Software Compensation

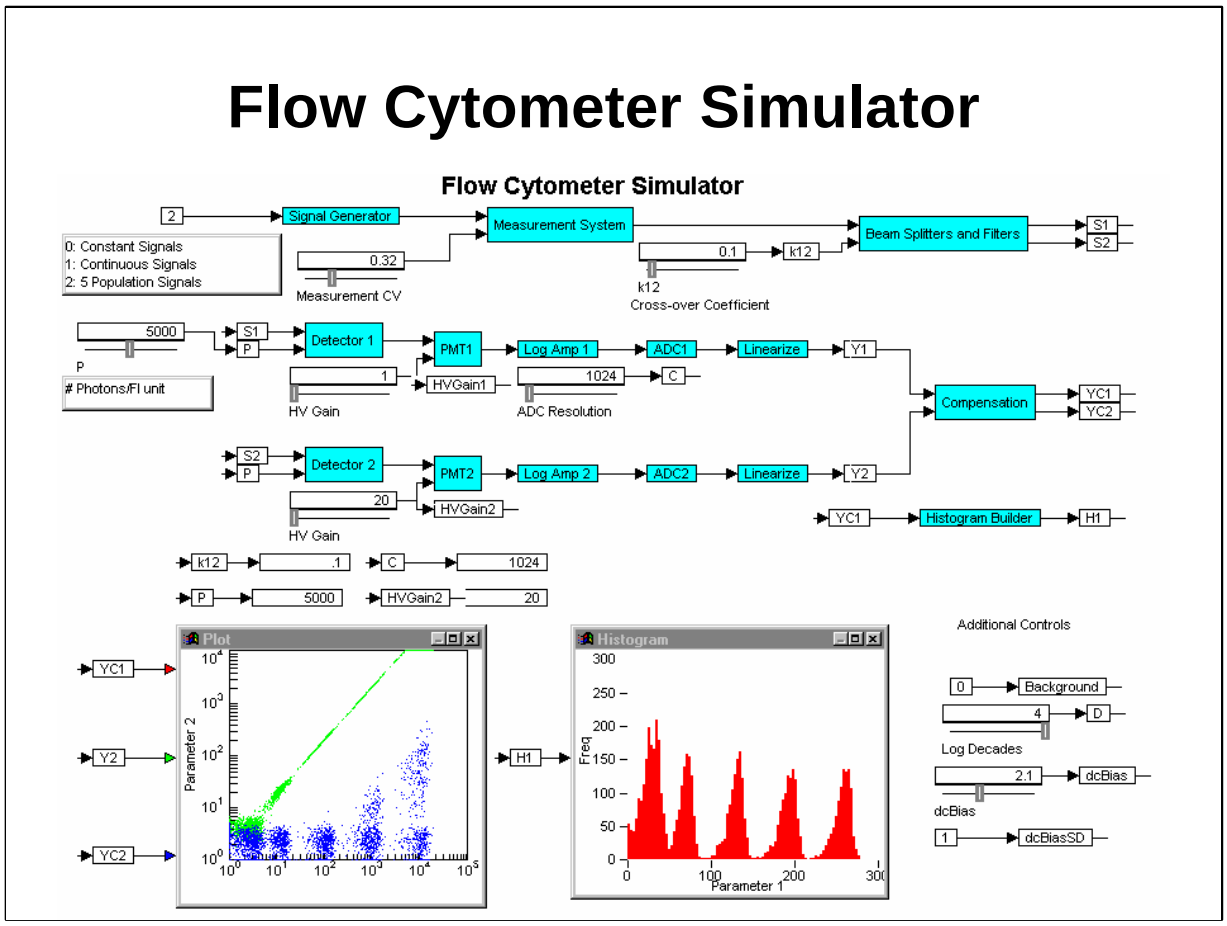
Laboratory

C. Bruce Bagwell MD, Ph.D.

25th Annual Course in Cytometry

Laboratory Overview

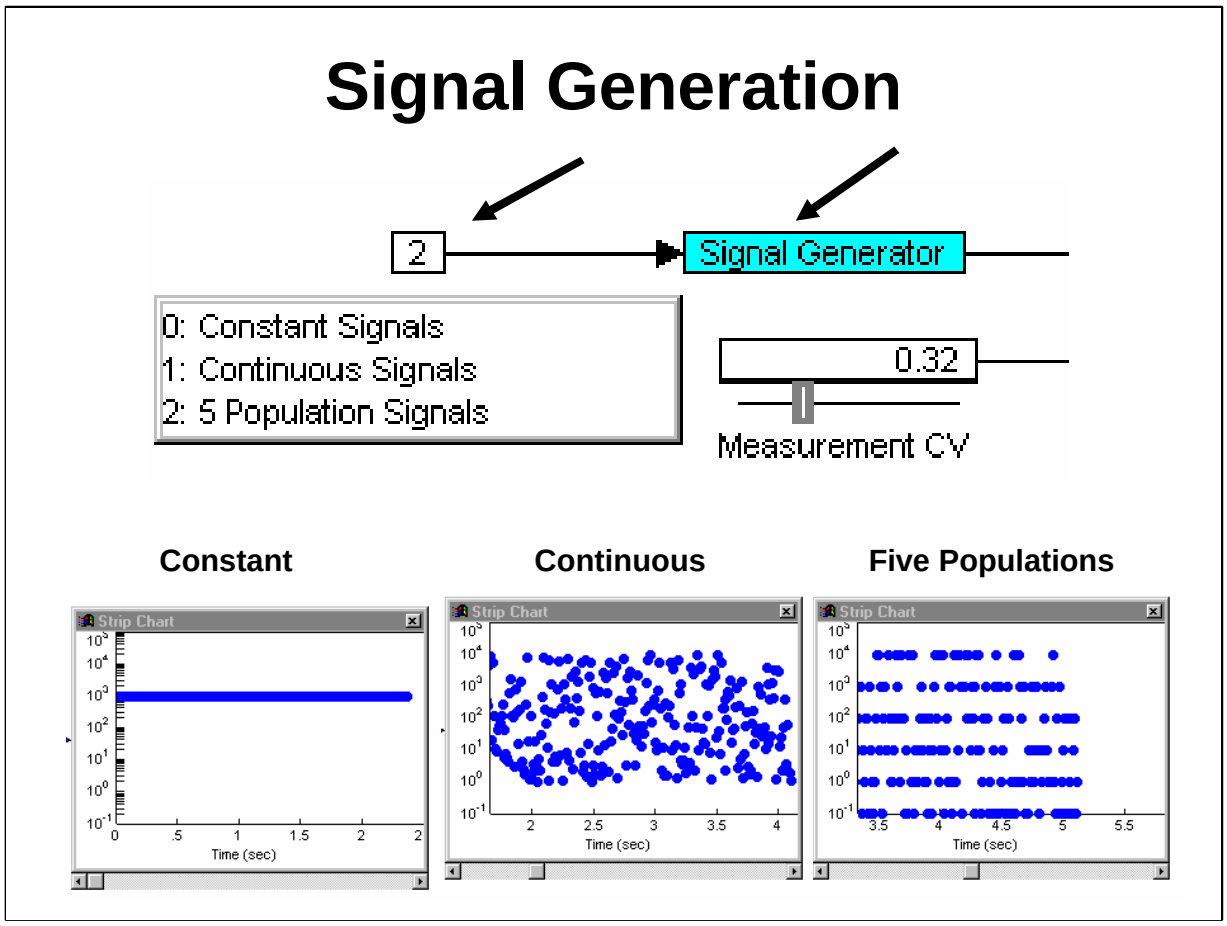
- **Part 1: Hands on Simulation Studies**
- **Part 2: Hands on Software Compensation Tutorials**



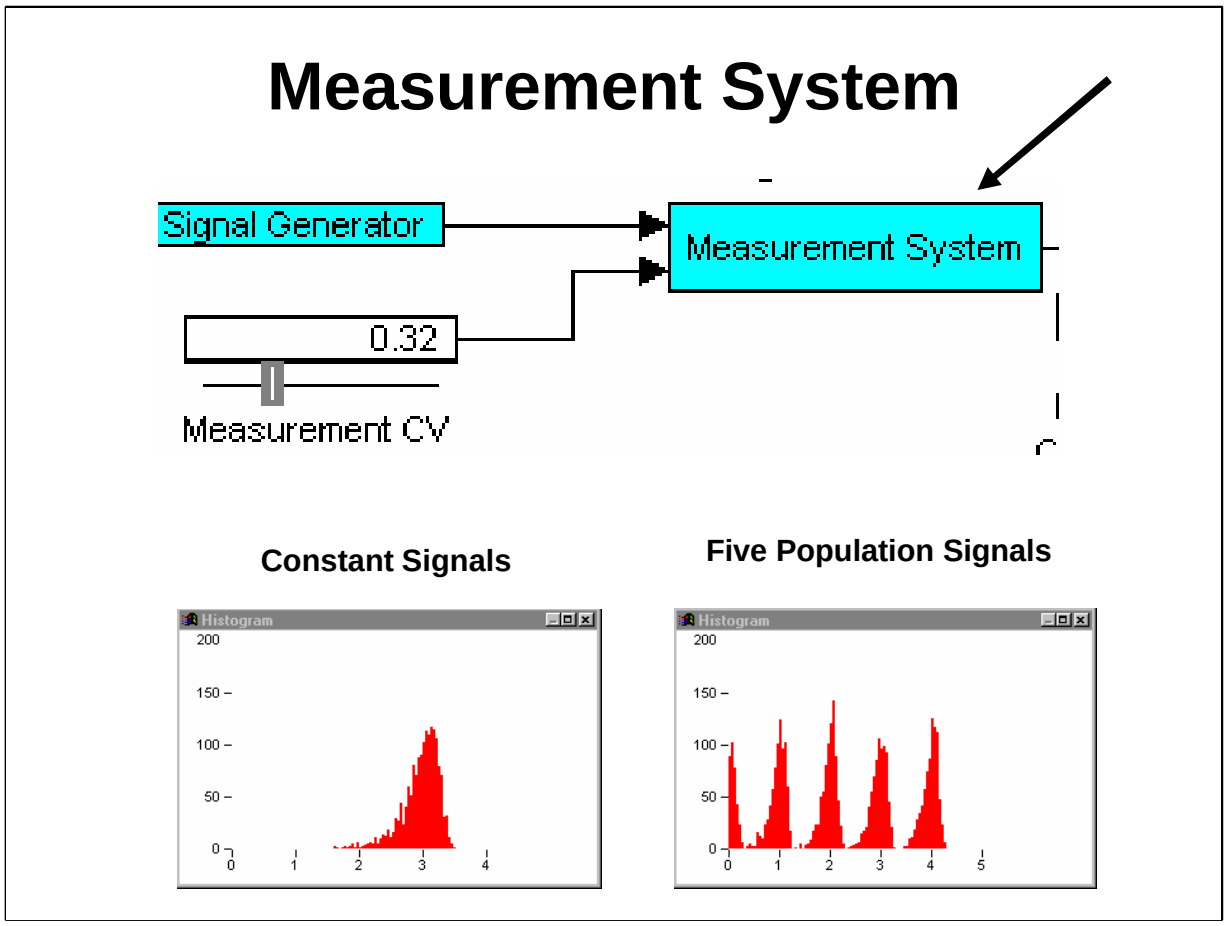
The above simulator will be used extensively in laboratory to better appreciate how factors such as ADC resolution, signal cross-over coefficients, and photon counting statistics affect compensation, particularly software compensation. By understanding how these factors interrelate, it is possible to avoid some pitfalls associated with compensation.

If you really want to understand any process, you should simulate it and then methodically study how each element of the process works dynamically. In the past few years, simulators are playing a more important part in our understanding of complex systems.

In this laboratory we will be using the VisSim 4.5i simulator made by Visual Solutions Incorporated to better understand the principles of flow cytometry and how they relate to compensation.



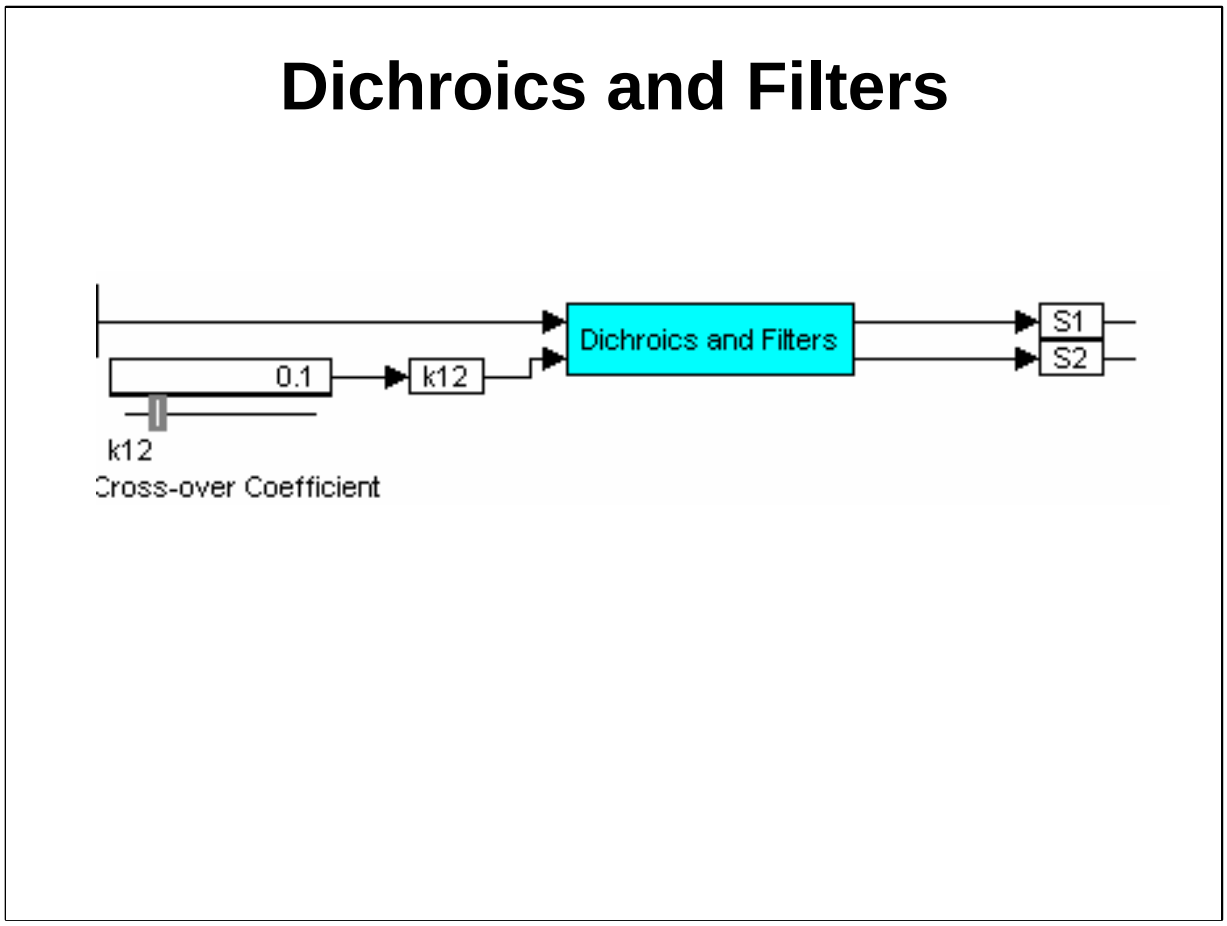
Three different types of signals can be generated in the simulator: constant, continous (four-decades), and five populations (one decade intervals). For the most part, we will use the five population mode for our experiments.



Usually in the process of measuring something, error is introduced. In this simulator, we assume the signals are broadened with constant CV. If a constant signal is used, we can appreciate the broadening with a histogram (lower-left inset graph). The distribution isn't exactly Gaussian in nature because we are viewing the histogram of the log signals rather than the linear signals. The advantages of log signals are described further below.

In the Five Population Signals graph we can see the five populations at integer log decade intervals. Notice that all the widths of the peaks seem identical.

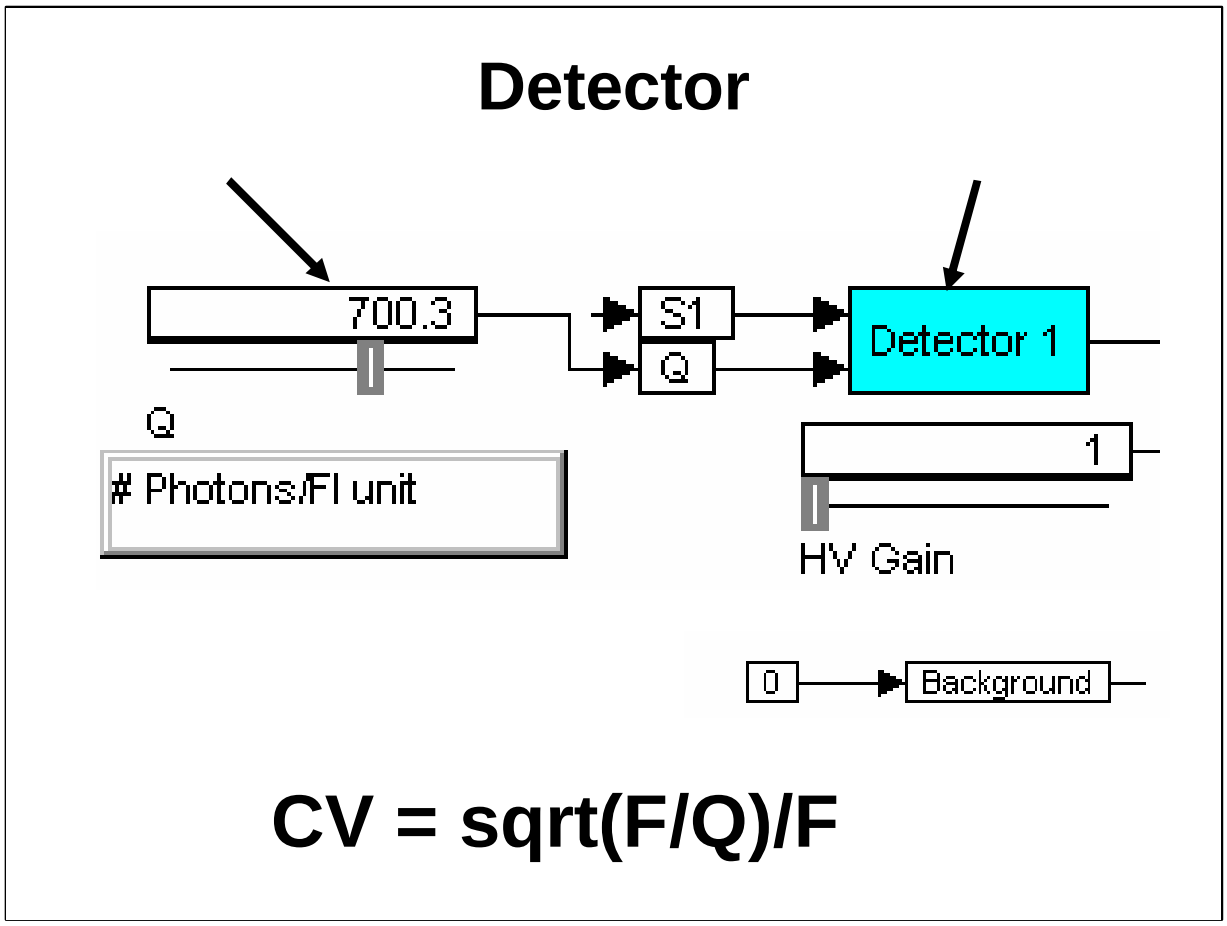
The reasons we generally display our signals in the log domain are well represented in this figure. The log transformation allows us to view a large dynamic range of population fluorescence intensities. In this simulation, we are viewing five populations that span four-decades. Another advantage of the log transformation is that measurement error tends to normalize such that it remains constant throughout this large dynamic range. In other words, data that has constant CV in the linear domain, will have constant SD in the log domain.



Flow cytometers generally have a system of filters and dichroics to separate one fluorescence color from another. Because the fluorescence emissions of most of our common fluorescence probes are broad and overlap each other, this signal separation is only partially successful.

If our signal is generated by fluoroscein molecules, for example, some of the signal will generally be detected by another detector. The fraction of signal that "crosses over" into the other detector is called the cross-over coefficient.

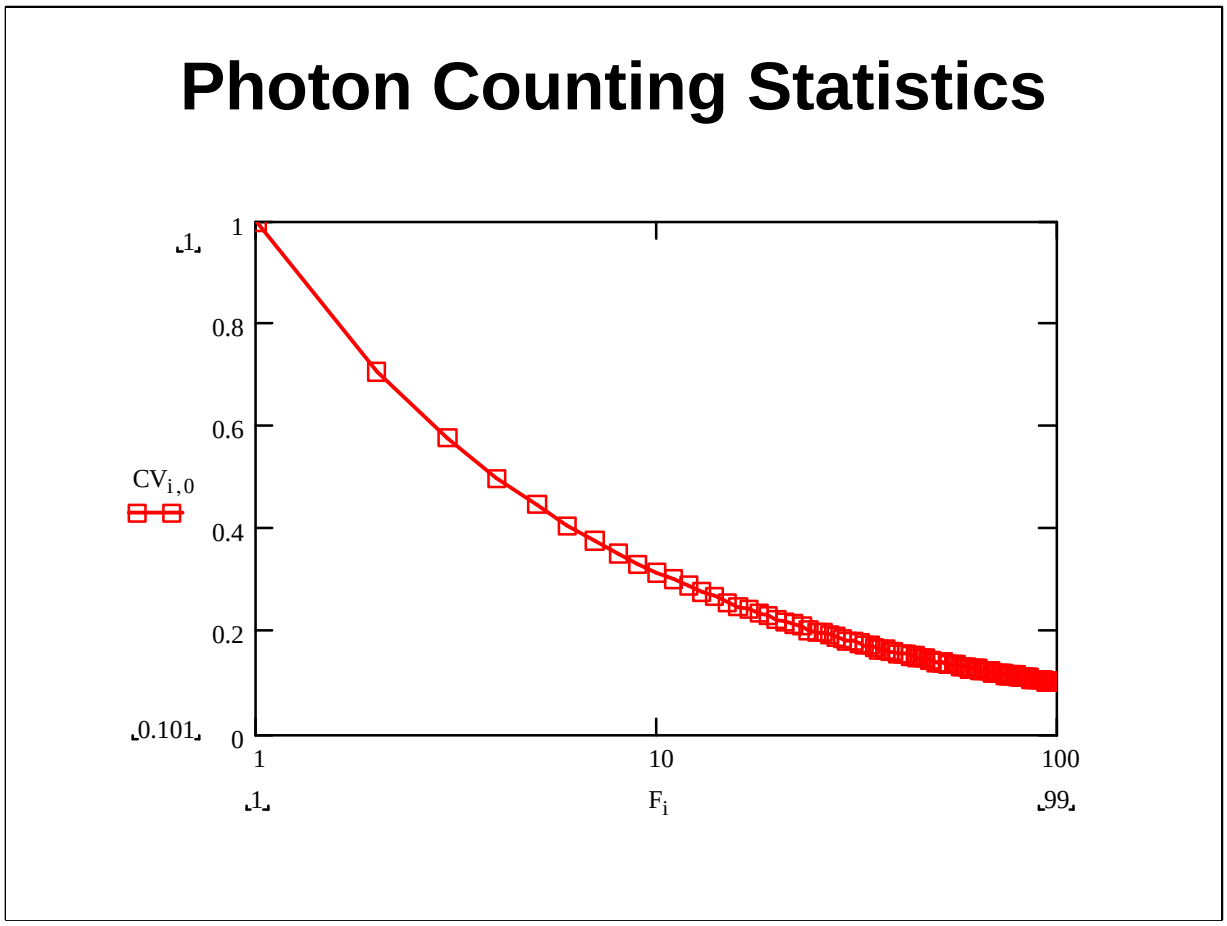
The cross-over coefficient is controlled in the simulator by a scrollbar labeled k12 (fraction, k, of signal 1 going into detector 2).



As a cell or particle traverses the flow cytometer laser beam, fluorescence molecules emit photons in all directions. The optics of the cytometer routes some of these photons to the detectors. The proportionality constant that relates number of fluorescence molecules, F, to number of captured photons, is commonly referred to as Q. If you think about it, Q is an important measurement of a cytometer's sensitivity.

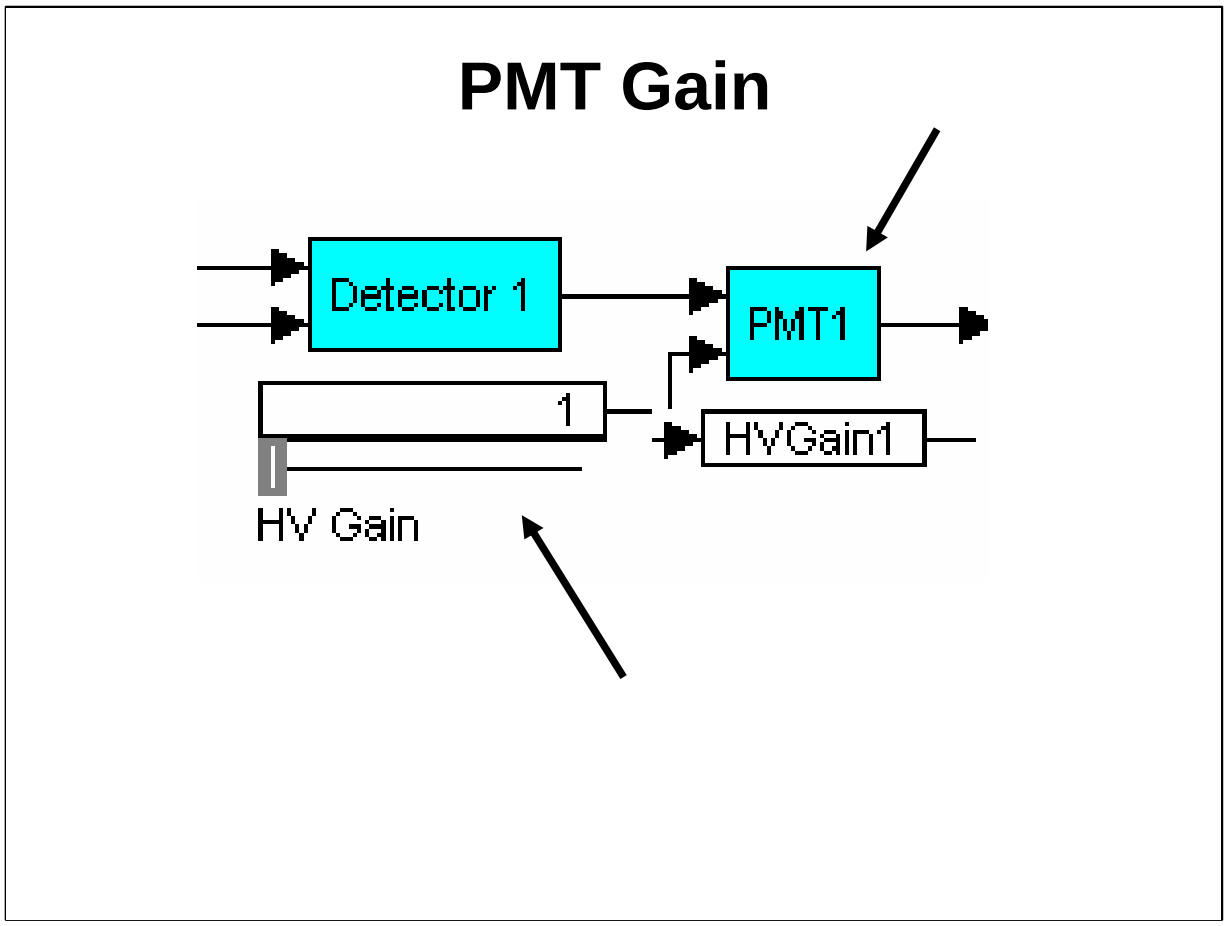
For dim signals, the number of captured photons can be small enough that counting statistics can appreciably affect the cytometer's ultimate quantitation of these signals. The standard deviation of the cytometer's measurement is proportional to the square root of F*Q (number of captured photons). The CV, which is SD divided by mean intensity, is given by CV = sqrt(F*Q)/F*Q. If we divide the numerator and denominator by Q, we end up with CV = sqrt(F/Q)/F.

In the simulator, Q is controlled by a scrollbar labelled Q. Also included in the simulator is the means to introduce background light to the detector (see above Background control).



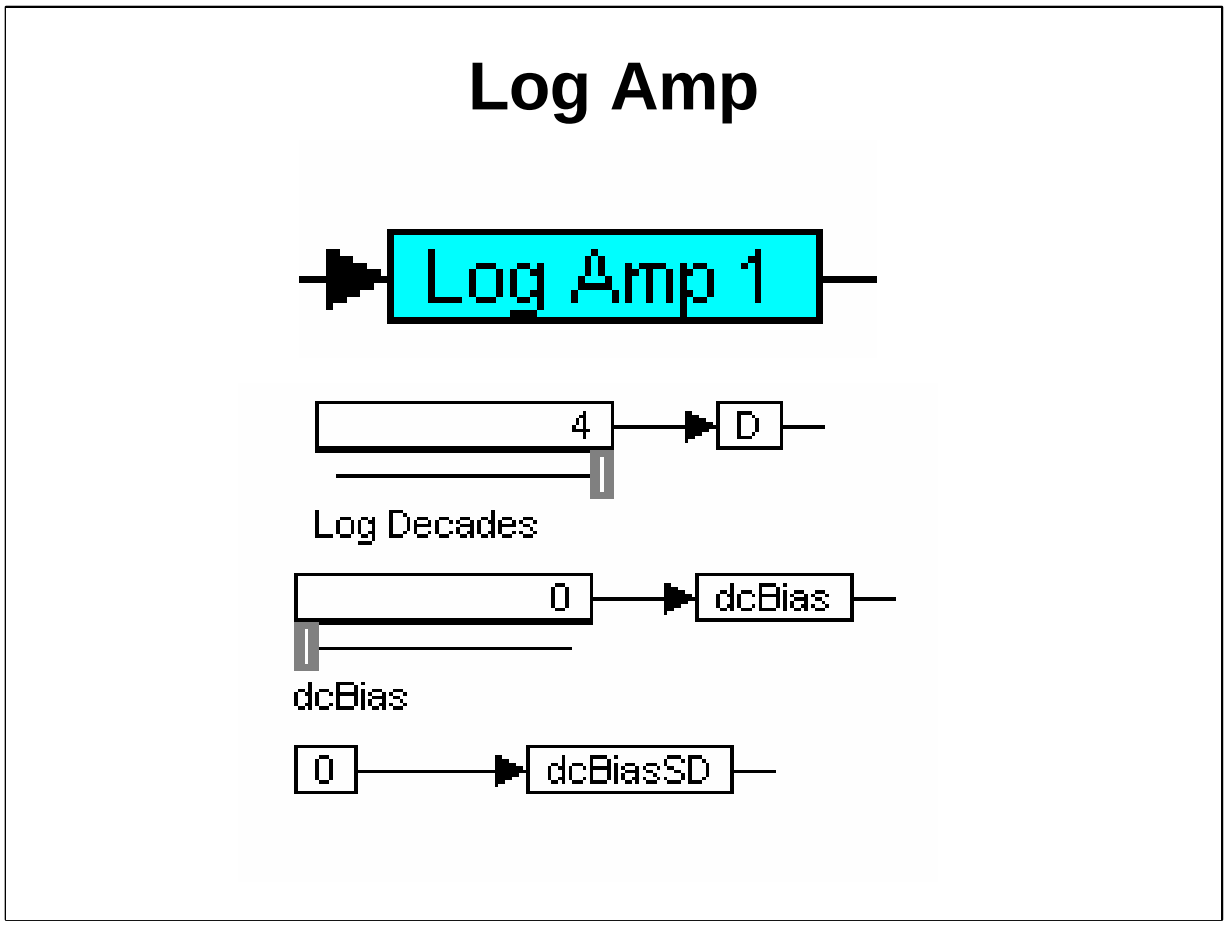
Because the square root function increases less rapidly than F as a function of F, the above equation predicts that the CV will decrease as F increases (see above graph). The Standards laboratory will discuss this effect more fully and show proposed methods for estimating Q.

Later, we will use the simulator to show how Q relates to this CV broadening effect as well as compensation.



In the simulation, the PMT simply provides a means of amplifying the signal from a particular detector. Scrollbars are available in the simulator to control the magnitude of these gains.

Later, we will see that PMT gain settings can dramatically affect the software compensation system.

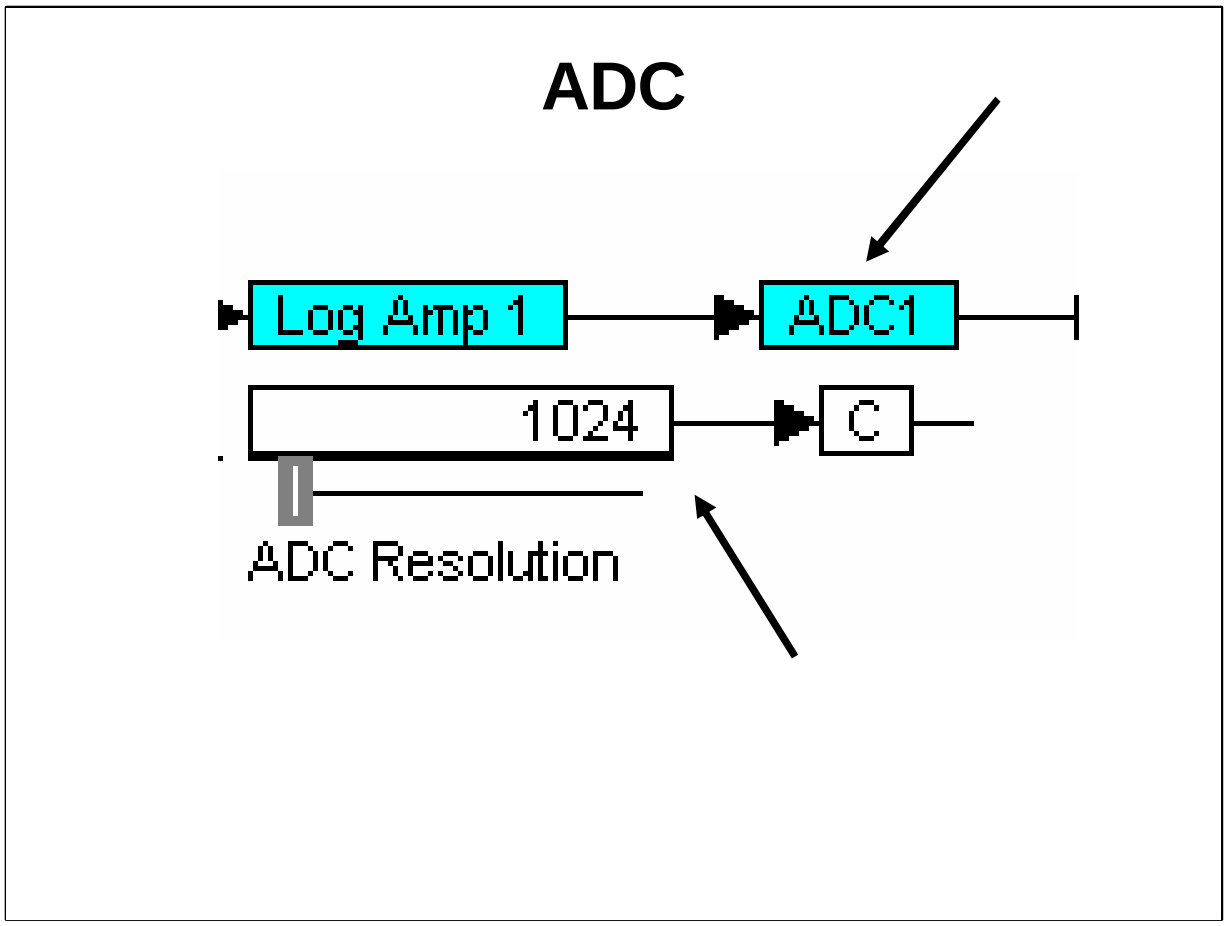


The log amp transforms linear signals to logarithmic signals. The advantages of using a log transform were previously discussed in the Measurement System.

In the simulator, we control the number of decades for the log amp by the above "Log Decades" scrollbar. It has a range of 1 to 6 decades.

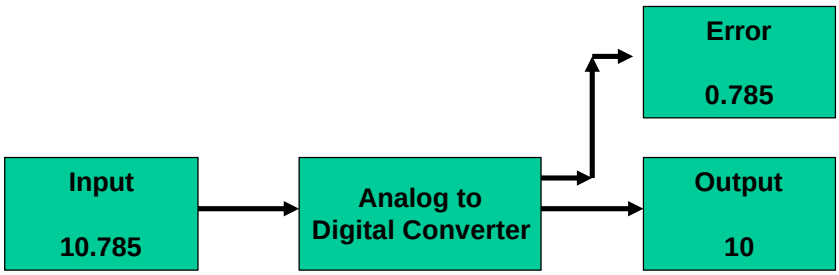
Typical Log Amps have a background noise and offset that can dramatically affect the visualization of populations. The simulator controls the offset with a "dcBias" control and the degree of noise with the "dcBiasSD" control.

Later, we will see how the Log Amp affects compensation and how the dc bias and noise in the log amps affects our displays.



A critical component to the flow cytometers is the analog to digital converter. The ADC converts an analog signal to a digital signal at some defined resolution, C. In the process of performing this conversion, digital error is introduced into the system (see next slide).

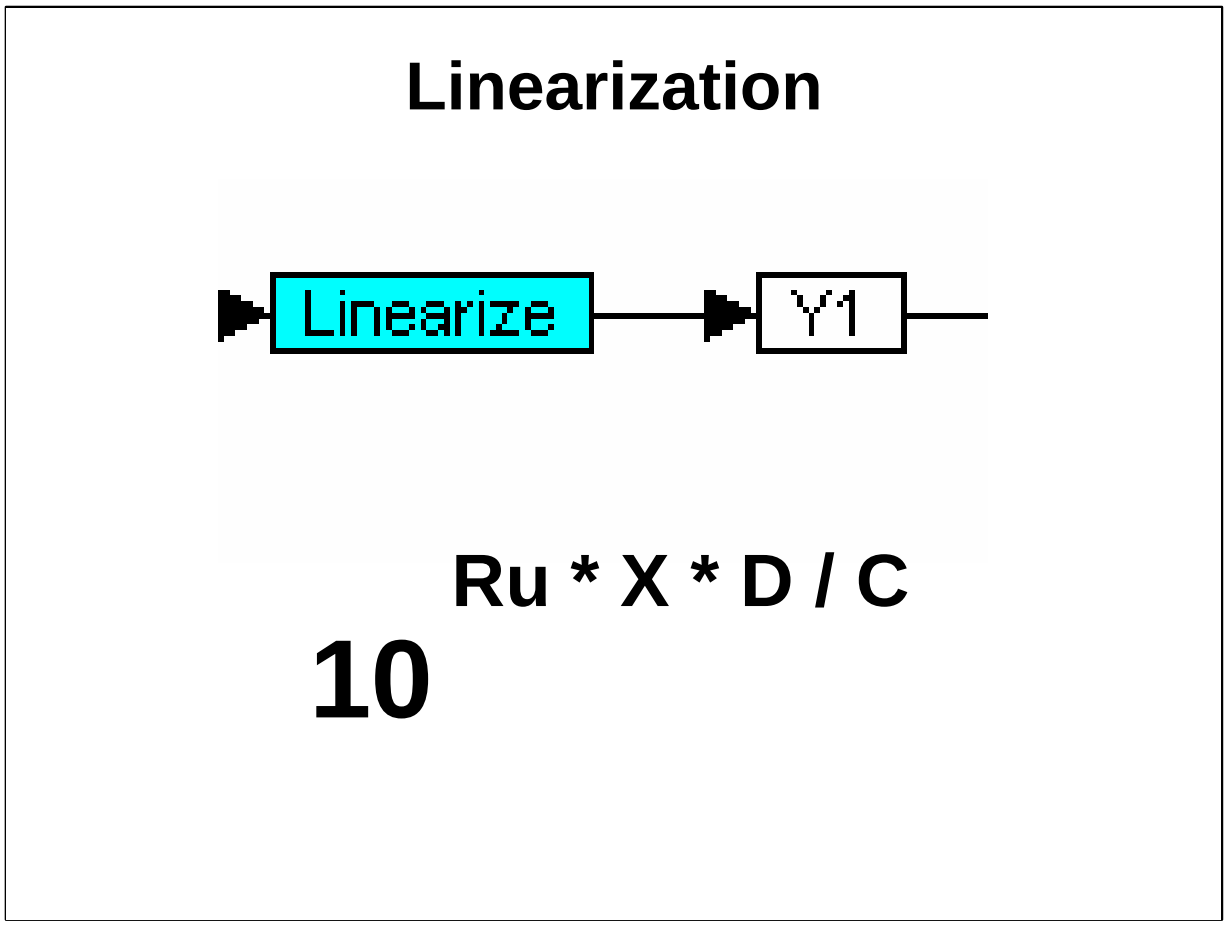
What is Digital Error?



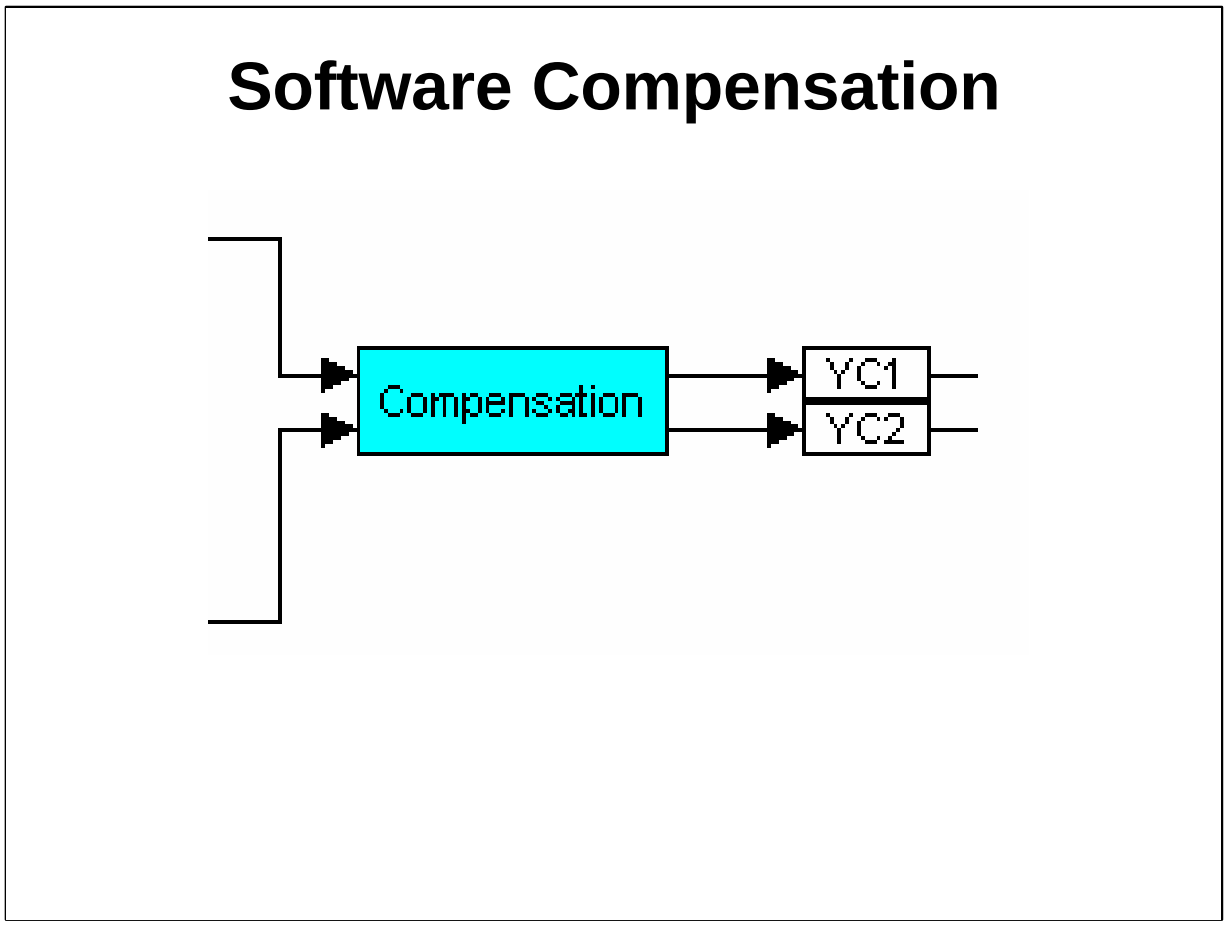
For 1024 channel ADC's the error averages 0.5/1024 or 0.049%

The above flow chart demonstrates what happens when an analog signal is digitized by the ADC. Upon digitalization, the number is truncated and the error made in this truncation is the decimal or fractional part of the input signal. For a 1024 channel ADC, this error averages about a 0.05% and, at worse, 0.1%. Normally, this level of error is reasonable and doesn't affect our interpretation of data; however, when the data is compensated, these relatively small errors are quite obvious, especially when four-decade log transforms are used on the data.

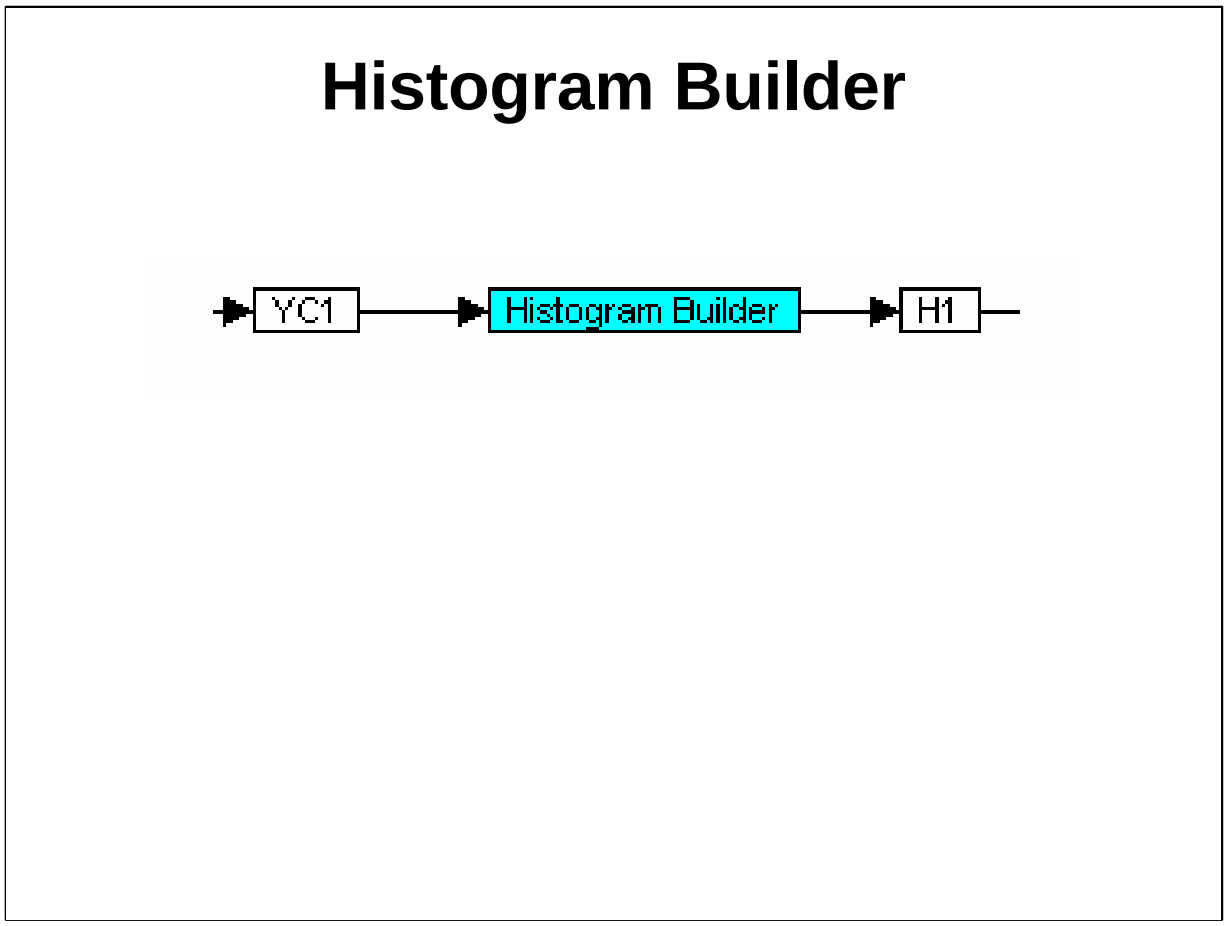
Later we will use the simulator to appreciate these digital errors.



The log data needs to be linearized in software applications that will attempt to perform software compensation. The linearization algorithm assumes that the log amp is truly logarithmic over its dynamic range. The ADC resolution, C, and number of decades, D, convert the log signal, X, to a linearized signal (see above formula). The Ru term in the formula is a uniform random variable ranging from 0 to 1 that eliminates distracting gap patterns in the data.



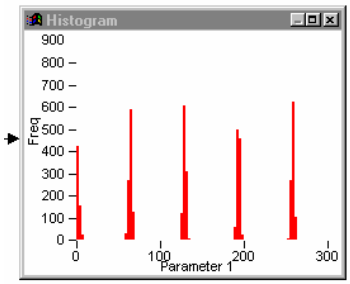
Based on the signal cross-over coefficients and the PMT amplifier gains, the Compensation system subtracts the signal due to signal cross-over from the second parameter.



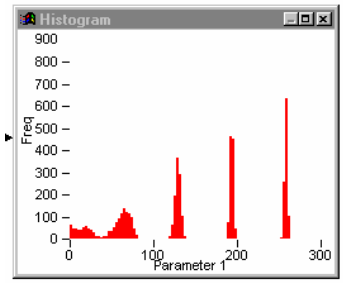
The histogram builder simulator control takes the compensated parameter 1 values and builds a 256 log channel histogram from them.

Effect of Q on Population CV

Q=100



Q=1



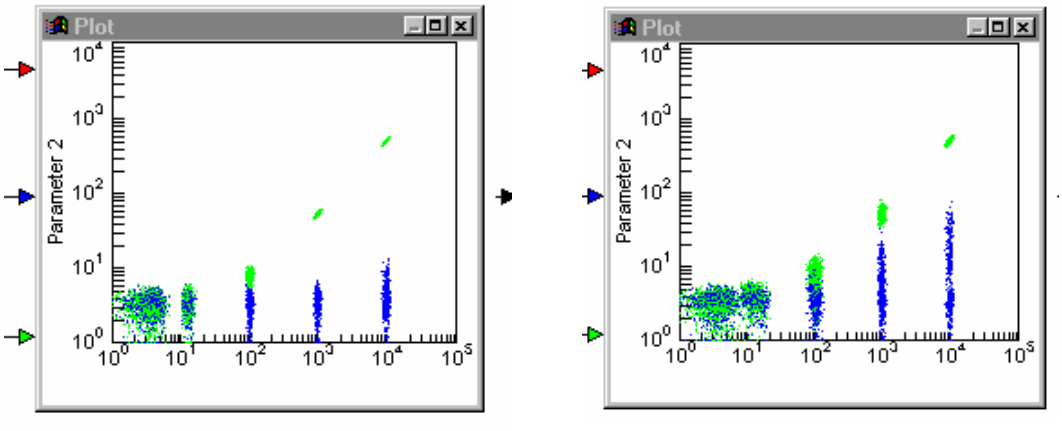
Experimental Design. We will set the simulator up to allow us to best visualize how Q affects dim population CV's.

Setup: 5 Pop Signal (2), Q=100, Measurement CV=0.05, k12=0.05, HV Gains=1, C=1024, 0 background, dcBias=0, dcBiasSD=0. Turn on simulator. Slowly move Q from 100 to 1 while visualizing the log histogram.

Discussion: Notice how the low intensity peaks widen with Q=1 (dim signals). Keep in mind that we are looking at a log histogram, where an increase in CV is represented as an increase in peak width.

Effect of Q on Compensation

Q=100 **Q=1**



Experimental Design. How does Q affect compensation?

Setup: Same as previous experiment, except set the dcBias to 3 and dcBiasSD to 1. Reset Q to 100.

Note. By adding some noise in the amplifier, our populations will be easier to visualize (more on that later).

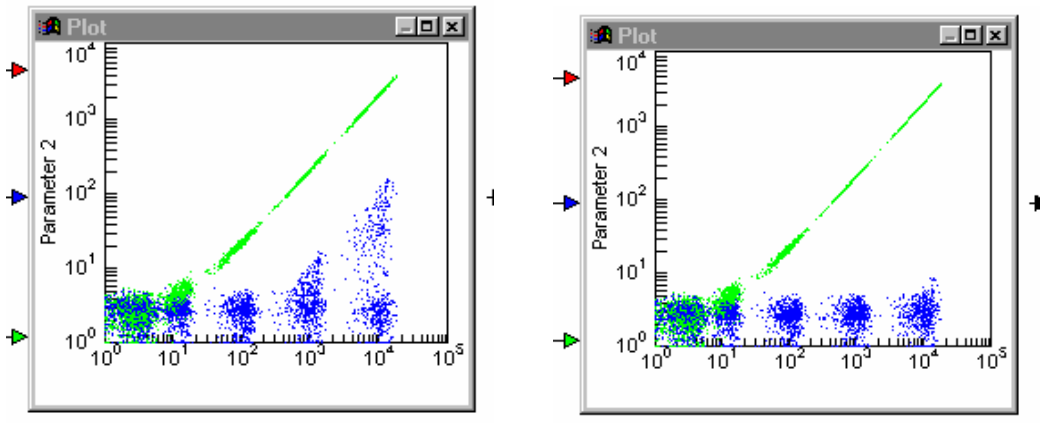
Turn on simulator. Slowly move Q from 100 to 1 while visualizing the two parameter dot plot. Also move the ADC resolution to higher resolutions. Also move the dcBias scrollbar to see how that affects our ability to visualize populations.

Discussion: Notice how the higher intensity compensated population is affected by Q. This effect is not very intuitive since we have just shown that the CV's of the lower intensity populations are greater than the higher intensity populations. Why is the apparent variance of the compensated data higher in for higher intensity populations? When you changed the ADC resolution, you didn't see the system change much. This is because this particular type of error is not due to digital error. This effect is intrinsic to the measurement process and affects both hardware and software compensation systems (see Roederer, Cytometry, 45, pp 194-205).

This experiment should convince you that creating orthogonal regions for gating or statistical analyses with dim compensated signals can result in significant errors.

ADC Resolution and Digital Error

C=256 **C=10000**



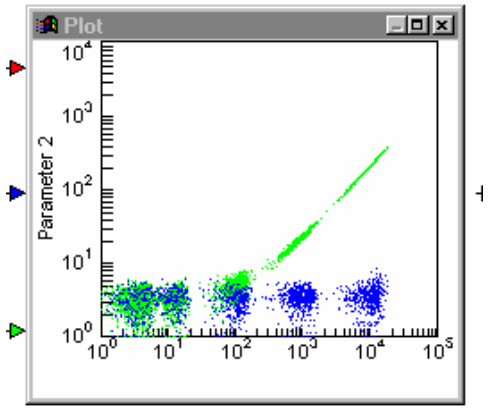
Experimental Design. We will now explore the complex effects associated with digital error. All these effects are present to some degree with software compensation since, by definition, software compensation presently uses digitized data.

Setup: 5 Pop Signal (2), Q=1000, Measurement CV=0.3, k12=0.2, HV Gains=1, C=256, 0 background, dcBias=3, dcBiasSD=1. Turn on simulator. Slowly move C from 256 to 10000 while visualizing the two parameter dot plot.

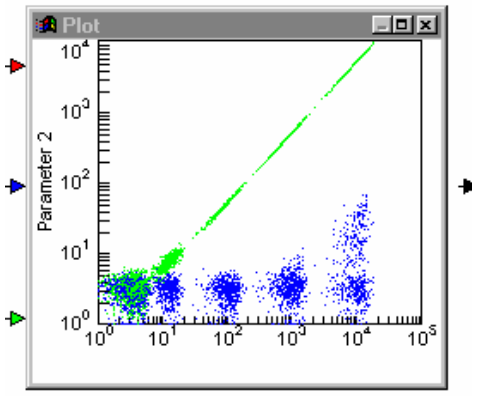
Discussion: Notice how the higher resolution ADC's reduce the effects of digital error. The newer cytometers with higher resolution ADC's will make software compensation much more accurate. If you currently have a cytometer with 1024 channel ADC's, you must be careful not to inadvertently over compensate data because of these digital error effects.

Effect of Signal Cross-over on Software Compensation

k12=0.02



k12=0.5



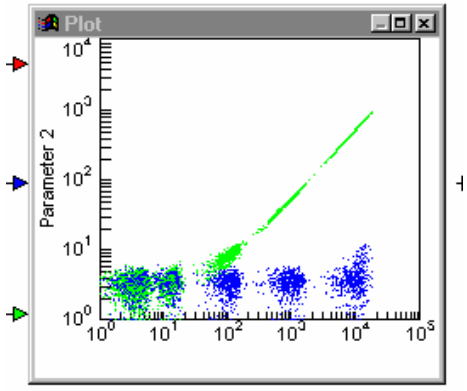
Experimental Design. How does the magnitude of the signal cross-over coefficients affect software compensation?

Setup: 5 Pop Signal (2), Q=1000, Measurement CV=0.3, k12=0.02, HV Gains=1, C=1024, 0 background, dcBias=3, dcBiasSD=1. Turn on simulator. Slowly move k12 from 0.02 to 0.5 while visualizing the two parameter dot plot.

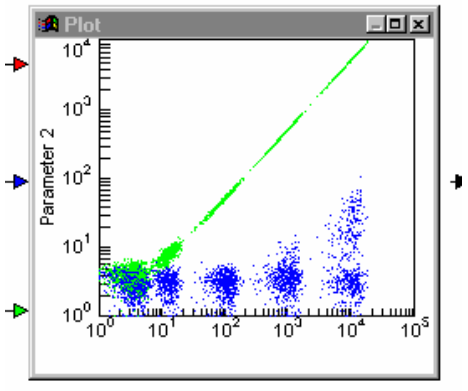
Discussion: The effects of digital error are proportional to the magnitude of the signal cross-over coefficients. You will always be better off by selecting fluorochromes and filters to minimize signal cross-talk.

Effect of Differential ADC Gain Software Compensation

HV Gain2 =1



HV Gain2=10



Experimental Design. How does the magnitude of the second parameter's PMT gain affect software compensation?

Setup: 5 Pop Signal (2), Q=1000, Measurement CV=0.3, k12=0.05, HV Gain 1=1, HV Gain 2=1, C=1024, 0 background, dcBias=3, dcBiasSD=1. Turn on simulator. Slowly move HV Gain 2 from 1 to 10 while visualizing the two parameter dot plot.

Discussion: The effects of digital error are proportional to the differential magnitude of the PMT gains, not a widely appreciated fact. From a software compensation perspective, you will always be better off by using similar PMT gains for all parameters.

Software Compensation Tutorials