Compensation: An Instrumental Perspective

Why Digital? Visualization Issues

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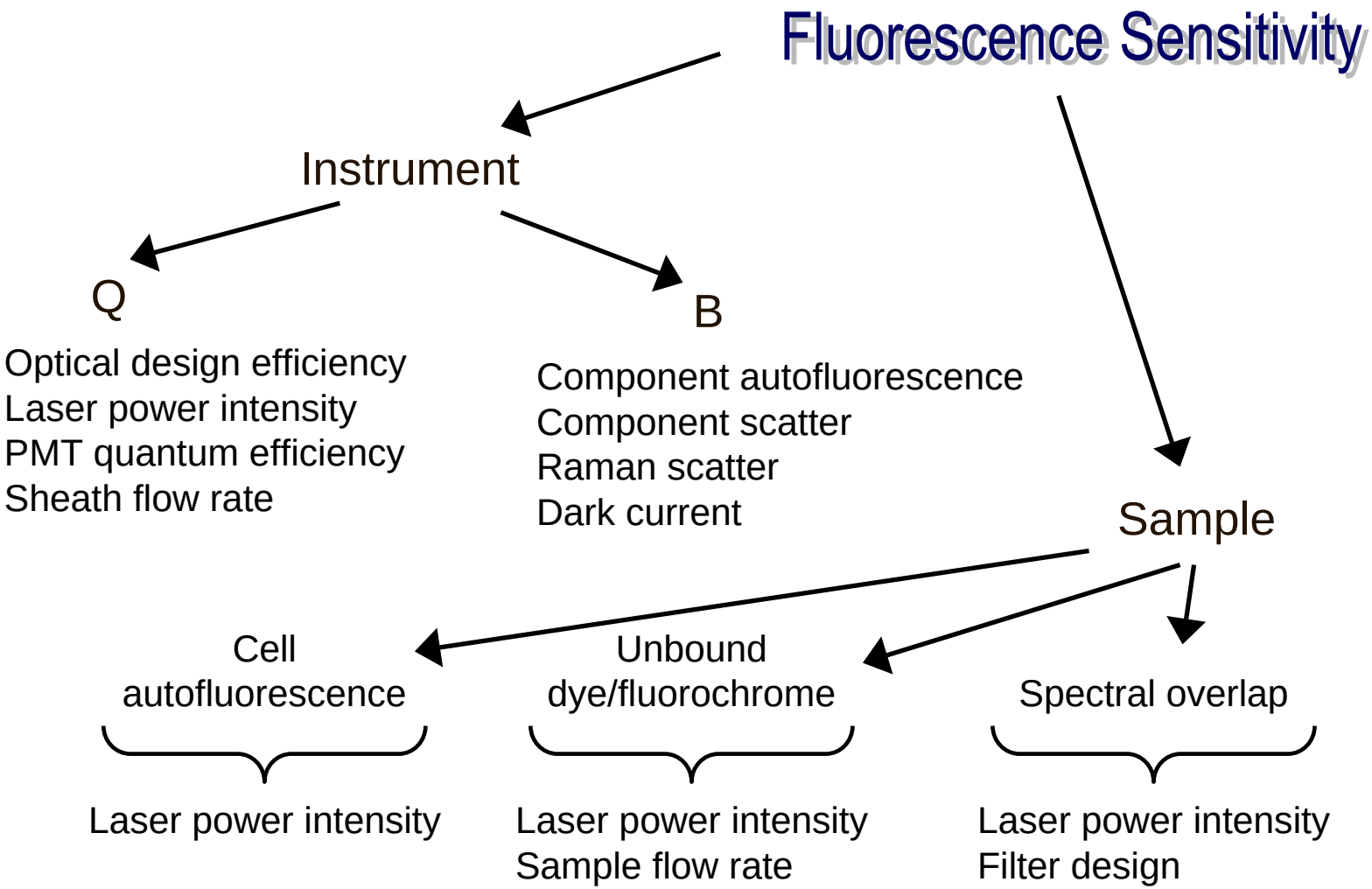
Review: Instrument Sensitivity

- Measuring Sensitivity - 2 definitions
  - **Threshold** - Degree to which a flow cytometer can distinguish dimly stained from particle free background. Usually used to distinguish means/medians.
  - **Resolution** – Degree to which a flow cytometer can distinguish unstained and dimly stained in a mixture. Can be very complicated in a polychromatic scenario.
- Good Sensitivity?
  - We have generally improved instrument sensitivity – threshold definition (50 – 200 MESF FITC, for example).
  - Good threshold sensitivity does not necessarily guarantee good resolution.

Things that impact sensitivity



Fluorescence Sensitivity is a Function of Detection Efficiency - Q

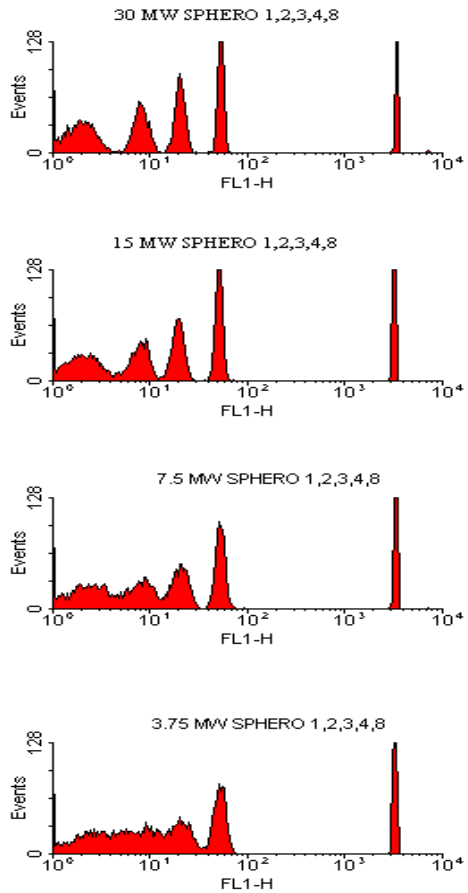
Fluorescence sensitivity:

a measure of the ability to resolve dimly stained cells from noise

Detection Efficiency (Q):

a measure of the ability of the instrument to excite and capture photons of interest

10 fold increase in laser power



30 mW
Q= 0.030

15 mW
Q= 0.018

7.5 mW
Q= 0.0076

3.75 mW
Q= 0.0040

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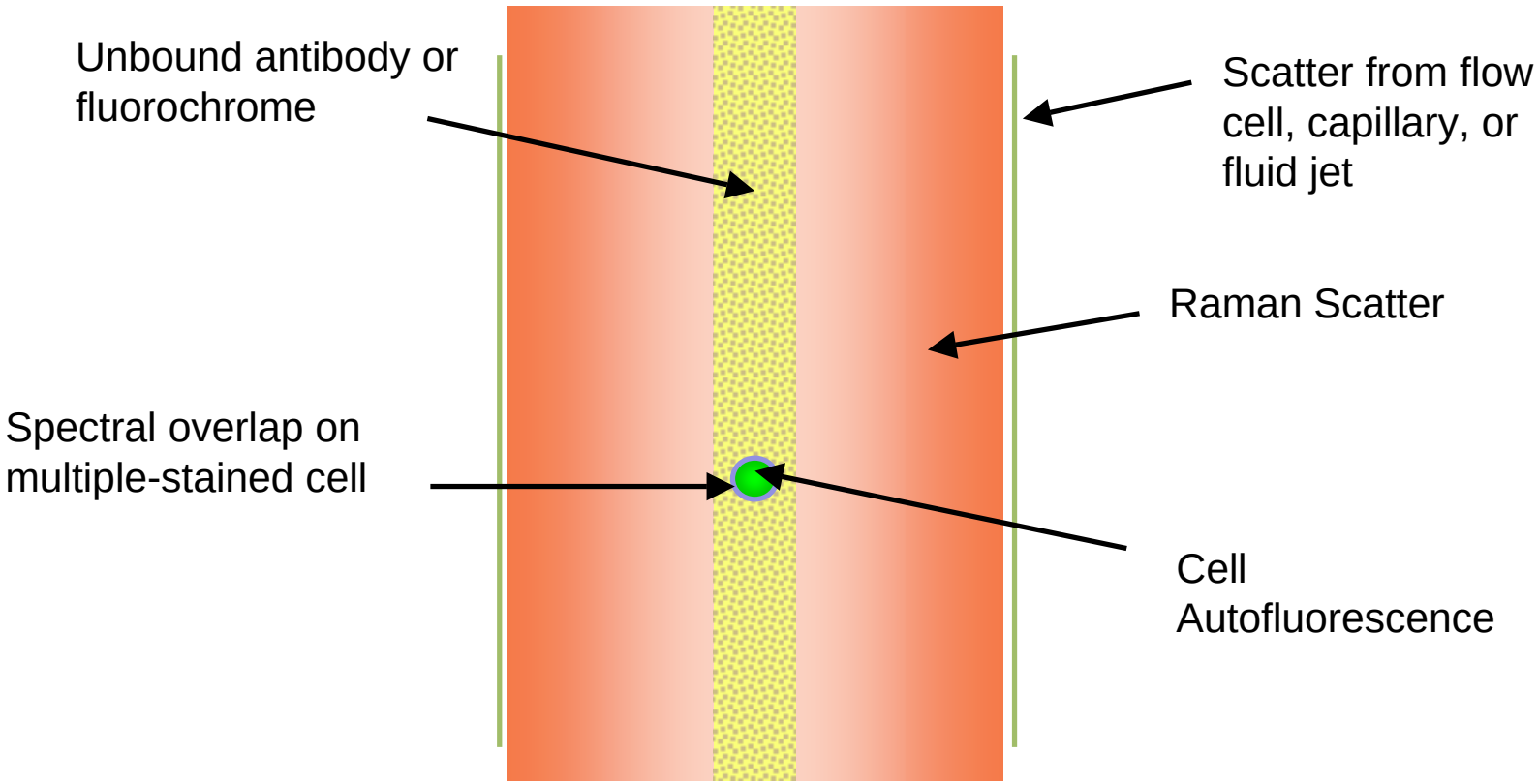
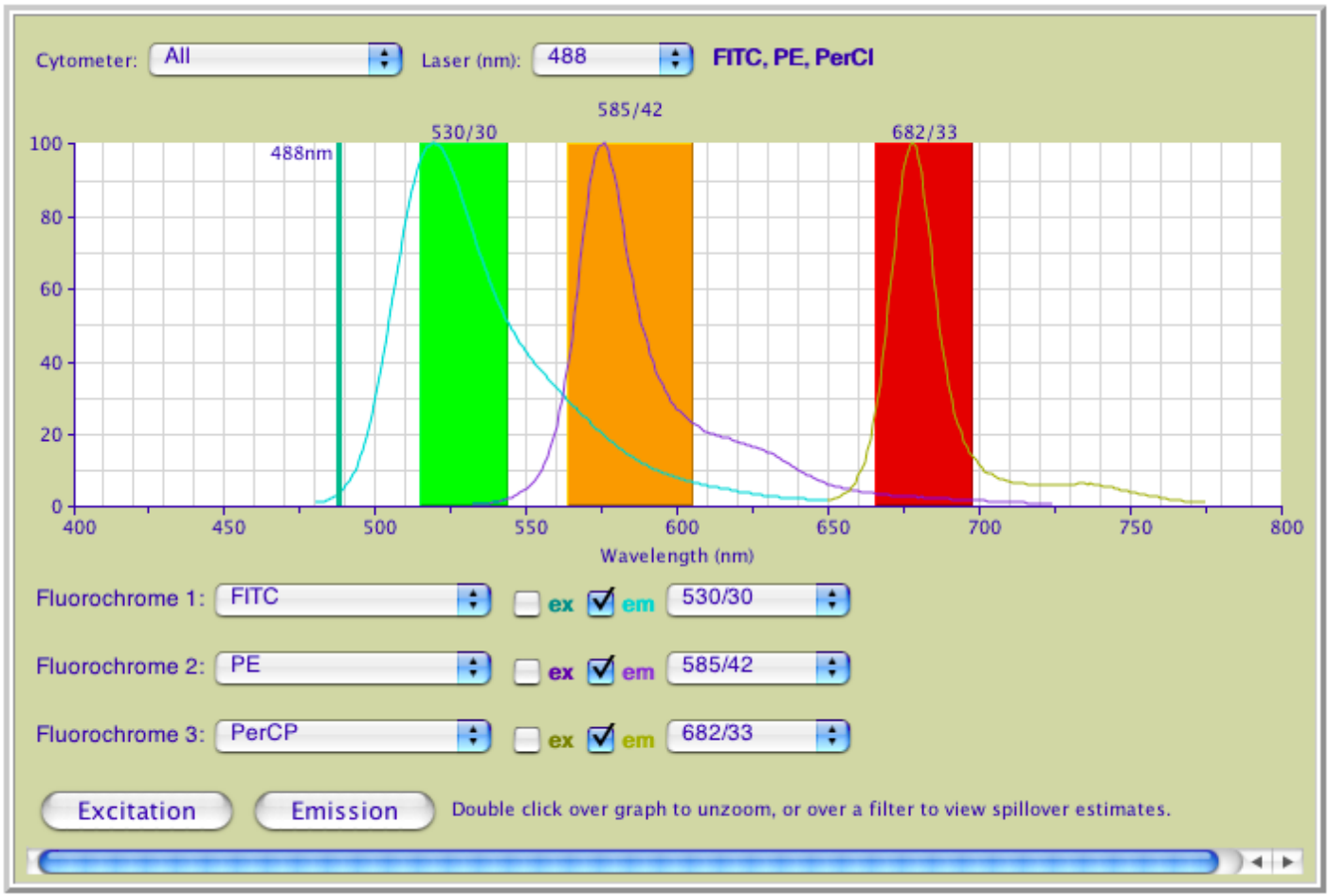
Sources of Background Light That Reduce Fluorescence Sensitivity - B



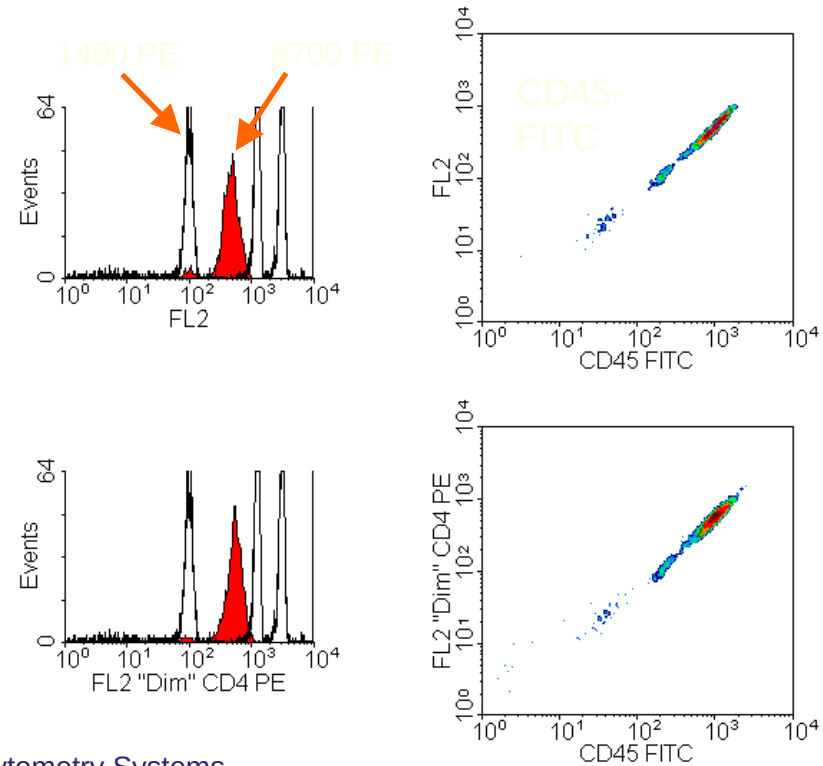
Fluorochromes emit in other channels

Spillover into other detectors increases background



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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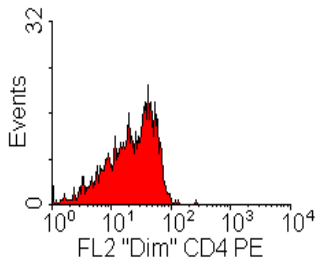
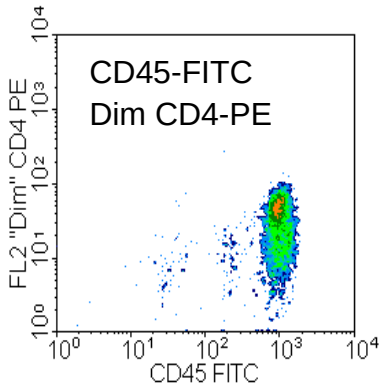
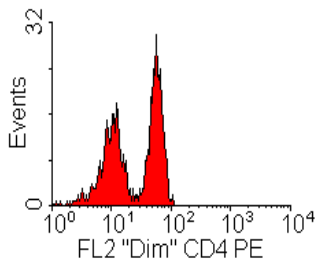
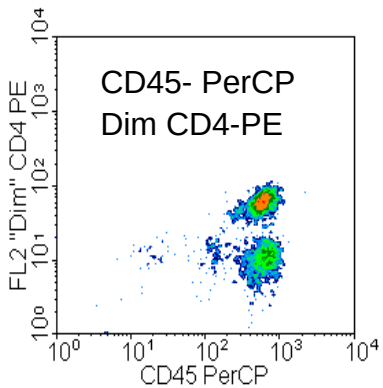
Effect of Spillover on Double Stained Cells





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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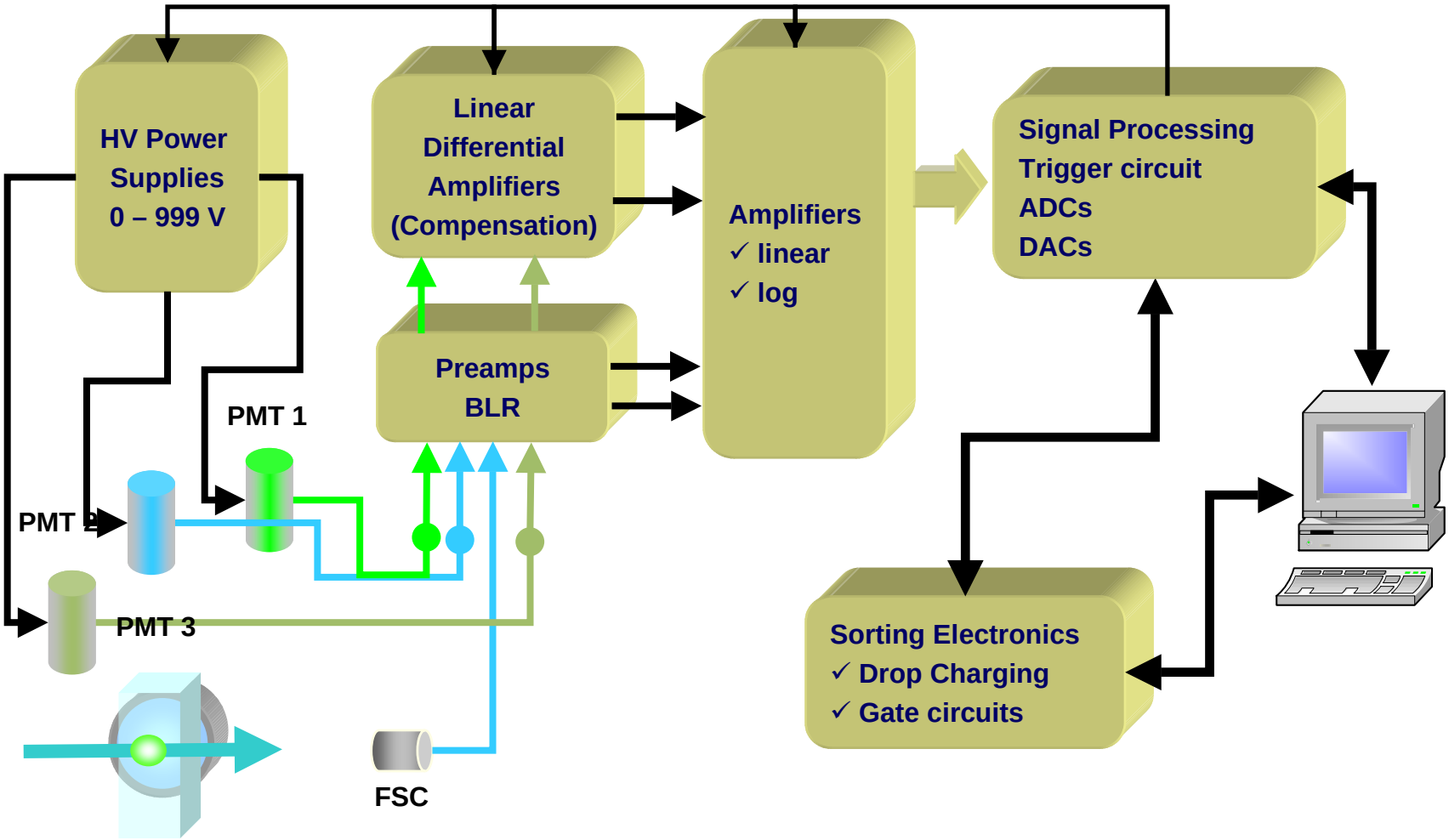
Analog Cytometers

- They have set the standard from the 1970s to now.
  - 2 stage linear display 0 – 10V:
    1. Preamp
    2. Amplifier/integrator
  - 3 stage logarithmic Display:
    1. Preamp (current to pulse)
    2. Differential amp(s) (compensation by pulse subtraction)
    3. Logarithmic amp
       a. Response error ±0.5dB from true log
       b. Linear error 6% to 10% over range
       c. Largest errors are from offsets

Simple 4 detector analog example



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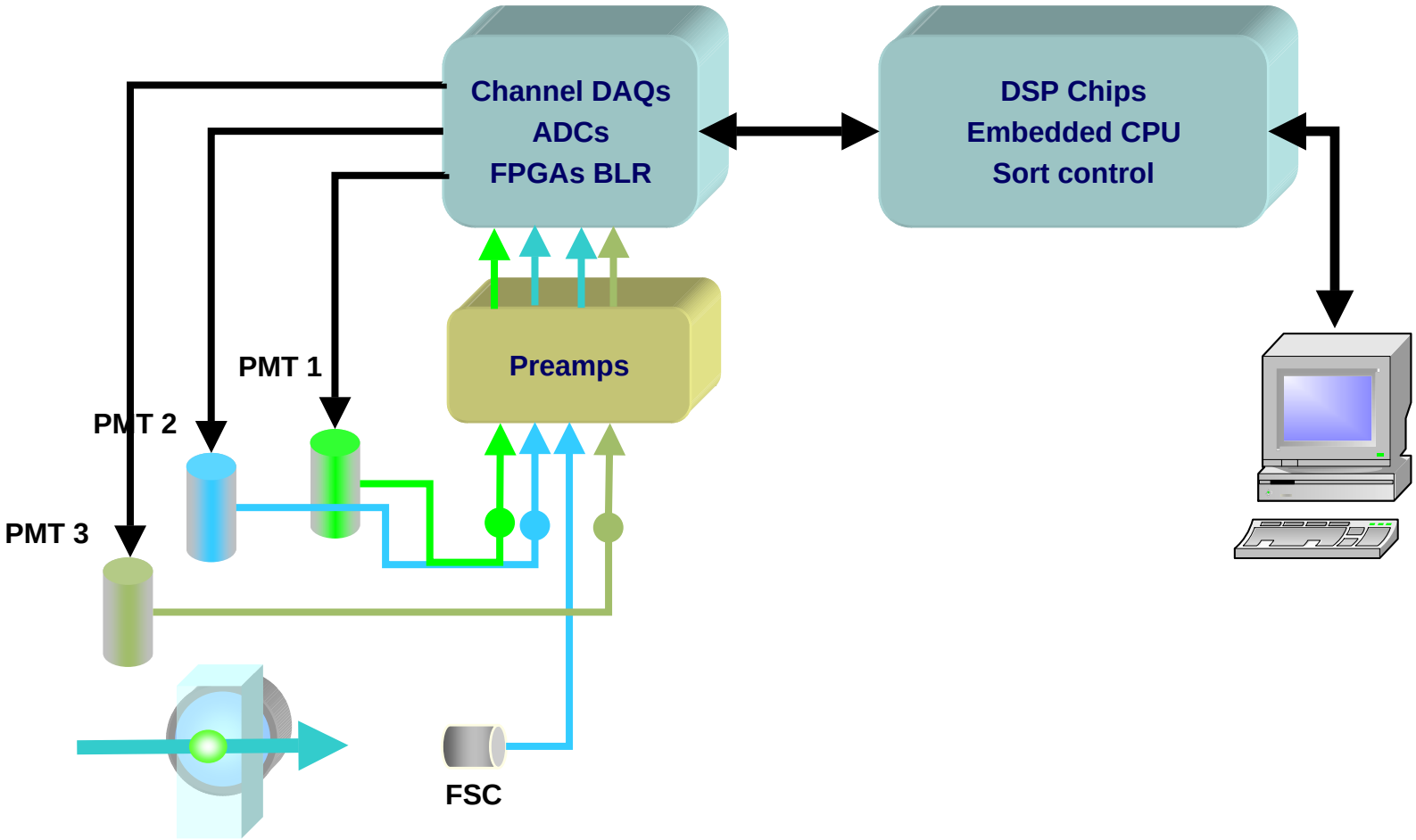
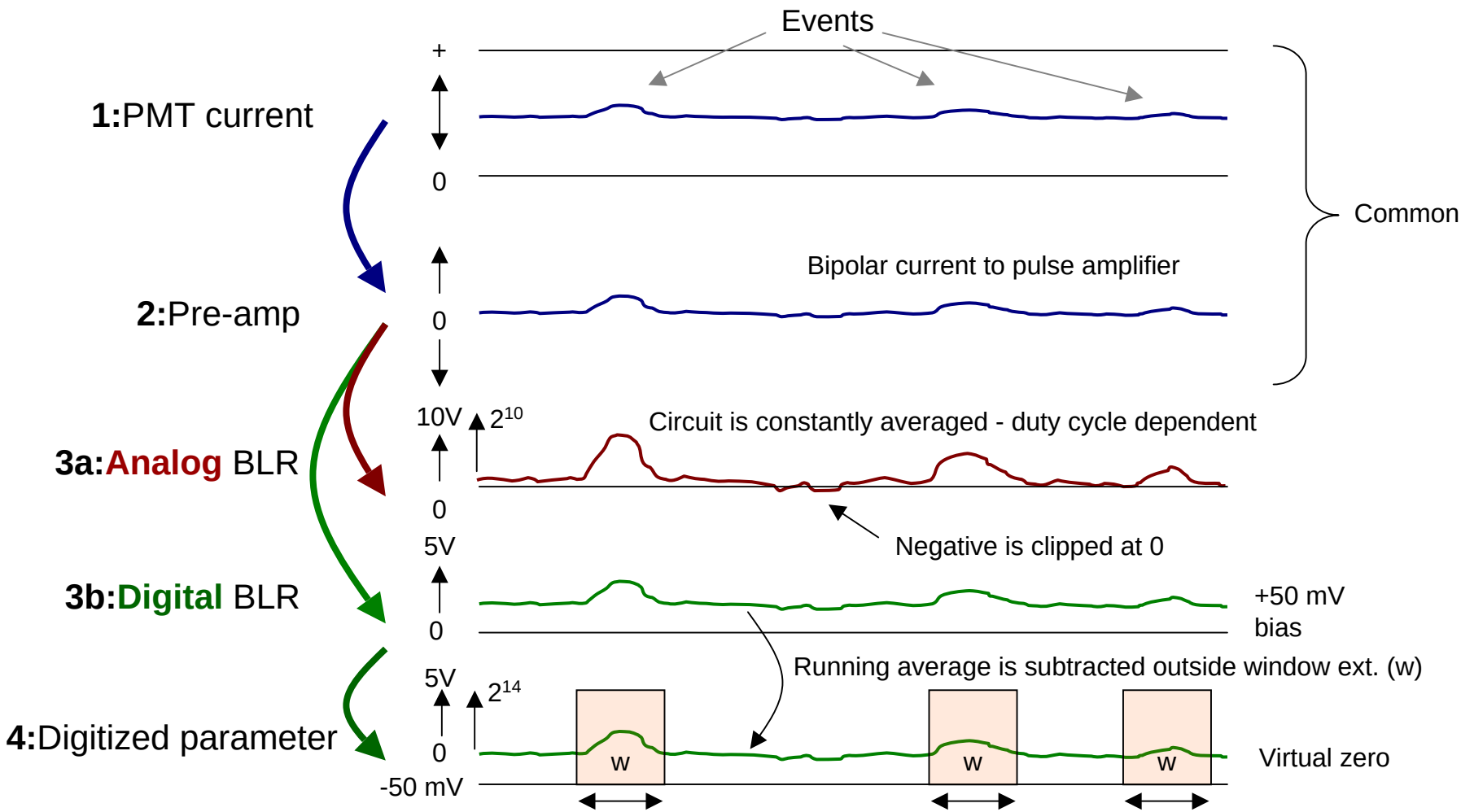
Simple 4 detector digital example



Base Line Restore - Analog vs Digital



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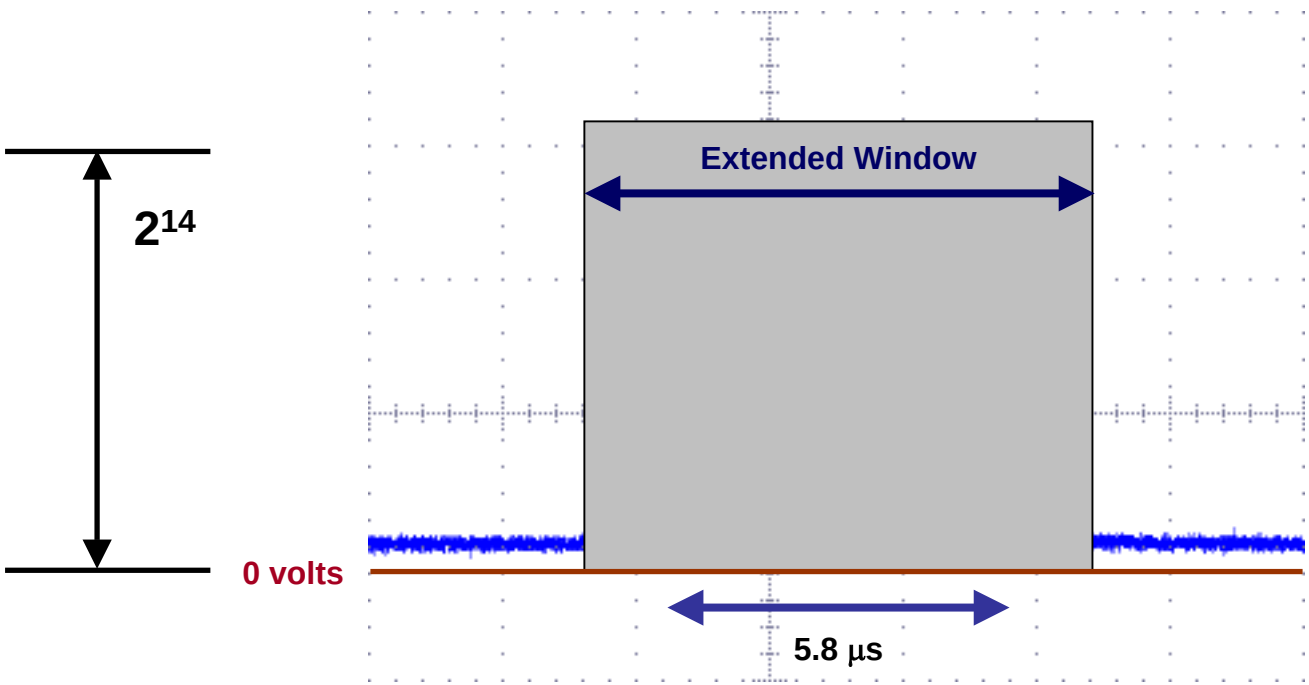
A Continuously Digitizing Cytometer

FACSAria 10 MHz ADC: 5.8 μsec 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)

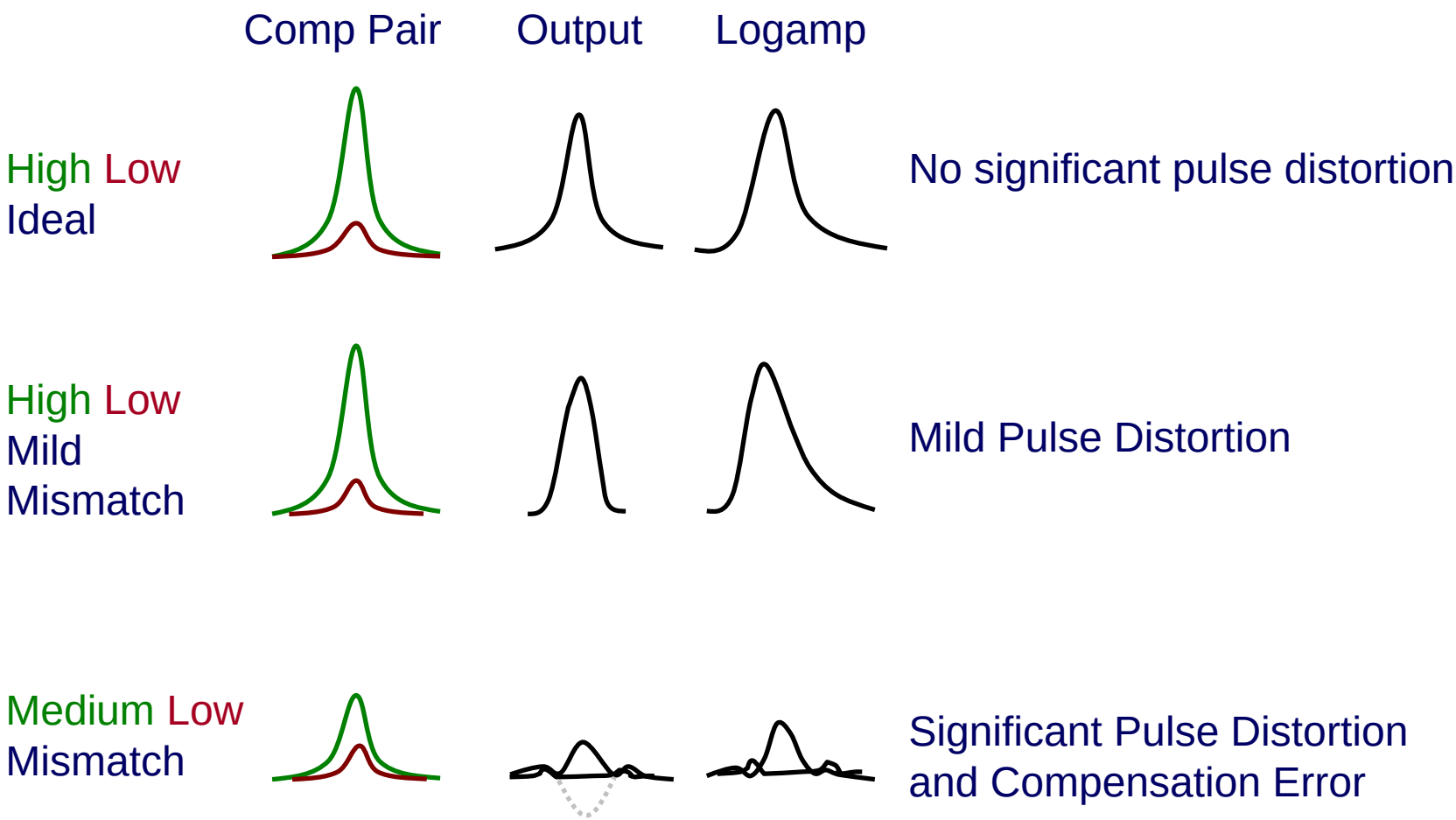
We live in an imperfect world

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) and matrix algebra.
- Compensated parameters exhibit spread.
  - Nonlinear error from photon counting statistics[1]
  - Worse in red and far red (fewer photons)
    - Sampling error is function of SQRT(number of photons)
  - Analog systems dampen spread due to errors in compensation circuits and logamp nonlinearity

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

Analog Compensation Errors



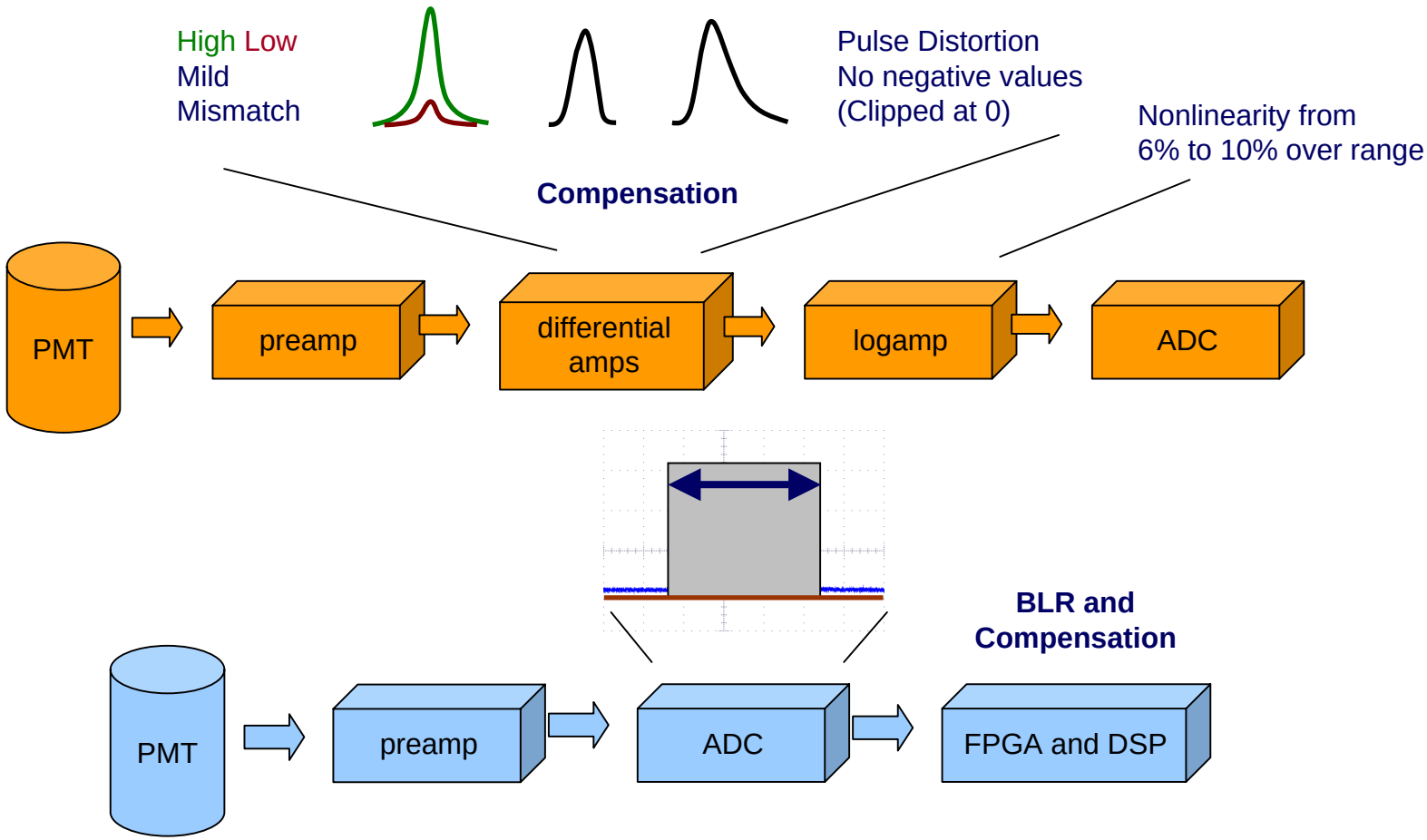
Cytometer Quantitative Characteristics

Quantitative Characteristics Over a 4 Decade Range[1]

Decade	Log amp output	Log amp input	Cathode photons/ 10 μsec	CV (%)	MESF	% error 1.0 mV offset	ADC channel (10 bit)	ADC channel (18 bit)
	10.0 V	10.0 V	4430000	0.13	1000000	0.01	1023	262143
4	7.5 V	1.0 V	443000	0.4	100000	0.1	102	26214
3	5.0 V	100 mV	44300	1.3	10000	1.0	10	2621
2	2.5 V	10 mV	4430	4.0	1000	10	1	262
1	0.0 V	1 mV	443	12.9	100	100	0	26

[1] Shapiro H, Perlmutter N and Stein P: A Flow Cytometer Designed for Fluorescence Calibration, Cytometry 33:280-287, 1998.

Analog vs Digital Electronics



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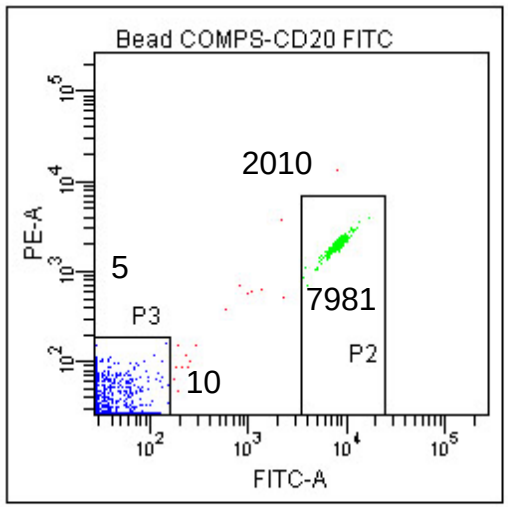
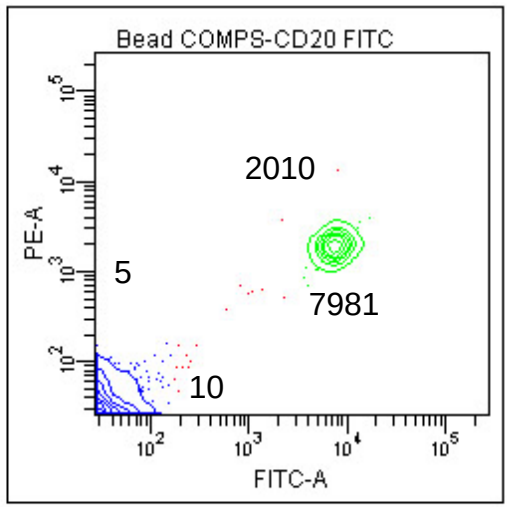
Digital Cytometer Numerical Range

- BLR zero is really brought to “0.0” numerically
  - An asymptotic logarithmic scale can never get there…
    - 100,000 - 10,000 - 1,000 - 100 -10 - 1 - 0.1 - 0.01…
- Range is from 1% below 0 to maximum value
  - 18 bits: That means from -2621 to 262144 in uncompensated data, more negative values appear after compensation due to spread
  - Typical range is from -300 to 200000 before compensation
- BD digital 4 decade range plots from 26.0 - 262143.0
  - Raw values < 26.0 are visualized at 26.0
  - Statistics use the actual raw IEEE 32 bit FP values from less than zero up to 18 bit range of 262143.0

Uncompensated CD20 FITC on capture beads





Specimen Name: Bead COMPS
Tube Name: CD20 FITC
$FIL: CD20 FITC.fcs

Population	#Events	FITC-A Median	FITC-A Mean	PE-A Median	PE-A Mean
P1	5,447	29	2,753.6	28	698.4
P2	1,867	7,981	8,003.2	2,010	2,016.8
P3	3,563	10	10.9	5	5.0

Spillover calculation AutoCompensation method

Compensation coefficient = slope

1. $k_{12} = (2010 - 5) / (7981 - 10)$

$k_{12} = 0.2515$

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

3. k^{-1}

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

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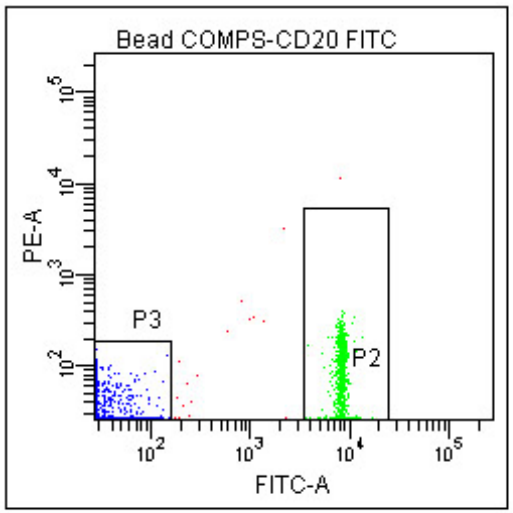
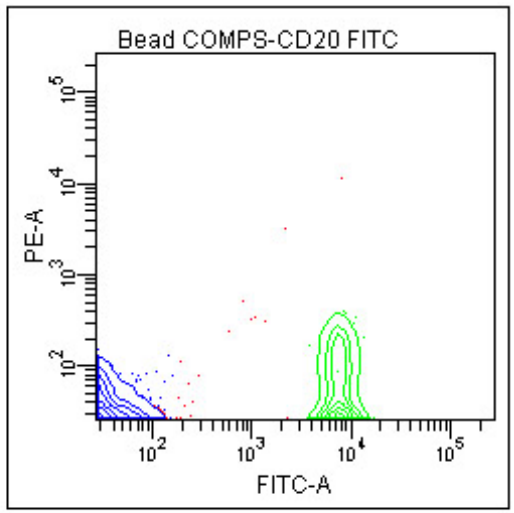
CD20 FITC Capture Beads Compensated





Specimen Name: Bead COMPS
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Population	#Events	FITC-A Median	FITC-A Mean	PE-A Median	PE-A Mean
P1	5,447	29	2,753.6	3	7.3
P2	1,867	7,981	8,003.2	7	8.0
P3	3,563	10	10.9	2	2.2

Populations are aligned In dye space

PE_c = PE + FITC x -0.25 using matrix algebra

Not a subtraction, rather a correction because we use compensation coefficients instead of spillover coefficients.

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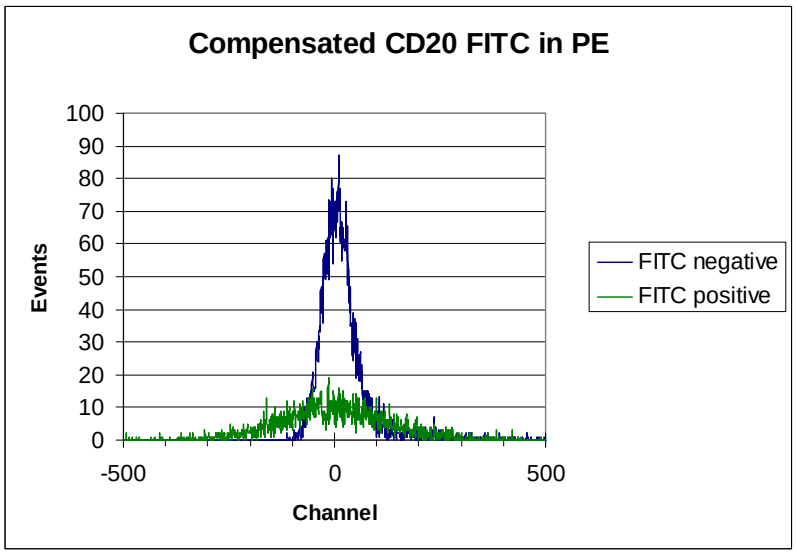
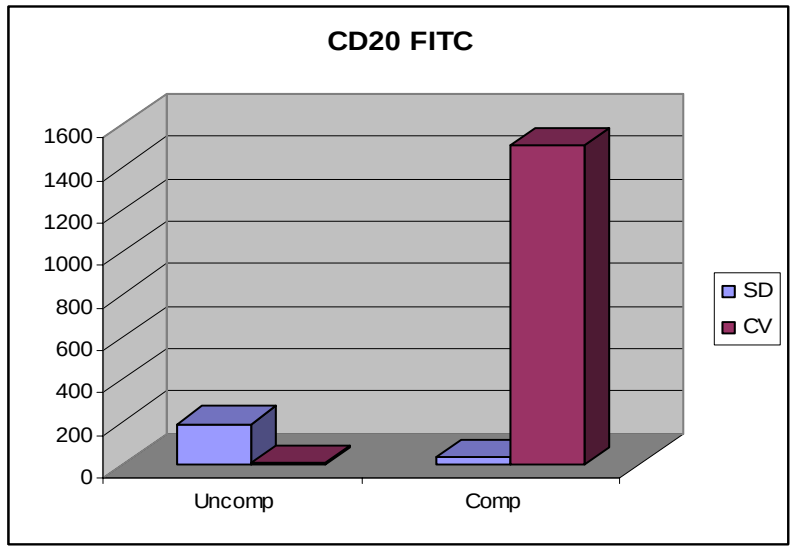
Compensation spread

The linear plot below shows the alignment of compensated CD20 FITC positive and negative capture beads in the PE dimension.

The CD20+ standard deviation decreases from 192 to 33.4 after compensation – yet the CV* goes to 1500% because the mean drops from 2010 all the way to 2.2.





**The Coefficient of Variation (CV) is inappropriate with compensated data. CV = 100 x Std/Mean, so as the mean approaches 0, the CV approaches ∞*

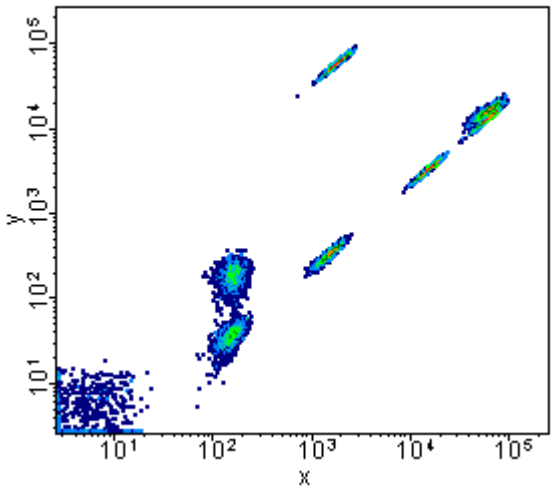
Generally accepted assumptions among the Asilomar 2002 flow community attendees[1]

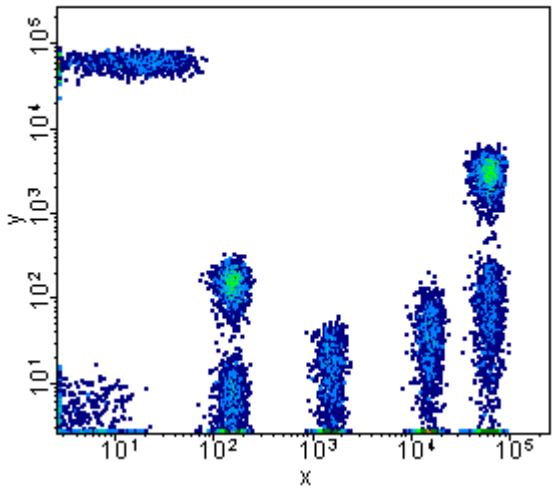
- Analog compensation error is responsible for the observed differences between analog- and digitally-compensated data
- Pure linear matrix compensation gives the best available estimates for the dye signals from each cell
- Statistical results should be computed on the matrix compensation output without 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

[1] October 2002 - Courtesy Dave Parks

Synthetic data example – log scale

8 modeled populations – 2 of which are double positive

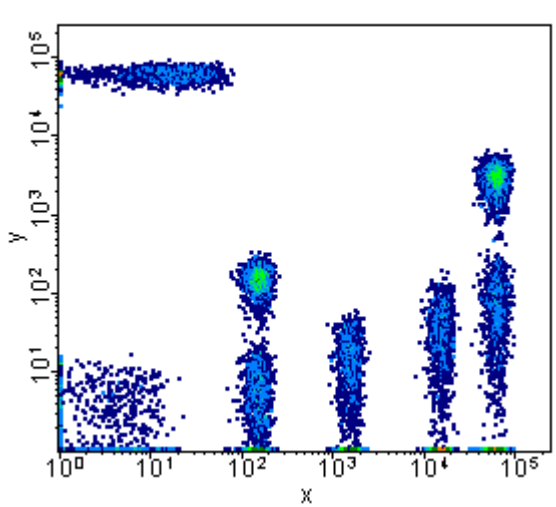




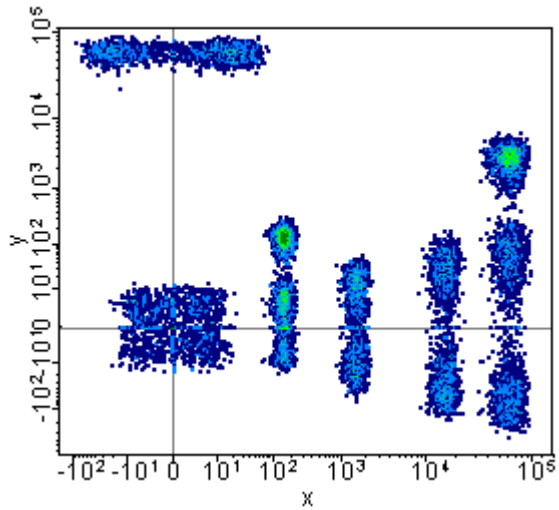
Difficult with low autofluorescence and compensation because high cross-over (22%) of X into Y, low cross-over (3%) of Y into X causes “high background” of X into Y on single positive bright X population, which inflicts significant data spread around zero after compensation

Full Log Display – Visualization Artifacts

Standard Log Scale



Blended Scale
95% Log with zero shown



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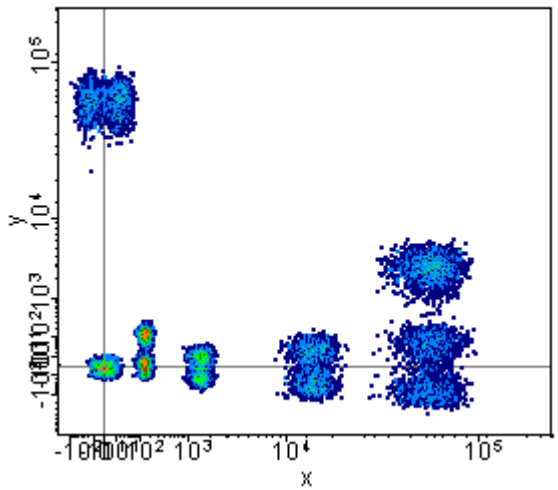
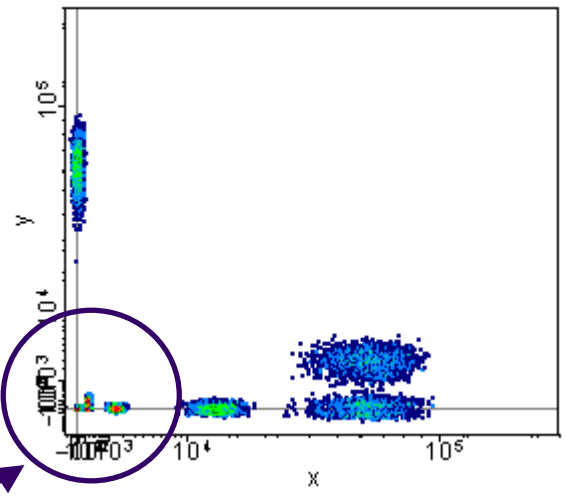
More Linearization minimizes bifurcation

20% Linear



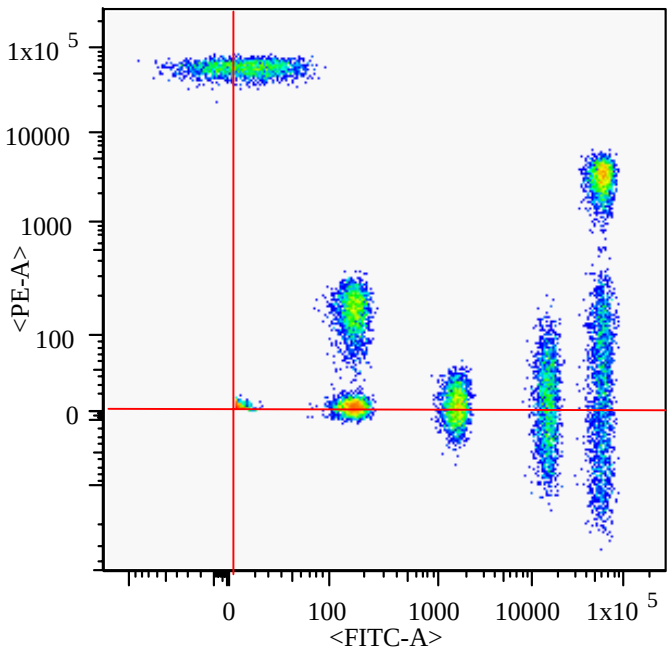
50% linear



Vanishing linear populations

Logicle: Compensated Biexponential Display

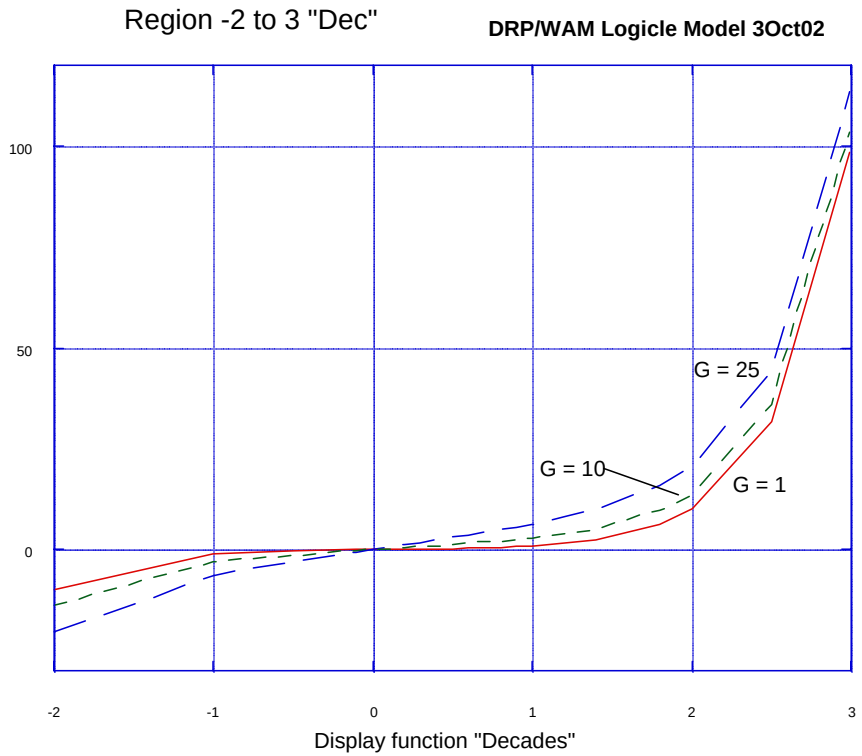
Upper decades are log, becomes linear near zero. Biexponential transform where data zero is shown by the crosshairs in the plot

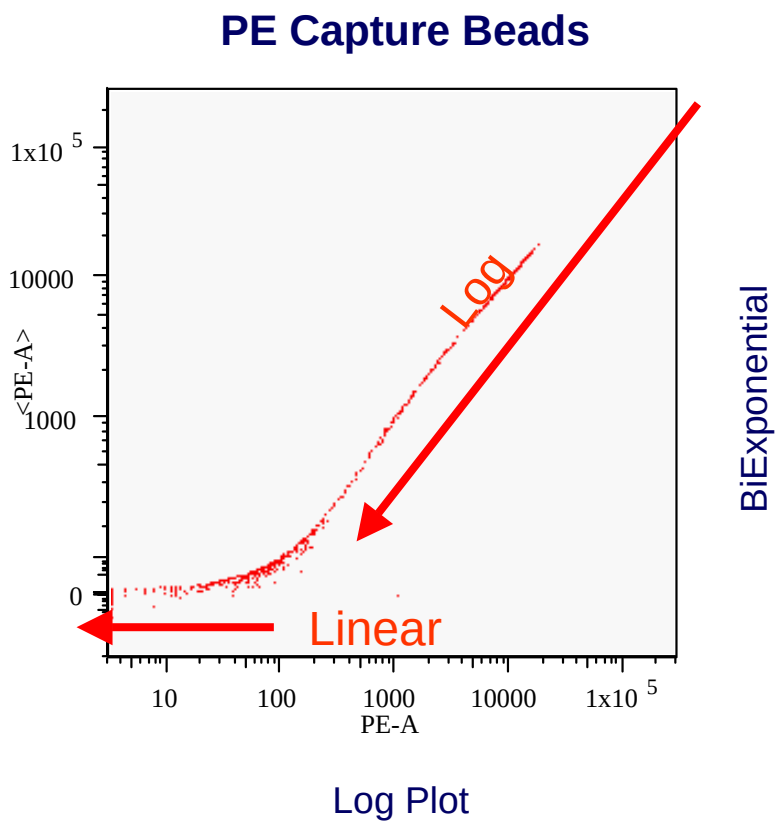


- This example shows the value of combining the blend with a mostly logarithmic scale on the upper such that the linear portion occupies a significant plot area over that in blend alone.
  - Compensated single pos are continuous
  - All populations are visible

Data dependant display capability

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





Log Plot

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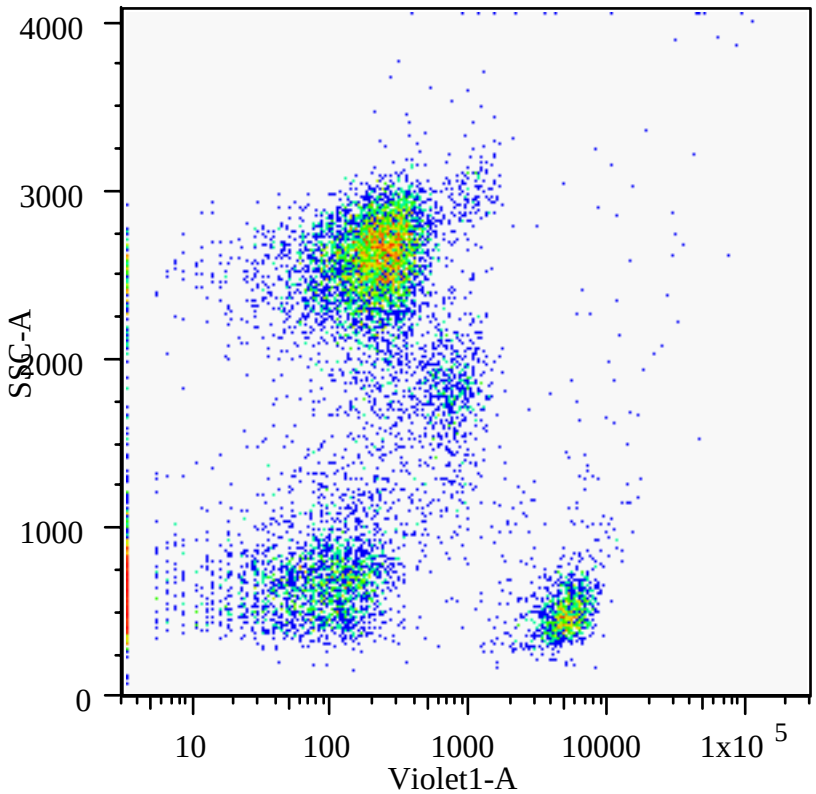
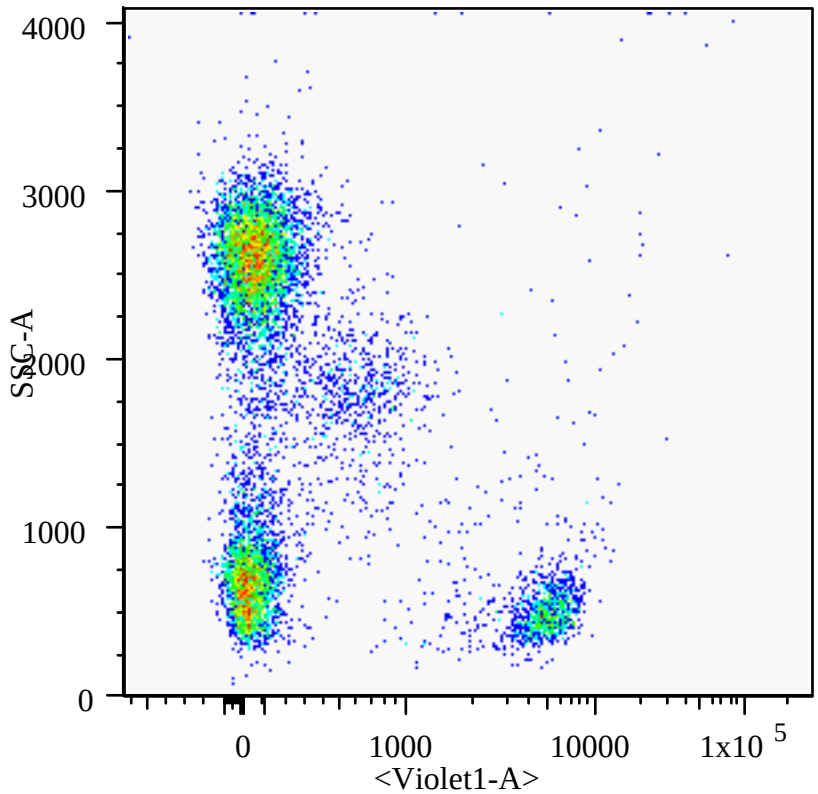
PacBlu-CD4 FACSAria example

5 decade pulse area

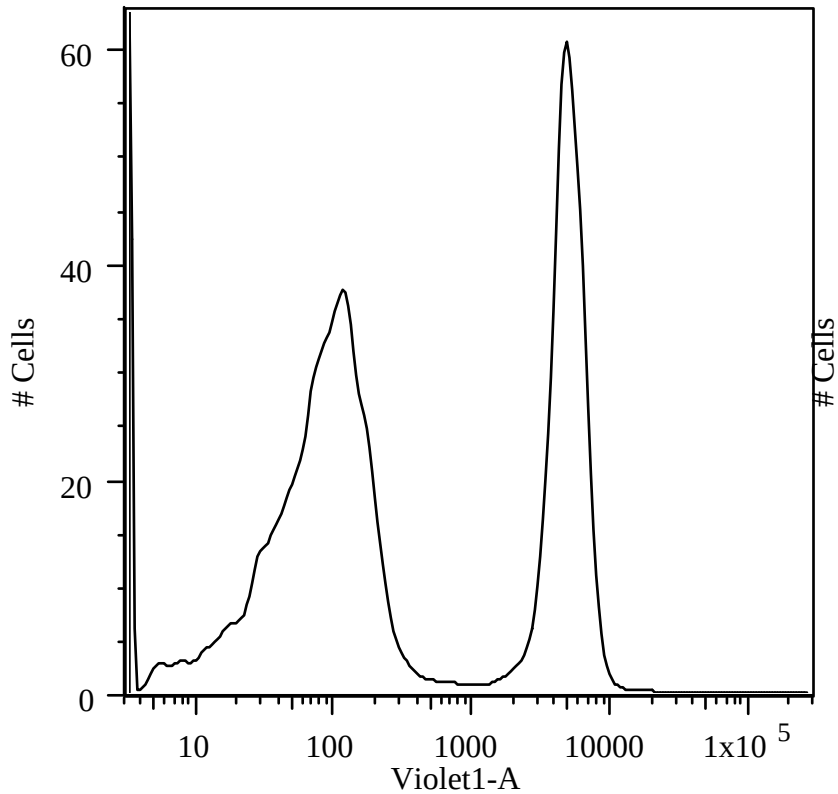


Transformed plot

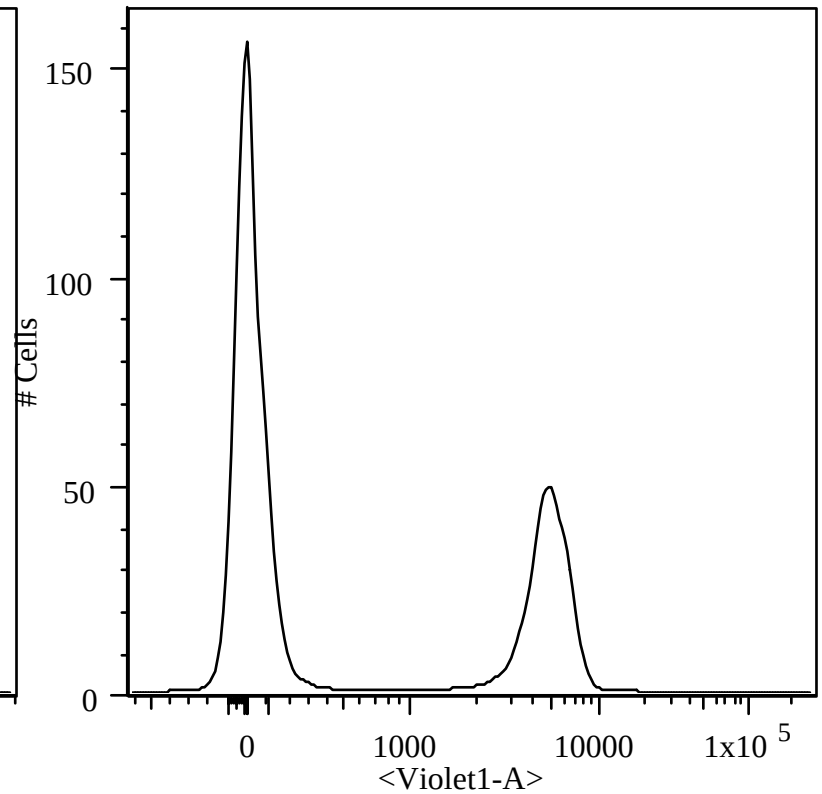


PacBlu-CD4 FACSAria with lymph gate

5 decade pulse area



Transformed plot



Summary: Current data display issues

- As pointed out by Mario Roederer, Dave Parks[1], 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.

[1]R Hardy data - Cytometry email thread 2002, DR Parks - Asilomar Workshop 2002

Can Compensated B Cells Correctly Align at Different Dye Concentrations?[1]

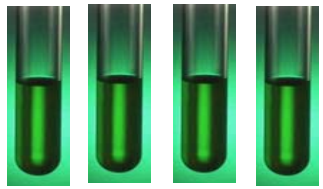
10^7 Murine Spleen Cells

Spleen

Stain B cells with B220 FITC
at 1/40 saturating concentration

Split each half into 4 samples and stain
with saturating CD19 Cy7-PE at:

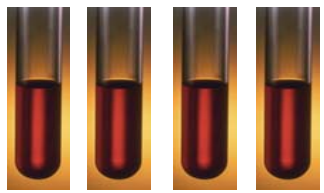
1X 1/10X 1/100X none

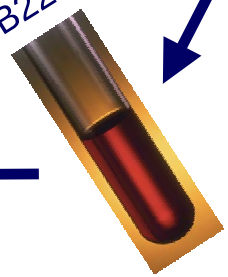


Wash ½

Wash ½ & Stain with
Saturating B220 Cy5-PE

1X 1/10X 1/100X none





Recombine so 50% of each
Cy7-PE CD19 concentration
is B220 Cy5-PE+

1X 1/10X 1/100X none

[1] Randy Hardy – Fox Chase Cancer Center

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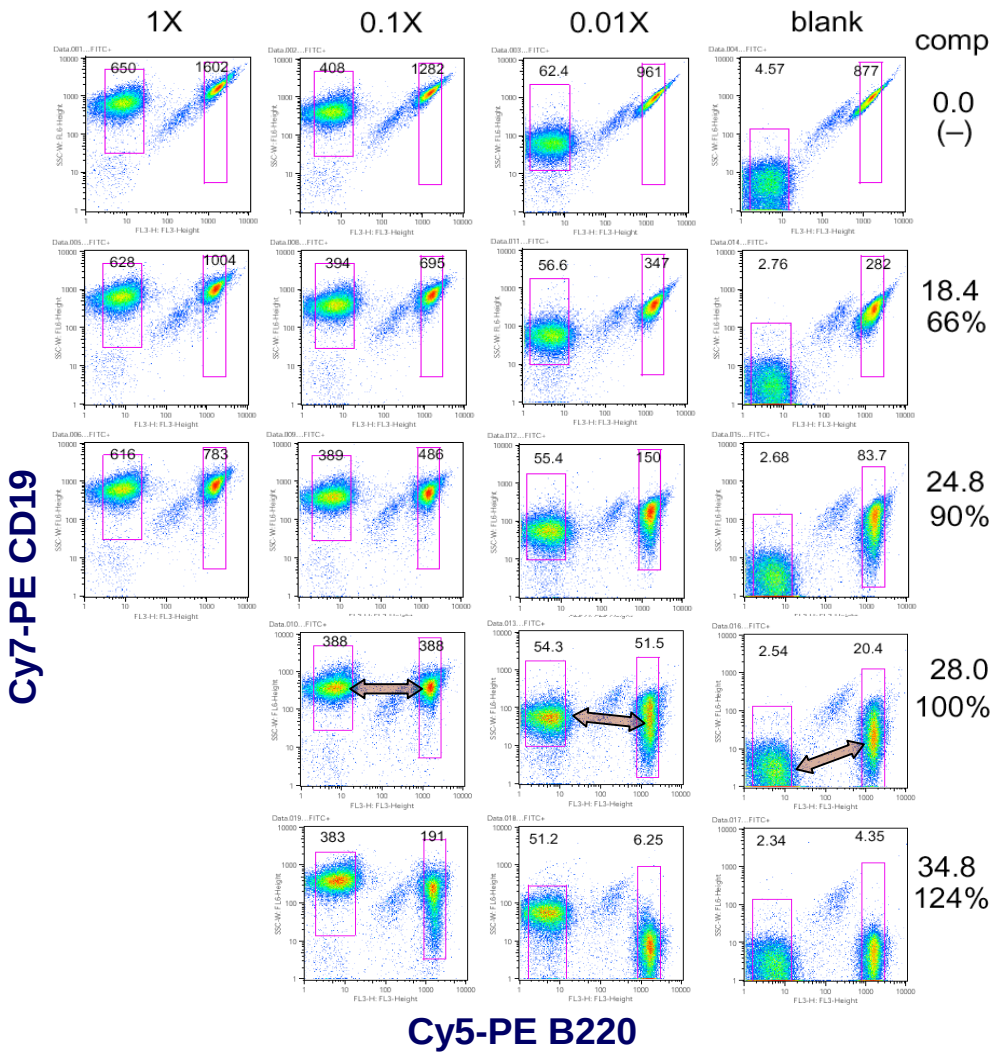
Analog Compensation Behavior



- "Y" reagent stained at high, medium, low and blank levels (CD19 Cy7-PE).
- Half are stained with the "X" reagent (B220 Cy5-PE) and half with mock stain (B220 FITC 1/40) only.
- Recombine the corresponding pairs and analyze at various compensation settings.
- No compensation value satisfies all tubes of the same stain.

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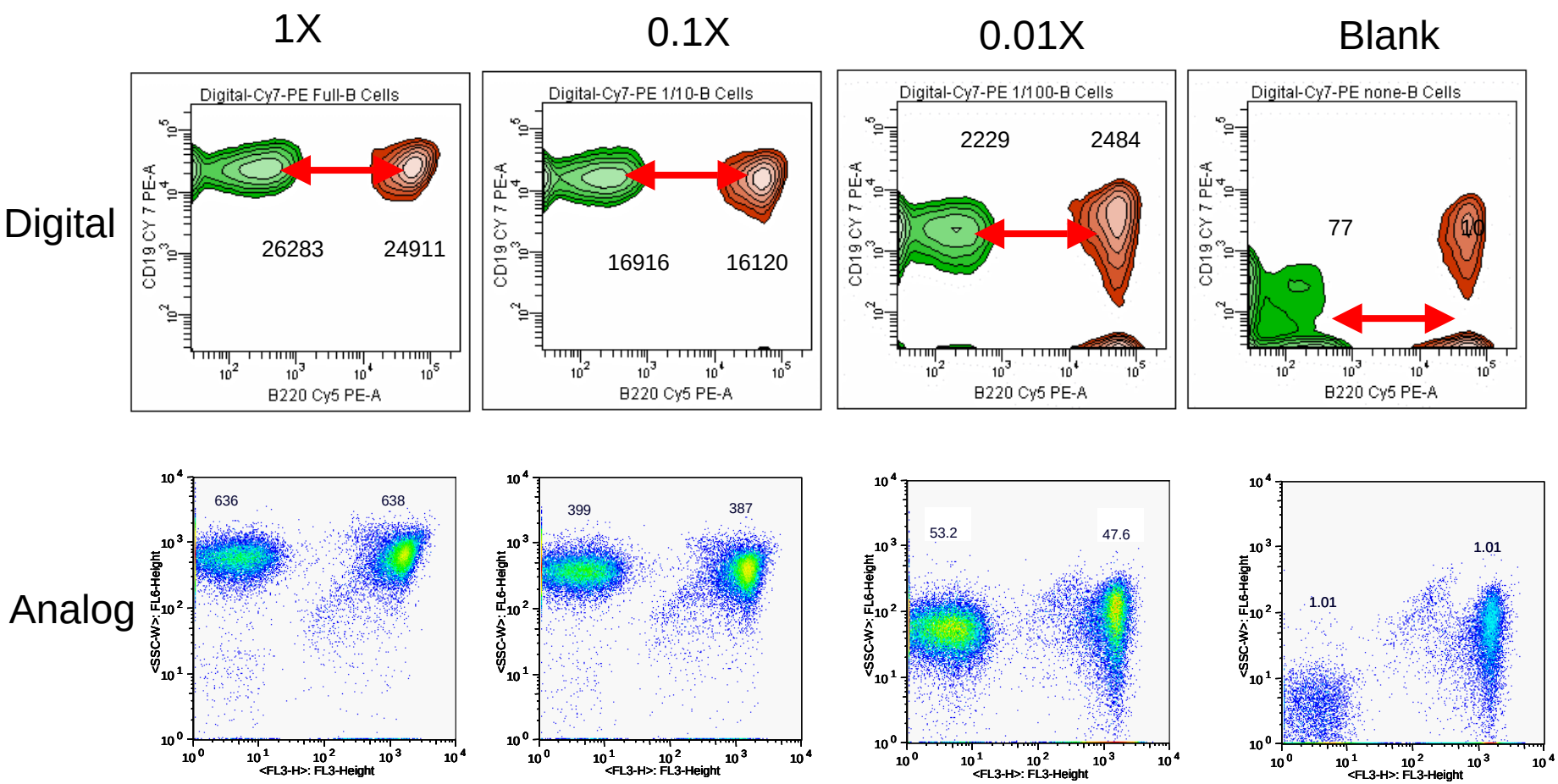
Digital & Analog: Software Compensation



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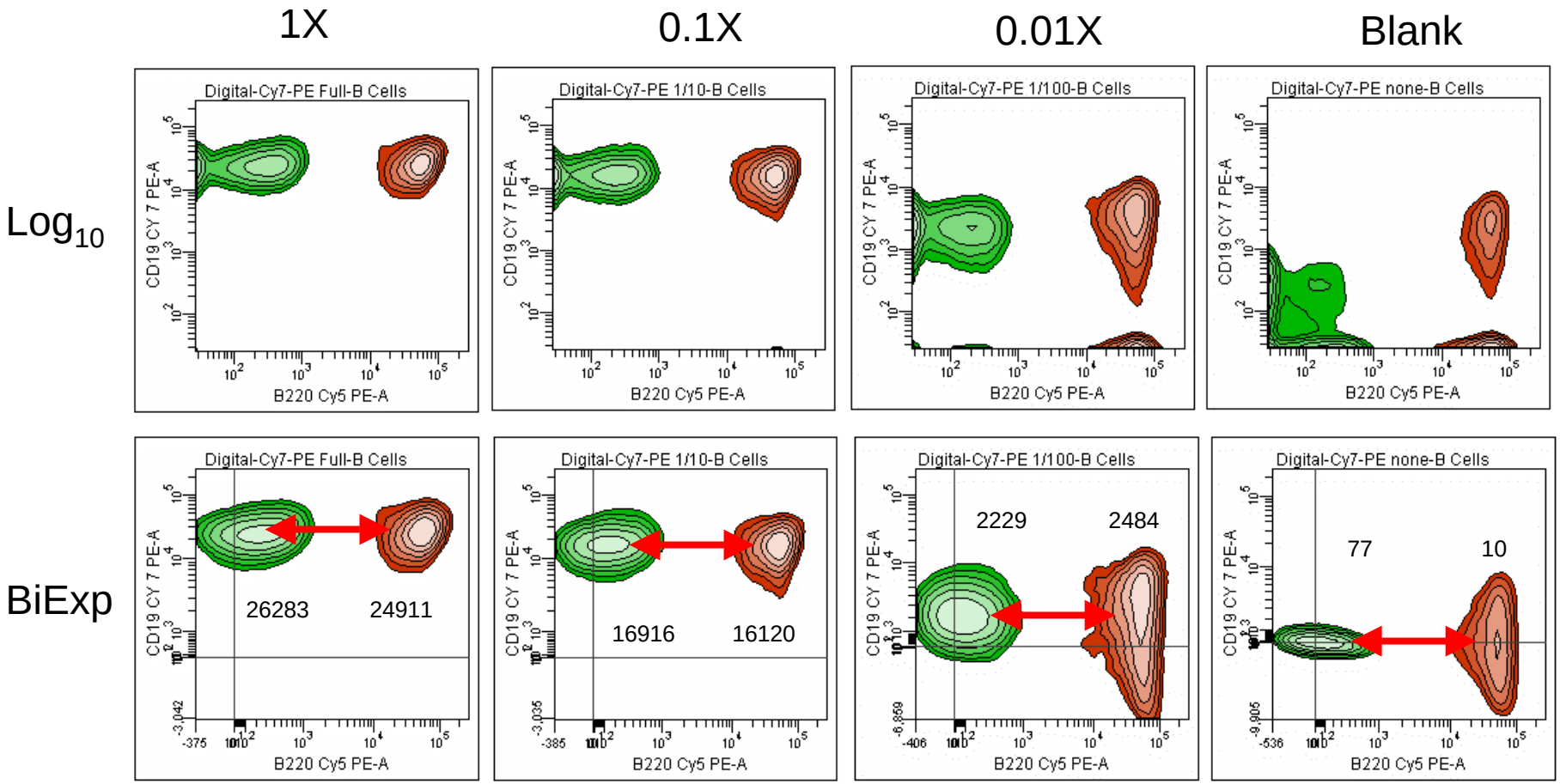
Correct Population Alignment



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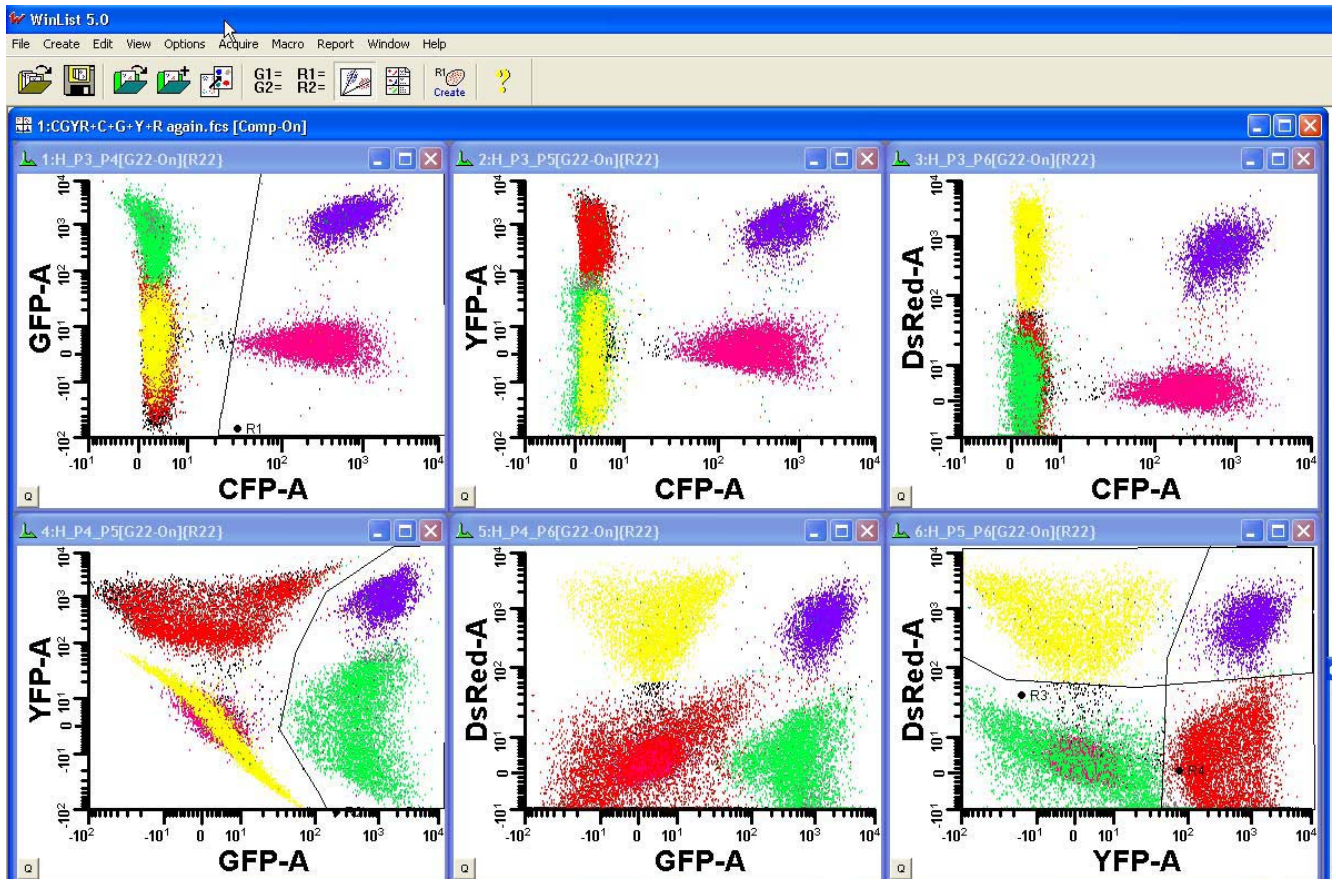
Hyper-Log: Another Solution[1]

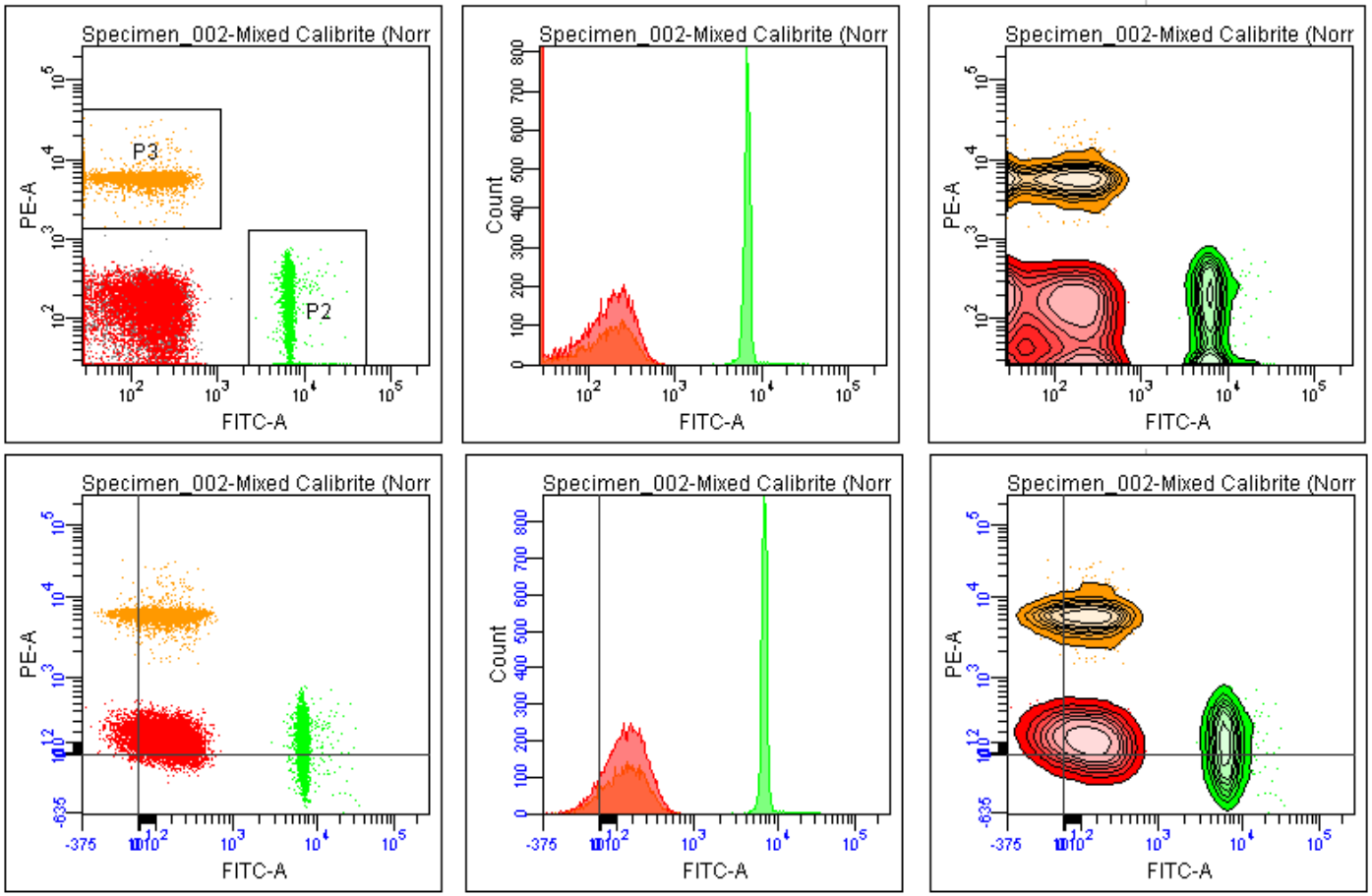
Four Fluorescent Proteins: ECFP, EGFP, EYFP, DsRed



[1] Courtesy Bruce Bagwell - Verity Software House

Optimized BiExponential

Algorithm self-adjusts and is automatically scaled



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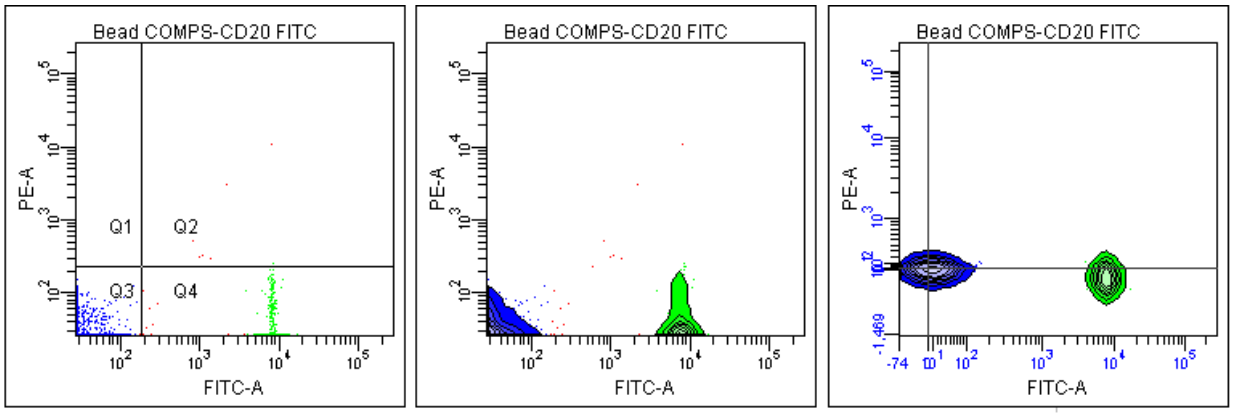
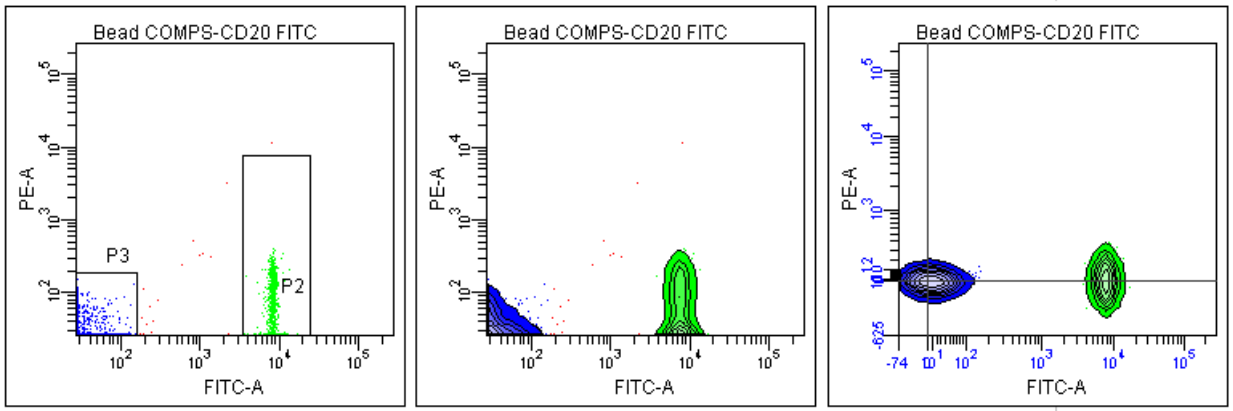
CD20 FITC Capture Beads Revisited

BiExponential display reveals proper compensation visually

2% over



Correct



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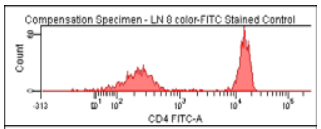
8-color antigen-specific immunophenotyping

Ab Conjugate	Laser λ	
CD28 PerCP-Cy5.5	488	
CD45RA PE-Cy7	488	
CD27 APC	633	Surface
CD8 APC-Cy7	633	staining
CD3 Pacific Blue	405	
CD4 AmCyan	405	
Anti-IFNγ FITC	488	Intracellular
Anti-IL-2 PE	488	staining

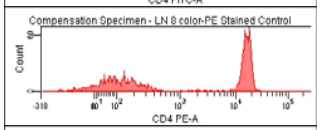
8 Color Compensation (LSR II)

Single-stained controls:

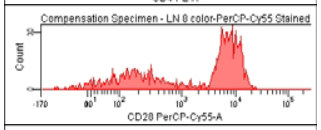
CD4 FITC



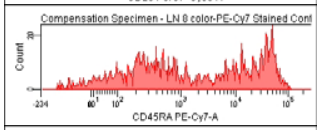
CD4 PE



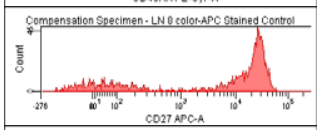
CD28 PerCP-Cy5.5



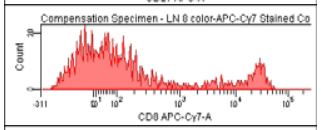
CD45RA PE-Cy7



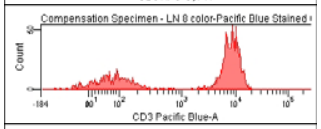
CD27 APC



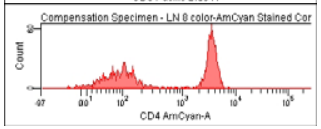
CD8 APC-Cy7



CD3 Pacific Blue



CD4 AmCyan



Auto-comp

	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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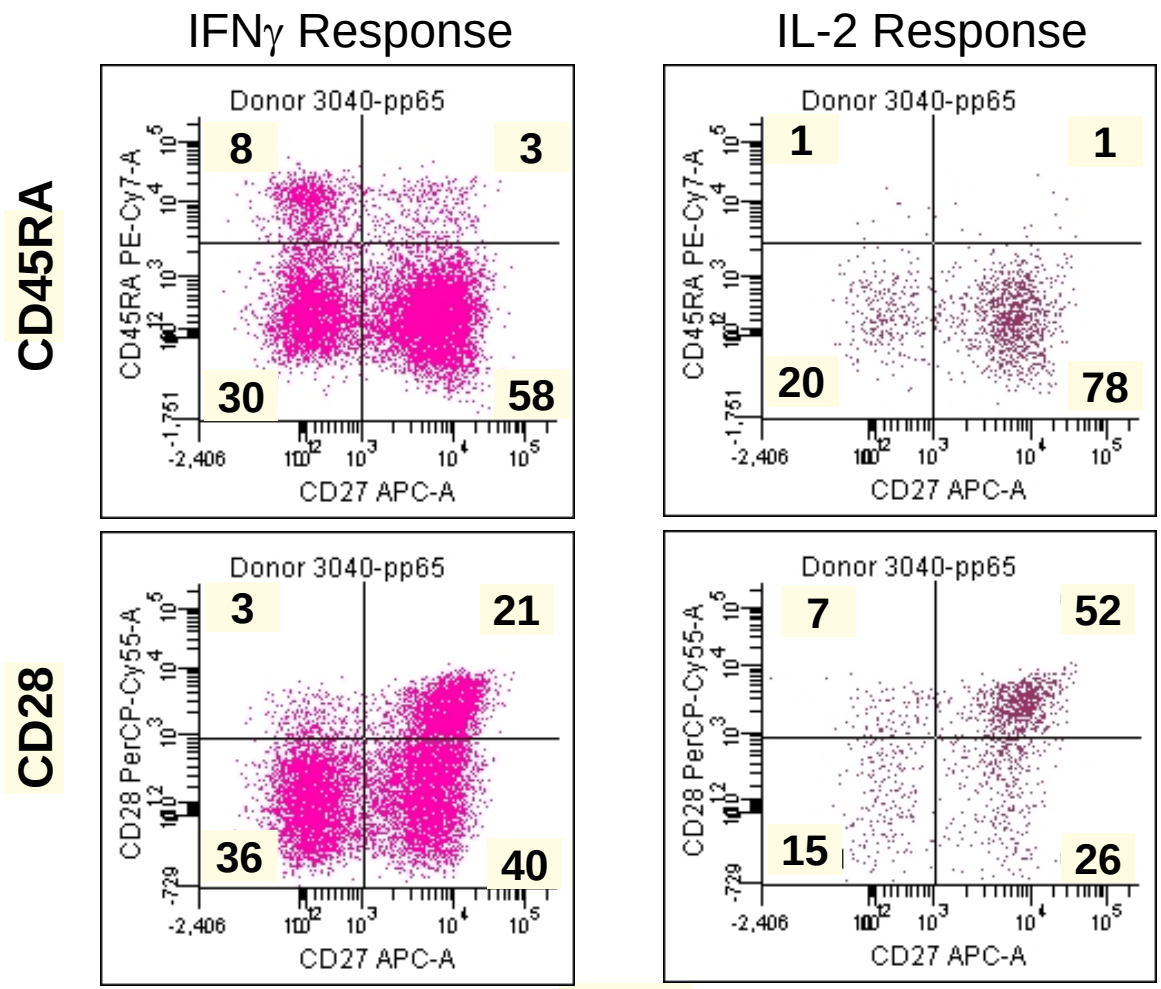
Phenotype of CMV-responsive CD8 T cells



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Reading: Compensation/Digital

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- Roederer M. Compensation. In: Robinson JP, Darzynkiewicz Z, Dean PN, Dressler LG, Rabinovitch PS, Stewart CC, Tanke HJ, Wheeless LL, editors. Current protocols in cytometry. New York: John Wiley & Sons, Inc.; 1999.

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