

COMPENSATION: AN INTRODUCTION

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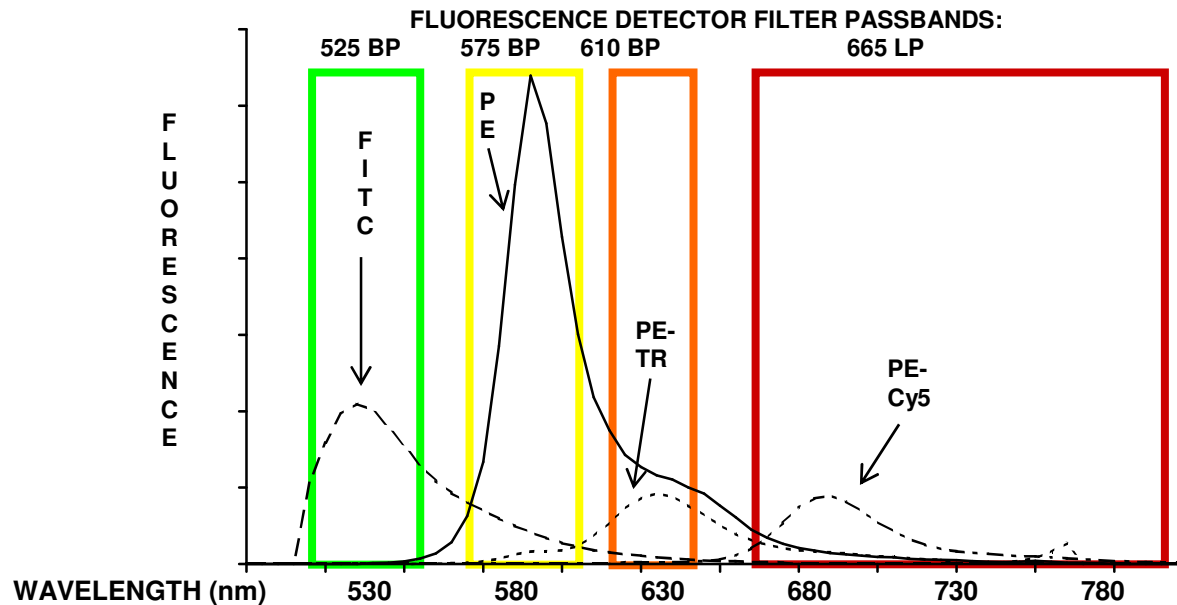
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Slides 2, 5, and 10-15 contain material from Shapiro HM: *Practical Flow Cytometry*, 4th Ed., © 2003 by John Wiley & Sons, Inc.

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WHY COMPENSATION IS NECESSARY



The emission spectra shown are for equal concentrations (mg antibody/ml) of mouse anti-human IgG directly conjugated with fluorescein (FITC), phycoerythrin (PE), and tandem conjugates of phycoerythrin with Texas red (PE-TR; ECD) and Cy5 (PE-Cy5), with excitation at 490 nm. Spectra are corrected for PMT responsivity differences at different wavelengths.

(After Shapiro HM, *Practical Flow Cytometry*, 4th Ed, Wiley-Liss, Hoboken (NJ), 2003, p. 37)

TWO-COLOR IMMUNOFLUORESCENCE USING A FLUORESCENCE-ACTIVATED CELL SORTER¹

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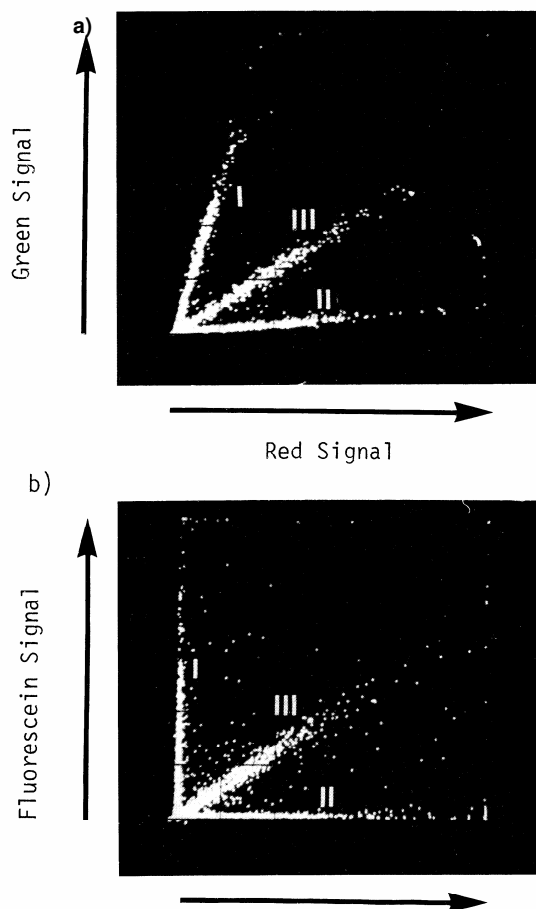


FIG. 2. Mouse spleen cells stained with anti-immunoglobulin. Group I cells were stained with fluorescein; Group II cells were stained with rhodamine; Group III cells were labeled with both fluorescein and rhodamine. An artificial mixture of these cells was analyzed (a) without, and (b) with the electronic correction for the spectral overlap of the two dyes.

The algebraic basis for this technique is simple. The signal in the green channel, $F1$, is a combination of light coming from fluorescein and rhodamine on a cell:

$$F1 = \alpha \cdot F + \beta \cdot R \quad (1)$$

where F and R are the number of photons emitted by fluorescein and rhodamine, α and β are the efficiencies for the detection of the two colors in this channel.

A similar equation can be written for the signal in the channel, $F2$:

$$F2 = \gamma \cdot F + \delta \cdot R \quad (2)$$

where F and R are defined as before, γ and δ are the efficiencies for the detection of fluorescein and rhodamine, respectively, in this channel. Subtracting an appropriate fraction of each signal from the other yields signals F' and $F2'$:

$$F1' = F1 - (\beta/\delta)F2 = \alpha \left(1 - \frac{\beta\gamma}{\alpha\delta}\right) \cdot F \quad (3)$$

and

$$F2' = F2 - (\gamma/\alpha)F1 = \delta \left(1 - \frac{\beta\gamma}{\alpha\delta}\right) \cdot R \quad (4)$$

By selecting the proper amount to subtract, the output signal $F1'$ is made proportional to F (fluorescein) and is unaffected by the presence of rhodamine emission. Likewise, $F2'$ can be made proportional only to rhodamine emission and will not be affected by fluorescein. After this correction the green channel can be referred to as the fluorescein channel while the end channel becomes the rhodamine channel.

The constants β/δ and γ/α are empirically determined. Their magnitude depends upon the spectral properties of the dyes, on the filter combinations used, and the spectral response of the photo tubes.

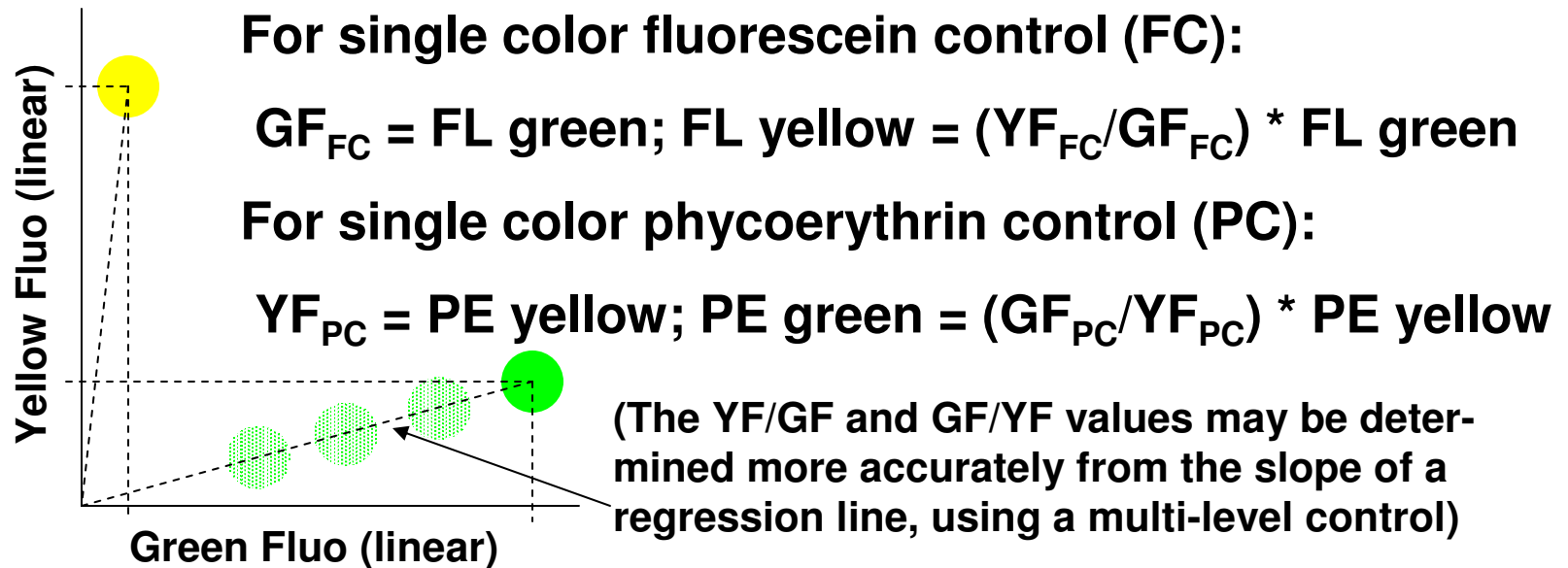
THE SUBTRACTIVE COMPENSATION “WORD PROBLEM”

(FL = fluorescein, PE = phycoerythrin)

Green Fluorescence (GF) = FL green + PE green

Yellow Fluorescence (YF) = FL yellow + PE yellow

Solve for FL green and PE yellow



Let $A = (YF_{FC}/GF_{FC})$; Let $B = (GF_{PC}/YF_{PC})$

$GF = FL \text{ green} + B (\text{PE yellow})$; $\therefore FL \text{ green} = GF - B (\text{PE yellow})$

$YF = A (FL \text{ green}) + \text{PE yellow}$; $\therefore \text{PE yellow} = YF - A(FL \text{ green})$

$FL \text{ green} = GF - B (YF - A (FL \text{ green})) = GF - B (YF) + A (FL \text{ green})$

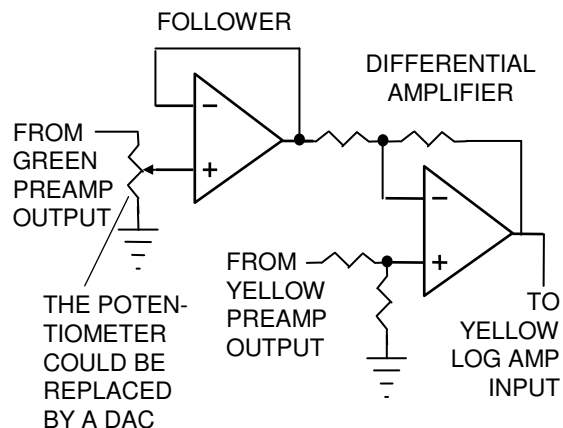
$(1-A) (FL \text{ green}) = GF - B (YF)$

$FL \text{ green} = (GF - B (YF))/(1-A)$ [FL green is proportional to FL total]

$\text{PE yellow} = YF - A (GF - B (\text{PE yellow})) = YF - A (GF) + B (\text{PE yellow})$

$(1-B) (\text{PE yellow}) = YF - A (GF)$

$\text{PE yellow} = (YF - A (GF))/(1-B)$ [PE yellow is proportional to PE total]



The circuit at left uses one potentiometer and two operational amplifiers and derives a compensated yellow fluorescence signal from green and yellow fluorescence inputs; a complementary circuit derives a compensated green fluorescence signal. Such circuits must operate on linear signals!!!

Fluorescence Spectral Overlap Compensation for Any Number of Flow Cytometry Parameters

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INTRODUCTION

In 1977, Michael Loken *et al.*¹ proposed compensating fluorescein and tetramethyl rhodamine spectral emission overlap with an electronic cross-coupling device that enabled adequate signal separation for flow cytometry analysis and sorting. The compensation circuitry involved two operational differential amplifiers (DIFFAMPs), two potentiometers, and simple cross-coupling connections to perform the real-time subtraction of signals (see FIGURE 1).² In the last 15 years, there has been little evolution in this concept and, for the most part, modern flow cytometers still employ equivalent circuitry for performing multiple fluorescence compensation. The system has worked well for two- and three-color compensation, but begins to fail at four or more colors. The reason for this color limitation is mainly a practical one. The number of potentiometer settings or coefficients (C) for performing full spectral overlap compensation is related to the number of chromophores (N) by the function, $C = N^2 - N$. For four- and five-color compensation, 12 and 20 coefficients are necessary for complete compensation. Most flow cytometers do not provide all the necessary inputs to perform complete four-color compensation and, even with the ability to enter all the potentiometer settings for four or more simultaneous colors, the settings become surprisingly complicated and confounding with greater than three parameters. Thus, flow cytometer systems that either force manual determination or limit the number of potentiometer settings practically constrain the number of simultaneous colors that can be measured and compensated.

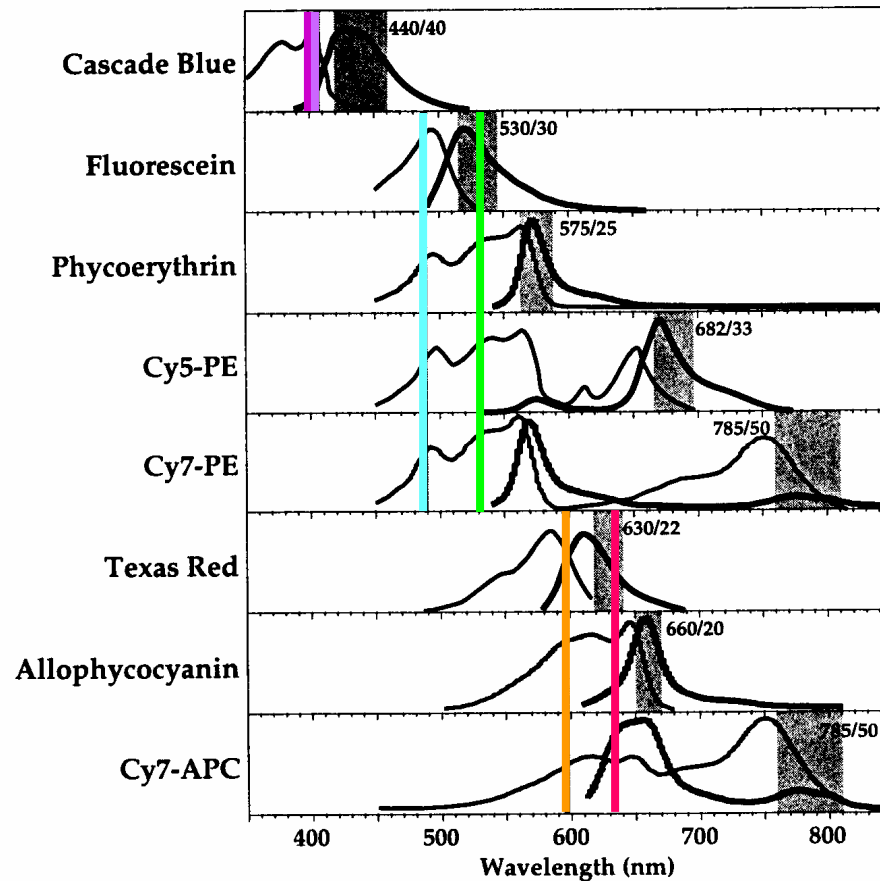
These compensation limitations as well as the paucity of suitable chromophores and antibody conjugates are significant factors in impeding the evolution of flow cytometry toward the routine use of more fluorescence parameters. Attempts have been made to ameliorate these compensation limitations by implementing spatially separated laser systems with time gating or by developing chromophores that have little or no spectral overlap.

This report examines another perhaps more general approach to compensation.

Bagwell and Adams (Ann N Y Acad Sci 677:167-84, 1993) pointed out that compensating for n -color fluorescence requires $(n^2 - n)$ coefficients, if software is used, and the same number of potentiometers (or DACs), and twice as many op amps, if hardware is used. That's 12 knobs for 4 colors, 20 for 5 colors, etc.

They described the matrix algebra of subtractive compensation for n colors, and also proposed an *additive* model, in which total fluorescence of each fluorochrome is derived from the sum of its overlap contributions to fluorescence in bands other than the primary emission region. The model also considers autofluorescence.

It now appears that subtractive compensation is preferable...



HIGH-TECH IMMUNOPHENOTYPING

Mario Roederer et al, starting in the Herzenberg lab at Stanford and now in a number of other places, have examined as many as 12 [maybe more] labels at once (8 are shown here), defining sub-subsets of lymphocytes as one practical consequence.

BEATING THE “MAINFRAME MENTALITY”

In the 1950’s, executives at IBM estimated the worldwide market for mainframe computers at under 100 units...



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1961

IBM 7094

160 kHz CPU

160 KB RAM

No HD

Power: kW

\$4,000,000

1981

IBM PC

4.77 MHz CPU

640 KB RAM

5 MB HD (\$500)

Power: 250 W

\$4,000

2001

IBM ThinkPad

1 GHz CPU

256 MB RAM

15 GB HD

Power: 25 W

\$2,000

This and cheap ADC’s from digital audio lets us keep up with 12 colors...

TRADITIONAL FLOW CYTOMETER:

**DETECTOR → PREAMP → COMPENSATION CIRCUIT
→ LOG AMP → INTEGRATOR/PEAK DETECTOR
→ 10-BIT ANALOG-TO-DIGITAL CONVERTER (ADC)**

SEMI-DIGITAL FCM (EPICS XL, CYTOMUTT):

**DETECTOR → PREAMP → INTEGRATOR/PEAK DET
→ 16 to 20-BIT ADC; compensation and logarithmic
conversion are done by digital computer**

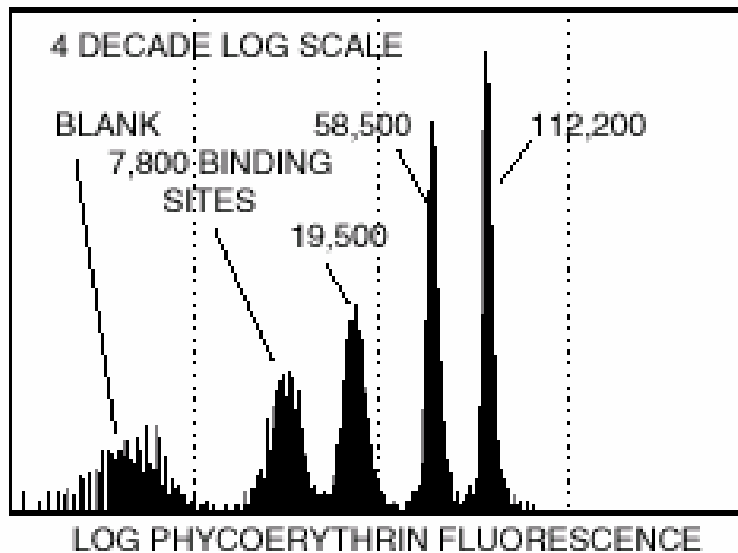
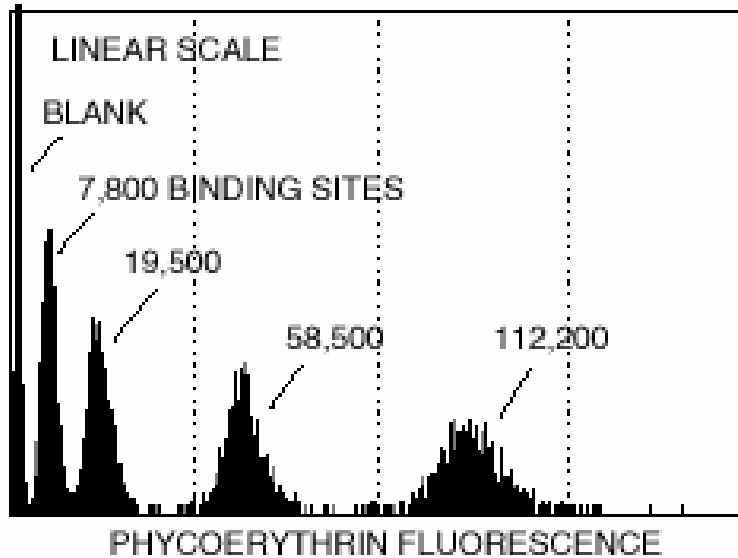
ALL-DIGITAL FCM (LUMINEX; B-D PROTOTYPE):

**DETECTOR → PREAMP → 14-BIT HIGH-SPEED ADC;
triggering, baseline restoration, integration/peak
detection, compensation and log conversion are done by
DSP chip or digital computer**

	LOG AMP OUTPUT	LOG AMP INPUT	ANODE CURRENT	CATHODE CURRENT	PHOTONS/ 10 μ s	ELECTRONS/ 10 μ s	CV (%)	CHANNEL (16 BIT ADC)
5	10 V	10 V	100 μ A	10 nA	4,430,000	620,000	0.13	65,535
4	8 V	1 V	10 μ A	1 nA	443,000	62,000	0.4	6,554
3	6 V	100 mV	1 μ A	100 pA	44,300	6,200	1.3	656
2	4 V	10 mV	100 nA	10 pA	4,430	620	4.0	66
1	2 V	1 mV	10 nA	1 pA	443	62	12.9	7
0	0 V	100 nV	1 nA	100 fA	44	6	40.8	0

WHAT THE DECADES MEAN (ELECTRONICS)

The bottom of the scale on a real flow cytometer is likely to represent only a few photoelectrons; if the minimal number of fluorochrome molecules detectable is 100, the top of a 4-decade scale represents 1,000,000 molecules.

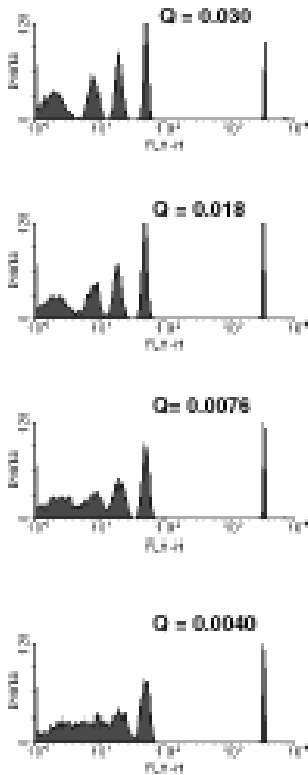


THE “PICKET FENCE”

The same data, taken from measurements of a PE-antibody bound to antibody-binding beads, are shown on a 256-channel linear scale in the upper plot, and a 256-channel (64 channels/decade) log scale in the lower plot. No log amps were used; the data were taken with a 16-bit ADC.

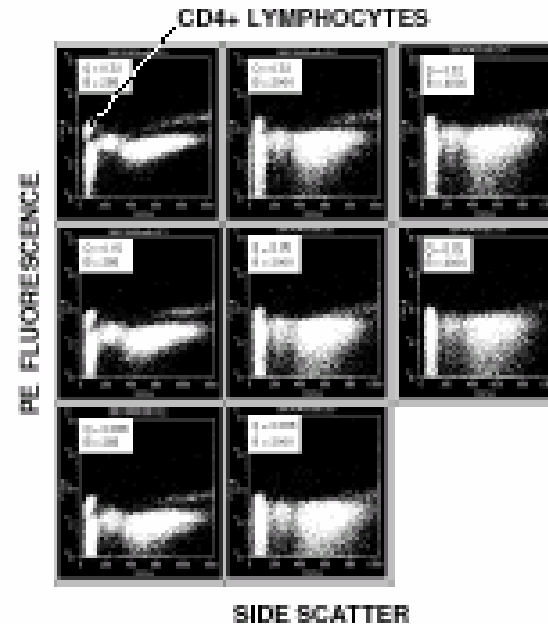
The “picket fence” arises because one cannot convert low 16-bit linear numbers to log values unambiguously; one would need a 20-bit converter. to “tear down the fence.” Look at the fence as if it had an attached sign saying “Beware of the Data.”

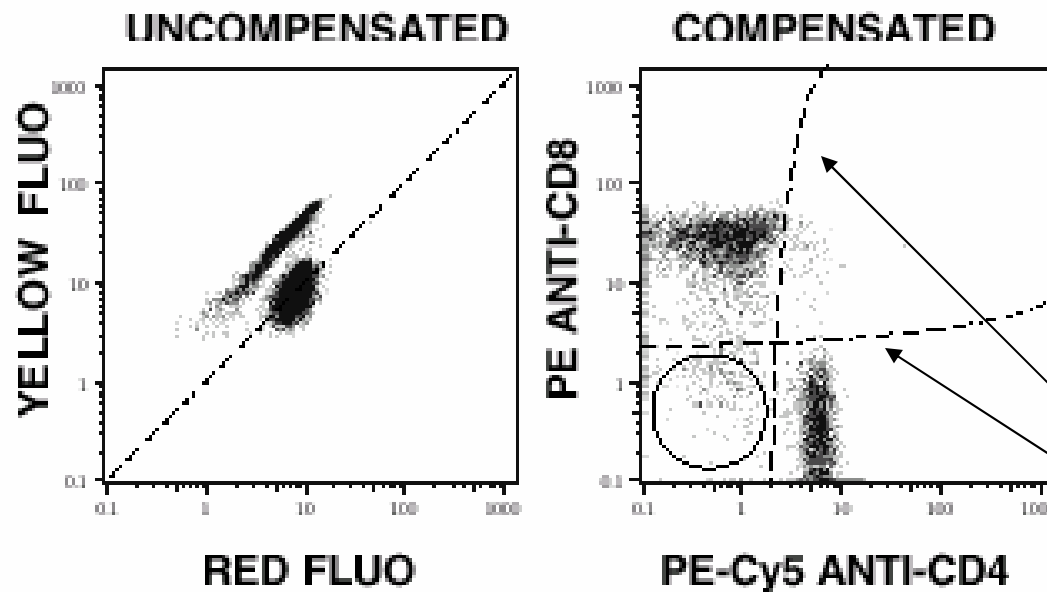
“Q and B”: Determinants of Cytometer Sensitivity



Bob Hoffman of BD Biosciences and Jim Wood, formerly of Beckman Coulter, have formulated the model of cytometer sensitivity now preferred by leading flow jocks. Q is the quantum efficiency of detection, typically 0.25 photoelectrons/MESF for PE and 0.012 photoelectrons for fluorescein on a modern benchtop cytometer. B is background; the picture below shows deleterious effects of increased background on resolution of dim objects.

CVs of dim objects are dominated by photoelectron statistics; lower Q means fewer photoelectrons are detected, CVs go up, and separation of dim populations gets worse.





“To get to
compensation
Heaven, you
must pass
through the
Curly
Gates...”

Figure 5-12. How compensation gets data to not quite fit into quadrants.

Photoelectron statistics dictate that the variance of a dim or “negative” signal will be higher than that of a positive signal. In the case of “double negatives”, the amount by which compensation changes the original signal is small; in the case of “single positives”, the variance of the compensated “negative” data will be greater than the variance of the “negative” data in the “double negatives”. The real data are probabilistic; compensation is deterministic. Spectral overlap is background (B); additive compensation therefore tends to lose more than it gains.

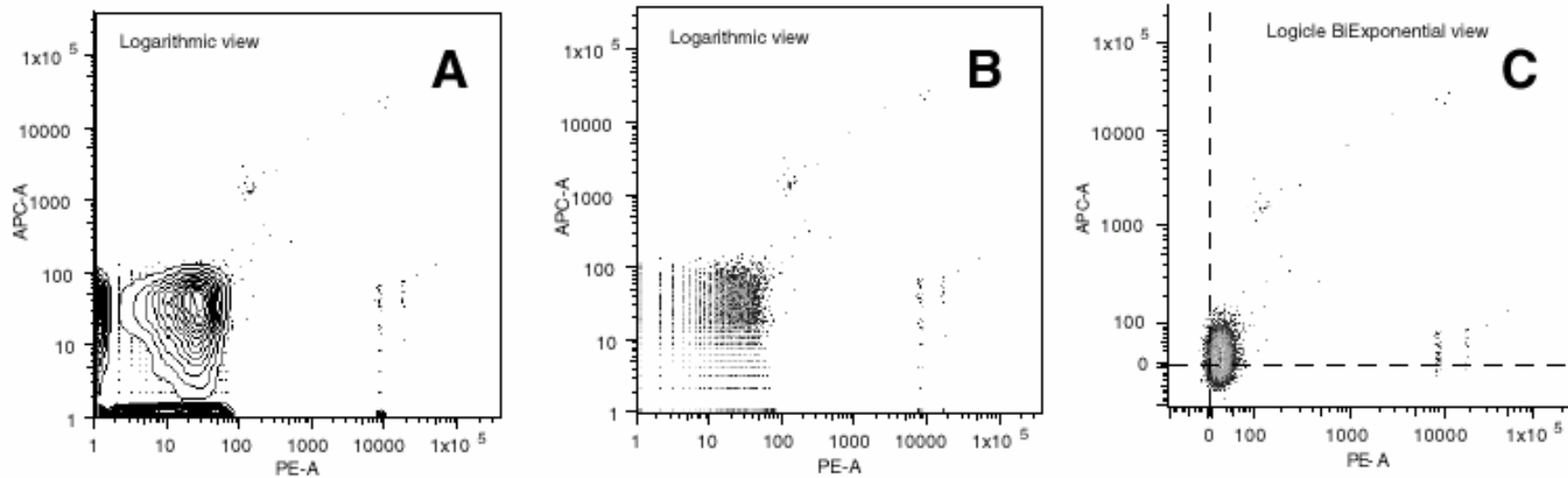


Figure 12-1. Tearing down the “picket fence” and reuniting the negatives using a BiExponential data transform instead of a logarithmic scale. Courtesy of David Parks and Wayne Moore (Herzenberg Lab, Stanford).

The log of a negative number is undefined, meaning that an algebraic (or electronic) fudge factor is needed to keep compensated data on a log scale. This can generate the appearance of multiple populations among the negatives, and, for those who can't read the signs on the picket fences, a transformation to a biexponential scale may help.

**As I'd say, one May in Montpellier,
There's more to know of flow
Than I wrote years ago.
Of compensation, polarization,
GFP and DSP and bead assays galore.**

**Lists of probes now tax our frontal lobes,
And we've got laser beams
Beyond our wildest dreams.
With my book slated to be updated,
I requested help in getting on, on with my chore!**

**Colleagues heard, and when they got the word,
They e-mailed files of cells
And piles of URL's,
Sent me plots of what they'd stained,
Told me what should be explained,
And all I've gained is here contained.**

**Edition Four is finally out the door,
Fully revised; now supersized.
Adding references and figures, I have stressed
How the field has progressed.**

**I've done as good a job as I could do,
All thanks to help from you!**

From you, from you, all thanks to help from you!

