CENTER FOR AIDS RESEARCH
University of Washington

CFAR
Flow Cytometry Workshop
June 27, 2007

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HIV Vaccine Trials Network
CFAR, Flow Core Director

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A LIFE OF SCIENCE

HIV VACCINE TRIALS NETWORK

Overview

- Topics chosen based on my experience working with and training scientists in the use of advanced flow cytometry
- Especially relevant for experiments requiring 6 or more colors
- Both practical and theoretical considerations
- Useful references included at the end of presentation and on UW CFAR website

http://depts.washington.edu/cfas/cfar/core_molecular.html

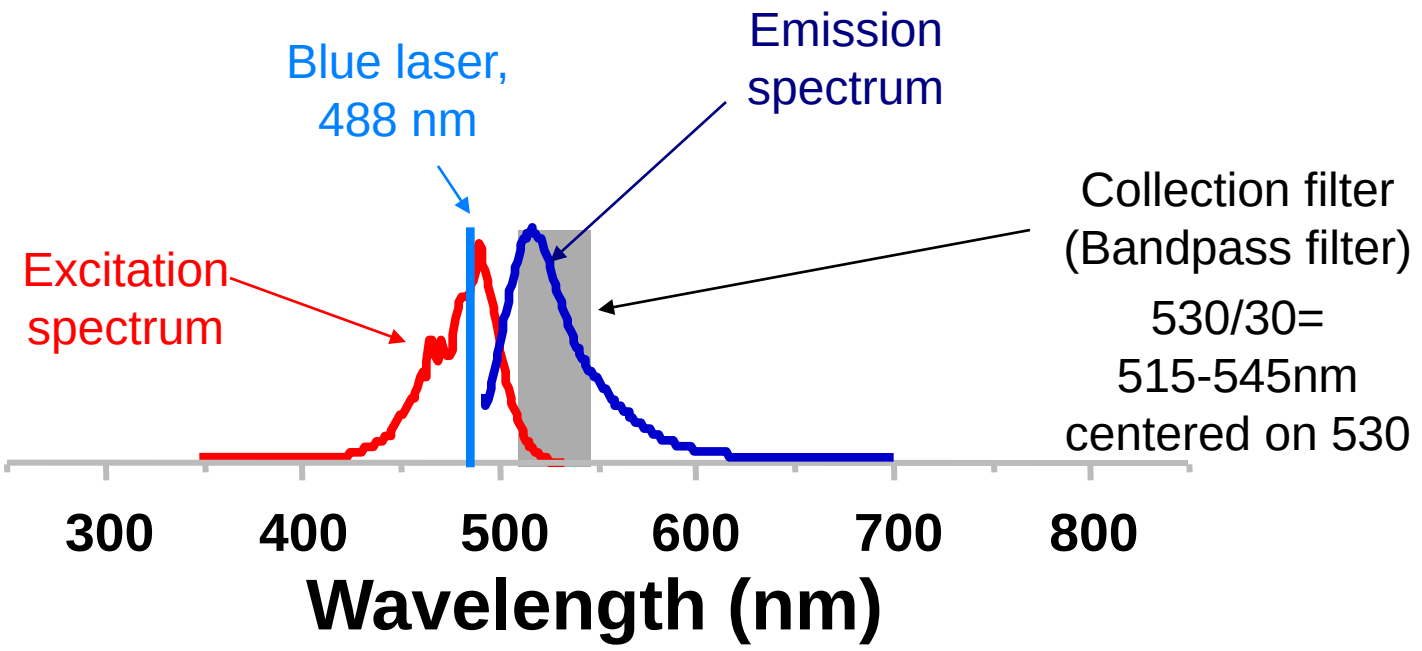
Topics

- Multicolor panel design based on dye spectra and instrument configuration
- Instrumentation - selected comments and local resources
- Compensation
- Antibody titration
- Flow data analysis not presented

Fluorescent dyes used in combination for multicolor FACS

- Dyes must be bright, have minimal spectral overlap, and there must be methods to conjugate to antibodies
- Multicolor FACS uses dyes that are either **excited** at different wavelengths or **emit** at different wavelengths
- Optimal excitation and emission wavelengths can be determined from excitation and emission **spectra**

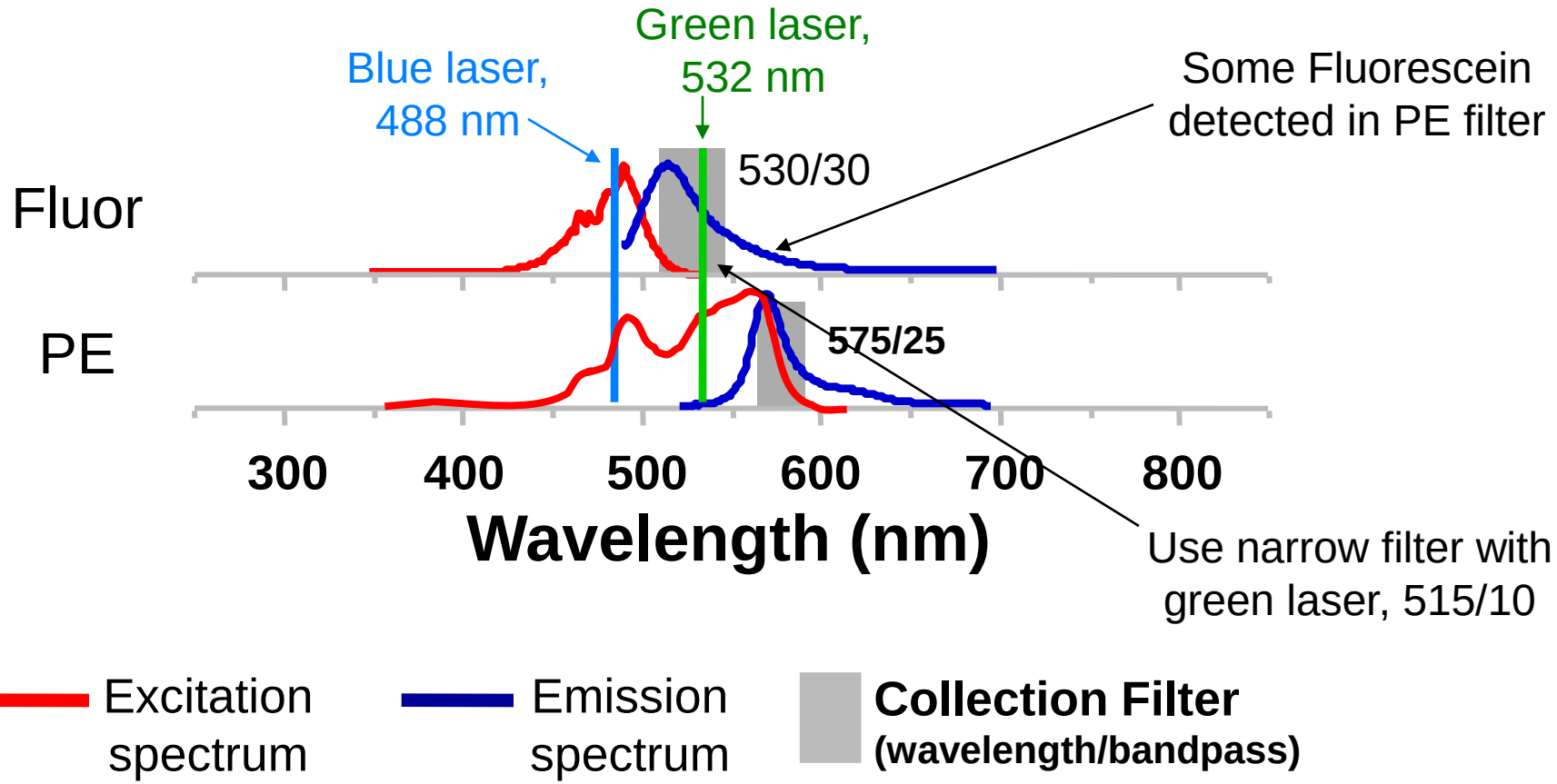
Spectra of Fluorescein



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

Spectra of dyes: 2-color example

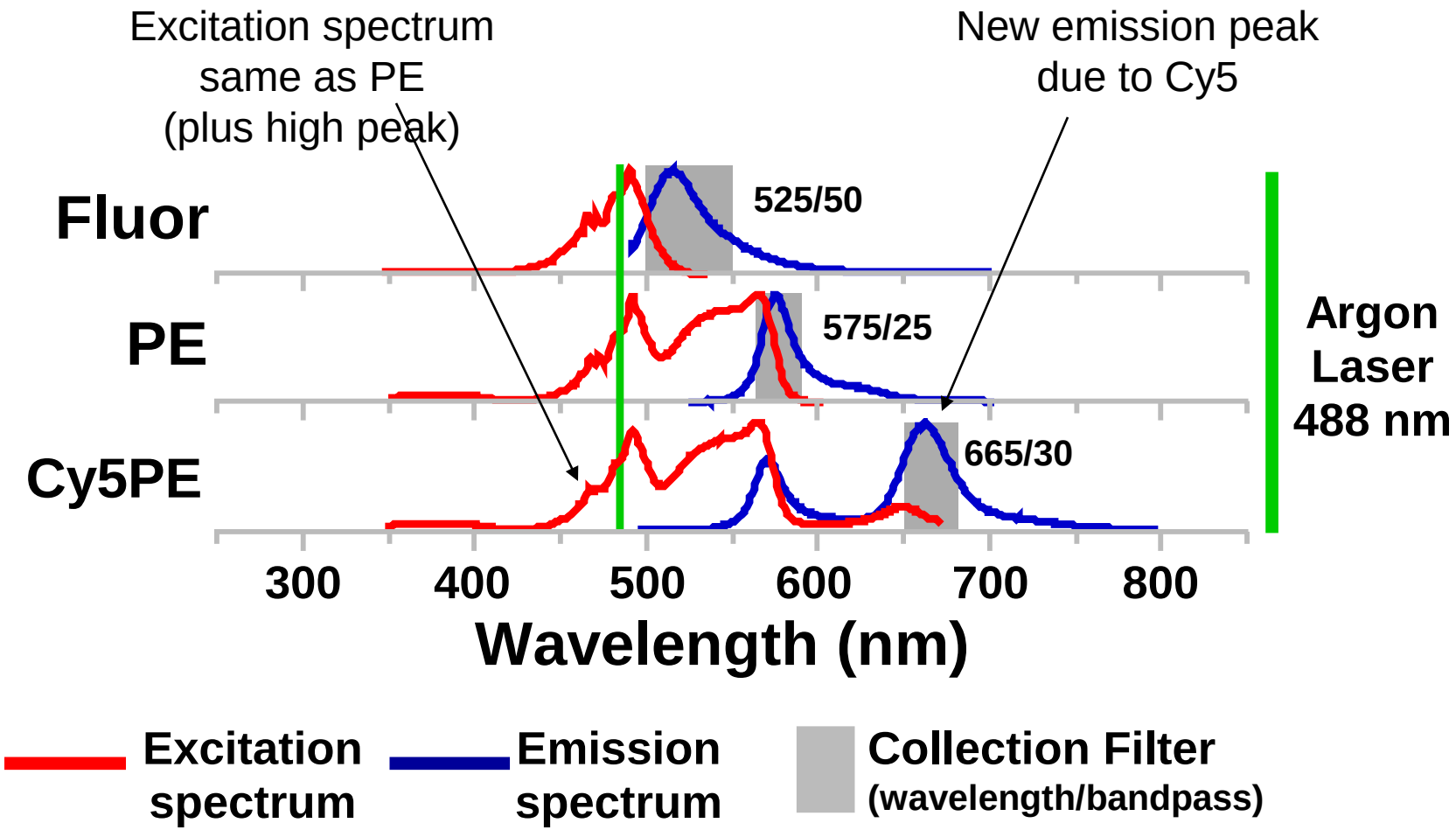
Different emission spectra allow signals to be distinguished



Tandem dyes

- Dyes covalently linked so that energy from one dye is transferred to the other (resonance energy transfer)
- Ideally, when the dye is excited, it emits mainly at the wavelength characteristic of the second dye (with little emission from the first dye)
- Examples: Cy5PE (cychrome), TRPE (ECD), Cy5.5PE, Cy7PE, Cy5.5APC, Cy7APC, Alexa700-PE

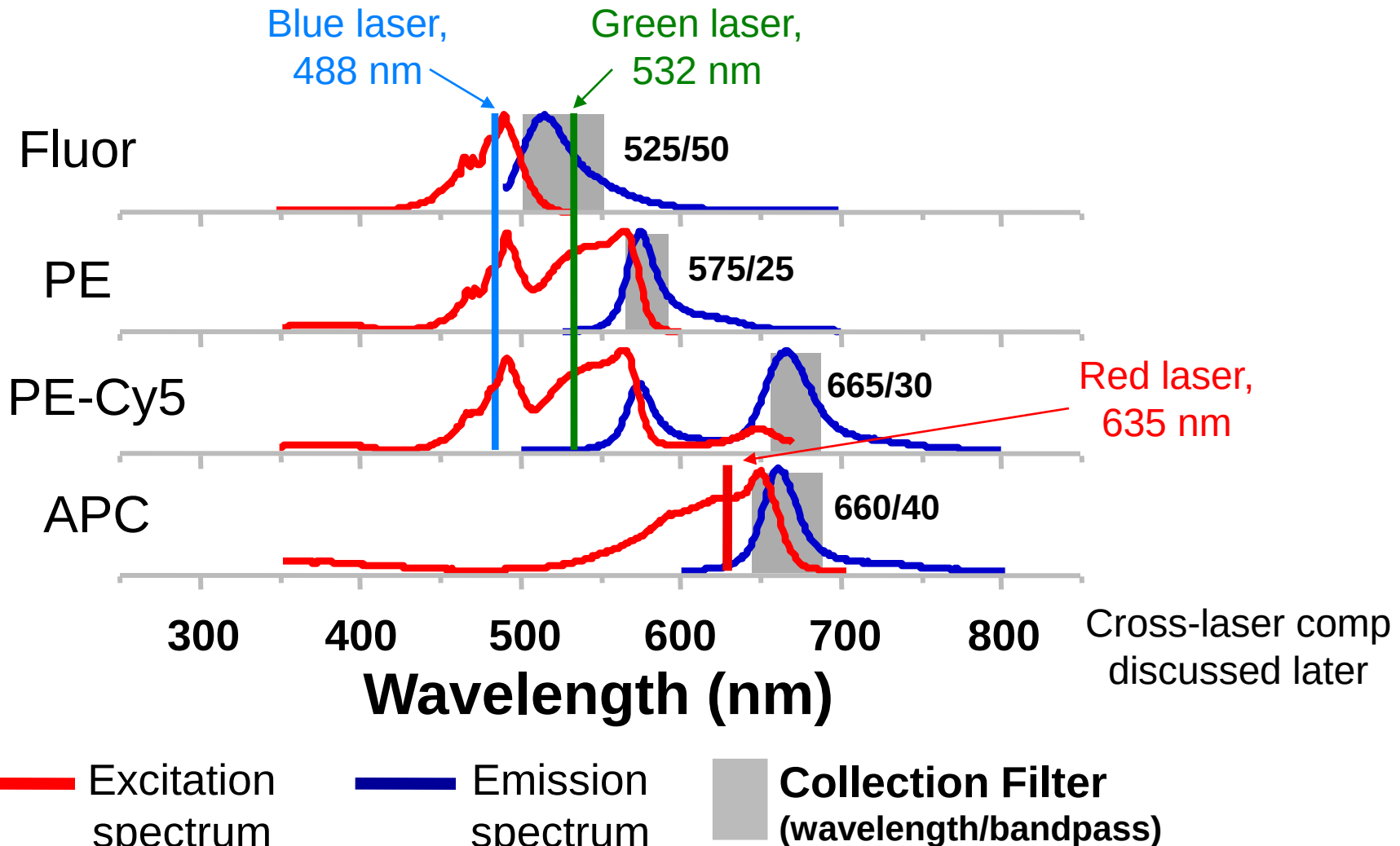
Spectra: 3-color example (one laser)



Additional lasers

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

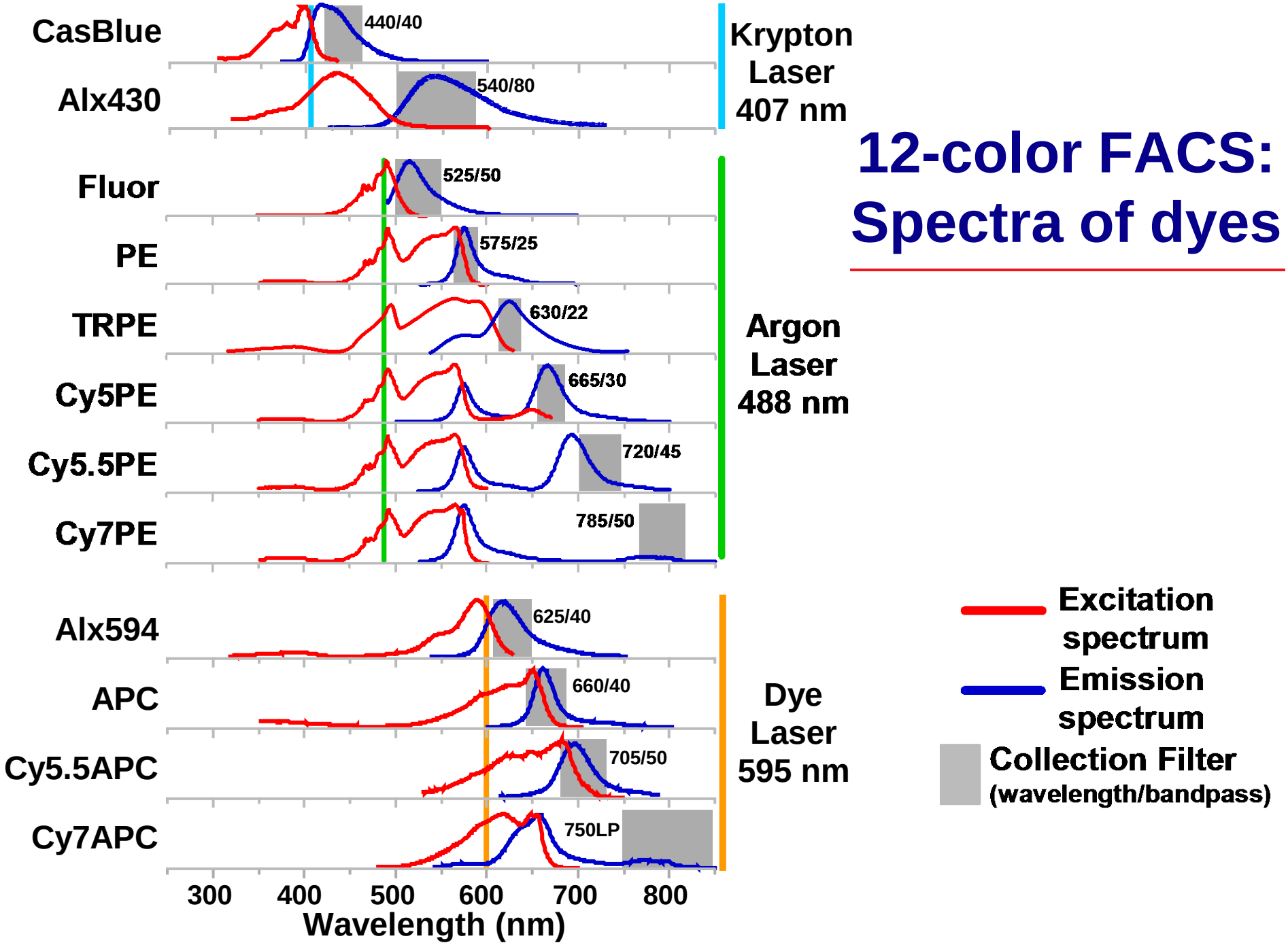
Spectra: 4-colors (2 lasers)



Up to 17-color FACS is a logical extension of 2- and 3-color FACS

Multiple lasers with multiple PMT detectors for each laser

12-color FACS: Spectra of dyes



There is flexibility in choice of dyes

- Consider instrument configuration and spectra of dyes
- Consider compensation requirements
- Consider other characteristics of dyes (susceptibility to photo-bleaching, stability)

Nomenclature note: a channel may be labeled with the name of a dye even though several different dyes can be used in that channel.

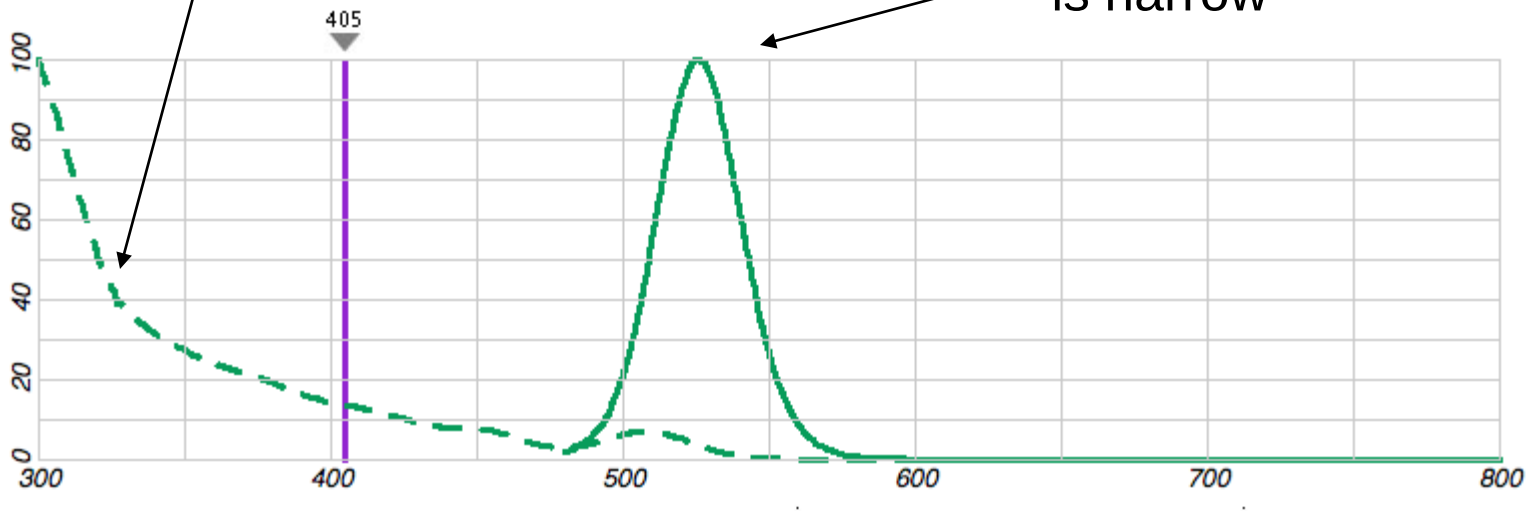
Quantum Dots

- Semiconductor nanoparticles
- Inorganic crystals of cadmium selenide (core) coated with zinc sulfide
- Roughly size of proteins (10 to 20 nm)
- Wavelength of light emitted depends on the size of the core (available in many different “colors”)
- Narrow emission spectra
- Excellent photostability

Quantum dot 525

Excitation spectrum
UV and violet excitation optimal

Emission spectrum
is narrow

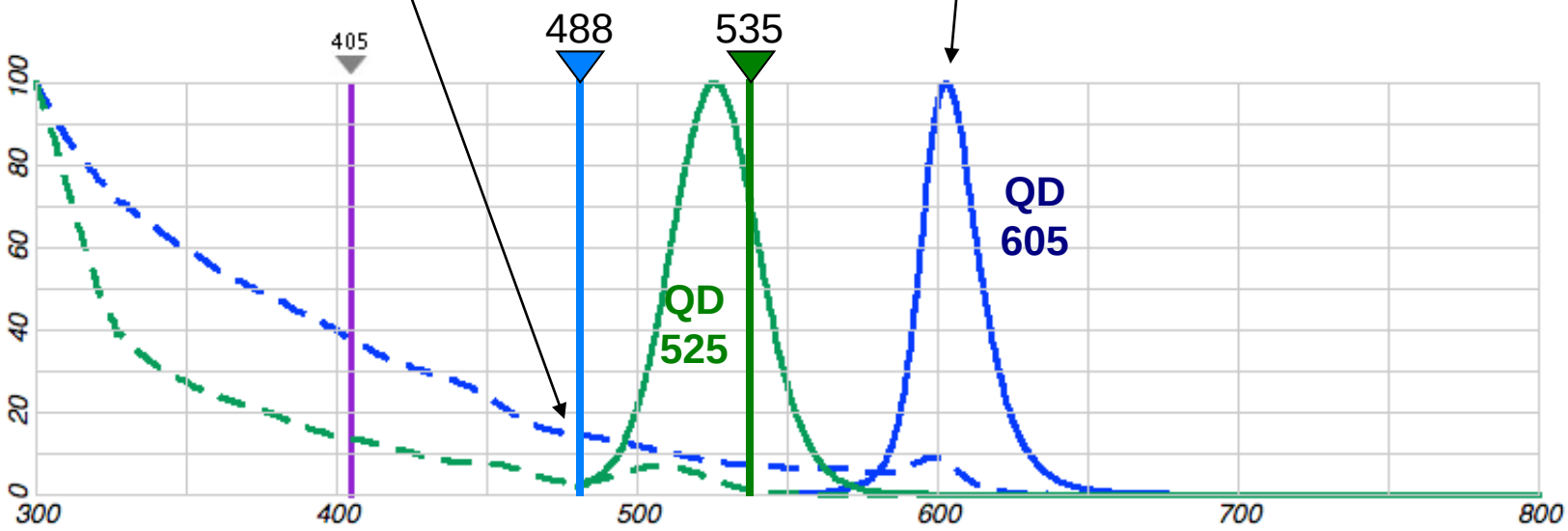


http://probes.invitrogen.com/resources/spectraviewer/

Quantum dots 525 and 605

QD605 also excited by 488 laser

Emission spectrum overlaps with PE-TR

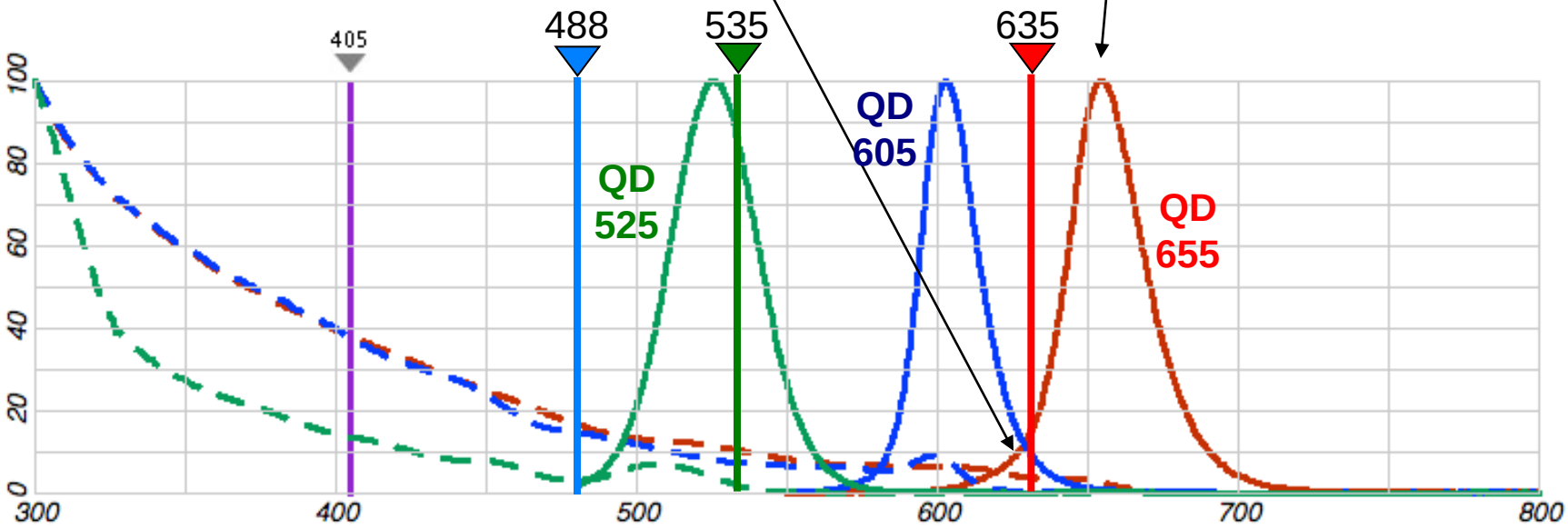


http://probes.invitrogen.com/resources/spectraviewer/

Quantum dots 525, 605 and 655

QD655 also excited by 635 laser

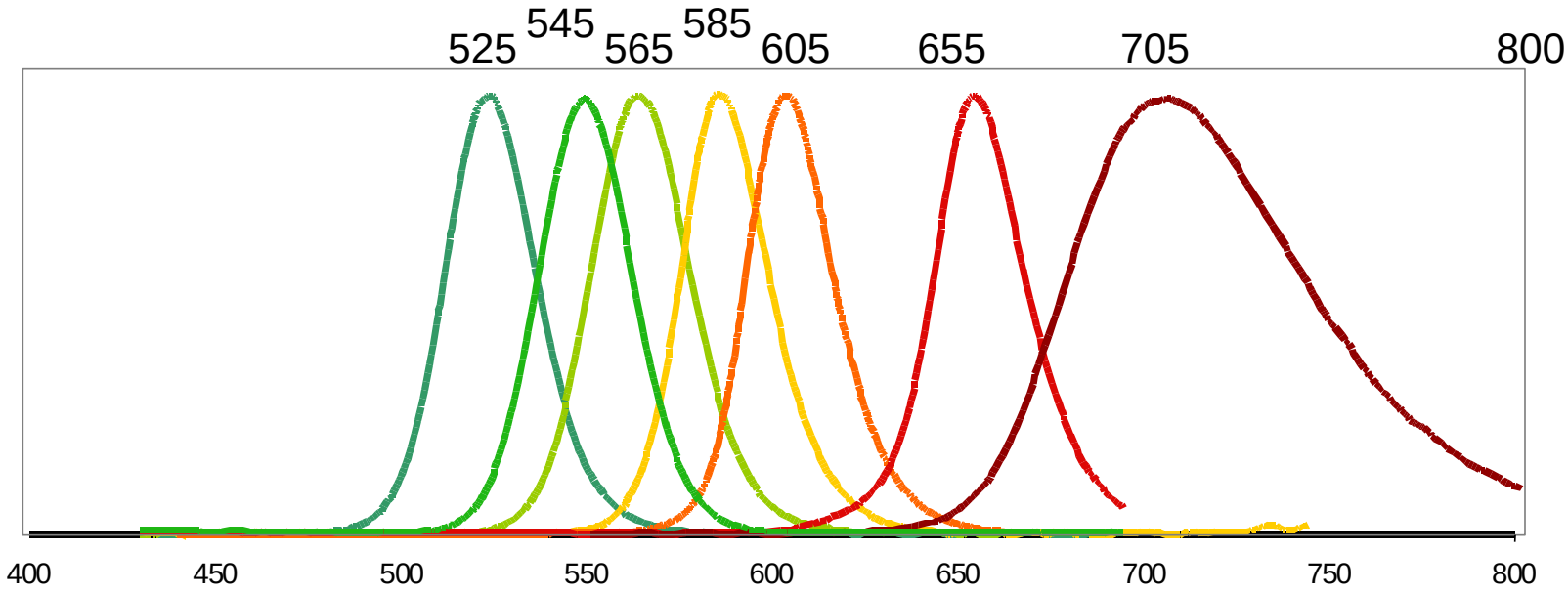
Emission spectrum overlaps with PE-Cy5 and with APC



http://probes.invitrogen.com/resources/spectraviewer/

Quantum dots

Available in many “colors”



Quantum dot emission spectra are *fairly* narrow. Adjacent dots have much overlap; but alternate dots do not

LSR
17 color

Violet laser 407nm

Cascade Blue, Pacific Blue, Alexa 405
AmCyan, Cascade Yellow, Alexa 430
Quantum Dot 545
Quantum Dot 565
Quantum Dot 585
Quantum Dot 605
Quantum Dot 655
Quantum Dot 705

Blue laser 488nm

FITC, CFSE, Alexa 488
PE
PE-TR, ECD
PE-Cy5, PerCP
PE-Cy5.5, PerCP-Cy5.5, PE-Alexa700
PE-Cy7, PE-Alexa750

Red laser 635nm

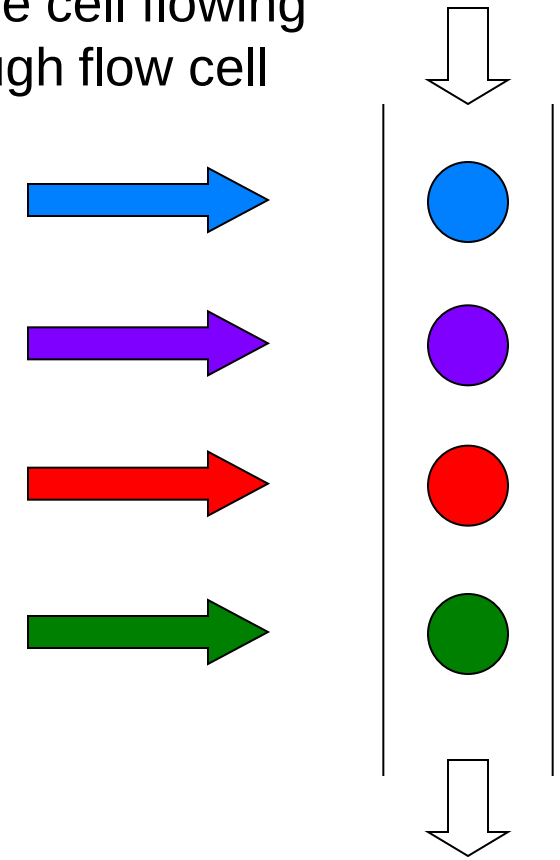
APC, Alexa 647
Alexa 680 or 700
APC-Cy7, APC-Alexa750

Cross-Laser Excitation

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

Laser Delay

A single cell flowing through flow cell



Signal collected at:

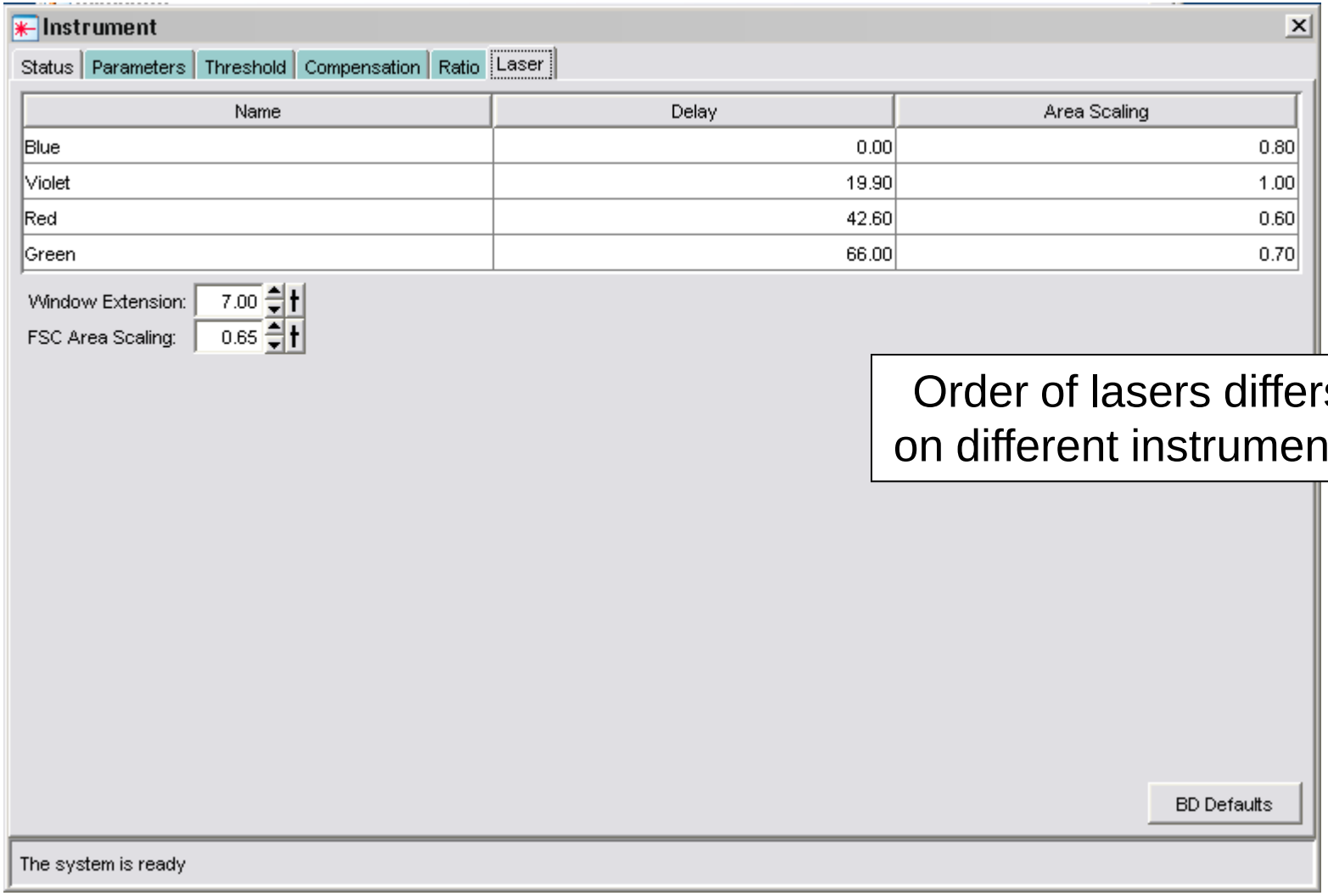
time T_{blue}

time T_{blue}+violet delay

time T_{blue}+red delay

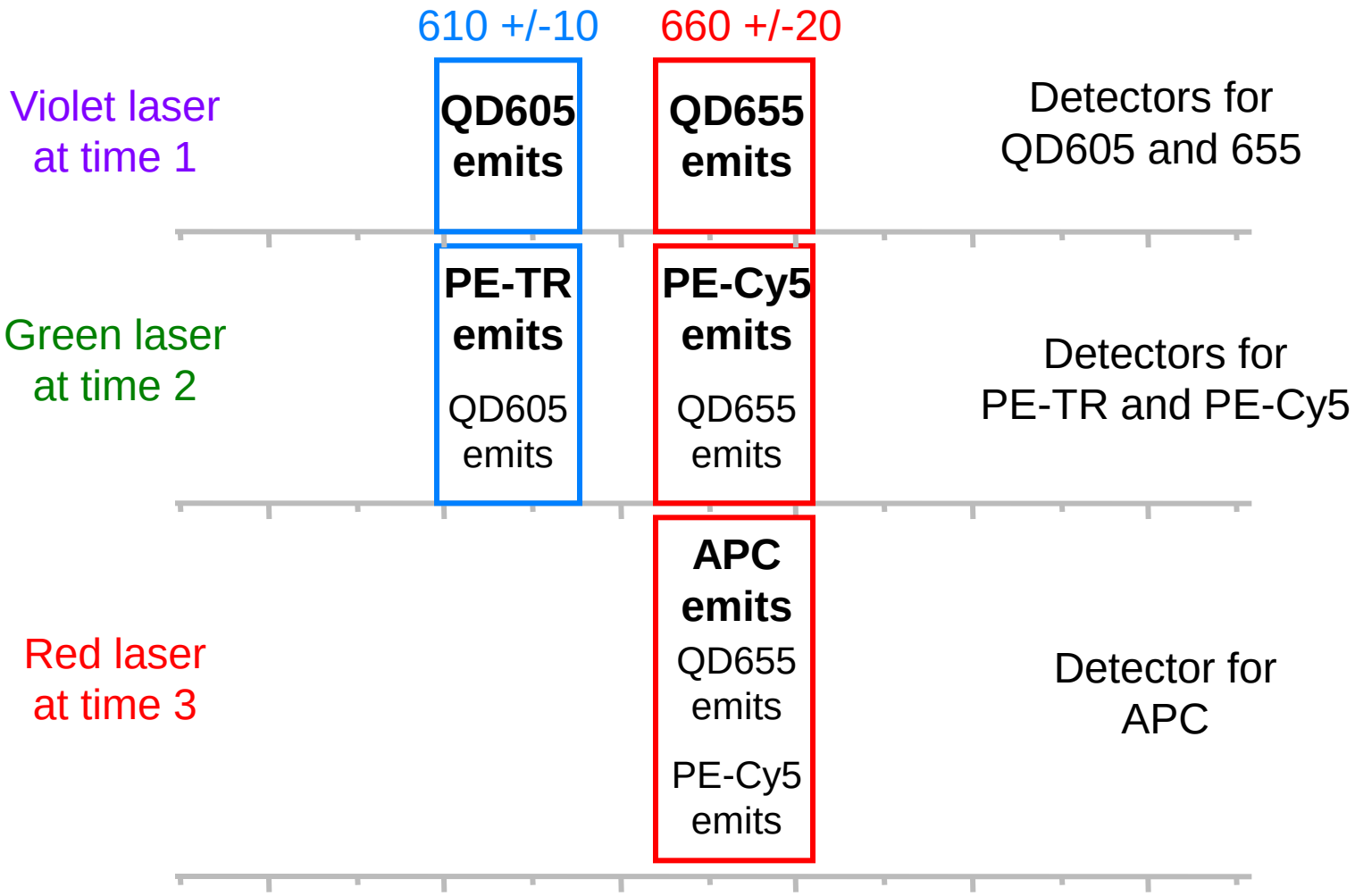
time T_{blue}+green delay

Laser Delay Settings



Order of lasers differs on different instruments

Cross-laser compensation



Staining Panel Optimization*

- List markers of interest
- Characterize staining profiles for markers:
  1. distinct bright positive populations (e.g., CD3, CD4, CD8)
  2. varying intensity from negative to positive (e.g., CD45RO)
  3. dim staining (e.g., IL4)

*Adapted from seminar given by Mario Roederer, VRC/NIH

Staining Panel Optimization: choice of colors

- Characterize each potential color in context of the multicolor combination
  - Brightness
  - Spread after compensation
  - Available for use with intracellular staining
- Panel design is partly theoretical and partly empirical
  - Dim markers restricted to bright colors with little compensation spread
  - Other markers fill in other colors

Practical considerations

- Optimization is a long, iterative process
- Requires flexibility in color choices for some markers
  - Requires some markers to be conjugated to several colors
  - We conjugate the more common markers ourselves (CD3, CD4, CD8, IFN-γ)
  - Commercial manufacturers are now selling more varieties of antibody/dye conjugates
- Plan to conduct at least 5 optimization experiments (Do not expect the first panel choices to work!)

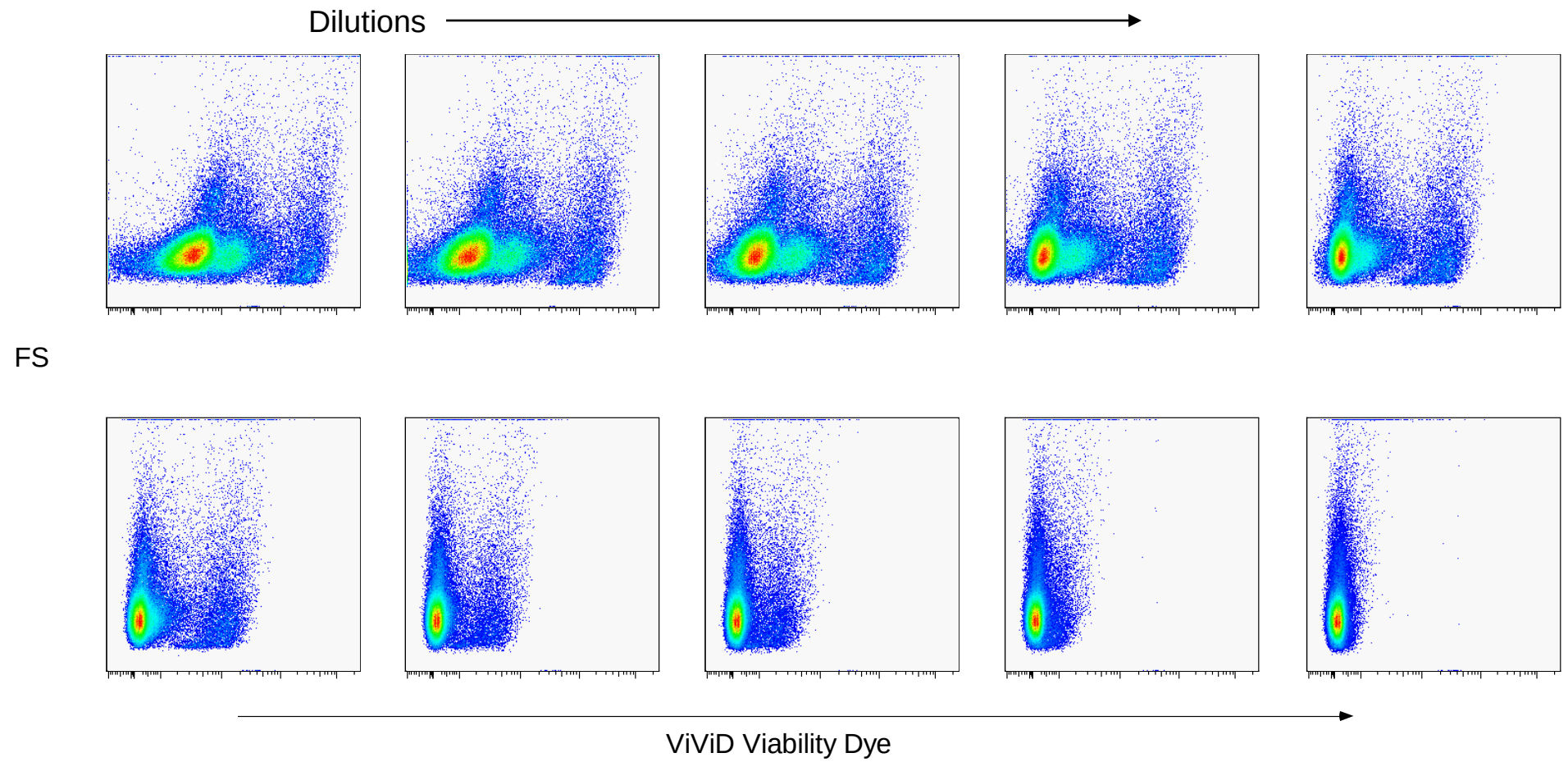
Practical considerations, cont

- It is often useful to start with only a few of the markers of interest used in combination
  - Gain experience with these markers
  - Will know if the staining pattern for these markers deteriorates as more colors are added to combination
- Test several different permutations for all staining panels
- Rank each combination, deriving rules about reagents and combinations
- Based on the staining results for first test panels, design modified panels, and repeat process as needed

Special note on viability markers

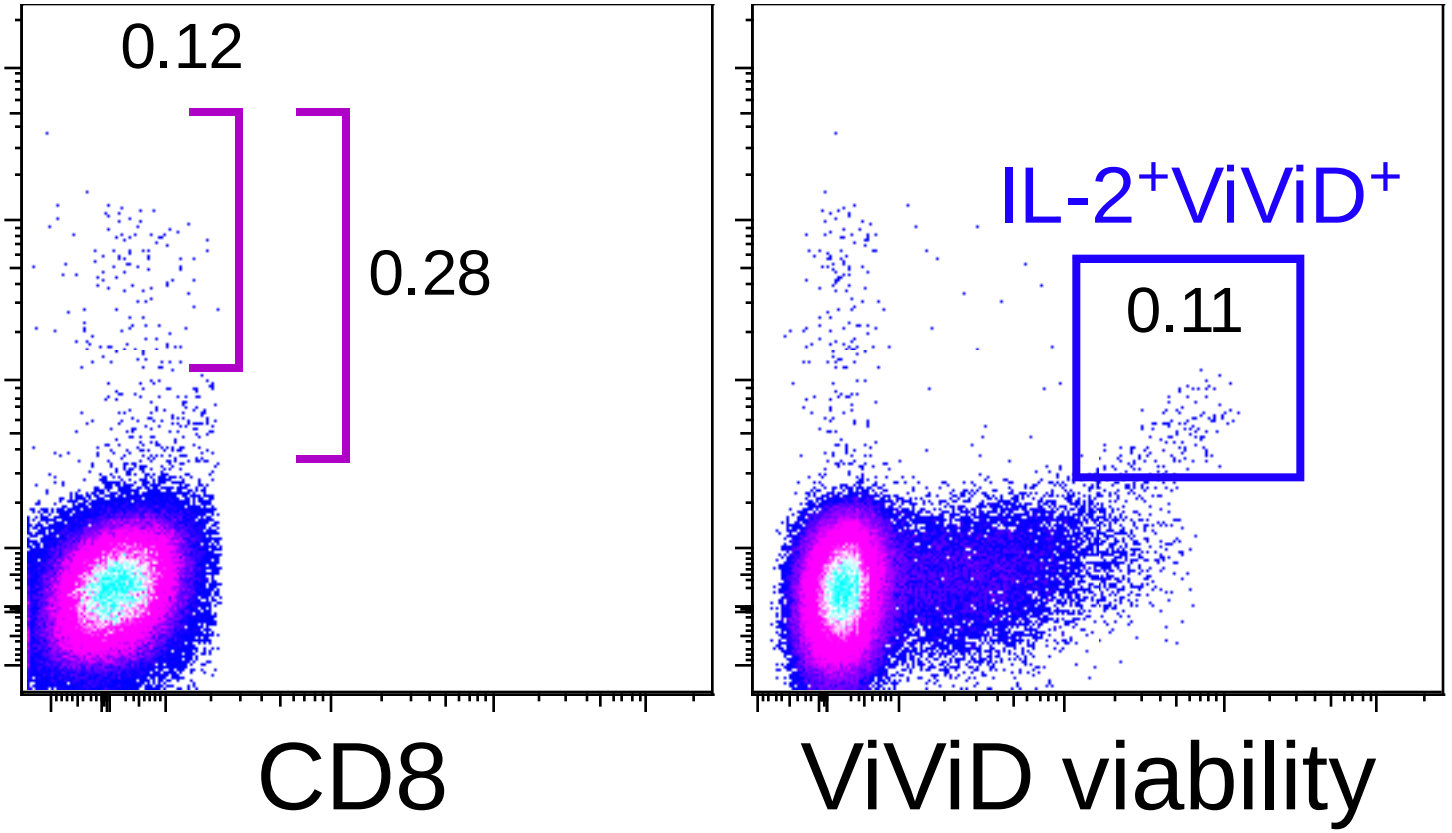
- A marker to exclude dead cells is necessary since scatter gating is often not sufficient
- With fixation/permeabilization procedures, viability dyes must be convalently linked inside cells
- EMA, ethidium monoazide bromide, covalently binds to DNA after light exposure
- Fixable Live/Dead staining dyes available from Molecular Probes/Invitrogen in multiple “colors”
  - Covalently link to amine groups on the cell and within the cell

ViViD Viability Dye Titration



032007

Including dead cells artificially increases IL-2 response



060602-008-0608
Horton et al, JIM, 2007

Instrumentation:
A few brief comments

The 2006 Kyoto Laureates
Advanced Technology Category
Biotechnology and Medical Technology

"Outstanding contribution to life sciences with the development of a flow cytometer that uses fluorescent-labeled monoclonal antibodies"

Dr. Leonard Arthur Herzenberg took the lead in developing a flow cytometer called the Fluorescence-Activated Cell Sorter (FACS) that automatically sorts viable cells by their properties. Combining fluorescent-labeled monoclonal antibodies as FACS reagents with this instrument, he made an enormous contribution towards the dramatic advancement of life sciences and clinical medicine.

稲盛財団
INAMORI FOUNDATION

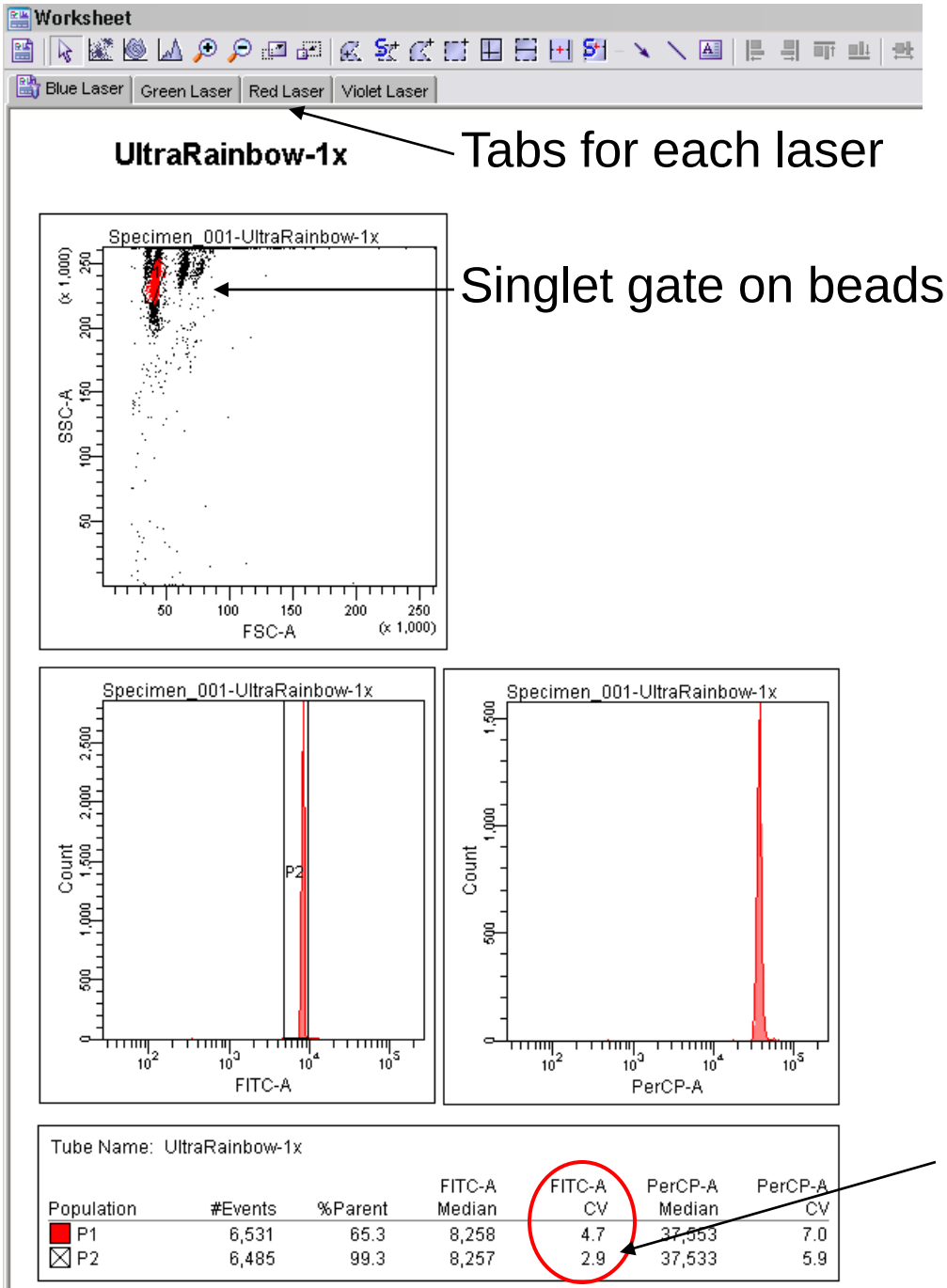
Instrument Set-up and Standardization

- Step 1: Ensure instrument alignment
- Step 2: Set PMT voltages
- Step 3: Collect standardization particles (used for trend analysis over time)
- Step 4: Begin sample collection - include unstained cells and compensation controls

Instrument Alignment

- Even though the FACS facility checks this each day, you should check this yourself before every experiment
- It is a simple procedure to check alignment:
  - Use fluorescent particles (beads), e.g. “rainbow” beads. These fluoresce in most channels.
  - Run beads at low flow rate and determine CV for every channel of interest (after gating on “singlets”)
  - It is useful to have a collection template showing histograms for each channel along with median fluorescence and CV
  - Acceptable upper limit of CV differs for different channels

Alignment Template

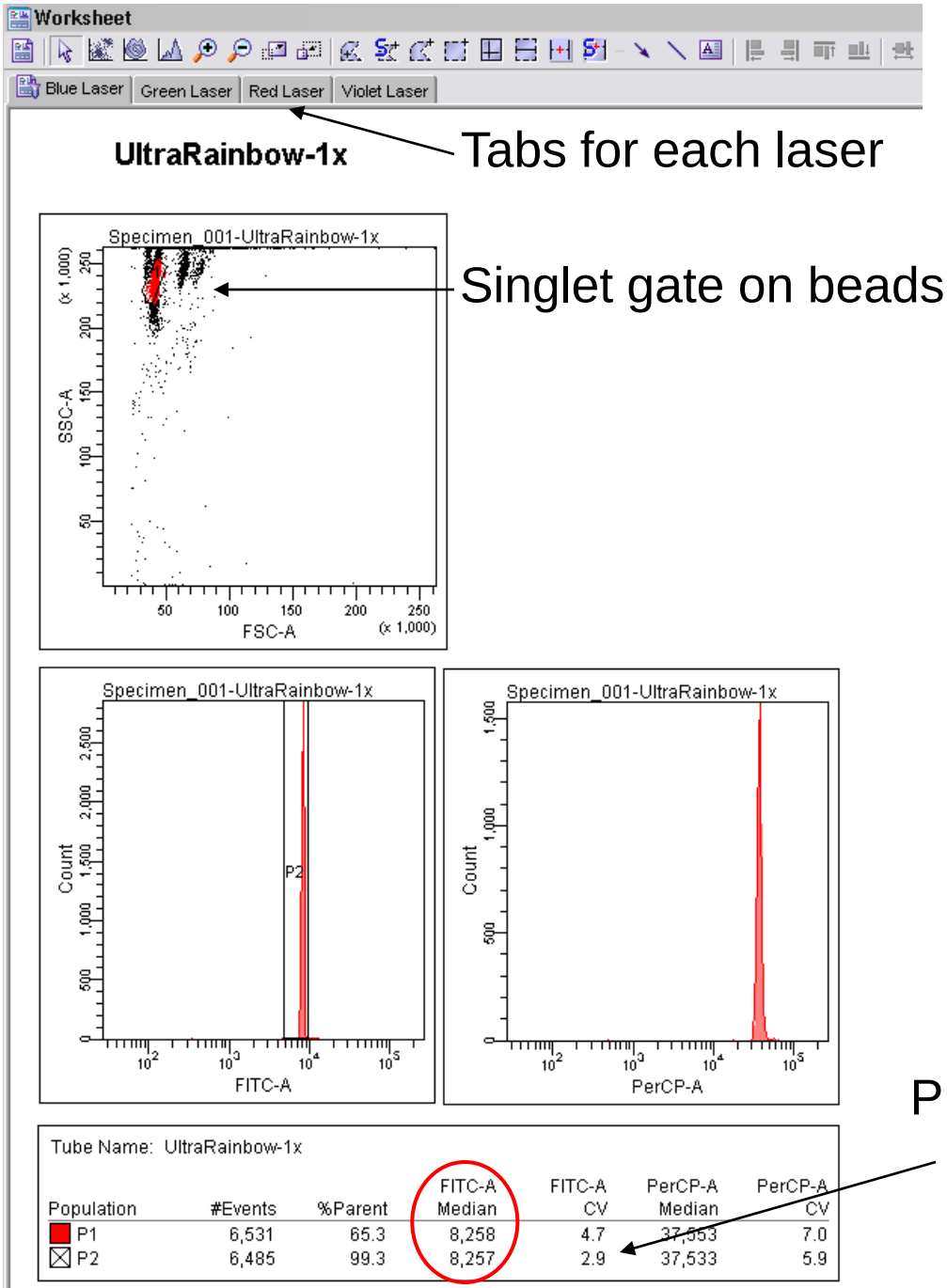


Collection template created in DiVa software for BD LSRII

Instrument Standardization

- Ensure data collected on different days are comparable
- A method to set PMT voltages:
  - Use fluorescent particles (beads), e.g. “rainbow” beads. These fluoresce in most channels.
  - At the beginning of a study determine the optimal target values for median fluorescence intensity (MFI) for the beads in each channel
  - Each time the instrument is used for that study, set the PMT voltages so that the MFI matches the targets (+/-10%)
  - Note: using the same PMT voltages for all experiments is not appropriate standardization, although PMT voltages across experiments should be similar

Alignment Template



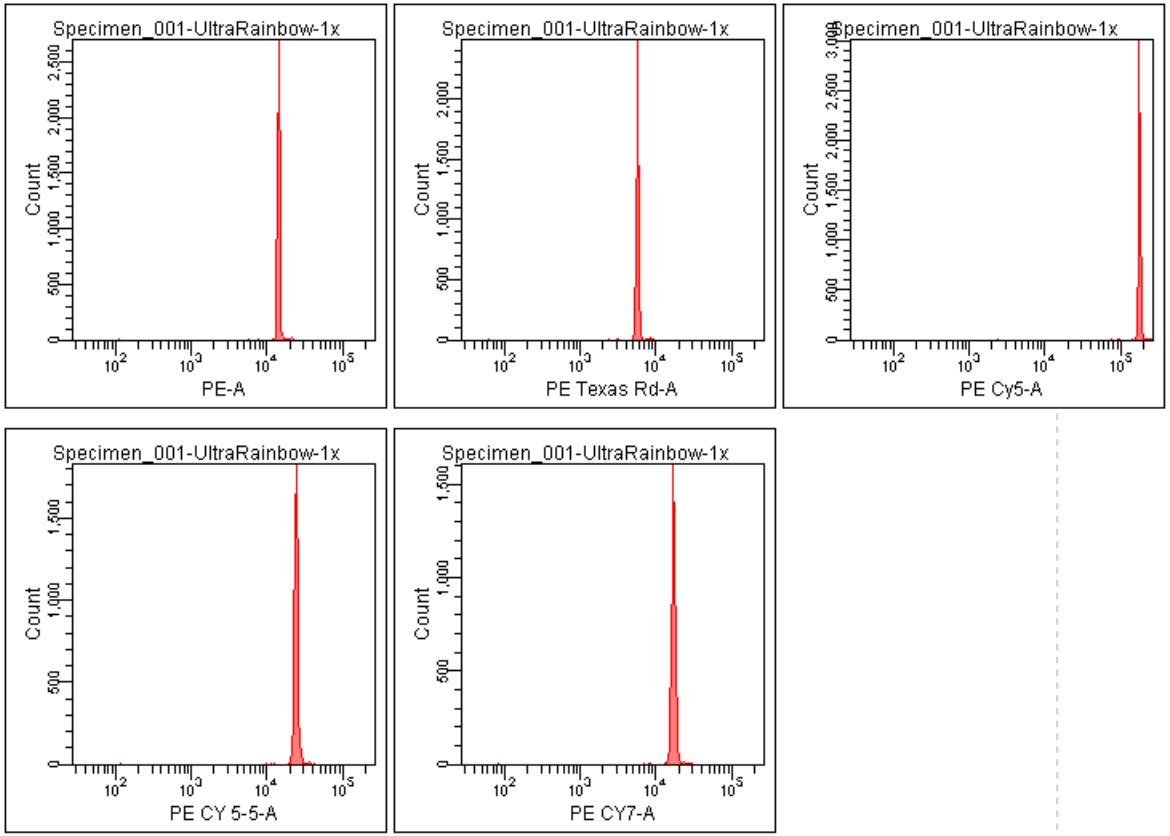
Same template used to set PMT voltages

Green Laser Alignment Template

Worksheet

Blue Laser | Green Laser | Red Laser | Violet Laser

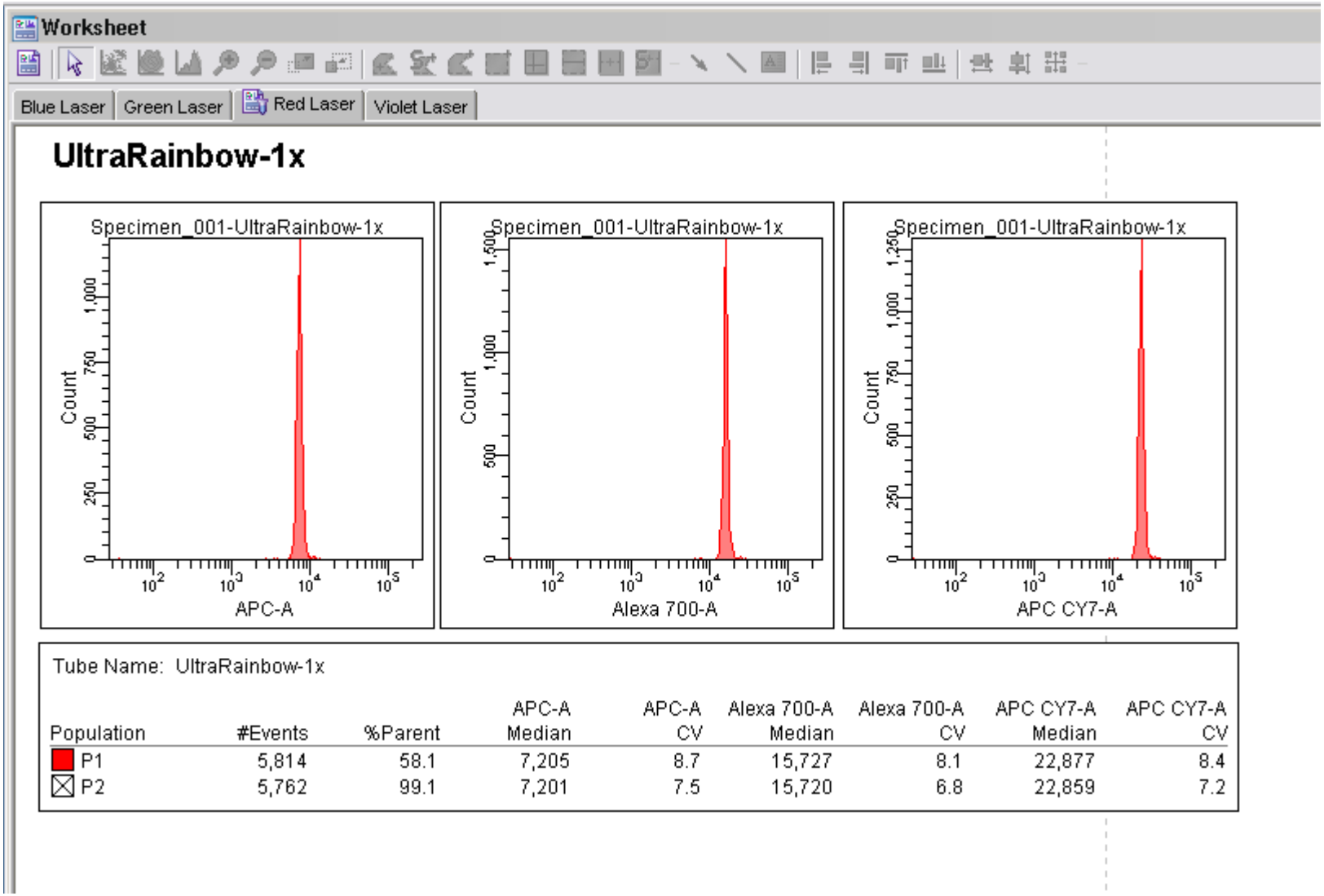
UltraRainbow-1x



Tube Name: UltraRainbow-1x

Population	#Events	%Parent	PE-A Median	PE-A CV	PE Texas R... Median	PE Texas R... CV	PE Cy5-A Median	PE Cy5-A CV	PE CY 5-5-A Median	PE CY 5-5-A CV	PE CY7-A Median	PE CY7-A CV
P1	5,814	58.1	14,279	5.3	5,706	5.2	180,430	4.7	24,351	6.7	17,155	7.4
P2	5,762	99.1	14,275	3.2	5,704	3.2	180,395	2.6	24,345	5.1	17,147	5.9

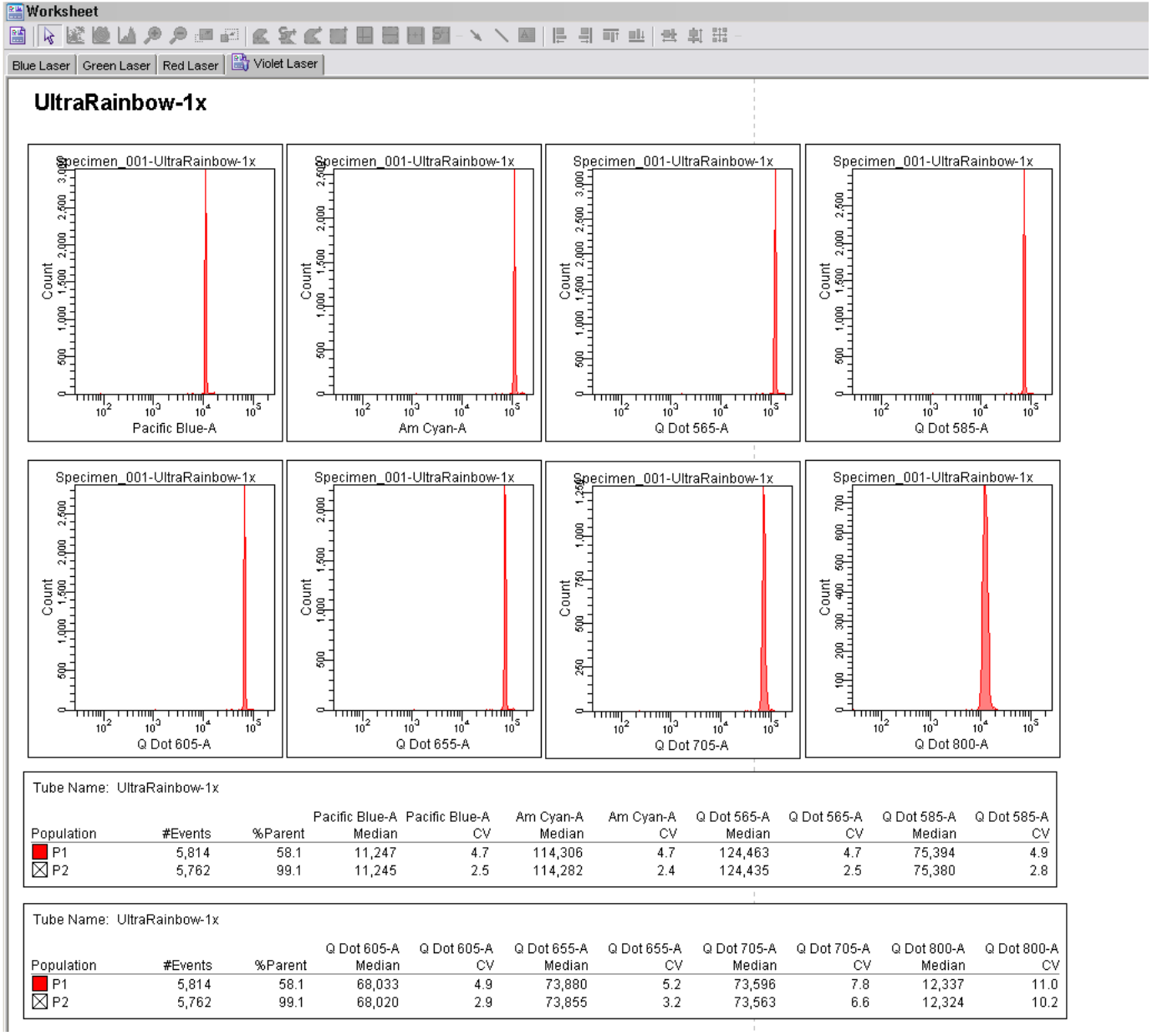
Red Laser Alignment Template



Tube Name: UltraRainbow-1x

Population	#Events	%Parent	APC-A Median	APC-A CV	Alexa 700-A Median	Alexa 700-A CV	APC CY7-A Median	APC CY7-A CV
P1	5,814	58.1	7,205	8.7	15,727	8.1	22,877	8.4
P2	5,762	99.1	7,201	7.5	15,720	6.8	22,859	7.2

Violet Laser Alignment Template



Tube Name: UltraRainbow-1x

Population	#Events	%Parent	Pacific Blue-A Median	Pacific Blue-A CV	Am Cyan-A Median	Am Cyan-A CV	Q Dot 565-A Median	Q Dot 565-A CV	Q Dot 585-A Median	Q Dot 585-A CV
P1	5,814	58.1	11,247	4.7	114,306	4.7	124,463	4.7	75,394	4.9
P2	5,762	99.1	11,245	2.5	114,282	2.4	124,435	2.5	75,380	2.8

Tube Name: UltraRainbow-1x

Population	#Events	%Parent	Q Dot 605-A Median	Q Dot 605-A CV	Q Dot 655-A Median	Q Dot 655-A CV	Q Dot 705-A Median	Q Dot 705-A CV	Q Dot 800-A Median	Q Dot 800-A CV
P1	5,814	58.1	68,033	4.9	73,880	5.2	73,596	7.8	12,337	11.0
P2	5,762	99.1	68,020	2.9	73,855	3.2	73,563	6.6	12,324	10.2

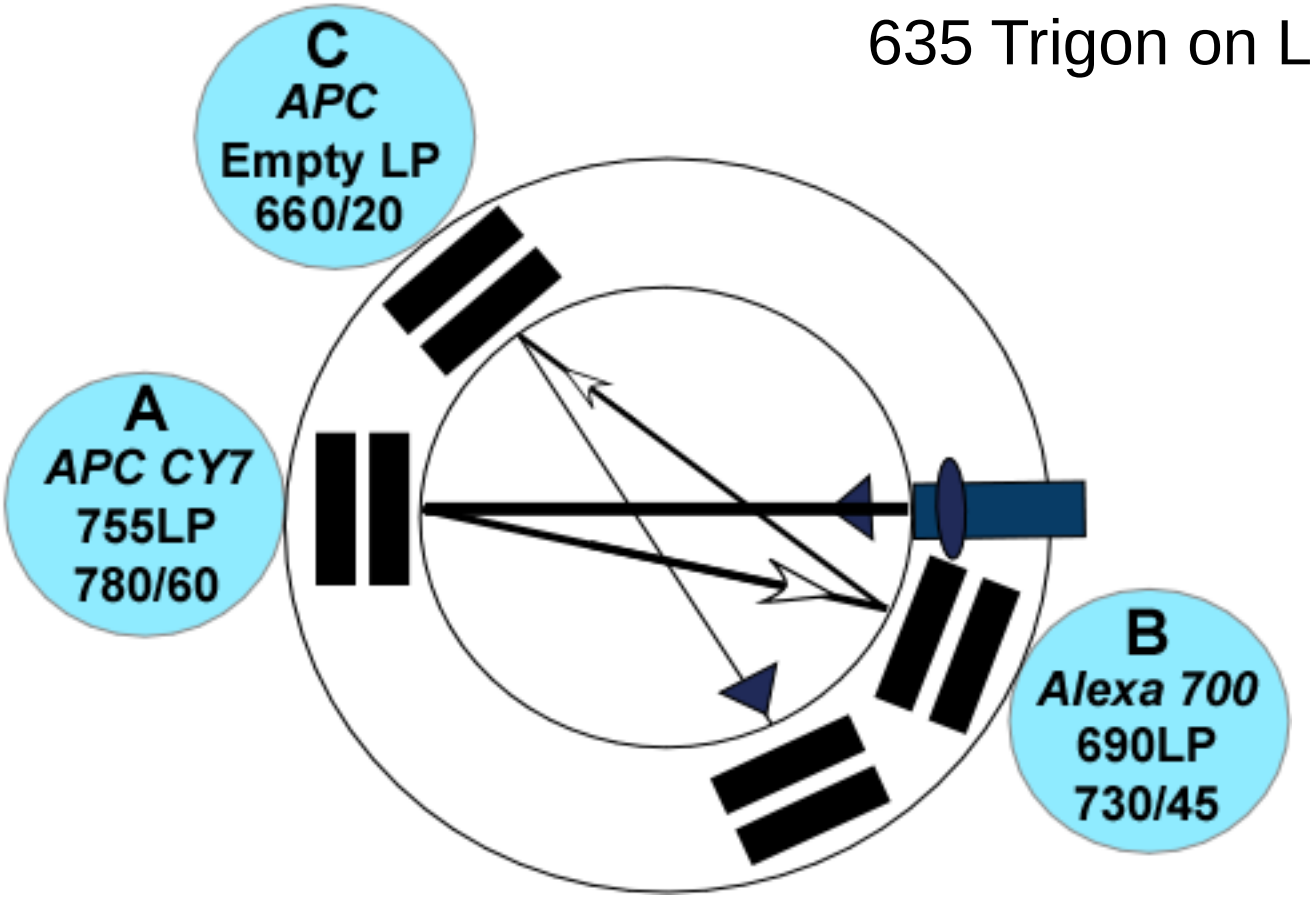
How to determine target MFI's

- Can be a complicated procedure
  - Perfetto et al, Nature Protocols, 2006
  - Joe Trotter and Bob Hoffman at BD have developed suggested protocols
- A simplified procedure is to ensure that all positive cells are on-scale and all negative cells are well above the lower scale
- Typically, we prefer to have the upper edge of the negative cells at about 100

Instrument Configuration

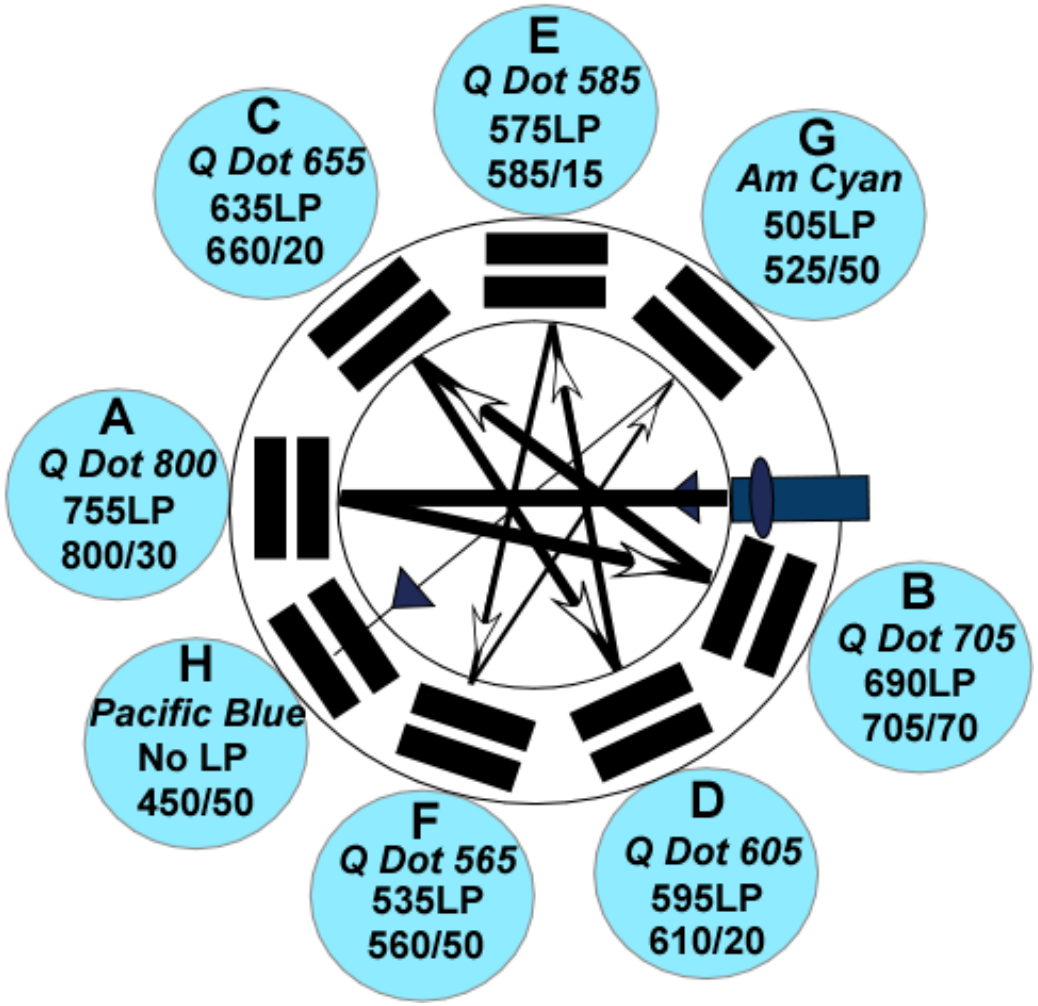
Octagons and trigons are common on BD instruments

635 Trigon on LSRII



Instrument Configuration

Octagons and trigons are common on BD instruments



405 Octagon on LSRII

Local Flow Cytometry Facilities

- FHCRC Shared Resources Flow Facility
- University of Washington Department of Immunology Cell Analysis Facility
- Seattle Biomedical Research Institute (SBRI) Flow Facility

SBRI Flow Facility

- Core Manager: Sean Gray
- Cytopeia Influx High Speed Cell Sorter
  - Three lasers (355, 488 and 635nm)
    - UV 355 1 color
    - Blue 488 5 colors
    - Red 635 2 colors

University of Washington Department of Immunology Cell Analysis Facility

- Director: Michele Black
- BD Aria cell sorter, 3 lasers, 13 colors
- BD FACScan and Calibur
- BD Canto
  - Blue 488 5 colors
  - Red 635 3 colors
- BD LSRI (3 lasers, including UV, 6 colors)
- BD LSRII
  - UV 355 2 colors
  - Violet 405 2 colors
  - Blue 488 8 colors
  - Red 635 3 colors

FHCRC Flow Facility

- Manager: Andrew Berger
- Sorters:
  - 3 BD FACS Vantages, up to 6 colors (One located in BL-3 suite)
  - BD Aria, 3 lasers
    - UV or 405 3 colors
    - 488 7 colors
    - 635 3 colors
  - Cytopeia Influx, 2 lasers, 5 colors
  - 2 Miltenyi AutoMACS bead sorters

FHCRC Flow Facility

- Analyzers
  - 3 BD FACS Caliburs (HTS on one)
  - BD FACS Canto
    - 488 4 colors
    - 635 2 colors
  - BD LSRI (3 lasers, including UV, 6 colors)
  - 2 BD LSR II's with HTS, 18 color
    - Violet 405 8 color
    - Blue 488 2 color
    - Green 532 5 color
    - Red 638 3 color

CFAR Flow Core

- Mission to support research involving HIV and HIV-related disorders
- Provides advice and training for experimental design, staining panel design, laboratory procedures, collection of samples on the instrument, analysis of data and interpretation of results
- Limited amounts of selected reagents available for testing
- No access to instruments through the CFAR core
- Contact: Steve De Rosa, Core Director

http://depts.washington.edu/cfas/cfar/core_molecular.html

Workshop Evaluation

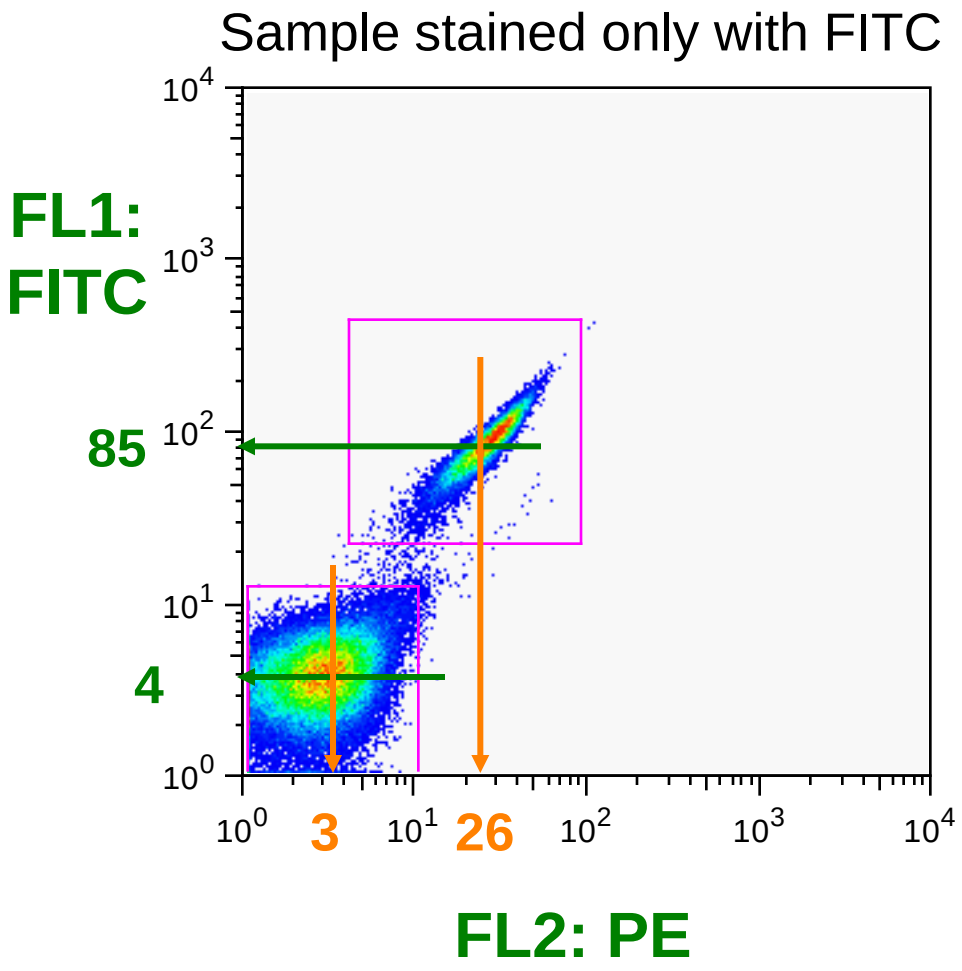
- Record of attendance - please check the registration list and add name/email if needed
- Will email an evaluation form to be returned anonymously
- This feedback is helpful and these evaluations are required as documentation of CFAR activities

http://depts.washington.edu/cfas/cfar/core_molecular.html

Compensation

- Spectral overlap between dyes results in the detection of the fluorescence from one dye in one or more other detectors (primary vs. spillover fluorescence)
- The spillover fluorescence must be subtracted from the total fluorescence detected in the secondary detector
- This spillover fluorescence is proportional to the level of the primary fluorescence
- For each cell, a specified percentage of the primary fluorescence is subtracted from the total fluorescence detected in the secondary detector

FITC into PE



% spillover of FITC into PE=

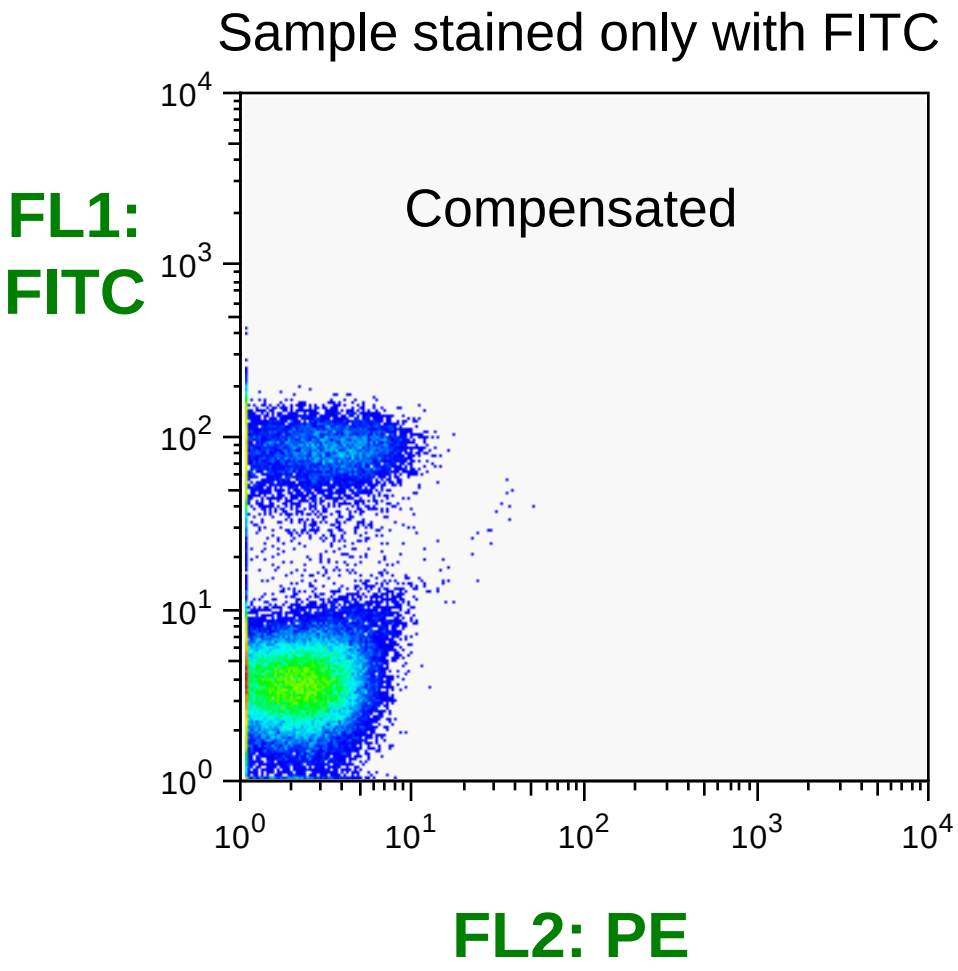
$$\frac{\text{Fluorescence}_{FL2}}{\text{Fluorescence}_{FL1}} \times 100$$

$$\frac{MFI_{FL2}(pos) - MFI_{FL2}(neg)}{MFI_{FL1}(pos) - MFI_{FL1}(neg)} \times 100$$

$$\frac{26 - 3}{85 - 4} \times 100 = 28\%$$

Data provided by J. Stucky, 041012

FITC into PE



% spillover of FITC into PE=

$$\frac{\text{Fluorescence}_{FL2}}{\text{Fluorescence}_{FL1}} \times 100$$

$$\frac{MFI_{FL2}(pos) - MFI_{FL2}(neg)}{MFI_{FL1}(pos) - MFI_{FL1}(neg)} \times 100$$

$$\frac{26 - 3}{85 - 4} \times 100 = 28\%$$

Data provided by J. Stucky, 041012

Compensation….

- For digital data collection, compensation on-line and compensation post-collection are identical.
  - This may not be true for analog data
- One advantage of compensating at collection is that you can then view compensated data during collection.
- However, if multiple staining panels are used requiring different compensation requirements, then it can be confusing at collection to assign the different comp requirements for the different panels

A few practical considerations for setting compensation

1. Be sure PMT voltages are set properly before collecting compensation samples
    - Changing PMT voltages will change compensation requirements
2. Make singly-stained compensation controls
    - One control for each fluorochrome
    - Must be as bright or brighter than the reagents used on that color in the multicolor panels (always is safe to use same reagent for comp as for multicolor panel)
    - Can use comp “beads” (note: check species and isotype)
    - Dyes like PI, EMA, CFSE require special comp samples

Practical considerations - tandems

Different lots of tandem dyes have different compensation requirements:

- Prepare single-stained controls for each tandem lot
- If tandem lot is unknown, then make a separate comp sample for each tandem-Ab conjugate
- When creating the comp matrix after collection, match the single-stained controls with the appropriate multi-stain panels

Another note on tandems

- Spectral properties change over time due to exposure to light and to fixation reagents
- Minimize exposure to light during staining and store stained samples in the dark
- Minimize concentration of fixative in final resuspension (0.5 to 1% PFA)
- Consider using tandem “stabilization” buffer?
- Ensure that compensation controls and test samples are treated the same, e.g., all receive fixation/permeabilization

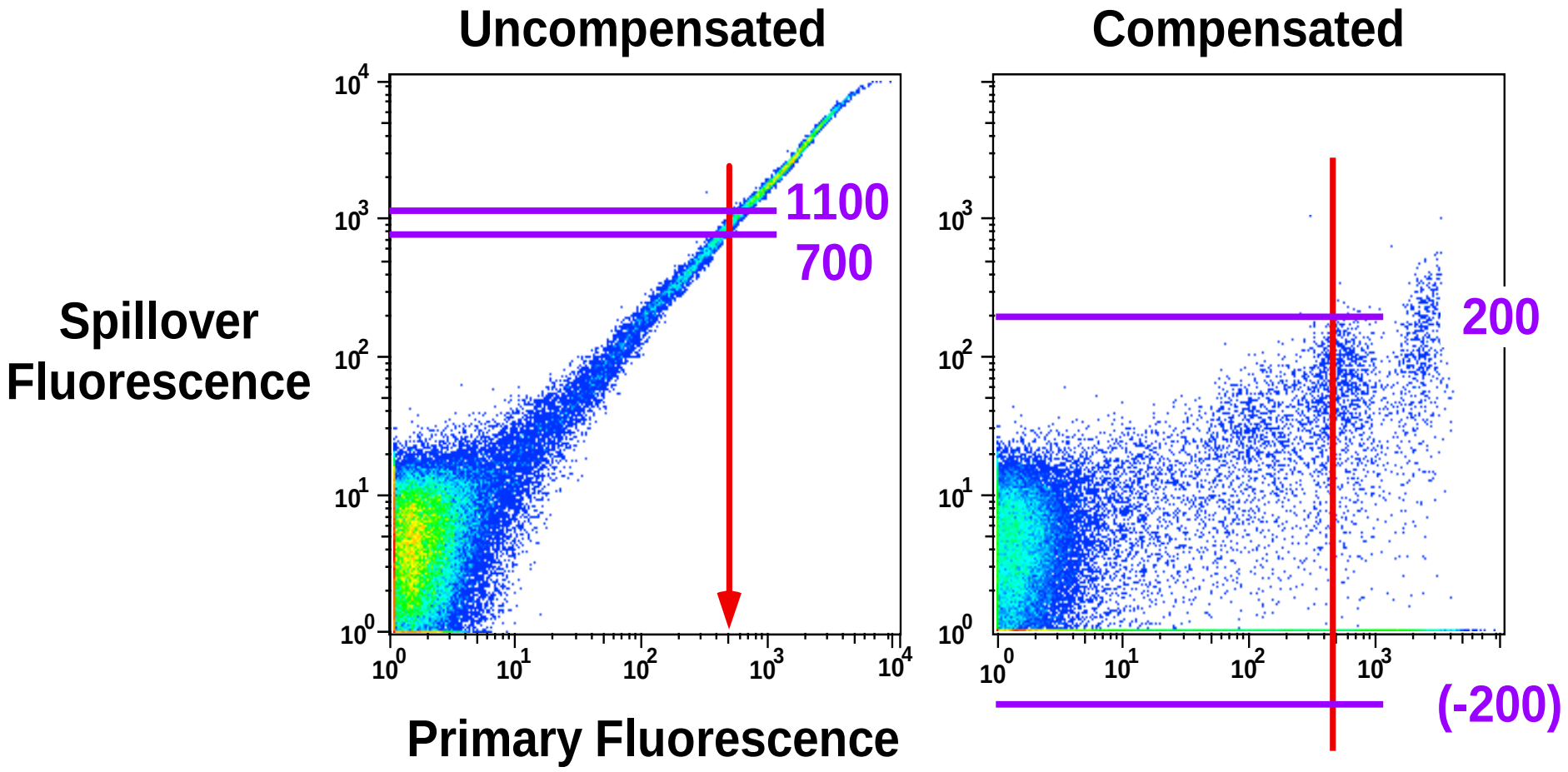
Advanced topics in Fluorescence Spillover Compensation

Mario Roederer, Cytometry 45:194-205 (2001)

Spectral Compensation for Flow Cytometry: Visualization Artifacts, Limitations, and Caveats

Nicole Baumgarth and Mario Roederer
Journal of Immunological Methods 243:77-97 (2000)
A practical approach to multicolor flow cytometry for immunophenotyping

Imperfect Measurement Leads to Apparent Spread in Compensation



Slide provided by M. Roederer, NIH

“Errors” in Compensation

- Properly compensated data may not appear rectilinear (“rectangular”), because of measurement errors.
- This effect on compensated data is unavoidable, and it cannot be “corrected”.
- It is important to distinguish between incorrect compensation and the effects of measurement errors.

Slide provided by M. Roederer, NIH

Compensation Does NOT Introduce or Increase Error:

Compensation Only Reveals It!

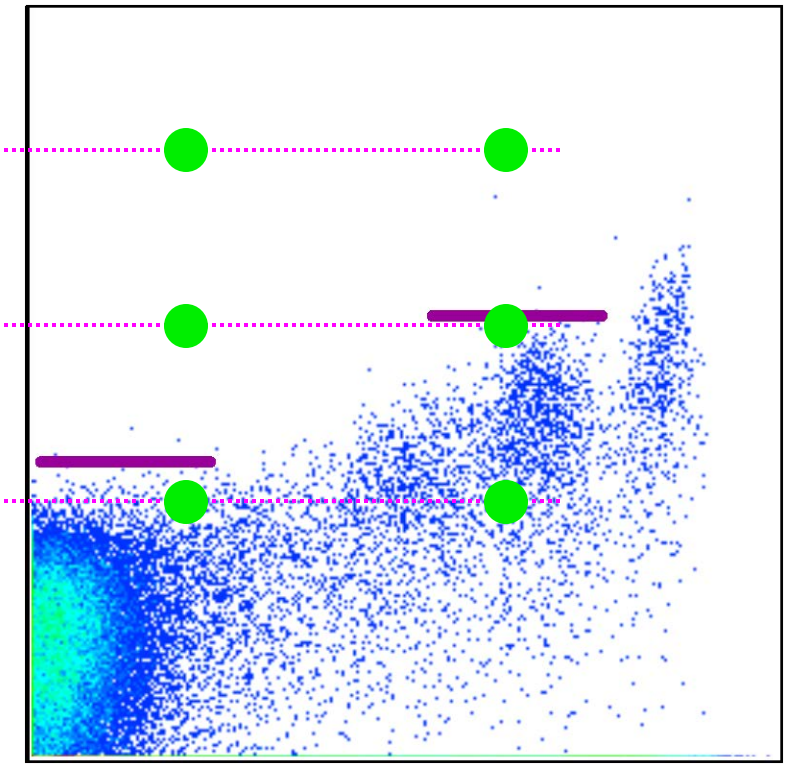
- The measurement error is already present. Compensation does not increase this error, it does not change it, it does not introduce any more error.
- Compensation simply makes the error more apparent by shifting it to the low end of the log-scale.

Slide provided by M. Roederer, NIH

Staining Controls

- Staining controls are necessary to identify cells which do or do not express a given antigen.

- The threshold for positivity may depend on the amount of fluorescence in other channels!



Slide provided by M. Roederer, NIH

Staining Controls

- Unstained cells or complete isotype control stains are *improper* controls for determining positive vs. negative expression in multi-color experiments.
- The best control is to stain cells with all reagents *except* the one of interest.

FMO Control

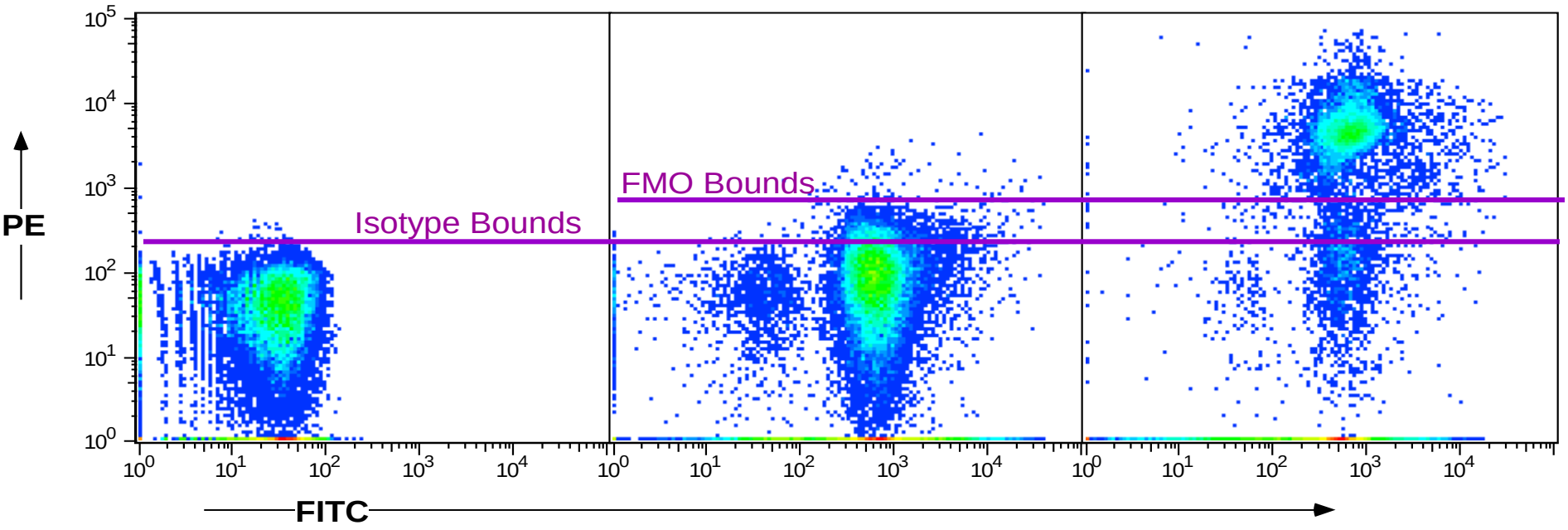
“Fluorescence Minus One”

Slide provided by M. Roederer, NIH

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	Ğ	CD3	CD3
PE	Ğ	Ğ	CD4
Cy5PE	Ğ	CD8	CD8
Cy7PE	Ğ	CD45RO	CD45RO

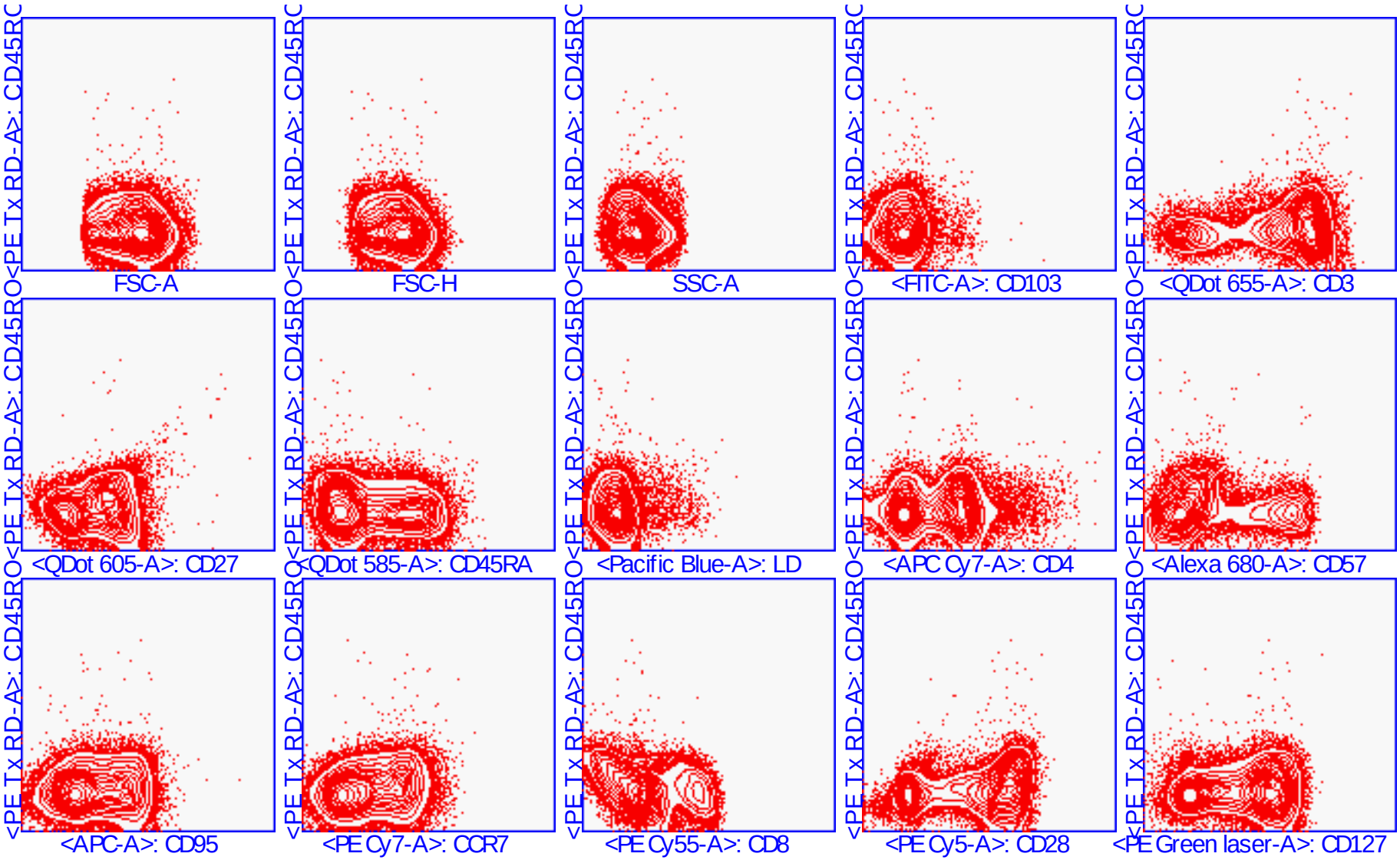


Slide provided by M. Roederer, NIH

FMO Controls

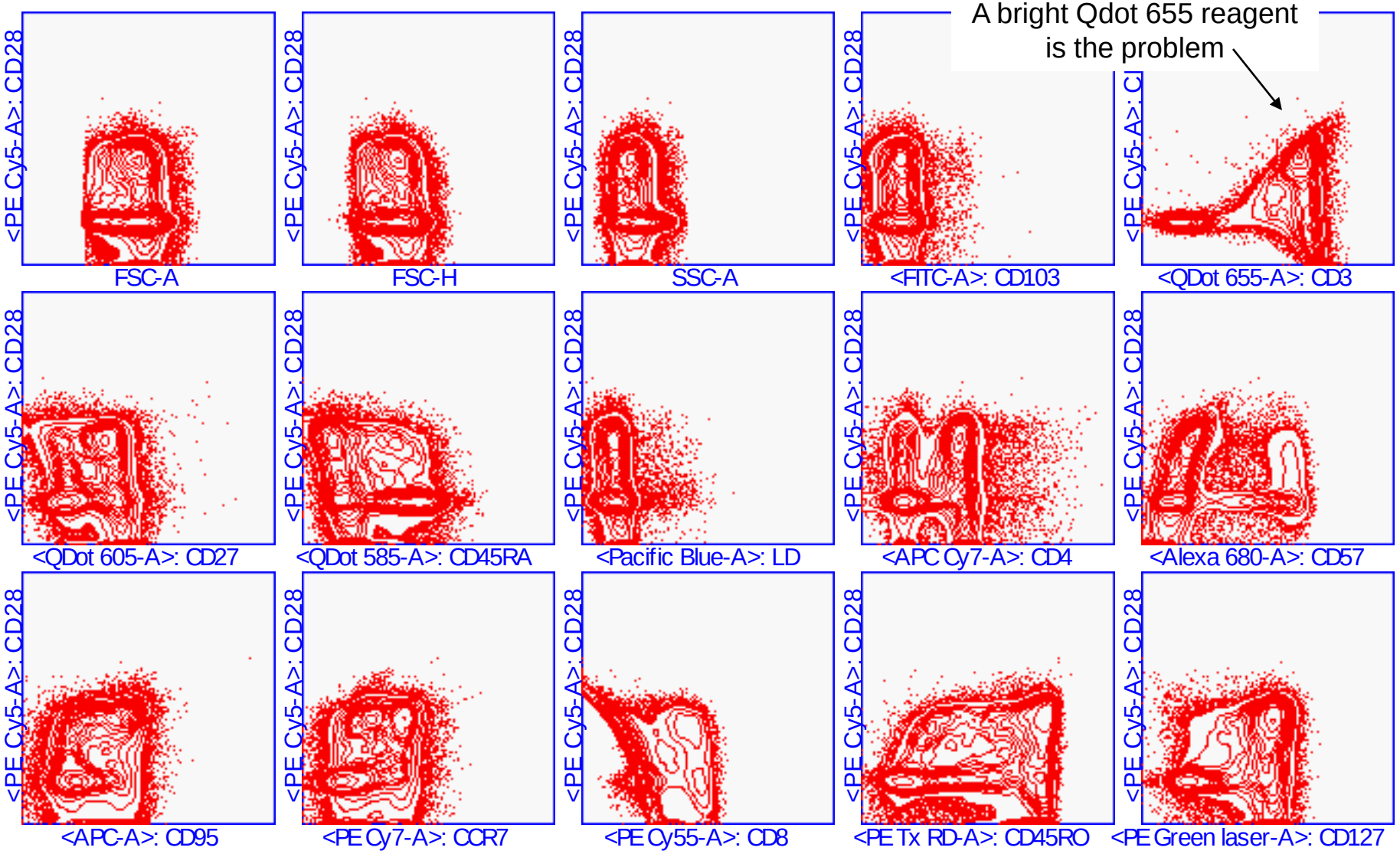
- FMO controls are a much better way to identify positive vs. negative cells
- FMO controls should be used whenever accurate discrimination is essential or when antigen expression is relatively low

FMO Example - missing PE-TR



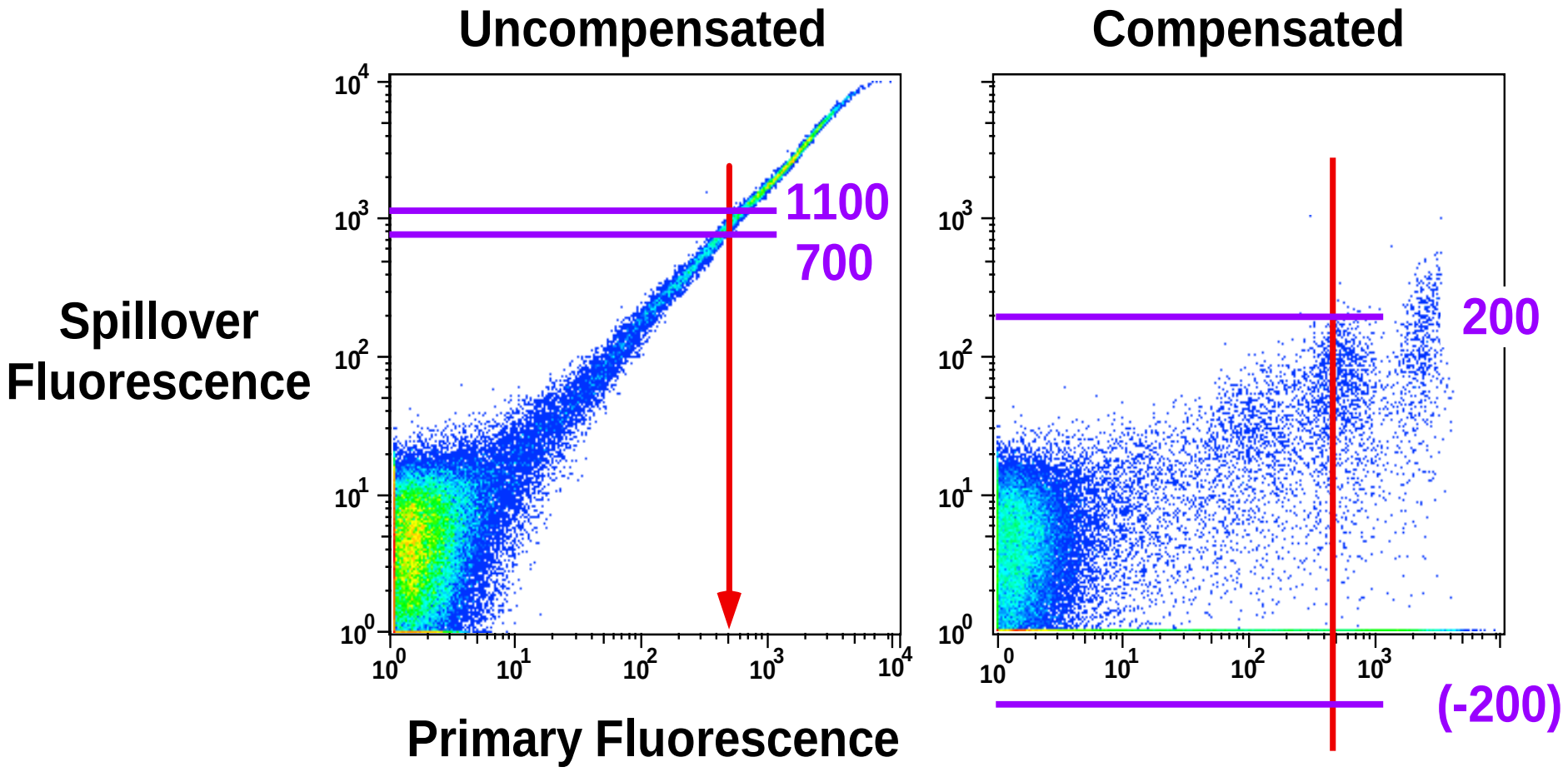
060908, Data provided by Emilie Jalbert

FMO Example - missing PE-Cy5



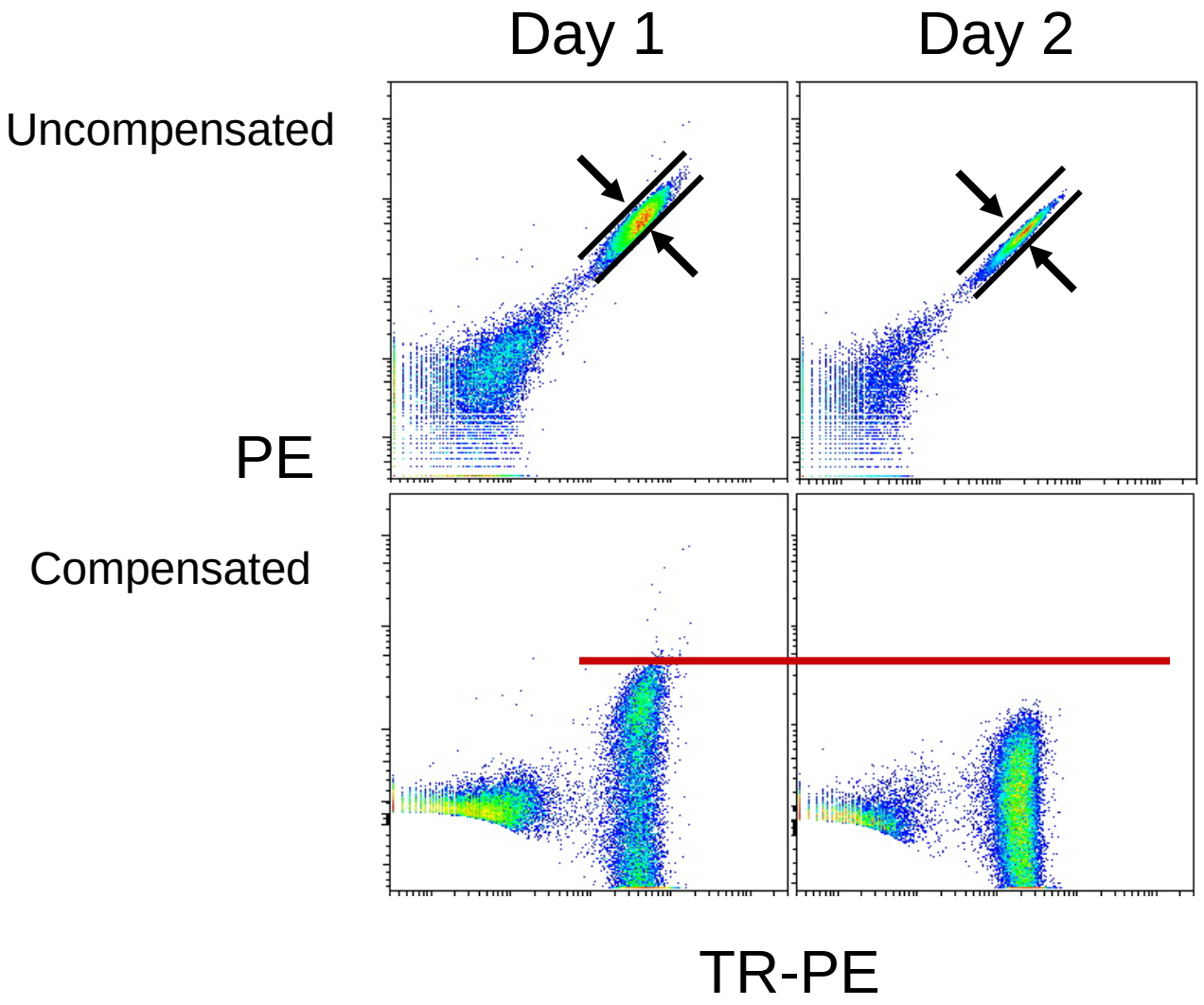
060908, Data provided by Emilie Jalbert

Imperfect Measurement Leads to Apparent Spread in Compensation



Slide provided by M. Roederer, NIH

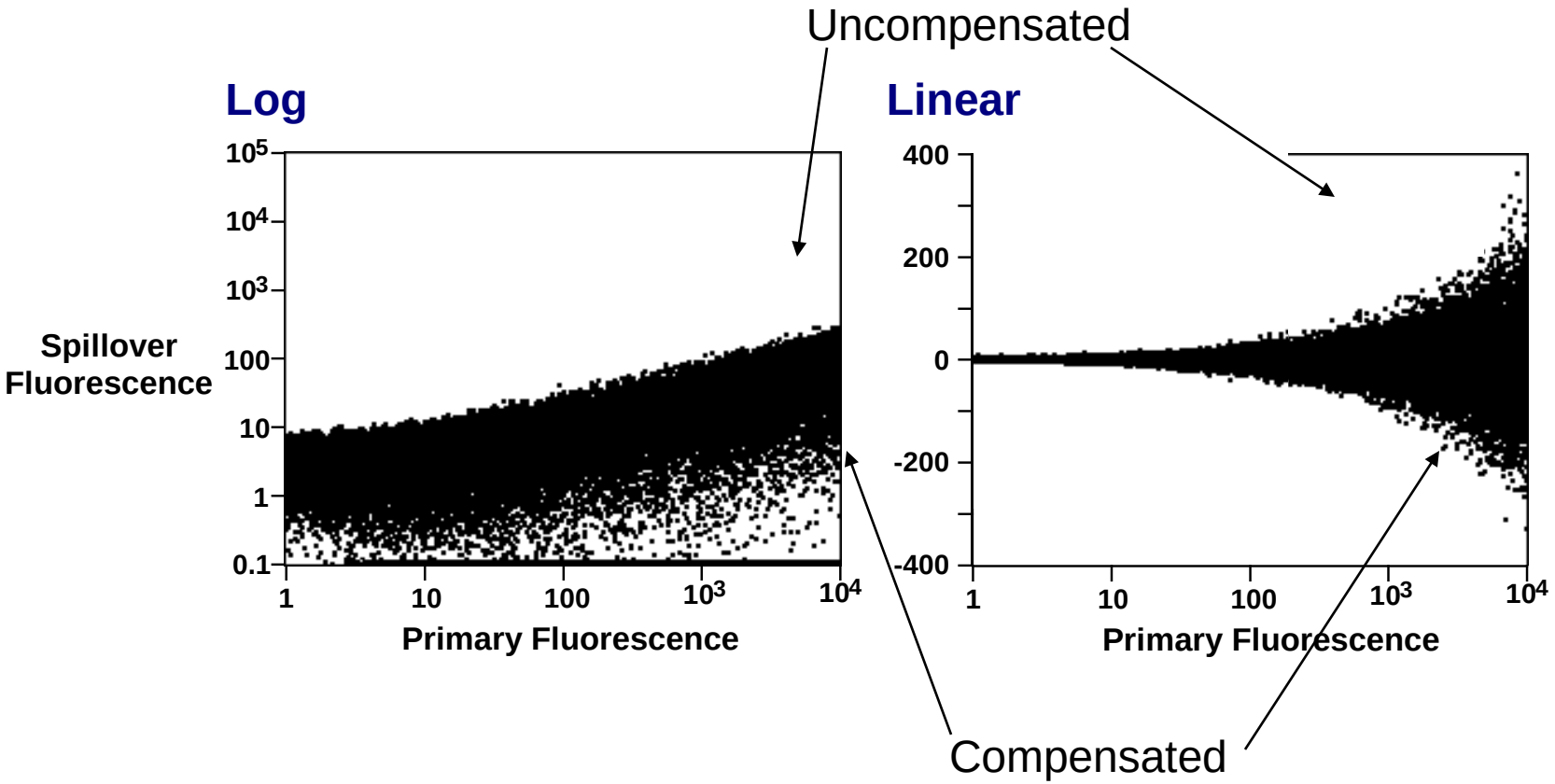
Good Instrument Alignment Is Critical!



While the amount of compensation did not differ, the measurement error (correlation) decreased leading to much better visualization of the population!

Slide provided by S. Perfetto

Log Transformation of Data Display Leads to Manual Overcompensation



Slide provided by M. Roederer, NIH

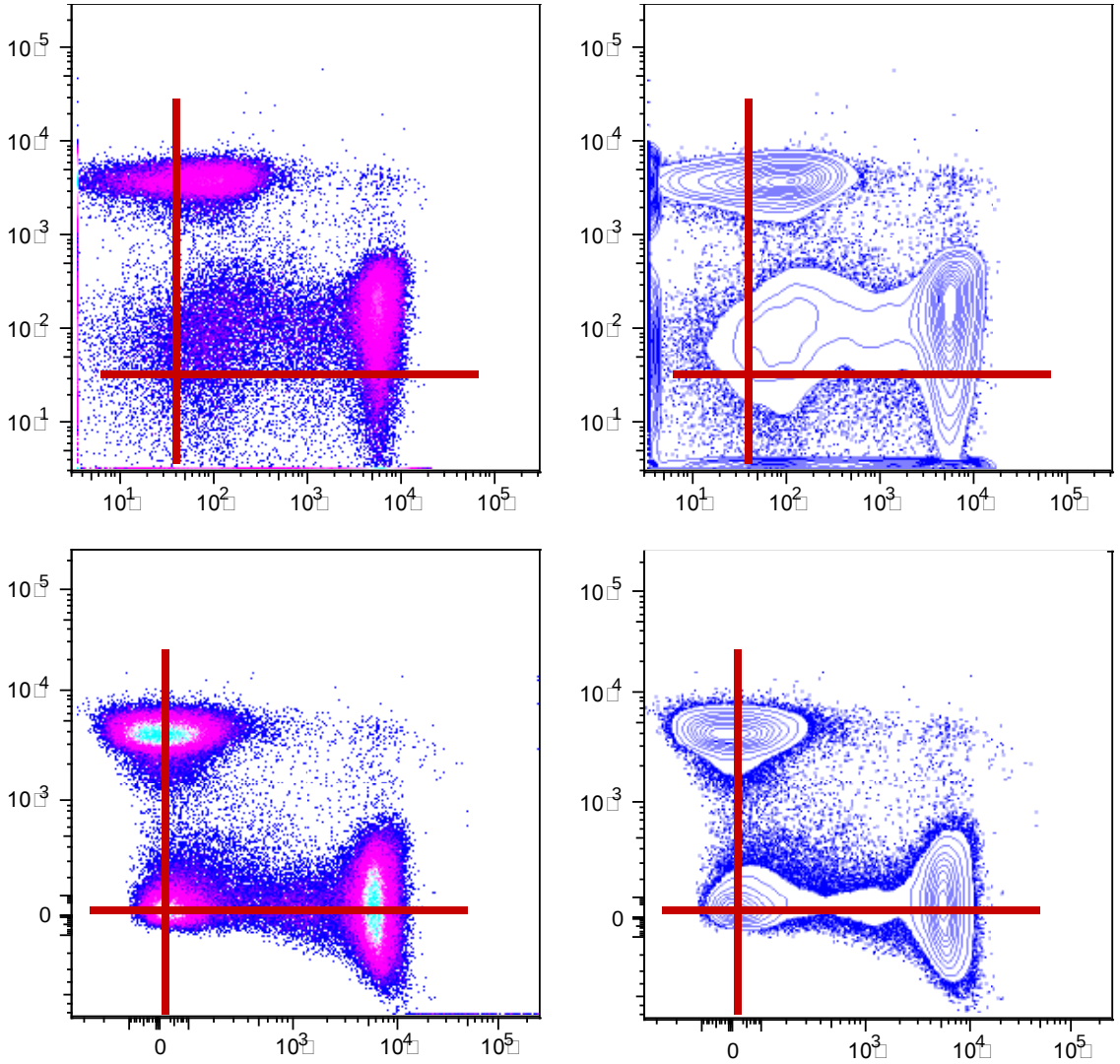
Bi-exponential or logicle transformation

- Transforms the log scale to display values below zero
- This allows better visualization of populations centered around zero
- This display feature is now available in most FACS analysis software

Transformation Confirms Compensation

Not Transformed

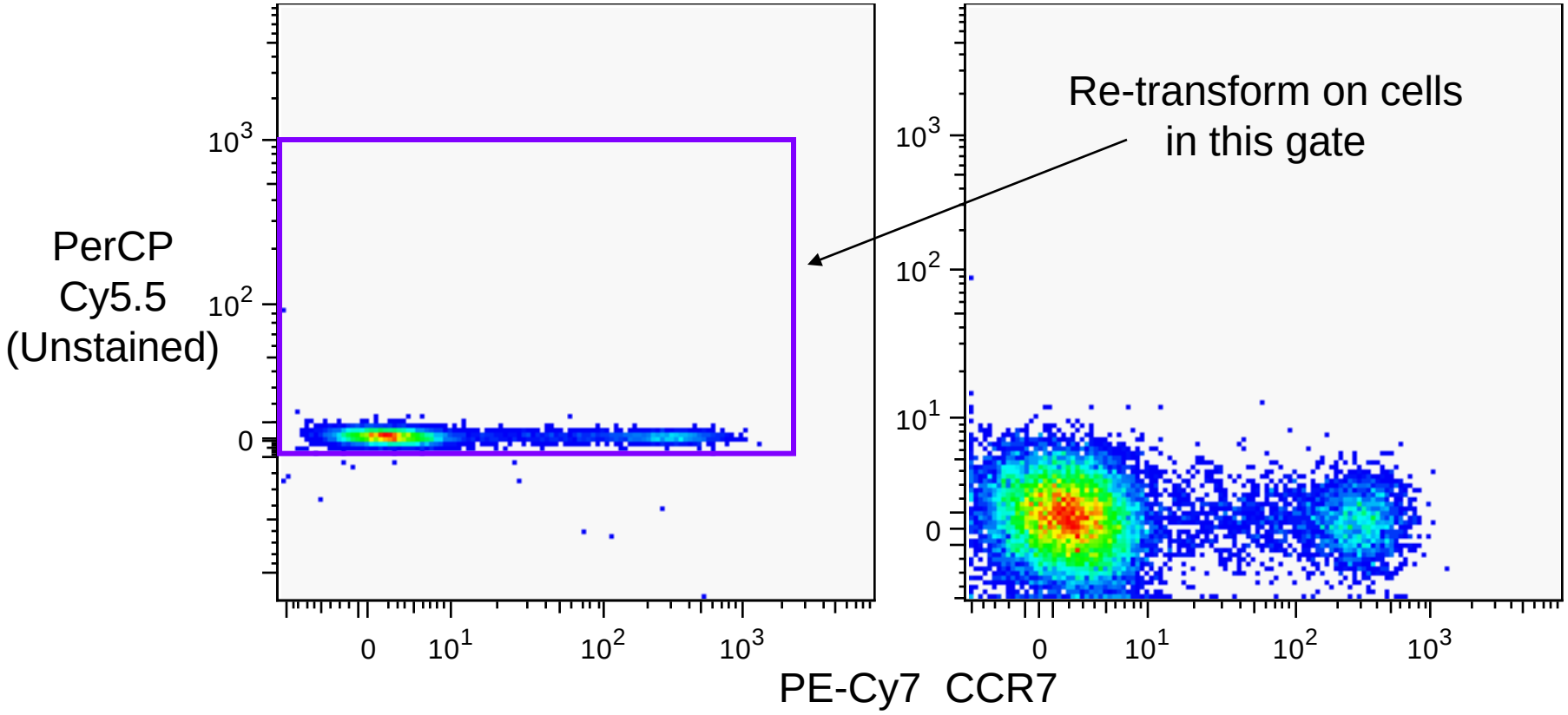
Transformed



Slide provided by M. Roederer, NIH

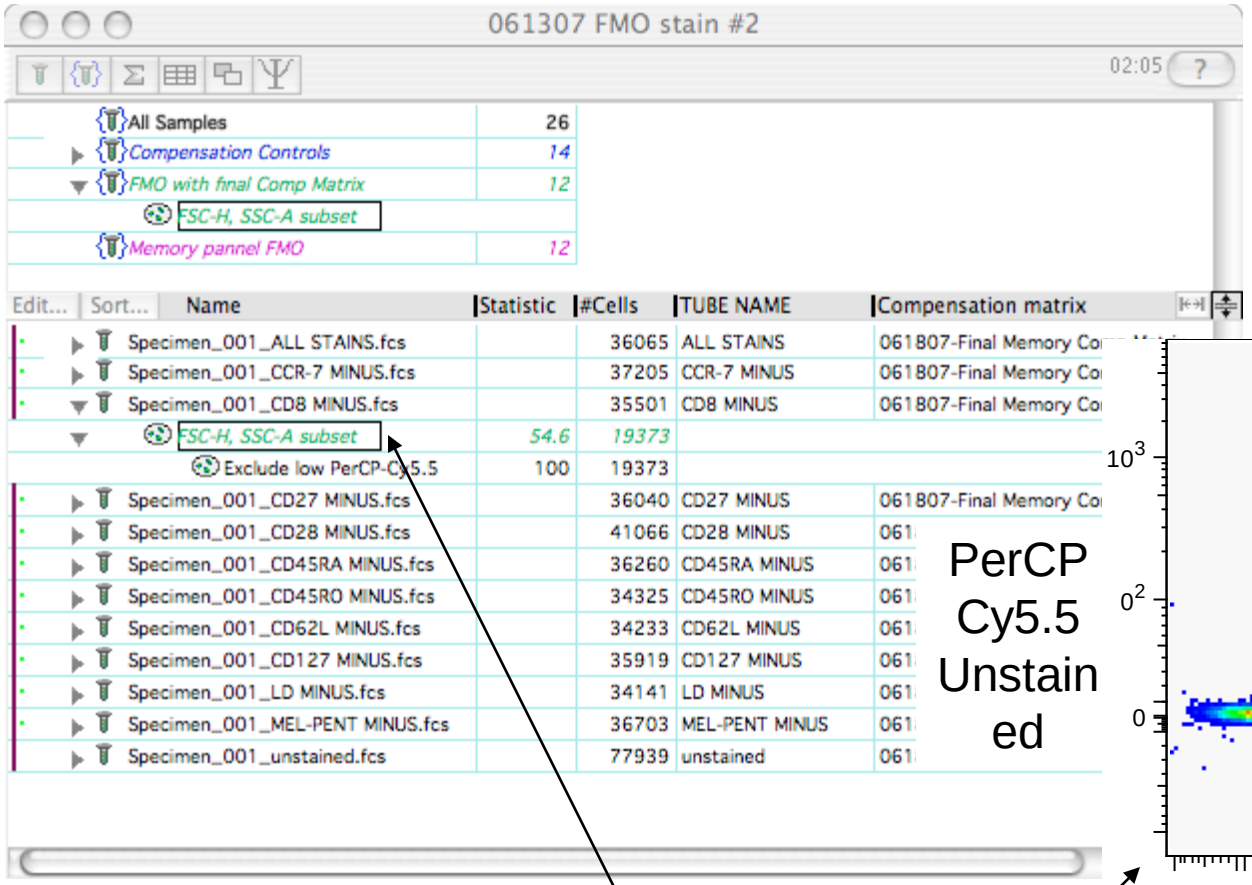
Re-Transformation

- Cells with large negative fluorescence values affect transformation
- Excluding these cells and re-transforming produces better results

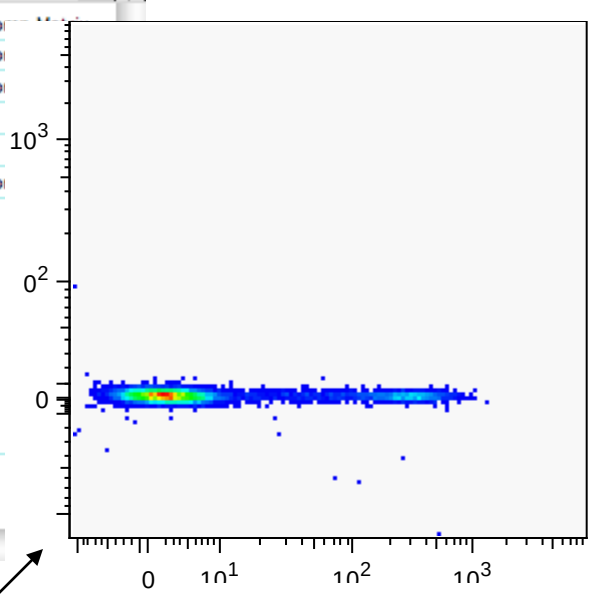


Data provided by Jeff Pufnock, 061307, CD8 minus FMO

In FlowJo, choose the appropriate gate when defining transformation



PerCP Cy5.5 Unstained

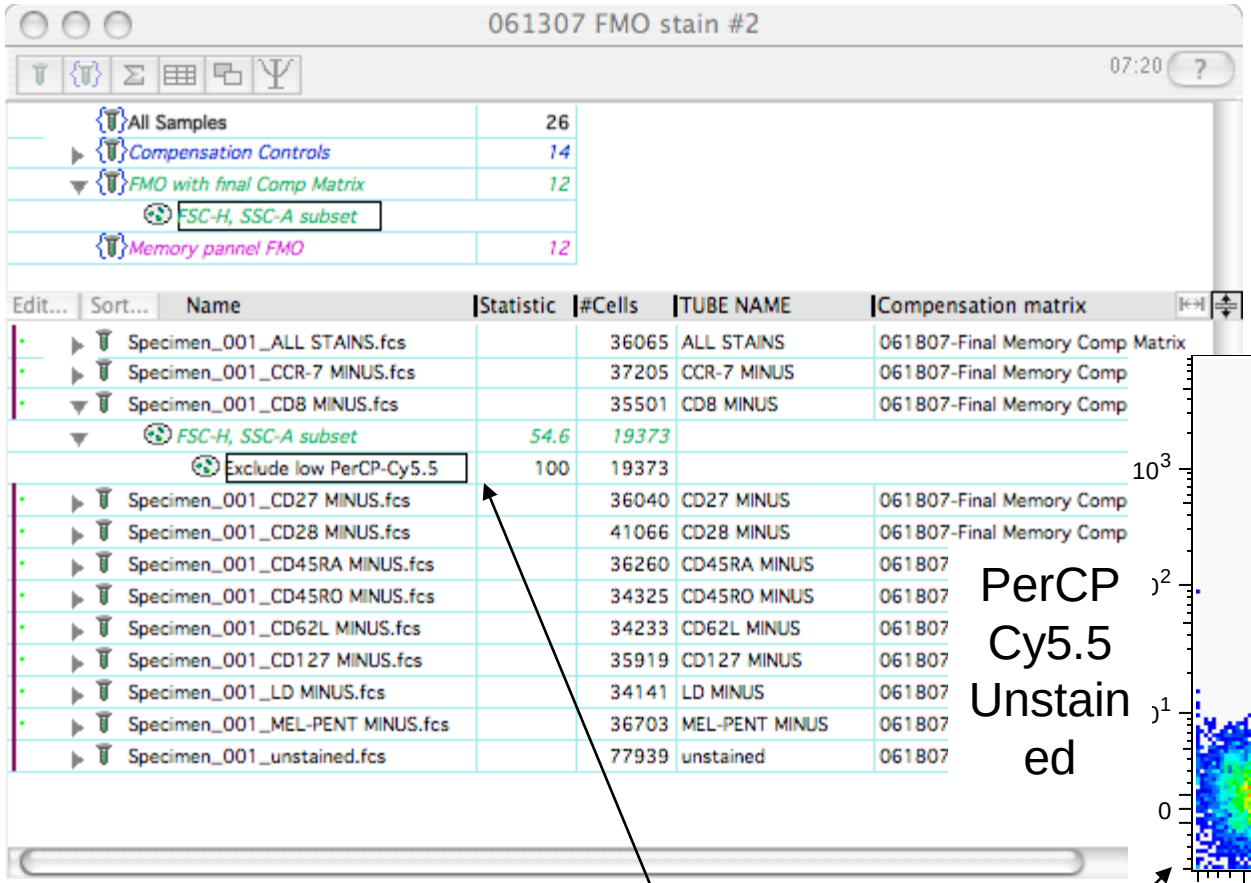


PE-Cy7 CCR7

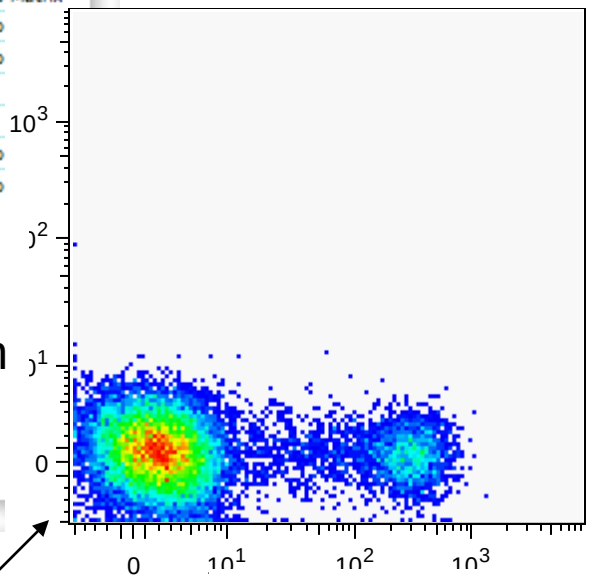
Transforming when this gate is selected produces this result

Data provided by Jeff Pufnock, 061307, CD8 minus FMO

Re-Transformation



PerCP Cy5.5 Unstained



PE-Cy7 CCR7

Transforming when this gate is selected produces this result

Data provided by Jeff Pufnock, 061307, CD8 minus FMO

Reagent Titer

- Amount to use per test
- Expressed as concentration (not per cell number)
- Saturating titer - the lowest titer that gives near maximal fluorescence
- Separating titer - useful titer that gives bright staining with low background

All FACS staining reagents need to be titrated by the user!

Even if a titer is supplied by the manufacturer.

Titration of FACS staining reagents

- Fluorochrome-conjugated mAb reagents (and other dyes) need to be titrated before use
- Must be titrated using same staining protocol under the same conditions used for experiment
- Same reagent may have different titers depending on staining protocol
  - Surface staining
  - Intracellular staining
  - Staining temperature (RT vs. 0deg)
  - Cell type or source or tissue (e.g., macaque vs. human)

Standardization

- Standardization of staining protocols allows titration information to be recorded in a database to be shared between individuals and groups
- Titration data should be recorded for a **standard staining volume** or as a **dilution**
  - E.g., use 5 ul per 100 ul final staining volume
  - Equivalent to 1:20 dilution
- Recording titers per volume has an advantage
  - For staining panels with multiple reagents, titers for each reagent simply need to be added together

Titration procedure

- 2-fold dilutions over range of expected titer
  (if suggested titer not supplied by manufacturer, then estimate starting titer as 1 to 2ug per 100ul staining volume)
- Optimally test 8 to 12 dilutions (to generate a saturation curve)
- Can titer multiple reagents at same time on the same cells
  - Must be different fluorochromes detected in different channels
  - Cannot use same Ab on different colors, or different Ab's directed to same Ag
  - May require compensation (collect single-stained compensation samples)
- Counter-staining may be beneficial (for markers expressed on rare cell populations)

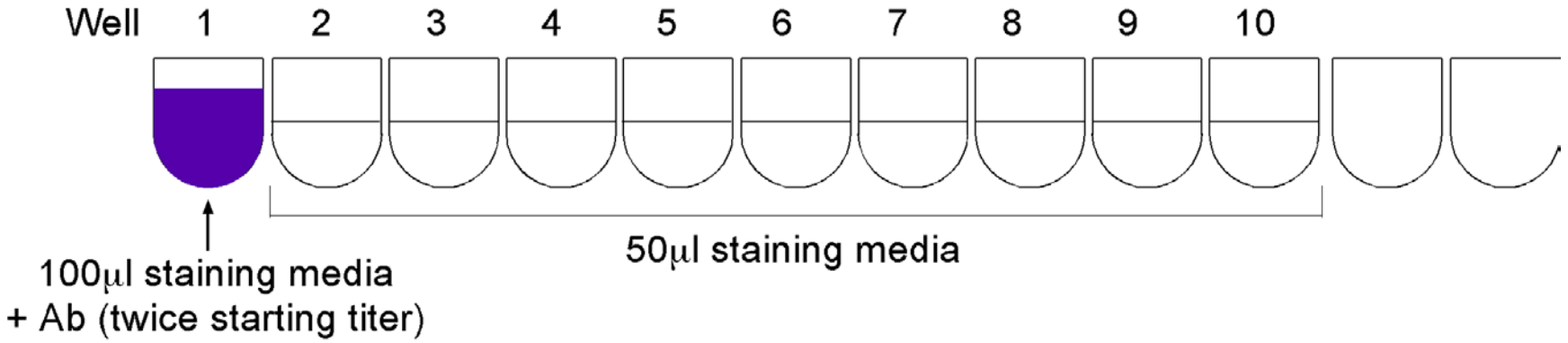
Example of titration protocol

- Staining protocol requires 50ul of cell suspension mixed with 50ul of Ab solution (100ul final staining volume)
- Prepare 2-fold dilutions in FACS tubes or 96-well plates (round-bottom or V-bottom)
  - Add 50ul of staining buffer* in each well or tube except first
  - For first tube or well, add twice the Ab amount for the first dilution and enough staining buffer to bring final volume to 100ul
- Gently mix first well, take 50ul and add to second well
- Gently mix second well, take 50ul and add to third well
- Repeat for all wells or tubes - discard last 50ul

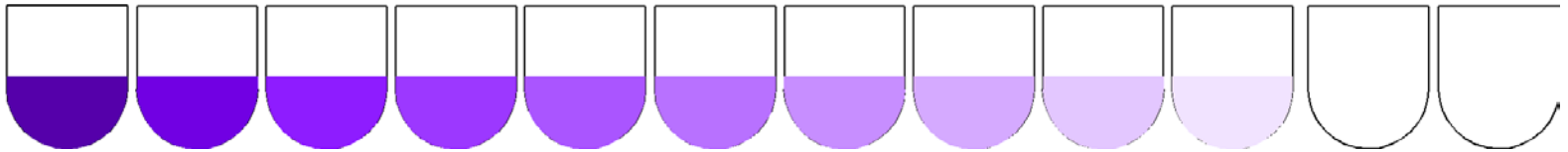
*Use appropriate staining buffer for experiment, e.g., intracellular staining may require saponin-containing buffer

2-fold dilution series for titration

Before dilution:



After 2-fold dilution:



Stain cells

- Prepare cells to use for titration, e.g.:
  - Prepare PBMC from whole blood or thaw frozen PBMC
  - Some reagents, such as cytokines, require cells to be stimulated, etc.
- For intracellular titrations, cells need to be fixed and permeabilized before staining
  - can fix/permeabilize cells in “bulk”
- Prepare cell suspension
  - Typically we use $1x10^6$ cells per test, so we prepare the cell suspension to provide $1x10^6$ cells per 50ul (=$20x10^6$ cells/ml).
  - If cells are limited, 100,000 to 250,000 cells can be used.
- Add 50ul of cell suspension to each well or tube
  - For titrations in plates, a multi-channel pipette can be used
- Follow staining protocol specific for study
- Final step is to re-suspend cells in a volume appropriate for FACS collection (typically ≥200ul with staining buffer including 0.5% paraformaldehyde)

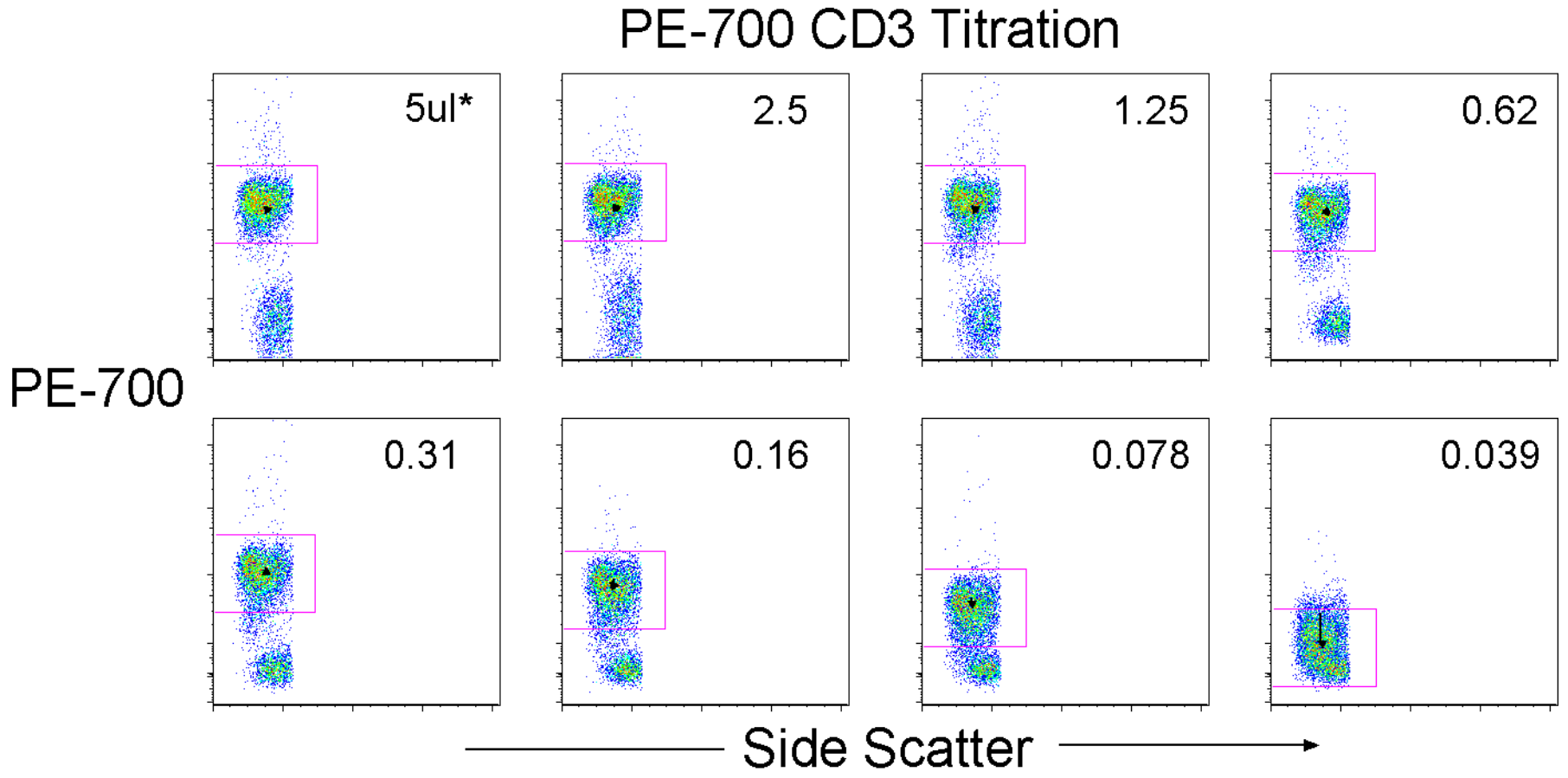
Analysis procedure

- Gate on population of interest, e.g., lymphocytes
- Gate on positive population
  - will need to move this gate for the different dilutions
- Print one (or more) graph from each dilution showing the staining profile for the reagent
  - This can be a histogram, or a bivariate plot
  - It is useful to print all graphs on one page

Statistical analysis

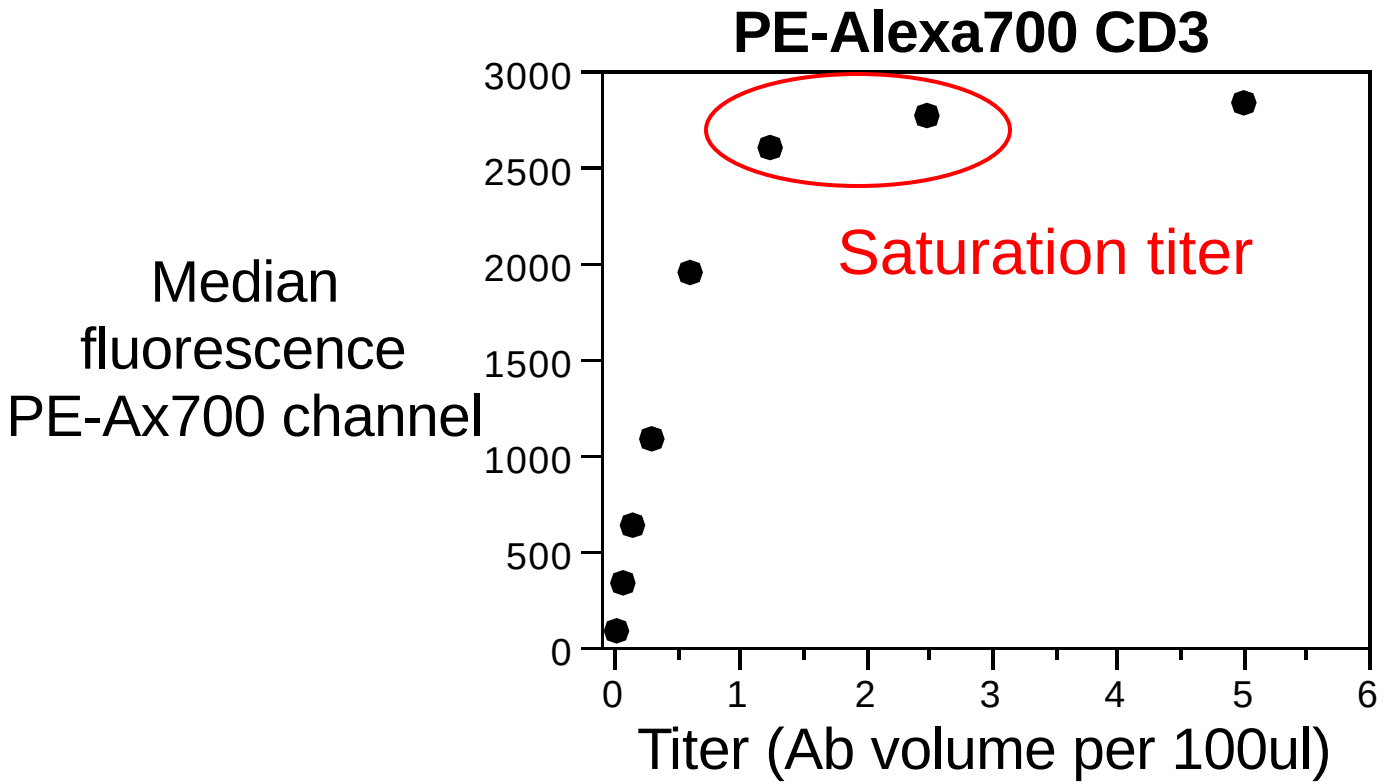
- Determine median fluorescence values for stained cells for each dilution
- Plot these medians vs. the dilution values (needs to be a linear scale)

Example - surface staining PBMC



*Antibody titer, amount of antibody per 100ul

Example of saturation curve surface titration

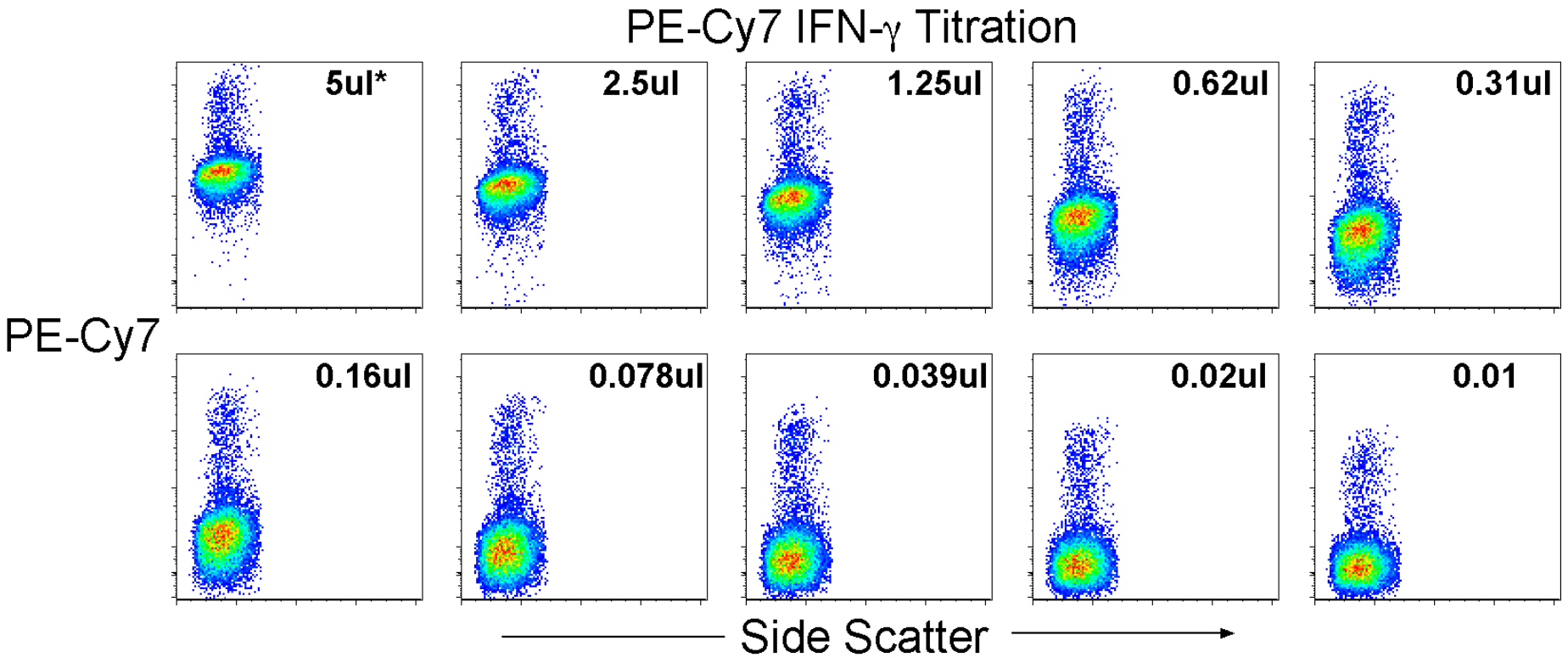


PE-Alexa700 is collected in the PE-Cy5.5 channel

Determine titration value at saturation

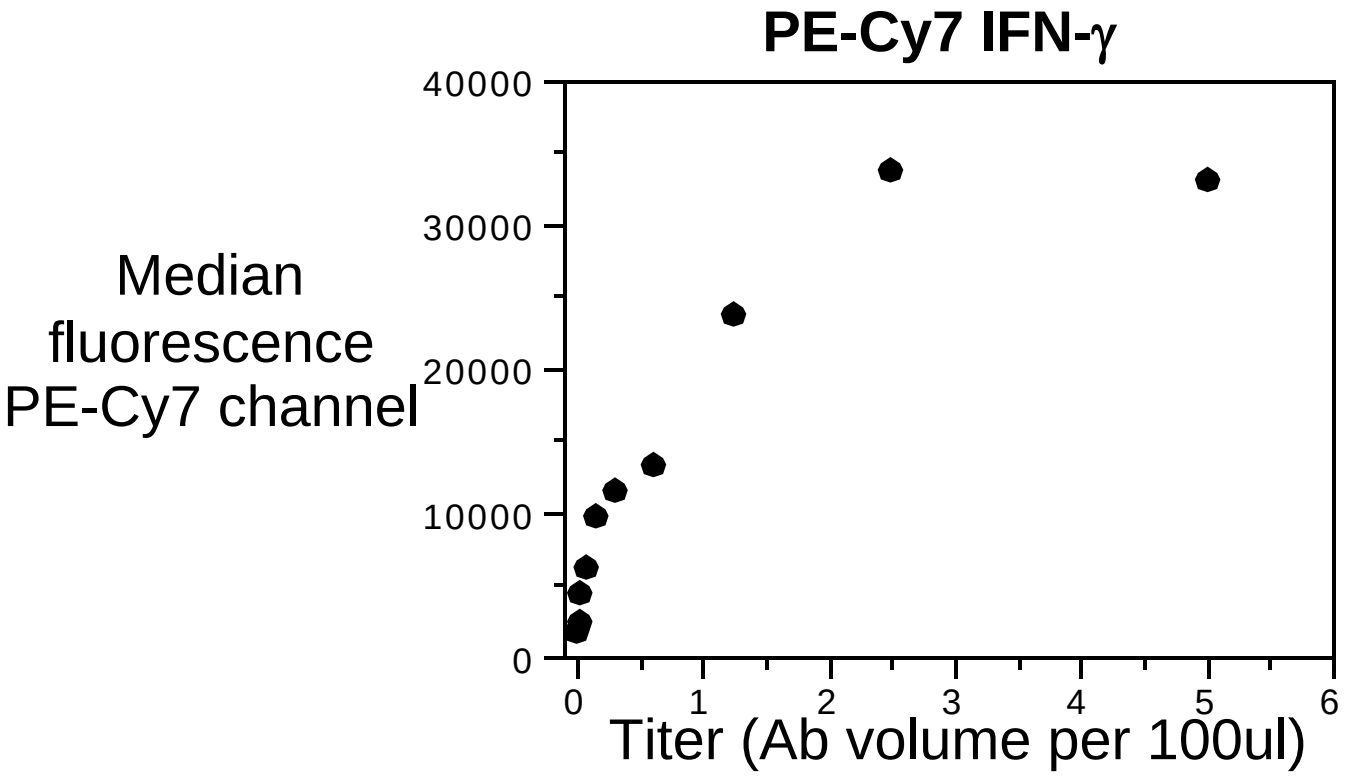
- For many reagents the saturation titer is the optimal titer
  - when background staining is minimal
  - for most surface staining
- Using reagents at saturating titers has advantages:
  - Variations in the staining conditions will have minimal effects on staining intensity (the reagent curve “flattens out” at saturation)

Example - intracellular reagent



*Antibody titer, amount of antibody per 100ul

Example of saturation curve intracellular titration



Determine titration value

- For many reagents, the saturating titer is not appropriate
- For these reagents the optimal titration value must be determined as:
  - Brightest staining (at or below saturation)
  - Lowest background
- This is often the case for intracellular reagents
  - Reagents saturate at a titer where background is very high
  - Examine FACS profiles to determine titer
- Other reagents do not saturate (while on-scale)
  - Can be doped with unconjugated Ab
- Reagents used at titers below saturation are subject to more variability in staining intensity (the reagent curve is steeper below saturation)

Consequences of non-saturating stains

- Final fluorescence will depend on:
  - Number of cells
  - Concentration of antibody
  - Amount of time of staining incubation
- Reasons for not saturating
  - Not enough antibody (antigen in excess over Ab)
  - Affinity constant very low
- Max amount of Ag that can be stained at saturation (K=binding or affinity constant)

  $[Ag_T] \leq [Ab_T] - 9/K$

Reference

For a theoretical discussion concerning Ab affinity and effect on titers:

Kantor, A. and Roederer, M. (1997)
FACS analysis of lymphocytes. In: Handbook of Experimental Immunology (Fifth Edition), Herzenberg, L. A., Weir, D. M., Herzenberg, L. A. and Blackwell, C. (ed.), Blackwell Science, Cambridge, pp 49.1-.13.

Available at:

Herzenberg.Stanford.edu; click on publications

Effect of cell number

- Typically the concentration of antibody far exceeds concentration of antigen (100-fold)
  - Larger numbers of cells (10^6 - 10^8 cells) can be stained at same Ab concentration with minimal decrease in fluorescence
  - When staining large number of cells for sorts - may only need to increase antibody concentration minimally (up to 5-fold)
- This can be tested by titrating with different cell numbers
- Note also, that when staining fewer than 10^6 cells, the amount of antibody cannot be decreased

Conjugation of antibodies to fluorochromes

- For some fluorochromes, the protocol is simple
  - Involves exchanging Ab into the correct buffer (dialysis, or exchange over desalting column)
  - Reaction for 1 hour
  - Exchange conjugated Ab into storage buffer
  - FITC, Alexa dyes, Pacific Blue
- Phycobiliproteins are a bit more complicated
  - Require a chemical linker to be attached to fluorochrome and reduction of the Ab to react with the linker
  - Pre-activated PE and APC available from Prozyme
- To make tandem dyes quite complex
- Quantum dots similar to phycobiliprotein procedure
  - Kits available, but expensive and only conjugate 2x 0.3mg Ab
- Conjugation protocols available at drmr.com

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Multicolor flow, FMO, Compensation

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- Roederer, M. (2001) Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. Cytometry 45, 194-205.

17-color flow, Green laser benefits

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- Perfetto, S.P. and Roederer, M. (2007) Increased immunofluorescence sensitivity using 532 nm laser excitation. Cytometry A 71, 73-9.

Quantum dots, Amine reactive viability dye

- Chattopadhyay, P.K., Price, D.A., Harper, T.F., Betts, M.R., Yu, J., Gostick, E., Perfetto, S.P., Goepfert, P., Koup, R.A., De Rosa, S.C., Bruchez, M.P. and Roederer, M. (2006) Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. Nat Med 12, 972-7.
- Perfetto, S.P., Chattopadhyay, P.K., Lamoreaux, L., Nguyen, R., Ambrozak, D., Koup, R.A. and Roederer, M. (2006) Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. J Immunol Methods 313, 199-208.

Transformation, data display

- Parks, D.R., Roederer, M. and Moore, W.A. (2006) A new "Logicle" display method avoids deceptive effects of logarithmic scaling for low signals and compensated data. Cytometry A 69, 541-51.
- Herzenberg, L.A., Tung, J., Moore, W.A. and Parks, D.R. (2006) Interpreting flow cytometry data: a guide for the perplexed. Nat Immunol 7, 681-5.

Instrument Standardization

- Perfetto, S.P., Ambrozak, D., Nguyen, R., Chattopadhyay, P. and Roederer, M. (2006) Quality assurance for polychromatic flow cytometry. Nat Protoc 1, 1522-30.
- Current Protocols in Cytometry Units 1.3 and 1.20

8-color ICS validation

- Horton, H., Thomas, E.P., Stucky, J.A., Frank, I., Moodie, Z., Huang, Y., Chiu, Y.L., McElrath, M.J. and De Rosa, S.C. (2007) Optimization and validation of an 8-color intracellular cytokine staining (ICS) assay to quantify antigen-specific T cells induced by vaccination. J Immunol Methods 323, 39-54.

Historical References: First FACS and First 2-color FACS publications

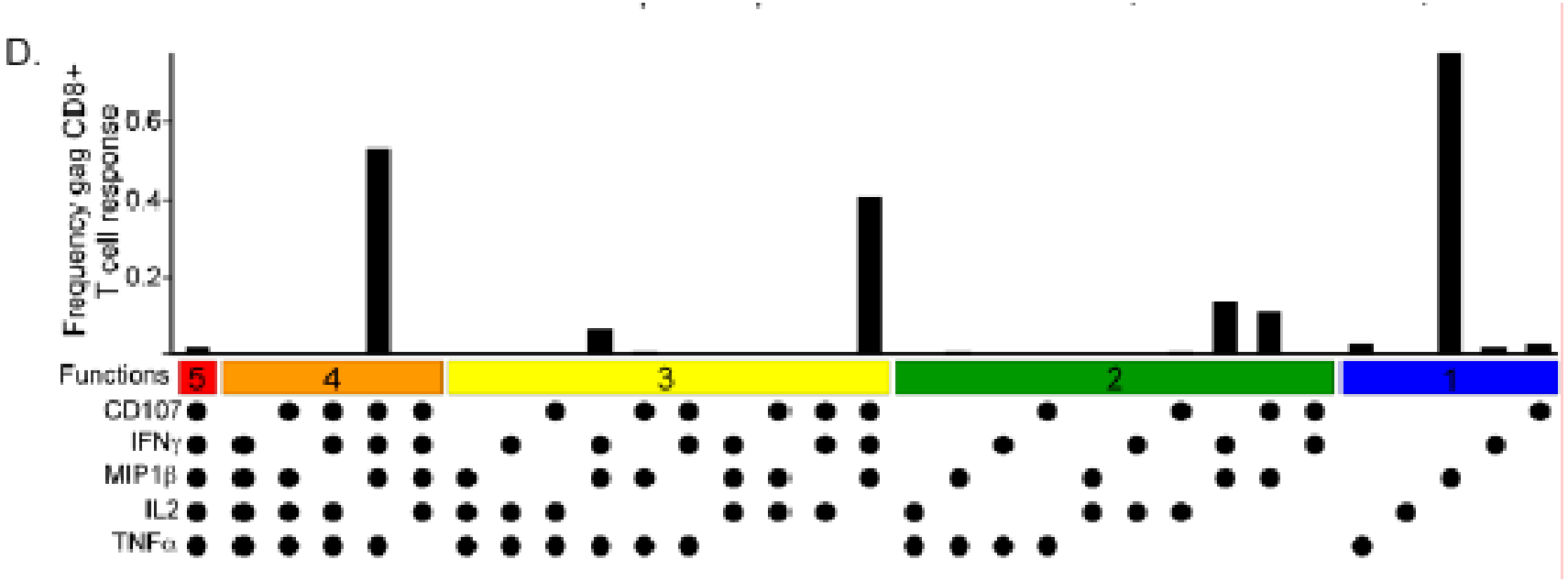
- Hulett, H.R., Bonner, W.A., Barrett, J. and Herzenberg, L.A. (1969) Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence. Science 166, 747-9.
- Loken, M.R., Parks, D.R. and Herzenberg, L.A. (1977) Two-color immunofluorescence using a fluorescence-activated cell sorter. J Histochem Cytochem 25, 899-907.

Beyond 6 colors - new challenges

- How do we visualize high dimensional data?

- Looking at 5 functions (IFN-γ, IL2, TNF-α, MIP-1β and CD107a) gives 32 possible flavors for each antigen-specific response….

Visualizing data



Betts et al; Blood 2005

Beyond 6 colors - New Challenges cont.

- Looking at 7 functions gives 128 possible flavors for each antigen-specific response….
  - Use of microarray-type analytical methods?

In summary

- New fluorophores and equipment advances (e.g. digital electronics) have allowed us to greatly expand flow cytometric phenotypic/functional panels.
- This allows detailed characterization of immune cell function with limited sample.
- Optimization of polychromatic panels is labor intensive and somewhat empirical.
- We face new challenges regarding visualization and display of high-dimensional data.