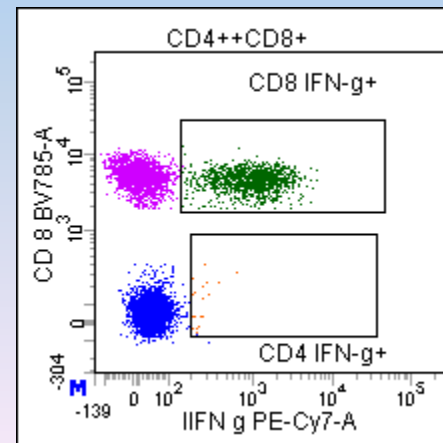
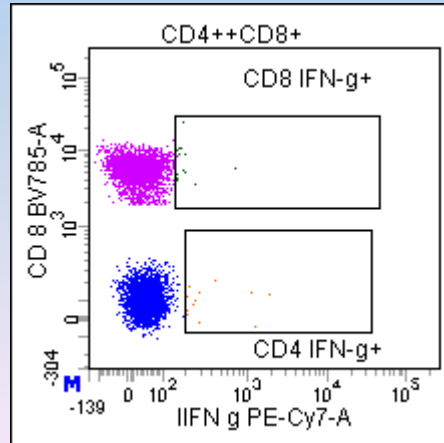
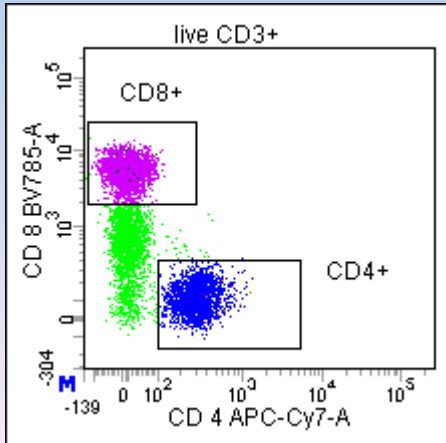
Two-color flow cytometric dot plots show IFN- γ vs CD69 expression by CD4 T cells that were either unstimulated (left panels), SEB-stimulated (as positive controls, center panels), and CMV pp65-stimulated (right panels) samples from two donors. Human whole blood was stimulated in the presence of brefeldin A before fixing, permeabilizing, and staining using the BD FastImmune™ 3-color CD4 intracellular cytokine detection kit. Data was acquired using a BD FACSVerse flow cytometer and a BD FACSuite™ software research assay.

ICS Staining

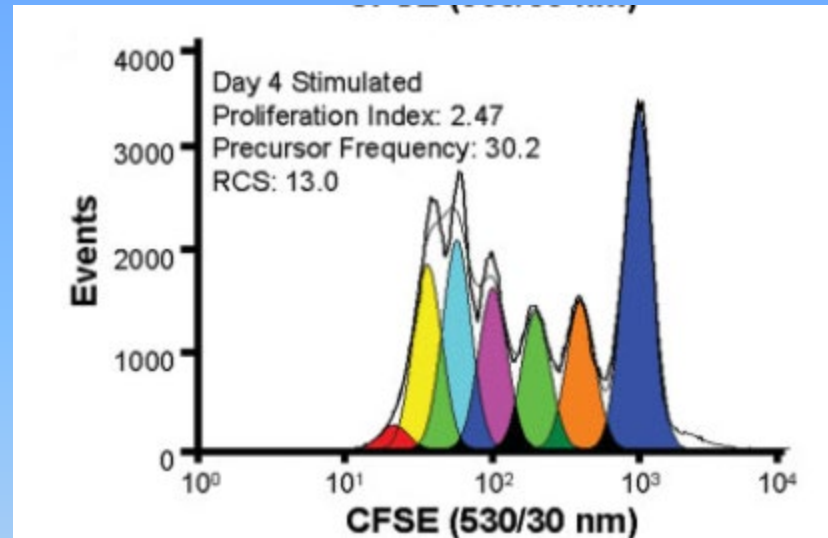
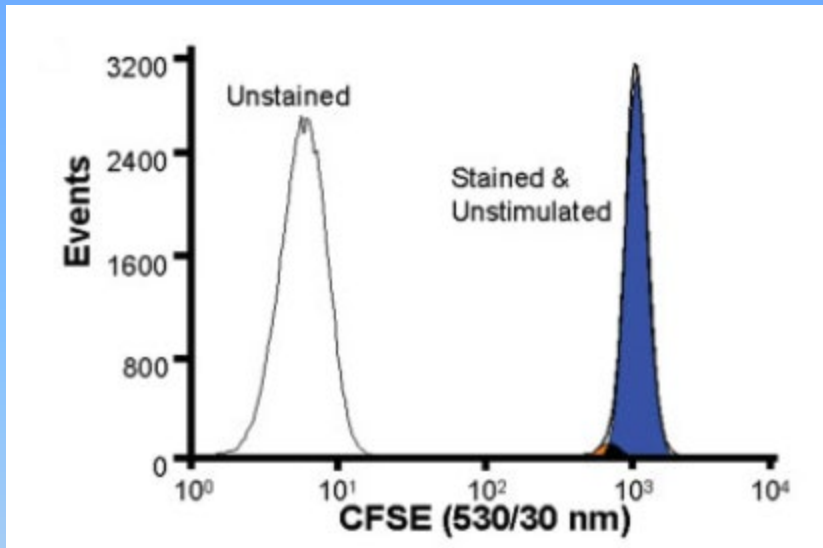
(“PIN Number”)



Tube:	Gag no stim		Gag Gag stim	
Population	%Parent	%Total	%Parent	%Total
All Events	####	100.0	####	100.0
lymphocytes	21.6	21.6	20.3	20.3
live CD3+	45.3	9.8	40.8	8.3
CD4+	31.6	3.1	28.3	2.3
CD4 IFN-g+	0.5	0.0	1.0	0.0
CD8+	37.9	3.7	42.7	3.5
CD8 IFN-g+	0.5	0.0	35.7	1.3



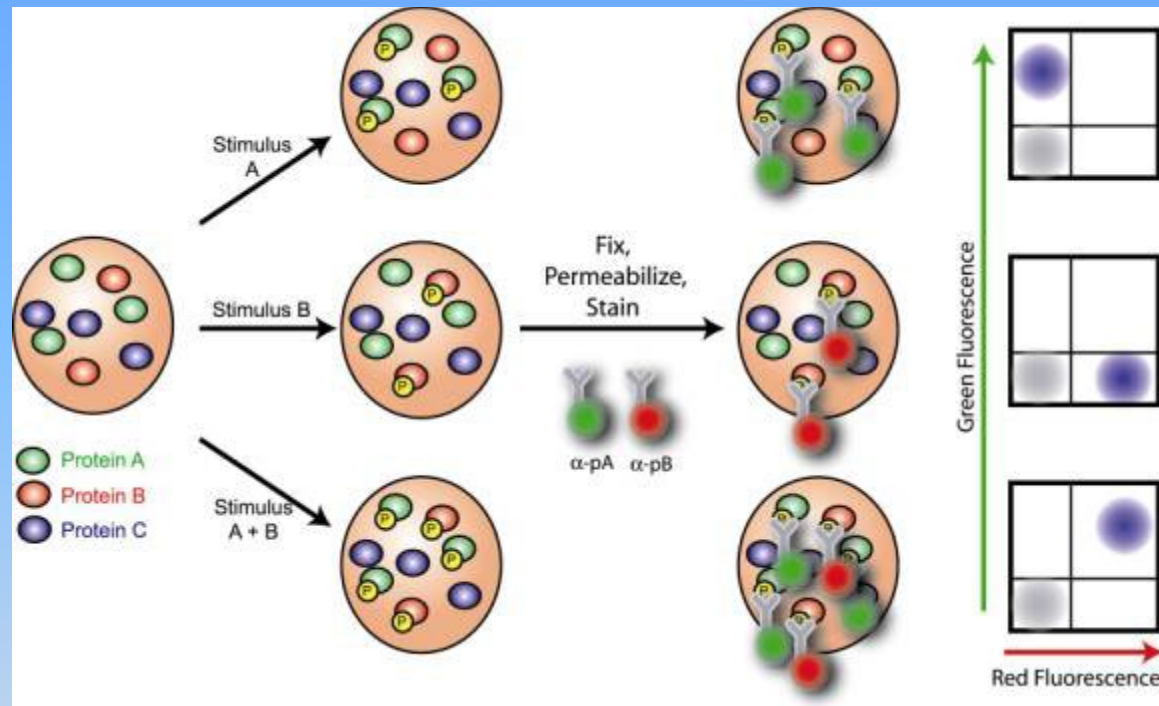
Proliferation



Cells are loaded with a non-fluorescent dye that becomes fluorescent and insoluble inside of the cell. The dye is divided among daughter cells upon division, resulting in a dilution of the dye and a reduction in fluorescence with each subsequent generation.

Tracking Antigen-Driven Responses by Flow Cytometry: Monitoring Proliferation by Dye Dilution. P.K. Wallace et.al. Cytometry Part A, 73A: 1019-1034, 2008

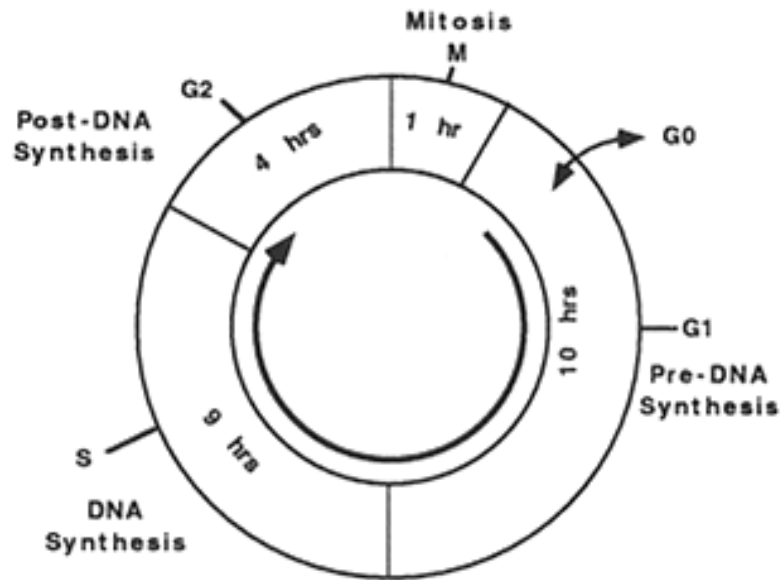
Protein phosphorylation



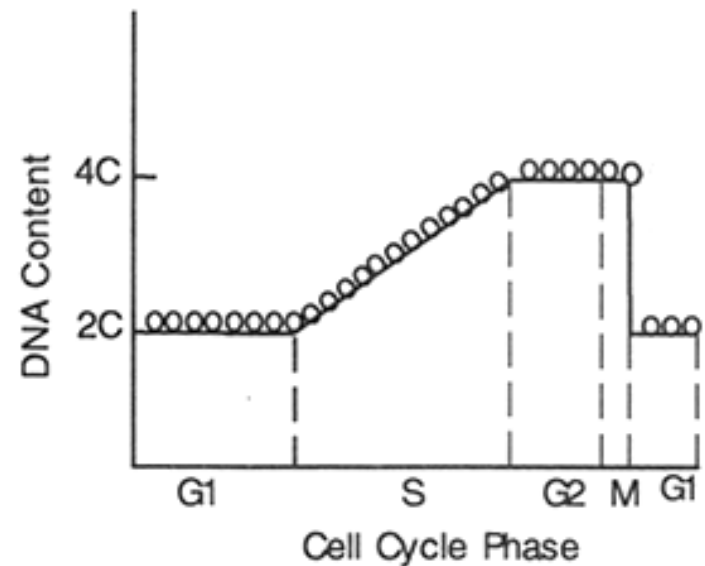
General phospho-protein staining technique for flow cytometry. Cells containing three proteins, A, B, and C, are treated with a stimulus that leads to the phosphorylation of protein A (Stimulus A), protein B (Stimulus B), or both simultaneously. The cells are then fixed, permeabilized, and stained with fluorophore-conjugated phospho-specific antibodies to the active forms of proteins A and B (α -pA and α -pB). When cells are analyzed by flow cytometry the individual inductions cause increases in fluorescence on one fluorescent channel, i.e. in the green or red channel, while the dual stimulation causes a shift in both channels.

This technique can also be applied to patient samples to help characterize aberrant signaling events that occur during disease progression, or determine the efficacy of signaling pathway-specific drugs *in vivo*.

Cell Cycle Analysis

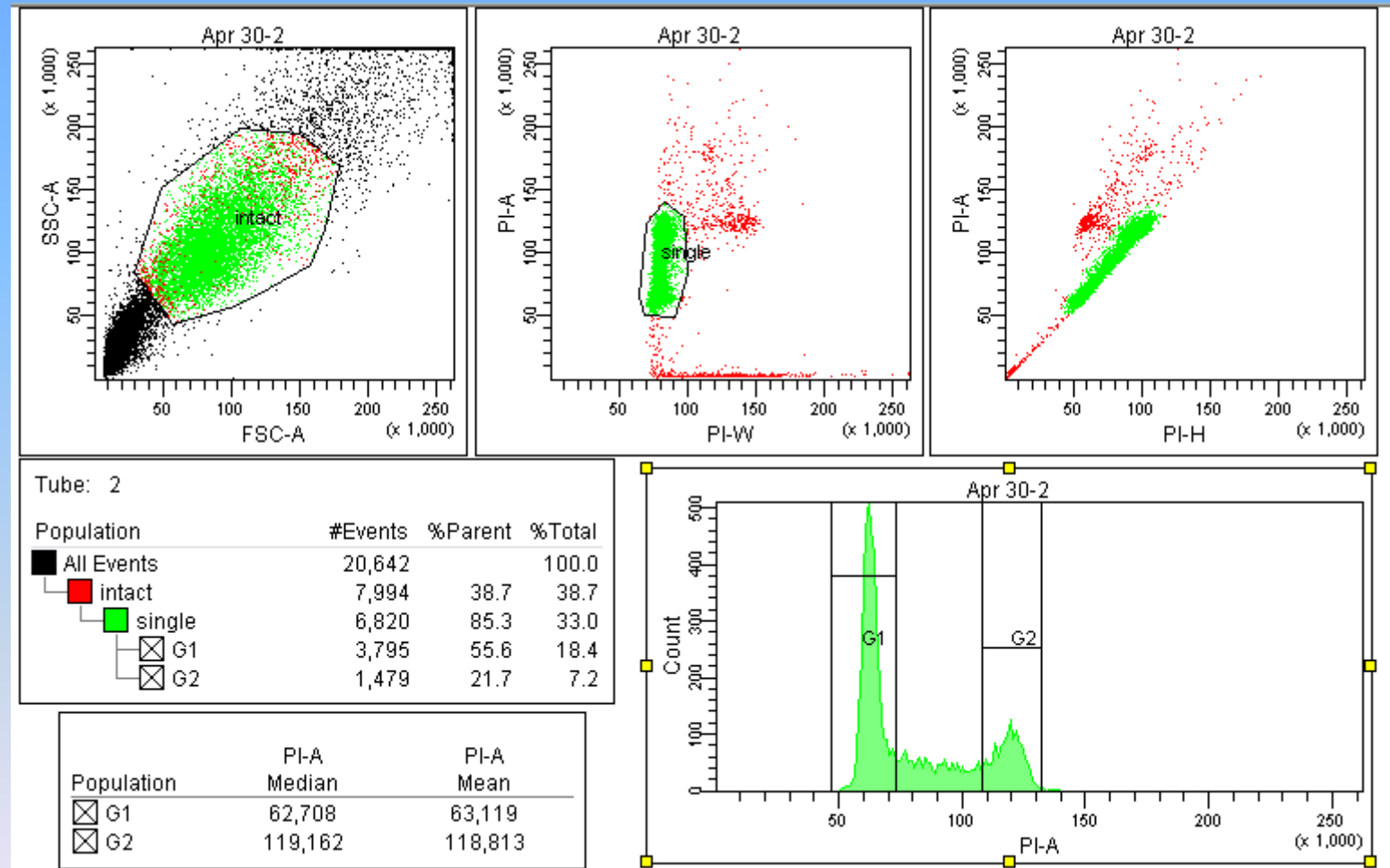


Cell Cycle Phases

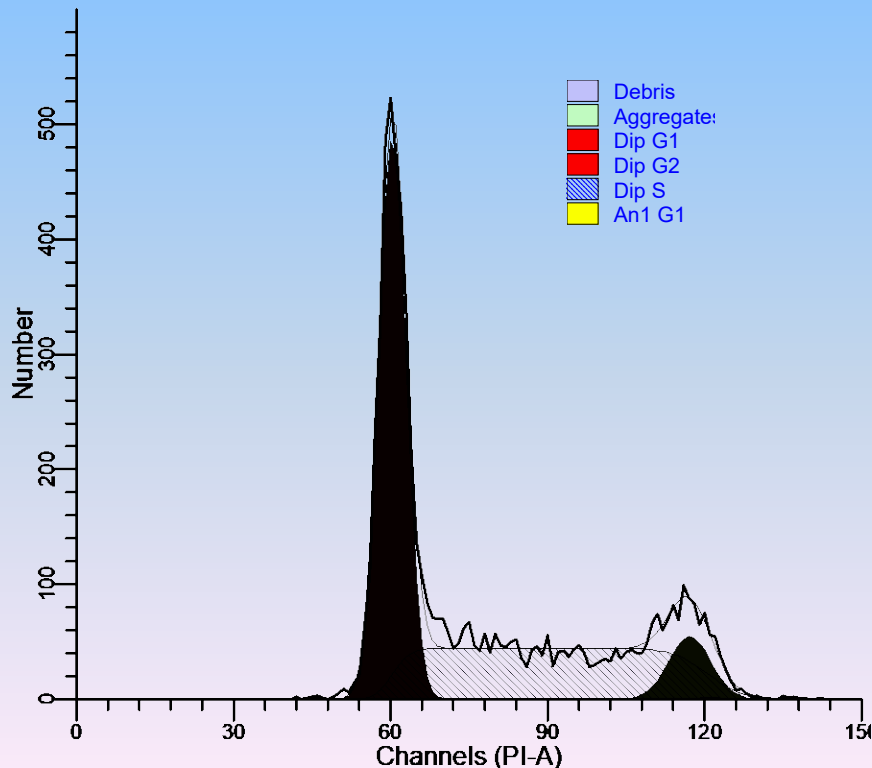
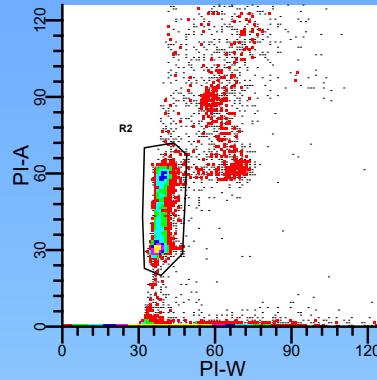
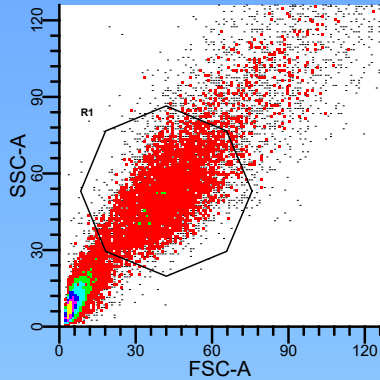


DNA Mass vs Cell Cycle Phase

Cell Cycle analysis



Modfit analysis



Diploid: 99.46 %

Dip G1: 50.56 % at 60.33

Dip G2: 8.00 % at 117.18

Dip S: 41.44 % G2/G1: 1.94

%CV: 4.34

Debris: 0.31 %

Aggregates: 0.40 %

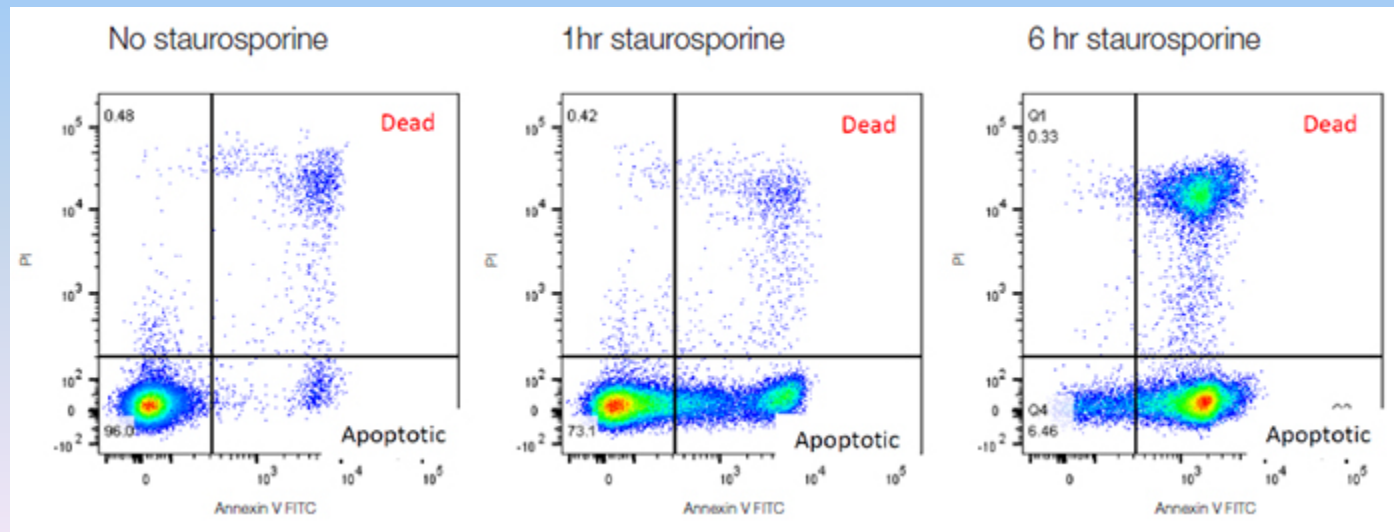
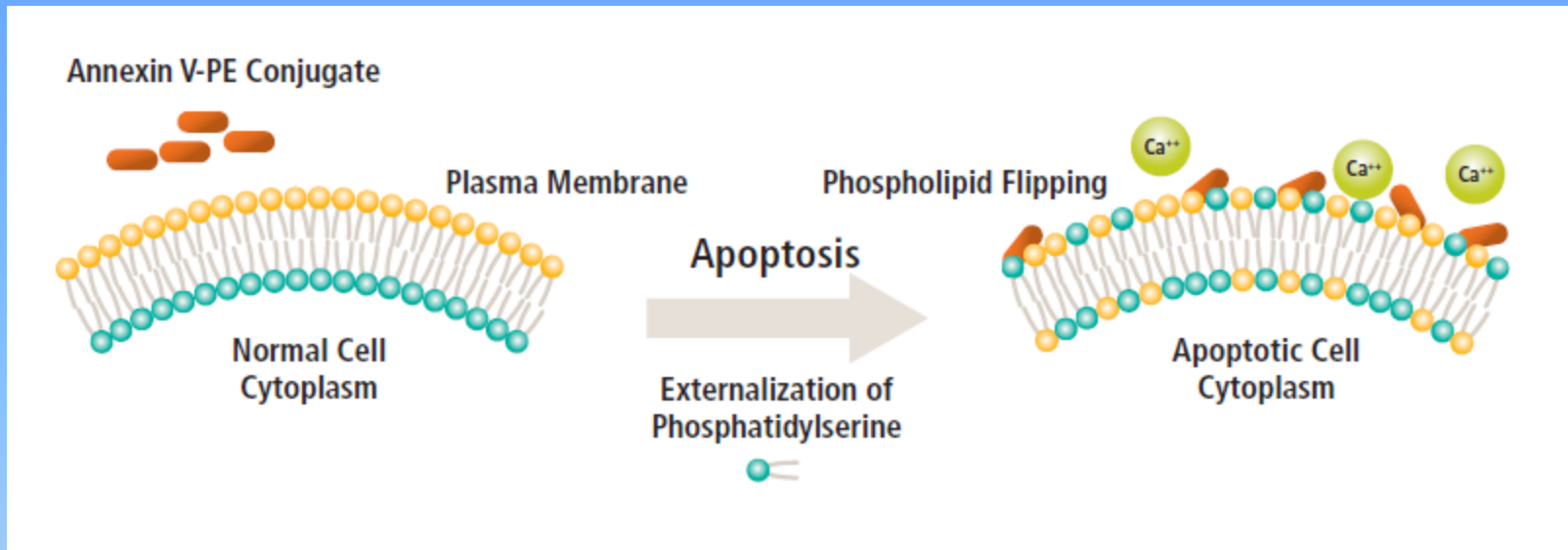
Modeled events: 6469

All cycle events: 6424

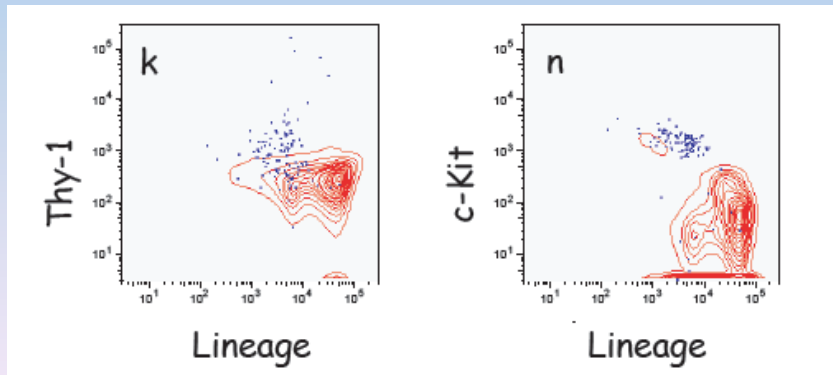
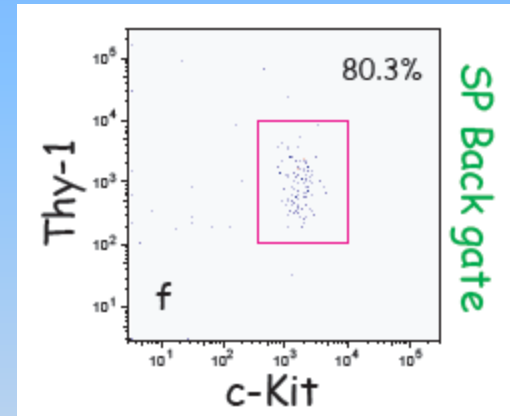
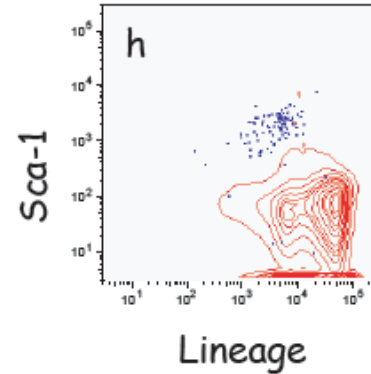
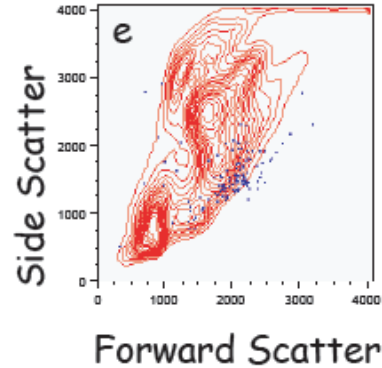
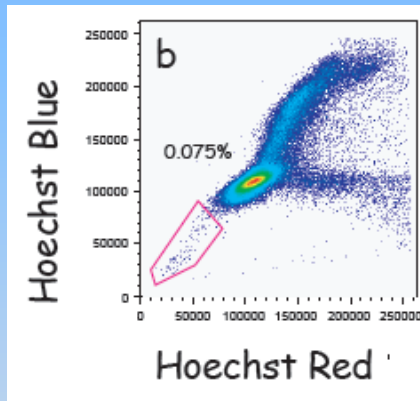
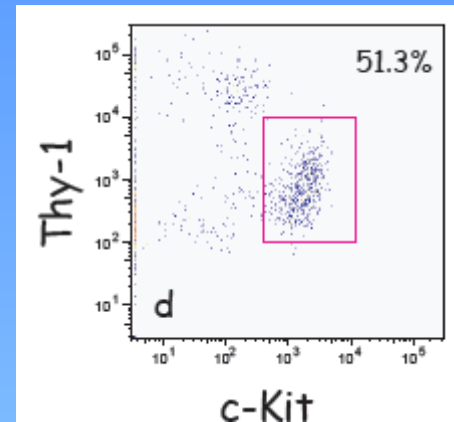
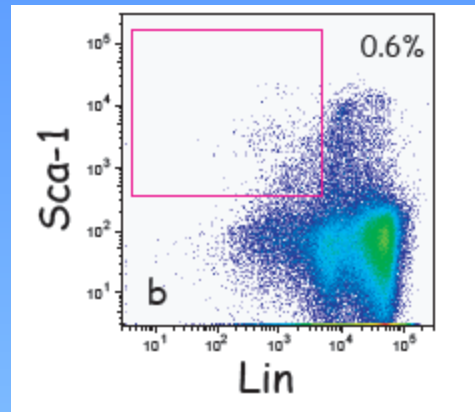
Cycle events per channel: 105

RCS: 1.289

Apoptosis



Stem Cells: KTLS and Side Population



<http://science.cancerresearchuk.org/sci/facs/>
Catherine Simpson, Derek C. Davies, Daniel Pearce, Dominique Bonnet.

General Flow Information

- We have multiple analyzers and sorters, descriptions can be found on the core's home page

<https://ragoninstitute.org/research-service/flow-cytometry-core>

- Sorters are run primarily by core personnel; select high-use Ragon researchers are also trained. We have 2 SONY SH800 sorters that are user-run, one in BL2 and one for yeast on the 4th floor.



Credits and Resources

Websites:

<https://ragoninstitute.org/research-service/flow-cytometry-core/>

-Core overview, link to user resources. READ “Ragon Core ABC’s”!

www.cyto.purdue.edu

-message board, other resources

-www.tinyurl.com/cytometry to search archives

New England Cytometry: www.newenglandcytometry.com

-Powerpoint presentations, local flow people

-useful flow links, blog posts

www.isac-net.org International Society for Advancement of Cytometry

-flow resources and training aids

Most vendors will have tons of resources on their websites.

Books (listed in increasing levels of complexity):

-Cytometry: First Principles- Alice Givan

-Flow Cytometry-A basic introduction—Michael Ormerod Online at

<http://flowbook.denovosoftware.com/>

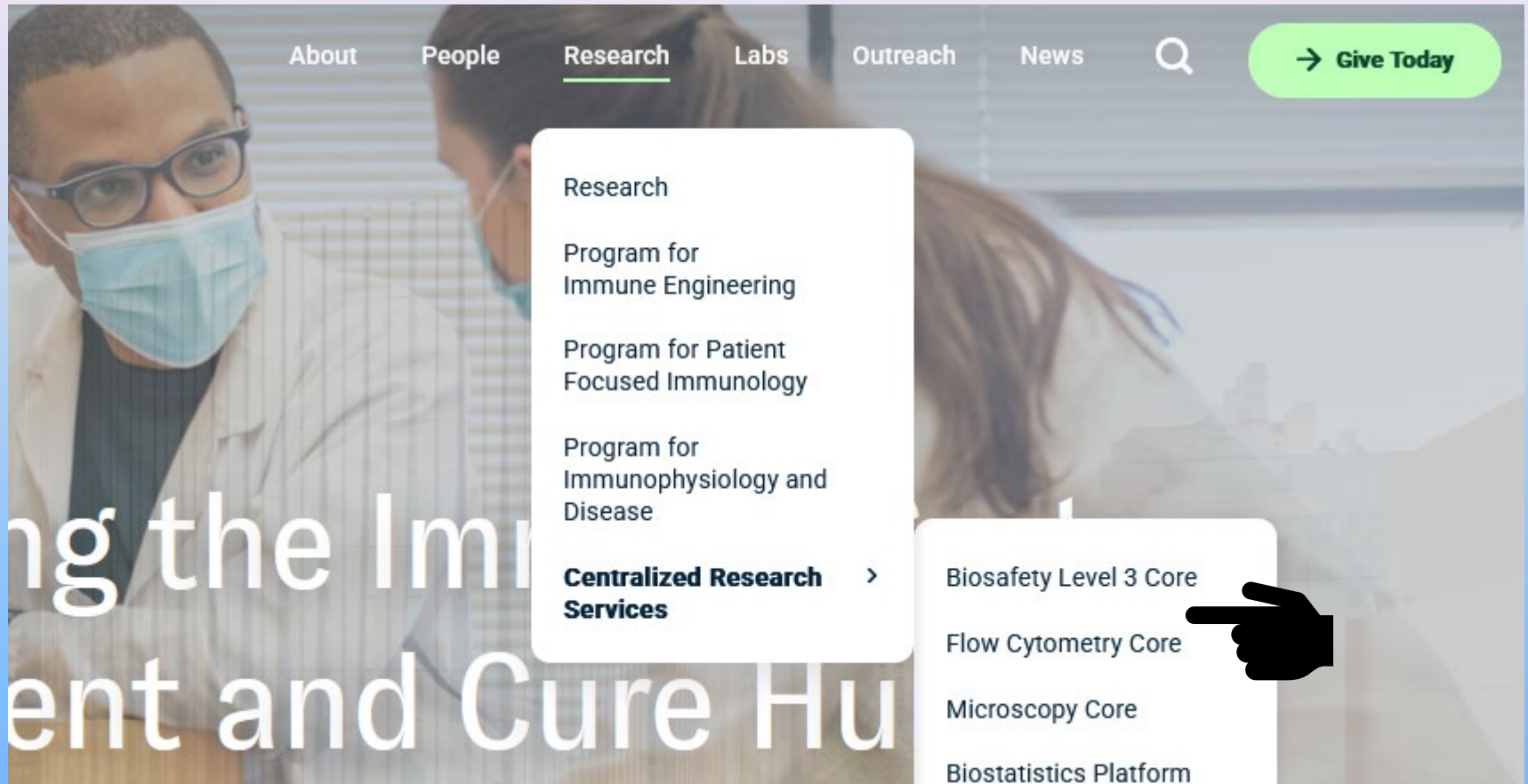
-In Living Color: Protocols in Flow Cytometry and Cell Sorting– Diamond and DeMaggio

-Practical Flow Cytometry-Howard Shapiro

Online: “google it” (Beckman used to have it on their site)

-Current Protocols in Cytometry (Wiley)—available in core lab 333.

Next steps! ragoninstitute.org:



How to get started

We welcome external users at the Ragon Flow Cytometry Core. Both internal and external users should review the guidelines on how to get started.

 [Guidelines for Core Facility Users \[PDF\]](#)

 [Usage rates FY2026 \[PDF\]](#)

How to set up your account in the RCMS calendar system

 [Internal users \(MGB fund numbers only\) \[PDF\]](#)

 [External users \(Non-MGB funding, even if you have an MGB network ID\) \[PDF\]](#)

Thank you!!

- Please make sure you checked your name off on the sign-in sheet!

Ragon Institute Flow Cytometry core booking system

We are part of the MGB Partners Core Management System and effective 10-1-2025 have switched over to the PCMS calendar system. To sign up, please visit:

<https://researchcores.partners.org>

and click on “Signup”

(Existing PCMS users can click on “login” to access your account)



MGB Internal users: Enter
Alias (your ID in the format “abc123”)

Internal: MGB ID, click
“lookup information”,
confirm its accurate
and then click
“Create Account”

User Information

Mass General Brigham ID *

Lookup Information

Will be used as your username

[Don't have a Mass General Brigham ID?](#)

External user:
click “Don’t have a Mass General
Brigham ID”

External users,
complete the following information. Your email
address will be your username:

User Information

Mass General Brigham ID *

mhw12 Change

Will be used as your username

Email *

mwharing@mhgh.harvard.edu

First name *

Michael

Institution

The General Hospital Corporation

Phone

Address

Alternative email

Separate multiple addresses by semicolon

Create Account Cancel

Home Cores Services Explore About Us Resources Login Signup

User Information

Email *

Will be used as your username

First name *

Last name *

New password *

New password confirmation *

Institution *

Department

Phone

Fax

Address

Alternative email

Send communication to

Primary Email

Separate multiple addresses by semicolon

Create Account Cancel

This site is protected by hCaptcha and its [Privacy Policy](#) and [Terms of Service](#) apply.