Playing Good Comp, Bad Comp

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Outline

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- Those pesky PMT settings
- Man *versus* machine
- Compensation standards
- Feng Shui for your protocols?
- Baseline offset
- 8-color gating

Those Pesky PMT Settings

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- Make unstained or negative controls “look good”
- Make important positive (especially dim) populations “look good”
- Once you have settings you like, run FlowSet beads and record MFI. These will serve as target channels for subsequent calibration with beads

Coming up with satisfactory PMT settings is an empirical science. In the 2 to 4 color days it was often sufficient to make unstained controls look good by tweaking voltage and gain until the negative populations came off axis, in the lower part of the first decade. Digital processing and the results of compensation often make this impossible in the multi-color world. It is probably more important to see that the positive populations of interest are properly placed, were the dynamic range is well used without putting the brightest cells off scale (where they can not be compensated).

Once you have settings that you like, you can make certain that they are reproduced in subsequent experiments by the use of calibration standards such as FlowSet beads.

Here is a simple 2-color experiment that we did to demonstrate the effects of different PMT gains on compensation.

We set the PMT gain with FlowSet beads. For the High Gain condition the beads were placed at channel 70 in the FITC and PE channels (this is channel 700 in the B-D and Cytomation Universes). For the Low Gain condition, the beads were placed in channel 30, and so on. The resulting voltage settings are shown in parentheses.

Correct 2-color compensation was then determined using single FITC and PE stained cells and the Beckman Coulter automatic digital compensation (ADC) software. (More on this later).

A PMT Experiment

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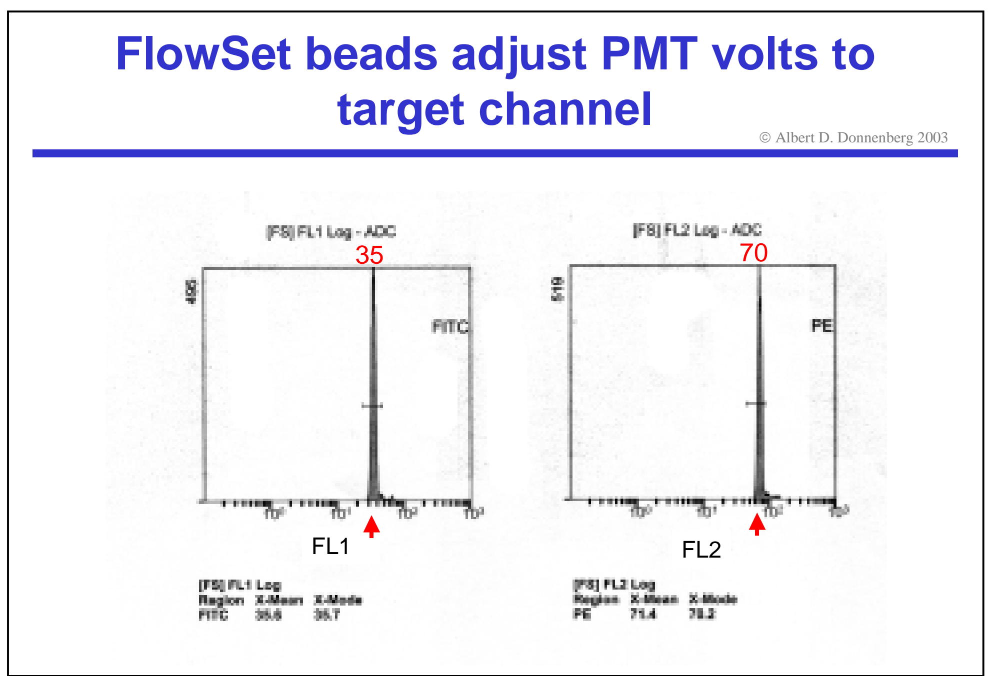
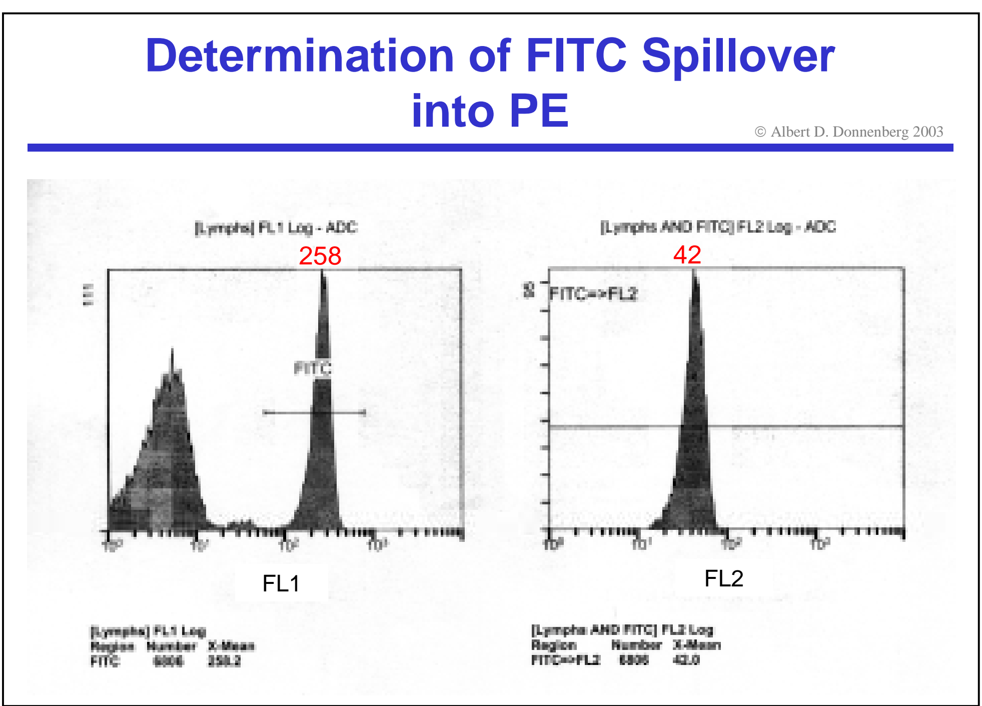
- 2-color (CD4 FITC, CD8 PE)
- PMTs set with FlowSet beads
- Compensation determined with ADC

	FL1 Ch (V)	FL2 Ch (V)
High gain	70 (815)	70 (780)
Low gain	35 (750)	35 (705)
Mixed	70 (815)	35 (705)
Mixed	35 (750)	70 (780)

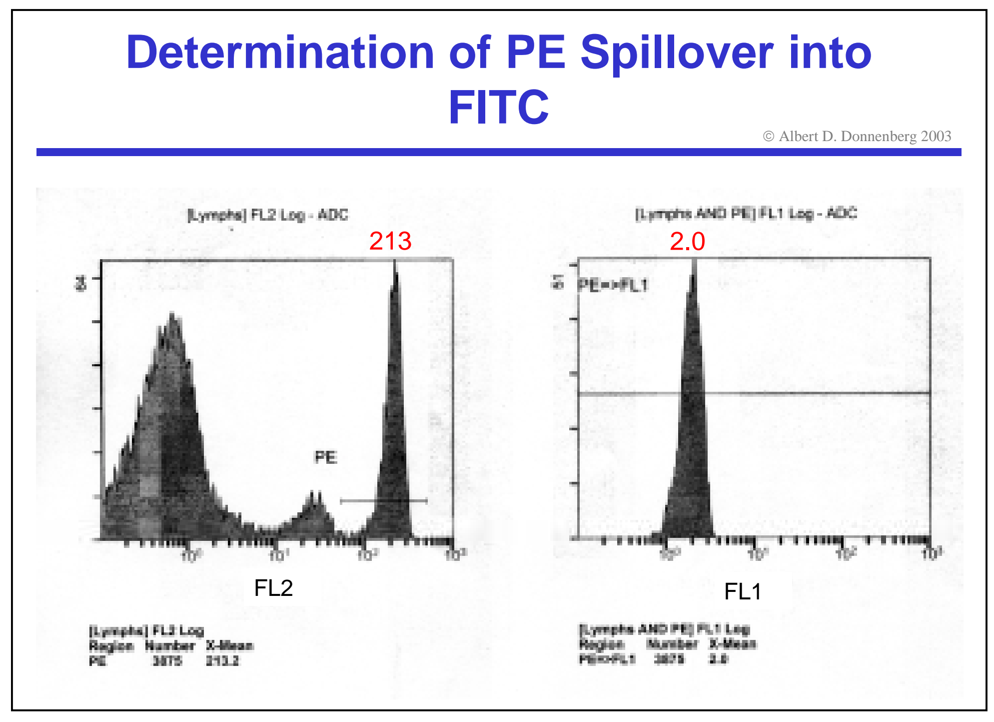


Here is an example of how we set the PMT voltages for one of the “mixed” conditions. The beads were placed at channel 35 in the FITC parameter, and at channel 70 in the PE parameter.

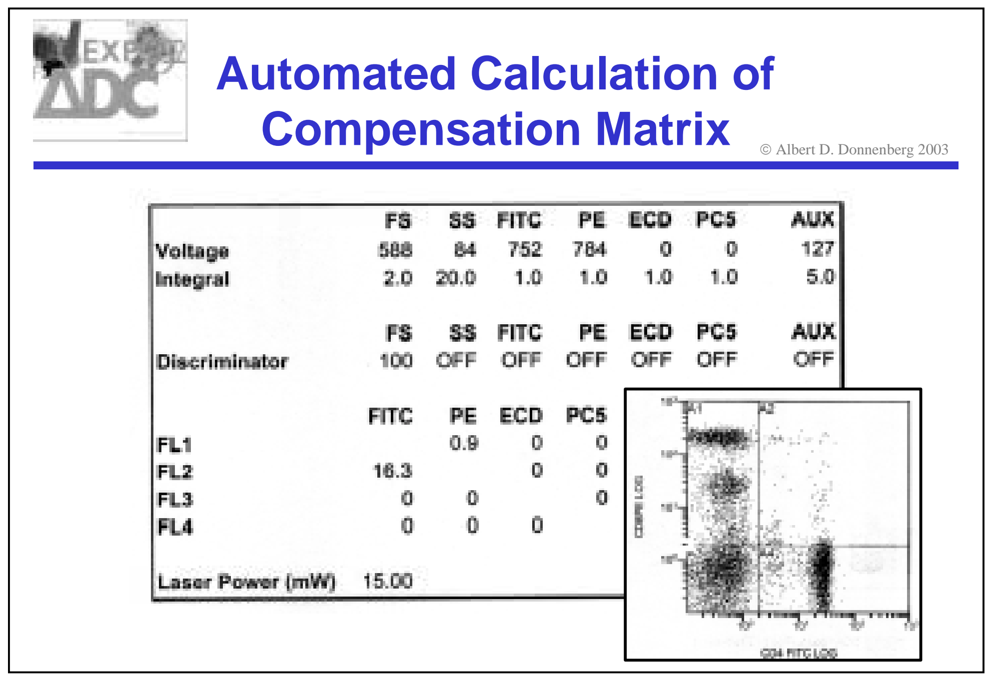


The B-C ADC software measures spillover of single stained preparations. Here, PBMC were stained with Anti-CD8 FITC. A gate was placed about the positive cells, and spillover was measured into the PE channel.

Similarly, PBMC were stained with anti-CD8-PE, a gate was placed about the positive cells, and spillover was measured into the FITC channel. The process is essentially the same for 5-color, continuing with single stained samples for each fluorochrome.



ADC automatically calculates the compensation matrix from the measured spillover. The procedure is no more difficult for 5-color than for 2-color. The inset shows this matrix applied to a “verify” tube (CD4-FITC, CD8-PE).



	FS	SS	FITC	PE	ECD	PC5	AUX
Voltage	588	84	752	784	0	0	127
Integral	2.0	20.0	1.0	1.0	1.0	1.0	5.0

	FS	SS	FITC	PE	ECD	PC5	AUX
Discriminator	100	OFF	OFF	OFF	OFF	OFF	OFF

	FITC	PE	ECD	PC5
FL1		0.9	0	0
FL2	16.3		0	0
FL3	0	0		0
FL4	0	0	0	

Laser Power (mW) 15.00

Here are the resulting compensation matrices, determined at the different PMT settings. You can see that altering PMT gain proportionately does not change the compensation matrix. Disproportionate changes between PMTs results in very different compensation.

Results

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Valid CD4 and CD8 determinations at all combinations of PMT volts, but very different compensation matrices for "mixed" settings

		FL1 into FL2	FL2 Ch into FL1
High gain	(70/70)	8.0	2.0
Low gain	(35/35)	8.0	1.8
Mixed	(70/35)	4.0	3.9
Mixed	(35/70)	16.3	0.9

Moral

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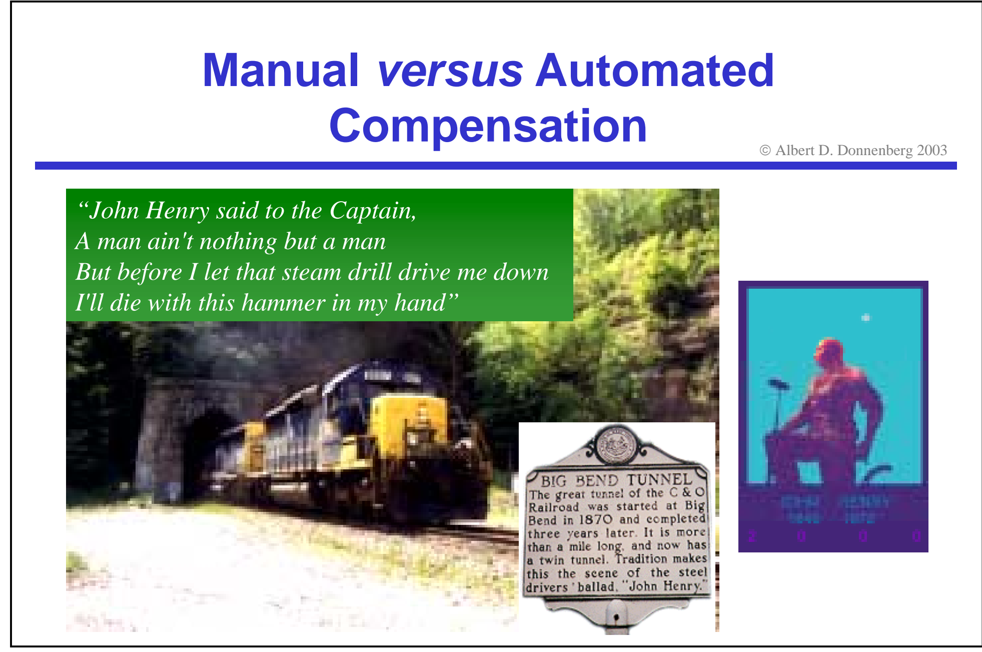
- Changing the PMT settings proportionately does not change the compensation (to the extent that the instrument is linear)
- Changing the PMT settings or gains disproportionately will change the required compensation
- Moral: You can't just dial up the voltage to bring up a dim population

Stories of man versus machine date to the dawning of the industrial revolution. The Big Bend Tunnel in rural West Virginia (closer to Pittsburgh than we care to admit), was the site of the legendary contest that pitted one John Henry against the newly invented steam drill. John Henry raced the steam drill to determine who could excavate more tunnel in a 12 hour shift. John Henry was victorious, but died of exhaustion shortly after the contest.



Our modern day John Henrys (Vera, Petar, and I) pitted ourselves against the B-C automatic digital compensation software (ADC).

Steam Drill Arm:

Each of us ran the same single stained controls using ADC, to arrive at a compensation matrix. PMT settings were held constant.

John Henry Arm:

Then, we ran the same single stained controls again, each of us manually determining a compensation matrix by matching the medians of negative populations. Again, using the same PMT settings.

"Nine pound hammer killed John Henry
And it's gonna kill me, and it's gonna kill me"

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Experiment

CD8 FITC/ CD8 PE/ CD8 ECD/ CD8 PC5/ CD8 PC7
CCR7 FITC/ CD62L PE/ CD45RA ECD/ CD27 PC5/ CD8 PC7

PMT settings

Lab favorite settings

Compensation

ADC and visual compensation (matching medians of negative pops within 0.2) performed on the same day by 3 different users (VSD, PJP, ADD)

These are the parameters that we compared.

Man *versus* Machine

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Compare

- Between user variability (within each compensation method)
- Correlation of Manual and ADC
- Time required for compensation
- Effect of “extreme” compensation matrices on the outcome variables

Here are the means of the 3 automatic and 3 manual compensation matrices determined in our experiment. The mean values are very close (we are old hands at manual compensation) but look at the CVs!

Steam Drill 1, John Henry 0.

Between User Variability

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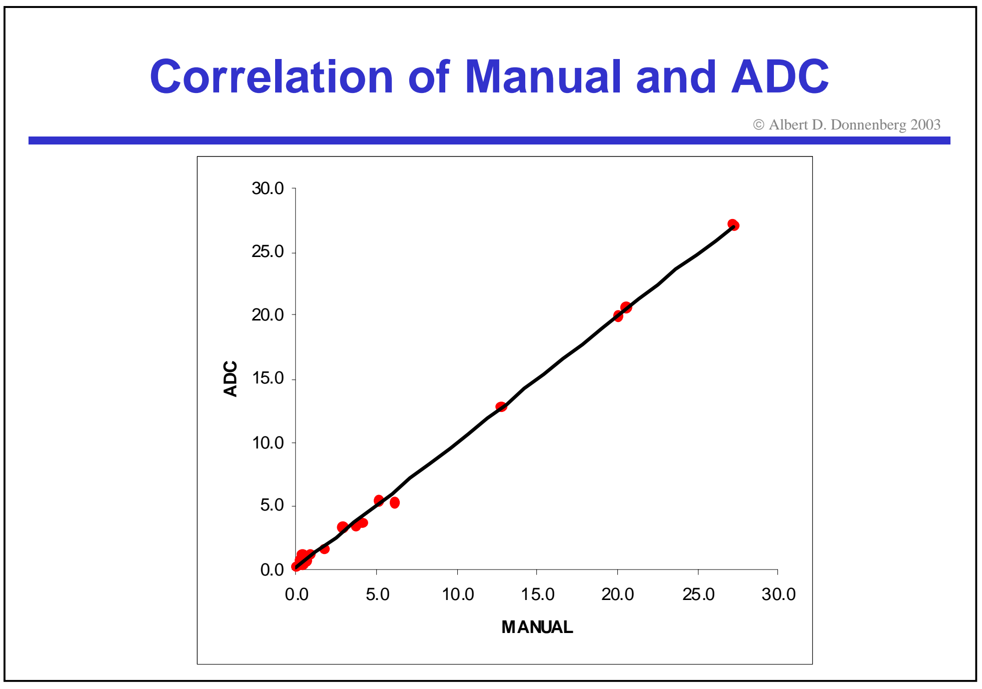
This =>	That	MEAN_ADC	MEAN_MAN	CV_ADC	CV_MAN
FITC	PE	20.7	20.6	0.3%	0.5%
FITC	ECD	5.5	5.2	1.1%	3.8%
FITC	PC5	0.7	0.7	0.0%	75.6%
FITC	PC7	0.2	0.0	0.0%	173.2%
PE	FITC	0.7	0.5	0.0%	12.4%
PE	ECD	27.0	27.3	0.0%	0.9%
PE	PC5	3.7	4.1	0.0%	19.0%
PE	PC7	0.3	0.2	17.3%	49.5%
ECD	FITC	1.2	0.4	0.0%	26.6%
ECD	PE	19.9	20.1	0.8%	2.0%
ECD	PC5	27.2	27.2	0.2%	1.7%
ECD	PC7	3.5	3.7	0.0%	44.3%
PC5	FITC	0.7	0.2	0.0%	132.3%
PC5	PE	3.3	2.9	1.7%	11.0%
PC5	ECD	1.2	0.9	0.0%	6.7%
PC5	PC7	12.8	12.7	0.4%	3.6%
PC7	FITC	0.7	0.4	7.9%	56.8%
PC7	PE	5.3	6.2	1.1%	17.3%
PC7	ECD	1.6	1.8	3.5%	19.9%
PC7	PC5	0.4	0.4	15.7%	15.7%
	MEAN			**2.5%**	**33.6%**

As suggested on the previous slide, the average manually and automatically determined compensation coefficients were indistinguishable.

Steam Drill 2, John Henry 1.



It took each of us 5 minutes to determine the 5-color compensation matrix using ADC. It took Vera and Petar 15 minutes to do the same thing manually. I was a little slower, partly because I was taught to run the 5 tubes once, determine a compensation matrix, and then rerun each single stained tube to tweak the matrix. This was not the only reason that I was slower.

Steam Drill 3, John Henry 1.

Time Required for Compensation

	Time (minutes)		
Method	VSD	PJP	ADD
ADC	5	5	5
Manual	15	15	30*

** Kept on falling asleep*

The goal here was to see how the different compensation matrices would effect the results of our 5-color memory/naïve protocol.

The three matrices that we determined by ADC were so close that we arbitrarily chose the first (Vera's) to use in our comparison. We took this as our "Gold Standard."

When we looked at the individual manually determined matrices, these were close too, at least to the human eye. In order to evaluate the effect of "extremes" in manually determined compensation, we constructed 2 artificially extreme matrices. The first took, for each pair of fluorochromes, the minimum compensation coefficient from each of our 3 John Henrys. The second used the maximal values.

Effect of "Extreme" Compensation

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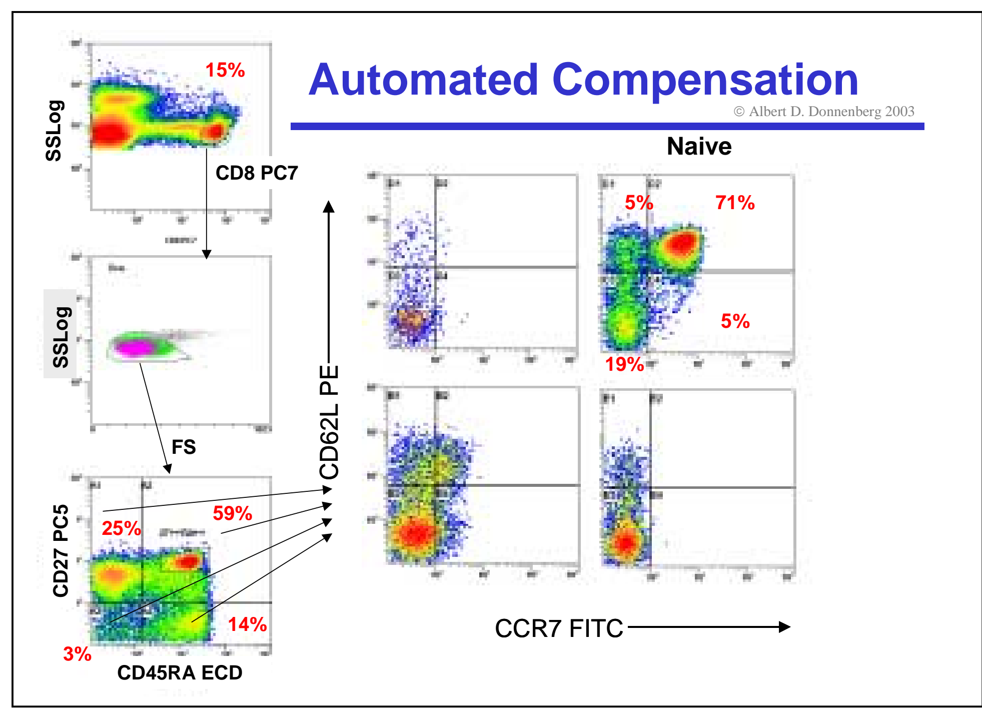
2 "extreme" compensation matrices were artificially created using the minimum and maximum values obtained by our 3 operators.

These turned out not to be so extreme

Here are results of our "Gold Standard" analysis using the ADC compensation values. First we gated on CD8+ events (CD8 vs log side scatter). Then we passed the CD8+ events through a FS by log side scatter gate to eliminate apoptotic cells (none here) and also troublesome events with high forward scatter and intermediate side scatter. This is especially important on the B-C FC500 which can not do doublet discrimination and 5-color at the same time. CD8+ cells falling within the scatter gate were then passed along to our primary subsetting markers, CD45RA and CD27. Each of the resulting quadrants was used in a compound gate. In this comparison we will focus on naïve cells, which comprised 59% of total CD8+ T cells. The major population within these was CCR7+/CD62L+, accounting for 71% of CD45RA+/CD27+ CD8+ cells.



How then did our “extreme” matrices affect the results? Here are the results with “minimum values” compensation matrix (the difference between the present results and our “Gold Standard” measurement are shown in parentheses.:

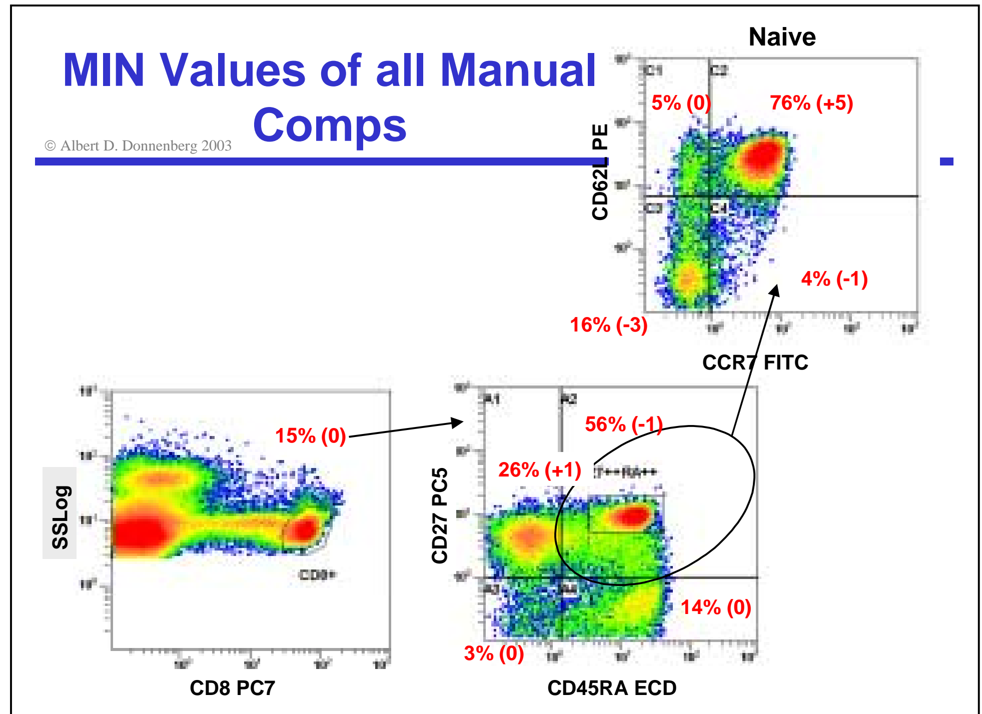
CD8s were exactly the same

There were small or no differences in CD45RA versus CD27

The results of undercompensation are visually apparent in CCR7 vs CD62L, but the results are not really that different in terms of % positive. Quantitative measurement of fluorescence would have been a different story.

For the purpose of this comparison, I kept regions and gates constant .

Steam Drill 4, John Henry 2.



Here are the results with "maximum values" compensation matrix (the difference between the present results and our "Gold Standard" measurement are shown in parentheses.:

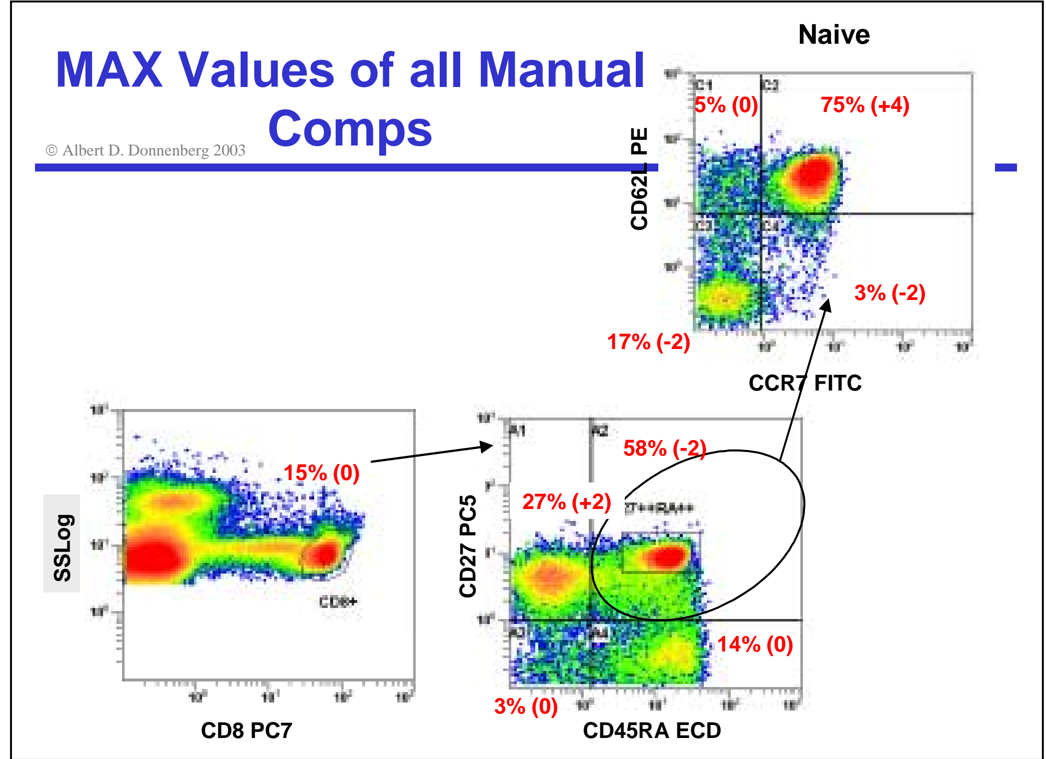
Again, CD8s were exactly the same

There were small or no differences in CD45RA versus CD27

The results of overcompensation are masked by the baseline offset (more on this later)

Again, CCR7 vs CD62L results are off, but not by much.

Steam Drill 5, John Henry 3.



Well, the steam drill won, but our John Henrys lived to compensate another day. The very small CVs between independent ADC compensation, the orthogonal appearance of the populations (but beware, see MRs slide set), and my inherent bias that this is a problem of math and physics and best left to an algorithm, all favor the Steam Drill.

But there is also a message of hope here. If you chose reasonable PMT settings, and reasonable combinations of reagents (more on this later), the analysis of percent+ cells is more robust than I would have imagined before conducting this empirical experiment.

If you are a reasonably skilled operator and run the correct controls (and are not concerned about quantitative fluorescence), chances are that your manually determined multi-color compensation is just fine.

However, our job is to remove sources of variability. In our facility, where we have 2 B-C instruments and 2 Dako Cytomation instruments, we routinely use ADC software on the former, and (on the basis of these results) we resolve to do offline automated compensation (Verity WinList) for the latter in the future.

Man *vs.* Machine Conclusions

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- The steam drill wins hands down
- Manual compensation is slower and more variable, but properly done, the variability is not sufficient to invalidate 5-color results
- John Henry lives to Flow another day

It seems that PE-Cy7 is particularly susceptible. You can prevent deterioration by protecting your antibodies (and stained cells) from light. We literally are working in the dark (well..in a dimly lit room).

Another alternative, preferred by MR, is to use antibody capture beads for compensation standards. The advantage of these standards is that you are compensating with the actual reagents to be used in your experimental tube, and unlike real cells, the beads are uniformly bright, independent of the antibody specificity.

About Compensation Standards

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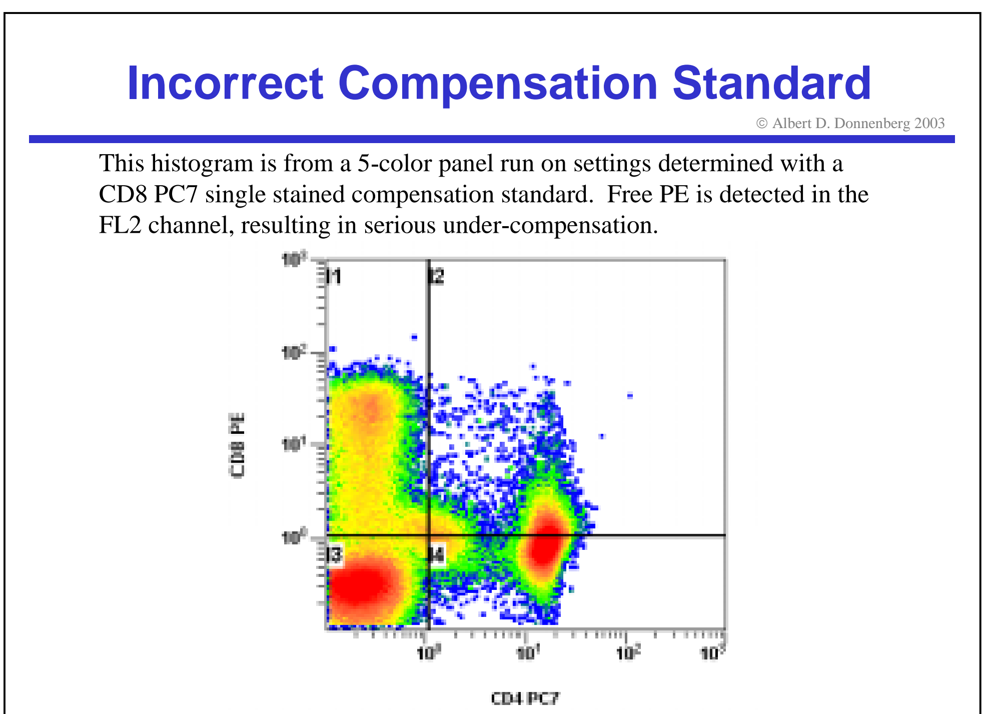
- In an ideal world its all about the fluorochrome; the antibody to which it's conjugated is irrelevant to compensation
- In the real world tandem dye-conjugated antibodies can deteriorate, leaving varying amounts of free PE.
- If this happens, the compensation determined by your standard will be wrong

Here is an example where capture beads would have been better! We compensated this 5-color combination with CD8 antibodies conjugated to our 5 fluorochromes. You can see that we were undercompensated for CD4-PE-Cy7, which spilled over into PE. The reason? Although this reagent was treated well, and was within its expiration date, there was clearly more free PE than in the CD8-PE-Cy7 reagent used for compensation.

How to detect such problems? Print out histograms for all of you pairs of fluorochromes (ungated) and use your eyes to confirm compensation (do not use baseline offset, see below).



Feng Shui is a Chinese art intended to maximize harmony, health and happiness by arranging the objects in one's surroundings to their best possible advantage. For example, according to Feng Shui, it might not be such a good idea to put your beautiful set of razor-sharp knives on prominent display in your kitchen.

In polychrome (multicolor) flow cytometry, the art of Feng Shui should be considered when deciding on antibody combinations.

Here we consider two different 3-color arrangements of CD45, CD25 and CD4, the object being to best visualize the sometimes elusive CD4 regulatory T cells (CD45+, CD4+, CD25dim).

According to the theory of Feng Shui, the first combination shown here should be unfavorable, since the bright marker (CD45) is labeled with FITC, and can spill over significantly into the dim maker (CD25-PE). Conversely, the second combination should be more favorable, as there is little spillover from the PE to FITC channels.

Feng Shui for Your Protocols?

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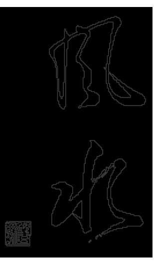
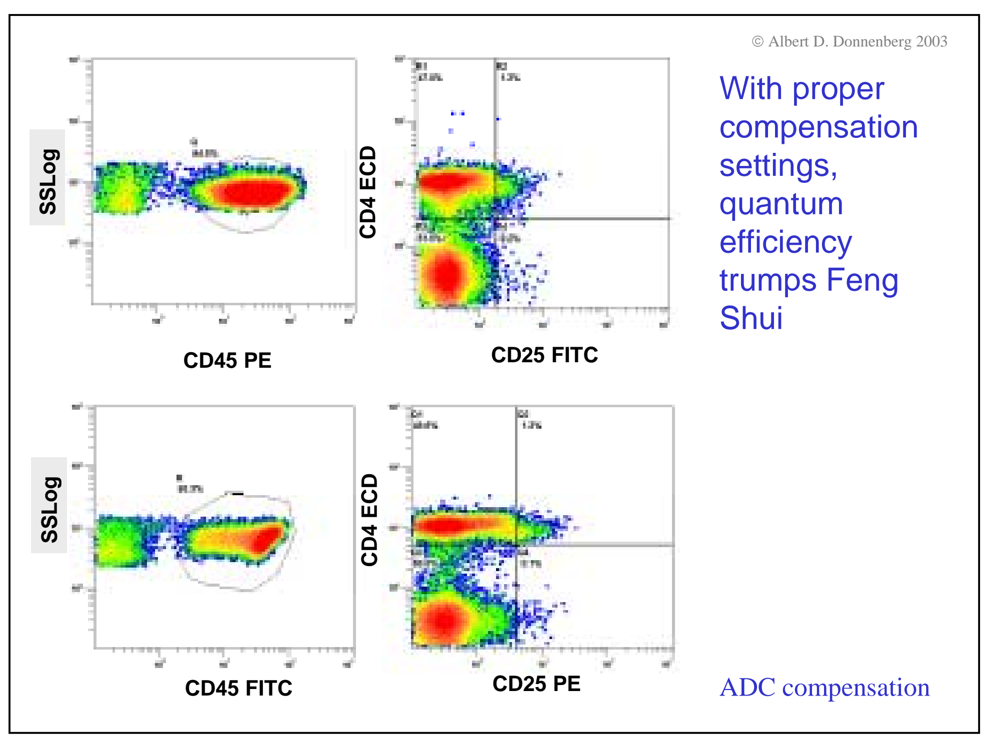


Decrease the flow of negative energy by avoiding antibody combinations that place a bright marker adjacent to a dim marker when there is significant spillover from the fluorochrome labeling the bright marker to the fluorochrome labeling the dim marker

	FITC	PE	ECD
Bad Feng Shui:	CD45	CD25	CD4
Good Feng Shui:	CD25	CD45	CD4

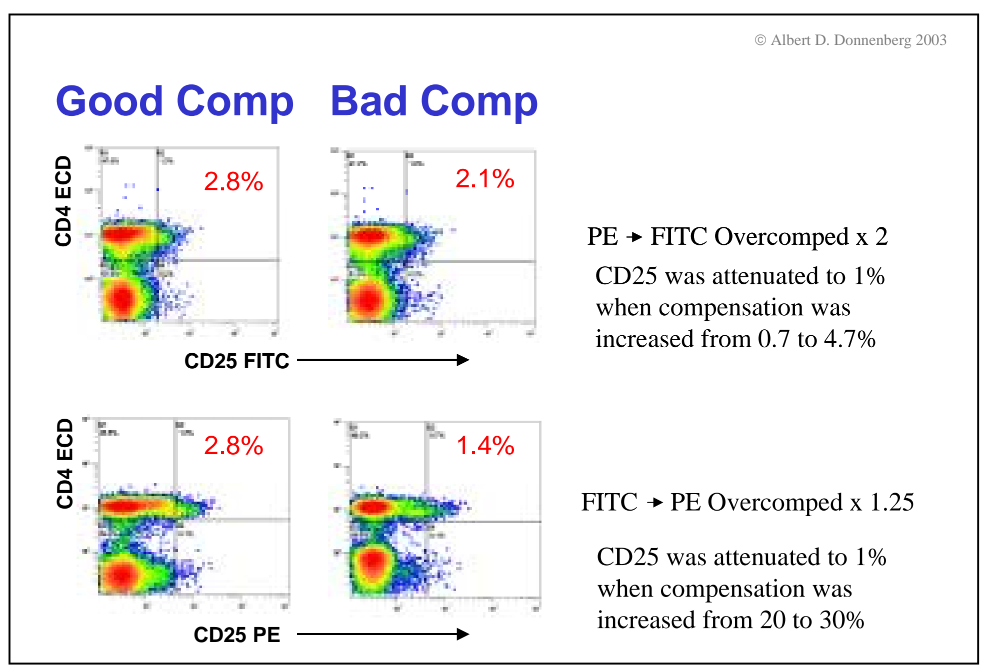
So much for theory. With proper compensation (as determined here by ADC) it is easier to visualize the CD25 dim population in PE! PE has much higher quantum efficiency than FITC, and so can provide better signal to noise.

A few other things worth noting: The FMO control recommended by MR (in this case CD45 plus CD4) would have been most helpful in determining exactly where the CD25 dim population begins. Here, I chose the leading edge of the CD4- population (mostly CD8+) as these are known to have few CD25+ cells. There are other tricks that can help. For example CD25dim cells have a little less side scatter than the majority of CD4+ T cells.



We enjoyed playing “good comp bad comp” with our data. The object of this exercise was to determine how resistant the two panels were to intentional overcompensation. It appears that the combination with the dim marker (CD25) in FITC was a little more robust, but I would still go with CD25 in PE!

PE-Cy5 and APC can be used together on instruments with noncolinear blue and red lasers. But beware, the Cy5 will be excited twice. First by the blue laser through the PE, and then by the red laser (direct excitation of the Cy5). This can usually (but not always) be compensated out. Feng Shui in the choice of markers for this fluorochrome combination, can be very important.



Feng Shui?

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Better to get the compensation right and match the dim marker with the best fluorochrome, but beware combinations like PE-Cy5 and APC.

Adherents of the graphic design principles espoused by Edward Tufte (The Visual Display of Quantitative Information, Graphics Press, Cheshire CN, 1983) all agree that a misleading graphic is worse than no graphic at all. Even when data is properly compensated it is possible to end up with many events plastered on the axes of bivariate plots. This can lead to very misleading graphics. For example double negative populations could appear visually to comprise a small proportion of cells, when in reality they are a major population.

In the most recent software releases, Expo 32 and RXP, Beckman Coulter has included the option of baseline offset for the display of bivariate scatter and density plots. Baseline offset introduces a small amount of normally distributed “noise” to values that are in the 0 channel. The data themselves are not affected, only the display. The effect is that data that formerly piled up on the axes now form a nice neat ball in the first decade.

Purists object to this, allegedly because real data in the first decade is being obfuscated by artifactual results. I have also heard it argued that baseline offset “adds noise” to measurements above the first decade, but this is a misunderstanding of the algorithm implemented by B-C.

There are some real downsides to baseline offset. First, inexperienced users are apt to leave it on during compensation (despite warnings to the contrary). With baseline offset on, overcompensation, even severe overcompensation, is not apparent in the least (see slide 27). Even if you (an experienced user) will never make this mistake, baseline offset proves a problem for evaluating the work of others: it all looks gorgeous, no matter how overcompensated it is.

A lot of the problems that baseline offset attempts to fix arise from the fact that zero is undefined in log space, and we almost universally use log transformations to visualize our data. MR and JT both have provided examples of a transformaton related to the hyperbolic sine function, that is symmetrical about zero, and rather linear close to zero and then increasingly logarithmic. Yes, symmetrical about zero means that it can accommodate negative numbers. Why on earth would I want to plot negative fluorescence, and what could this possible mean? Its really not all that counterintuitive. For well compensated data on a well calibrated instrument, the median fluorescence channel of a negative population could be close to zero. But remember, all measurements have error, and in compensating we are subtracting several measured values from the fluorescence value of interest. In our example where the median is zero, fully half of the values will be below zero (I.e. negative). The plots of well compensated data using this function are quite striking and intuitive, although for most of us the negative values will take some getting used to. In addition to B-D, Verity software plans to add a related “hyperlog” function to their next version of WinList.

Until these alternative transforms are widely available, baseline offset is in my opinion a good thing, and we will continue to use it for our publication graphics.

Baseline Offset

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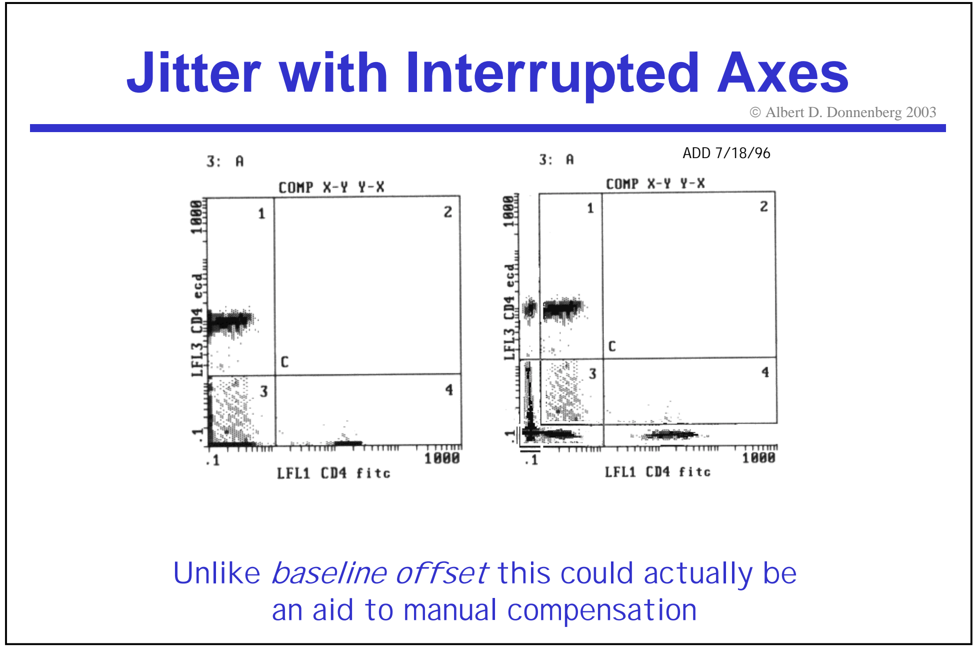
Addition of Gaussian noise to data in the first channel

- 2 Parameter histograms provide more accurate visualization of the data distribution
- Obfuscates data in first half of the first decade (no great loss)
- Many sins committed with baseline offset on during compensation (overcompensation is not evident)

Here is a data display trick that we pitched to B-C a number of years ago (without success). On the left is a typical example of properly compensated data where the FITC signal is plastered against the X axis. The histogram shows a mixture of single stained CD4+ cells (ECD and FITC). There is no way that you can tell from this graphic that there are equal numbers FITC and ECD labeled cells

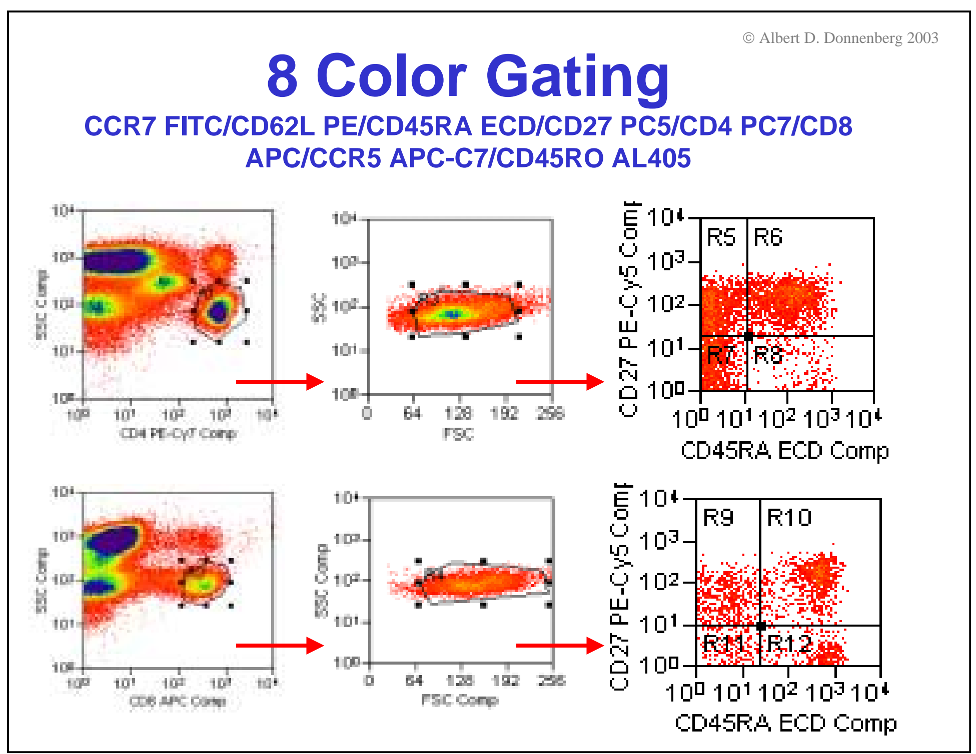
On the right is a Photoshop-created mockup of a data display where the events on the axis are projected to the left of the Y-axis and below the X-axis with a small amount of random noise added. This is just like baseline offset, except the results are not superimposed over data in the first decade. Unlike baseline offset, you could really tell if you were overcompensated, because the population(s) outside the axes would continue to grow as more events are placed in the zero channel.

Do you like this better than baseline offset?

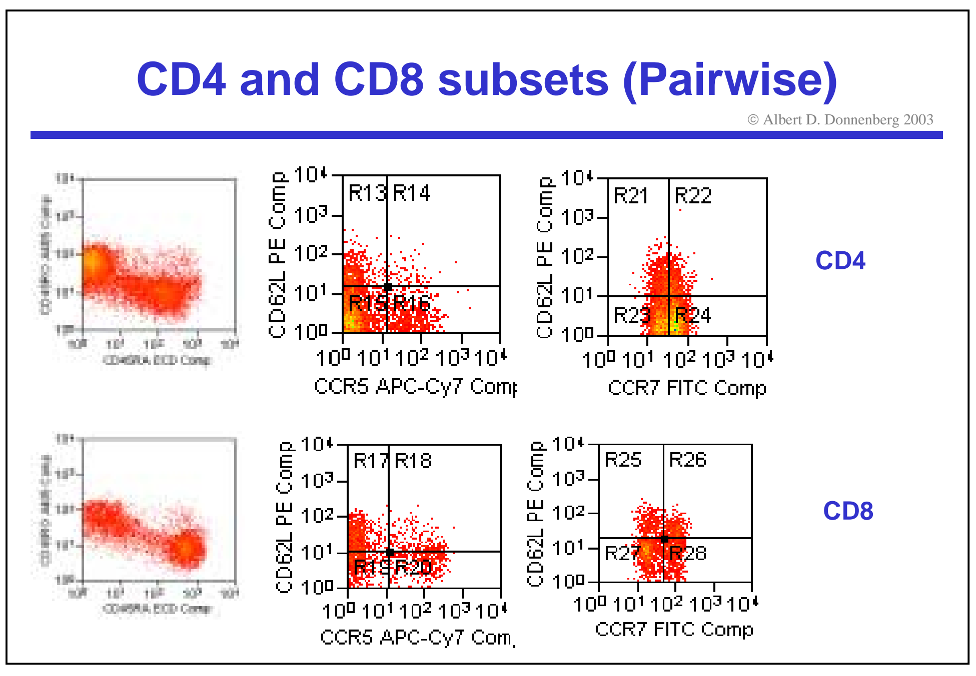


Here is our foray into polychrome cytometry (defined as almost as many colors are MR uses) using our Dako-Cytomation CyAn LX. The LX has 3 solid state lasers (violet, 488 and red) and we used them all for this 8-color analysis of naive/memory markers on CD4+ and CD8+ cells.

We started with our usual gating strategy: First CD4 or CD8 by log side scatter. Note how beautifully the CD4 and CD8 populations stand out. These are freshly isolated PBMC, but in stimulated cells, the activated and apoptotic populations virtually jump out of the page with this gating strategy. Next comes our customary live gate, then our first subsetting cut, on the basis of CD45RA and CD27. So far so good, but this is only 4 colors.



Looking at the markers pairwise is a bit of a diversion. With this analysis we might as well be doing 3 color staining. It is good for one thing though. If you are familiar with the staining patterns of the antibodies you can look for signs of correct or incorrect compensation. Are populations that are supposed to be orthogonal really orthogonal? In the case of CD45RA vs CD45RO, is there a nice diagonal?

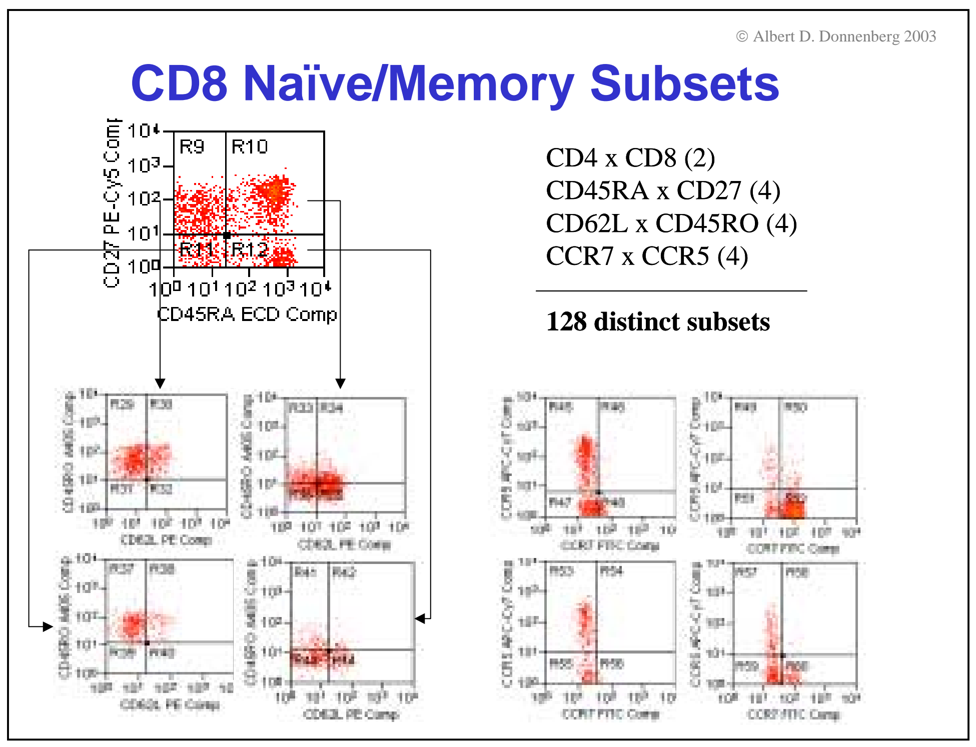


Now we are in trouble. If we want to look at all possible subsets in this panel, there are 128 populations to look at (counting the empty ones like CCR7+/CCR5+).

Clearly a hypothesis would be helpful. Here we are using our primary subsetting antibodies (CD45RA and CD27) to divide our CD8+ T cells into central memory, naive effector/memory and effector populations, and looking at CD62L, CD45RO, CCR7 and CCR5 expression on those four subsets.

Following naïve CD8 T cells (CD45RA+/CD27+) we see that they are CD45RO-, largely (but not exclusively) CD62L+, CCR7+ and CCD5-. We could do the same sort of analysis for CD4+ T cells (not shown).

We did compensation the hard way for this data set. Two experienced users performed manual compensation with single stained anti-CD8 for each fluorochrome. The compensation matrices that they arrived at were quite similar. In the future we will perform automated compensation offline with WinList.



I hope these exercises have helped you to compensate without decompensating, in the immortal words of Howard Shapiro.

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Compensate Without Decompensating

Howard M. Shapiro, PFC 3rd ed. 5.6, p. 2144

These are some of the members of Albert’s and Vera’s research labs, and the UPCI Flow Cytometry Facility. From left to right Cassy Singer, Erin McClelland, Vera Donnenberg, Melissa Merola (highest head), Noah Donnenberg (smallest head), Albert Donnenberg (cool sunglasses), and Anita and Petar Popovic.

We drove this beautiful Pitt van (a.k.a. the great white whale) from the ‘Burgh to Boston and back (1250 miles) to attend the BUG.

AVDLab/UPCI Flow Cytometry Facility