

Polychromatic Flow Cytometry

Analog to Digital

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Polychromatic Flow: Why Now?

- **Faster More Powerful Computers**
- **New Excitation Sources**
 - YAG Laser
 - Violet Diode
 - Sapphire Diode
- **New Methods**
 - Intracellular Flow Cytometry (neutral pH/reversible detergent)
 - Bead Based Assays (CBA)
- **New Reagents**
 - Tetramers/Dimers
 - Phosphospecific Antibodies
 - Alexa Fluors, PE-Cy7, APC-Cy7, etc.....
 - Destabilized Signaling Vectors
 - Fluorescent Proteins (eFP, RCFP)
 - Quantum Dots
- **New Digital Hardware & Software**
 - FACSDiVa
 - FACSaria
 - LSR II
 - FACSCanto
 - FACSArray
- **Filter Plate Based Sample Prep/Loaders**



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6 to 32 Fluorescence Parameters available across various platforms

Blue

1. FITC/Alexa 488/eGFP/ZsYellow
2. PE/eYFP/DsRed
3. Alexa 610-PE/PE-Texas Red
4. 7-AAD
5. PerCP/PE-Cy5
6. PerCP-Cy5.5/PE-Cy5.5
7. PE-Cy7
8. open

Red

10. APC/Alexa 647/HcRed
11. Alexa 700
12. APC-Cy7

Violet

13. Pacific Blue
14. AmCyan/eCFP
15. Alexa 430

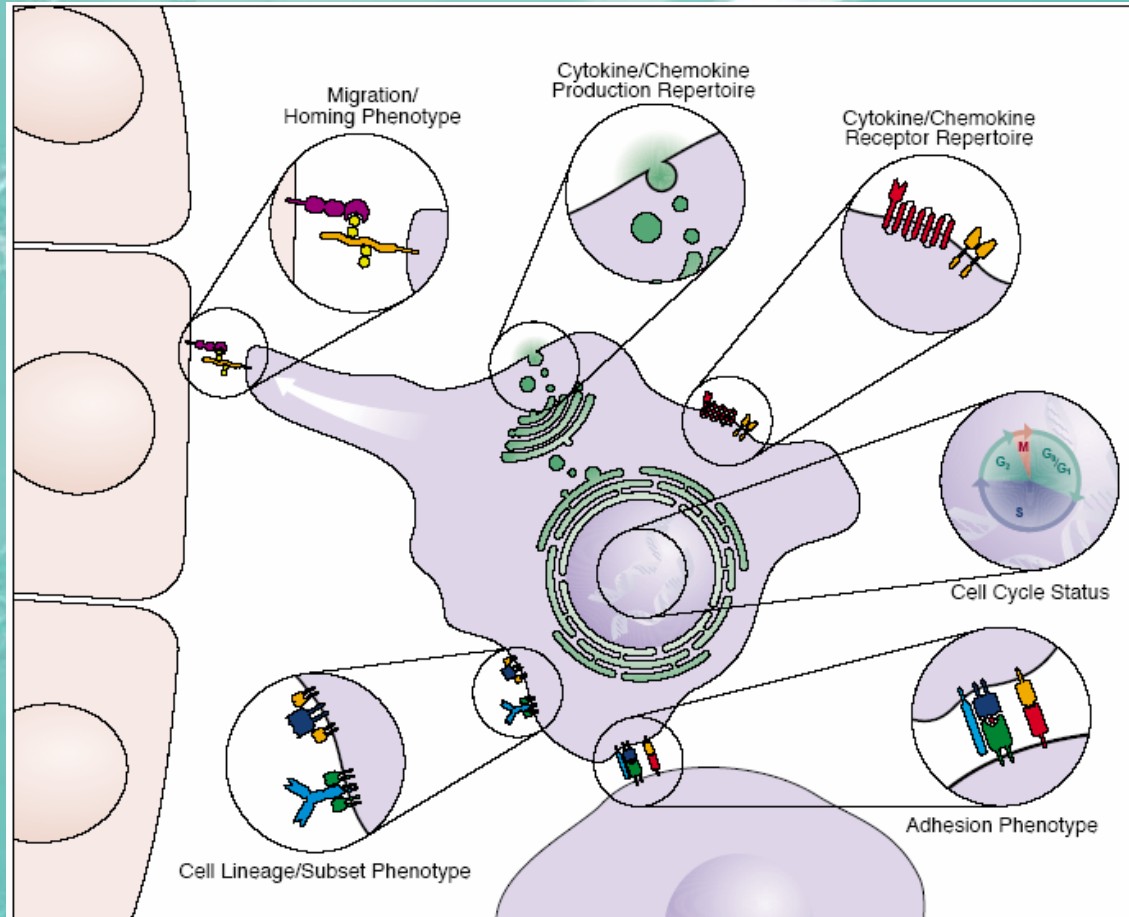
UV

16. DAPI
17. Hoechst
18. Alexa 305



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Why Polychromatic Flow Cytometry?

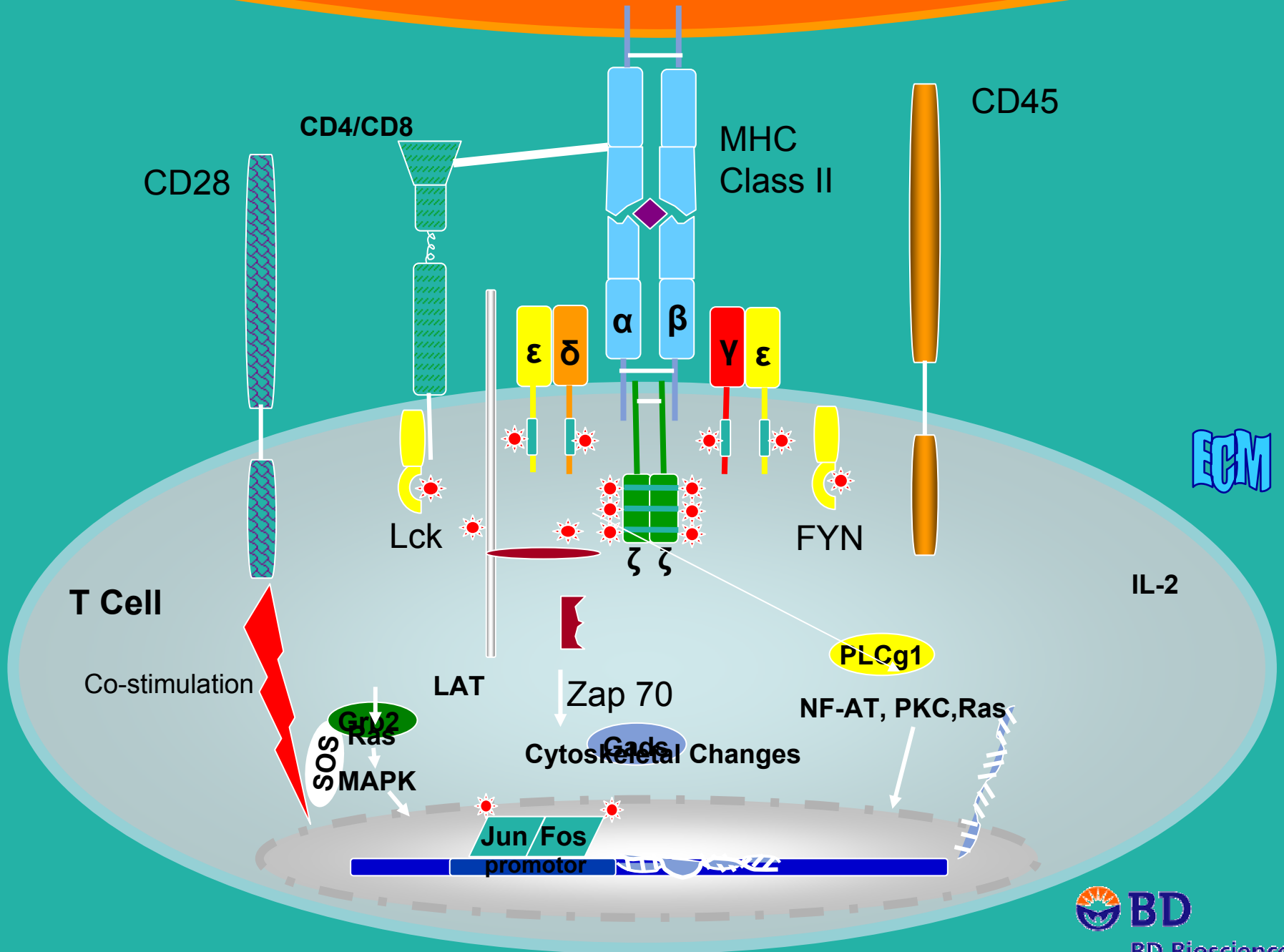


- Cell Lineage
- Subset identity
- Activation status
- Cell Cycle
- Chemokine Production
- Chemokine Receptor Repertoire
- Migration/Homing Phenotype



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APC



Polychromatic Flow Cytometry

- What Do We Need For PFC?
 - Chemistry – the fluorescent dyes
 - Must be bright (S/N)
 - Minimal spectral overlap
 - Straightforward conjugation to antibodies
 - Instrumentation
 - **More Light Sources:** Multi-laser (2- 4 or more)
 - **More Detectors:** 6 to 16 or more parameters
 - More Efficient Optical Pathway: **Higher sensitivity**
 - **Higher resolution & fast complex data handling :Digital Electronics & New Graphical User Interface**



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Polychromatic Flow Cytometry

Practical Considerations: Improving Resolution

- **Experimental design:**
 - **Make “Good” Fluorochrome/Antigen Density Choices/Matches**
 - Implementing multicolor panels is principally empirical and requires many iterations (time)
 - **Optimize Instrument Setup**
 - Photomultiplier Voltages
 - Compensation/Spillover
 - **Add necessary controls**
 - Fluorescence Minus One (FMO)
 - Autofluorescence Controls
 - Optimized Isotypic Controls
- **Hardware**
 - Digital Electronics: Eliminate Analog Artifacts
- **Software:**
 - Automate compensation
 - Enhance graphical user interface



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Building An PCF Assay: Fluorochrome Choices

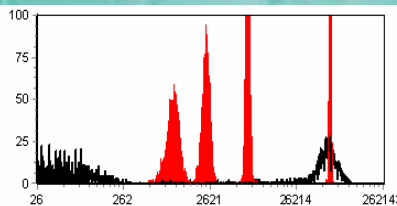
Order Available Fluorochromes By Relative Brightness

FACSArray

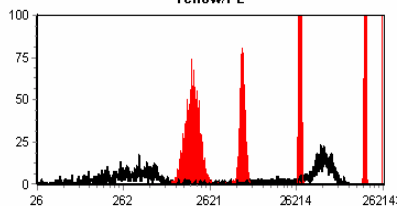
LSR II

PE > APC > PE-Cy7 > PerCP-Cy5.5 >
Alexa 700 > PE-Texas > PerCP > FITC >
Pacific Blue > AmCyan > APC-Cy7

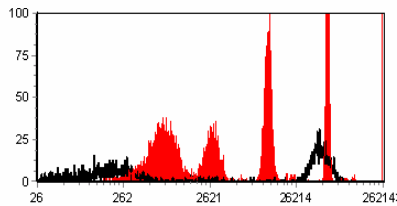
- This Is Instrument Dependent
- Varies with:
 - Laser Wavelength
 - Laser Power
 - Filter/Mirror Sets
 - Optical Alignment/Pathway
 - PMT/APD Sensitivity



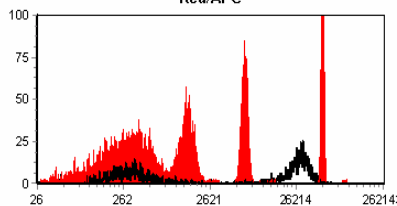
Yellow/PE



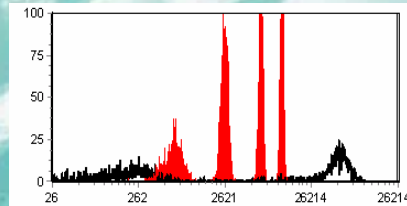
FarRed/PE-Cy7



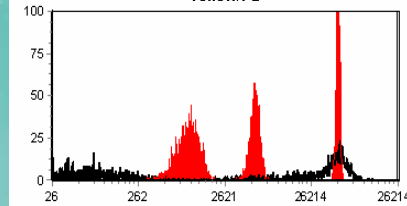
Red/APC



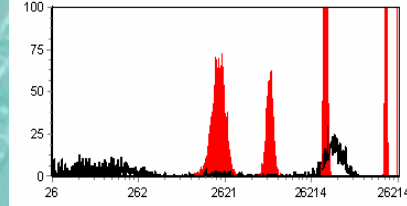
NIR/APC-Cy7



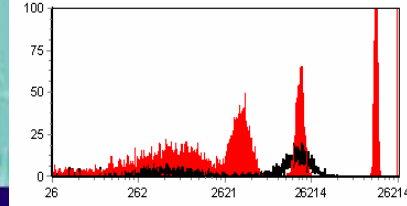
Yellow/PE



FarRed/PE-Cy7



Red/APC

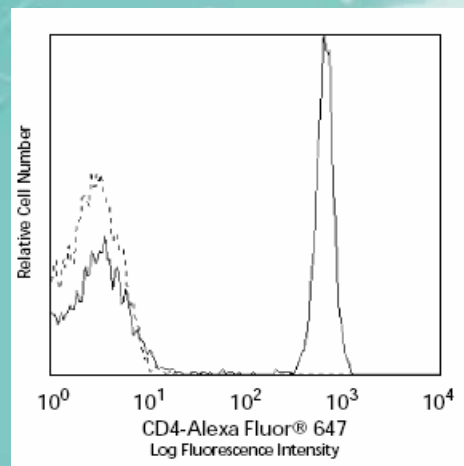
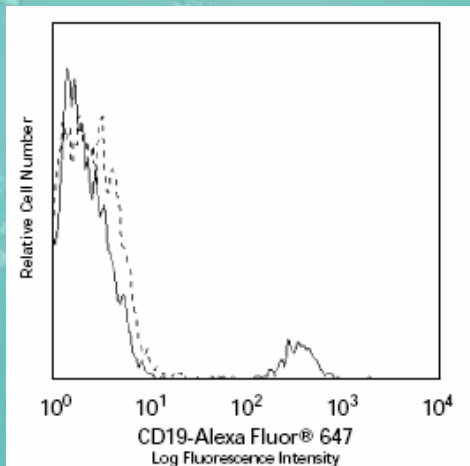
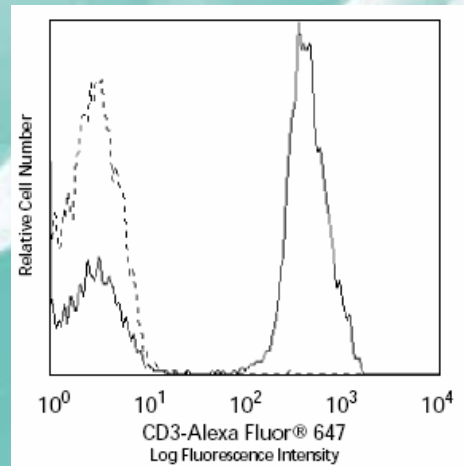
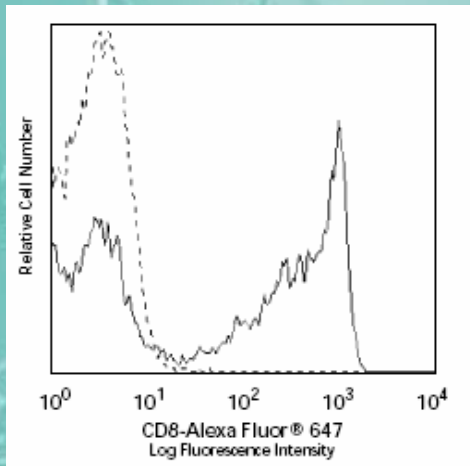


NIR/APC-Cy7



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Building An PCF Assay: Relative Antigen Densities



- **Approximate Relative Antigen Densities from Technical Data Sheets**
- **Match The Lowest Density Antigen To The Brightest Fluorochrome, etc.....**
 - Limited by conjugate availability
 - New cheaper custom conjugates available
 - Consider potential spectral overlap



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Building An PCF Assay: Laser Choices

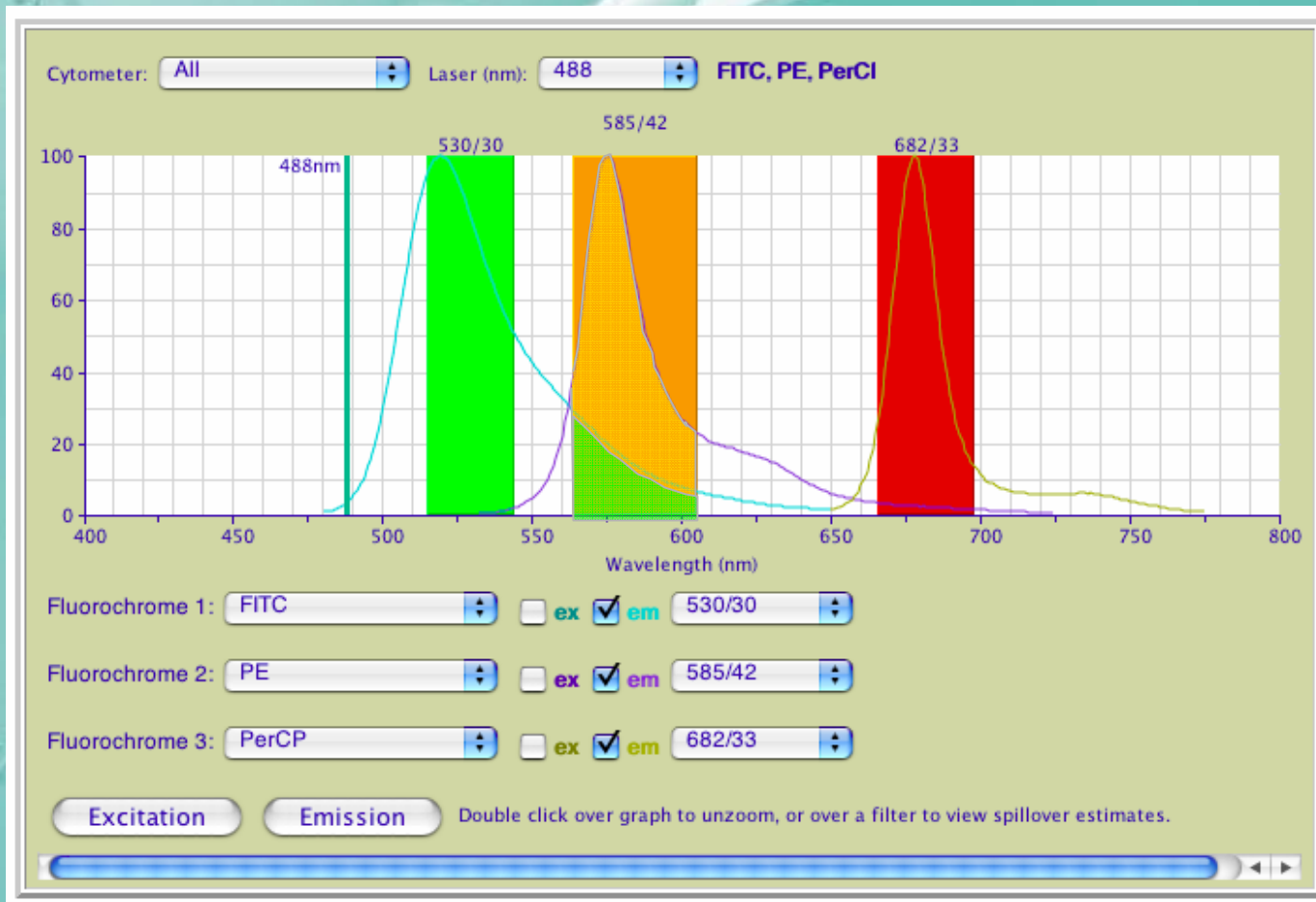
Optimize Your PCF Assay By:

- Using Multiple Laser Lines
- Don't "Pack" A Laser Line
- Choose "Optimal" Laser/Fluorochrome Combinations:
 - To Minimize Spillover Background
 - To Optimize Signal :Noise
- Optimize Filter Choices to Minimize Spillover
 - Use JAVA Applet on BD Website



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Building An PCF Assay: Spillover increases background



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Building An PCF Assay: Spillover increases background

- Fluorescence Compensation
 - We correct for dye spillover to align stained populations in dye space without bias from spectral overlap.
 - Analog system essentially subtracts pulses
 - Digital systems correct using a compensation matrix (inverted spillover matrix) using matrix algebra.
 - Compensated parameters exhibit spread.
 - Nonlinear error from photon counting statistics¹
 - Worse in red and far red (fewer photons)
 - Analog systems dampen spread due to errors in compensation circuits and log amp nonlinearity

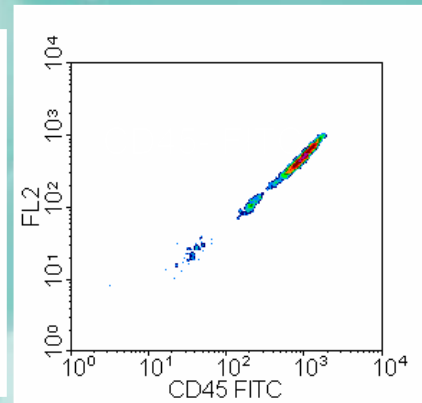
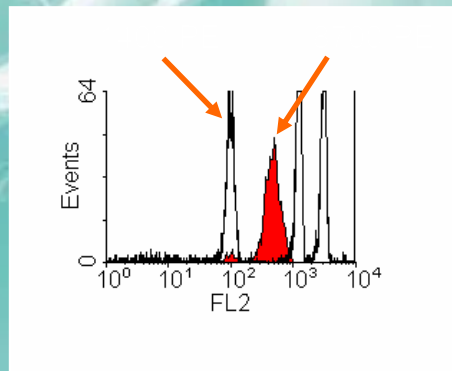
¹ Roederer M: Spectral Compensation for Flow Cytometry: Visualization Artifacts, Limitations, and Caveats. Cytometry 45:194–205 (2001).



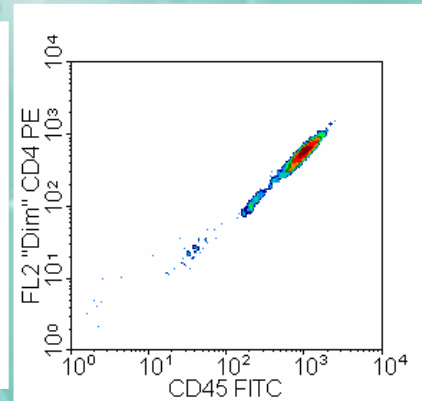
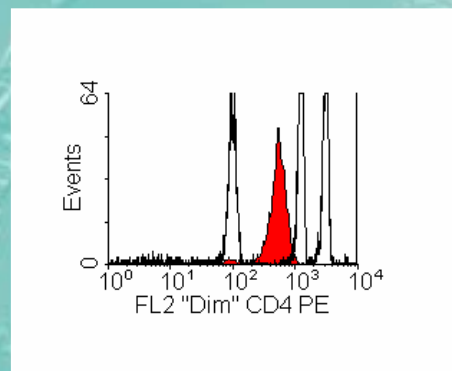
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Building An PCF Assay: PE Background Due to FITC Spillover

- Without compensation, the amount of PE MESF background contributed by bright CD45-FITC staining can be determined (8700 PE MESF).



**Analog Data:
CD45 FITC only**

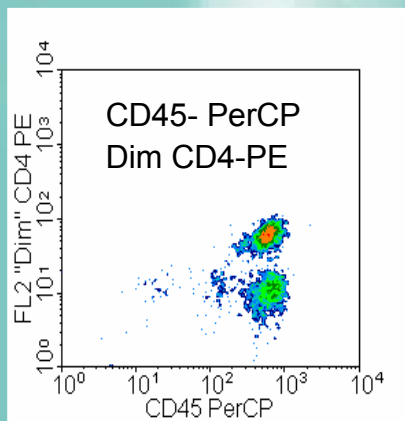
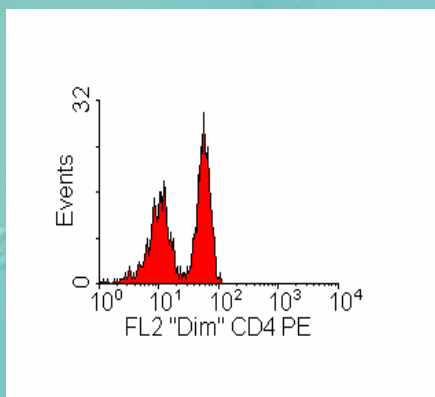
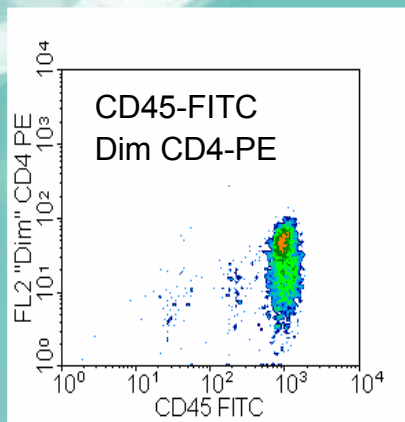
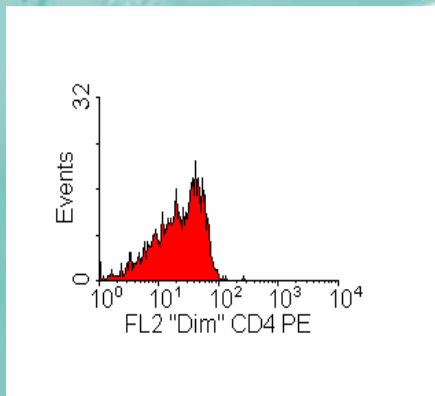


**Dim CD4 PE double
stained cells
not visible**



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Building An PCF Assay: Effect of Spillover on Double Stained Cells



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

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



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Building An PCF Assay: FMO (Fluorescence Minus One)

- Compensated data exhibits spread
- Bright single positives may change threshold levels between dim and background in other dimensions
- Use where autofluorescence and/or isotypic controls are “less than satisfying” when determining threshold over background
- The best control is one stained with all reagents *except* the one of interest

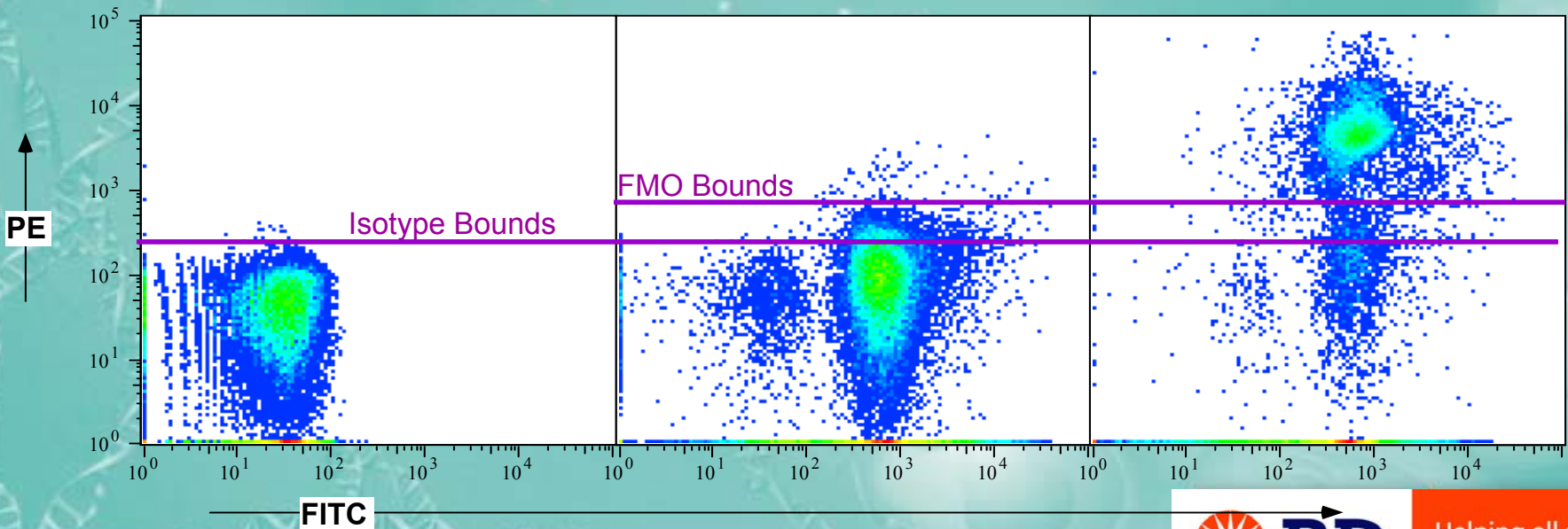


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Building An PCF Assay: FMO (Fluorescence Minus One)

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



Courtesy Mario Roederer



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Building An PCF Assay: FMO (Fluorescence Minus One)

- FMO controls are a much better way to identify positive vs. negative cells
 - No false negatives (better specificity)
 - May miss some positive (lower sensitivity)
- FMO controls can also 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 (“smears”)



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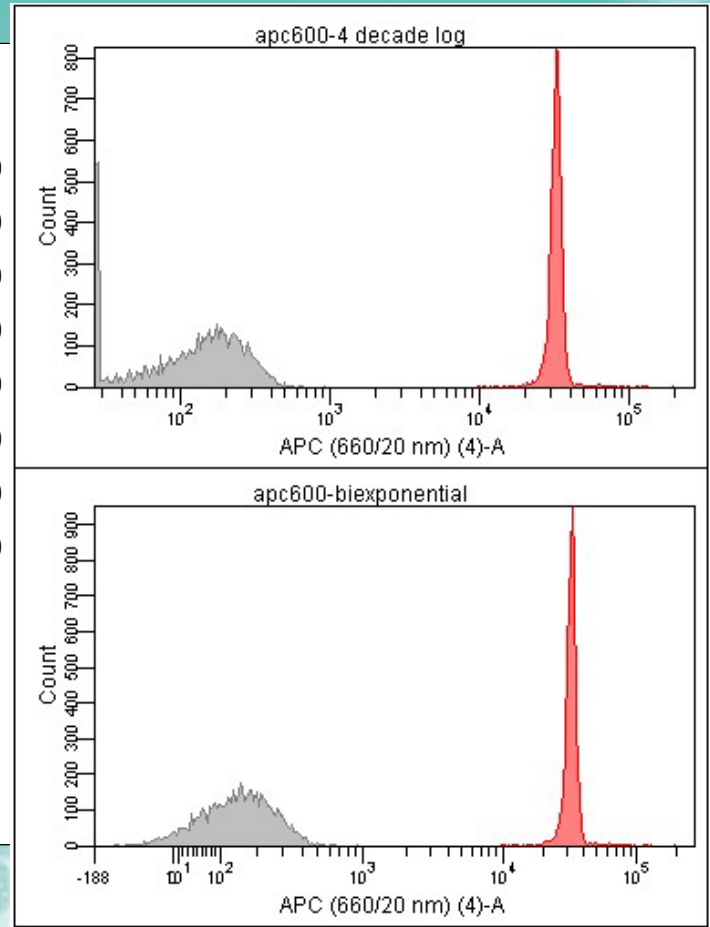
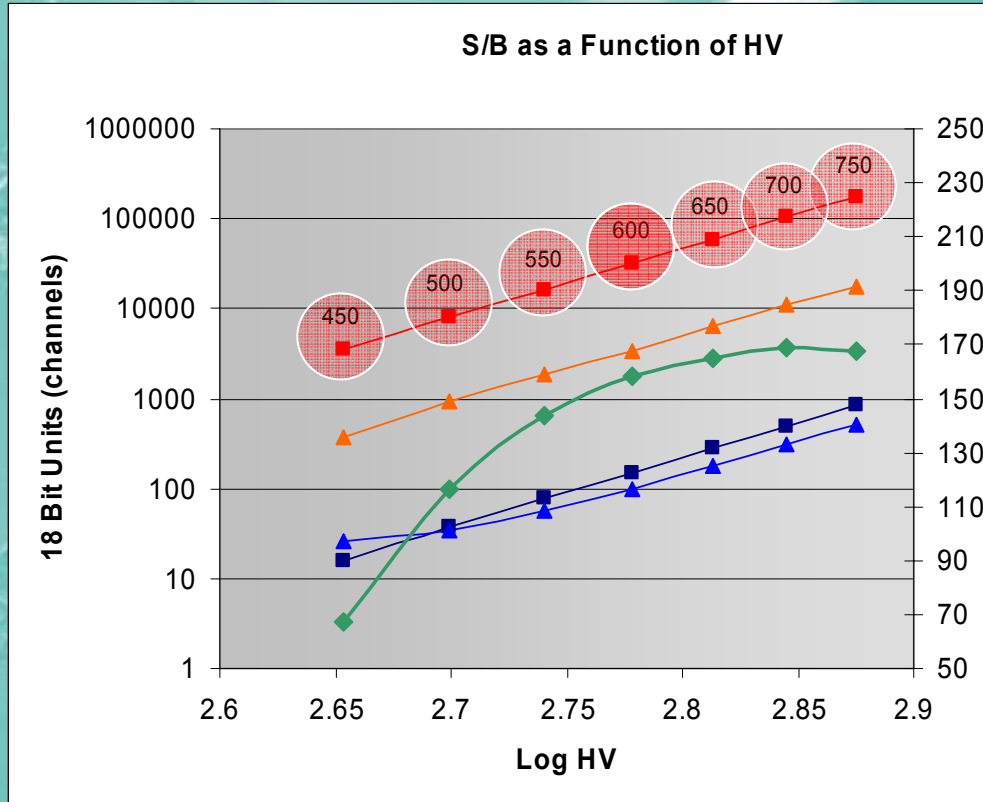
Building A PCF Assay

- Many iterations may be required
 - Start with major lineage markers
 - One per laser if possible
 - Add one reagent at a time
 - Look for spillover masking
 - Use FMO controls for “smears” and low density antigens
 - Try several combinations if possible
 - This should yield equivalent results



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Setting Up For A PCF Assay: High Voltage – Where should it be?



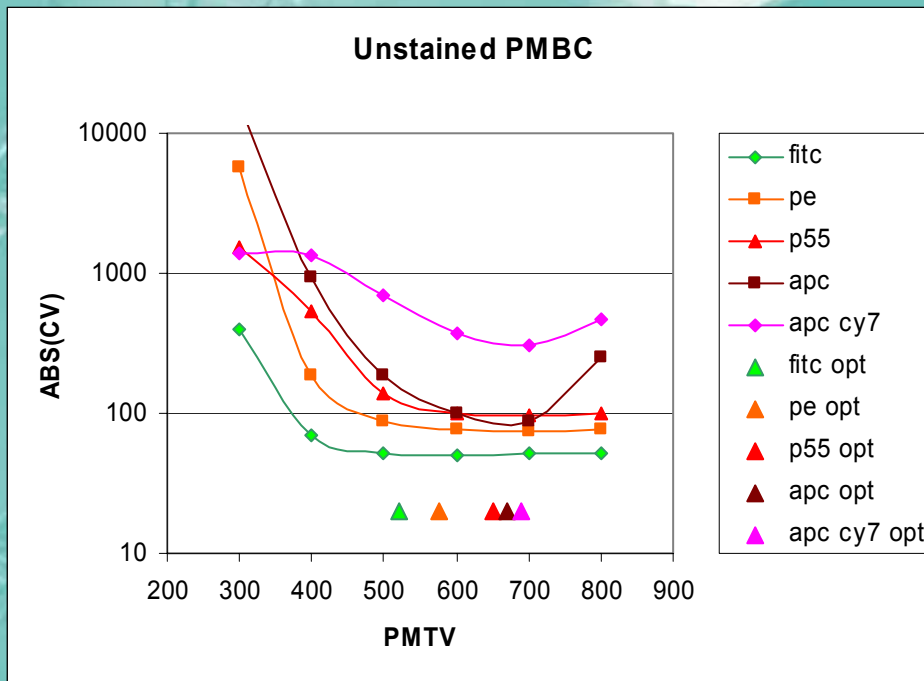
After ~ 600 Volts nothing very useful happens.
It is futile to attempt to put all bkg on-scale.



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Setting Up For A PCF Assay: Optimizing PMT Voltages

Using unstained particles/cells:



Graph CV versus PMT voltage

Specific to:

- Optical Alignment
- Laser Power
- Filter (All Optics)
- PMT

Acquire files for each 50 volt interval for all parameters



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Setting Up For A PCF Assay: Compensation: BD™ CompBeads

- Three Specificities
 - Anti-mouse Ig, kappa
 - Anti-rat Ig, kappa
 - Anti-rat/hamster Ig, kappa
- Negative Control Bead
- Supplied in sets: Positive & Negative Bead
- Stain with reagents used for PCF Assay
 - Optimal Spillover Control
 - 50% positive/50% negative Control



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Setting Up For A PCF Assay: Compensation: BD™ CompBeads

Method:

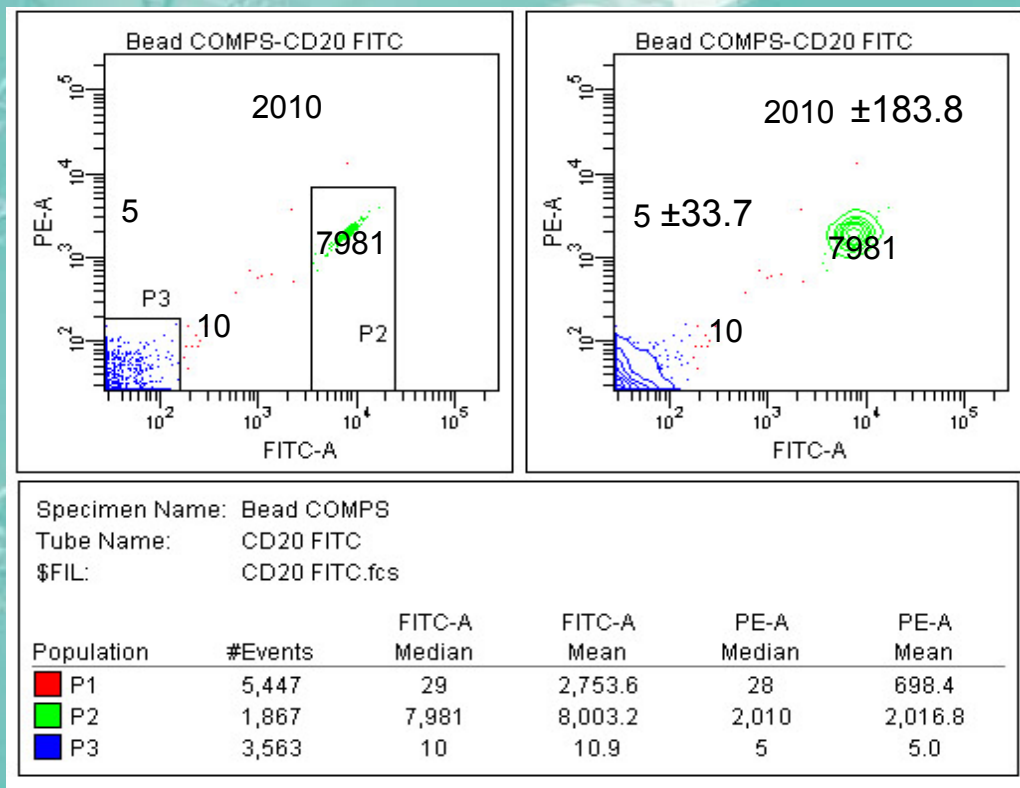
For Each Conjugate:

1. Add 1 drop (60 ul) of positive bead and 1 drop of negative bead to 100 ul of staining buffer in a tube or well
2. Add optimally titered antibody
3. Incubate 15-30 minutes RT
4. Wash with staining buffer
5. Resuspend pellet in staining buffer
6. Run according to instructions for automated spillover algorithm



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Setting Up For A PCF Assay: Compensation: BD™ CompBeads



FITC Spillover calculation
AutoCompensation method
Matrix algebra (PE = 0.83%)

Spillover coefficient = slope

$$1. \quad k_{12} = \frac{(2010 - 5)}{(7981 - 10)}$$

$$k_{12} = 0.2515$$

$$2. \quad k = \begin{bmatrix} 1 & k_{12} \\ k_{21} & 1 \end{bmatrix} = \begin{bmatrix} 1.0 & 0.2515 \\ 0.0083 & 1.0 \end{bmatrix}$$

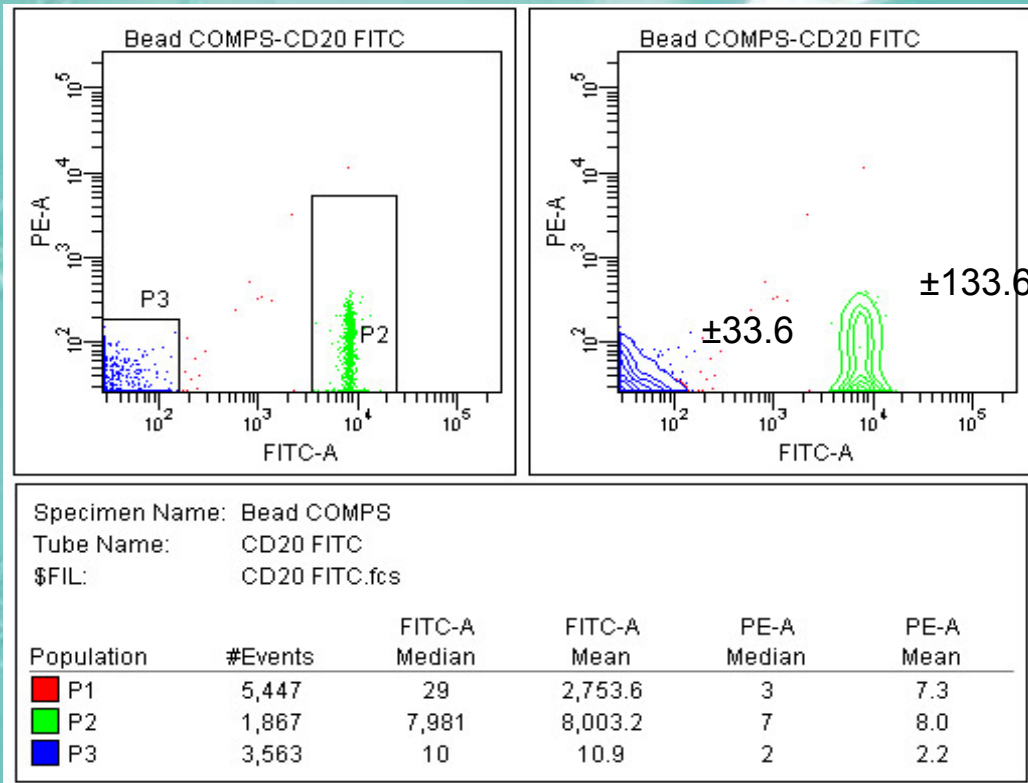
$$3. \quad k^{-1} = \begin{bmatrix} 1.00209 & -0.25203 \\ -0.00832 & 1.00209 \end{bmatrix}$$

$$4. \quad PE_{comp} = PE \times k^{-1}$$



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Setting Up For A PCF Assay: Compensation: CD20 BD™ CompBeads



Populations are aligned
In dye space

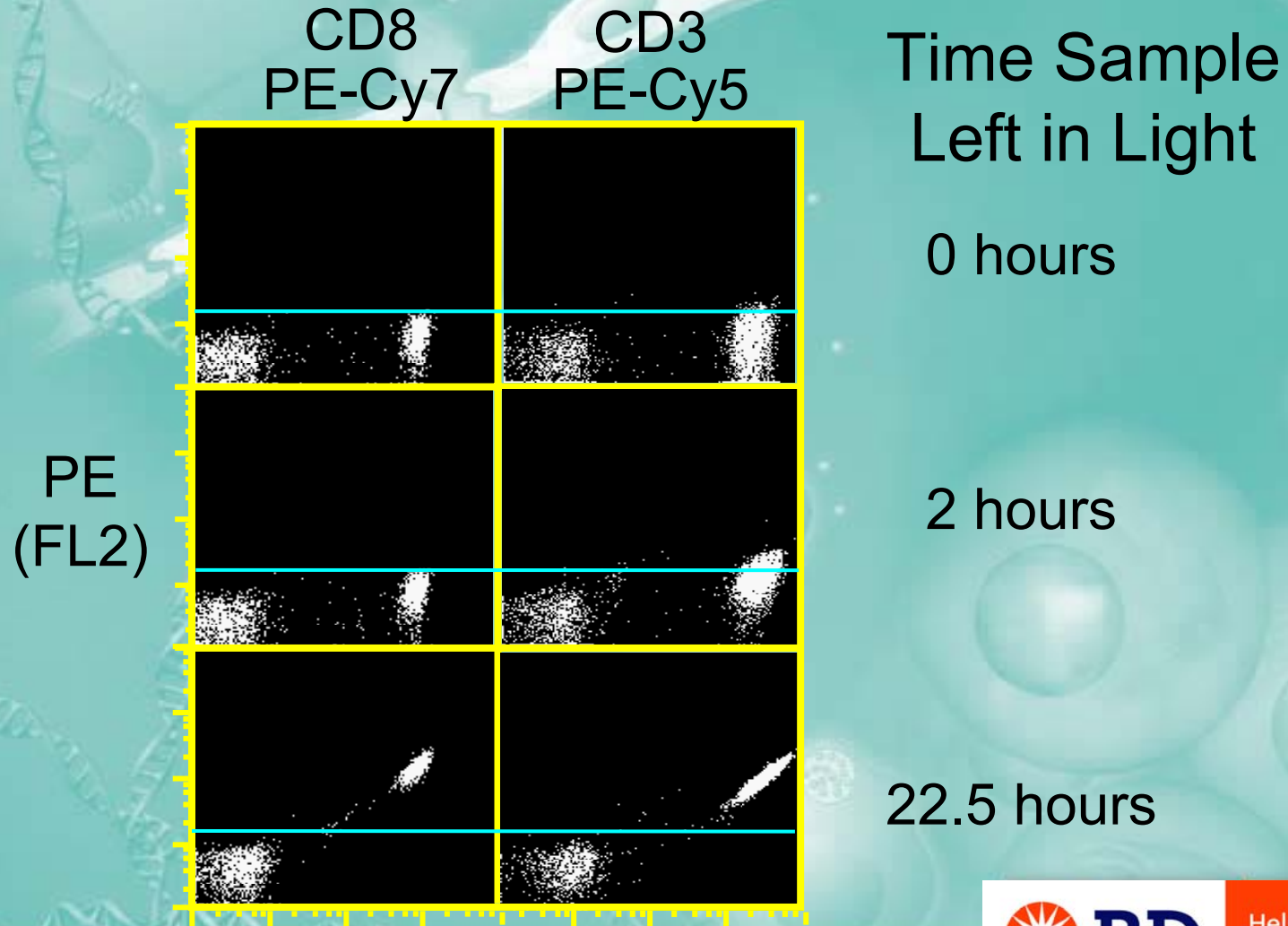
$$PE_C = PE \times 1.00209 + FITC \times -0.25203$$

Not a subtraction, rather a correction because we use matrix algebra and compensation coefficients.



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Setting Up For A PCF Assay: Compensation: Tandems



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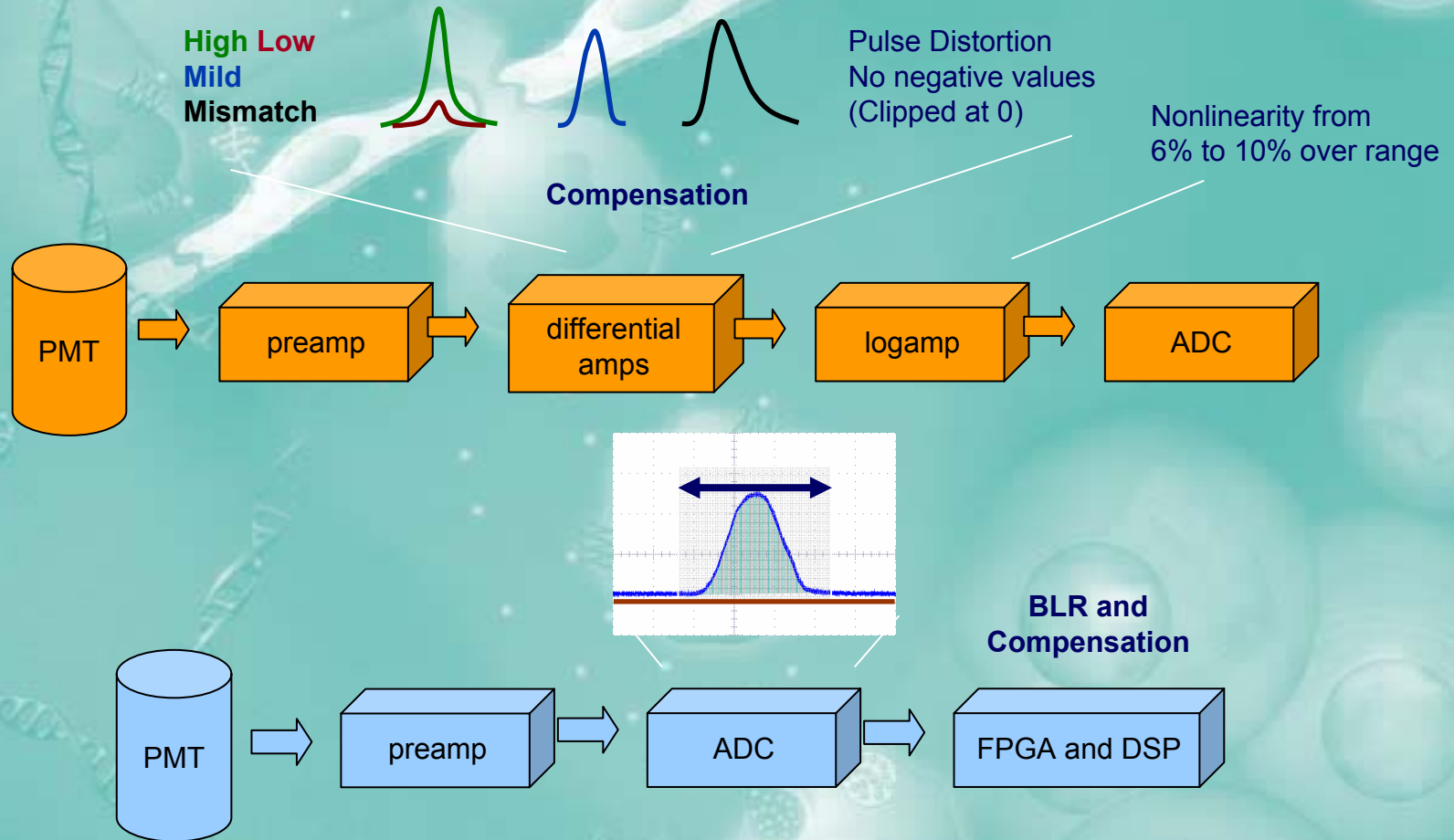
PCF: Current data display issues

- As pointed out by Mario Roederer, Dave Parks¹, Randy Hardy and others, what often looks like properly compensated analog data tends to be overcompensated, “leading to systematically biased dye level estimates”.
- Compensated digital data does not systematically bias dye level estimates.
- Logarithmic display of flow cytometry immunofluorescence data can be misleading and often difficult to interpret.
- Digital immunofluorescence data, with its virtual zero and floating point database, is more vulnerable to log distortion than analog, and many events cannot be visualized on a log scale even before compensation.

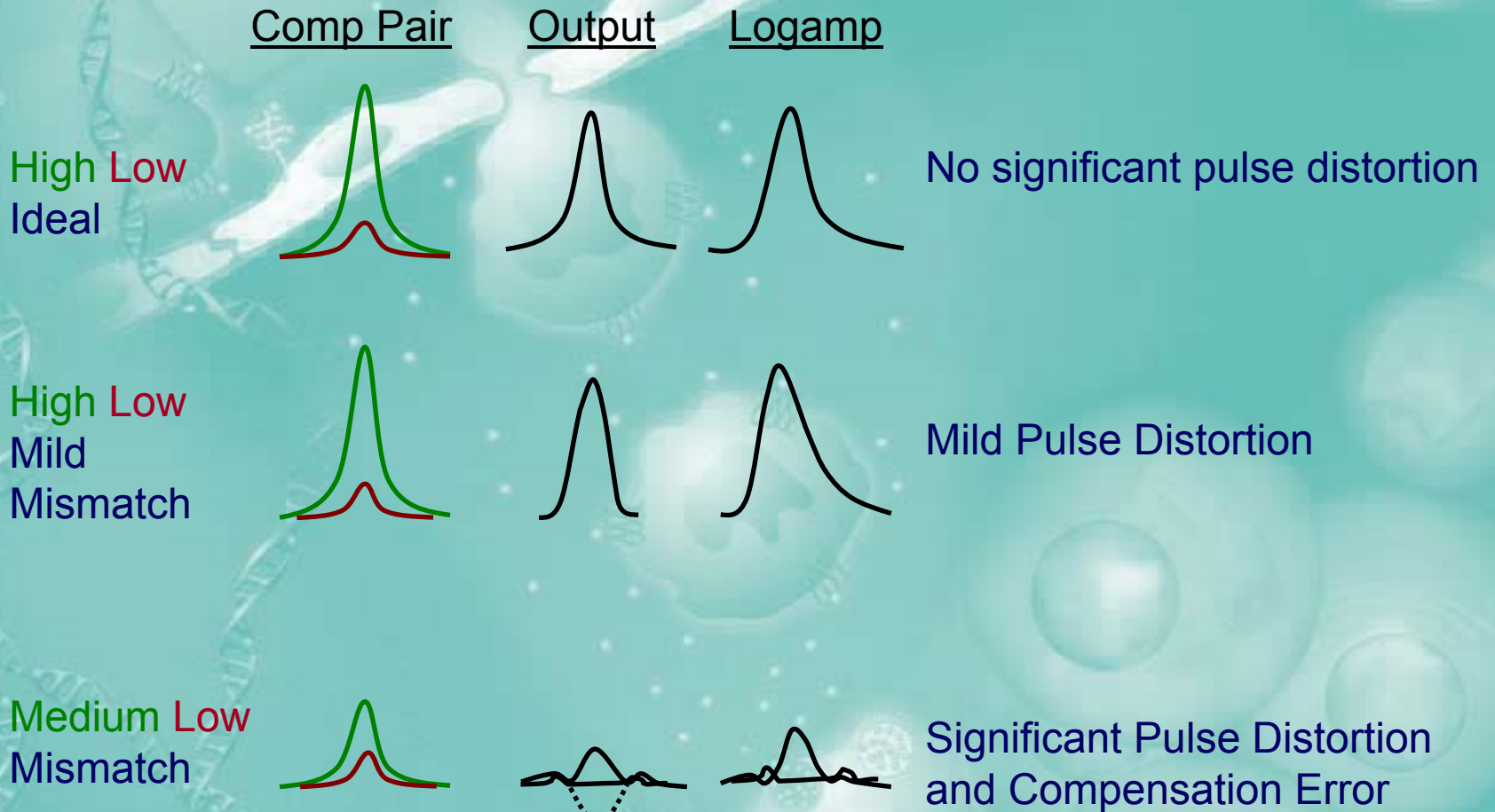


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PCF Requires Digital Electronics

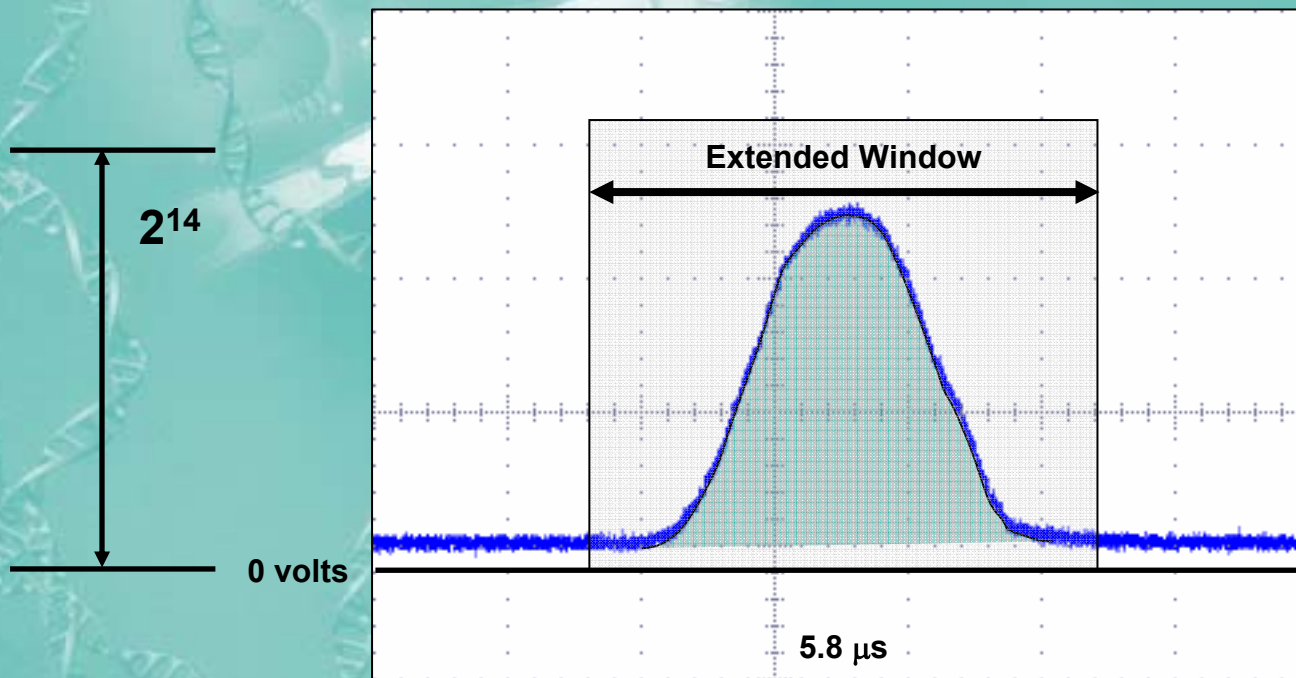


Analog Pulse Matching



A Continuously Digitizing Cytometer

FACS Aria 10 MHz ADC: 5.8 μ sec pulse has ~ 58 observations



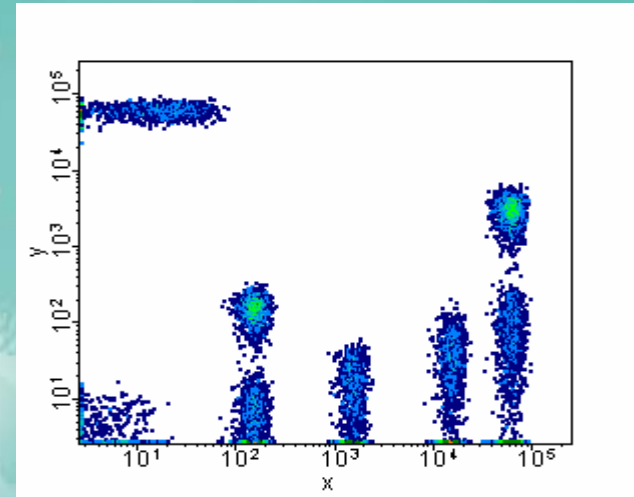
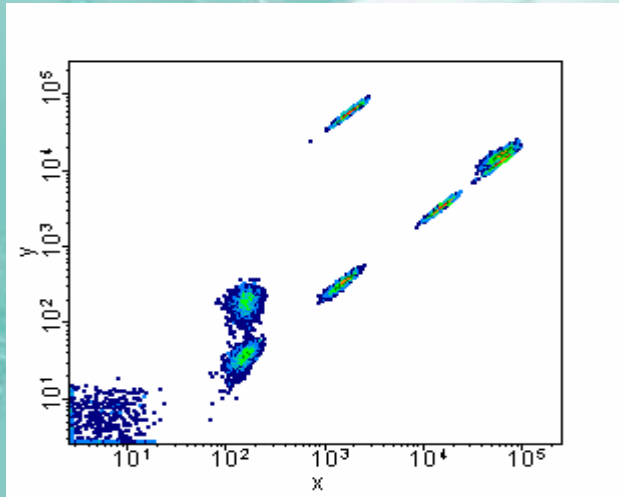
Sum 14 bit height measurements into area as IEEE 32 bit floating point
Pulse area is a measurement of *total* fluorescence (18 bit resolution)



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Synthetic data example – log scale

8 modeled populations – 2 of which are double positive



Difficult with low autofluorescence and compensation because of high spillover (22%) of X into Y, low spillover (3%) of Y into X causes “high background” of X into Y on single positive bright X population, which inflicts significant data spread after compensation.



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Stanford: Biexponential Transform¹

- Start with the sinh function:

$$\sinh(x) = (e^x - e^{-x})/2$$

- Stanford generalized this as a biexponential function:

$$f(x) = a e^{bx} - c e^{-dx}$$

- The first parameter b is the number of decades (onto the original scale) where the transition from the negative to the positive exponential occurs. By default the same value is used to set the range of negative values shown. The second parameter d is optional and if present, indicates the number of extra decades worth of space added to negative range

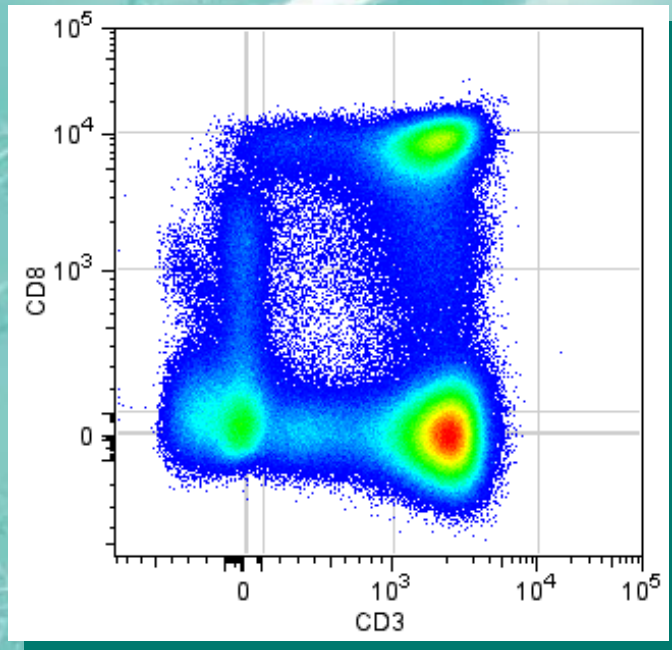


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¹ Logicle Display Transformation: Dave Parks & Wayne Moore, Stanford

Logicle: Compensated Biexponential Display

Log at the upper end, linear at the low, and symmetrical about zero. Biexponential transform where data zero is shown by the crosshairs in the plot



- This FlowJo example shows the value of a mostly logarithmic scale on the upper end, and a lower linear region occupies a reasonable plot area compared to that in the blended scale.
 - Compensated single pos are continuous
 - All populations are visible



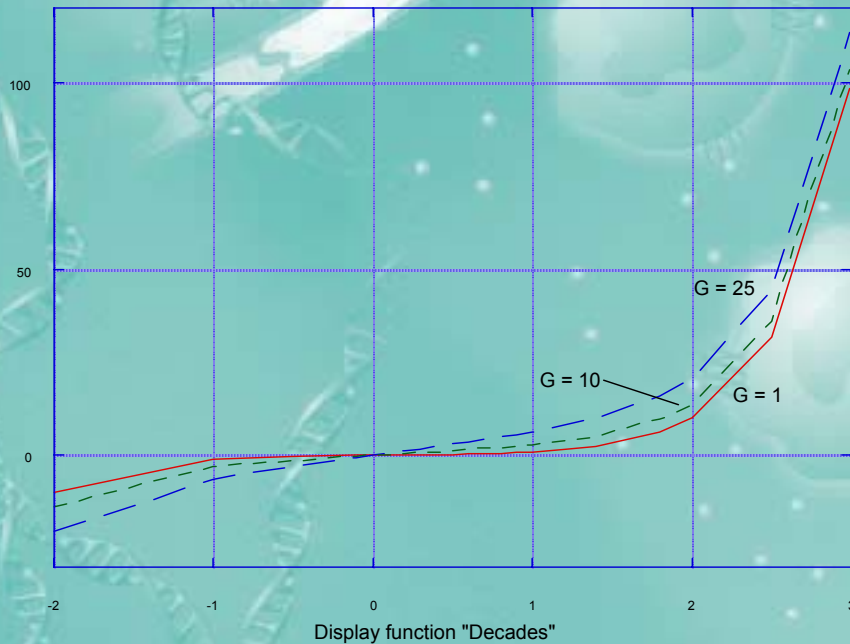
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Data dependant display capability

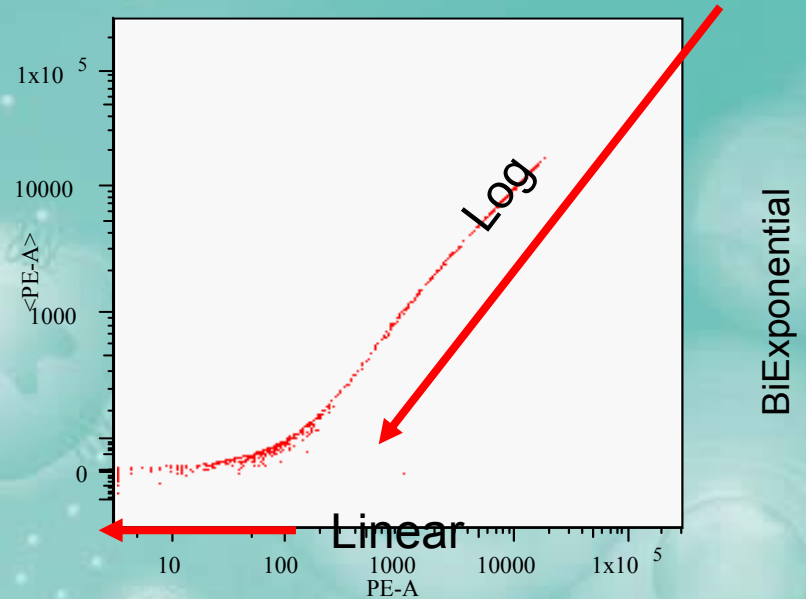
Input variables may be adjusted based on sampled population variance to optimize the display for any particular data set

Region -2 to 3 "Dec"

DRP/WAM Logicle Model 30Oct02



PE Capture Beads



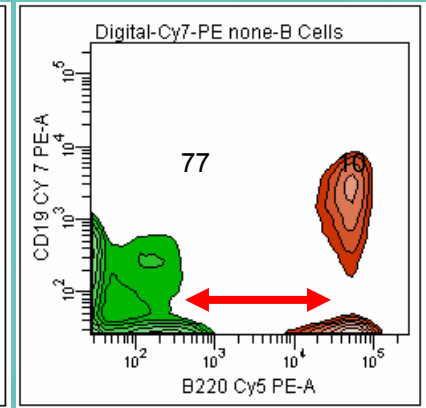
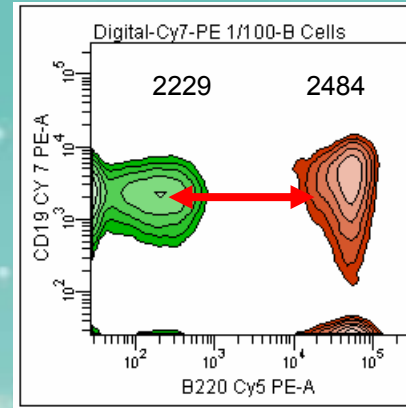
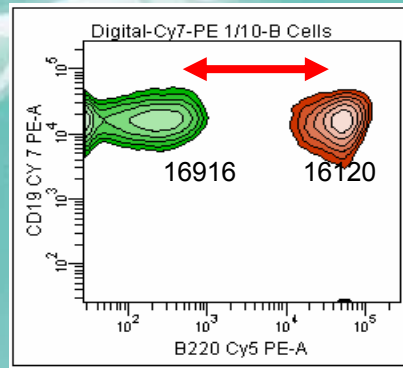
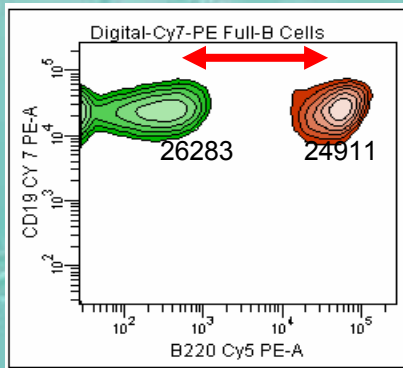
Log Plot



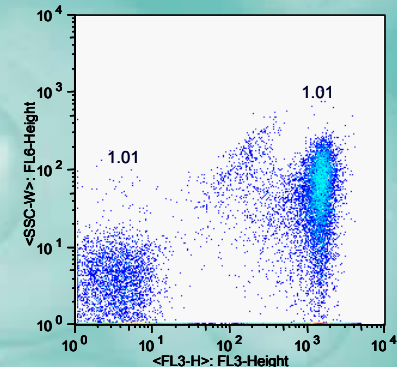
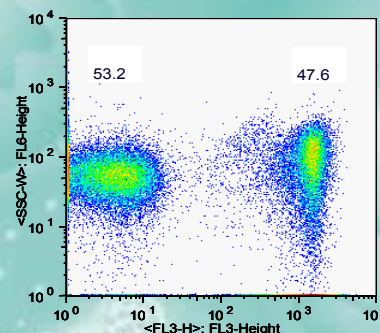
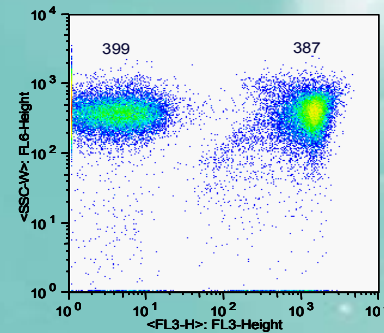
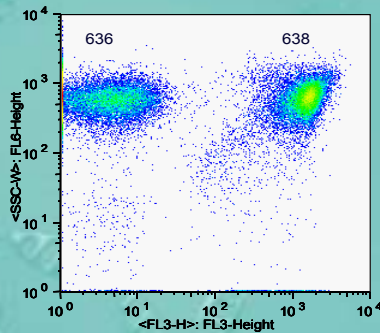
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Digital & Analog: Software Compensation

Digital



Analog



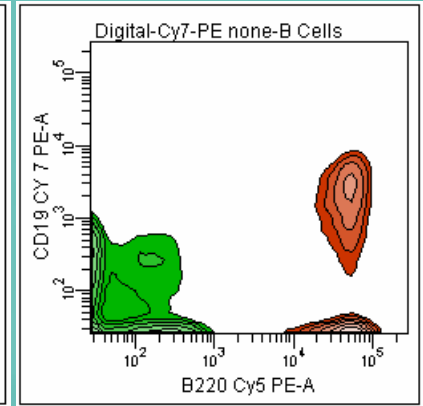
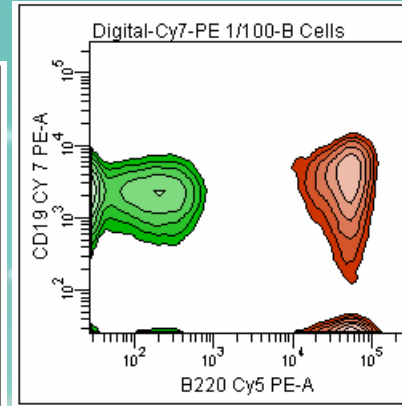
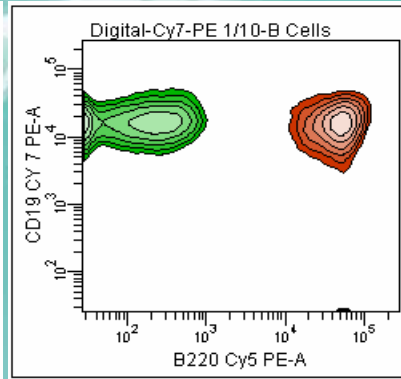
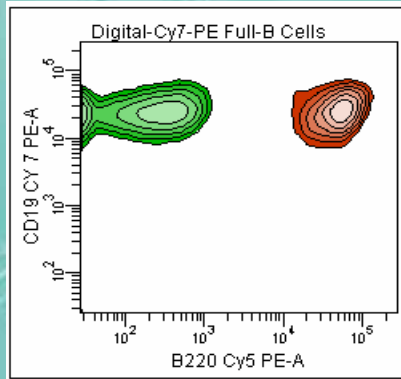
Digitally compensated analog data bears a striking resemblance to digital data



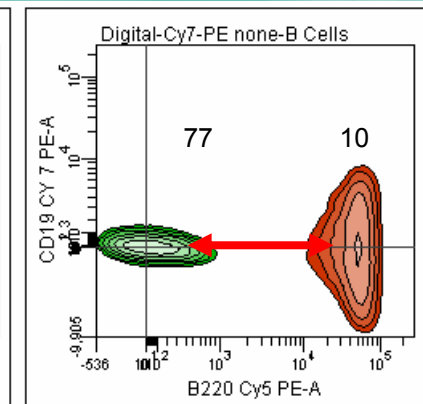
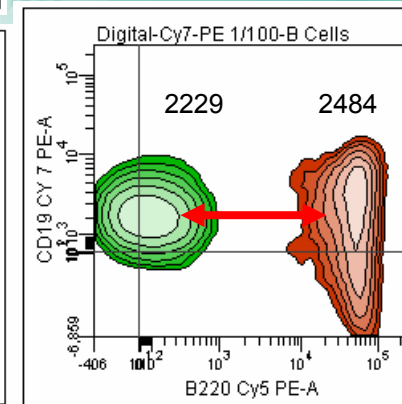
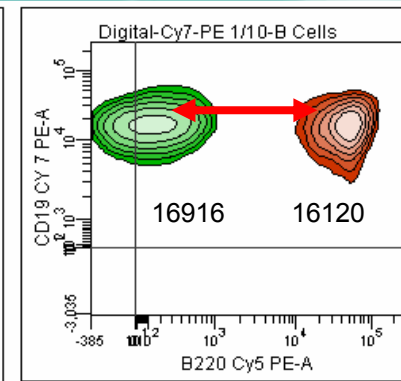
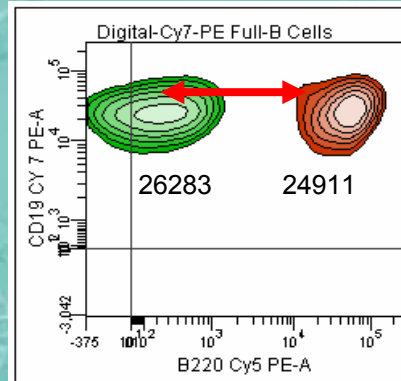
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Correct Population Alignment

Log₁₀



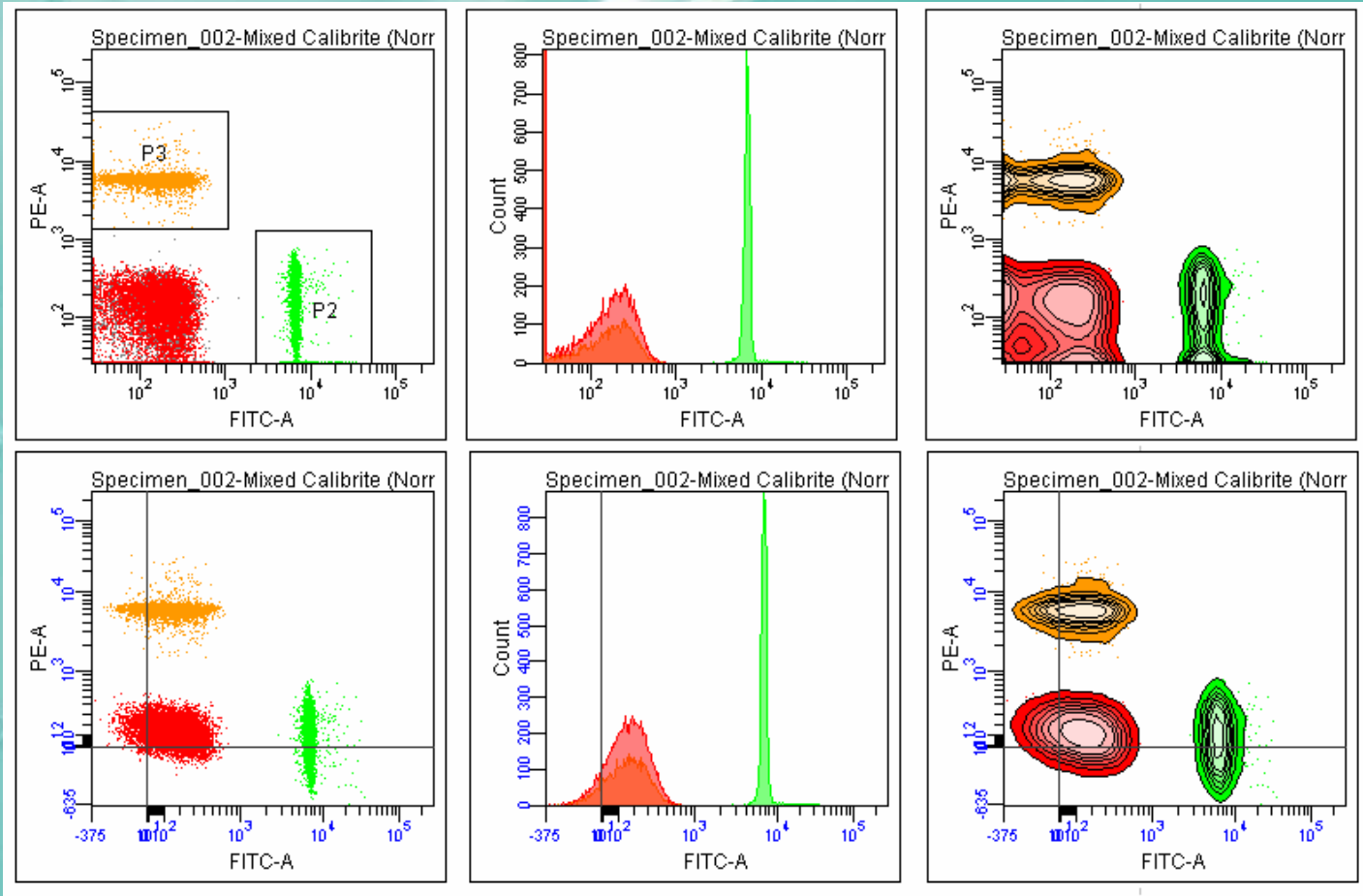
BiExp



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Optimized BiExponential

Algorithm is automatically scaled

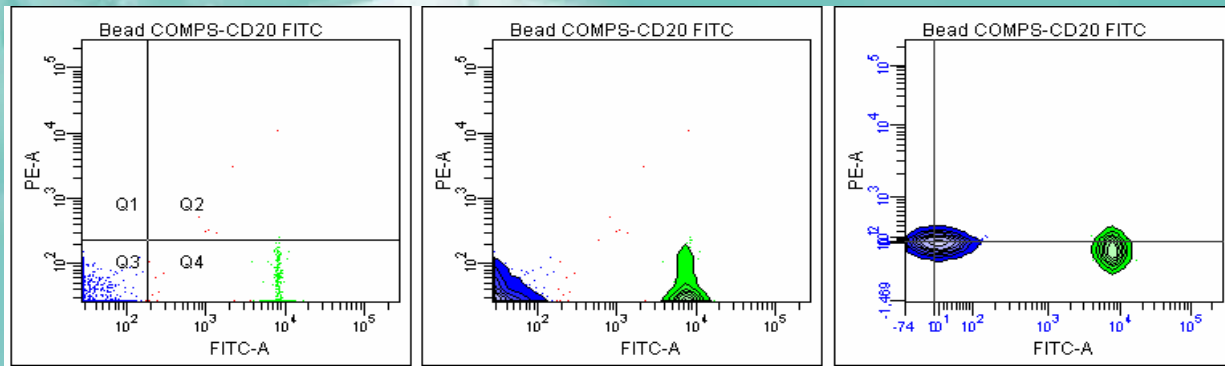


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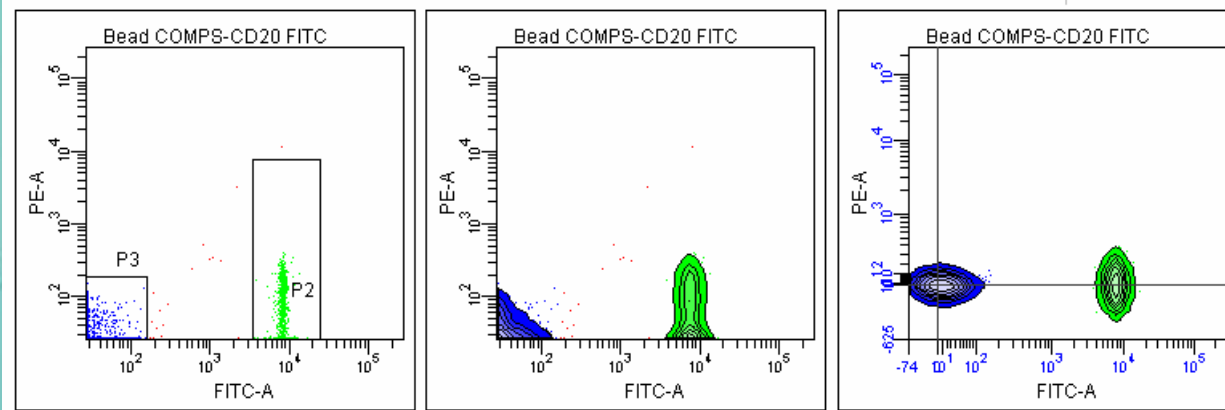
CD20 FITC Capture Beads Revisited

Biexponential display reveals proper compensation better than log

2% over



Correct



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Asilomar 2002 Cytometry Development Workshop¹

- Analog compensation error is responsible for the observed differences between analog- and digitally-compensated data
- Pure linear matrix compensation gives the best available estimates for the dye signals from each cell
- Statistical results should be computed on the matrix compensation output without further manipulations
- Logarithmic display of compensated data interferes with proper interpretation of samples with populations that include low and negative data values
- Data display transformations that are not simply linear or simply log would provide better and more interpretable visualizations

PCF: Questions of T Cell Differentiation

Questions of T Cell Differentiation that can be Addressed with Polychromatic Flow Cytometry

- What is the CD45RA/CD27/CD28 phenotype of antigen-specific CD4 and CD8 T cells?
 - In IFN γ + versus IL-2+ cells?
 - In CMV- versus HIV-specific cells?
 - In CMV-specific cells of HIV- versus HIV+ donors?



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8-color antigen-specific immunophenotyping

Ab Conjugate

Laser λ

CD28 PerCP-Cy5.5

488

CD45RA PE-Cy7

488

CD27 APC

633

CD8 APC-Cy7

633

CD3 Pacific Blue

405

CD4 AmCyan

405

Anti-IFN γ FITC

488

Anti-IL-2 PE

488

Surface
staining

Intracellular
staining

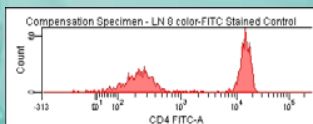


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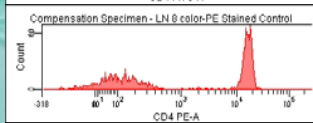
8 Color Compensation (LSR II)

Single-stained controls:

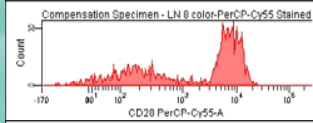
CD4 FITC



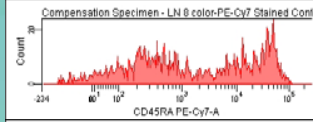
CD4 PE



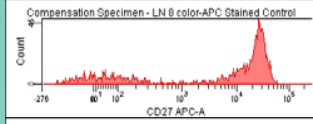
CD28 PerCP-Cy5.5



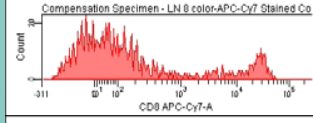
CD45RA PE-Cy7



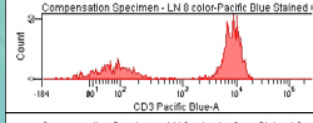
CD27 APC



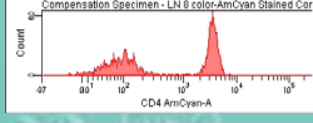
CD8 APC-Cy7



CD3 Pacific Blue



CD4 AmCyan



Auto-comp

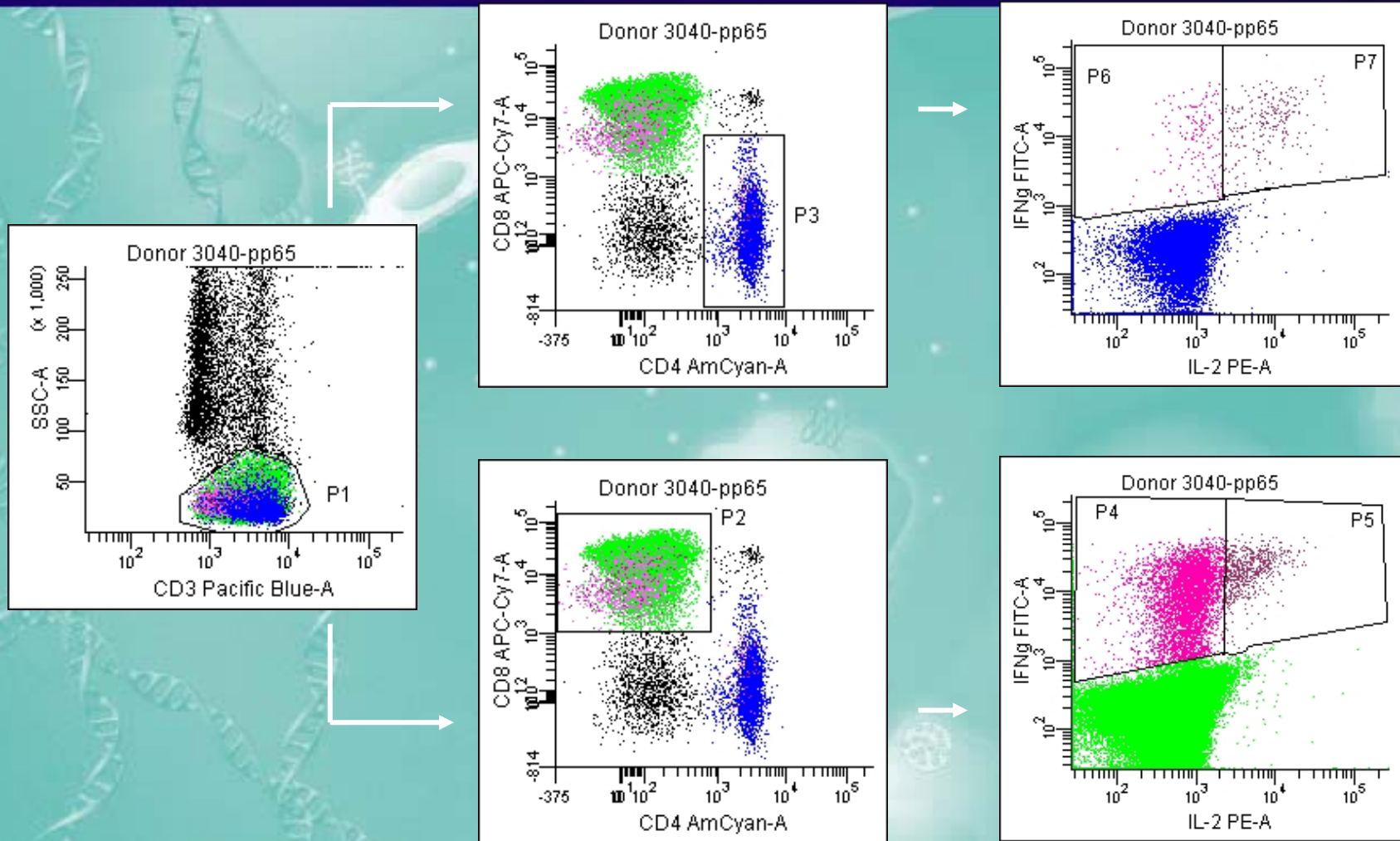
Spillover Matrix

	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7	Pacific Blue	Am Cyan
FITC	100.0	23.5	2.1	0.7	0.0	0.0	0.0	3.0
PE	1.6	100.0	12.3	2.7	0.0	0.0	0.0	0.0
PerCP-Cy5.5	0.2	0.1	100.0	43.0	2.5	5.6	0.0	0.0
PE-Cy7	0.0	0.6	0.1	100.0	0.0	3.6	0.0	0.0
APC	0.1	0.0	0.3	0.2	100.0	2.7	0.0	0.0
APC-Cy7	0.0	0.0	0.1	3.9	19.9	100.0	0.0	0.1
Pacific Blue	0.1	0.0	0.0	0.1	0.0	0.0	100.0	18.1
Am Cyan	38.1	7.0	1.1	0.6	1.5	0.0	17.1	100.0



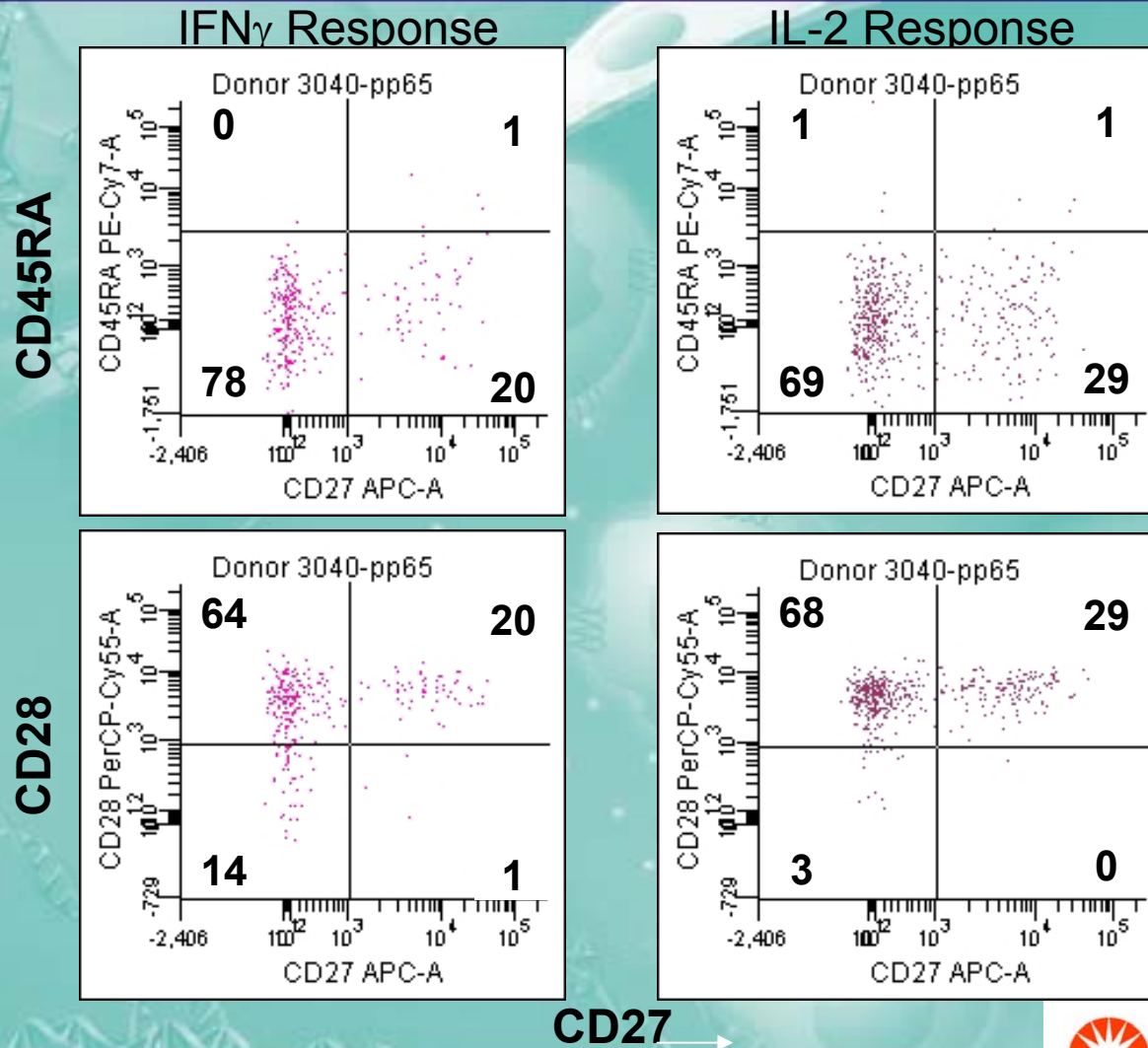
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Hierarchical Gating Strategy



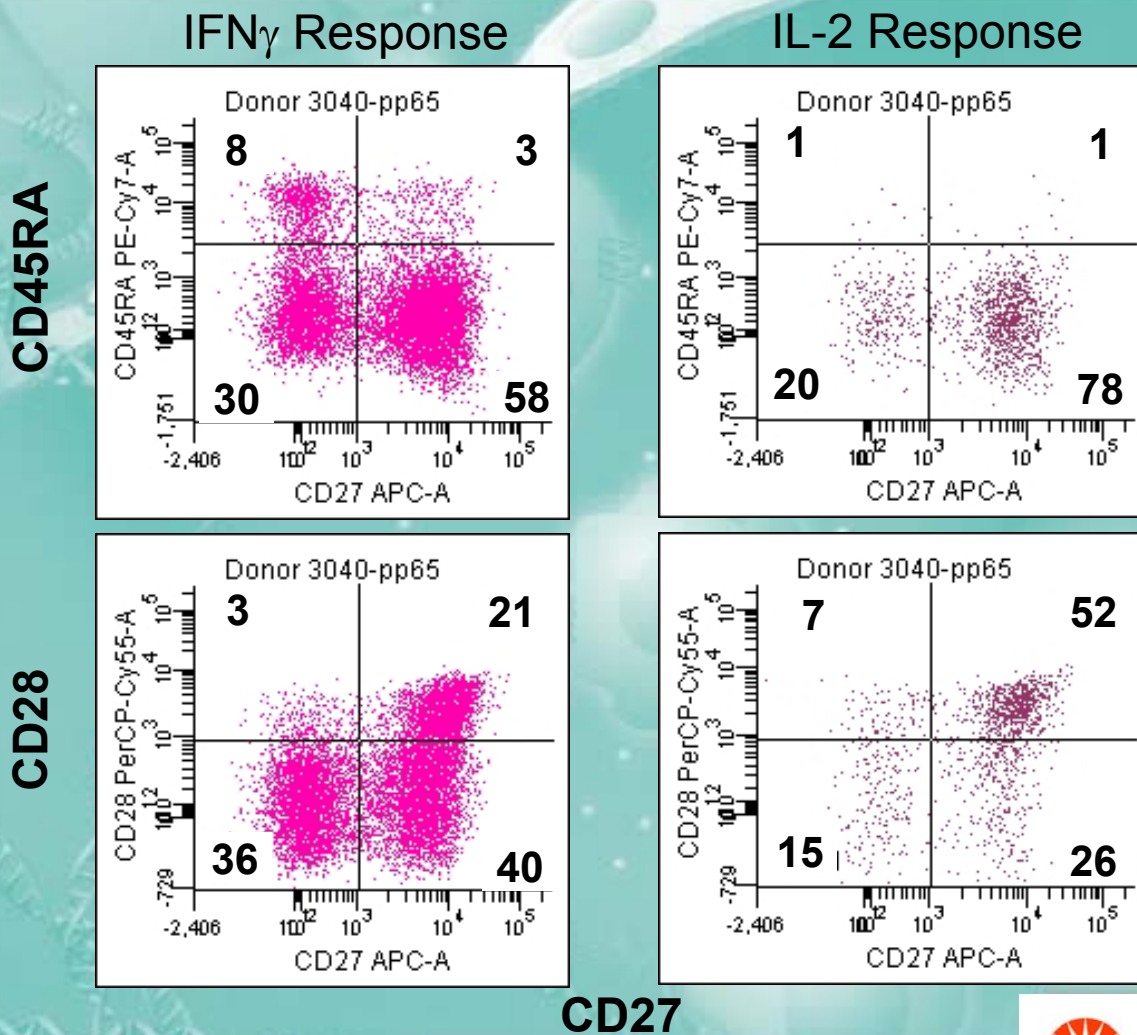
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Phenotype of CMV-responsive CD4 T cells



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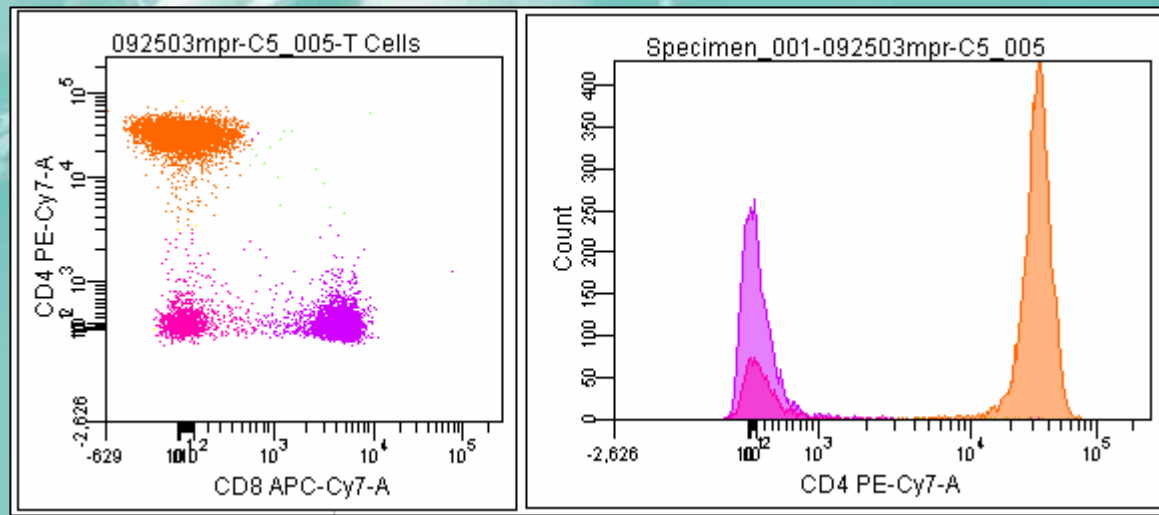
Phenotype of CMV-responsive CD8 T cells



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6 Color Example – Murine LN

- CD44 FITC, CD122 PE, B220 PE-Cy5.5, CD4 PE-Cy7
- CD25 APC, CD8 APC-Cy7



Bright B220 PE-Cy5.5 spills heavily into CD4 PE-Cy7 (8.69%) and is correctly compensated and appears to have a larger variance



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Polychromatic Flow Cytometry

– new tools are needed

- Automated spillover compensation (done)
- Shape GUI tools and algorithms to work with more sophisticated data (in progress)
 - New scale of linear and log to deal with negative numbers (done). Other multidimensional displays with clustering?
 - Fit compensated data spread over controls with nonlinear function – define tool shape (Quadrants, Polygons, etc..)
 - Then deal with multidimensional spillover effects
- Current efforts on multicolor digital data analysis
 - Mario Roederer, Dave Parks, Wayne Moore, Bruce Bagwell



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Reading: Compensation/Digital/Sensitivity

- Roederer M: Spectral Compensation for Flow Cytometry: Visualization Artifacts, Limitations, and Caveats. *Cytometry* 45:194–205 (2001).
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- Shapiro HM, Perlmutter NG, Stein PG: A Flow Cytometer Designed for Fluorescence Calibration. *Cytometry* 33:280–287 (1998).
- Hoffman RA and Chase ES, Resolution of Dimly Fluorescent Particles: A Practical Measure of Fluorescence Sensitivity. *Cytometry* 33:267–279 (1998).
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Acknowledgements

- Bob Hoffman
- Holden Maecker
- Laurel Nomura
- Ken Davis
- Barney Abrams
- Scott Gaumer
- Dave Parks
- Marty Bigos
- Randy Hardy
- Wayne Moore
- Jane Gray
- Dennis Sasaki
- Diether Recktenwald
- Mike Lock
- Mario Roederer
- Howard Shapiro
- Bruce Bagwell
- Donna Gandour
- Dwayne Yount
- Alan Stall
- Pierce Norton
- Joe Trotter
- Pat Collins
- Gil Reinin



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