

Multicolor Flow Cytometry

Principles of Panel Design

Presented by
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Key Concepts for the Design of Multicolor Flow Cytometry Panels

- Multicolor flow cytometry is a powerful tool enabled by development of advanced flow cytometers.
- Robust assays can detect and monitor multiple analytes to produce a tremendous amount of data while conserving sample.
- Availability of different conjugates and putting the colors in the appropriate channels makes developing multicolor (>6) panels challenging.
- Data quality (results) depends greatly on proper panel design and optimization of instrument setup.



Principles of Panel Design

- 1** Match fluorochromes by brightness (values from the stain index) to the lowest density and smeared antigens (published values or TDSs).
- 2** Minimize spillover.
- 3** Use tandem dyes with consideration of their technical limitations.
- 4** Know your instrument and use the BD™ CS&T system.
- 5** Use appropriate controls.



1

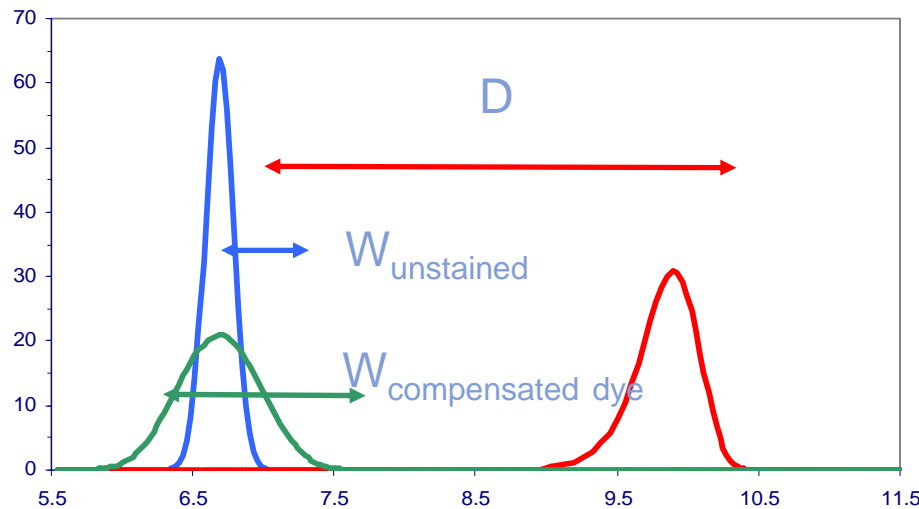
Match Fluorochromes by Brightness

- Know the stain index of the instrument for which you are designing a panel.
 - Specific to a set of lasers and filters
- Refer to the ranking of fluorochromes by stain index for a platform.



Normalized Signal to Background: Stain Index

$$\text{Stain Index (SI)} = \frac{D}{W}$$



Goal: Normalize the signal to the spread of background where background may be autofluorescence, unstained cells, or compensated cells from another dye dimension.

$$\text{SI} = \frac{1.645 \times (\text{positive} - \text{background})}{\text{background}_{95\%} - \text{background}_{5\%}} = \frac{\text{positive} - \text{background}}{2 \times \text{SD}_{\text{background}}}$$

Stain Index: metric used by David Parks, Stanford, presented at ISAC 2004



CD4 Conjugates – Average Stain Index on the BD™ LSR II and BD FACSCanto™ II for CD4 Antibody

Fluorochrome	Stain Index
PE-Cy™5	353
PE	302
APC	278
Alexa Fluor® 647	214
PE-Cy™7	139
PerCP-Cy™5.5	107
BD Horizon™ V450	85
Pacific Blue™	80
Alexa Fluor® 488	73
Alexa Fluor® 700	61
FITC	56
APC-Cy7	37
PerCP	37
AmCyan	25
APC-H7	24



Antigen Density

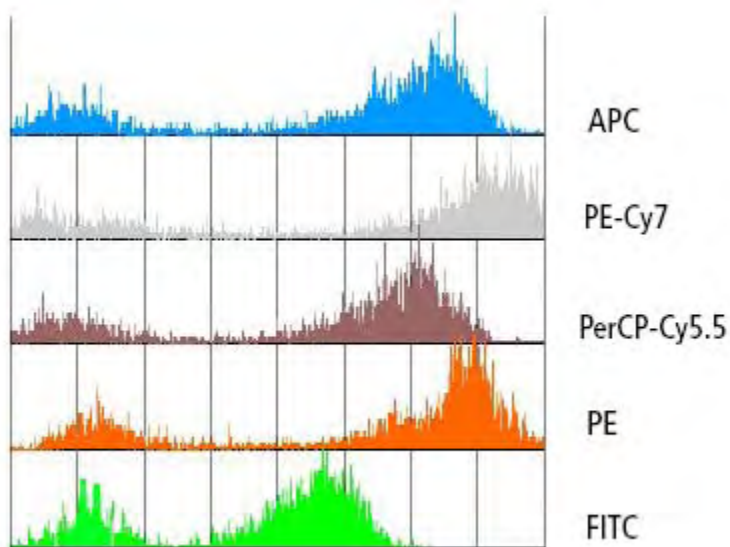
- Level of antigen expression on a cell:
 - Antigen expression can vary due to cell activation level and functional differences
 - Antigen density can be a range (ie, smeared population)

Cell	Antigen	Molecules per Cell
T cell	TCR	100,000
	CD2	55,000
	CD3	124,000
	CD5	90,000
	CD7	20,000
	CD45	>200,000
CD4+ T cell	CD4	100,000
	CD28	20,000
	CCR5	4,000-24,000
CD8+ T cell	CD8	90,000
	CD28	15,000
B cell	CD19	18,000
	CD20	109,000
	CD21	210,000
	CD22	14,000
	HLA-DR	85,000
	CD11a	10,000
	CD40	2,000
	CD86	16,000
	CD80	2,000
Dendritic cell	CD11a	27,000
	CD40	17,000
	CD80	132,000
	CD86	208,000
Monocyte	CD14	110,000
	CD32	21,000
	CD64	13,000
Neutrophil	CD14	3,500
	CD16	225,000
NK cell	CD56	10,000
Red Blood Cell	Glycophorin A	340,000
Basophil	CD23	15,000

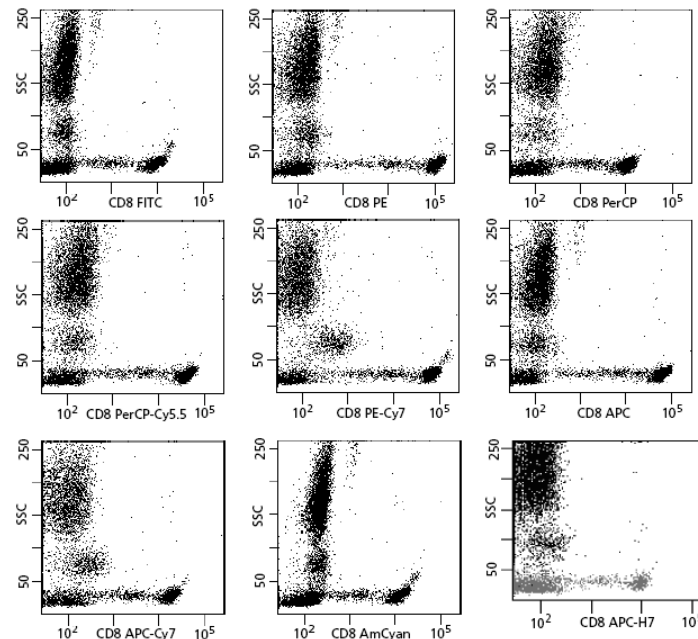
1 Determine Antigen/Fluorochrome Combos

- Match brightest fluorochromes with dimmest antigens.
 - Antigen density
 - Intracellular antigens are usually dimmer and/or less discrete populations than surface antigens.
- Review antibody/fluorochrome combinations in TDSs.
 - Visually compare all antigens conjugated to same fluorochromes.

CD5



CD8



1 Example

Bright antibodies go on dim fluorochromes

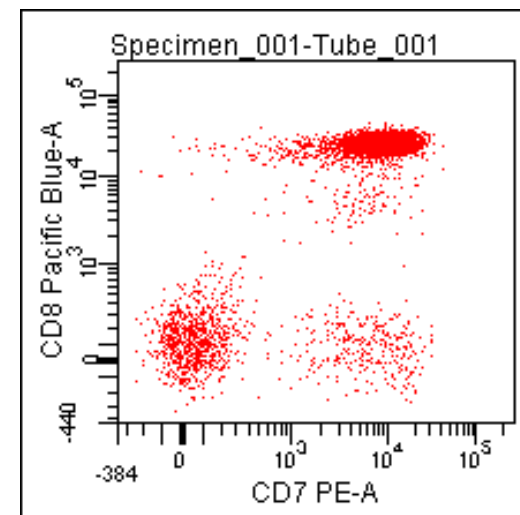
Example:

CD8 bright → Pacific Blue™ (SI = 80)

CD7 less bright → PE (SI = 302)

CD8 = 90,000 molecules per cell

CD7 = 20,000 molecules per cell



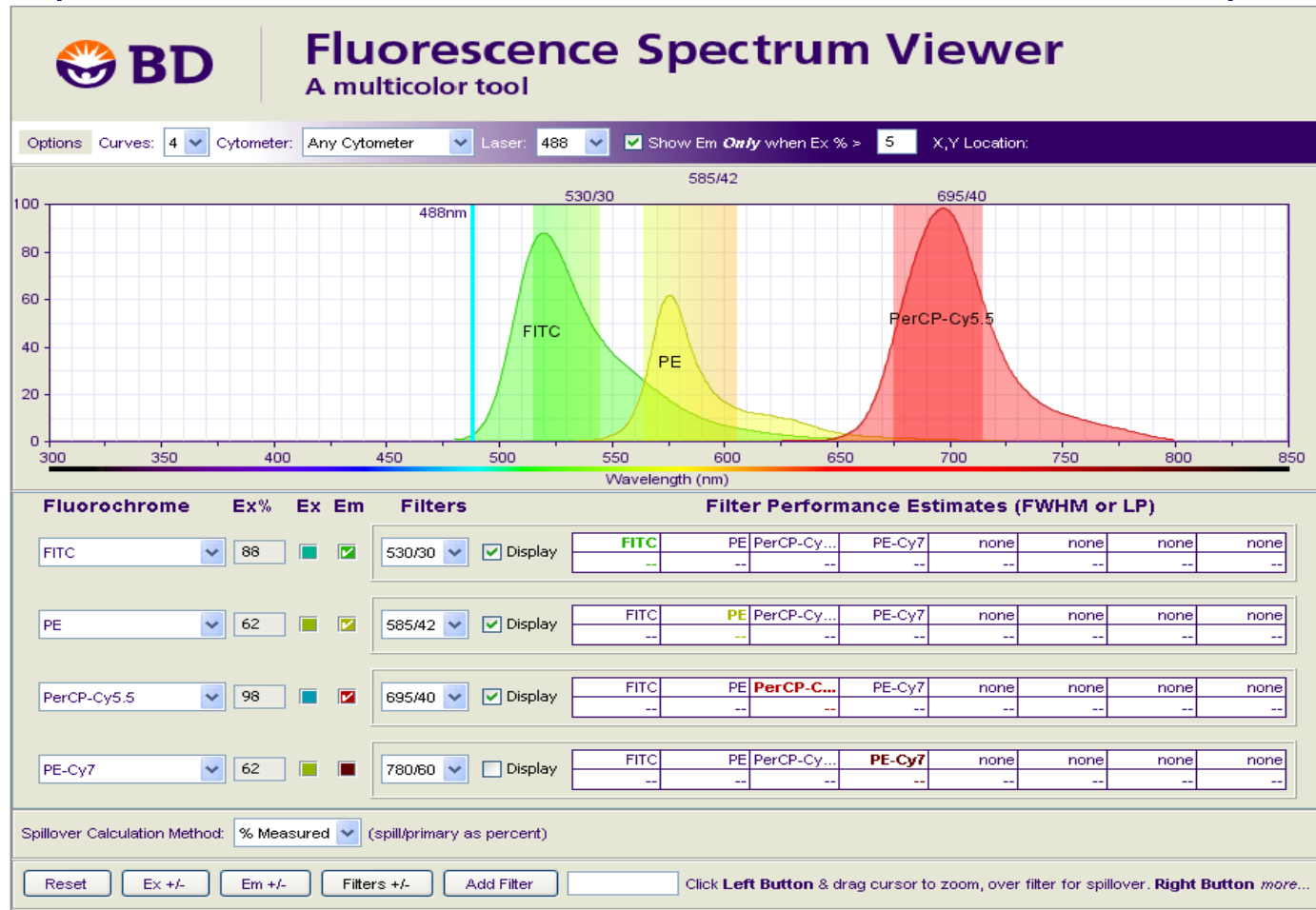
Principles of Panel Design

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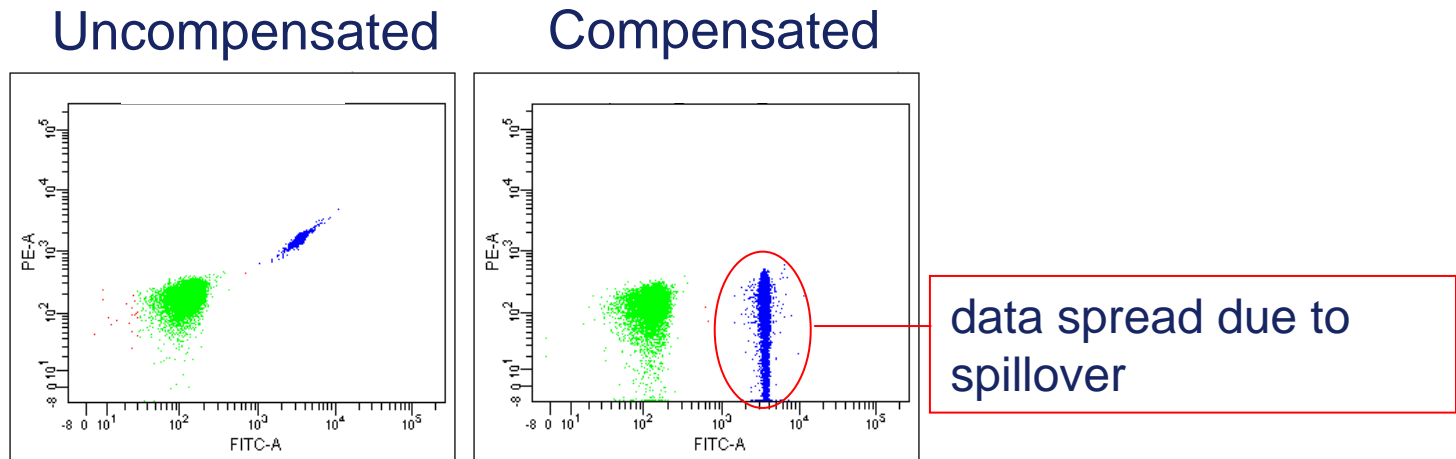
2 Minimize Spillover

- Minimize the potential for spectral overlap
 - Spillover estimates available in the BD fluorescence spectrum viewer



2 Data Spread Due to Spillover

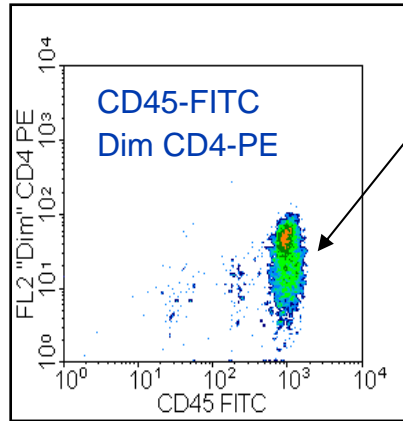
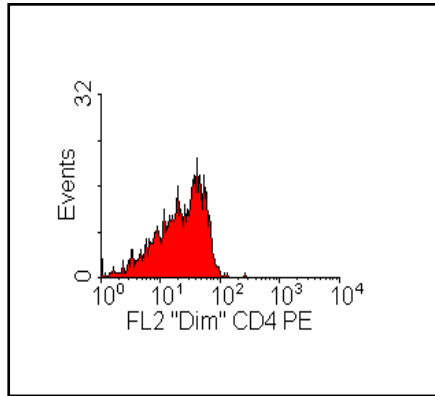
↑ spillover = ↓ resolution



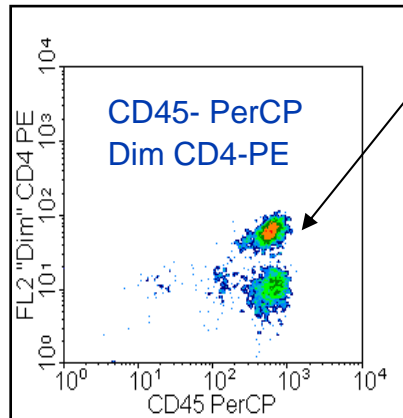
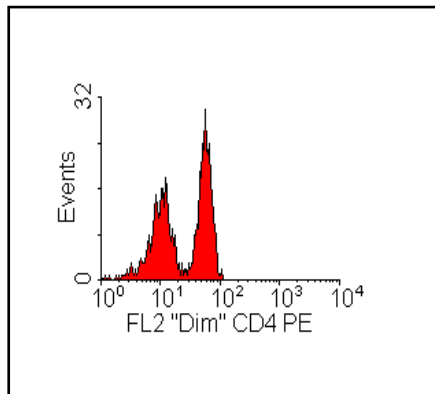
Maecker HT, Frey T, Nomura LE, Trotter J. **Selecting fluorochrome conjugates for maximum sensitivity.** *Cytometry A* 2004; **62**:169-173.



2 Population Lost Due to Spillover



CD45 FITC spills over into the PE detector.
CD4 PE dim cells cannot be separated.



CD45 PerCP DOES NOT spill over into
the PE detector.
Dim CD4 cells to be separated from
background

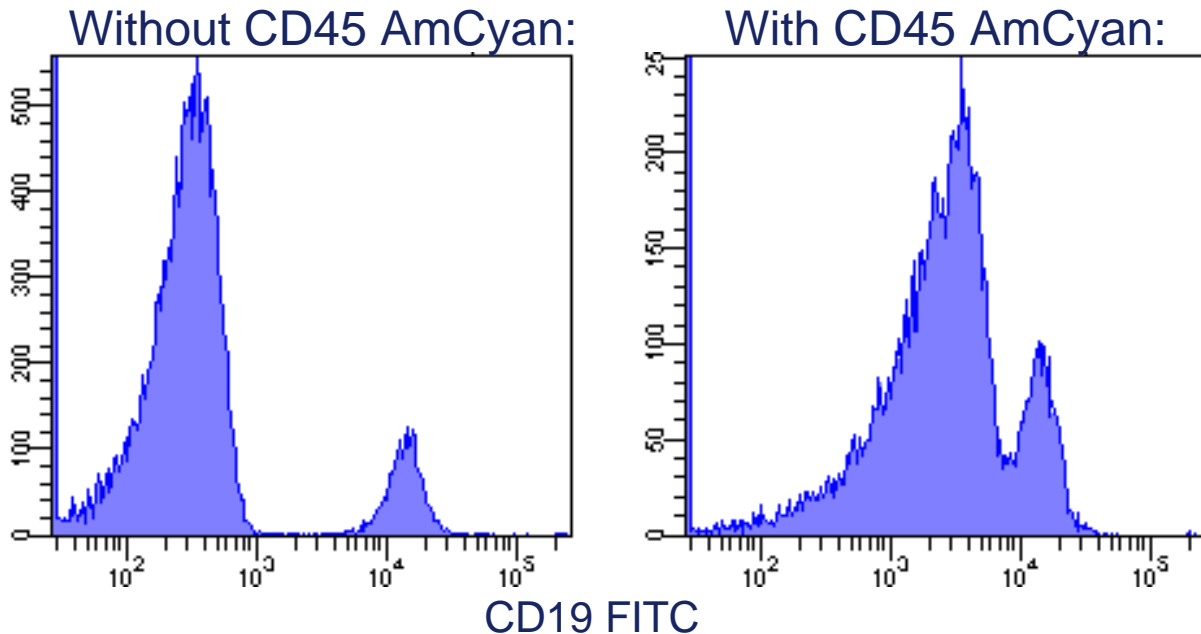


2

Dual Excitation Reduces Resolution

Fluorochromes that are excited by more than one laser cause high spillover.

- AmCyan excited by the violet and blue lasers spills into the FITC detector.
- PE-Cy5 excited by the blue and red lasers spills into APC detector.



2

Spread Antigens Across Lasers

If multiple antigens are present on a cell, spread them across as many lasers as possible to minimize spillover.

Example:

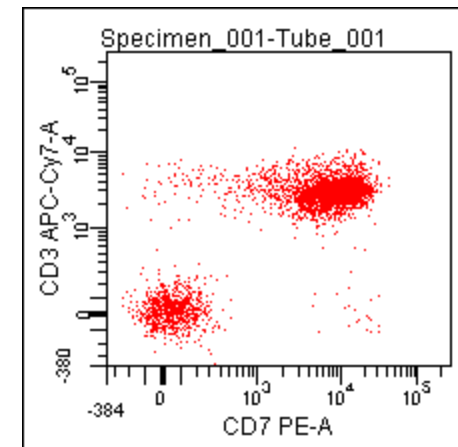
CD3 bright APC-Cy7 (SI = 42.2)

CD7 less bright PE (SI = 356.3)

Both antigens expressed on same cell, low spillover of CD3 into CD7 and vice versa.

CD3 = 124,000 molecules per cell

CD7 = 20,000 molecules per cell



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3

Use Tandem Dyes with Consideration of Their Technical Limitations

- Compensation requirements for tandem dye conjugates can vary, even between two experiments with the same antibody.
 - Require compensation that is: lot specific, experiment specific, and label specific.
- Certain tandem dye conjugates (APC-Cy7, PE-Cy7) can degrade with exposure to light, elevated temperature, and fixation.
 - Minimize exposure to these conditions.
 - Use BD™ Stabilizing Fixative for final fixation.



3

Using Tandems with Intracellular Staining

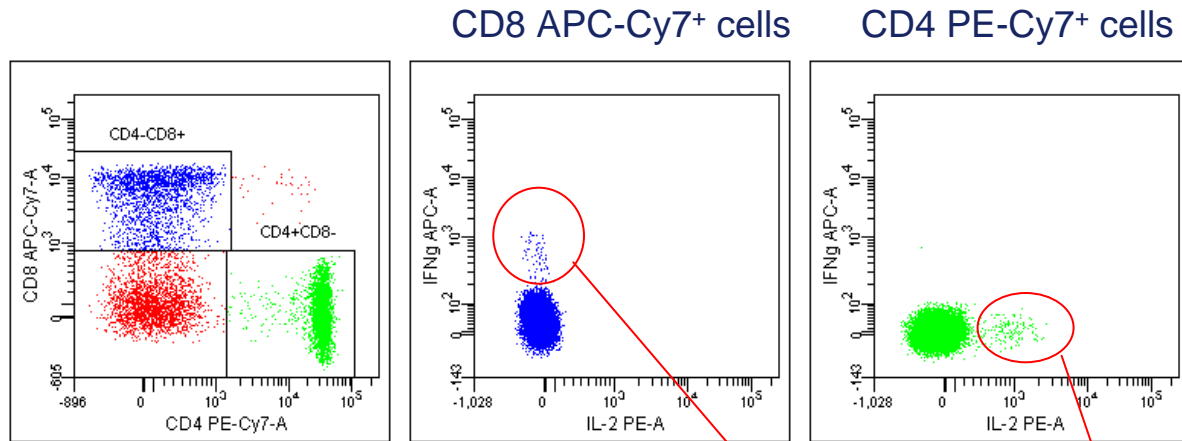
- The fixation/permeabilization protocol of intracellular staining makes spillover of all Cy5 and Cy7 tandems unpredictable unless you:
 - Treat compensation controls the same as sample cells.
 - In this case you would not use BD™ CompBeads for compensation as you compensate with fixed/permed cells.
 - Stain with tandems post-fixation and permeabilization.
 - BD CompBeads can be used for compensation.



3

False Positives Due to Tandem Degradation

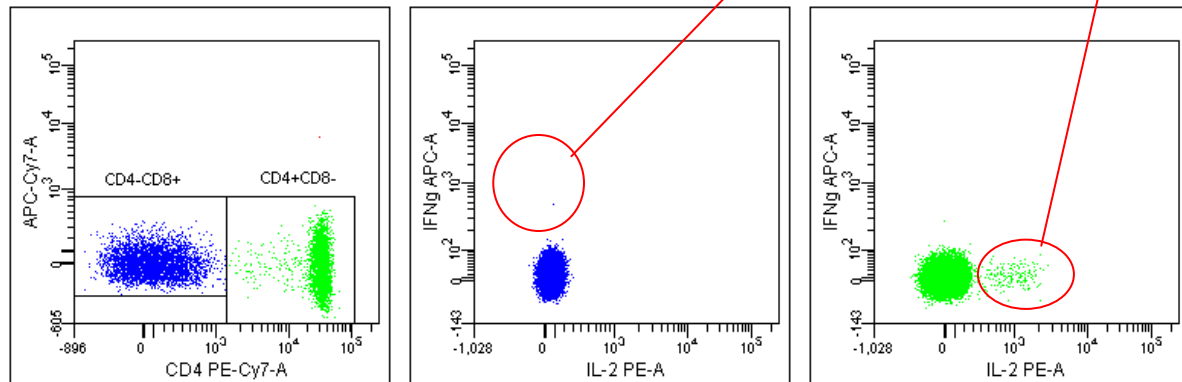
A. With CD8 APC-Cy7 and CD4 PE-Cy7



False positives in APC channel reduced in absence of APC-Cy7

False positives in PE channel remain

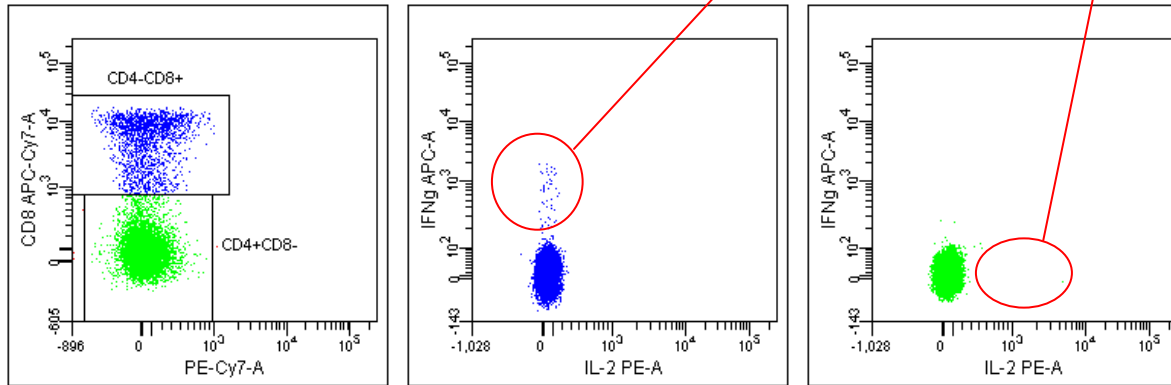
B. Without CD8 APC-Cy7



3

False Positives Due to Tandem Degradation

C. Without CD4 PE-Cy7

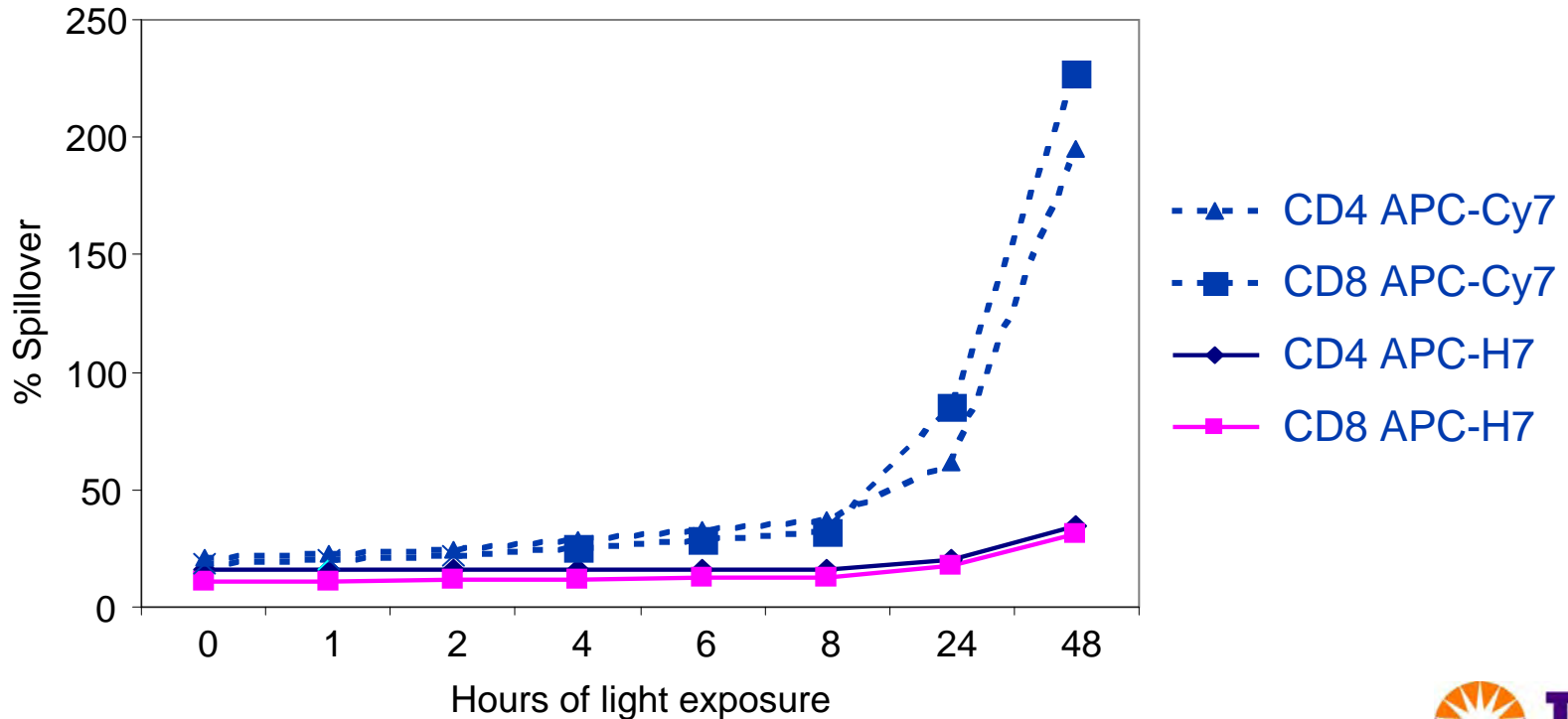


3

New Tandems Are More Stable

APC-H7 to replace APC-Cy7:

Comparison of Sample Stability
(in BD Stabilizing Fixative at RT)



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BD FACSDiva™ 6.1 Software with BD CS&T System: Automatic Instrumentation Optimization and Setup

- A revolutionary new tool that automates cytometer setup and optimizes all the key parameters for superior multicolor performance.
- The BD Cytometer Setup and Tracking (CS&T) system results in consistent instrument setups day-to-day and decreased data variability due to instrument setup.
- Application settings within BD CS&T allow you to adjust the instrument PMT voltage and laser power settings optimally for a particular assay.
 - Reproducible instrument setup and assay settings will result in superior data quality (reproducibility), especially in higher order multicolor assays.



4 Know Your Instrument

BD CS&T Values:

- Linearity
- Q_r
- B_r
- Electronic Noise (SD_{EN})



4

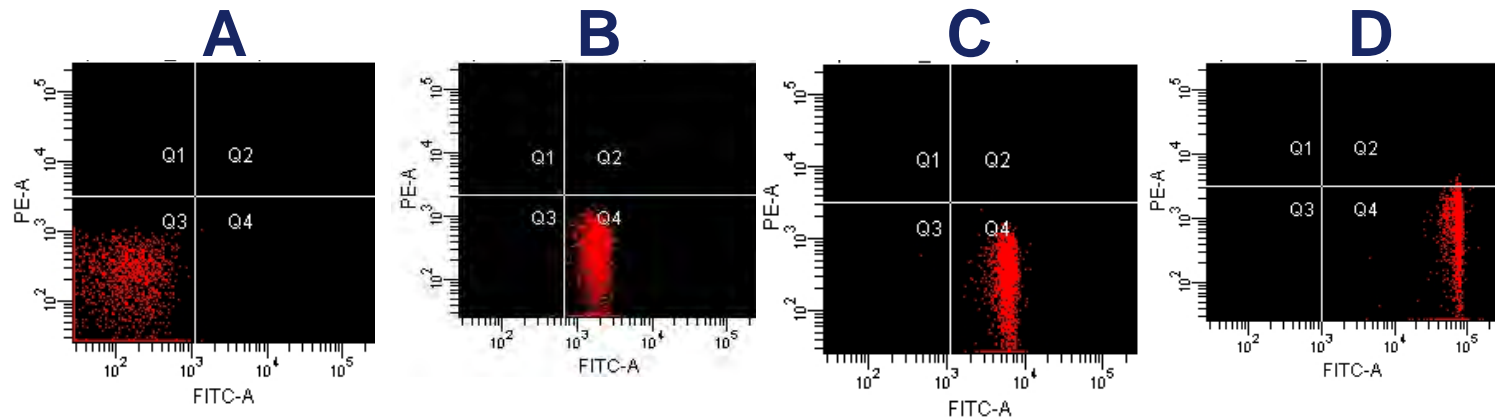
Performance Parameter: Linearity

- Defined as the proportionality of output to input (Signal:number of photons)
- Important for fluorescence compensation
- Important for quantitative measurements
 - DNA measurements
 - Antigen to antibody binding



4 Linearity: Effect on Compensation

- Compensation of data in the last decade involves subtraction of large numbers.
- Errors (non-linearity) in one or both large numbers can cause a large absolute error in the result.



Detector	Median Fluorescence Intensity (MFI)			
FITC	68	1796	5921	73,000
PE	80	75	79	365

BD CompBeads stained with varying levels of FITC-Ab.

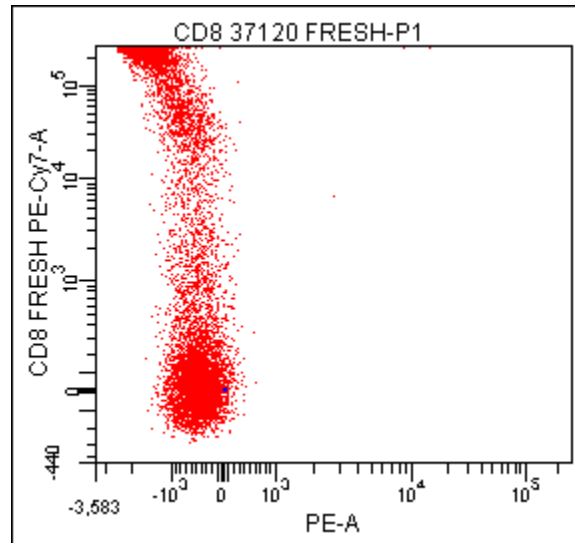
Compensation was set using samples A and C.

This cytometer had a 2% deviation from linearity above 50,000 units.



4 Keeping Signals within Linear Range

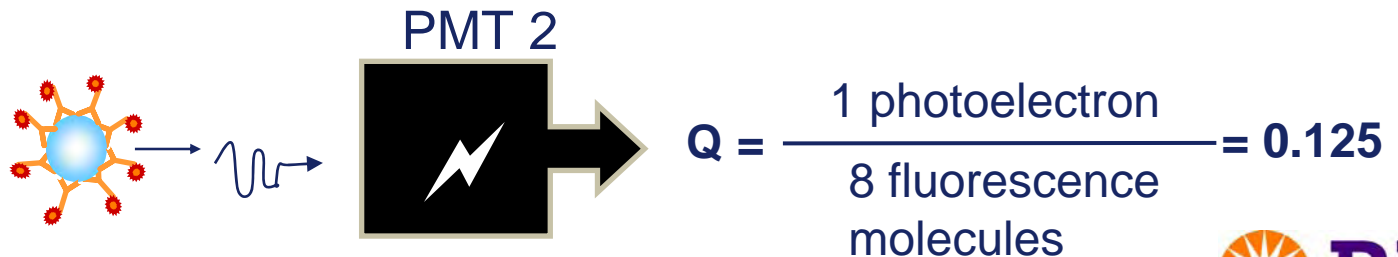
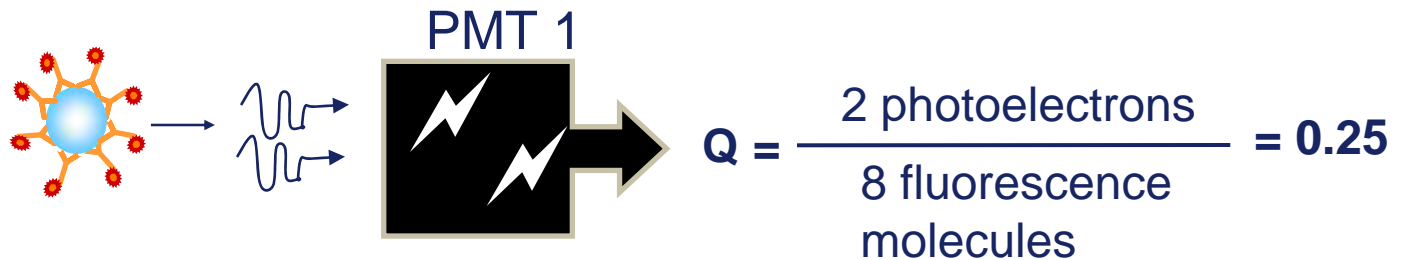
- Run the brightest staining samples for each channel.
 - If the brightest populations are off scale, lower the PMT voltage to put the population on scale within the linear range.
- Save application settings.



4 What is Q?

Q is the efficiency of the system to detect fluorescence.

$$Q = \frac{\text{No. of photoelectrons}}{\text{No. of fluorescence molecules}}$$



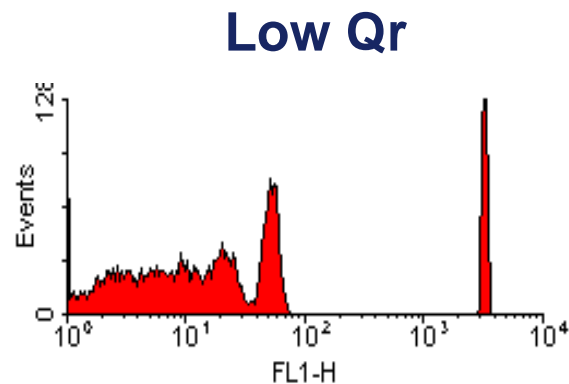
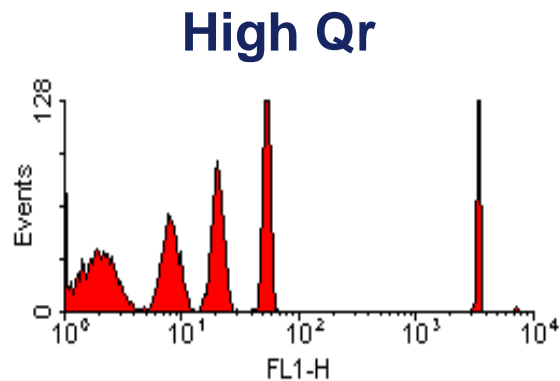
4 Why is Qr Important?

Qr is the relative efficiency of each channel to detect fluorescence.

A system with a higher Qr has a better resolution than a system with a lower Qr.

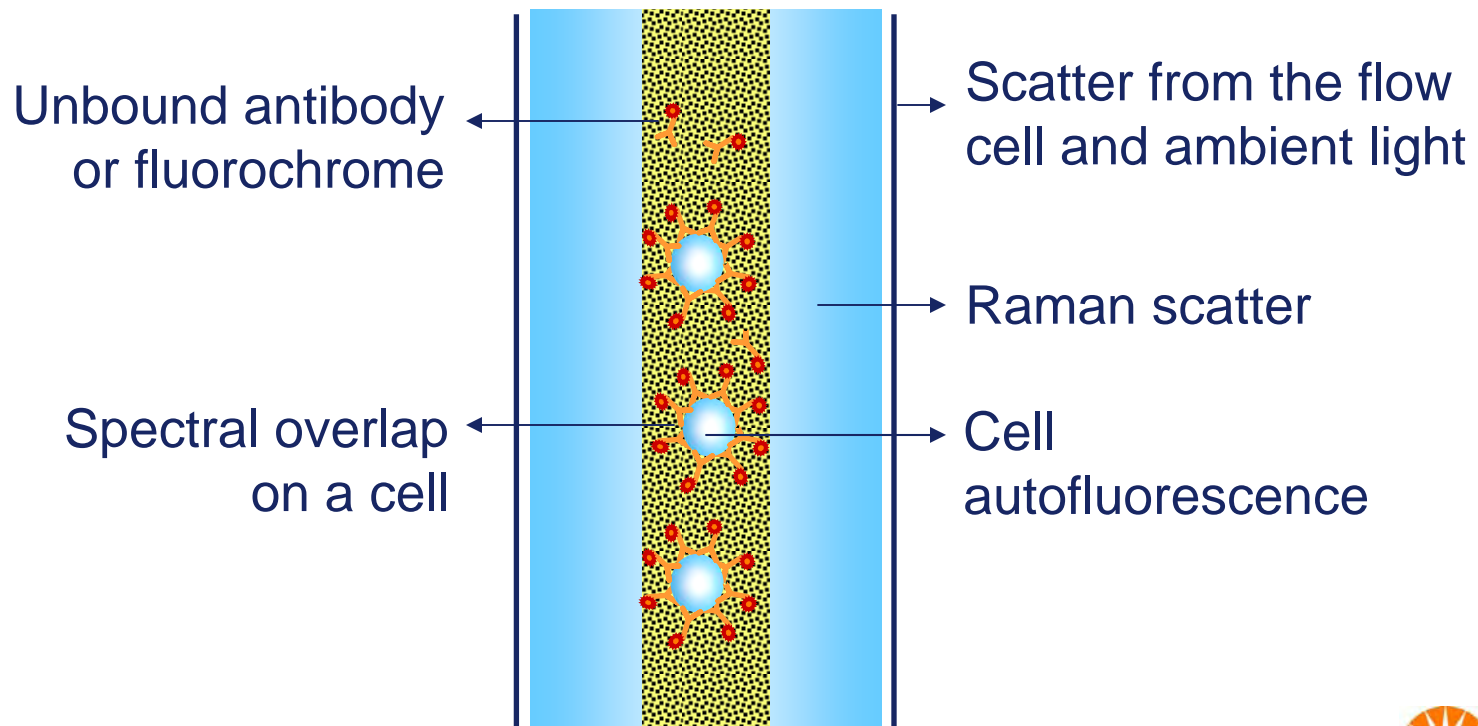
Low Qr value = high CV = lower resolution

High Qr value = low CV = higher resolution



4 What is Relative Background (Br)?

Relative B (Br) is a measure of the true optical background in the fluorescence detector.



4 What Factors Affect Br?

- Dirty flow cell
- Damaged optical component

Laser	Detector	Parameter	Br
Blue	FSC	FSC	N/A
Blue	F	SSC	N/A
Blue	E	FITC	135.09
Blue	D	PE	320.66
Blue	B	PE-Cy5	3.94
Blue	A	PE-Cy7	29.88
Red	C	APC	6.24
Red	A	APC-Cy7	0.00
Violet	B	Pacific Blue	1,137.38
Violet	A	AmCyan	1,212.19

High Br due to Raman light scatter



4

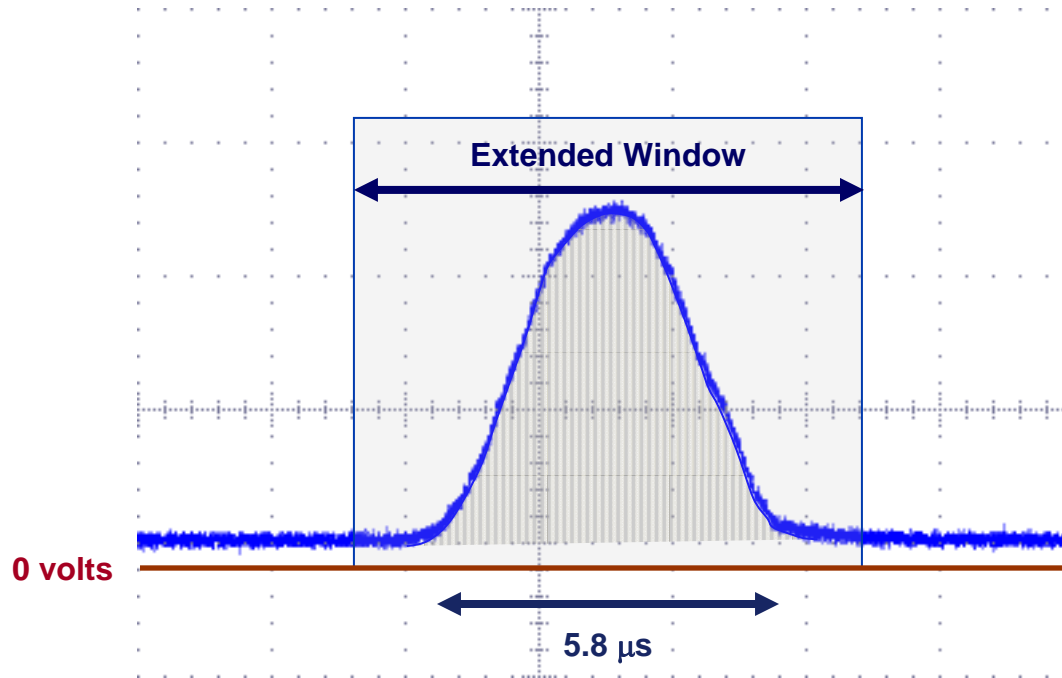
What is the Standard Deviation of Electronic Noise (SD_{EN})?

Electronic noise(SD_{EN}) is the constant low level background noise contributed by the electronics system.

Laser	Detector	Parameter	Electronic Noise Robust SD
Blue	FSC	FSC	N/A
Blue	F	SSC	N/A
Blue	E	FITC	23.19
Blue	D	PE	23.24
Blue	B	PE-Cy5	24.17
Blue	A	PE-Cy7	25.07
Red	C	APC	21.02
Red	A	APC-Cy7	22.73
Violet	B	Pacific Blue	22.90
Violet	A	AmCyan	28.99



4 Decreasing Window Extension Lowers Electronic Noise



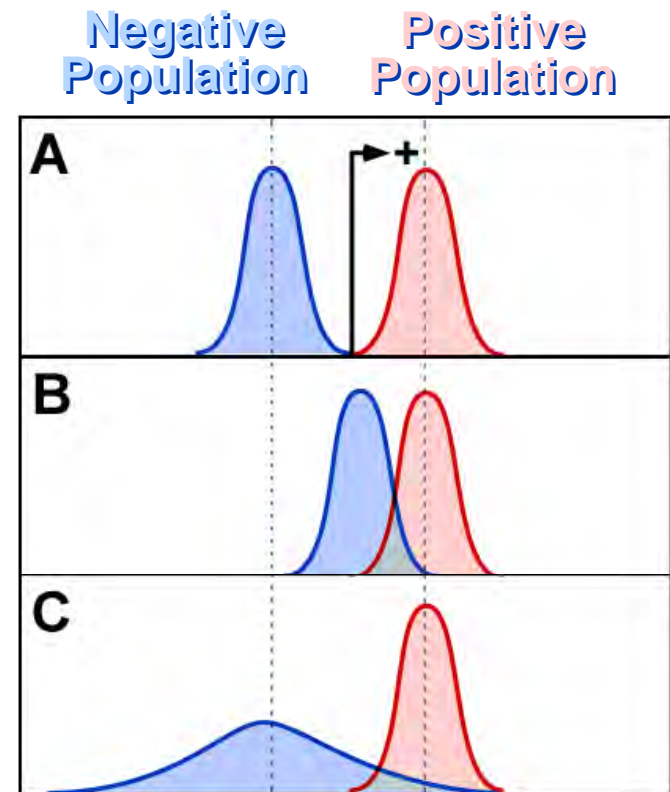
4

Sensitivity: Resolution vs. Background

Negative population has low background
Populations well resolved

Negative population has high background
Populations not resolved

Negative population has low background and high CV
Populations not resolved



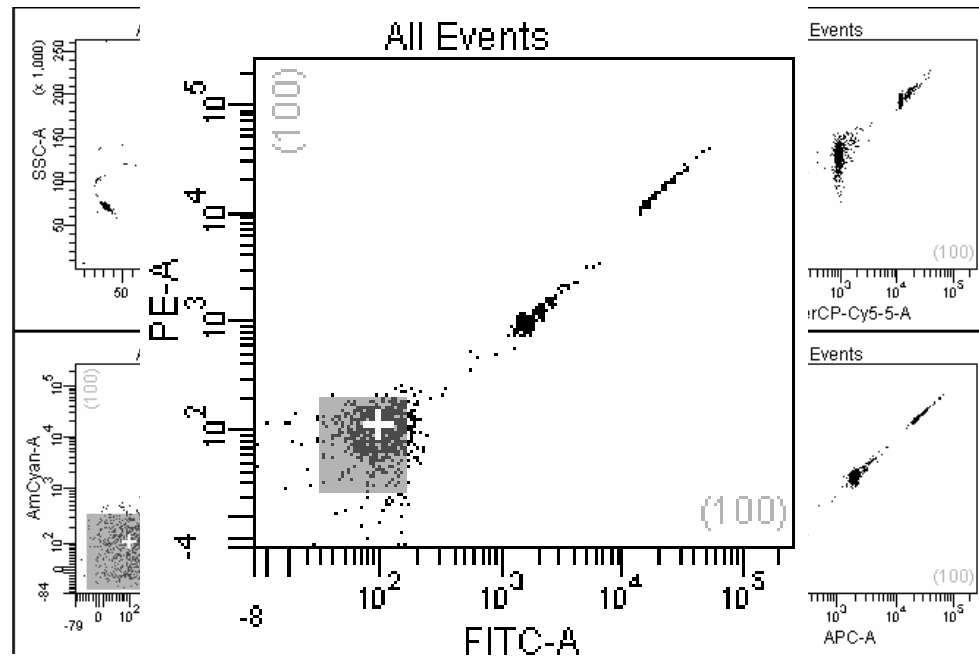
The ability to resolve populations is a function of both background and spread of the negative population.



4 Cytometer Setup – Application Settings

A pre-defined application settings worksheet provides a guide to setting PMT voltages based on a baseline definition.

- Crosshair indicates the target value for the negative population based upon $10 \times SD_{EN}$.
- Adjust the negative population to fit:
 - Within the gray box
 - Center at the crosshair



4 Why is PMT Optimization Important?

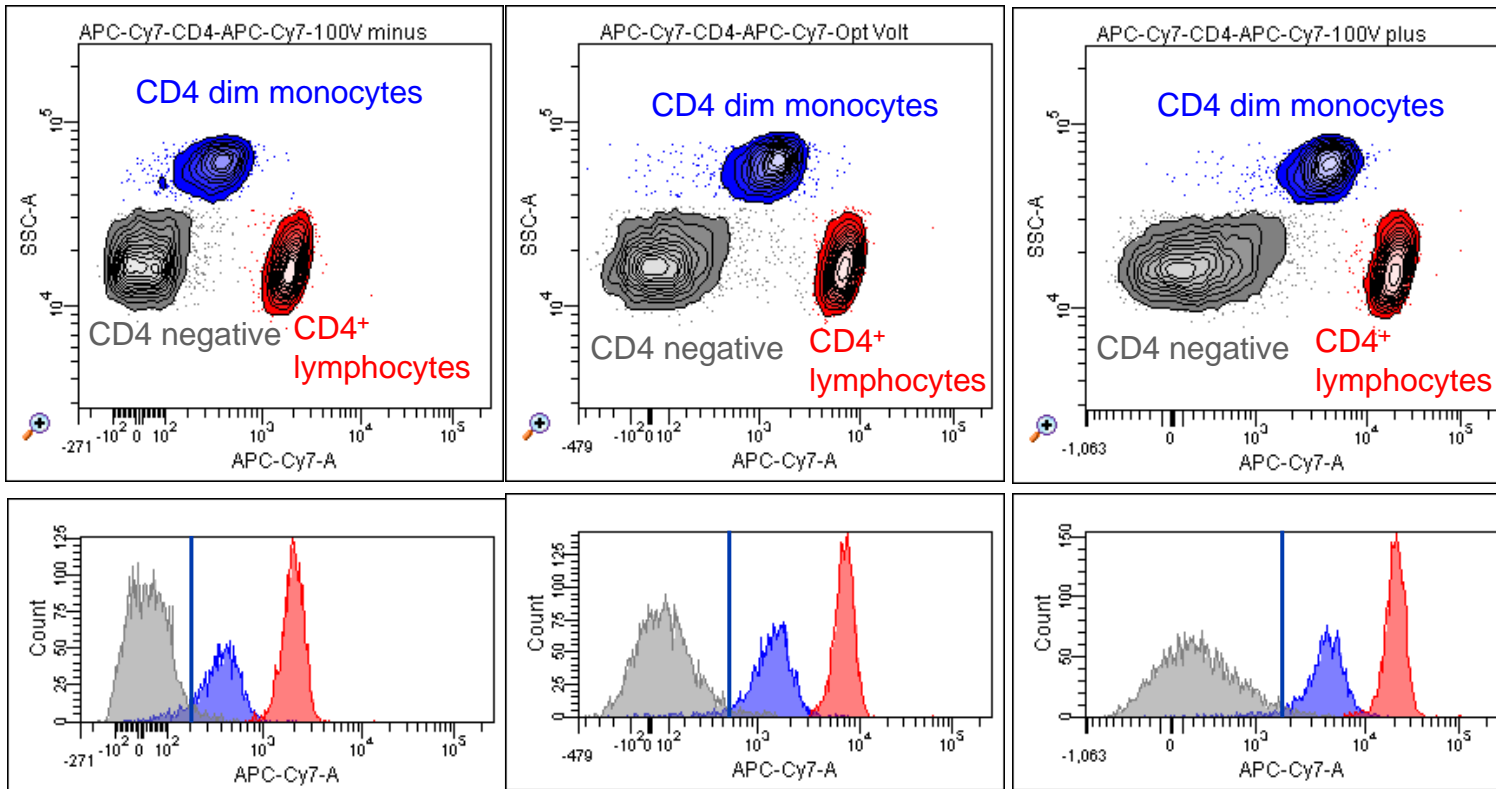
Finding PMT settings that maximize resolution sensitivity for each experiment:

Move fluorescent populations out of electronic noise

550 volts

650 volts

750 volts



4

BD CS&T Workflow with Application Settings

1. **Run** the BD CS&T Baseline Definition and Performance Check procedures to set the voltages.
2. **Run** fully stained cells using the Application Settings Worksheet.
3. **Run** single-stained BD CompBeads to see if each bead is brightest in its primary detector. Make a spill index calculation if necessary.
 - If not, increase the voltage in the primary detector.
4. **Record** single-stained BD CompBeads and calculate compensation.
5. **Run** samples.



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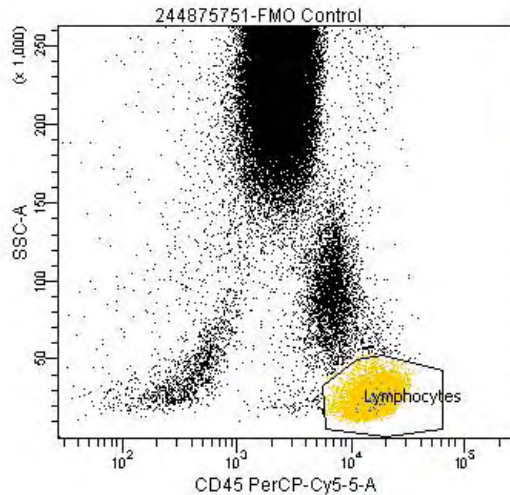
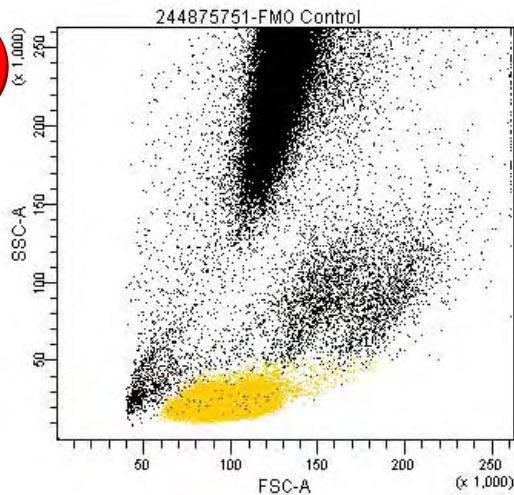
5

Use FMO Controls for Accurate Data Analysis

- Fluorescence Minus One (FMO) controls contain all the lineage markers except the one of interest.
- For low density or smeared populations (eg, activation markers), FMOs allow accurate delineation of positively vs negatively stained cells.
 - Regulatory T cells (Tregs) are a good example.

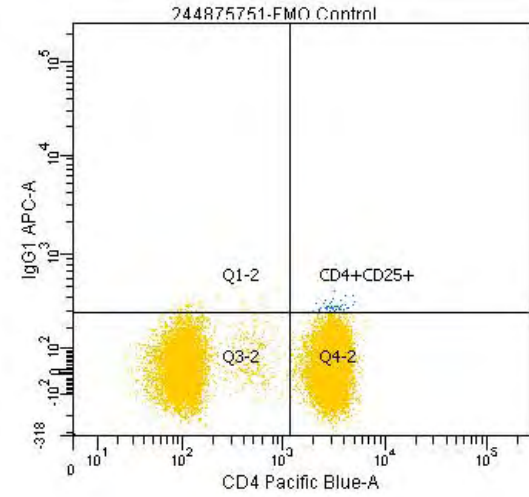
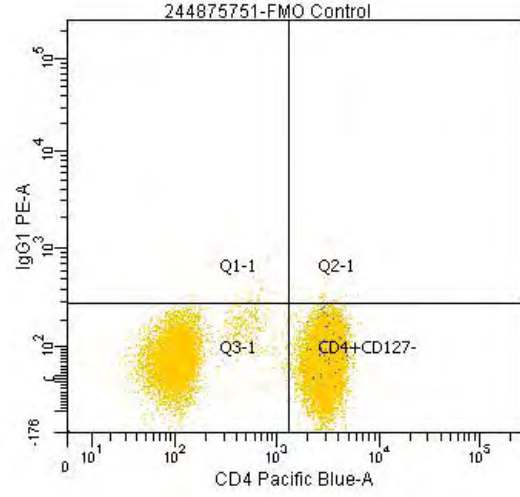
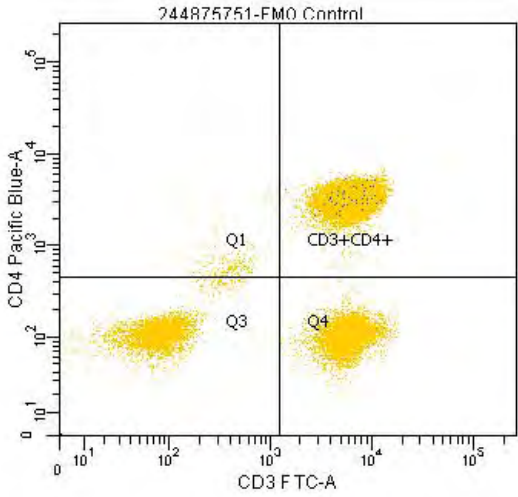


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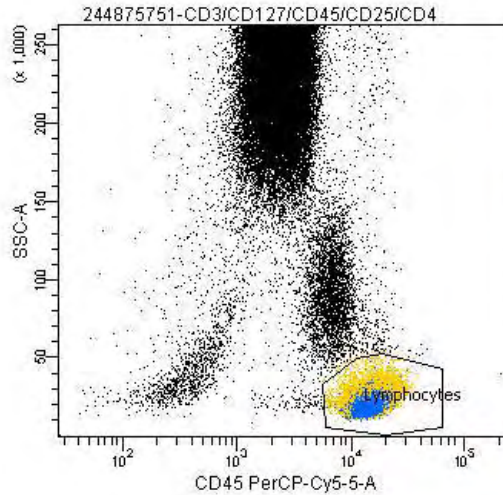
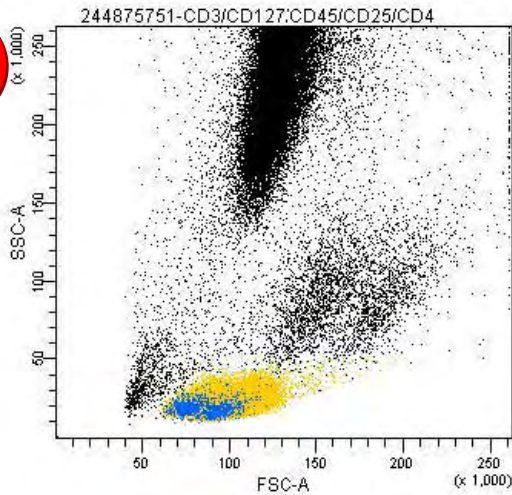


Experiment Name: T Reg #1 ... Tube Name: FMO Contr...
 Specimen Name: 244875751 Record Date: Oct 22, 20...

Population	#Events	%Parent
All Events	91,706	###
Lymphocytes	20,192	22.0
Q1	177	0.9
CD3+CD4+	11,955	59.2
Q3	2,833	14.0
Q4	5,227	25.9
Q1-1	33	0.2
Q2-1	27	0.1
Q3-1	8,233	40.8
CD4+CD127-	11,899	58.9
Q1-2	48	0.2
CD4+CD25+	52	0.3
Q3-2	8,199	40.6
Q4-2	11,893	58.9
Regulatory T Cells	48	0.2

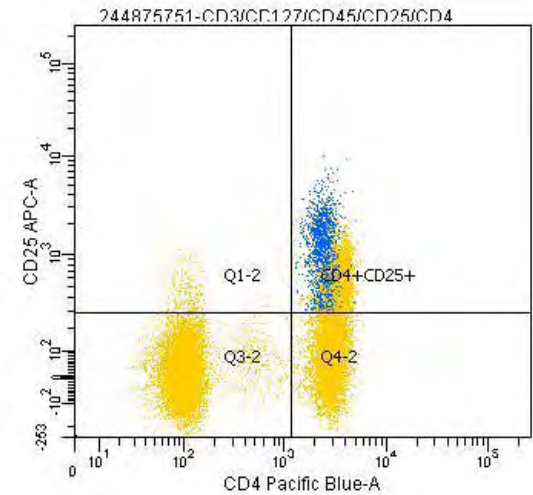
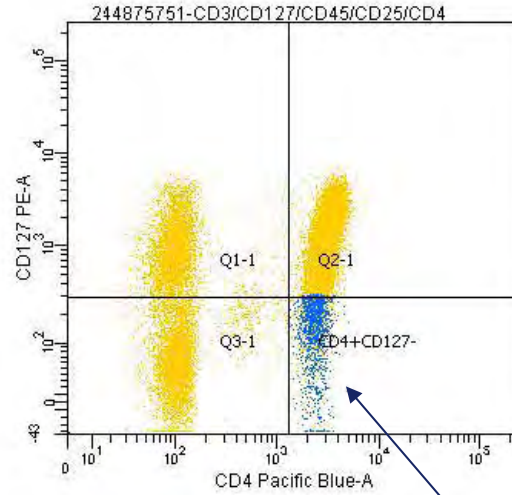
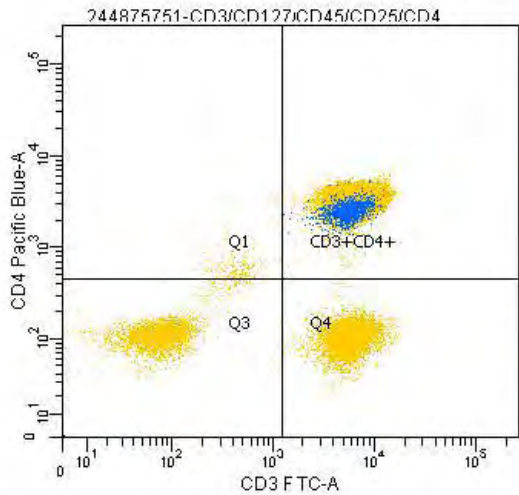


5



Experiment Name: T Reg #1 ... Tube Name: CD3/CD1...
 Specimen Name: 244875751 Record Date: Oct 22, 20...

Population	#Events	%Parent
All Events	88,477	###
Lymphocytes	20,176	22.8
Q1	155	0.8
CD3+CD4+	11,929	59.1
Q3	2,795	13.9
Q4	5,297	26.3
Q1-1	3,855	19.1
Q2-1	9,721	48.2
Q3-1	4,453	22.1
CD4+CD127-	2,147	10.6
Q1-2	231	1.1
CD4+CD25+	4,937	24.5
Q3-2	8,049	39.9
Q4-2	6,959	34.5
Regulatory T Cells	1,241	6.2

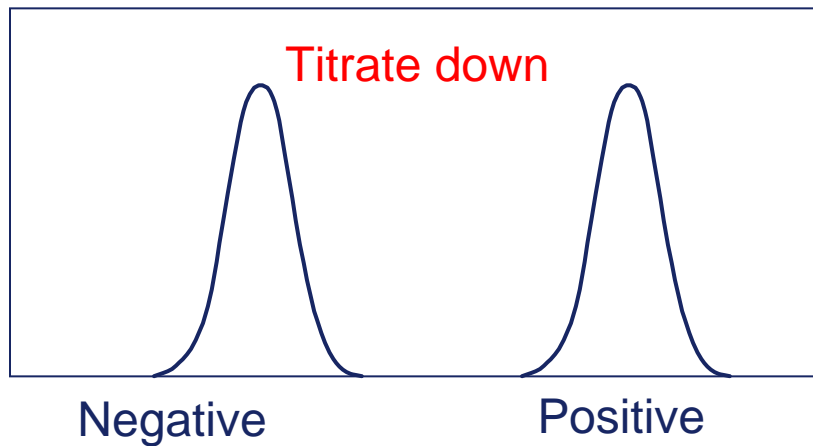


Blue = Tregs



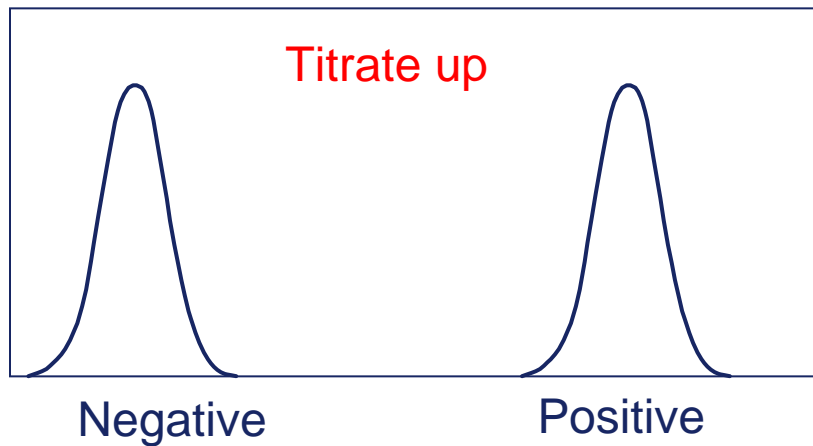
How to Titrate Antibodies?

Oversaturated



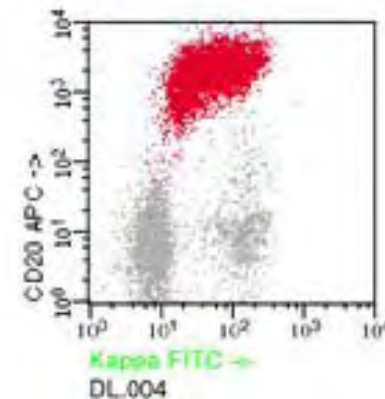
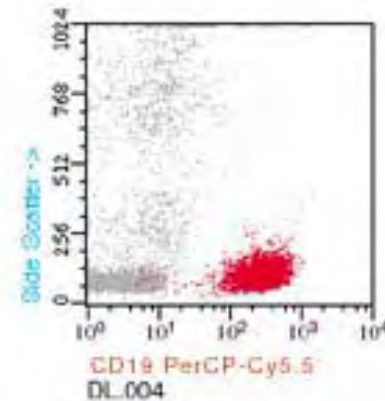
How to Titrate Antibodies?

Saturated



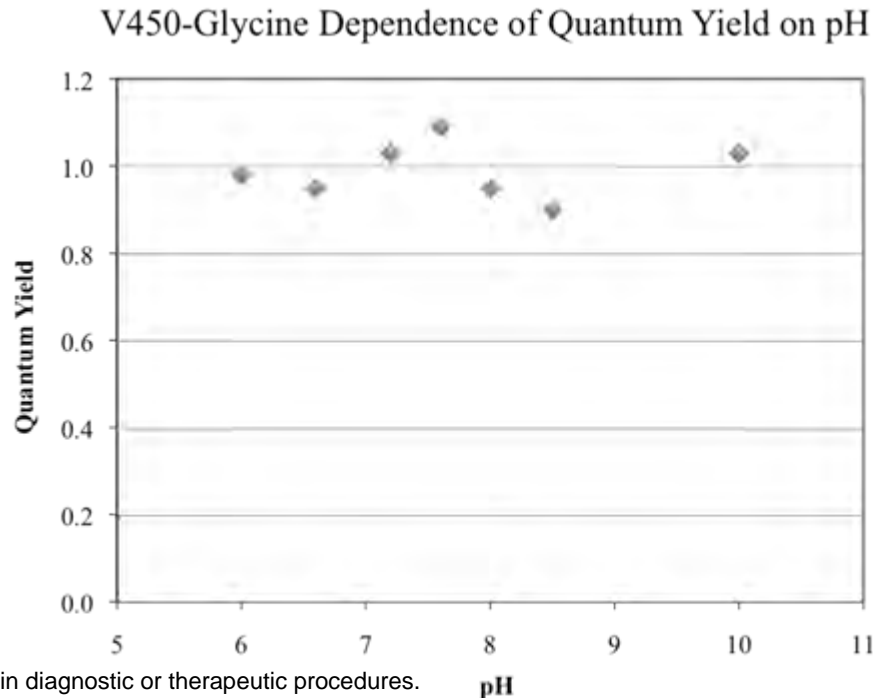
When Not to Titrate?

- Do ***not*** titrate antibodies when the cell concentration expressing the marker is unknown.
 - Leukemias and Lymphomas
 - Many activated cells



One Last Caveat: Quantum Yield

- All fluorochromes have a potential quantum yield.
- This yield can vary with conjugation.
- This means tube combinations must be validated.



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BD Horizon™ V450 Dye



A New Violet-Excited Fluorochrome

- Several violet-excited dyes have been developed over the last few years and are currently in limited use.
- As a part of a new dye development program, BD has synthesized several new violet fluorochromes.
- One of these, BD Horizon™ V450, proved to be as bright or brighter than any of the currently existing violet-excited organic fluorochromes.
- The BD Horizon V450 dye matched 405-nm excitation better than any other current violet-excited fluorochrome.



Excitation and Emission

BD Horizon V450 better fits the laser excitation line and emission filter sets on current flow cytometers.

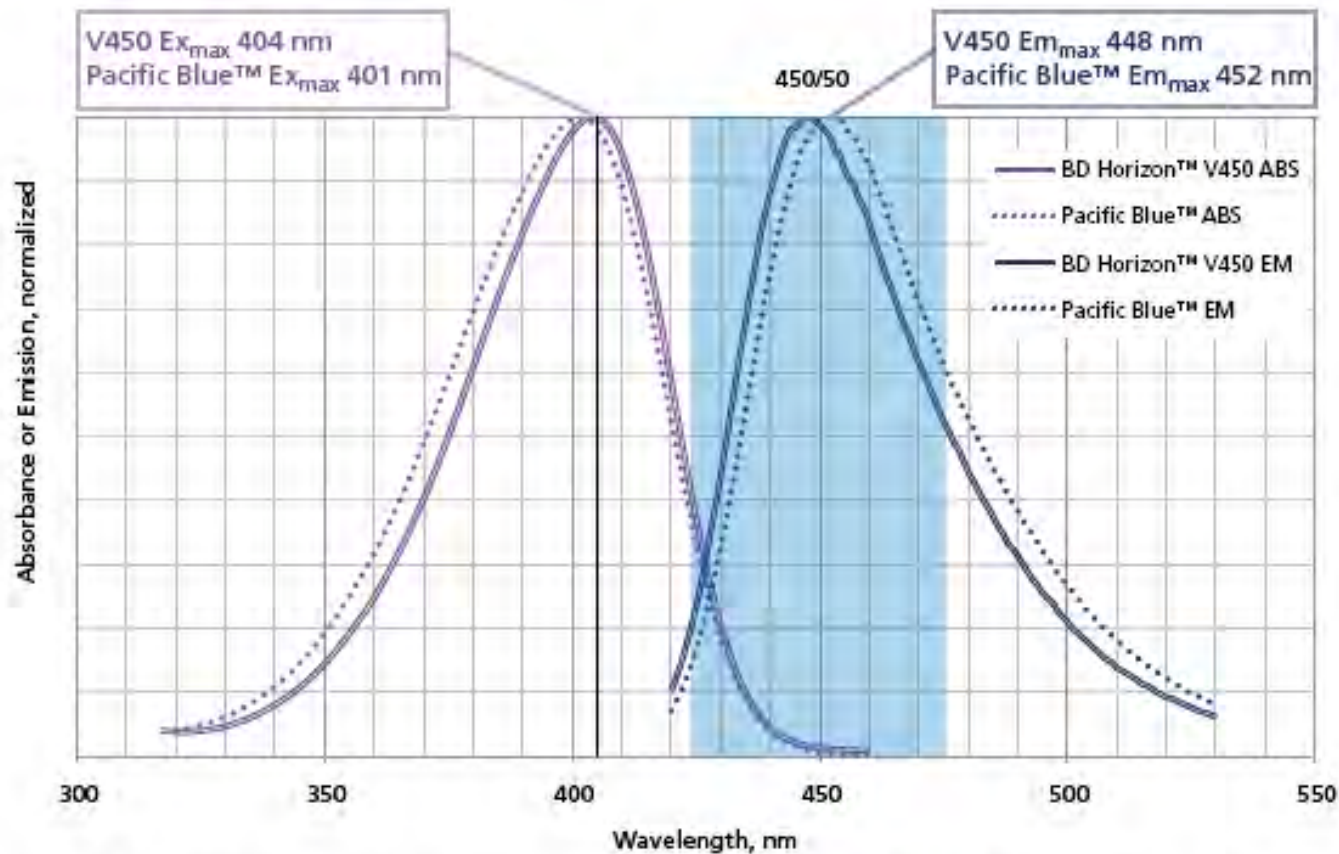
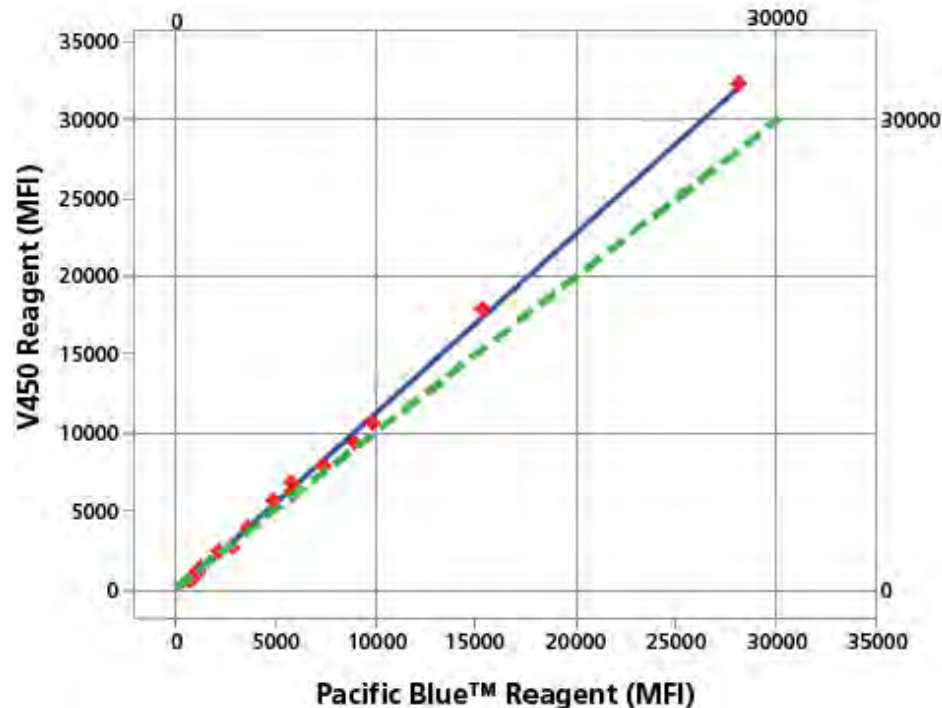


Figure 1. Spectral overlay comparison of BD Horizon V450 and Pacific Blue™



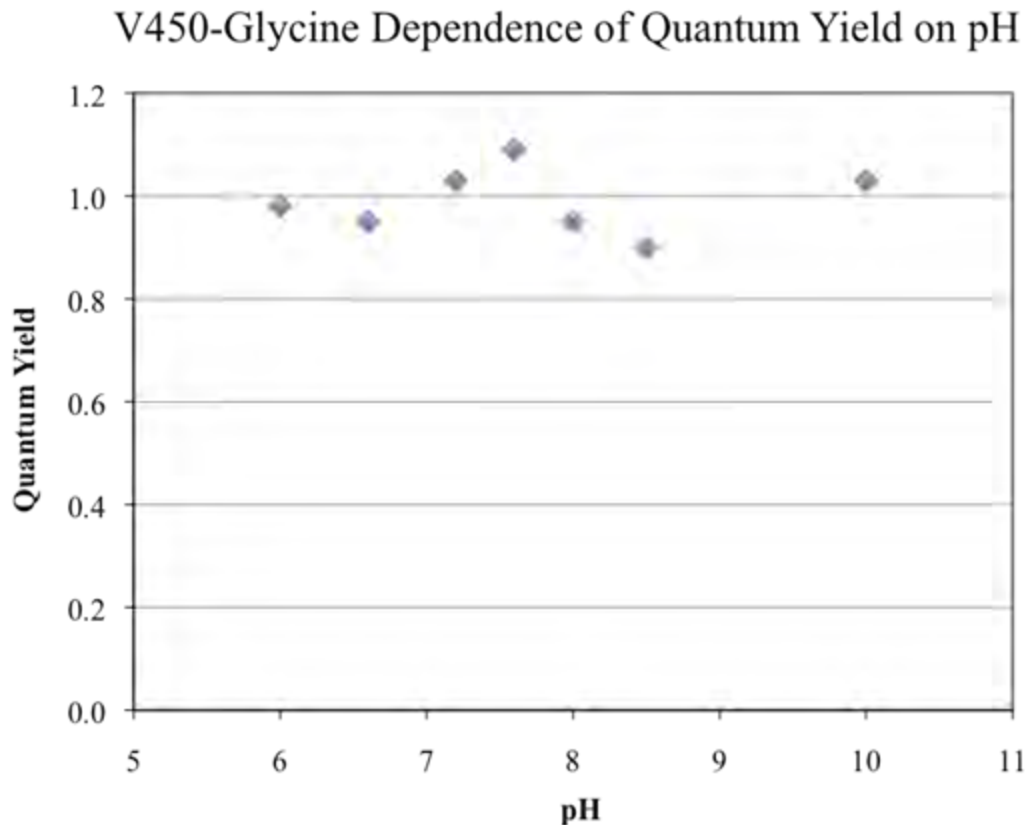
Brighter Than Pacific Blue™ by 15%

- Eighteen matched pairs of BD Horizon V450 vs Pacific Blue™ conjugates.
- Peripheral blood prepared by lyse/wash method with BD FACS™ Lysing solution.
- On average, V450 reagents are 15% brighter than corresponding Pacific Blue™ conjugates.



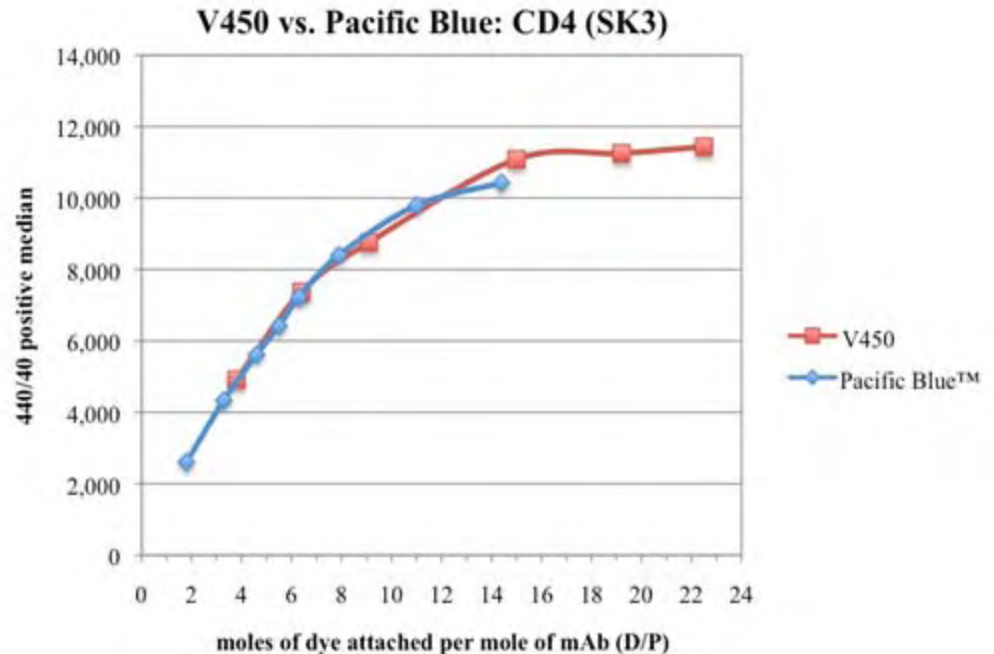
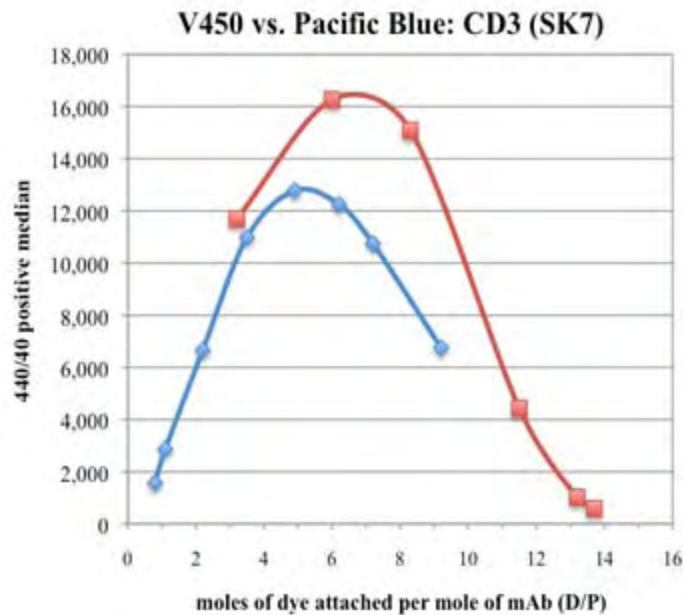
Stable pH-Independent Fluorescence

Brightness is pH independent over the biological range, 6–10.



BD Horizon V450 vs Pacific Blue™ Conjugates of CD3 (SK7) vs CD4 (SK3)

Brightness is clone specific.



BD Horizon V450: Photostability Data

V450 is as photostable as Pacific Blue™ and much better than other commonly used fluorochromes.

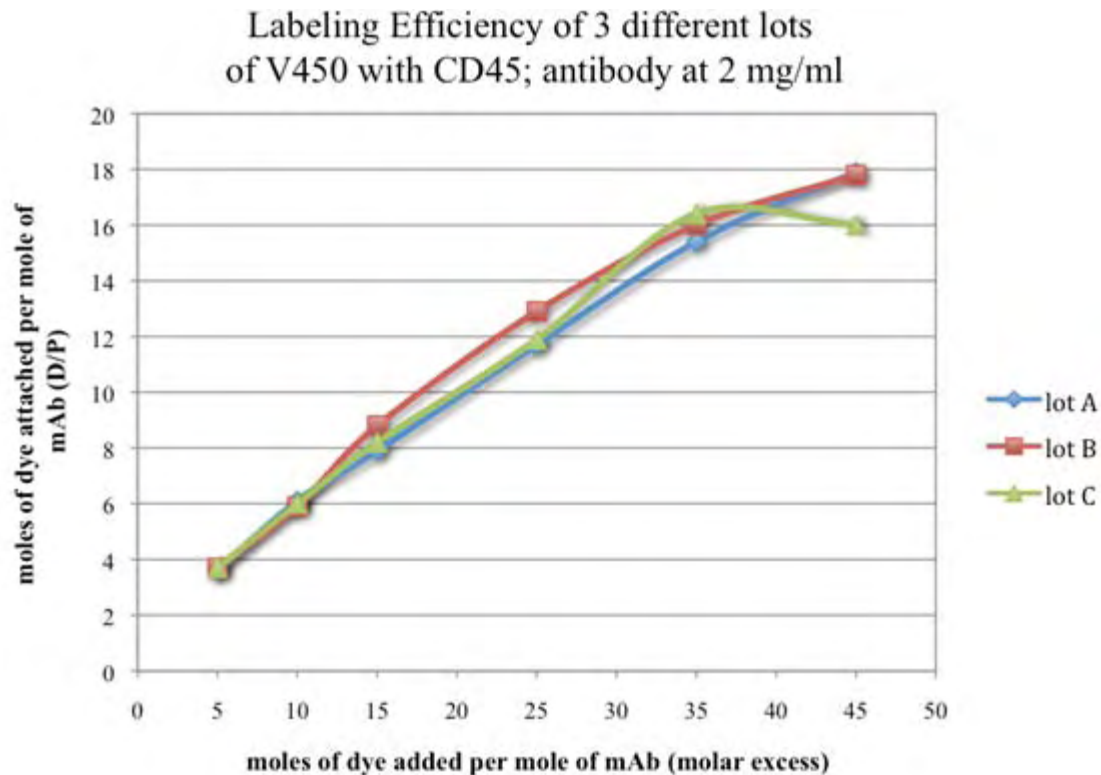
Concentration	Pacific Blue	V450
9.4 μM	96.3 ± 1.02	98.4 ± 0.35
4.7 μM	97.6 ± 0.32	98.0 ± 0.36

Comparative evaluation of photostability for BD Horizon V450 vs Pacific Blue™ after 12 hours of illumination (ambient light) in PBS, pH 7.4 (average of 3 percentage values ± SD)

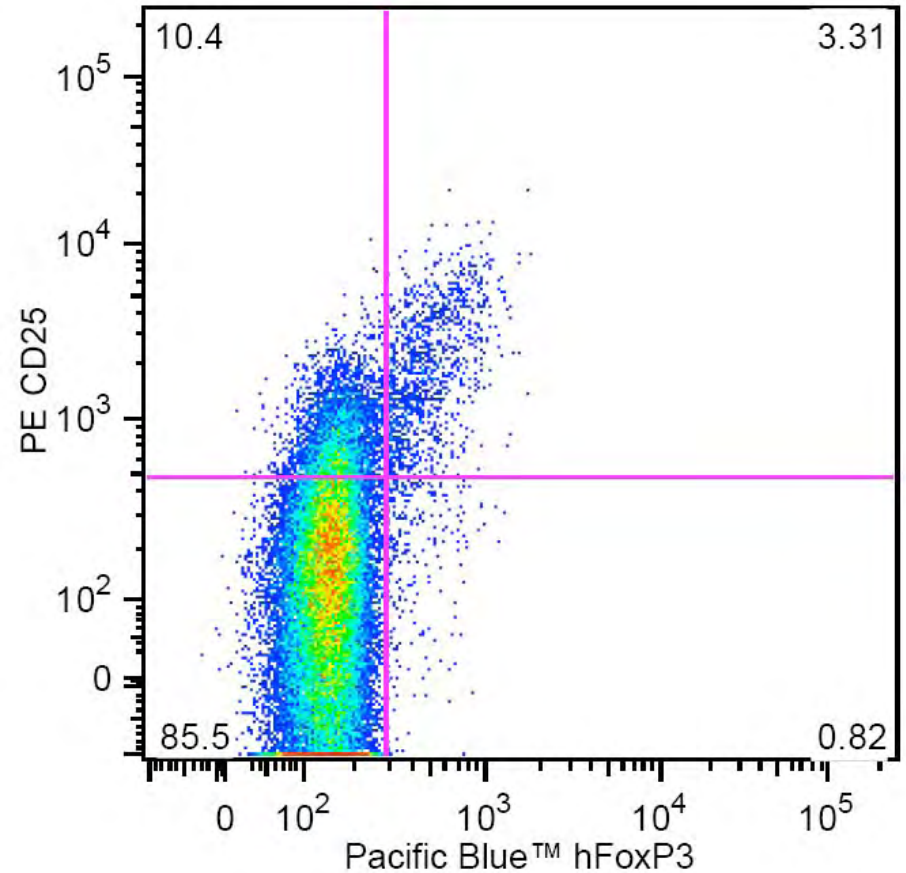
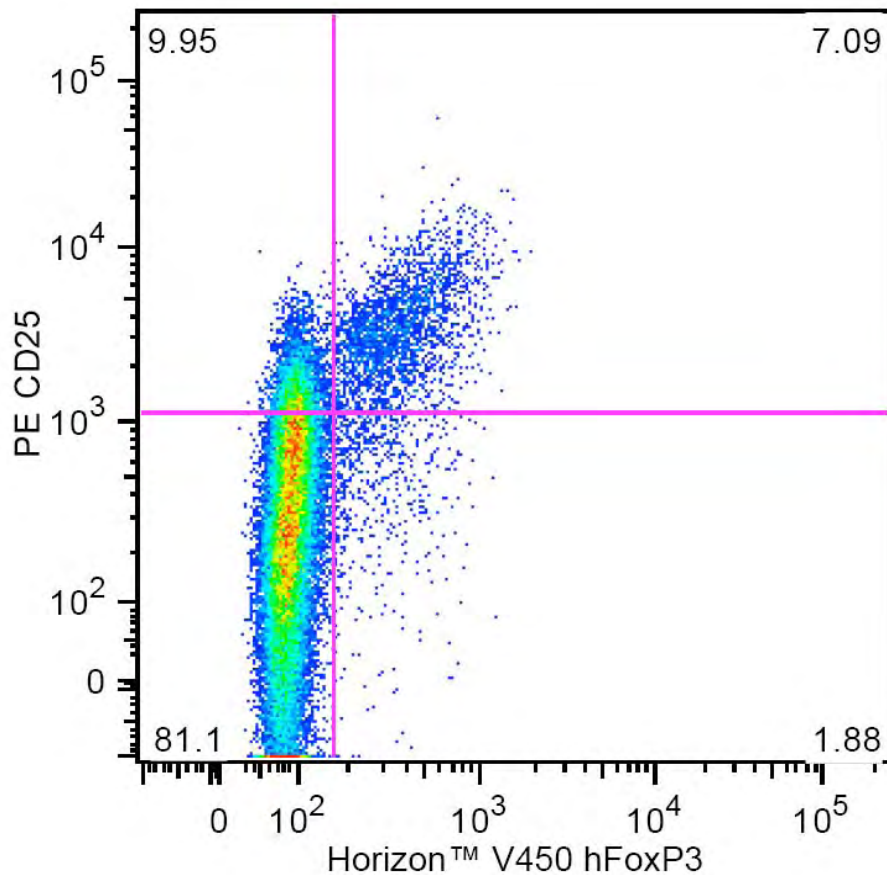


Minimal Lot-to-Lot Variation

Results suggest that there will be minimal lot-to-lot variation.



Sample Data: BD Horizon V450 hFoxP3 (259D) vs Competitor's hFoxP3 (259D) Pacific Blue™ Both Gated on CD4⁺



If you have further questions:

**Contact your US Reagent Sales Rep
or e-mail: ResearchApplications@bd.com**

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Class I (1) laser product.

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