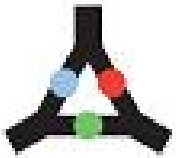


Ragon Institute
of MGH, MIT and Harvard

Michael Waring

Associate Director

Ragon Institute Imaging Core Facility

Cambridge, Massachusetts, USA

Ragon Institute Imaging Core

- Maintain and oversee operation of the Institute's flow cytometry equipment (Cytometers and cell sorters, as well as Luminex, SPR, and Imaging Cytometry technologies)
- Provide training and guidance in experimental design in flow cytometry.

- Core resources also include confocal and fluorescent microscopy and slide scanners

Ragon Institute Imaging Core
Flow Cytometry training syllabus

- "Introduction to Flow Cytometry" 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

- Today, 12/14/16—Diva experienced users only!
- Friday 12/16/16 from 2 to 4
- Mon 12/19/16 from 1 to 3

- Additional sessions in the new year.

If you cannot make any of them, let me know your schedule limitations and we will make accommodations.

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

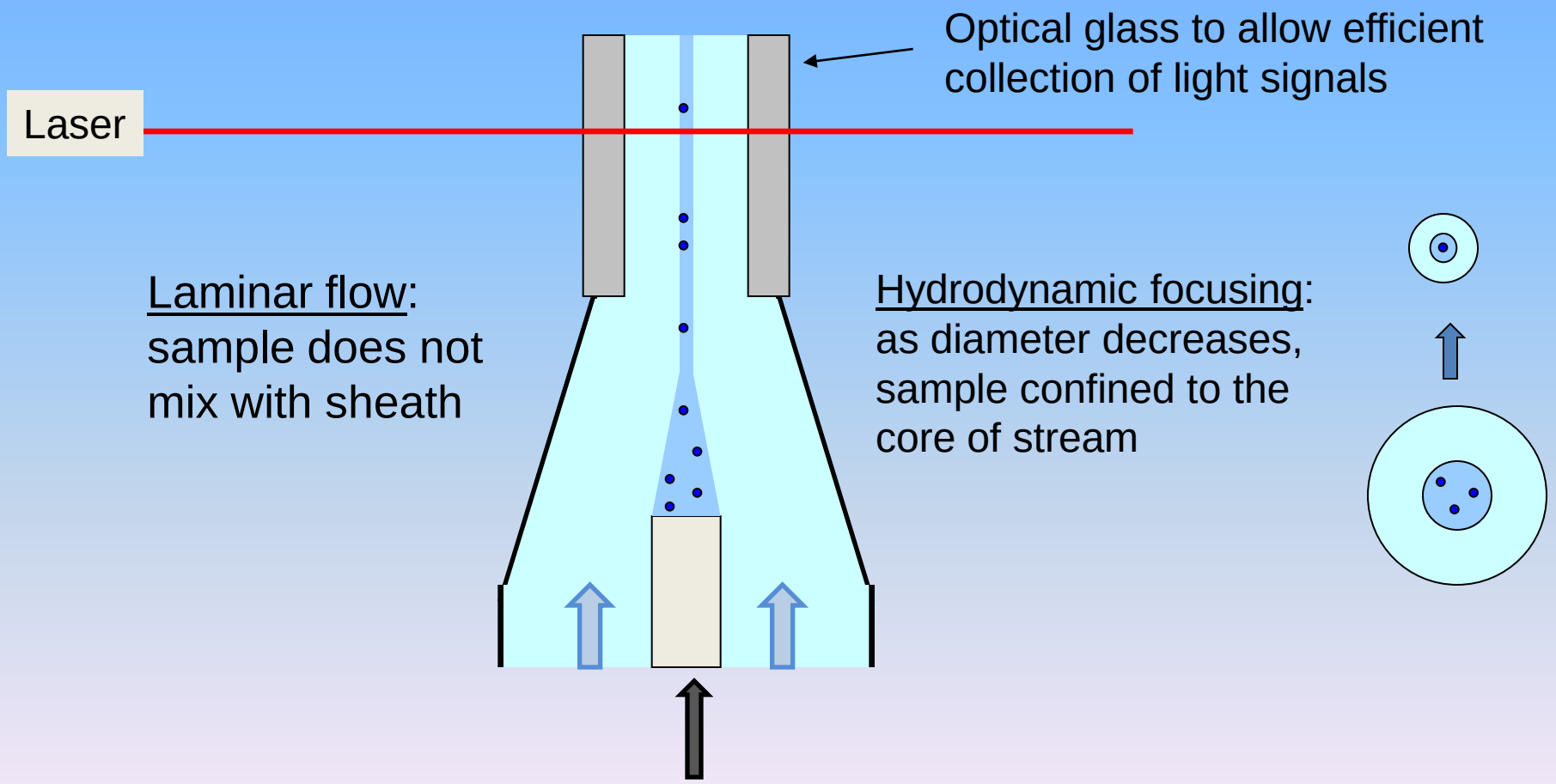
Information We Get From a Flow Cytometer

- 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
  - (FITC, PE, GFP, etc.)

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).



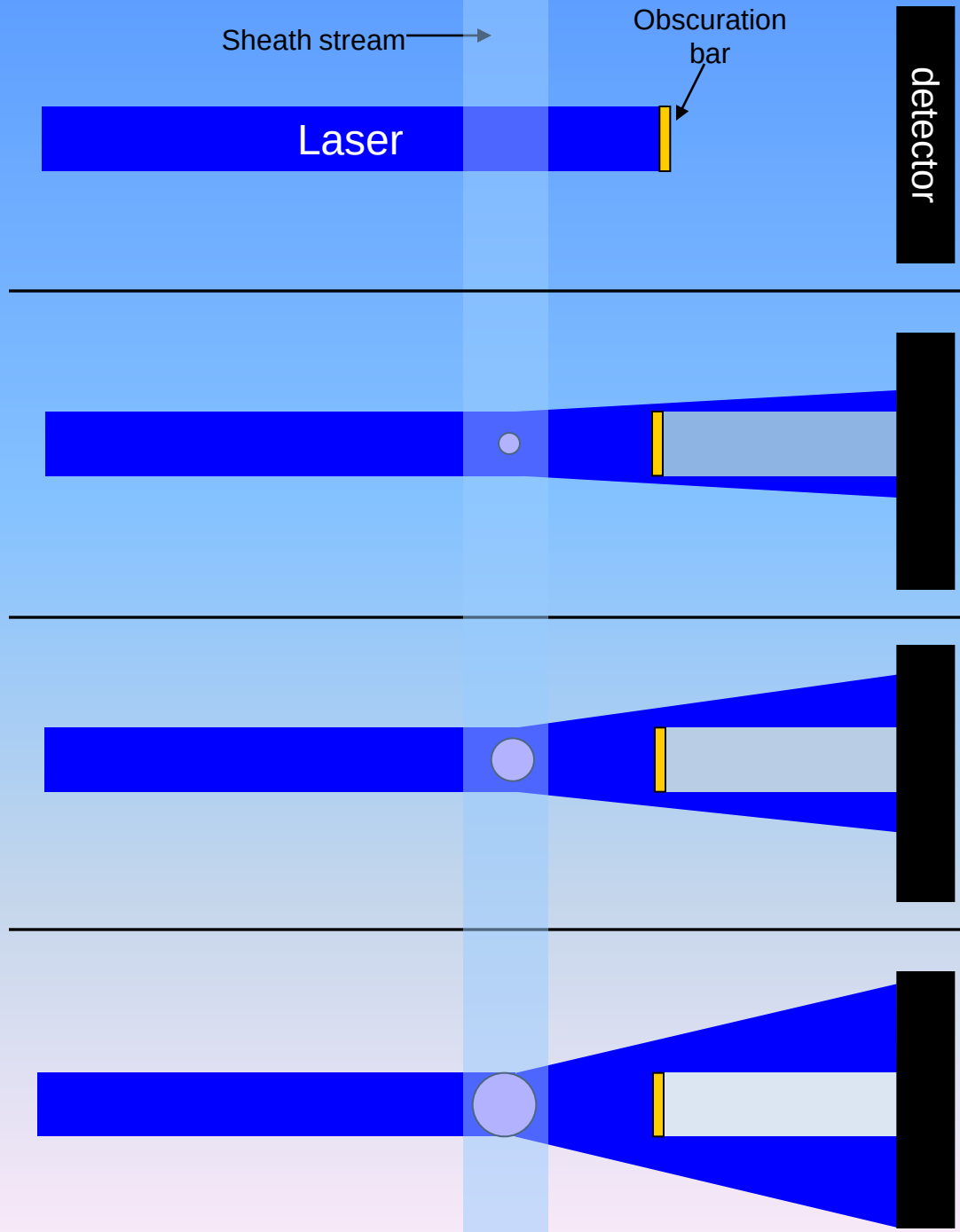
Forward scatter, small angle scatter, FSC

Diffracted light

Related to particle's surface area and refractive index

Detected along axis of incident light in forward direction.

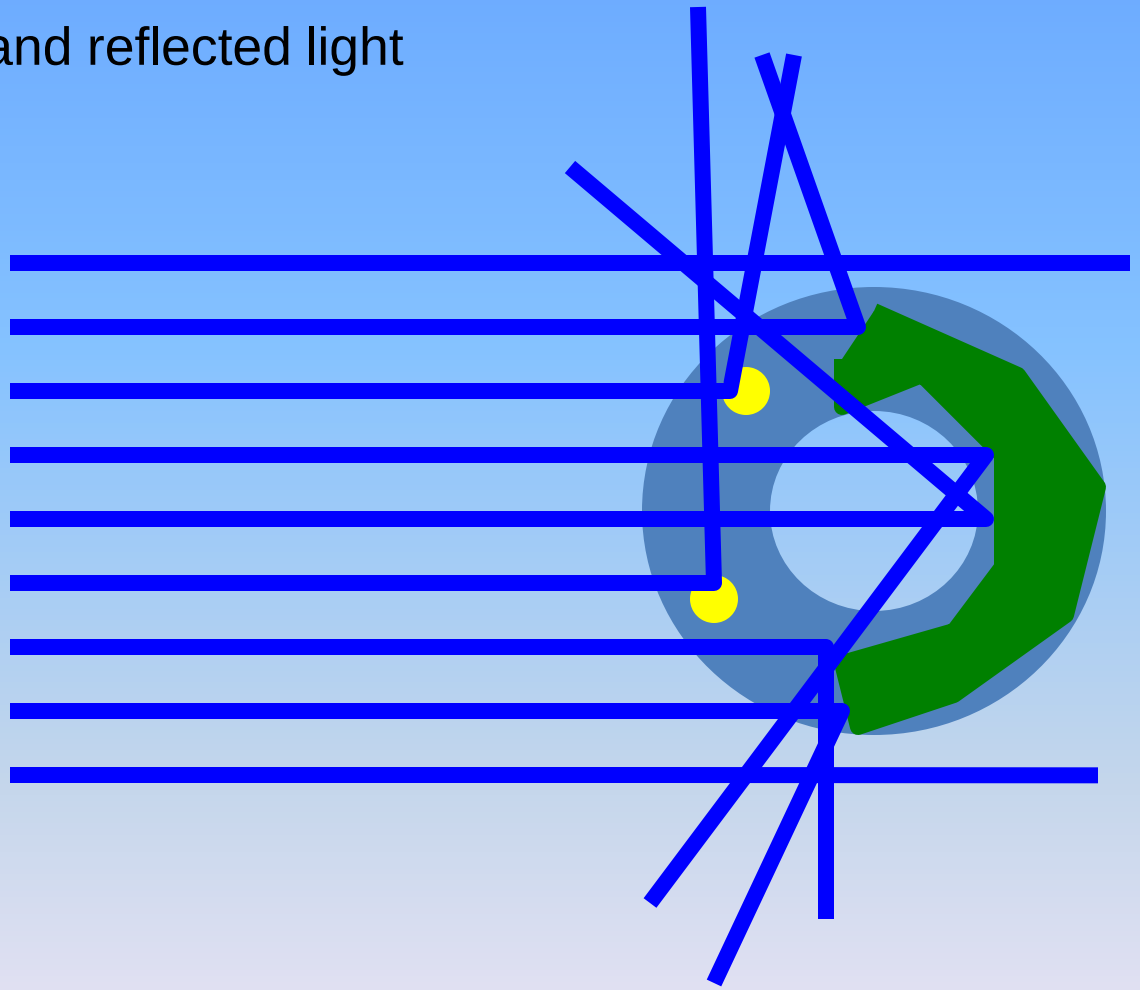
5um bead ≠ 5um cell



Side Scatter, Wide angle scatter, SSC

Measure of cell granularity and complexity

Refracted and reflected light



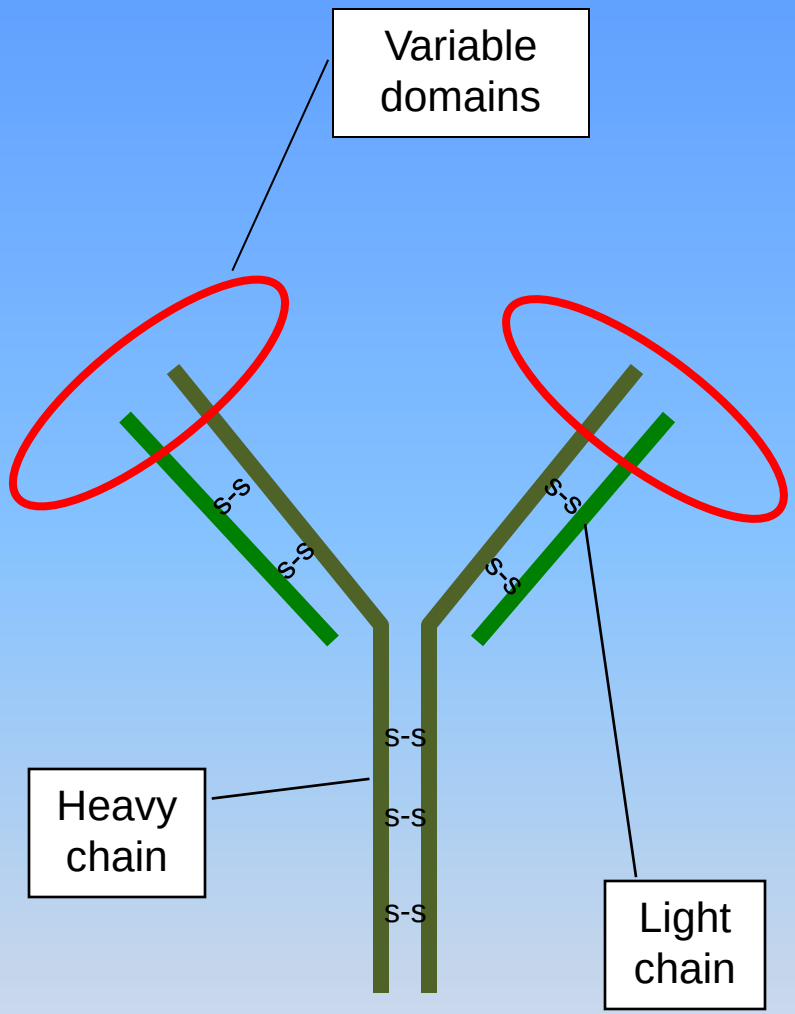
Light scatters in all directions, but SSC usually measured 90 degrees from incident light

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.

Antibodies

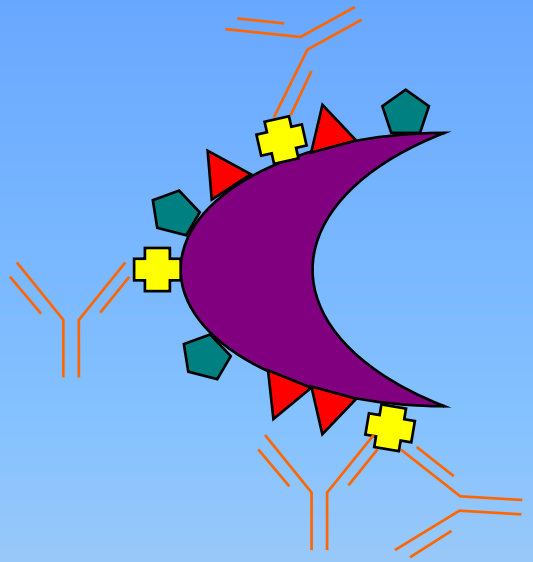
- Bind to specific proteins or epitopes on a cell
- Immunoglobulins come in 5 classes
  - IgG, IgA, IgM, IgD, IgE
- IgG-most often used in cytometry.
  - Heavy chain determines class and subclass (1, 2a, 2b, 3, 4)
  - Light chain either Kappa or Lambda
  - Constant domain gives the structure, variable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



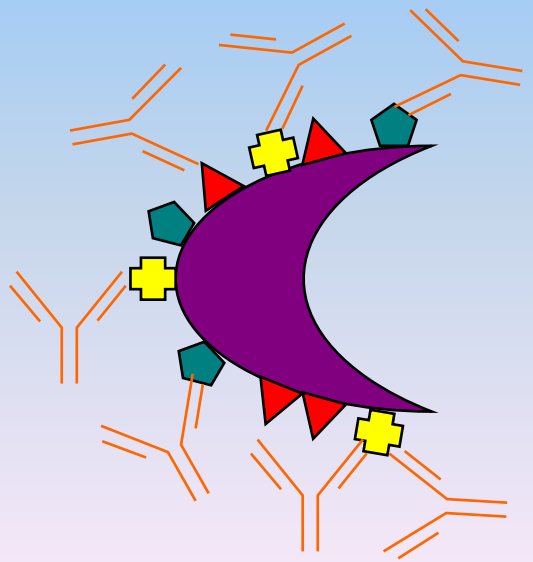
IgG

Antibodies 2

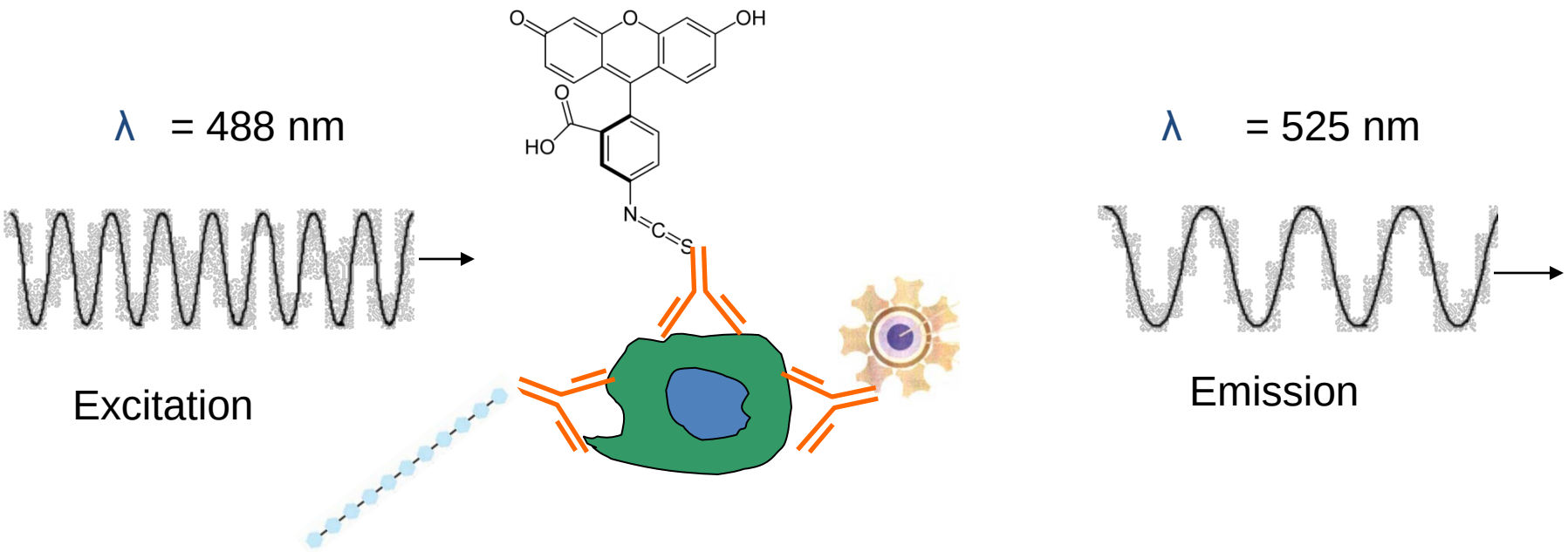
- Monoclonal
  - all antibody molecules are immunochemically identical and recognize the same epitope on an antigen
  - High specificity, no lot to lot variability in affinity
  - May not recognize fixed samples.



- 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.



Antibodies labeled with a fluorescent marker



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.

The absorbed energy is released by:

- -Vibration and heat dissipation (results in loss of some of the stored energy)
- -Emitting photons of a longer wavelength (lower energy than incident light)

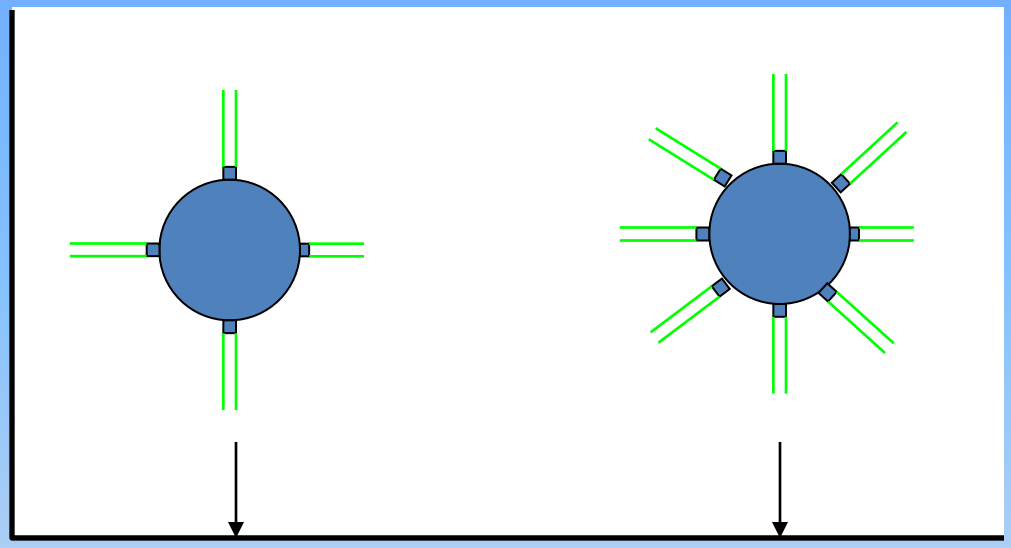
Magnitude of shift="Stokes Shift", specific to each fluorochrome

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.

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

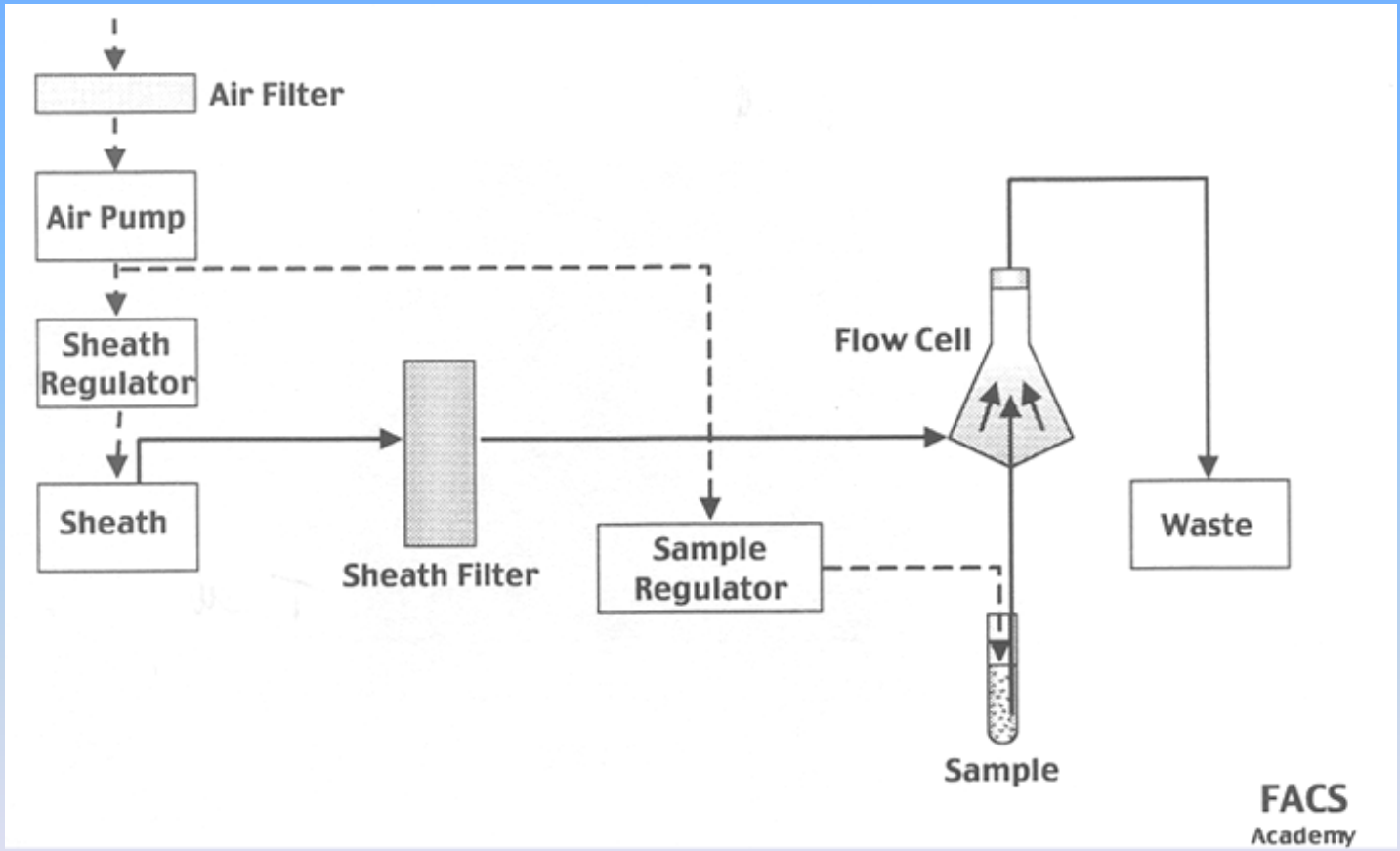


Fluorescence Intensity

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

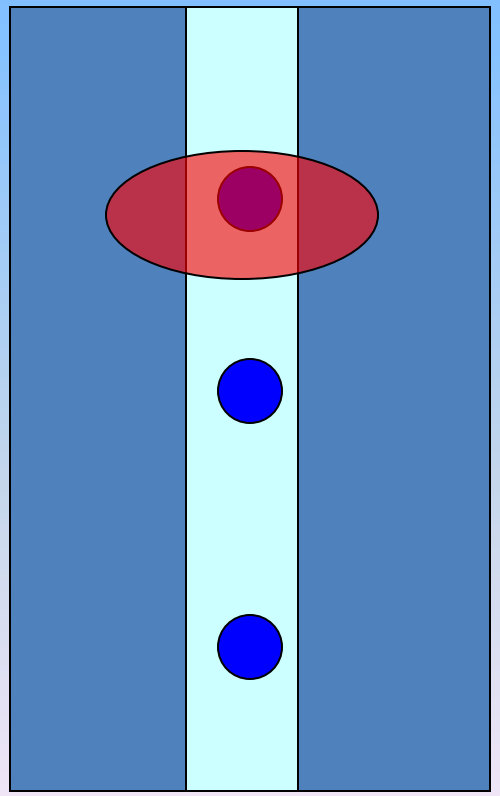
Fluidics



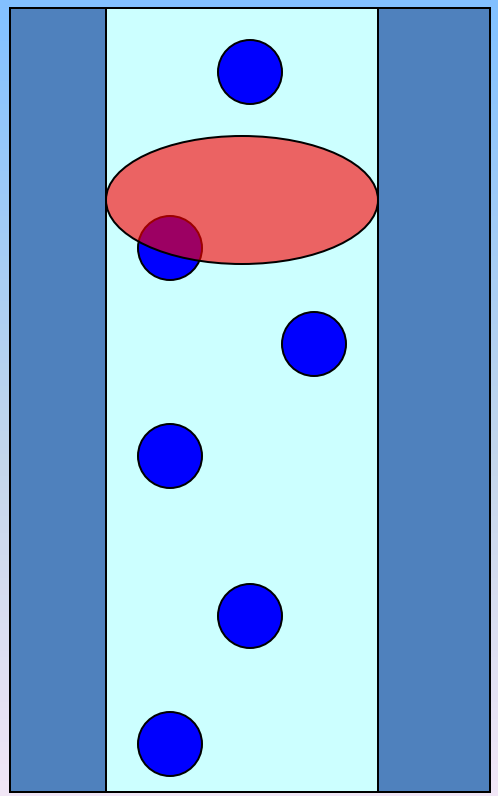
Most cytometers set up same way—sheath fluid supply, inject sample into flowing stream, quartz cuvette for interrogation.

Effect of flow rate on particle placement

"Low" flow rate



"High" flow rate



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

Optics can be divided into 2 parts

-Excitation Optics consist of:

- One or more lasers
- Lenses to shape and focus the beam(s)

-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

Lasers

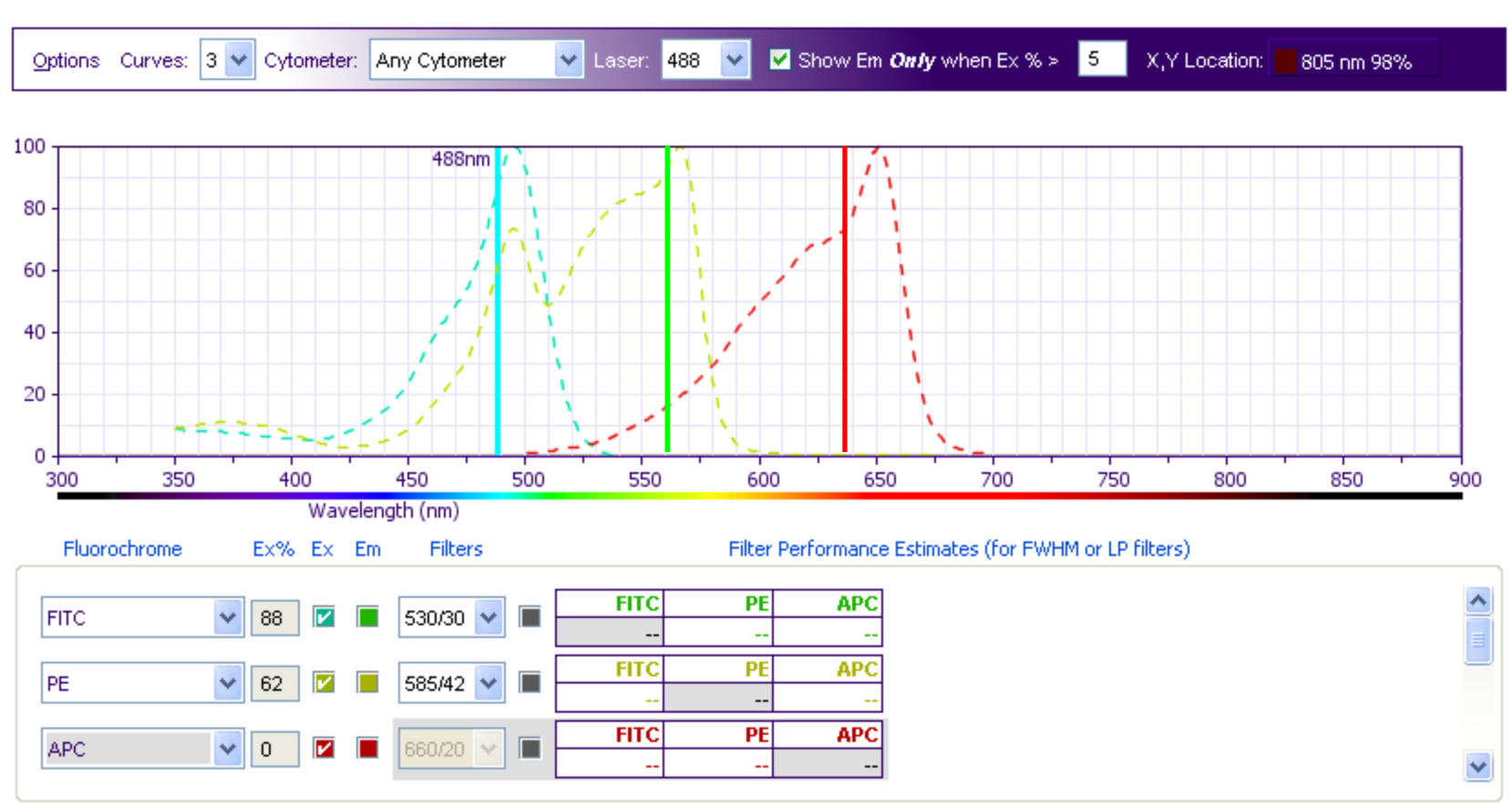
Cytometers will have one or more lasers:

- The laser color determines what fluorochromes can be used on that instrument, unlike a microscope that uses white light with an “excitation filter” to choose the optimum wavelength.

- Common excitation wavelengths:
  - 488 (blue)
  - 635 (red)
  - 405 (violet)
  - 532 (green)
  - 350 (UV)
  - 561 (yellow-green)

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

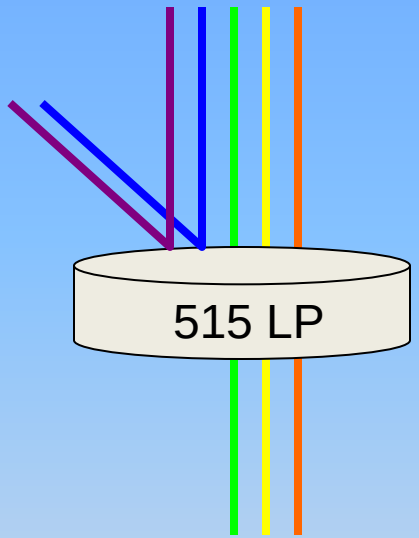
-PE near max with 561 (yellow-green).

-APC requires 635 excitation

-Multiple laser lines add to the # of parameters that can be measured simultaneously

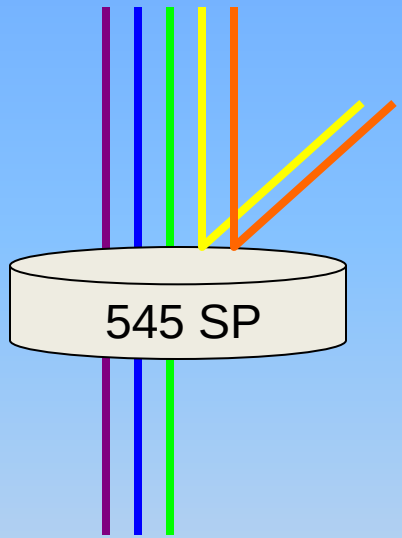
Collection Optics: Separating the Spectrum

Optical filters--3 main types



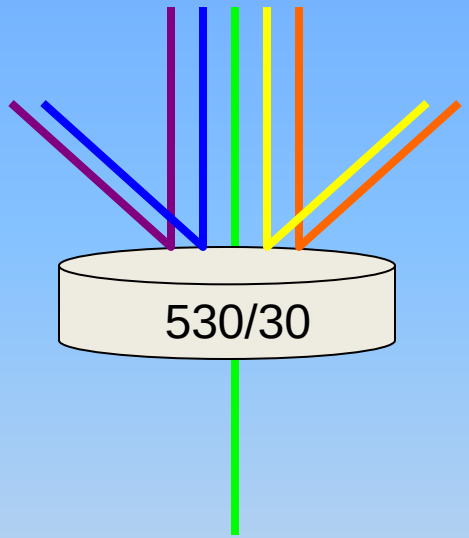
Long pass

Longer than cutoff passes through, shorter is reflected



Short pass

Shorter than cutoff passes through, longer is reflected

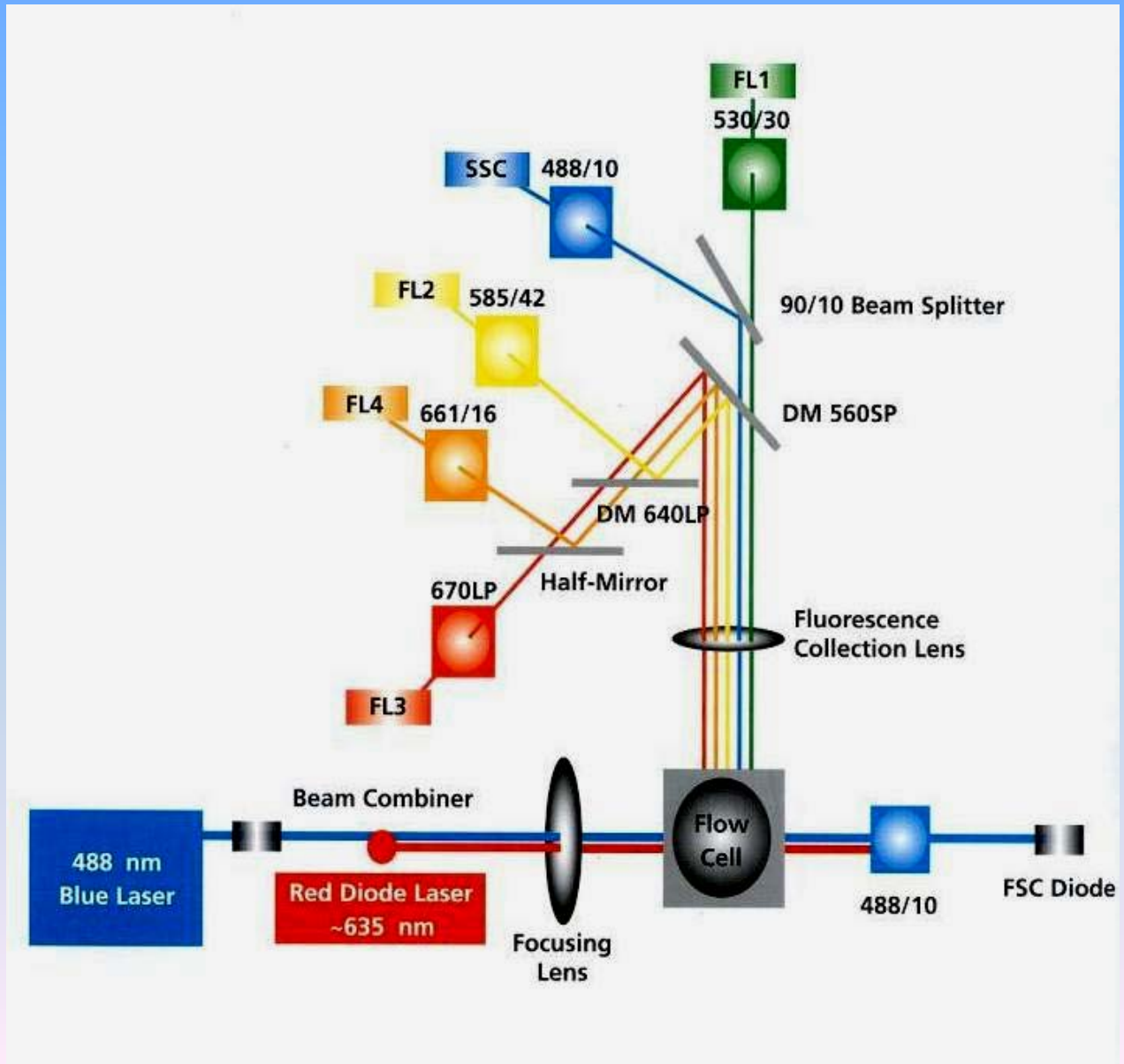


Band pass

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

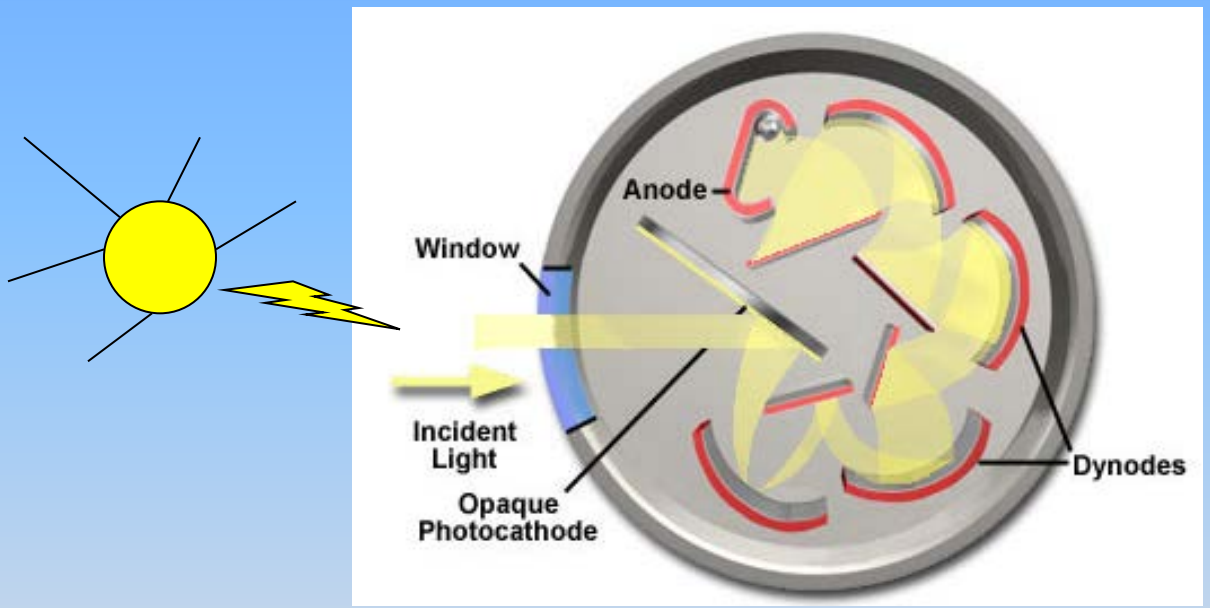
Optical Bench



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



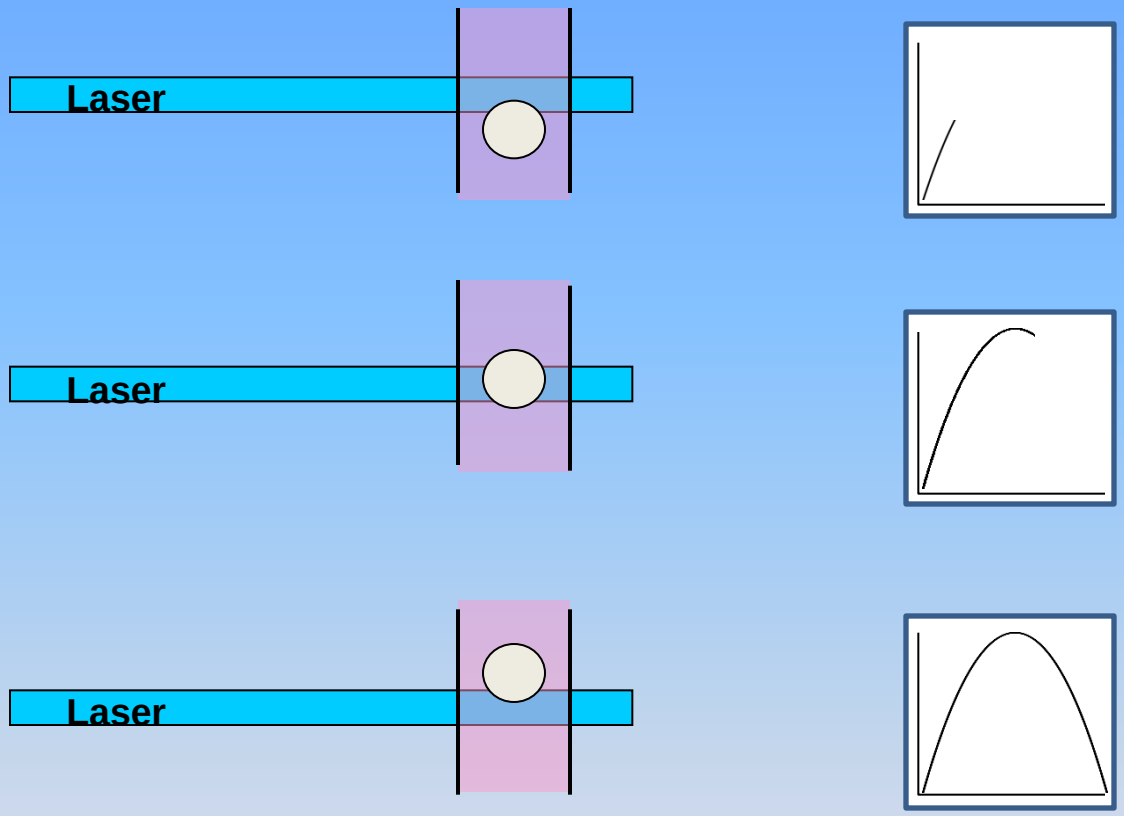
PhotoMultiplier Tube (PMT) and photodiode—generate voltage when struck by photons. PD used for FSC which produces more light.

PMT amplifies the signal depending on voltage supplied.

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

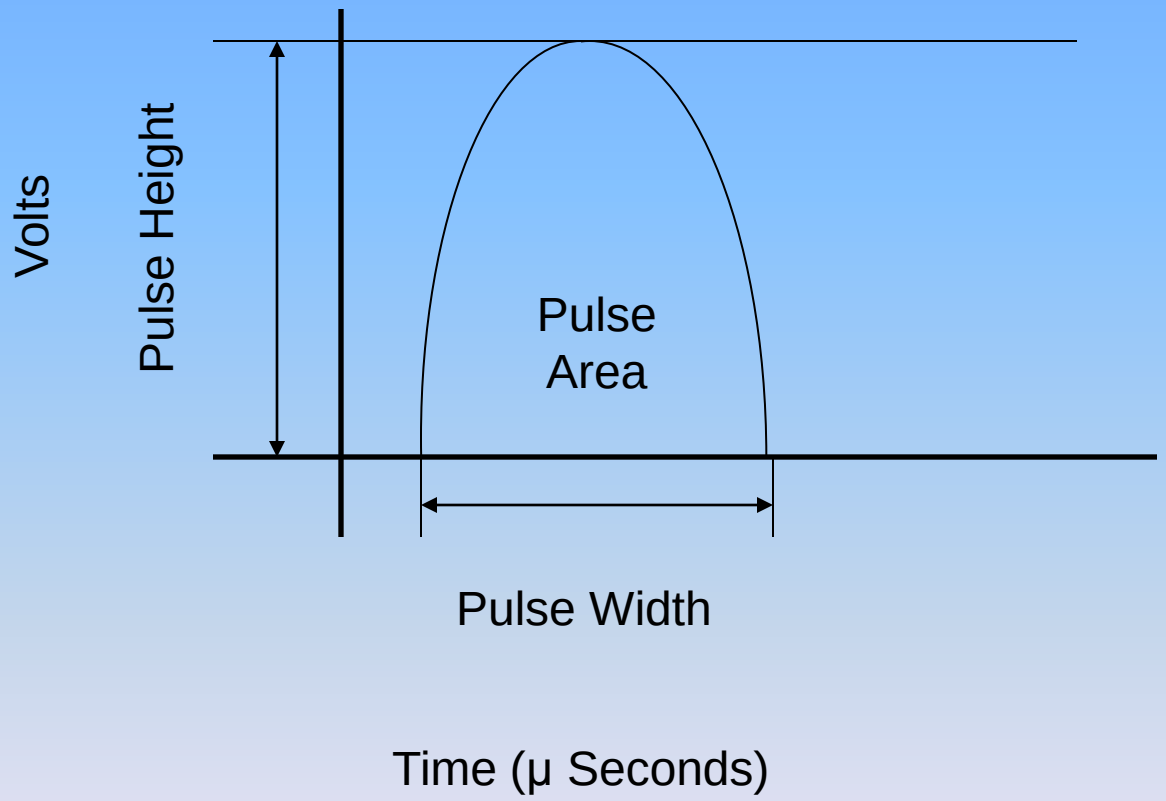
http://micro.magnet.fsu.edu/primer/java/digitalimaging/photomultiplier/sideonpmt/index.html

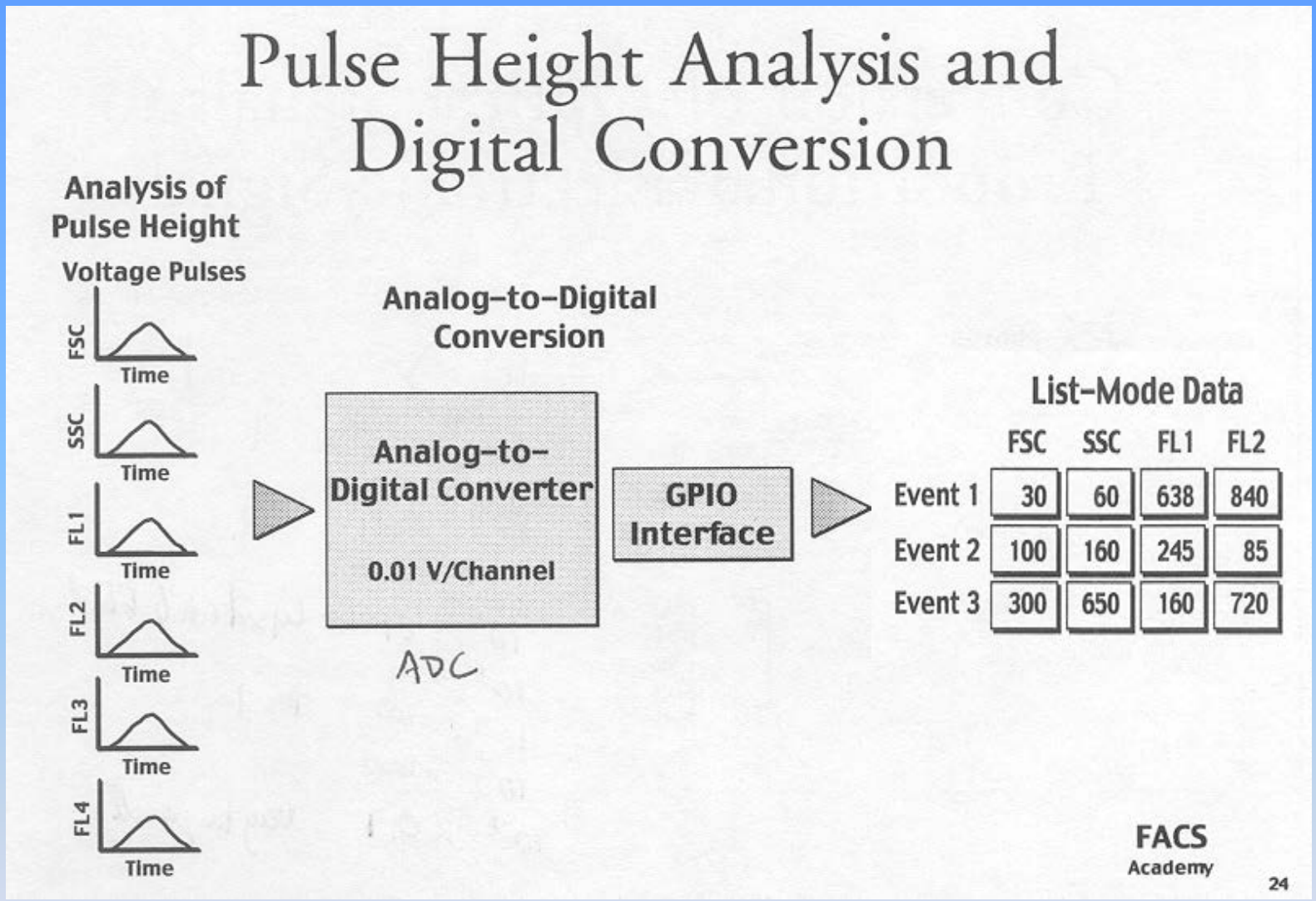
Creation of a Voltage Pulse



-Maximal signal when particle is entirely within the laser beam.
-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

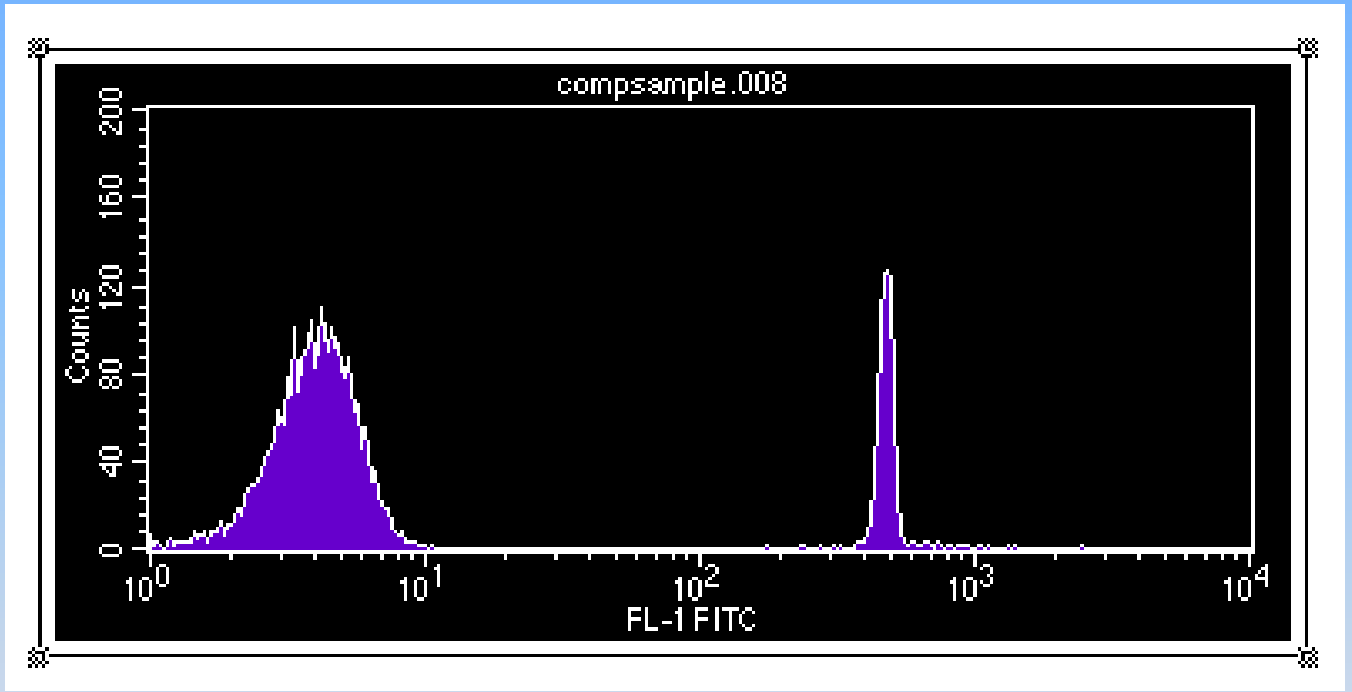




Voltage signals are converted to a value and sent to a computer.
Data stored in “list-mode” format, where each event has a value for each parameter being measured.
Files are saved as “.fcs” (Flow Cytometry Standard)

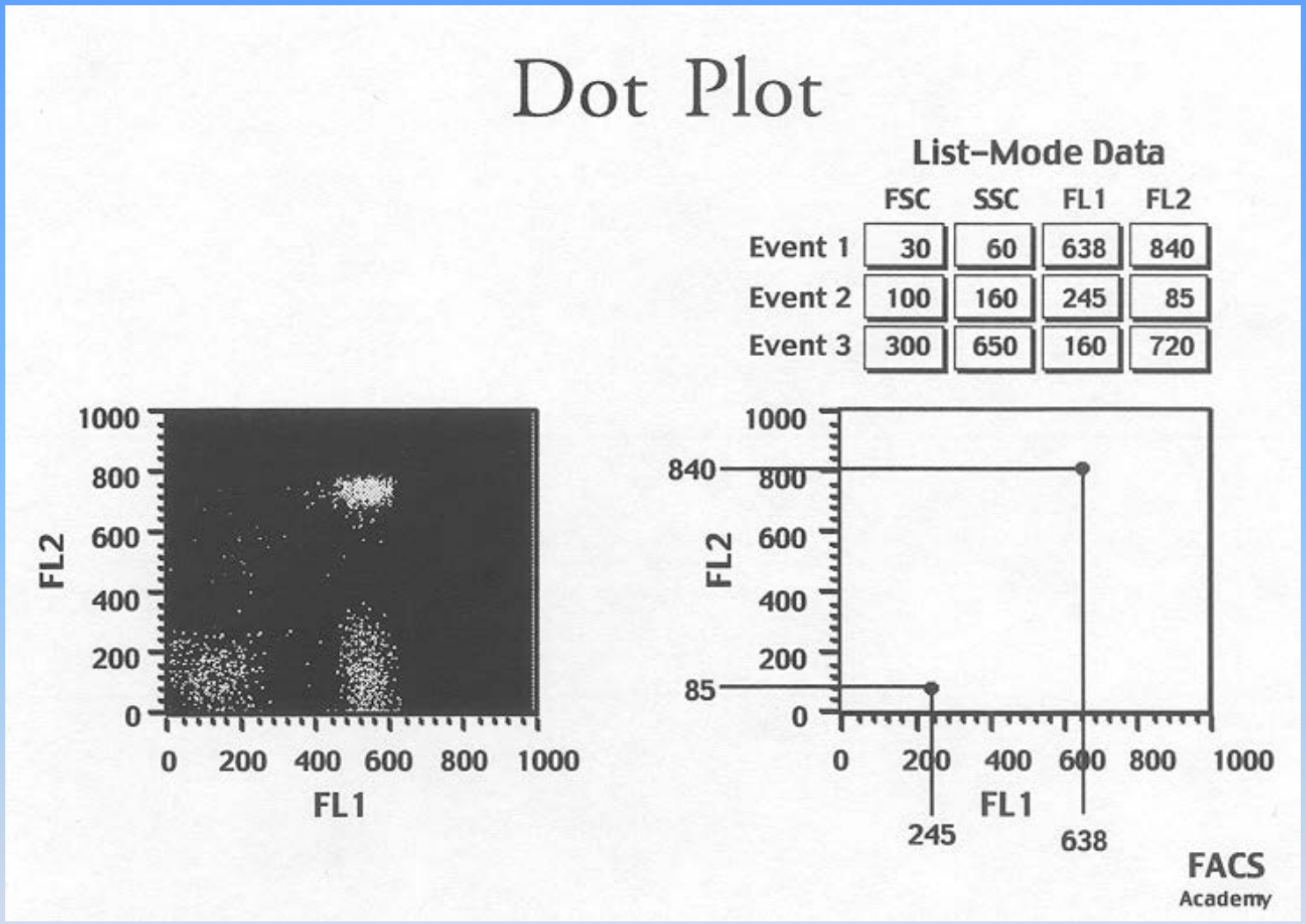
Histogram

Single Parameter



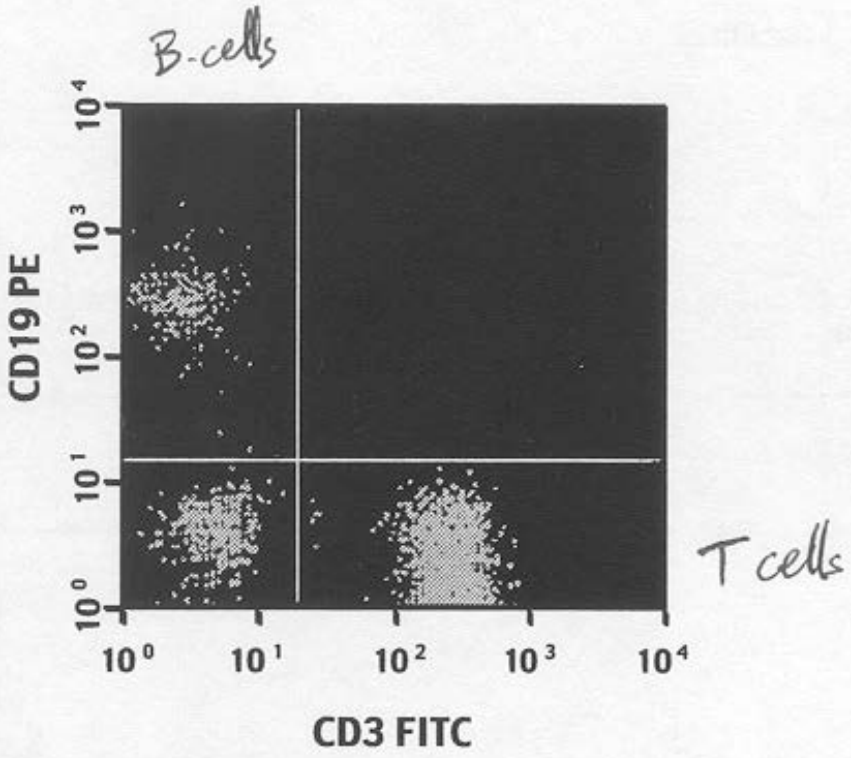
Histogram displays the number of events with a given signal intensity

Dot Plot



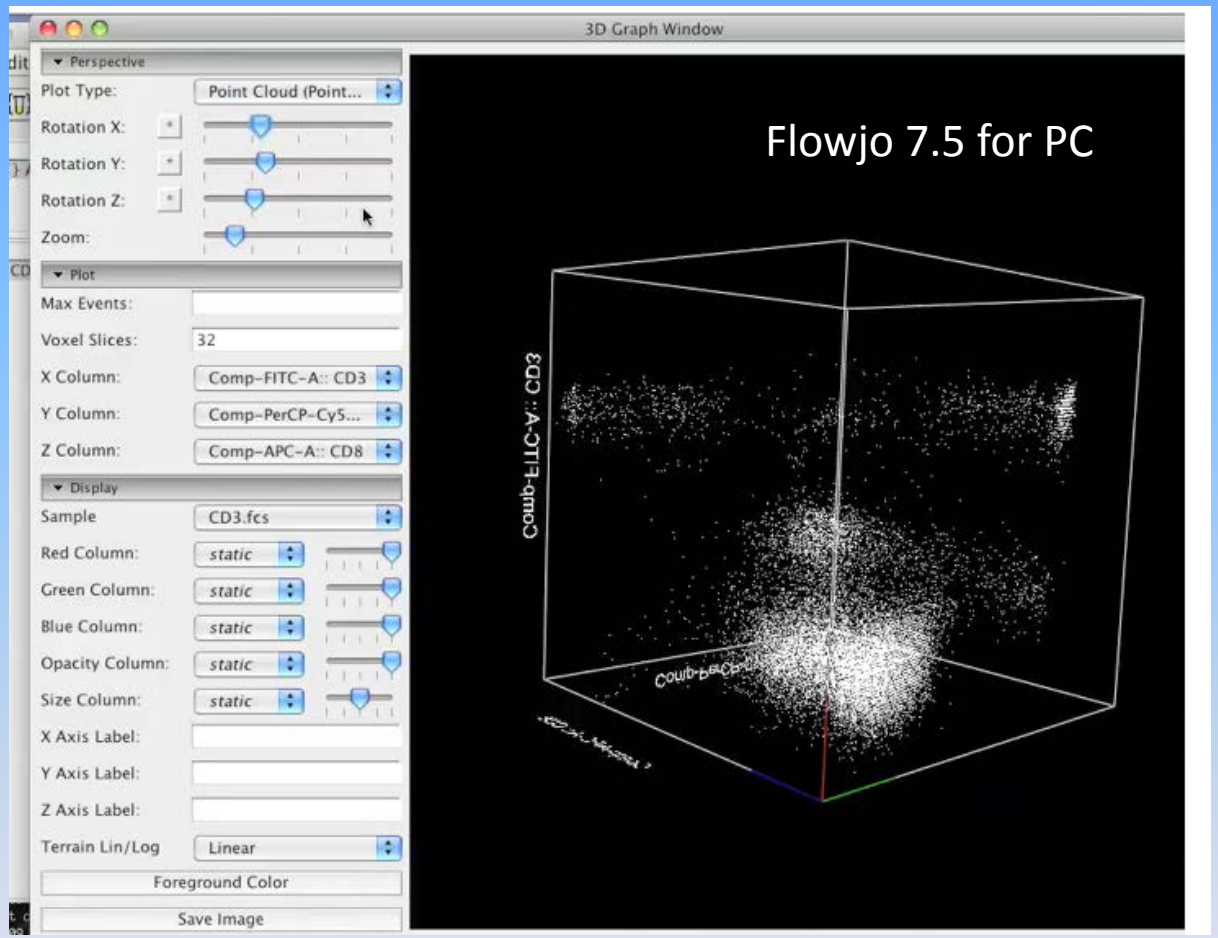
Bivariate plot or bivariate histogram, events map to regions of the graph. Allows you to visualize different populations among particles– look at 1 parameter would see only + or -, looking at 2 you can identify ++.

Two-Color Cell Analysis



Classify cells based on signal as B or T cells.
Can then look at only T cells and use other parameters to further classify them.
Identify CD4, CD8, GFP+, G1 cell cycle, etc…

3D plots in Flowjo



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

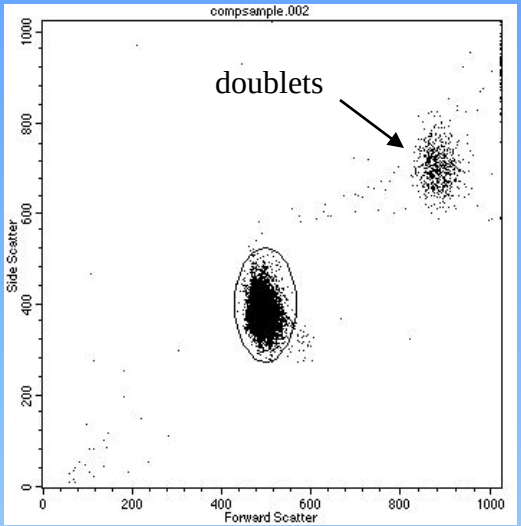
https://www.youtube.com/watch?v=_kBUoSj0C6k

Instrument Setup

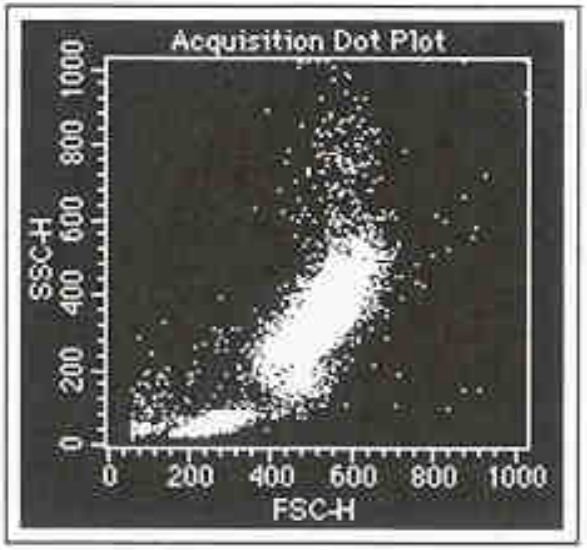
- User adjusts sensitivity of detectors so that:
  - Events of interest are on scale
  - “Negative” fluorescence on the left/bottom, providing maximum dynamic range for positive signals

Setting FSC and SSC

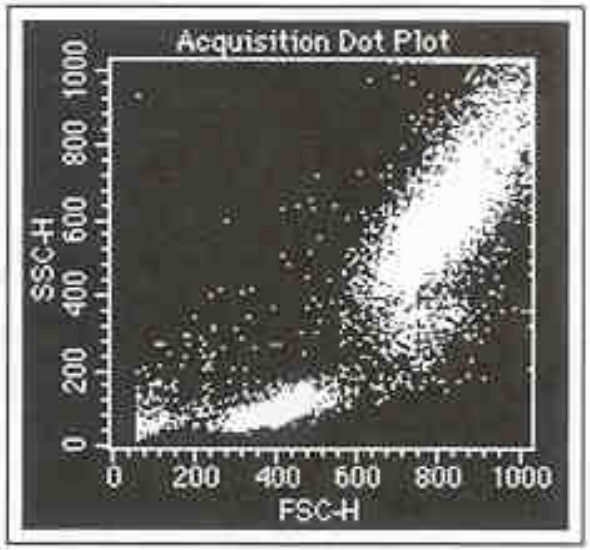
Beads



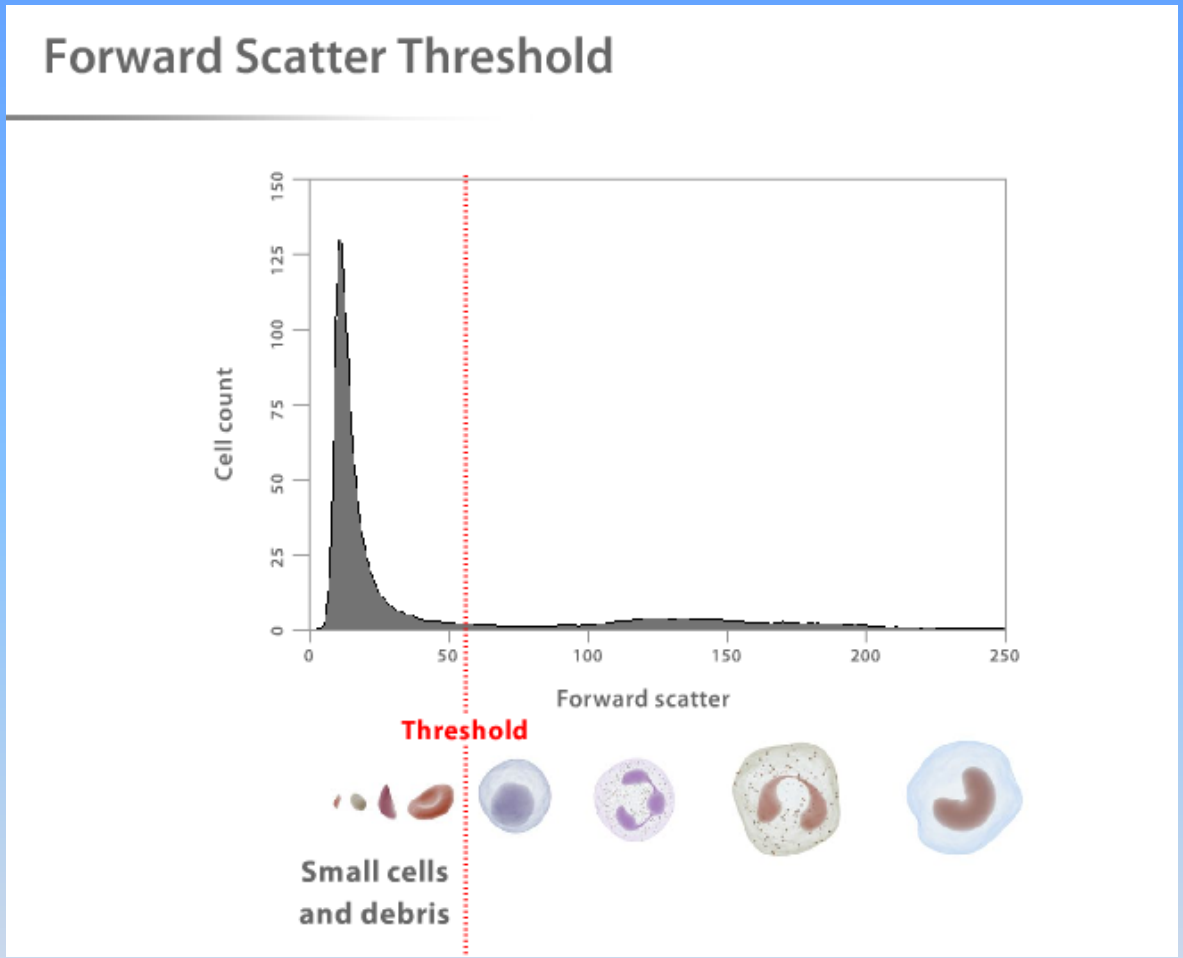
Lysed Whole Blood



Before optimizing

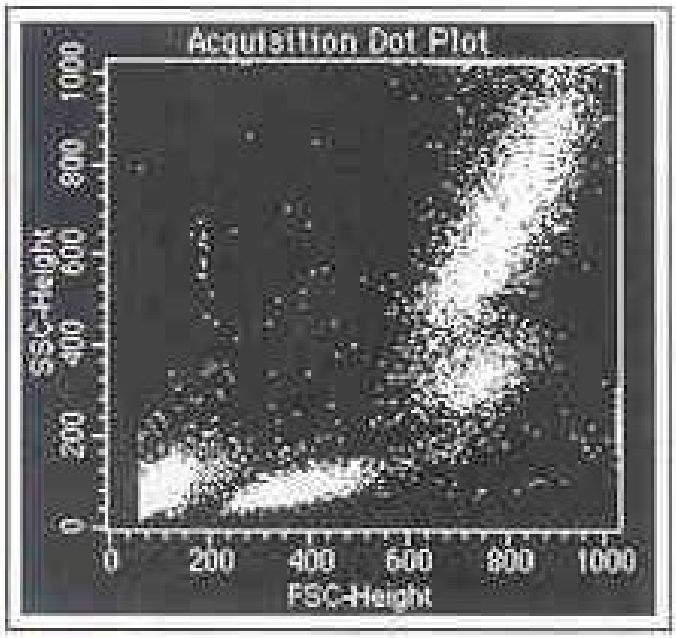


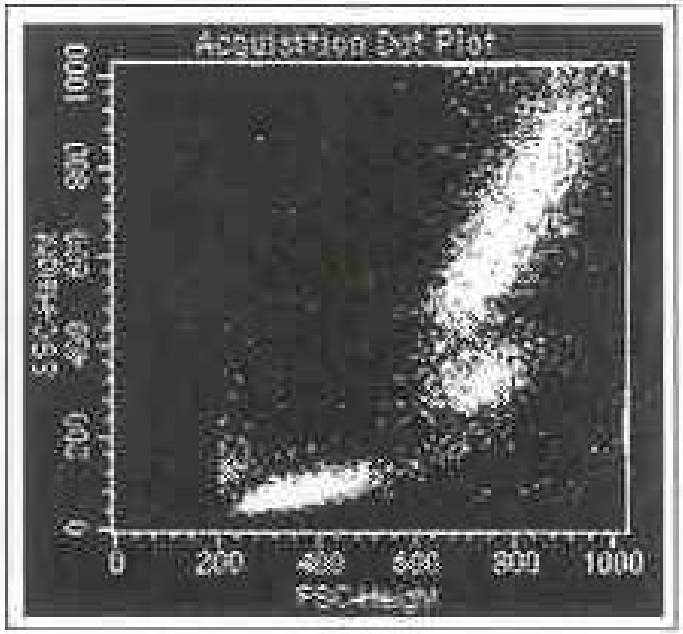
Optimized



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

Adjust FSC Threshold





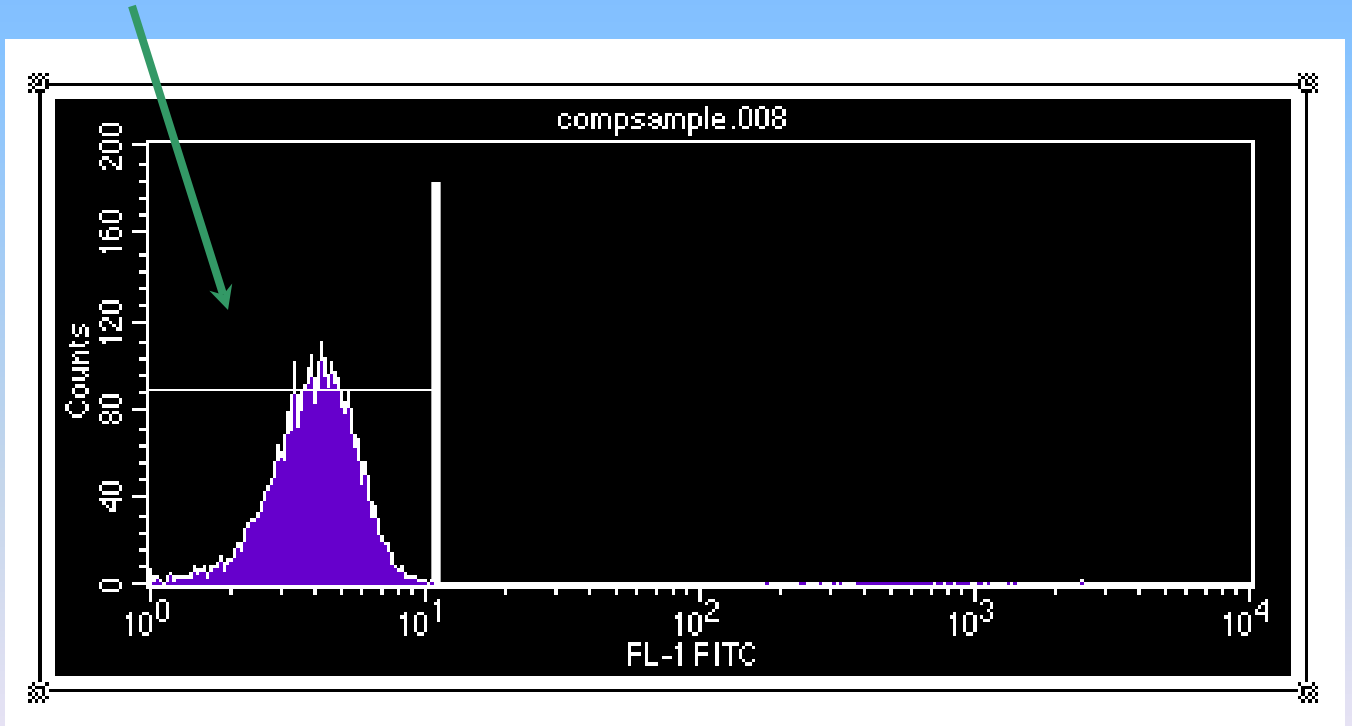
Before

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

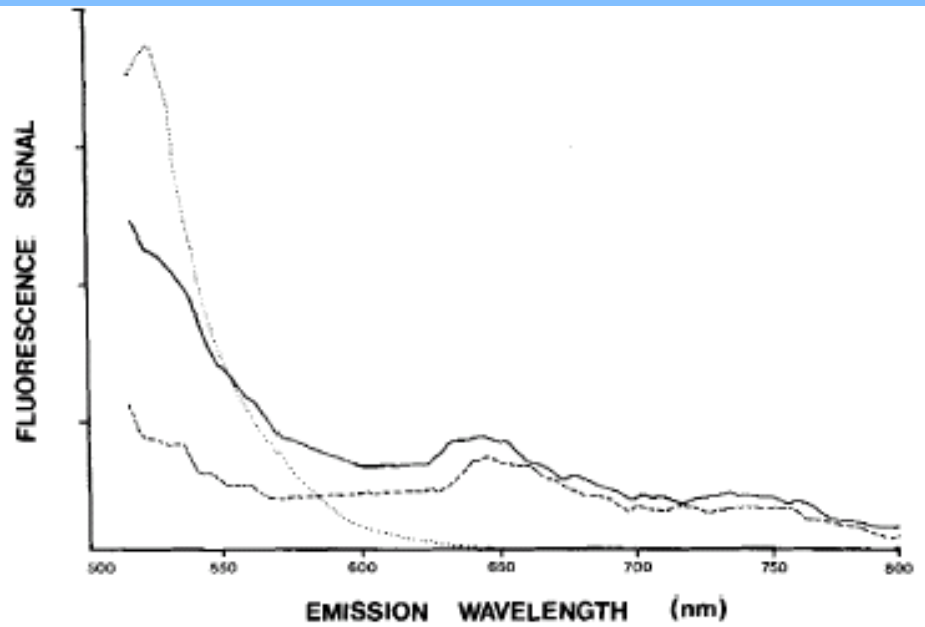
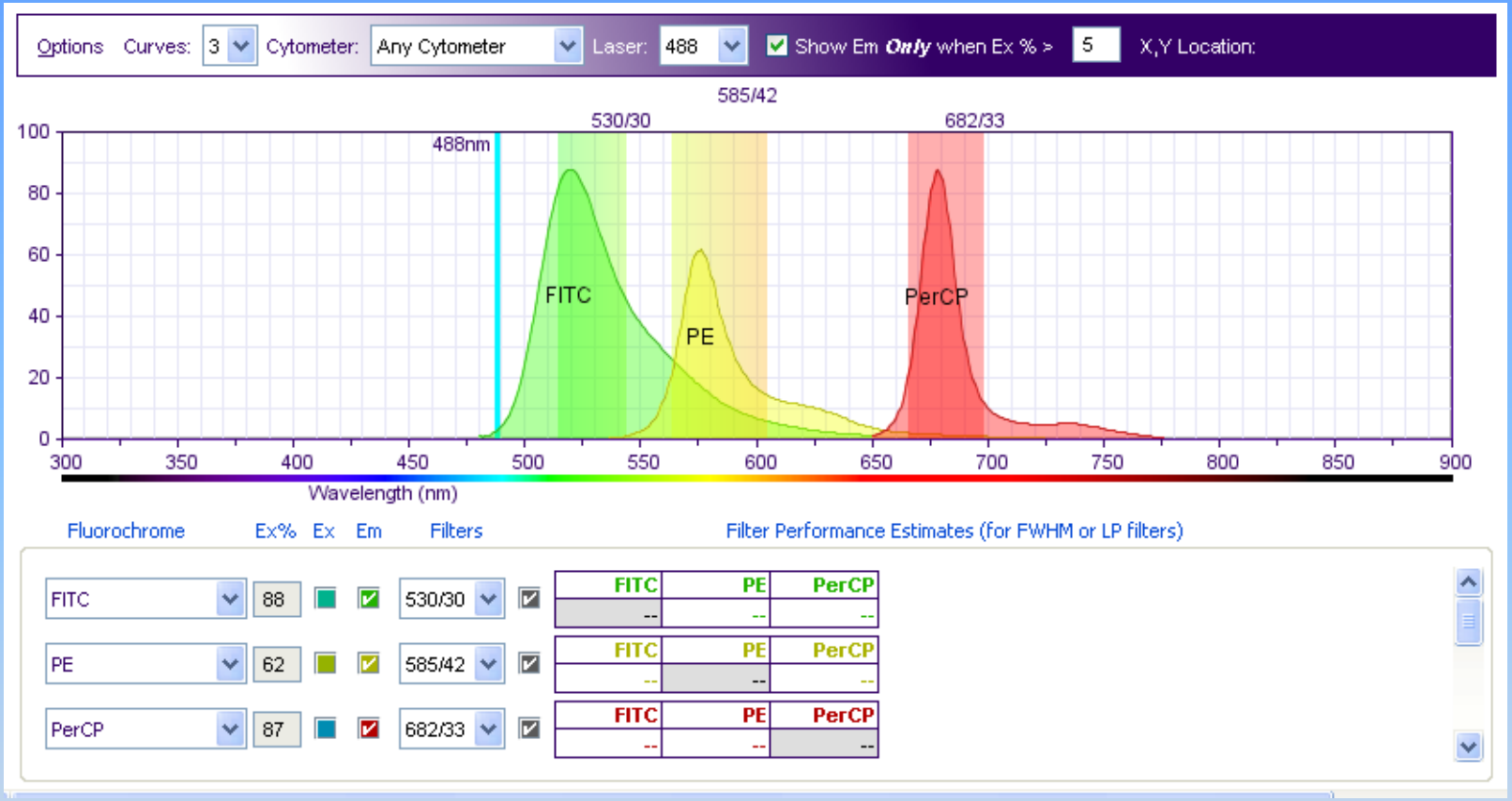


FIG. 1. Fluorescence emission spectra of L cells (solid line), 143 cells (broken line), and FITC-conjugated antibody to Trop-1 (dotted line) excited at 488 nm. Excitation window = 8 nm. Measurements were taken in 5-nm steps. The Raman scatter peak (570–600 nm) has been removed from the spectra. The spectra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

From Cytometry Part A,1987 Mar;8(2):114-9

Emission Spectra



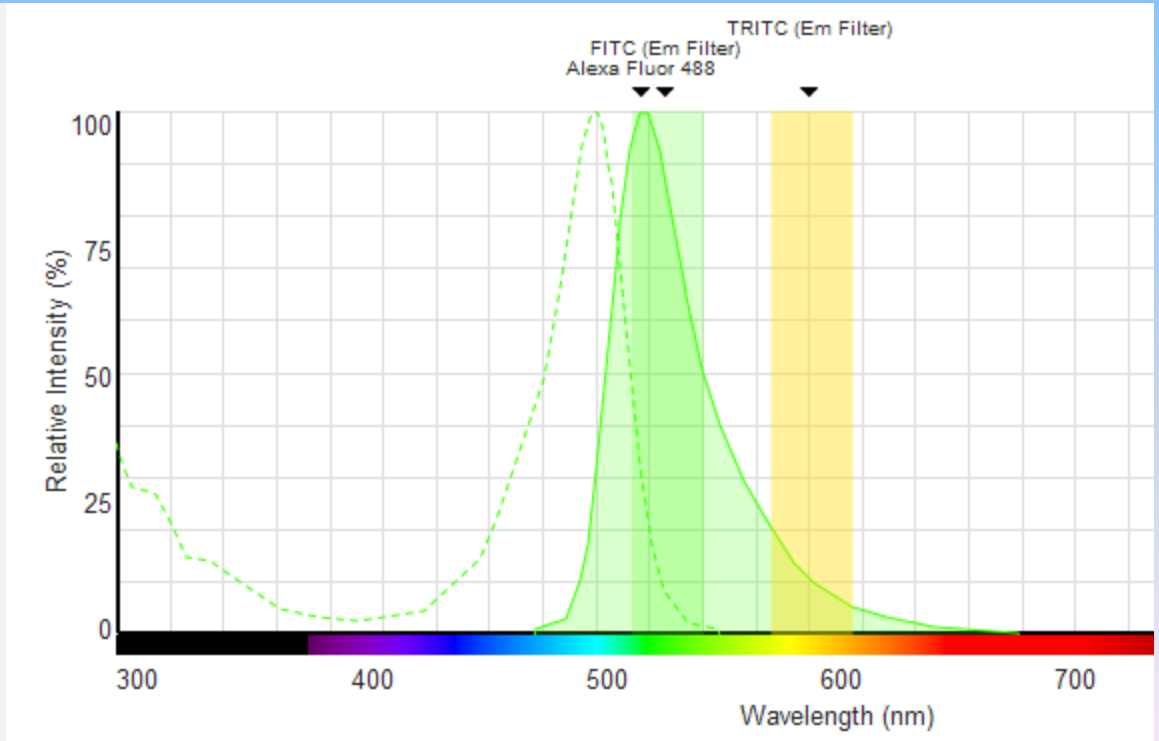
Excitation and Emission spectra of fluorochromes can be viewed using Spectraviewers linked from newenglandcytometry.com, “Helpful Links>Spectraviewers”

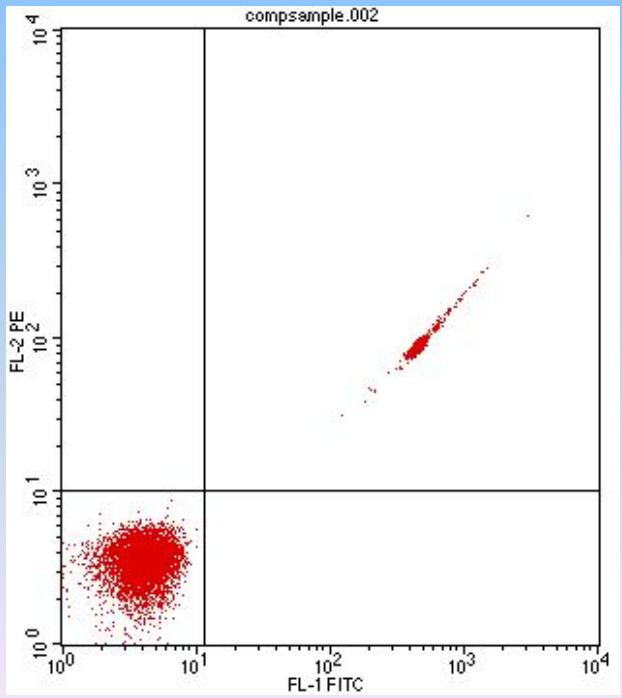
Emission occurs over a range of wavelengths, with measurements usually made at the peak of signal.

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.

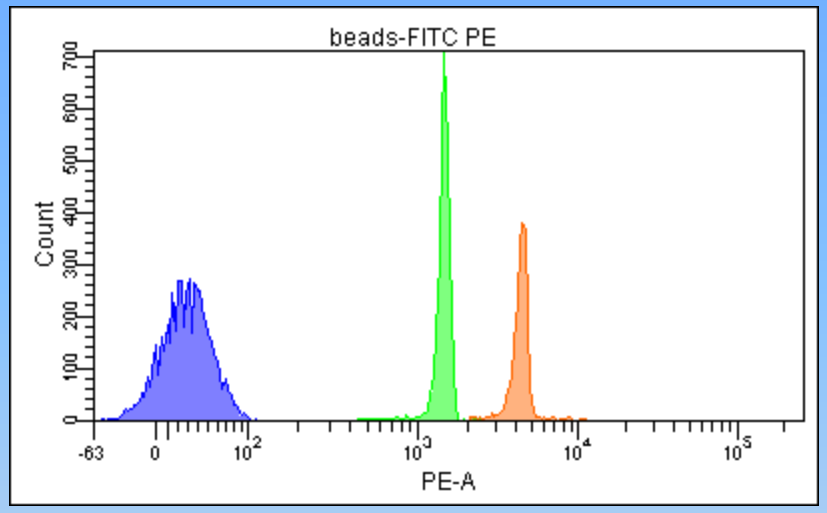




Compensation—FITC and PE

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



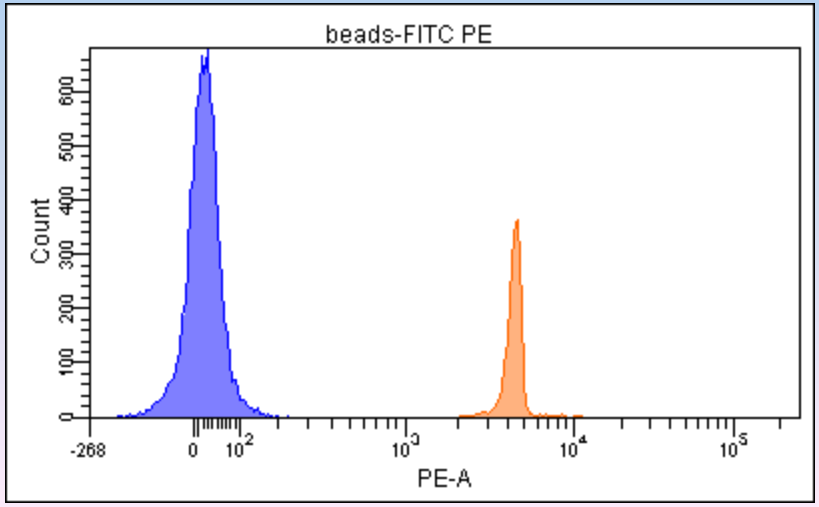
negative

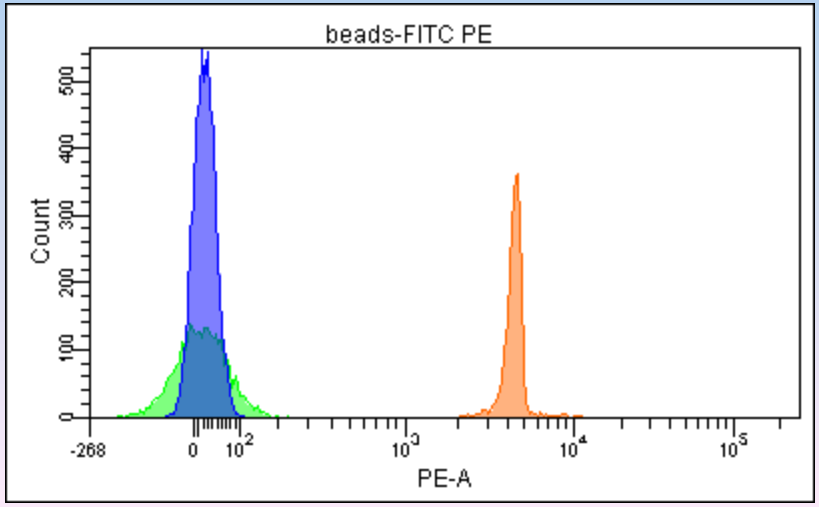
PE labeled

FITC labeled

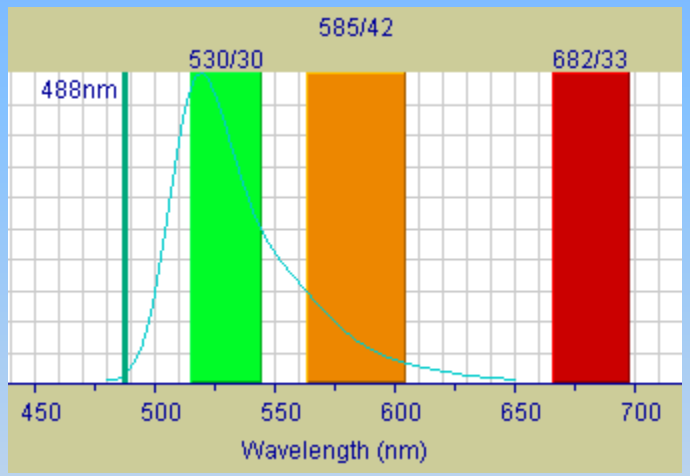
When compensation is properly applied, a single-stained particle will have the same mean signal in all other channels as an unstained particle.

Compensated

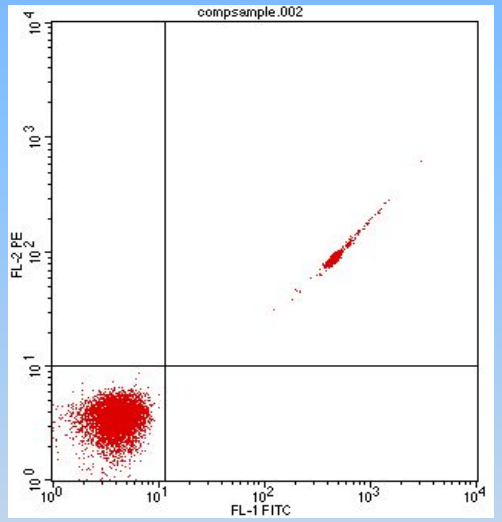




FITC fluorescence Overlap



PE

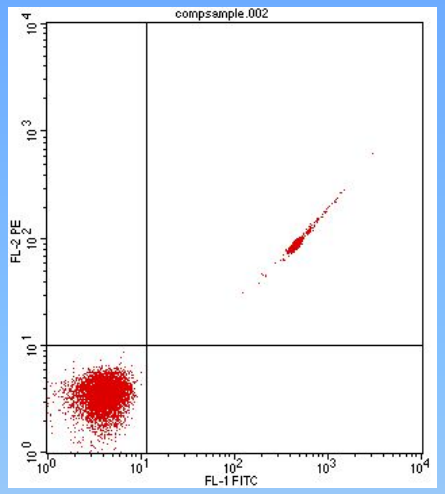


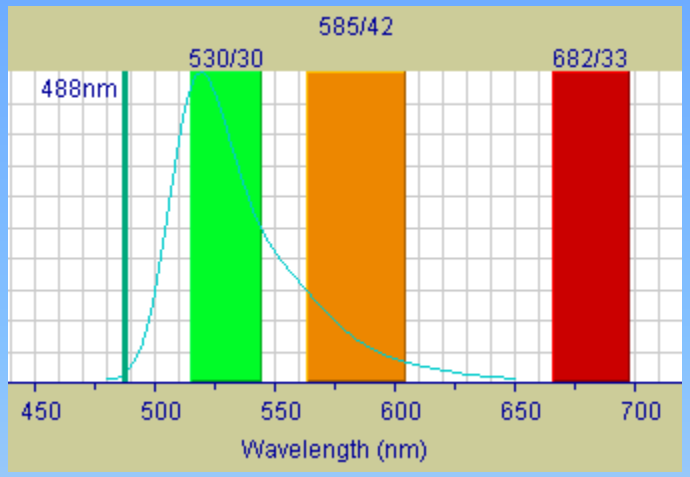
FITC

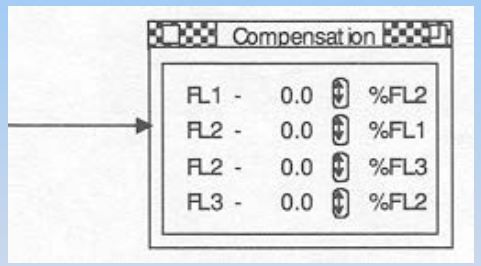
At given voltages, the amount of signal in the spillover detector is proportional to the signal in the primary detector:
100 FITC→20 PE, then 50 FITC→ 10 PE

Set up a ratio between the signals

FITC Compensation

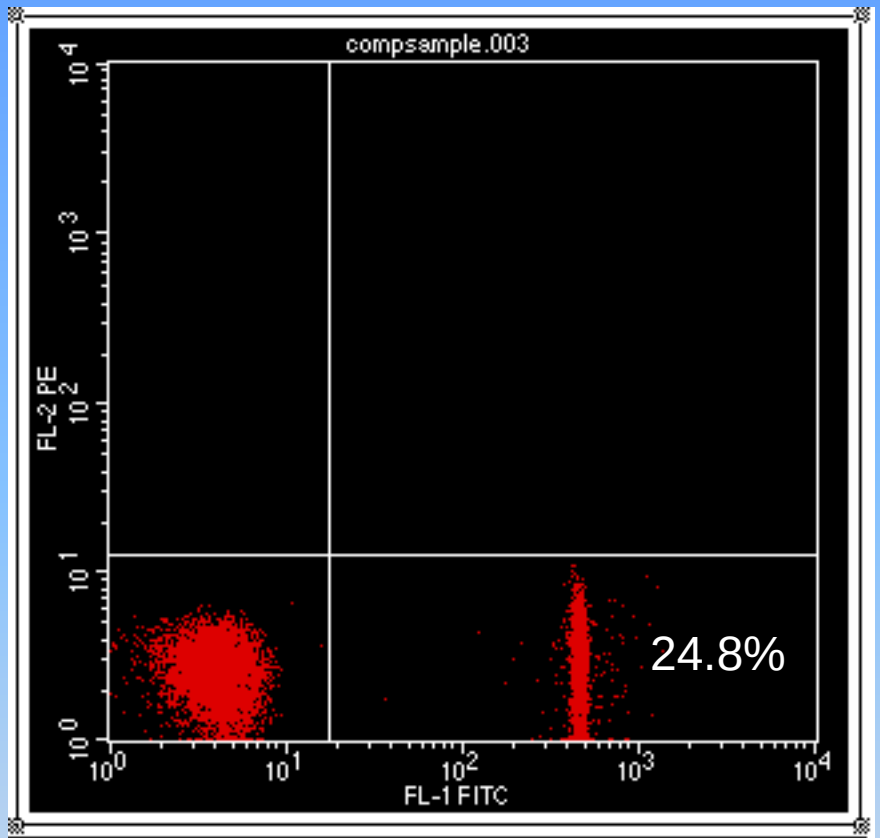






Subtract % of signal in primary channel **from** signal detected in the overlap channel so that the mean signal of + and – populations in overlap channel is equal

Goal:
mean signal equal



Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	0	0.00	0.00	***	***
UR	0	0.00	0.00	***	***
LL	5843	63.46	58.43	4.15	2.68
LR	3365	36.54	33.65	463.87	2.52

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!)

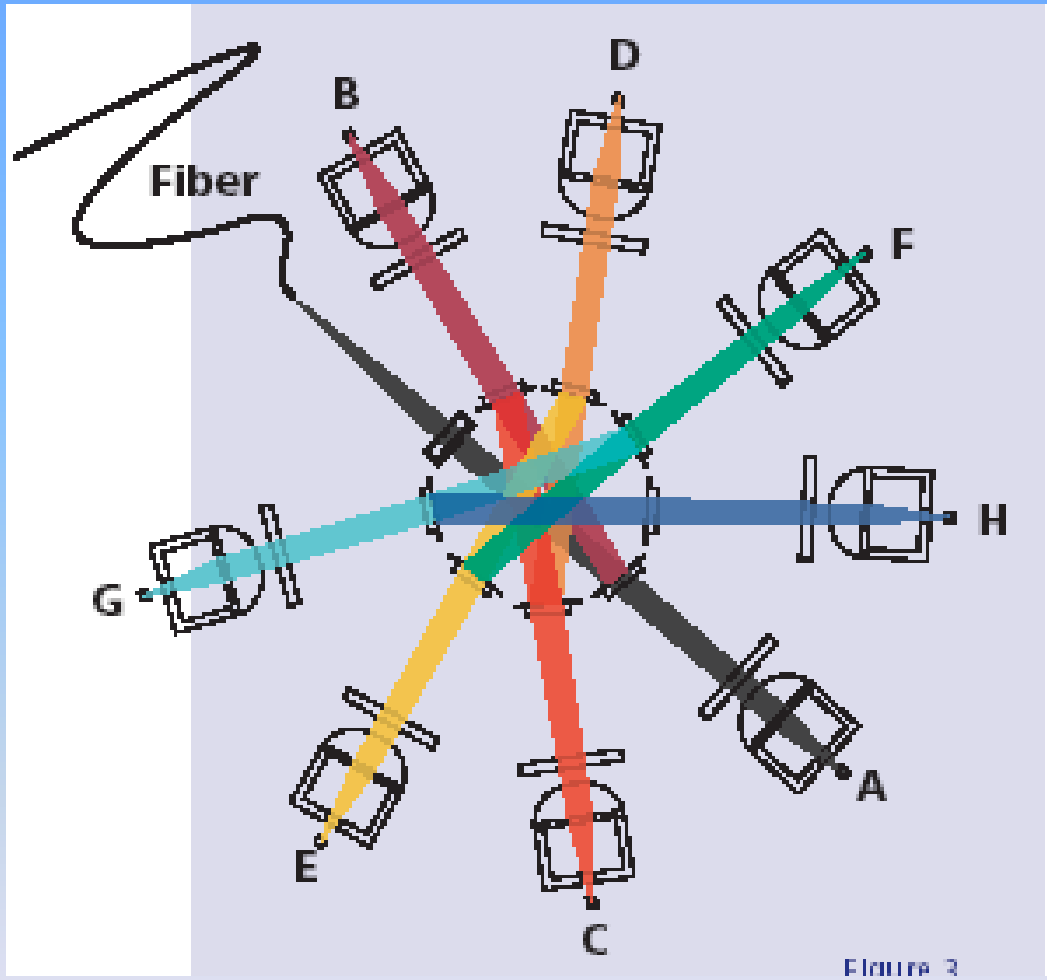
2 Flow Cytometer designs

Analog (FACSCalibur)	Digital (LSR II, Aria)
Older system, constrained by computer capabilities	Newer system, faster computers
Most of processing done with circuits (compensation, Lin vs. Log)	Processing done in computer (Log/Lin display modes, compensation modified post acquisition)
Manual Compensation	Automated Compensation
Fixed optical setup	Configurable optical setup
3000 events per second --"Dead time" between events to clear circuits for new measurement	20,000 events per second

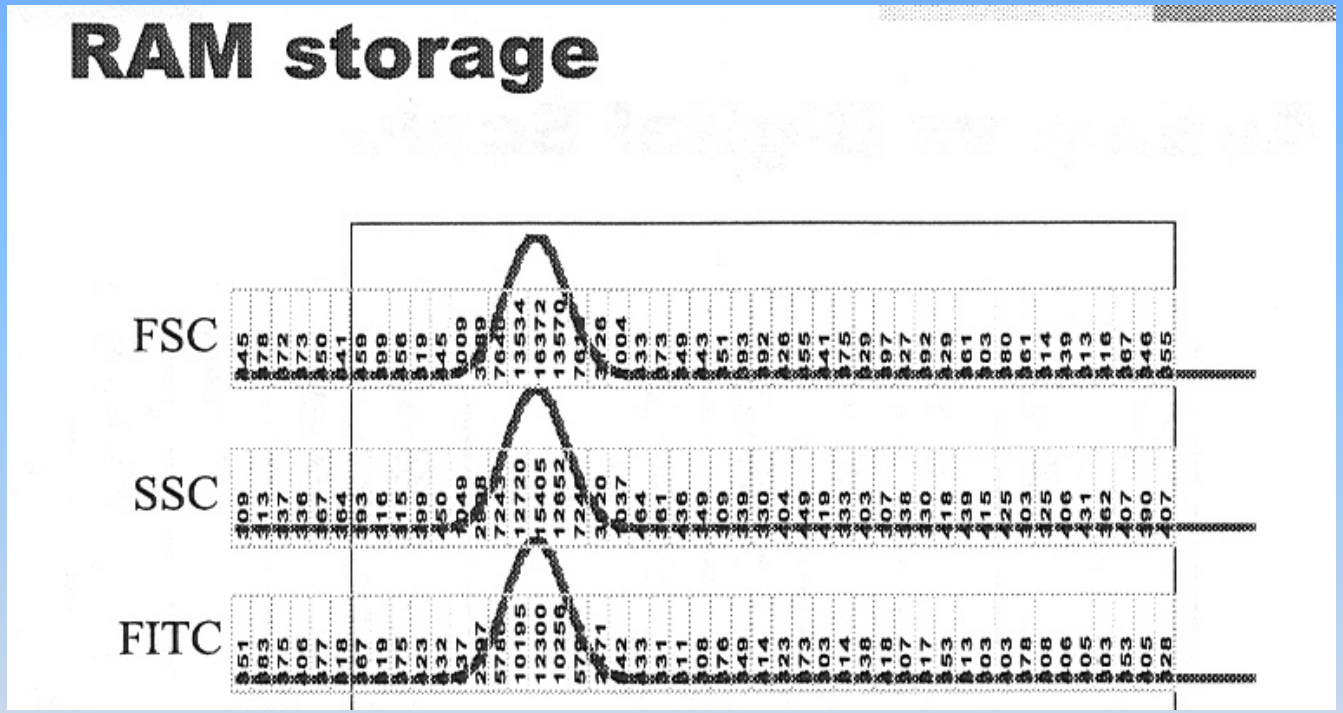
LSR II and LSR Fortessa: Detector array

•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 interchagable

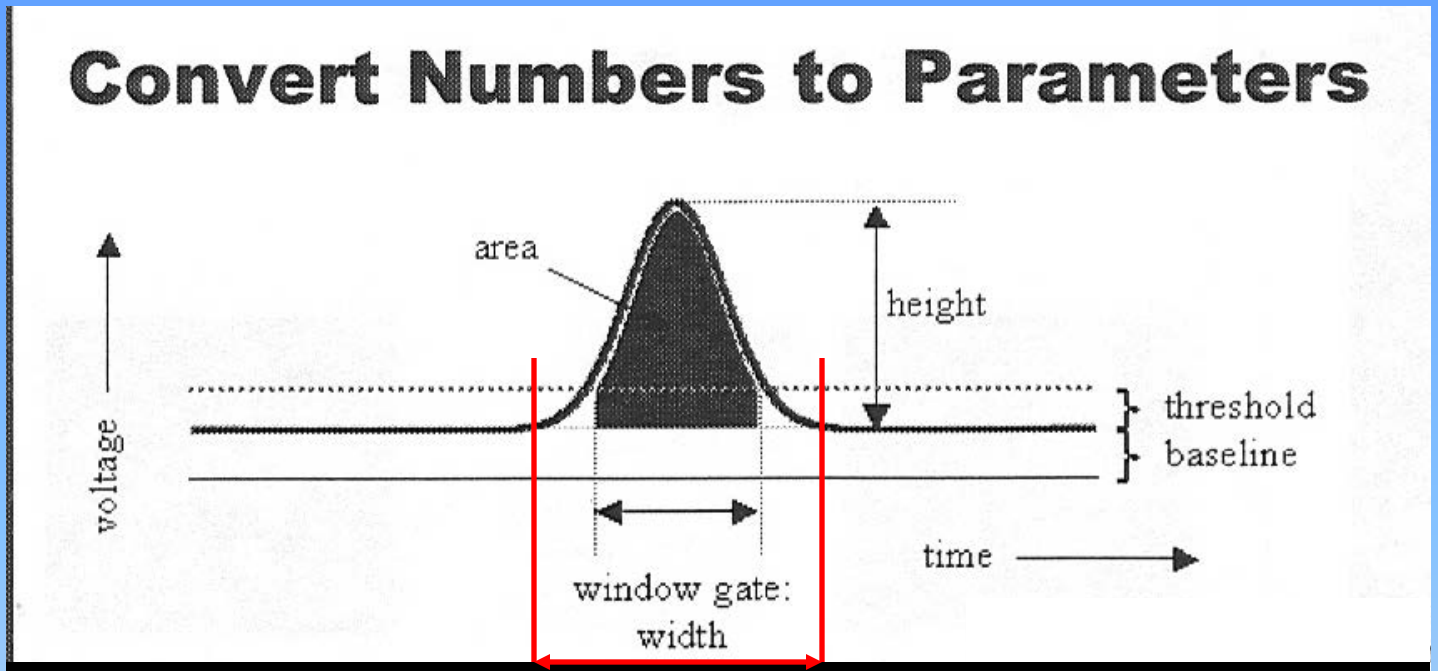


Digital systems



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: area/height x 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

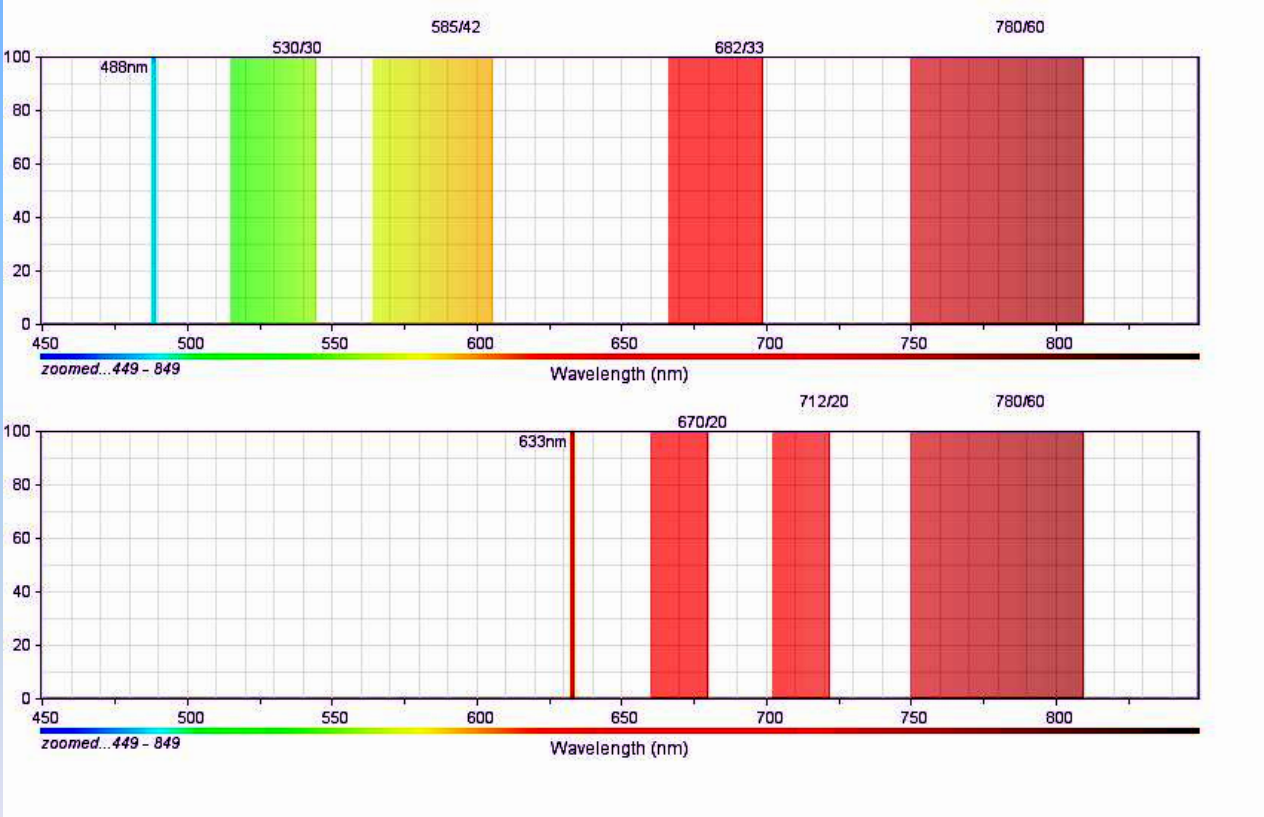
- FACSCalibur (analog)
  - Light signal converted to voltage pulse
  - Pulse subtraction for compensation
  - Log amp converts voltage pulse to proportional log pulse
  - Analog-to-Digital converter assigns value from 0-1023
  - Values for parameters sent to computer
  - Event displayed on monitor

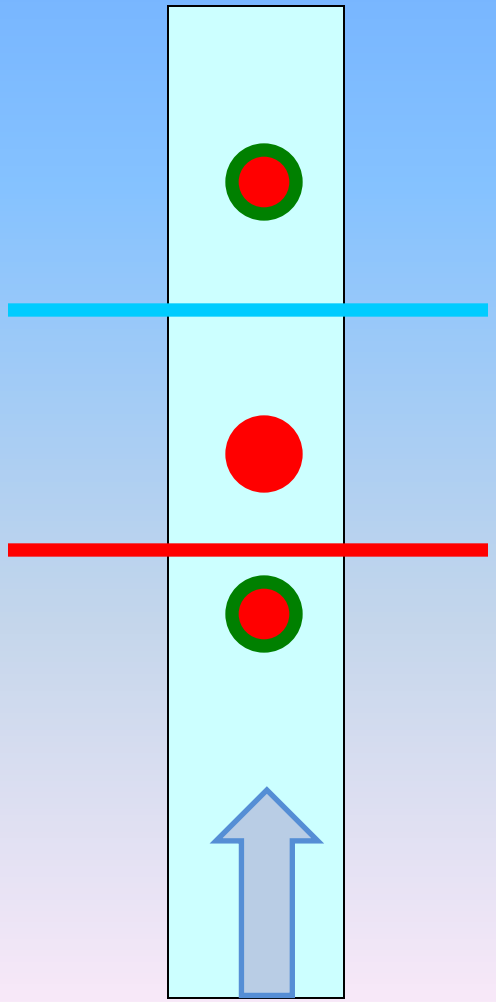
- FACSDiva (digital)
  - Light signal converted to voltage pulse
  - Analog-to-Digital converter assigns value from 0-16,384
  - “Digitized” signals sent to computer, values converted to parameters (A, H, W)
  - Compensation applied mathematically
  - User defines display mode (log, lin, biexponential)

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

Multiple Lasers are spatially separated

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

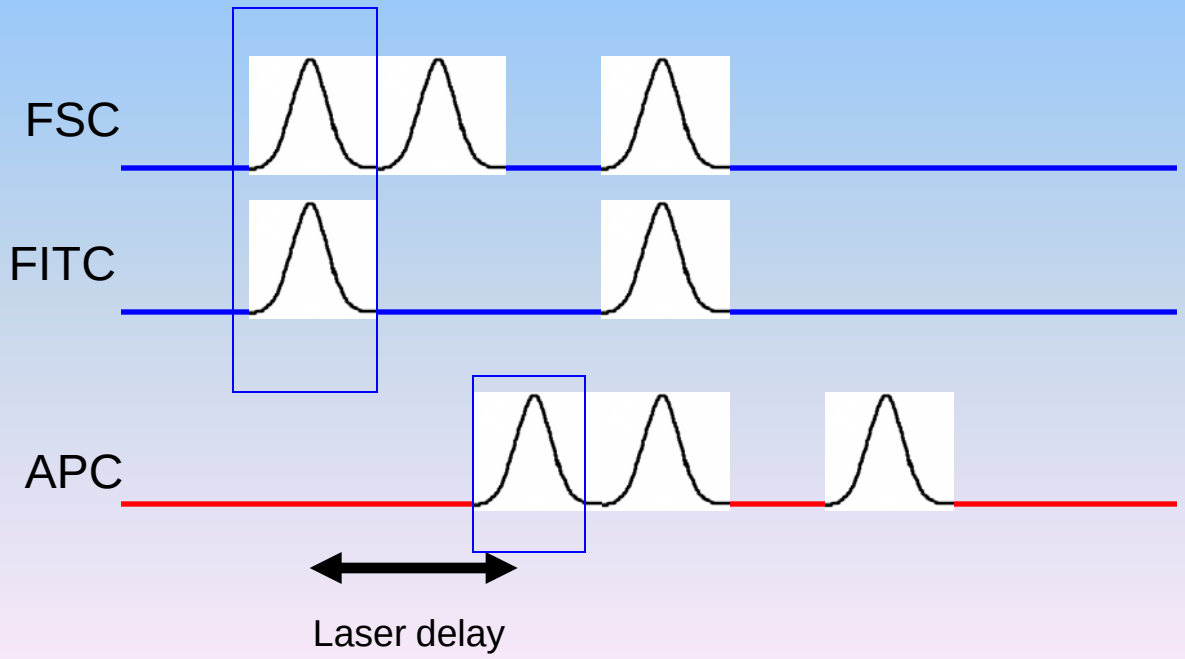


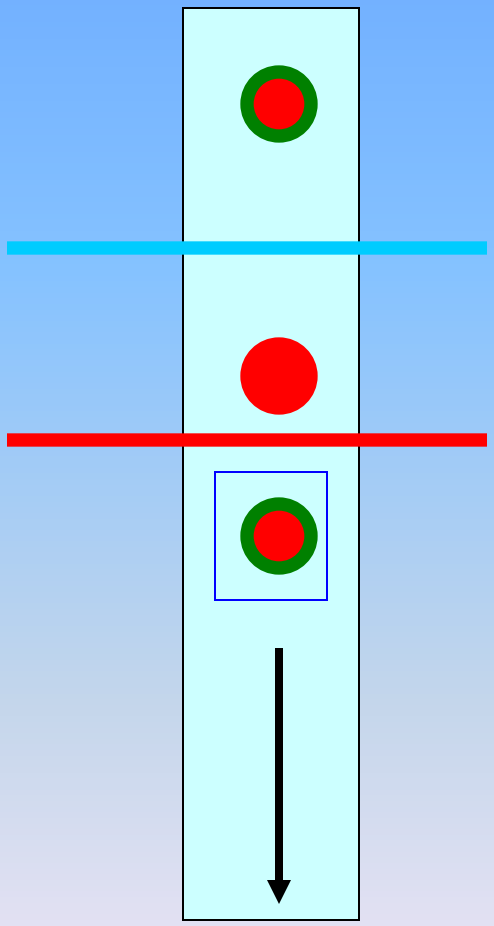


Fully equipped labs can run 5 laser, 18 parameter systems.
New instruments now running 7+ lasers, 30+ parameters.

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.

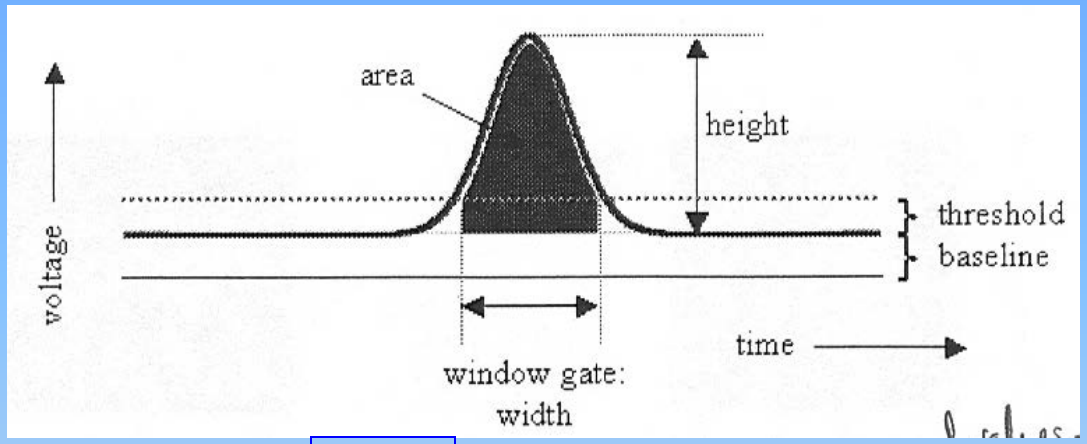


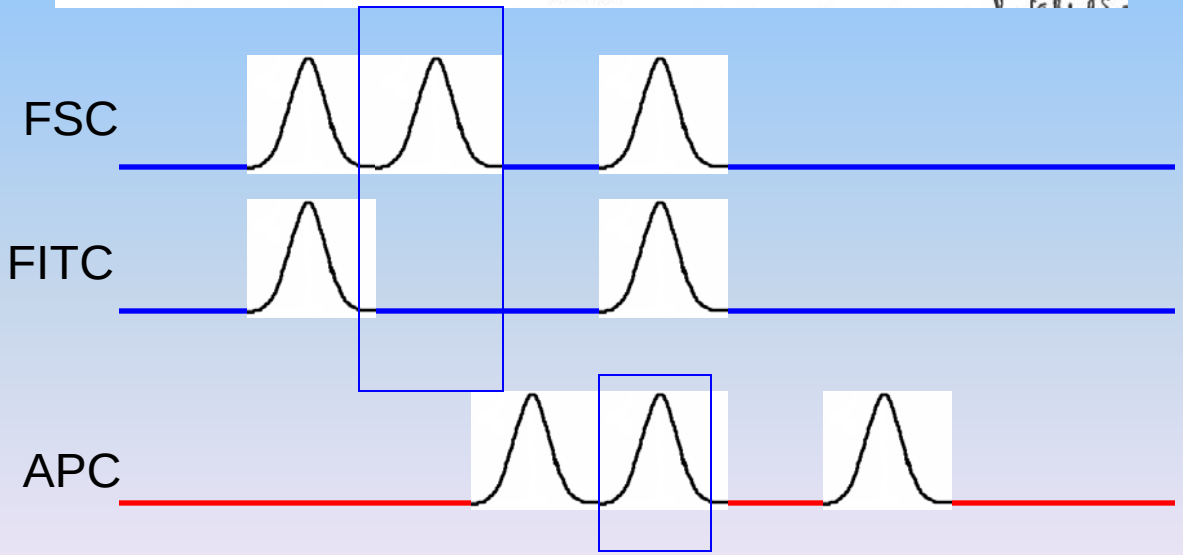


Baseline Correction

- The software averages the signals between events, and subtracts this from the total signal.

- If there is no signal for that parameter, this can sometimes result in a negative value for that event.

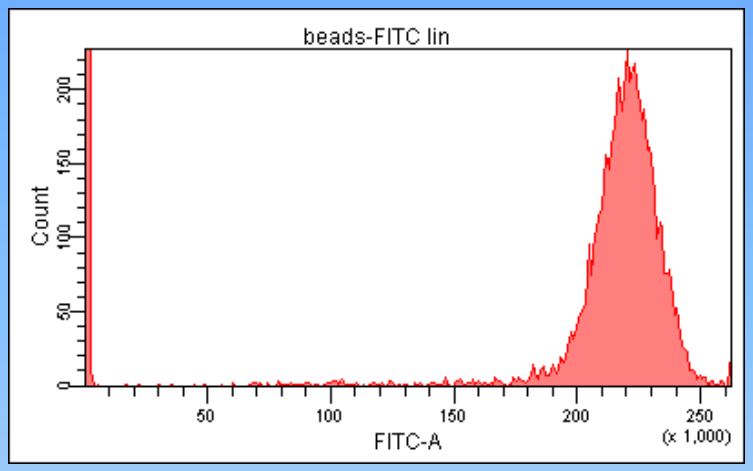




Basic Setup

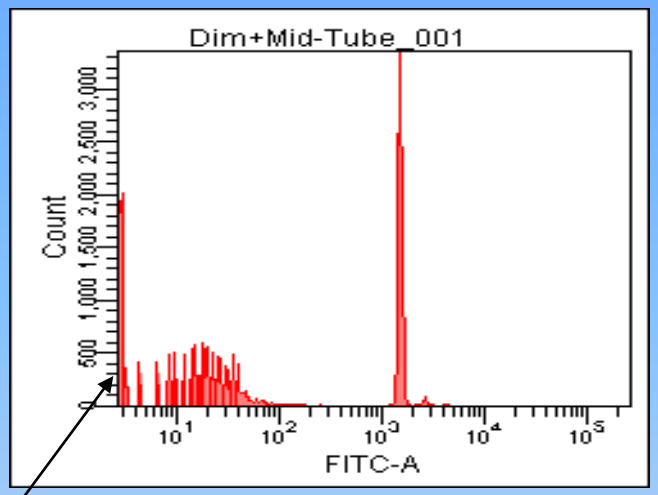
- In addition to the experimental samples, you will need an unstained sample and single stained samples for each color in the experiment.
- Use the unstained sample to set voltages so that the negative population is in the lower quarter of the plot
- Sequentially run and record each single stained sample,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

Linear



Compresses low values, high values are spread out

Log

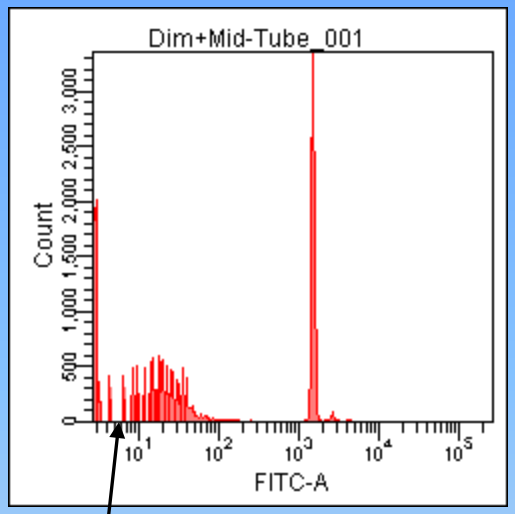


Compresses high values, low values are spread out

Events on the axis

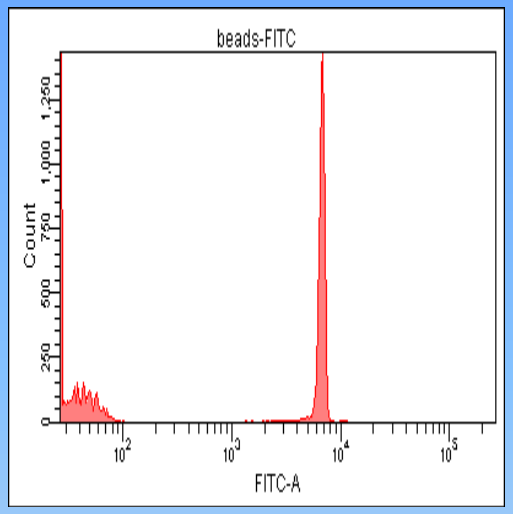
Can switch between linear and log (but signals won't be optimized for both!) Anything below the first displayed chanel would be displayed on the axis but retain its value.

Log scale 5 decades

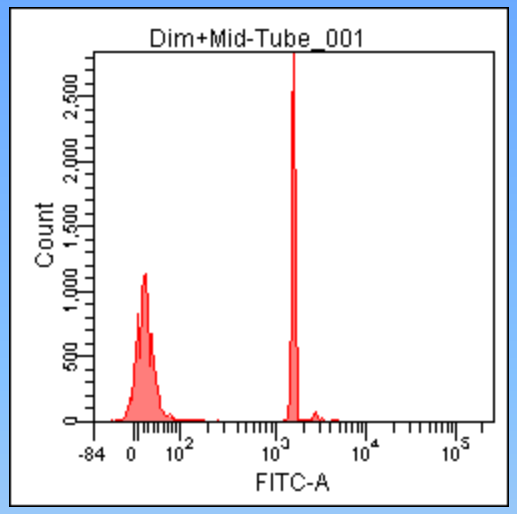


“picket fence”

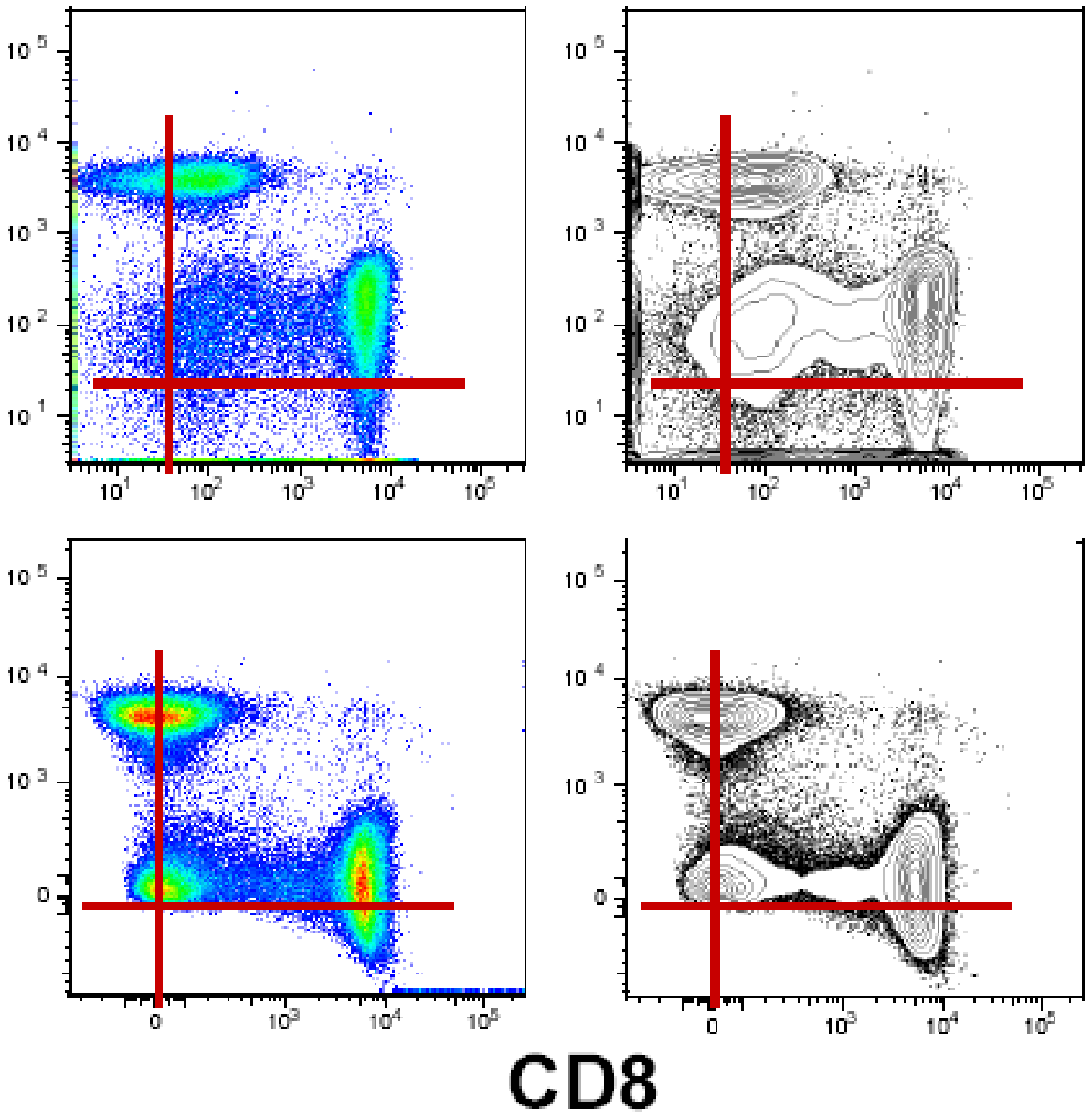
Log scale 4 decades



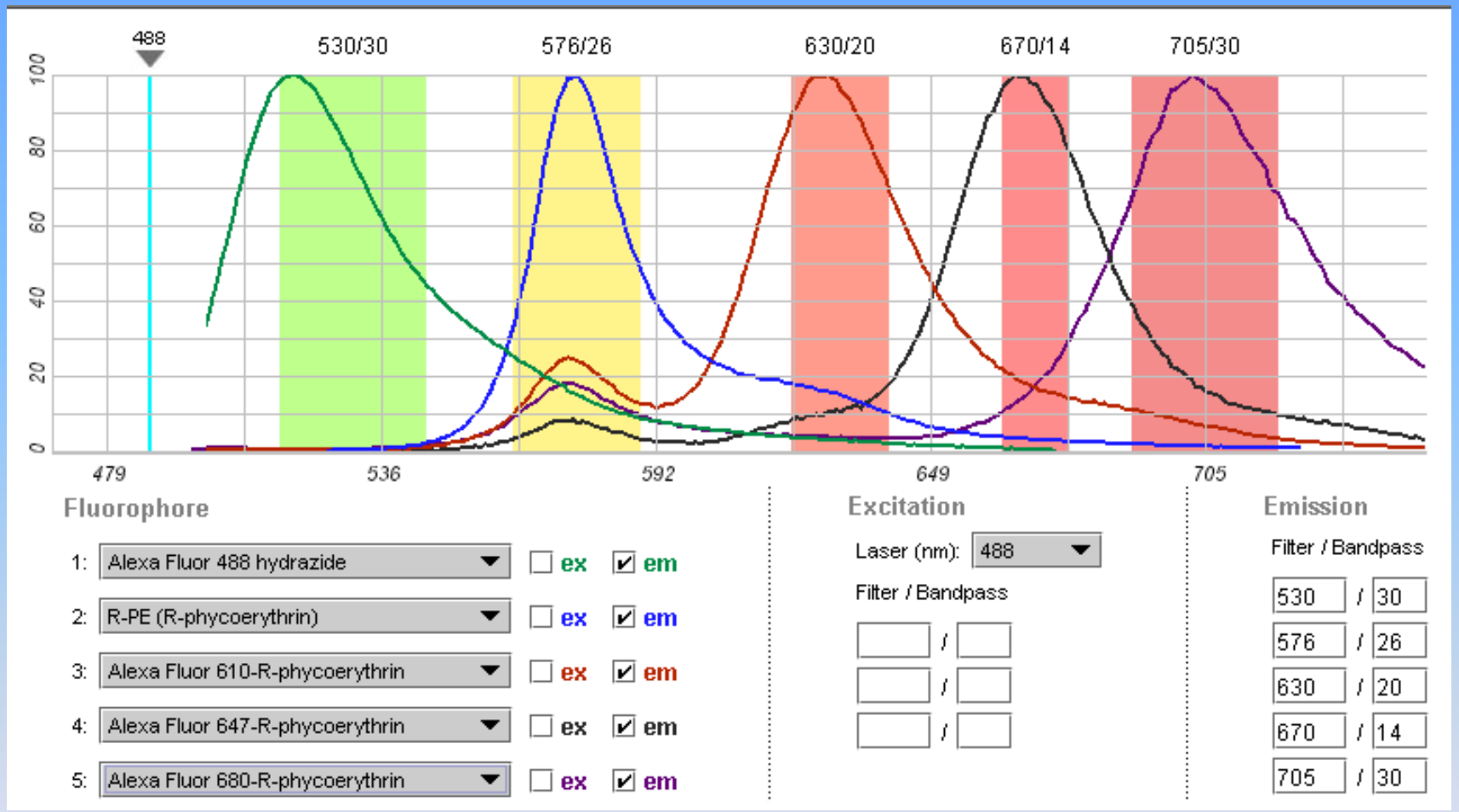
Biexponential



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.


Median
Transformed
CD4
CD8

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

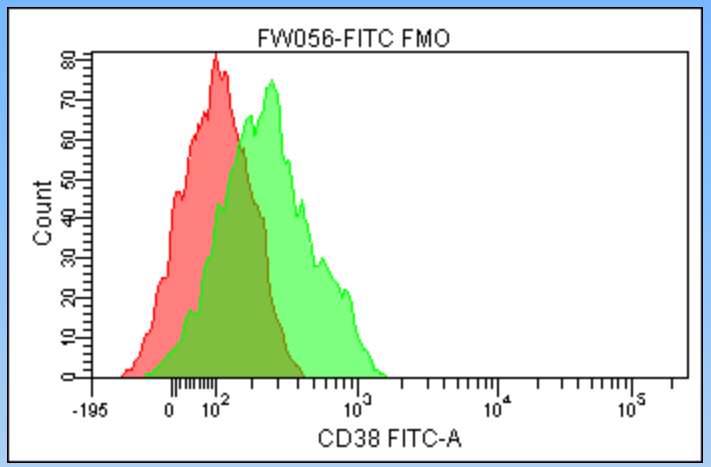
- For single color fluorochromes (FITC, PE, APC, etc), a surrogate marker can be used when target antigen is expressed in low levels or on a small population of cells.
- Because efficiency of transfer can differ between tandem molecules, compensation tube MUST be same as reagent in sample.
- Spillover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

Three types of staining controls

- Instrument setup and validation
  - Compensation, brightness
- Biological
  - “Normal” sample to verify proper detection of populations
  - Run identical sample with each experiment for daily variation
- Staining/gating
  - Nonspecific binding, FMO

Compensation Controls

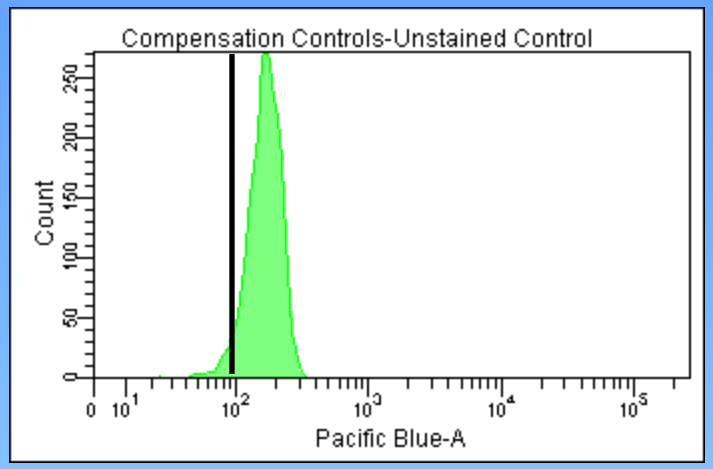
- Single color samples at least as bright as the reagent in your experiment (GFP counts!)
- One sample for every color, and one for each unique lot of tandem label (PE Cy5, APC Cy7, etc)
  - For tandems, amount of escaped light will vary from lot to lot, antibody to antibody, and possibly experiment 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.



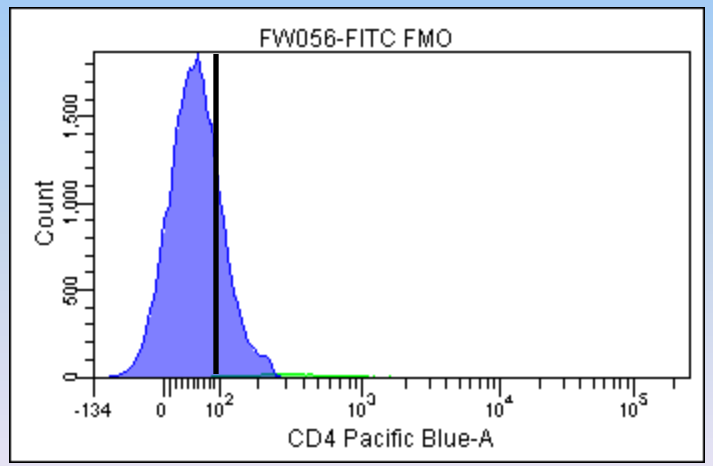
Lymphocytes

Monocytes

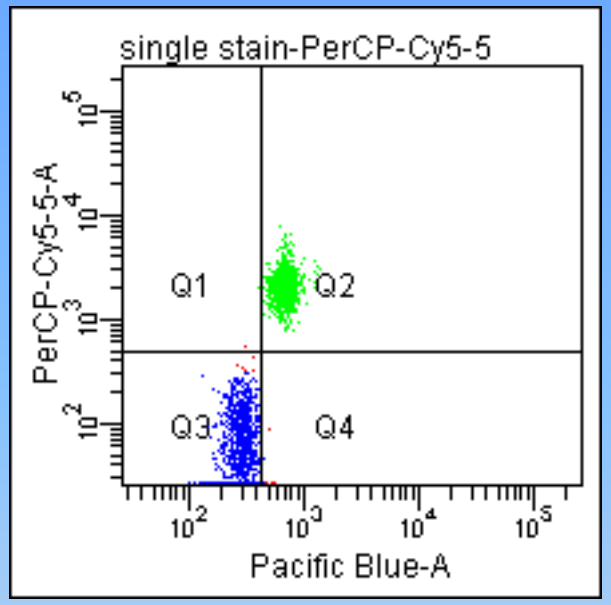
Beads



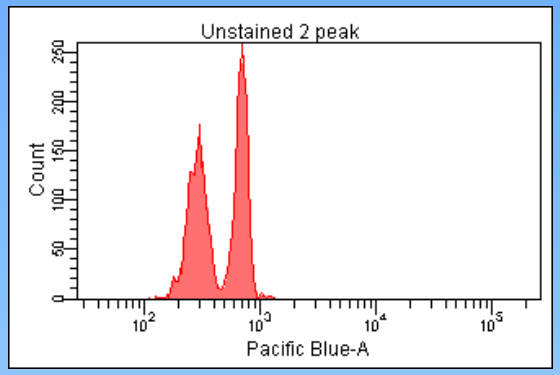
Cells



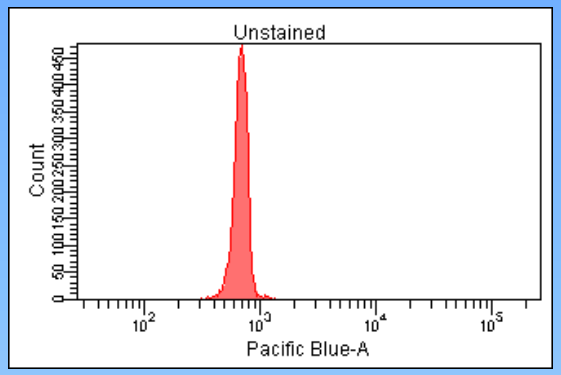
Mis-matched positive and negative compensation controls

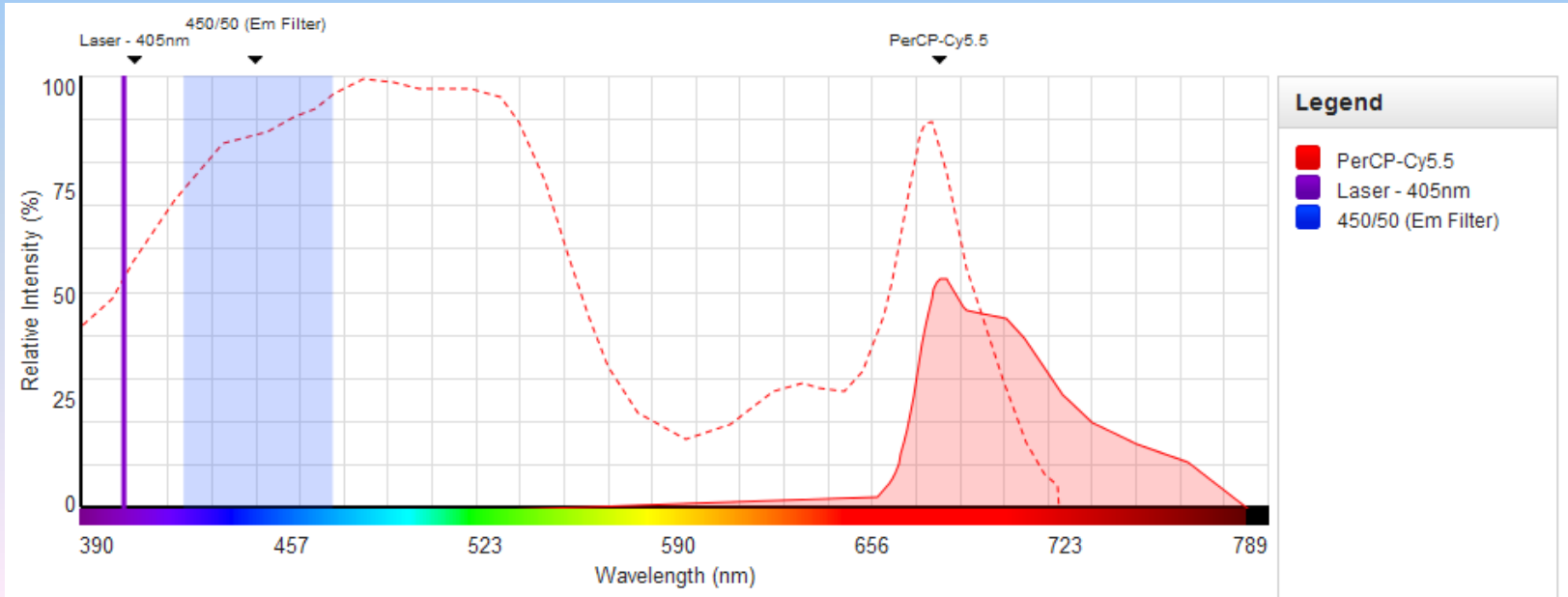


Mixed unstained

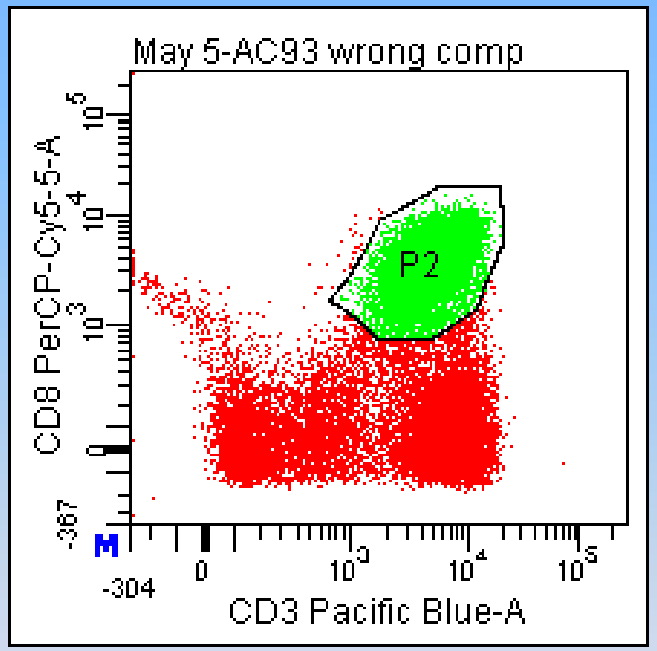


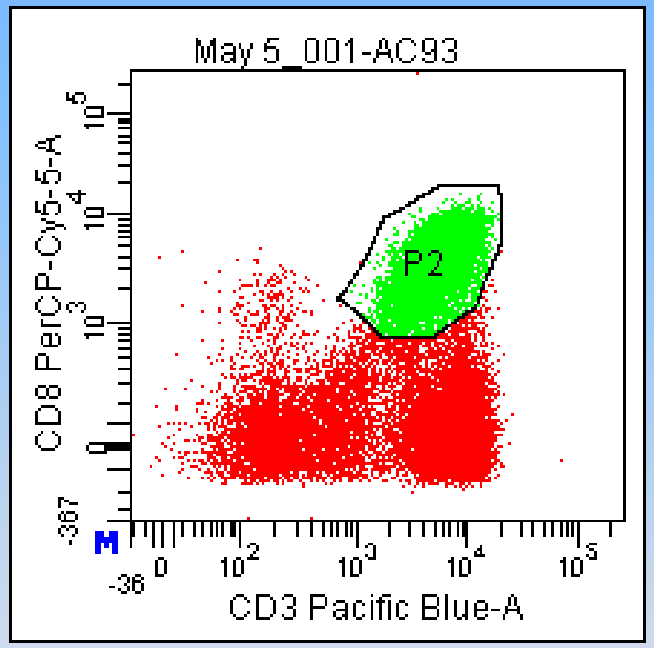
Unstained “binding beads”





Mismatched controls lead to improper compensation



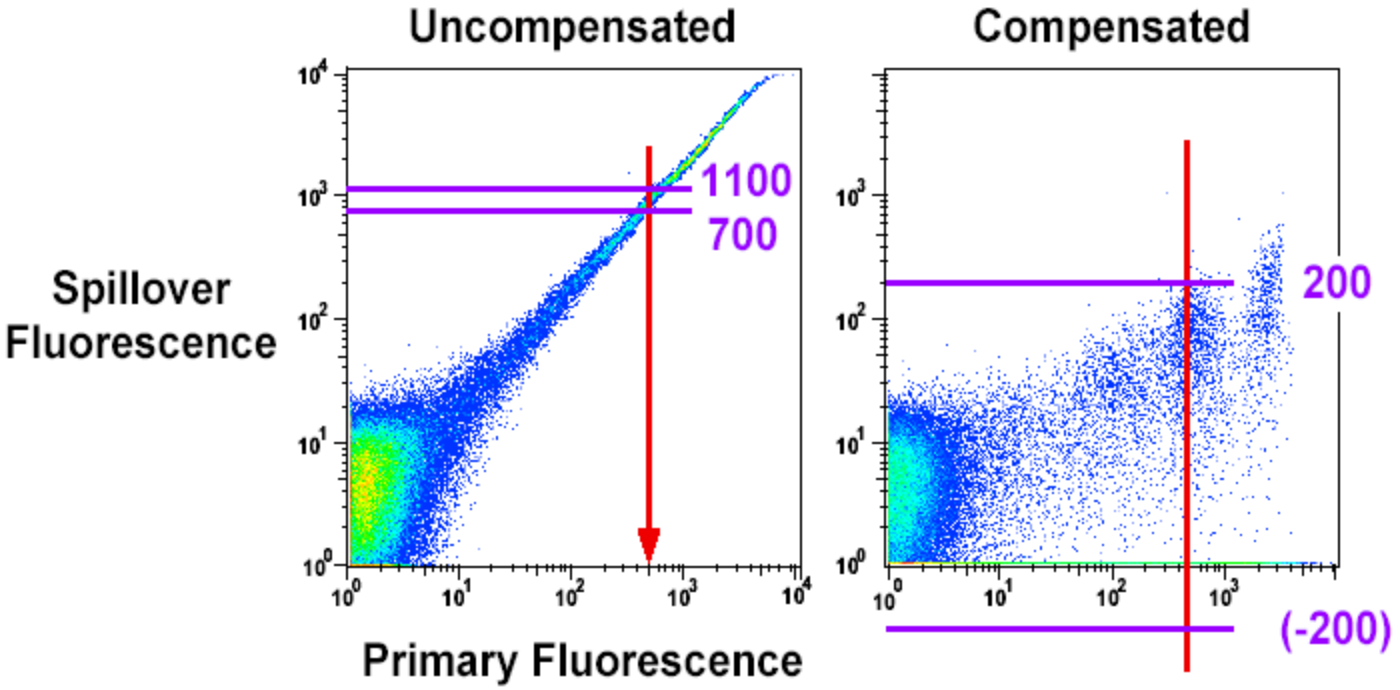


Pac Blue -% PerCP-Cy5.5
19.2 vs 0.17

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

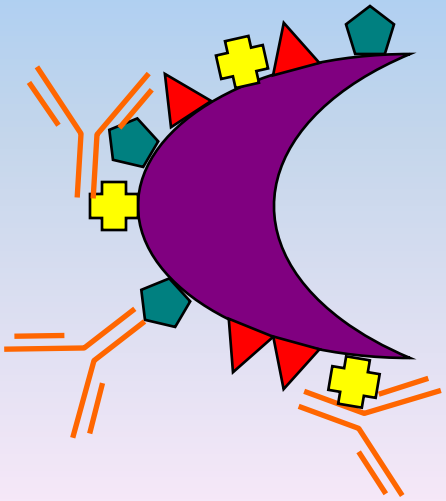


Why is there a 400-unit spread? Photon counting statistics.

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.

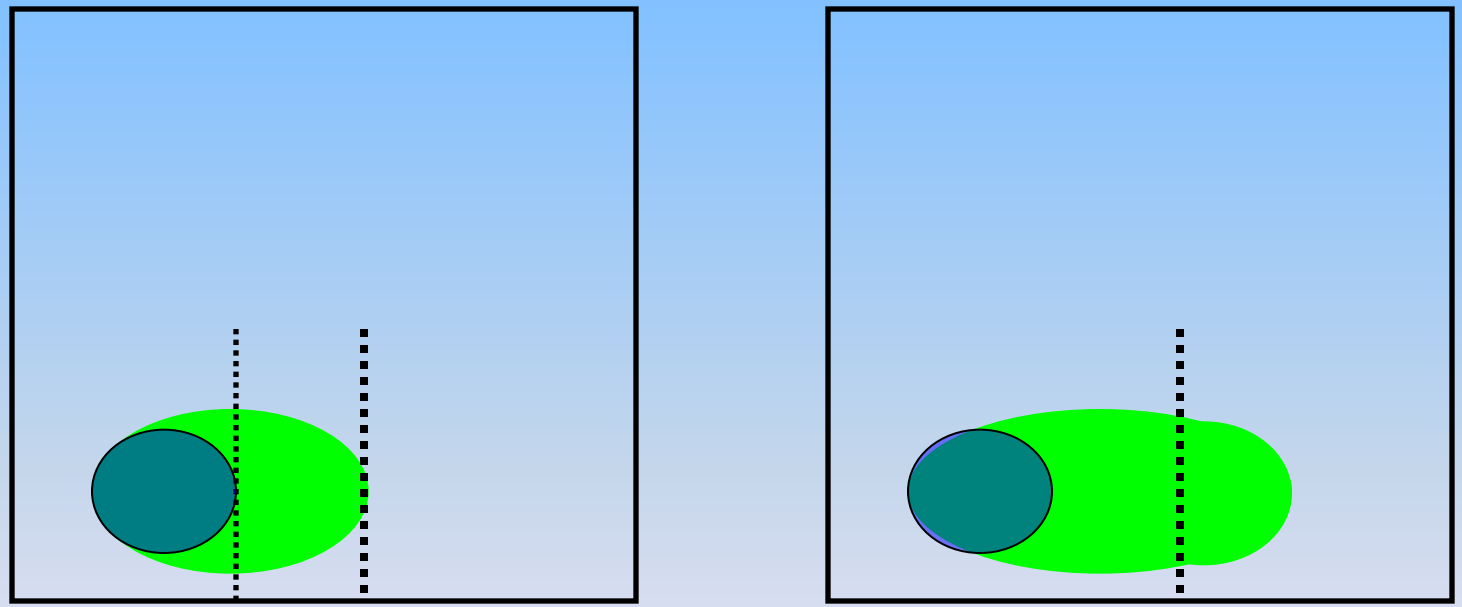
Nonspecific binding

- 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.



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



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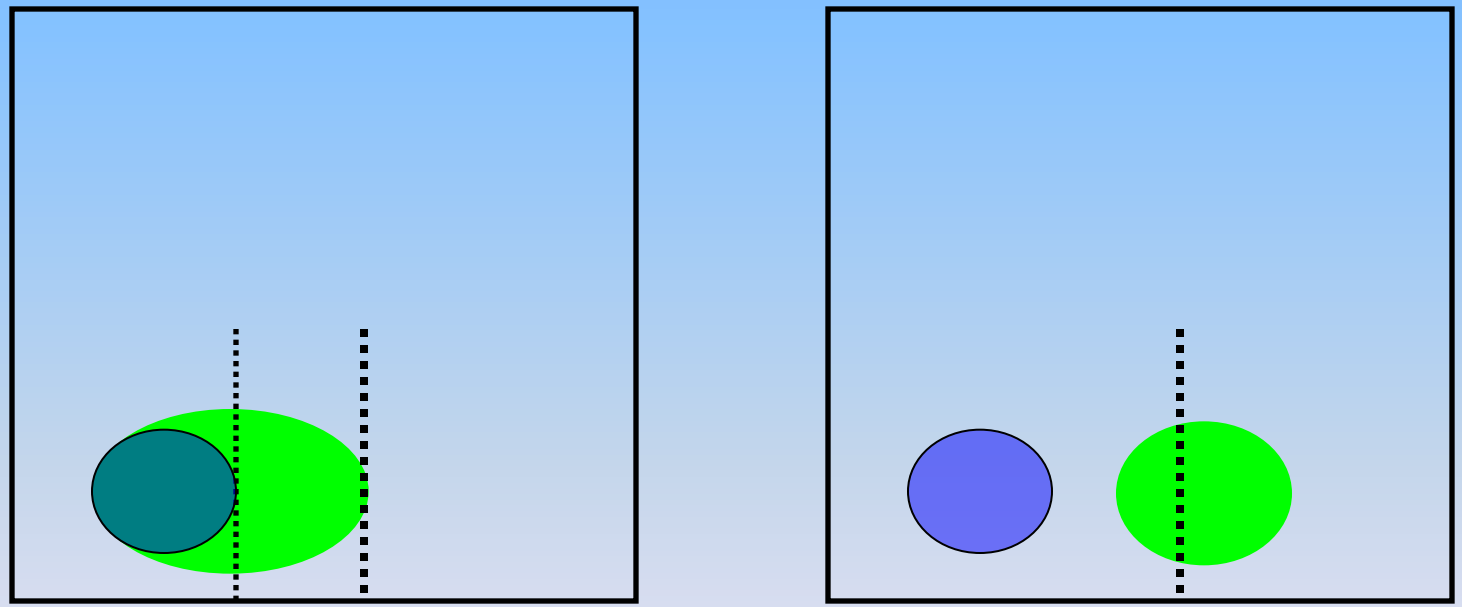
- Use to verify that there is no background, NOT to set gates



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

Staining Controls

- Unstained cells or complete isotype control stains are improper controls for determining positive vs. negative expression in multicolor experiments.
  - All isotype or unstained—changing more than 1 variable, no longer a control!

- The best control is to stain cells with all reagents except the one of interest.

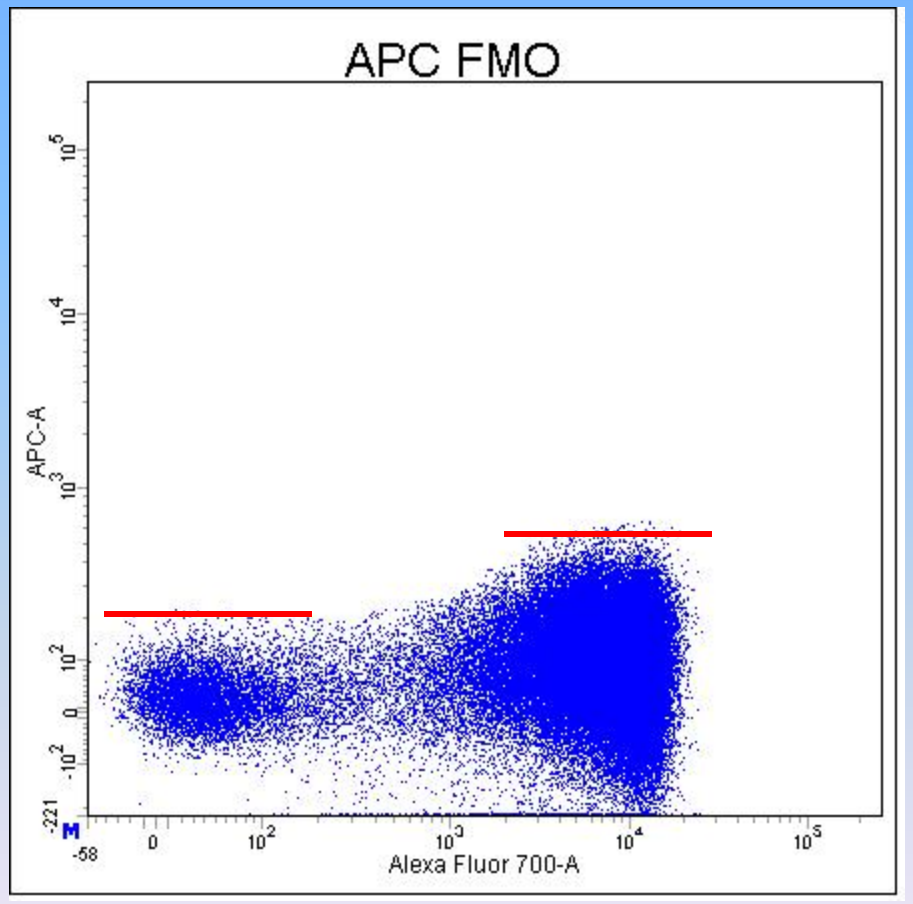
FMO

"Fluorescence Minus One"

Staining Controls

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

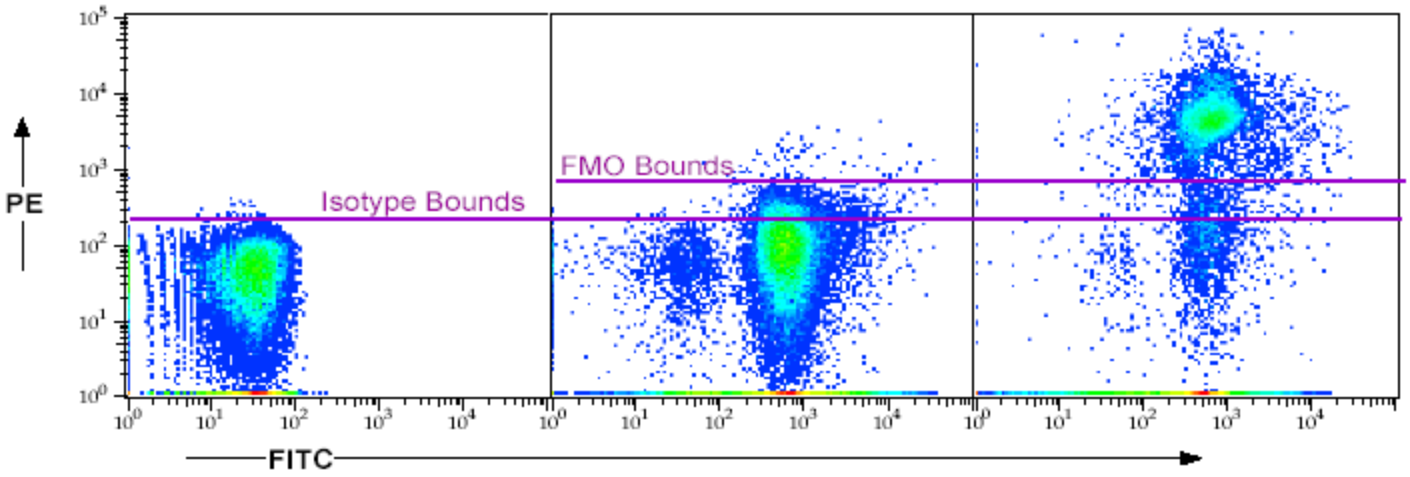
All the other colors may contribute small amounts of signal to the measured color, thus increasing the cutoff for “negative”.



Identifying CD4 cells with 4 colors

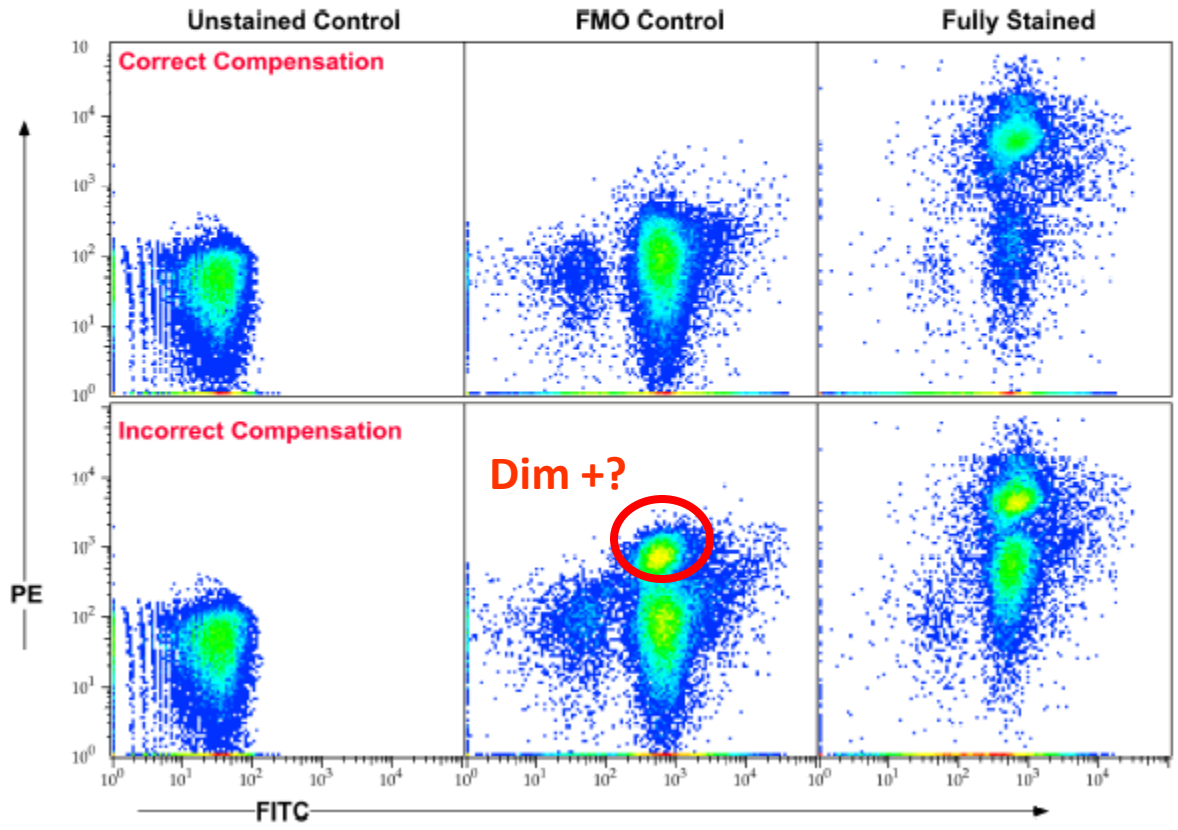
PBMC were stained as shown in a 4-color experiment. Compensation was properly set for all spillovers

	Unstained Control	FMO Control	Fully Stained
FITC	–	CD3	CD3
PE	–	–	CD4
Cy5PE	–	CD8	CD8
Cy7PE	–	CD45RO	CD45RO



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

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

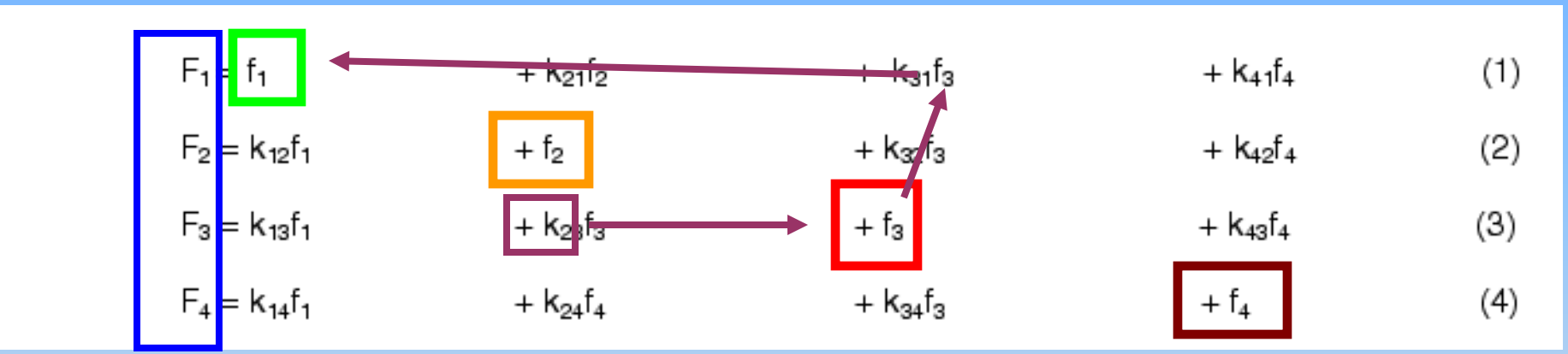
Observed uncompensated signal is the sum of the primary fluorescence plus the spillover of all other colors in the sample.

Compensation: The Calculation

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

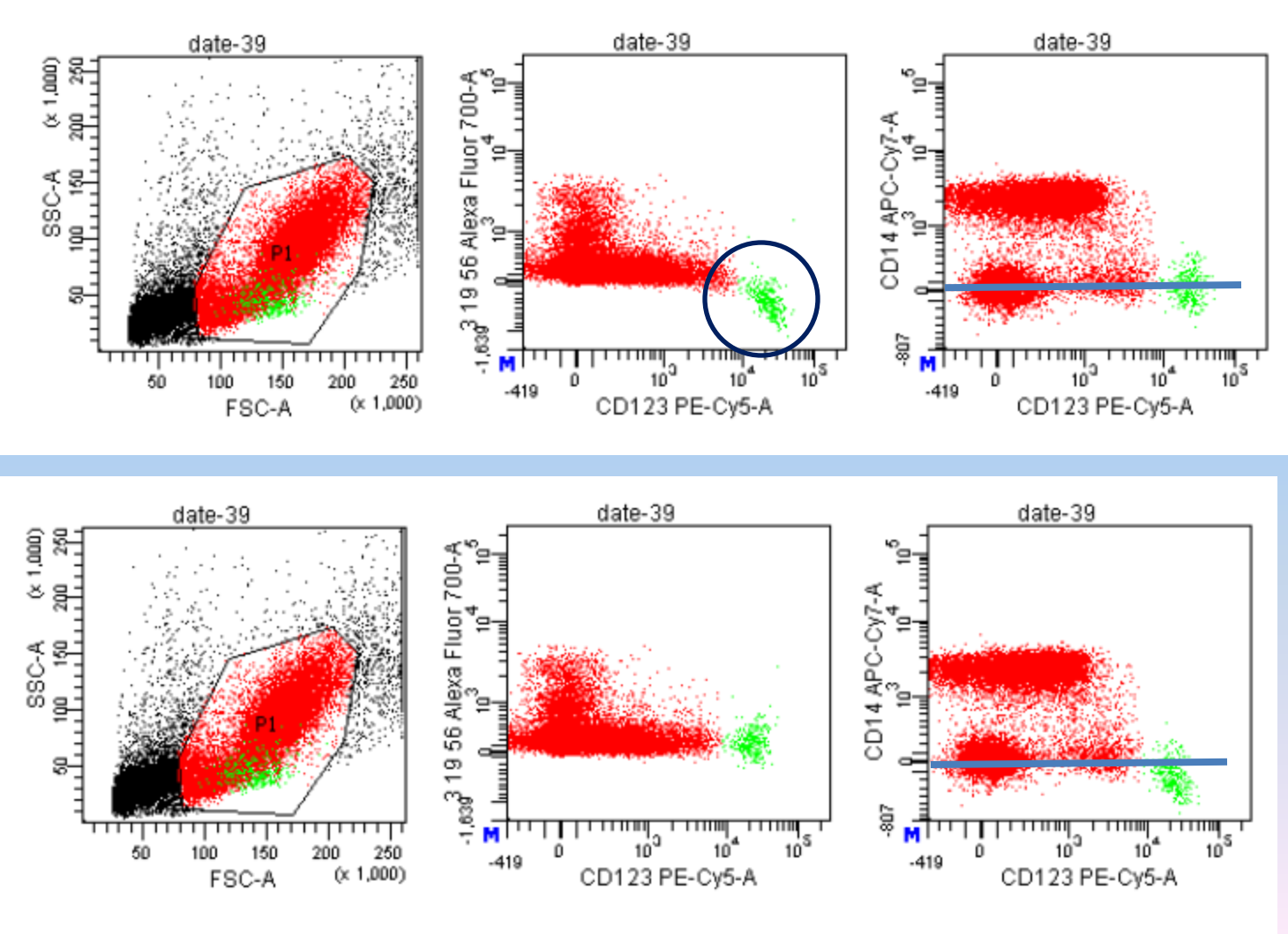
Using a single stained control allows you to determine the contribution of each color to all of the other channels—any signal above background in the other channels is known to be from that one fluorochrome and “k” for that pair can be determined.

growing frustrated in their pursuit of compensation



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



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

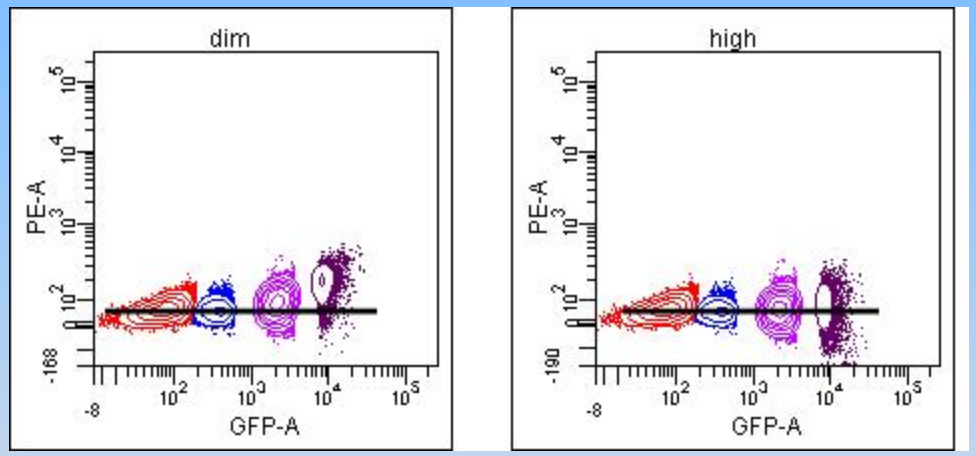
**If you were to do FMOs for all colors in a 10 color panel, that would be 10 single-stained controls (10 tests of antibody) and 10 FMOs (9 tests of antibody each, or 90)=100 tests of antibody before a single sample tube is stained.

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

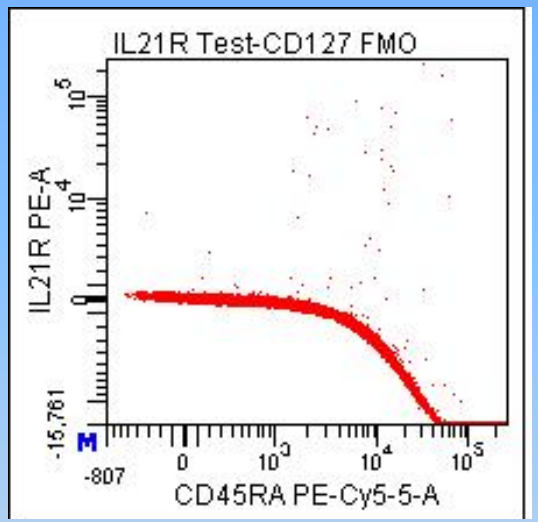
- Antibody capture beads are an excellent way to set compensation
  - -BD's CompBeads only recognize IgG k, limited by species

Compensation controls must be matched to your experiment and at least as bright as any of your reagents.

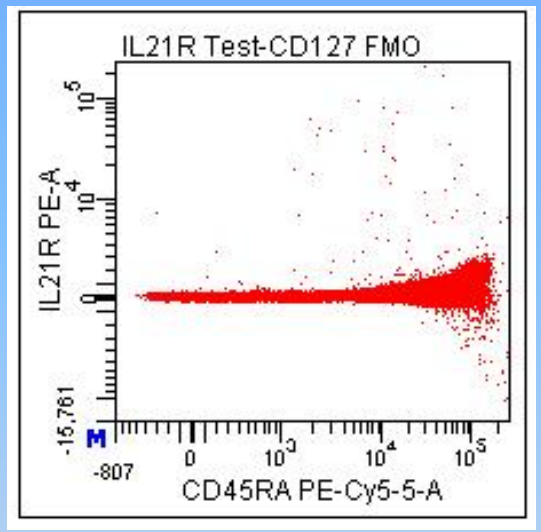


http://newenglandcytometry.com/2013/01/15/as-bright-or-brighter/

Compensating with the wrong tandem dye



42.6%



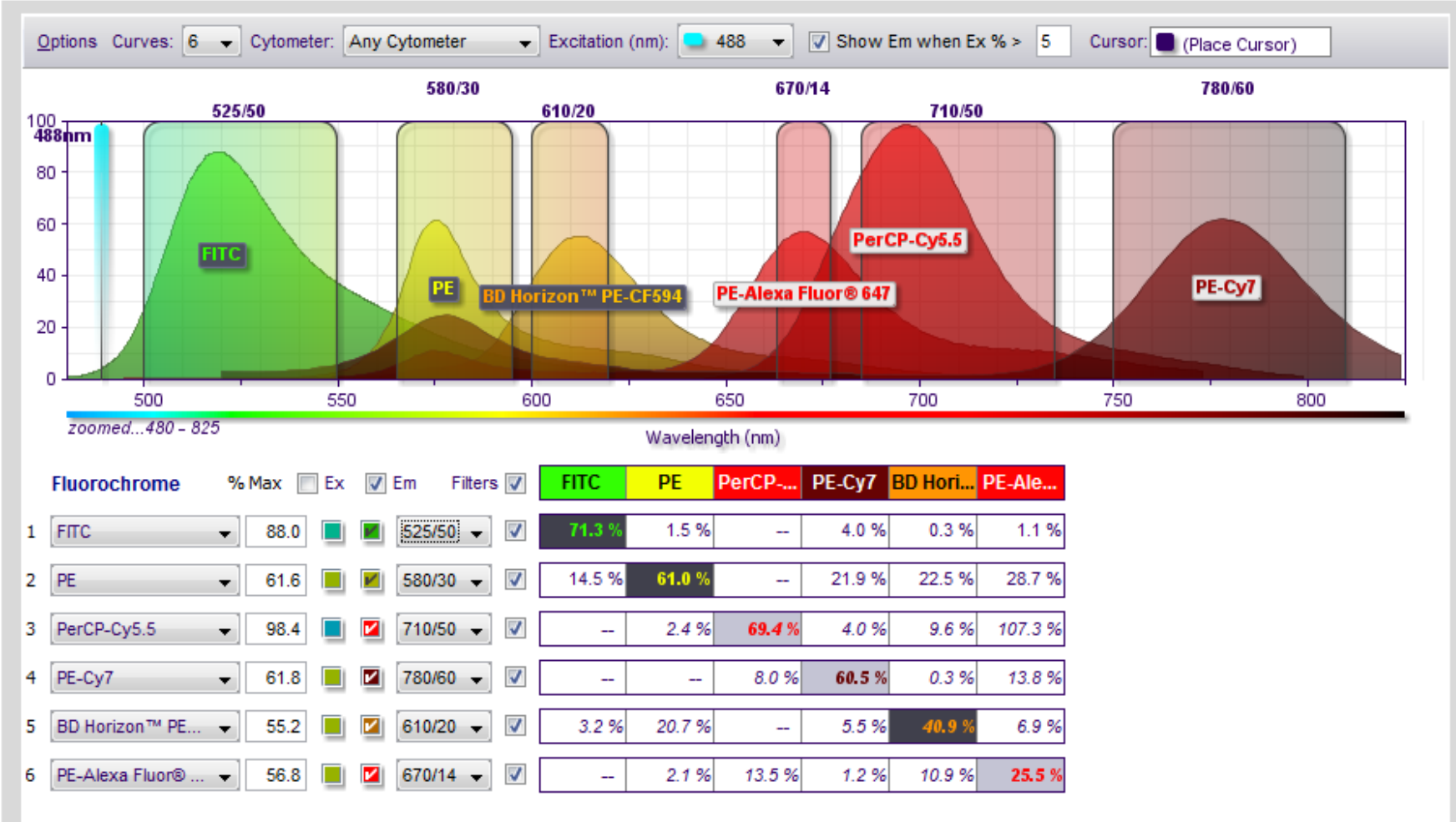
7.59%

Must use identical tandem dye to ensure proper compensation.

(example is compensated with an old vial of same lot/order of label)

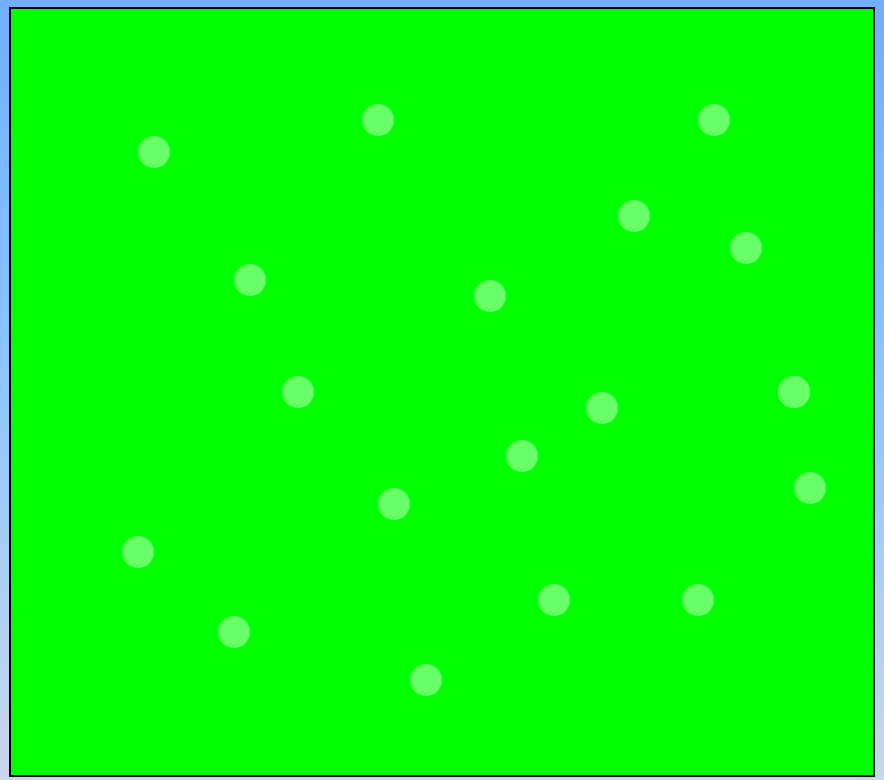
Challenges in flow: Spectral overlap

BD FLUORESCENCE SPECTRUM VIEWER A MULTICOLOR TOOL



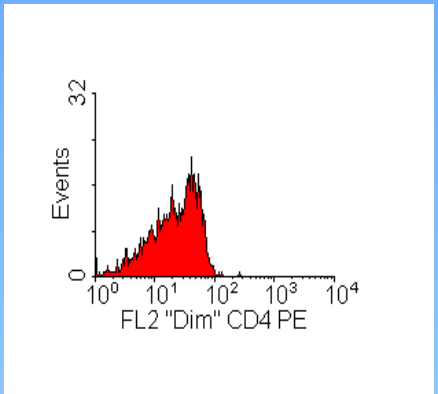
	Fluorochrome	% Max	Ex	Em	Filters	FITC	PE	PerCP-...	PE-Cy7	BD Hori...	PE-Ale...
1	FITC	88.0			525/50	71.3 %	1.5 %	--	4.0 %	0.3 %	1.1 %
2	PE	61.6			580/30	14.5 %	61.0 %	--	21.9 %	22.5 %	28.7 %
3	PerCP-Cy5.5	98.4			710/50	--	2.4 %	69.4 %	4.0 %	9.6 %	107.3 %
4	PE-Cy7	61.8			780/60	--	--	8.0 %	60.5 %	0.3 %	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 %

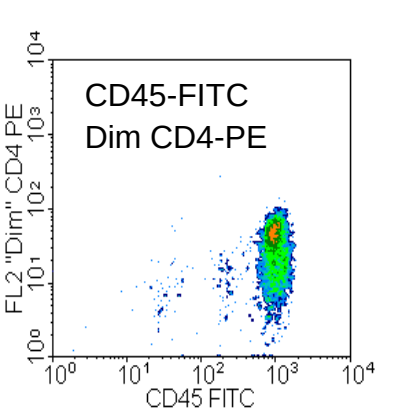
It is more difficult to detect signal where there is a lot of background light…



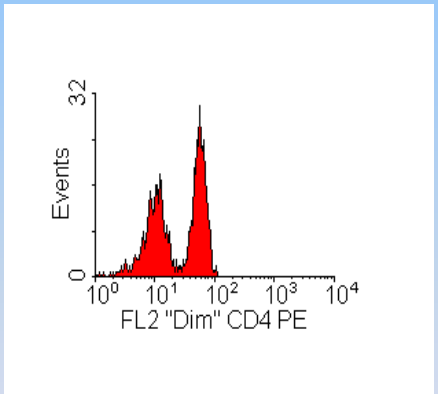
High Spillover from other channels—less sensitivity

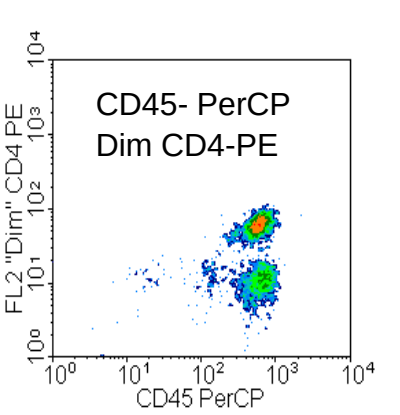
Effect of Spillover on Double Stained Cells





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

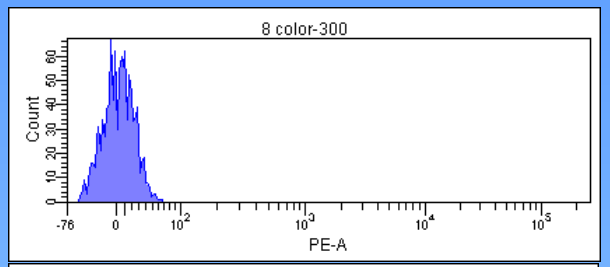
- Avoid using a dim marker in a channel adjacent to a bright marker with high spillover
- Separate reagents as much as possible (3 colors, use 3 different laser lines)
- Titrate your antibodies! Optimizes signal and saves reagent.
- Do NOT run a full experiment without validating the panel first!

Optimizing settings

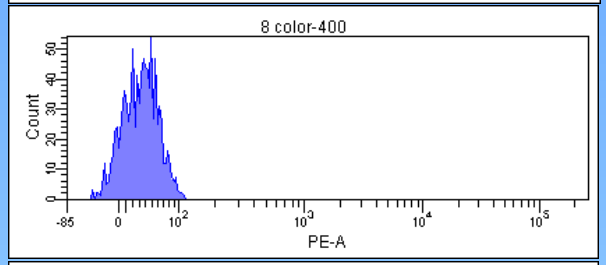
Same sample of 2 beads of similar fluorescence, recorded at different voltages.

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

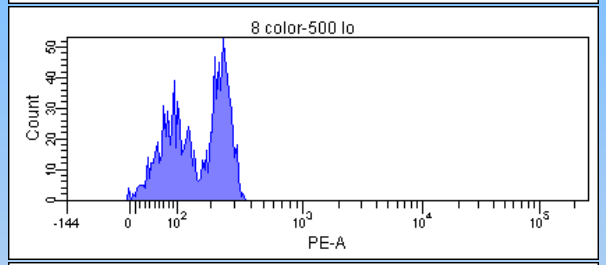
Higher voltage will not improve resolution but WILL increase overlap from other channels.



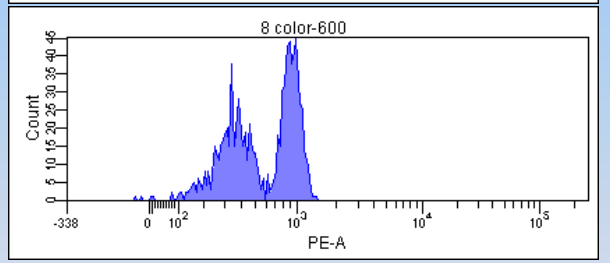
300V



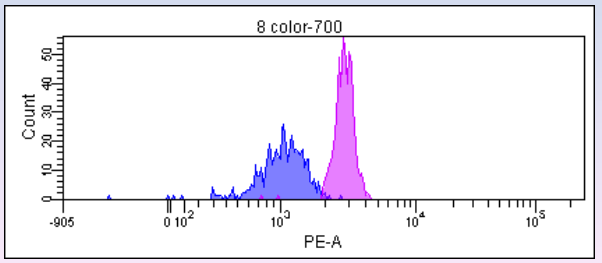
400V



500V



600V

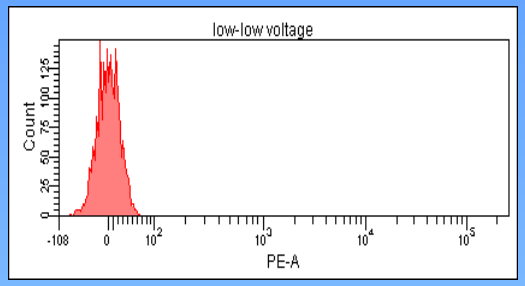


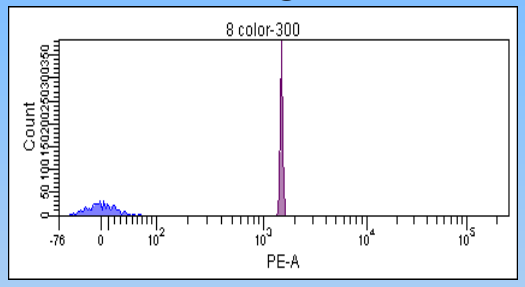
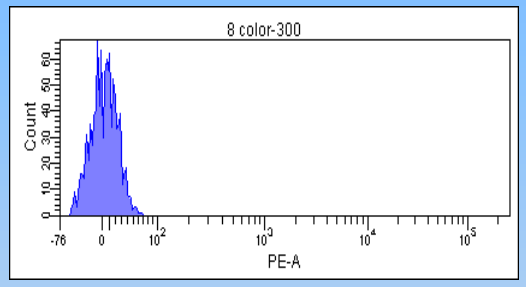
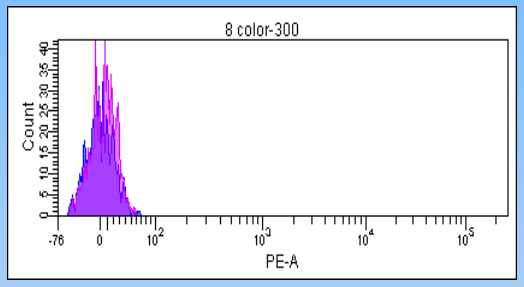
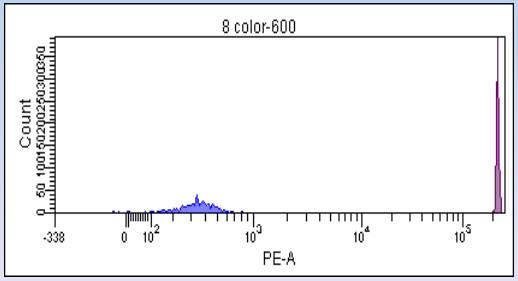
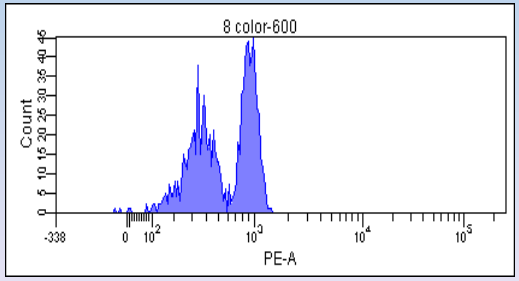
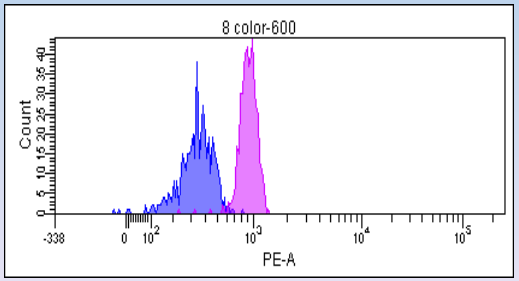
700V

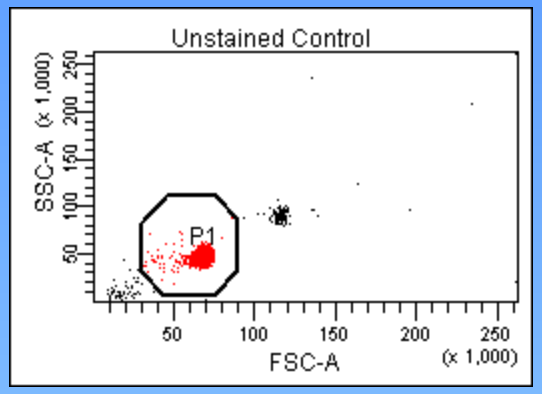
The correct voltage to use will depend on brightness of target population

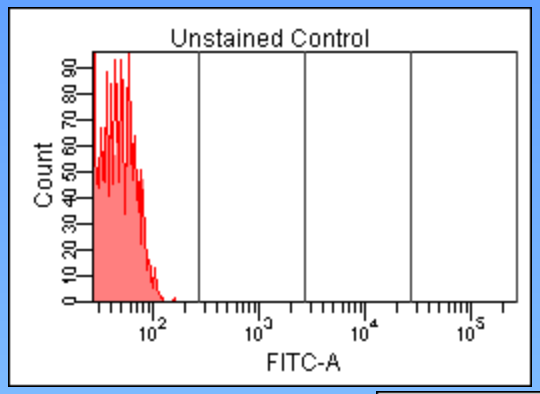
Only Noise

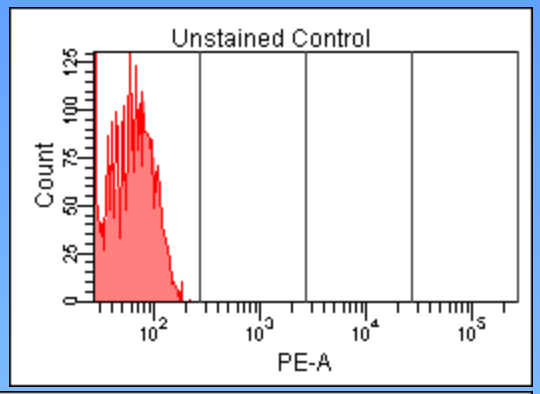
Light to PMT is blocked



	Bright	Dim	Dim (overlay)
300V Good separation for bright No separation for dim			
600V Brights off scale Maximum separation for dim			

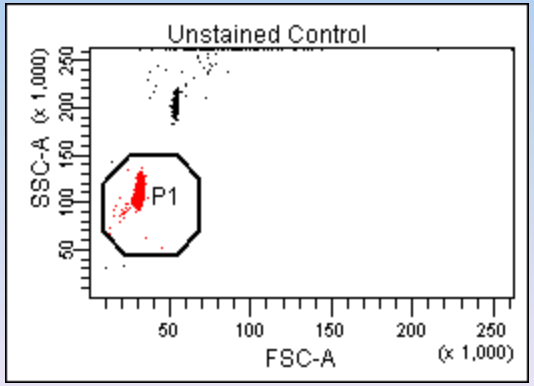


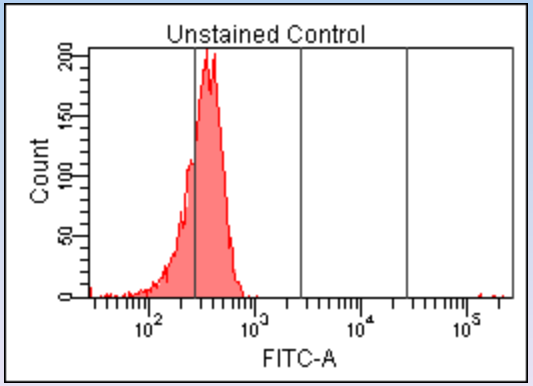


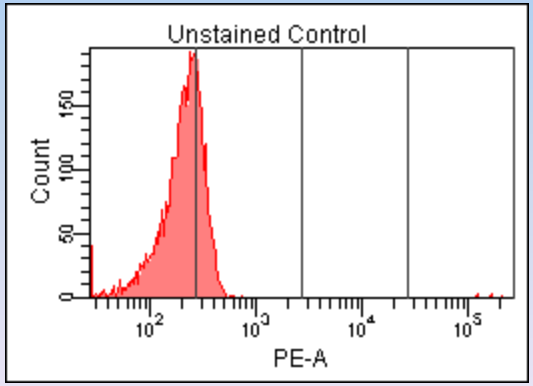


Population	#Events	%Parent	FITC-A Median	PE-A Median
P1	4,839	96.8	18	42

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)

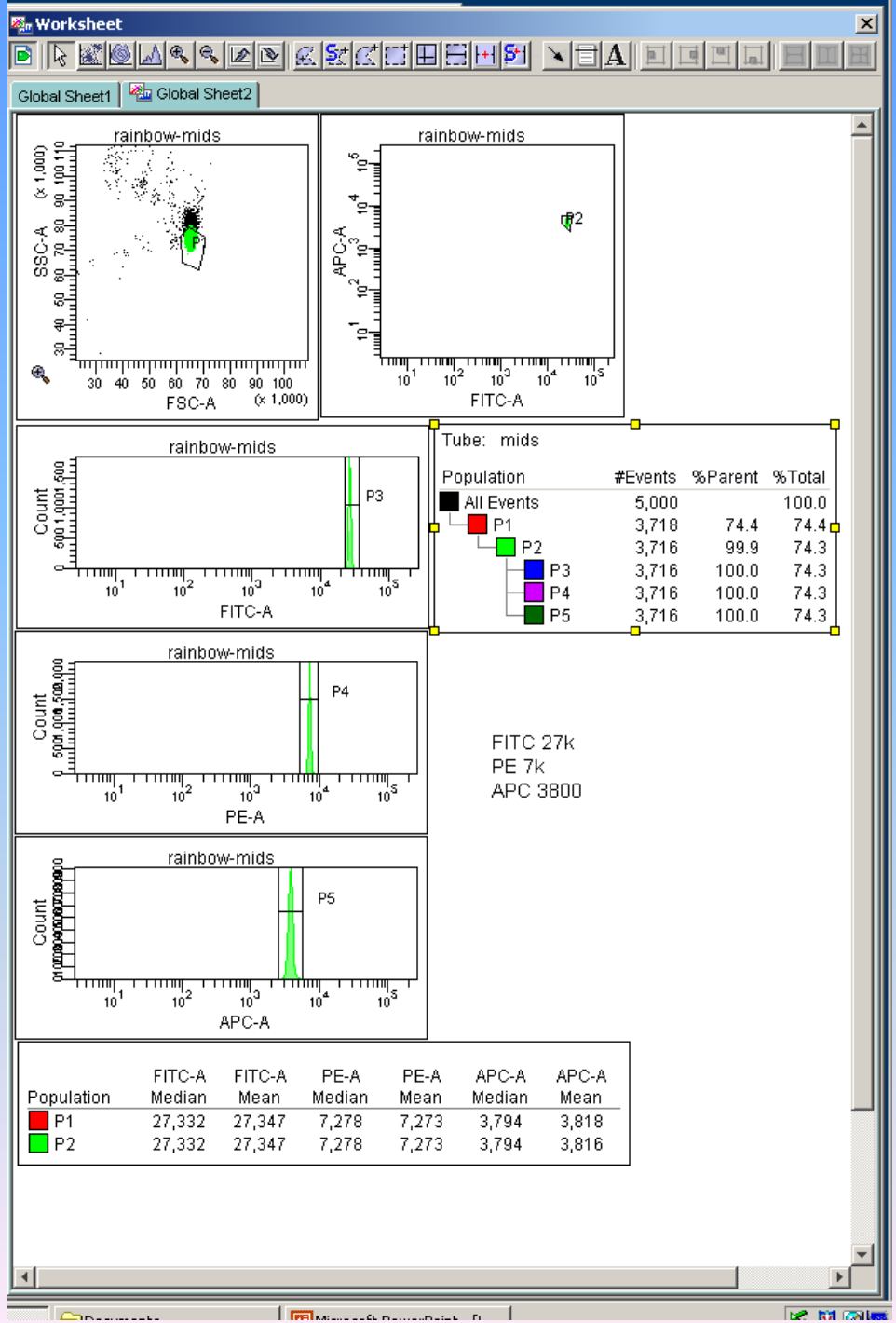
Track settings with beads

After determining settings for your sample, run a mid-range bead (such as Spherotech RFP-30-5A) and record *uncompensated* median signals.

Either:

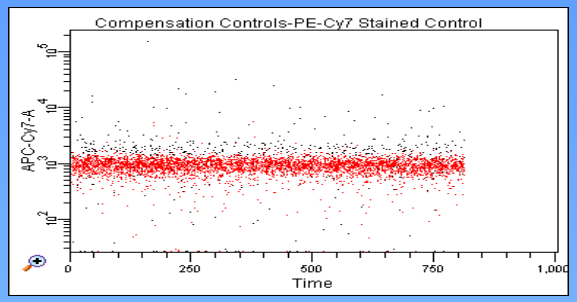
- Draw gates on histograms to aim for
- Create a statistics view and record median or mean on worksheet
- Be sure to use UNCOMPENSATED VALUES!

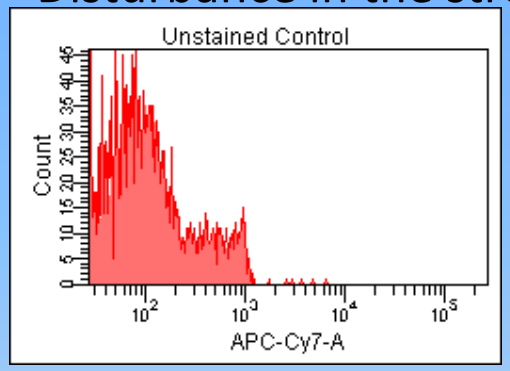
Match these values in future sessions with the same lot of bead to ensure detector output is consistent.

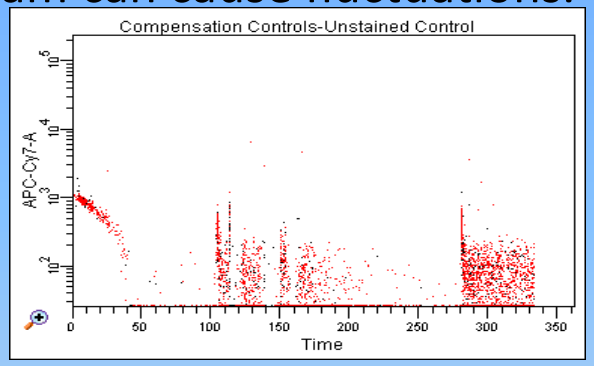


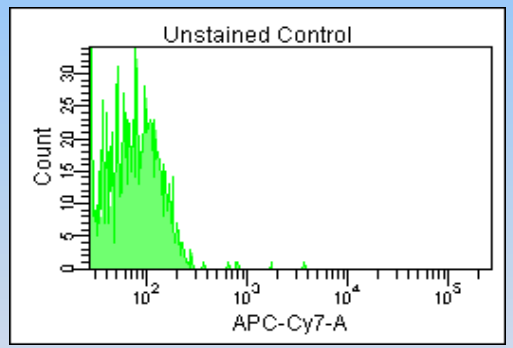
“Time Gating”

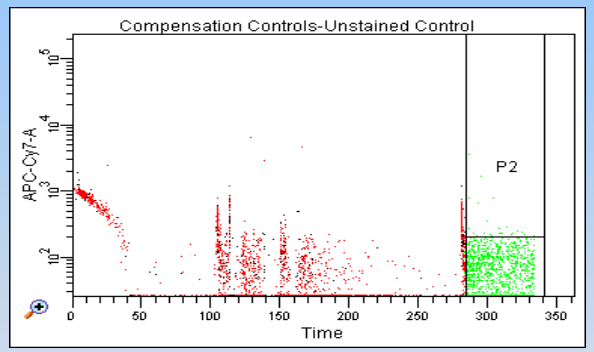
- View dot plot of time (x axis) vs parameter
- Signals should be consistent for duration of data file.
- Disturbance in the stream can cause fluctuations.





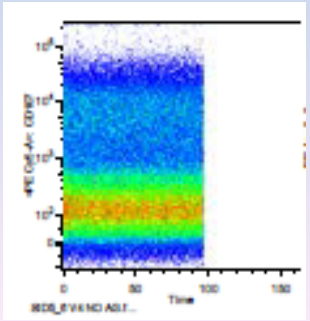


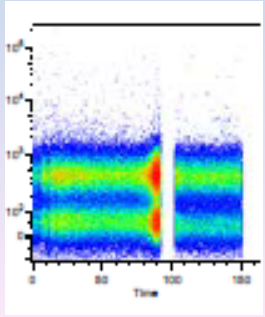


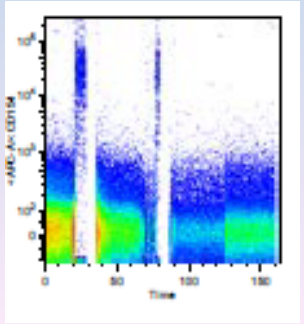


If it does fluctuate, you can draw an interval gate against “time” to exclude the initial events.

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







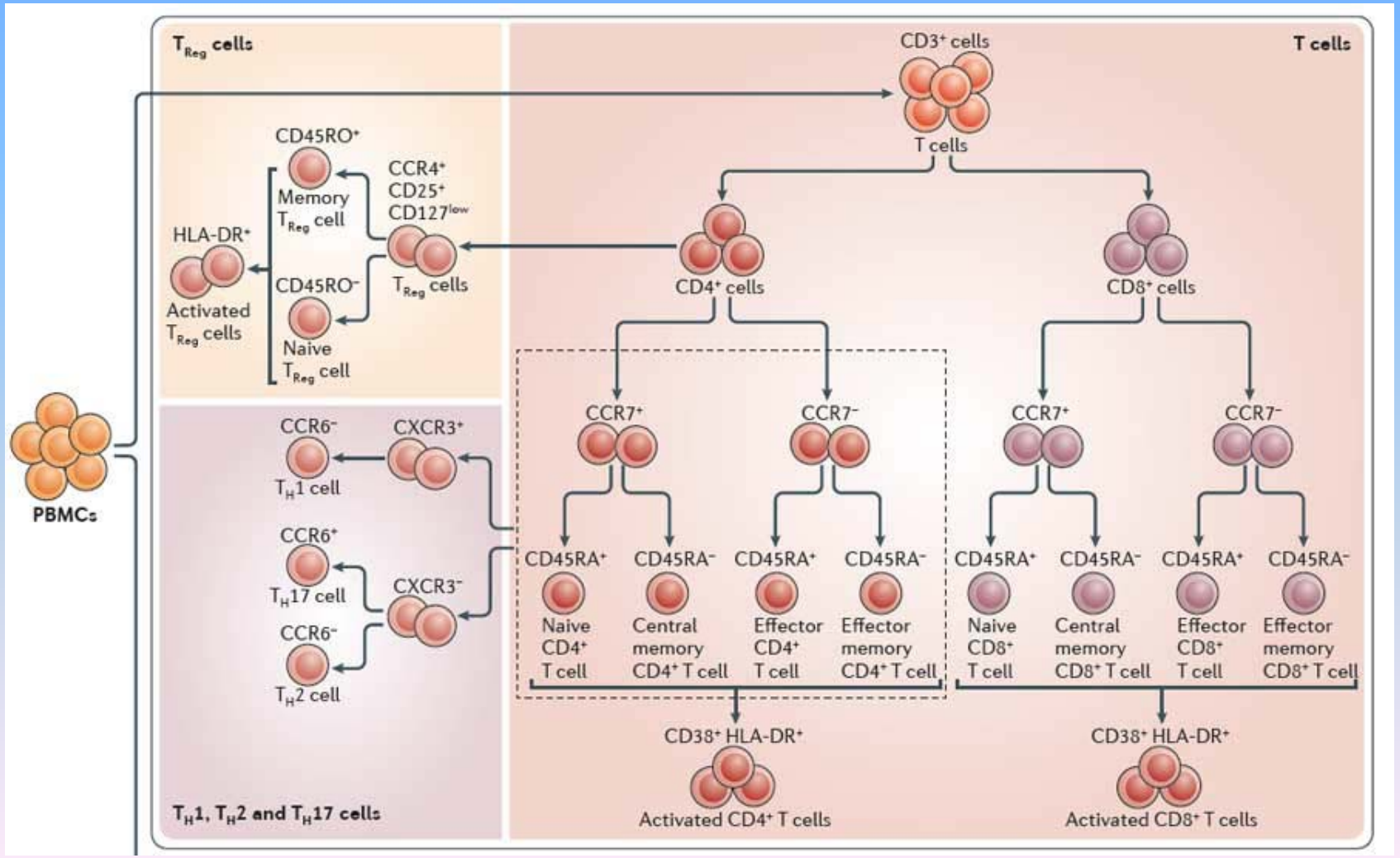
Applications

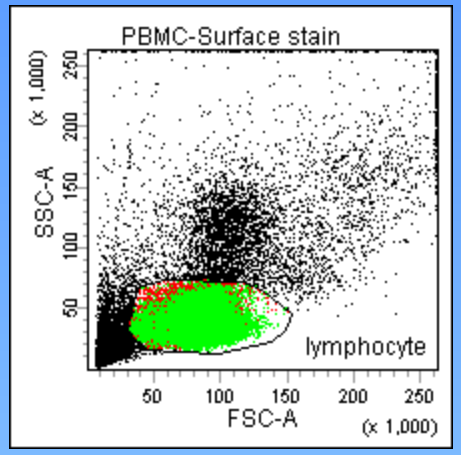
- Immunophenotyping
- ICS
- Proliferation
- Protein phosphorylation
- Cell Cycle
- Apoptosis
- Stem Cells

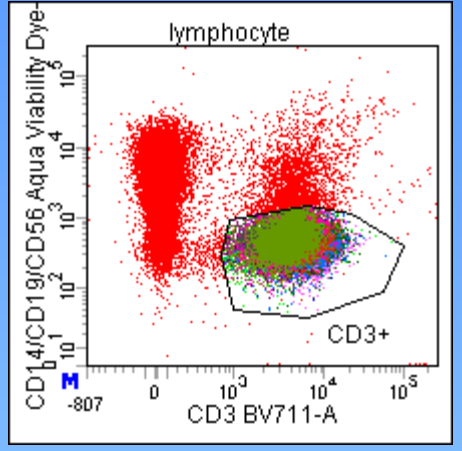
Standardizing immunophenotyping for the Human Immunology Project

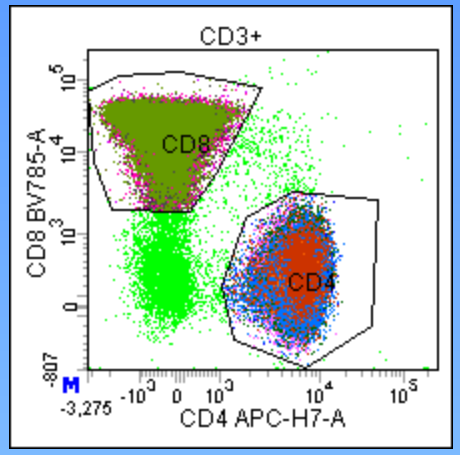
Holden T. Maecker, J. Philip McCoy & Robert Nussenblatt

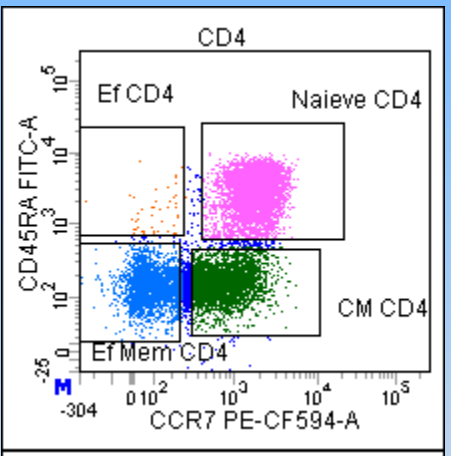
Nature Reviews Immunology 12, 191-200 (March 2012)

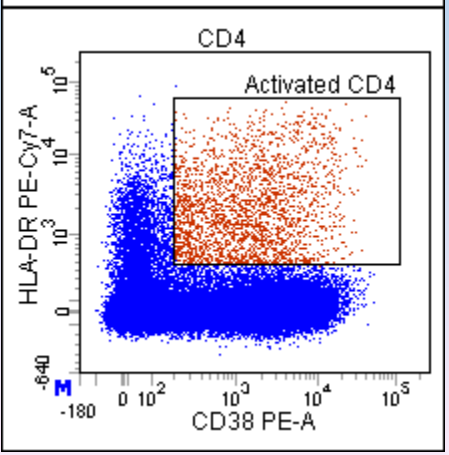








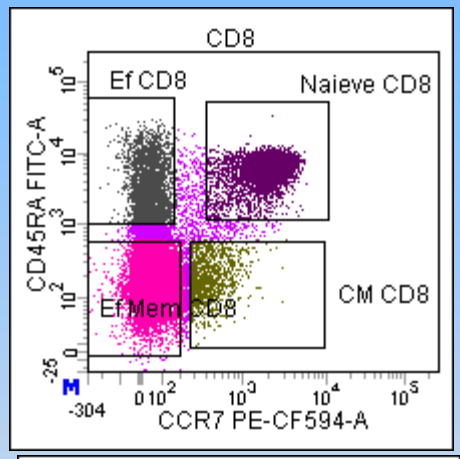


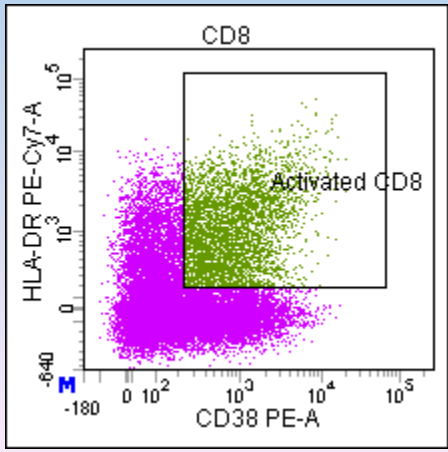


Phenotyping

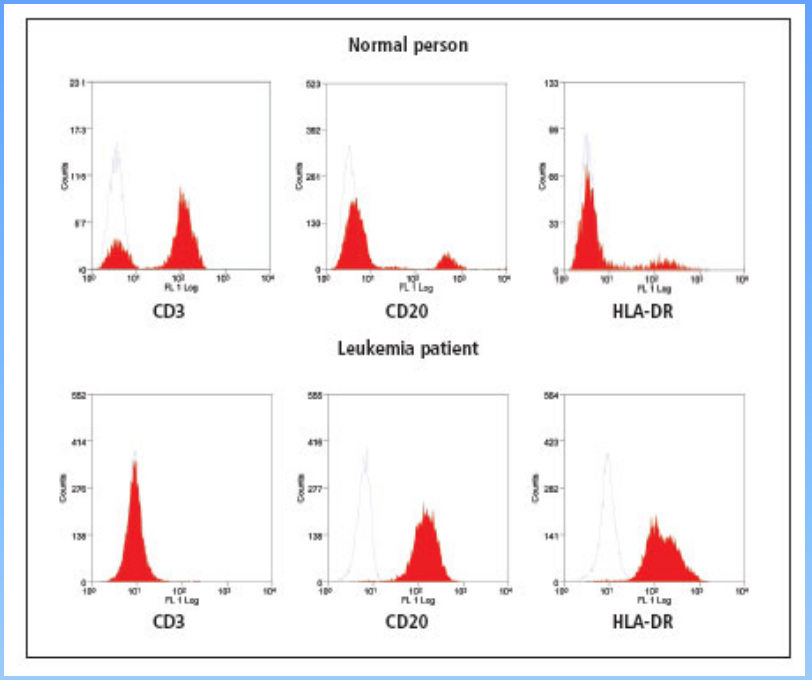
Tube: Surface stain

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
Naieve 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
Naieve 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



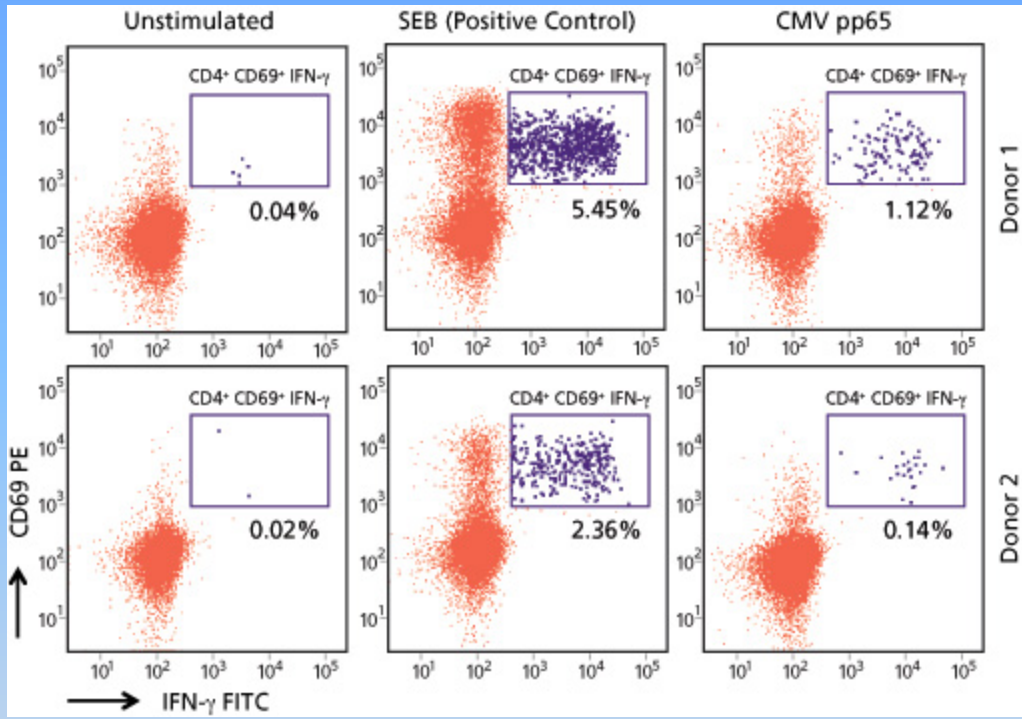
CD3-A normal person has a significant proportion of CD3-positive lymphocytes. In the patient with leukemia, staining for CD3 is absent.
CD20-In the leukemia patient there are a large number of cells staining positive for CD20. In the healthy person only a few stain positive.
HLA-DR-The leukemia patient 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.

http://www.abdserotec.com/flow-cytometry-immunophenotyping.html

Intracellular Cytokine Staining; ICS

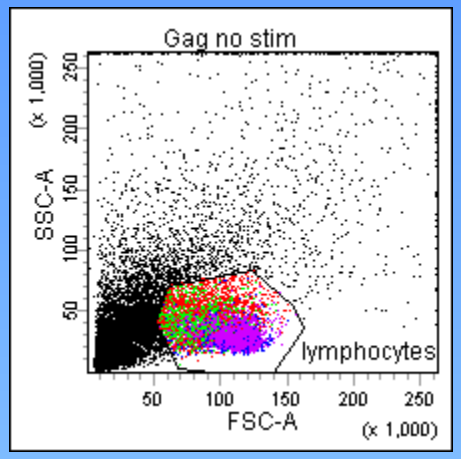
Antigen-specific IFN-γ production by cytomegalovirus (CMV) pp65- stimulated $CD4^+CD69^+$ T lymphocytes

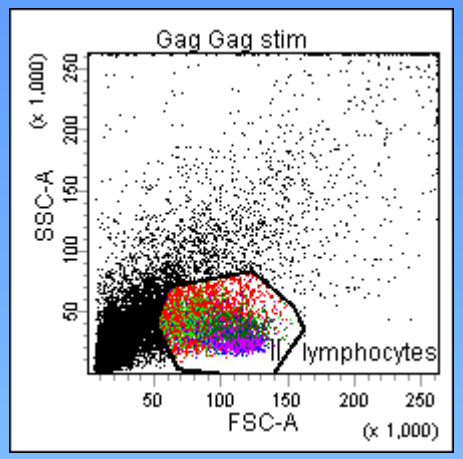


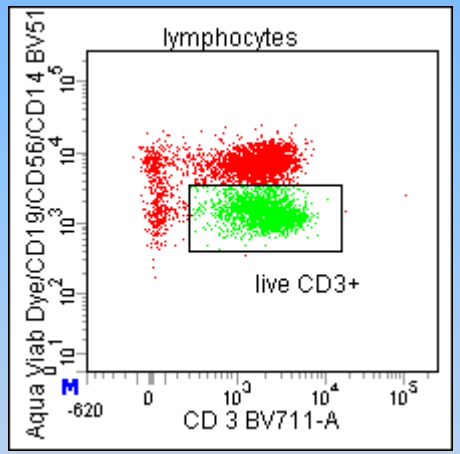
Two-color flow cytometric dot plots show IFN-γ vs CD69 expression by CD4 T cells that were either unstimulated (left panels), SEB-stimulated (as positive controls, center panels), and CMV pp65-stimulated (right panels) samples from two donors. Human whole blood was stimulated in the presence of brefeldin 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

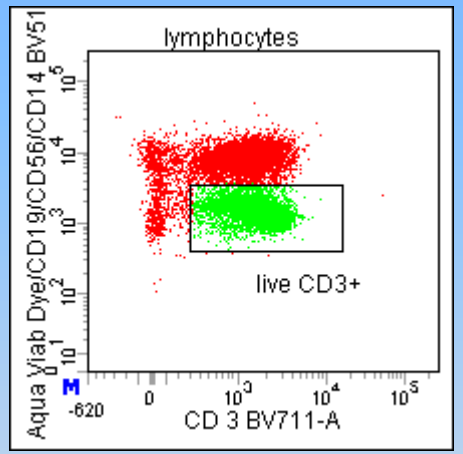
("PIN Number")

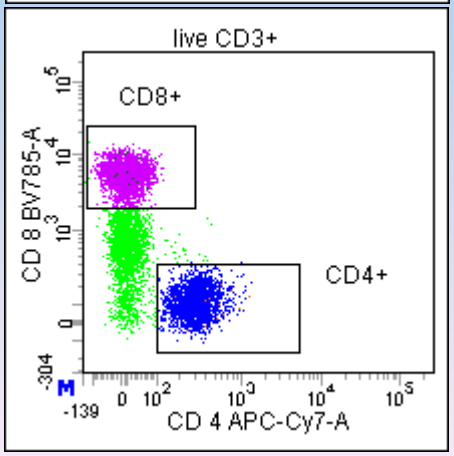


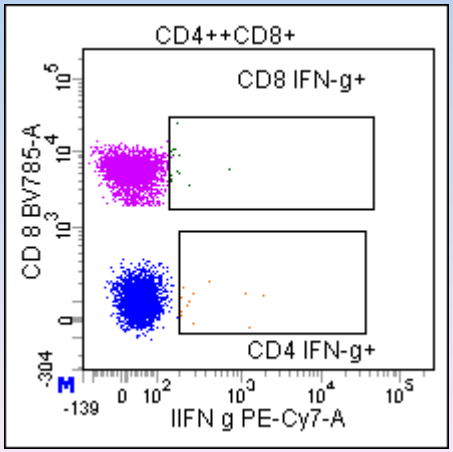


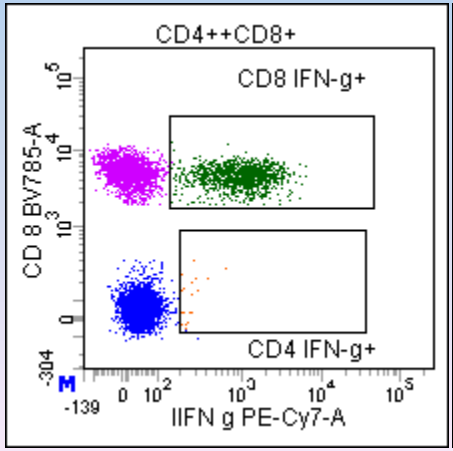


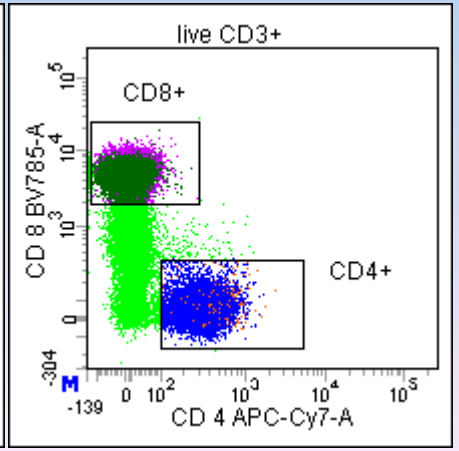
Tube:	Gag no stim		Gag Gag stim	
Population	%Parent	%Total	%Parent	%Total
All Events	####	100.0	####	100.0
lymphocytes	21.6	21.6	20.3	20.3
live CD3+	45.3	9.8	40.8	8.3
CD4+	31.6	3.1	28.3	2.3
CD4 IFN-g+	0.5	0.0	1.0	0.0
CD8+	37.9	3.7	42.7	3.5
CD8 IFN-g+	0.5	0.0	35.7	1.3



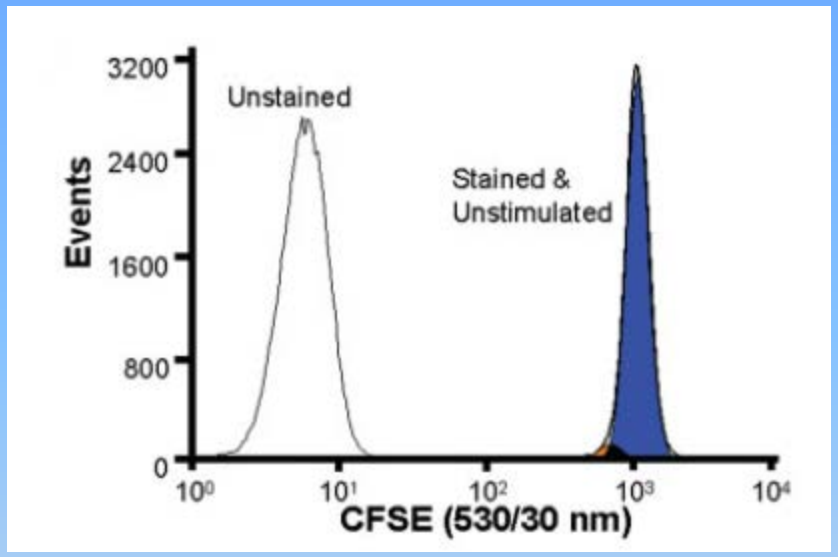


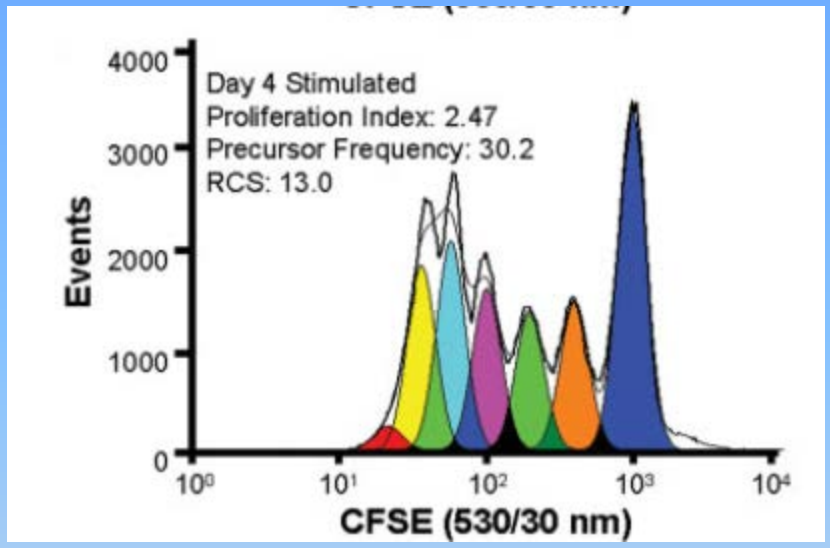






Proliferation

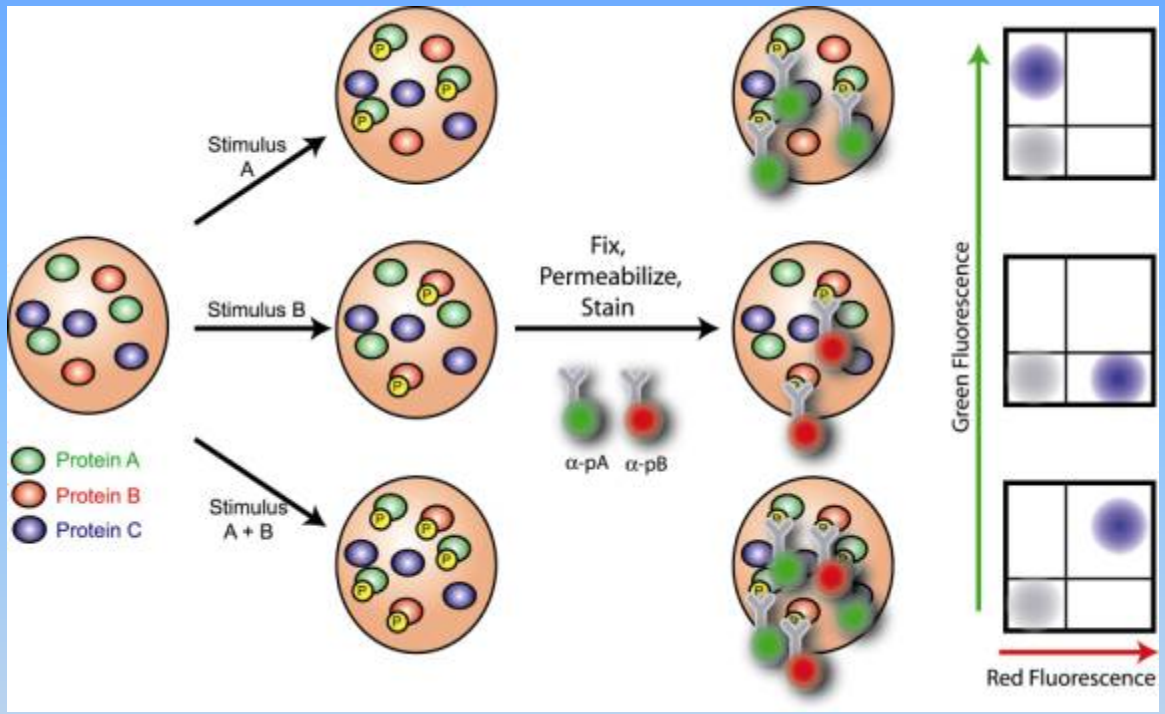




Cells are loaded with a non-fluorescent dye that becomes fluorescent and insoluble inside of the cell. The dye is divided among daughter cells upon division, resulting in a dilution of the dye and a reduction in fluorescence with each subsequent generation.

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

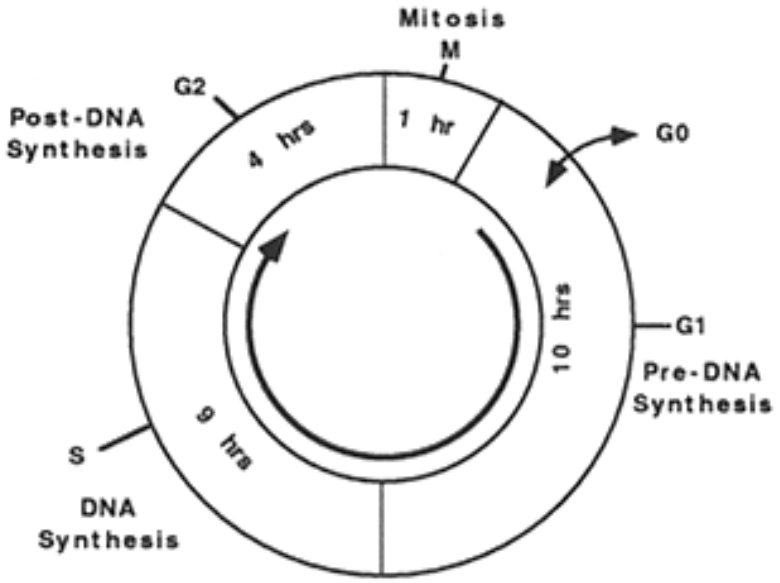


General phospho-protein staining technique for flow cytometry. Cells containing three proteins, A, B, and C, are treated with a stimulus that leads to the phosphorylation of protein A (Stimulus A), protein B (Stimulus B), or both simultaneously. The cells are then fixed, permeabilized, and stained with 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 in shift in both channels.

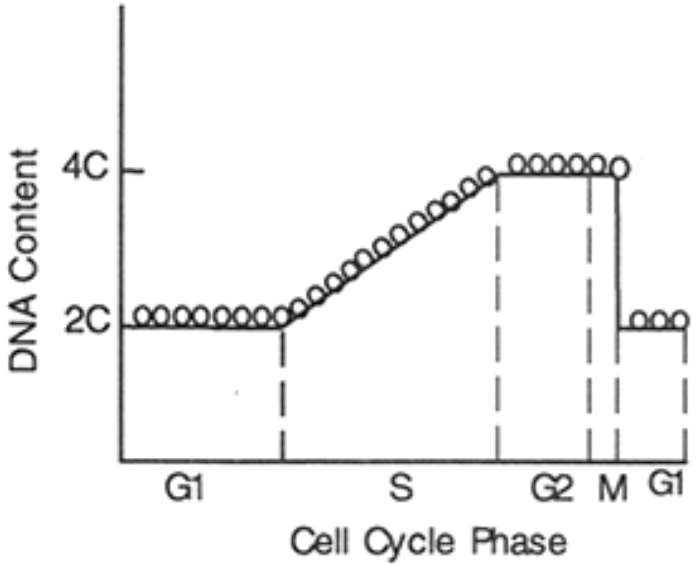
This technique can also be applied to patient samples to help characterize aberrant signaling events that occur during disease progression, or determine the efficacy of signaling pathway-specific drugs in vivo.

http://web.stanford.edu/group/nolan/protocols/proteomics/protocol2.htm

Cell Cycle Analysis

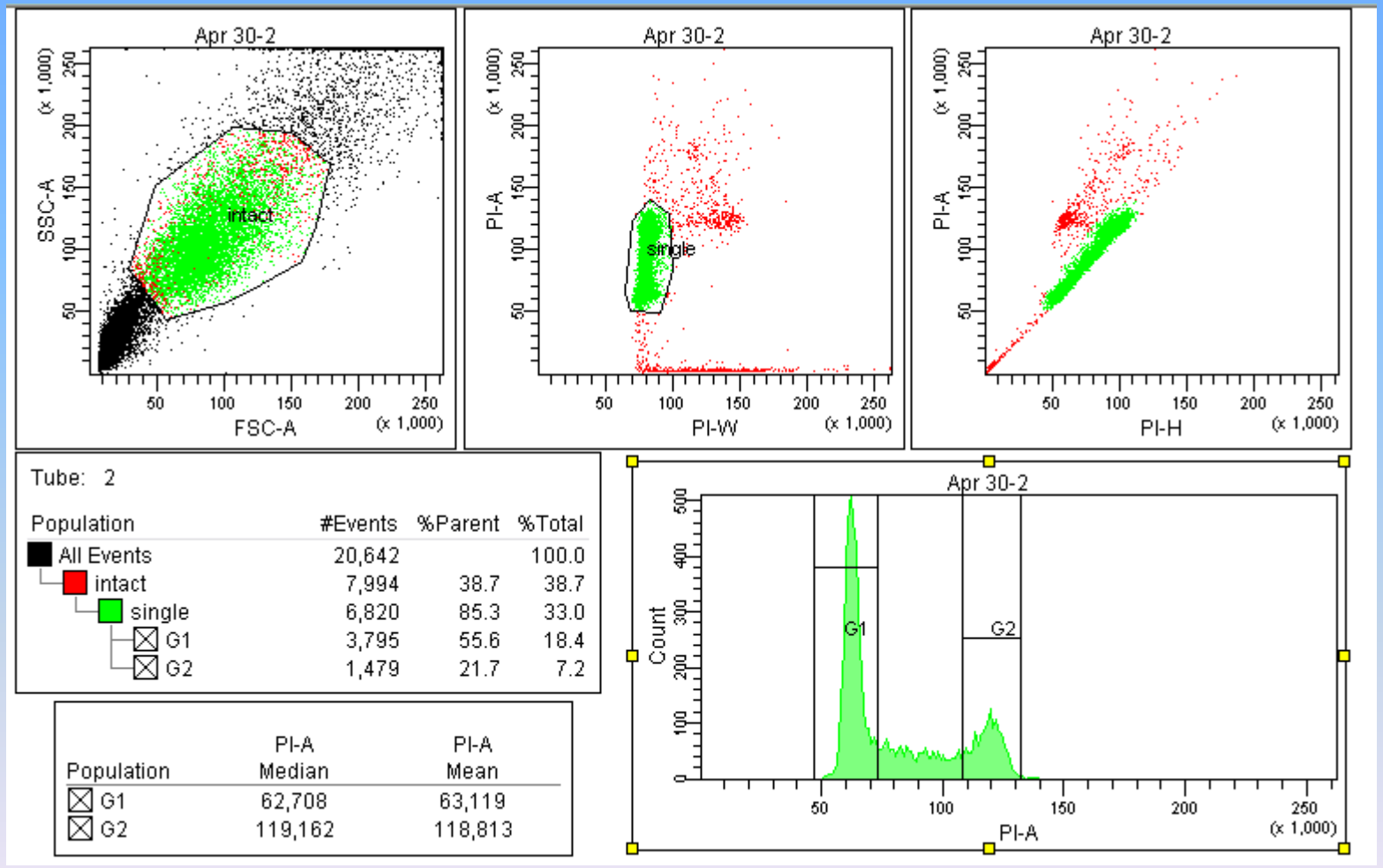


Cell Cycle Phases

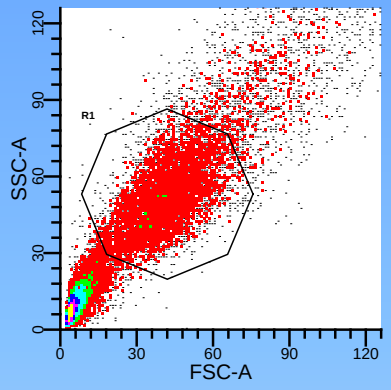


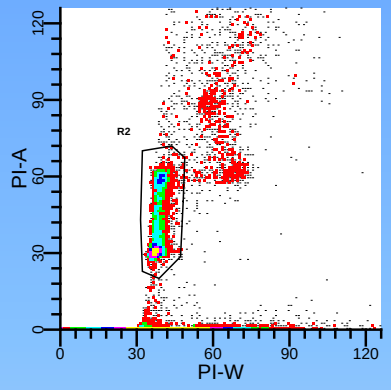
DNA Mass vs Cell Cycle Phase

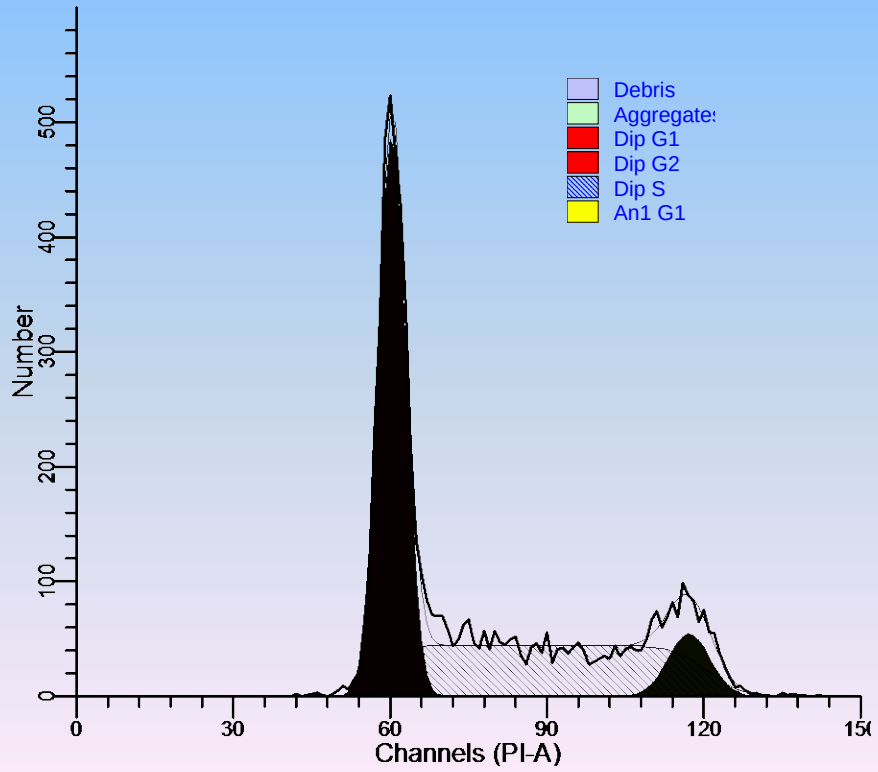
Cell Cycle analysis



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 %

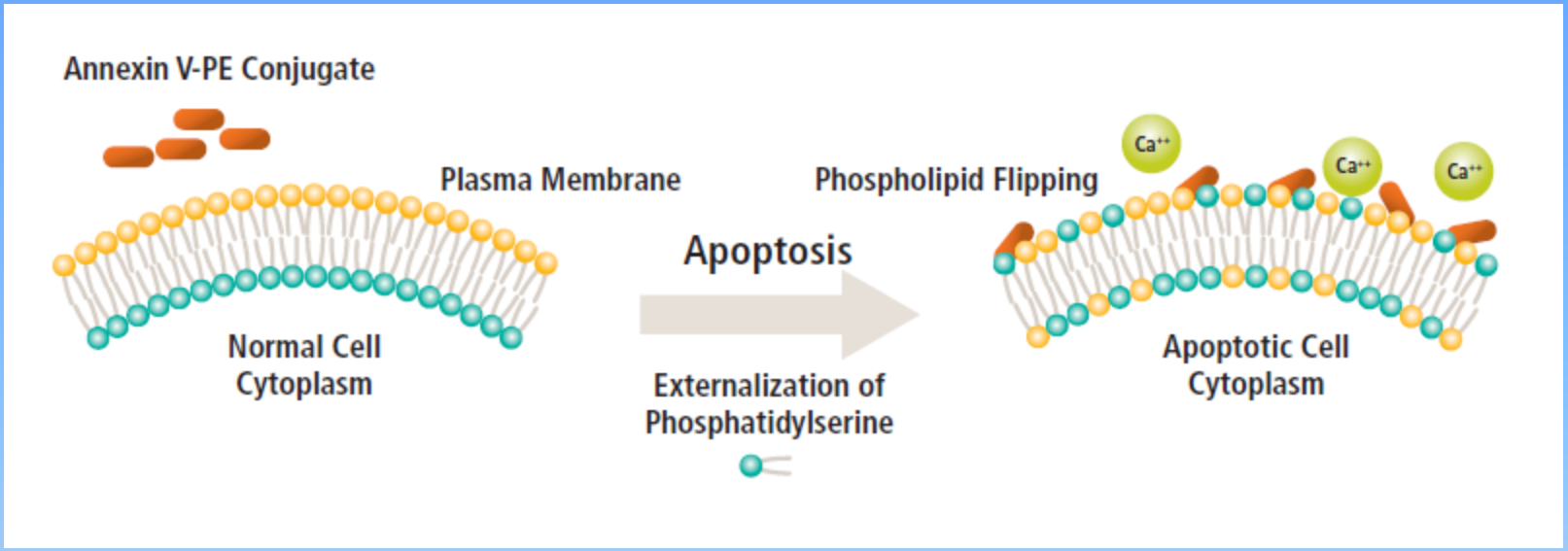
Modeled events: 6469

All cycle events: 6424

Cycle events per channel: 105

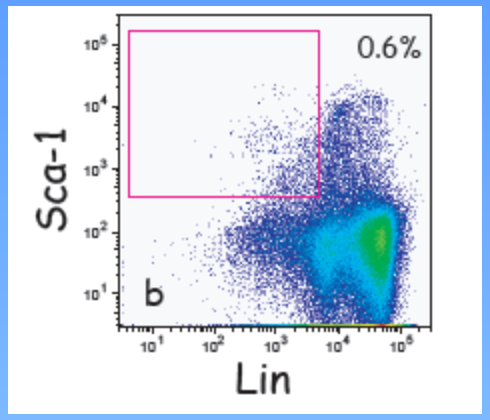
RCS: 1.289

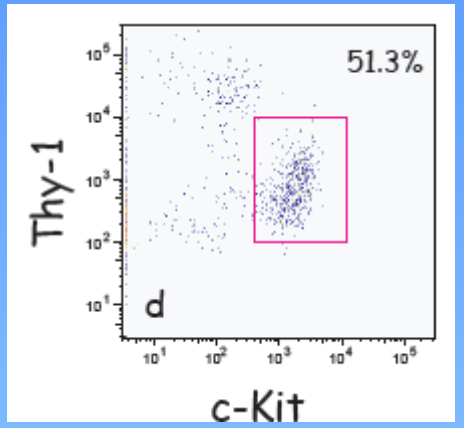
Apoptosis

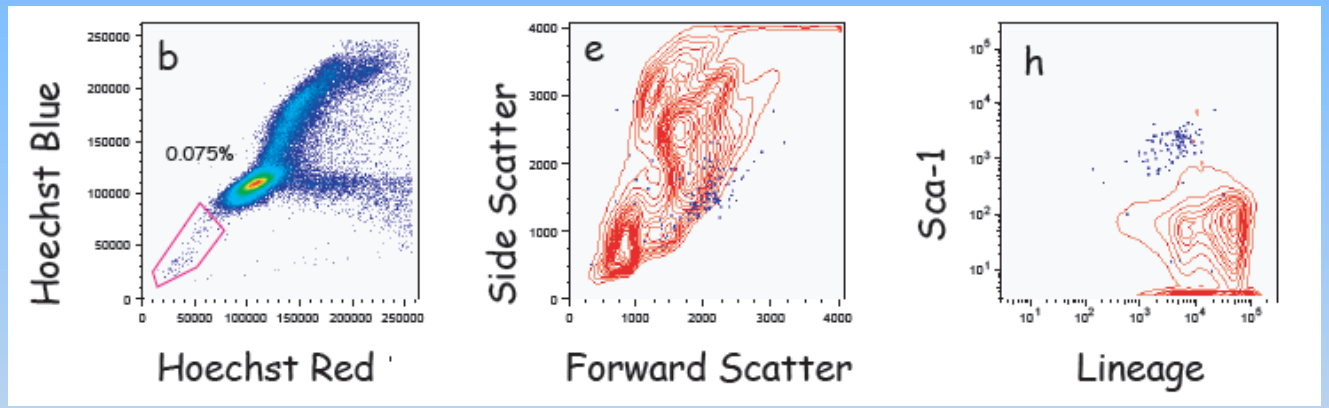


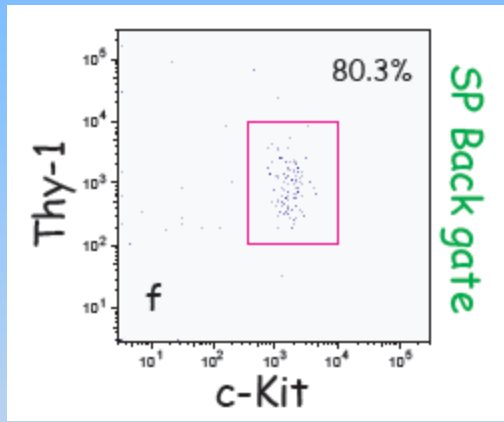
Annexin V–A Key Protein in Apoptosis Signaling Changes in the plasma membrane are one of the first characteristics of the apoptotic process detected in living cells. Apoptosis can be detected by the presence of phosphatidylserine (PS), which is normally located on the cytoplasmic face of the plasma membrane. During apoptosis PS translocates to the outer leaflet of the plasma membrane and can be detected by flow cytometry and cell imaging through binding to fluorochrome-labeled Annexin V when calcium is present.
Since intracellular Annexin V is also exposed if the plasma membrane is compromised, a membrane-impermeant dye such as 7-AAD is commonly used to distinguish between apoptotic and dead cells to exclude the dead cells. The populations of cells that are stained with Annexin V only represent the apoptotic cell populations.

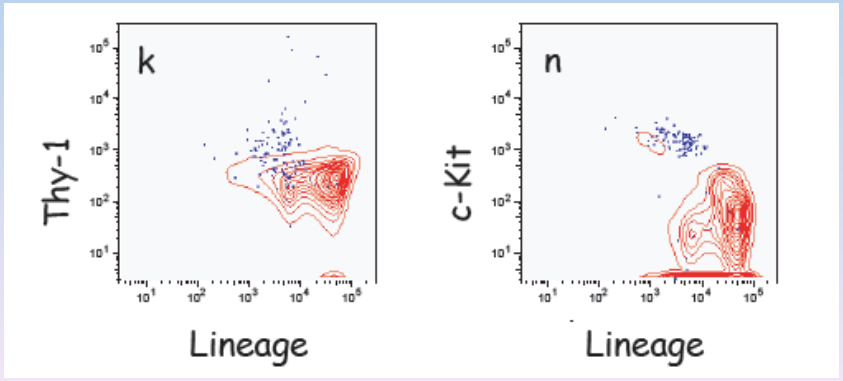
Stem Cells: KTLS and Side Population











http://science.cancerresearchuk.org/sci/facs/

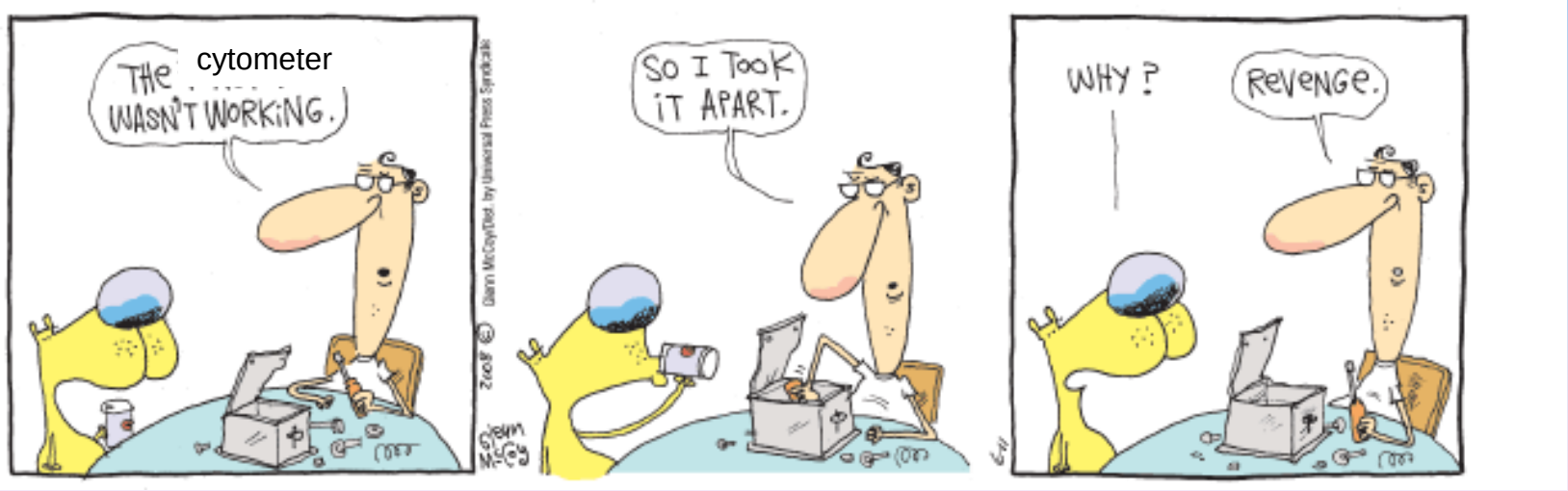
Catherine Simpson, Derek C. Davies, Daniel Pearce, Dominique Bonnet.

General Flow Information

•We have a 3 Laser LSR2, two 4 Laser LSR2s, a 5 Laser LSR, and a FACSCalibur 4 color instrument available, as well as a 5 Laser FACSAria sorter which is run solely by core personnel. Configurations on the website, along with non-Flow Cytometry resources that the core can provide access to.

•I strongly encourage you to sit with someone else in your lab that is using the instruments already, before scheduling an instrument orientation session with me.

• Please review the user documents on the ragonmanagement website



Credits and Resources

Slides borrowed from Mario Roederer, Howard Shapiro, Joe Trotter, BD Biosciences, Invitrogen and DAKO (/Cytomation: an Agilent Technologies company), or made by Andrew Cosgrove or me. Headlines from the Boston Globe.

Websites:

http://www.ragoninstitute.org/research/services/flow-cytometry/
-Core overview, link to user resources

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

www.bdbiosciences.com

www.invitrogen.com

Books:

Practical Flow Cytometry-Howard Shapiro
Available online at http://www.beckman.com/coulter-flow-cytometry/practical

Cytometry: First Principles- Alice Givan

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

Flow Cytometry-A basic introduction—Michael Ormerod

Current Protocols in Cytometry (Wiley)—available in core office room 996