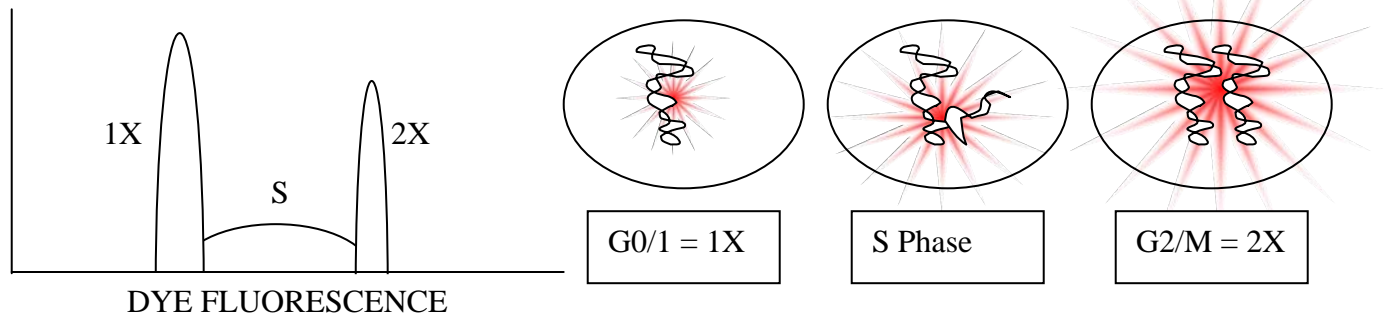


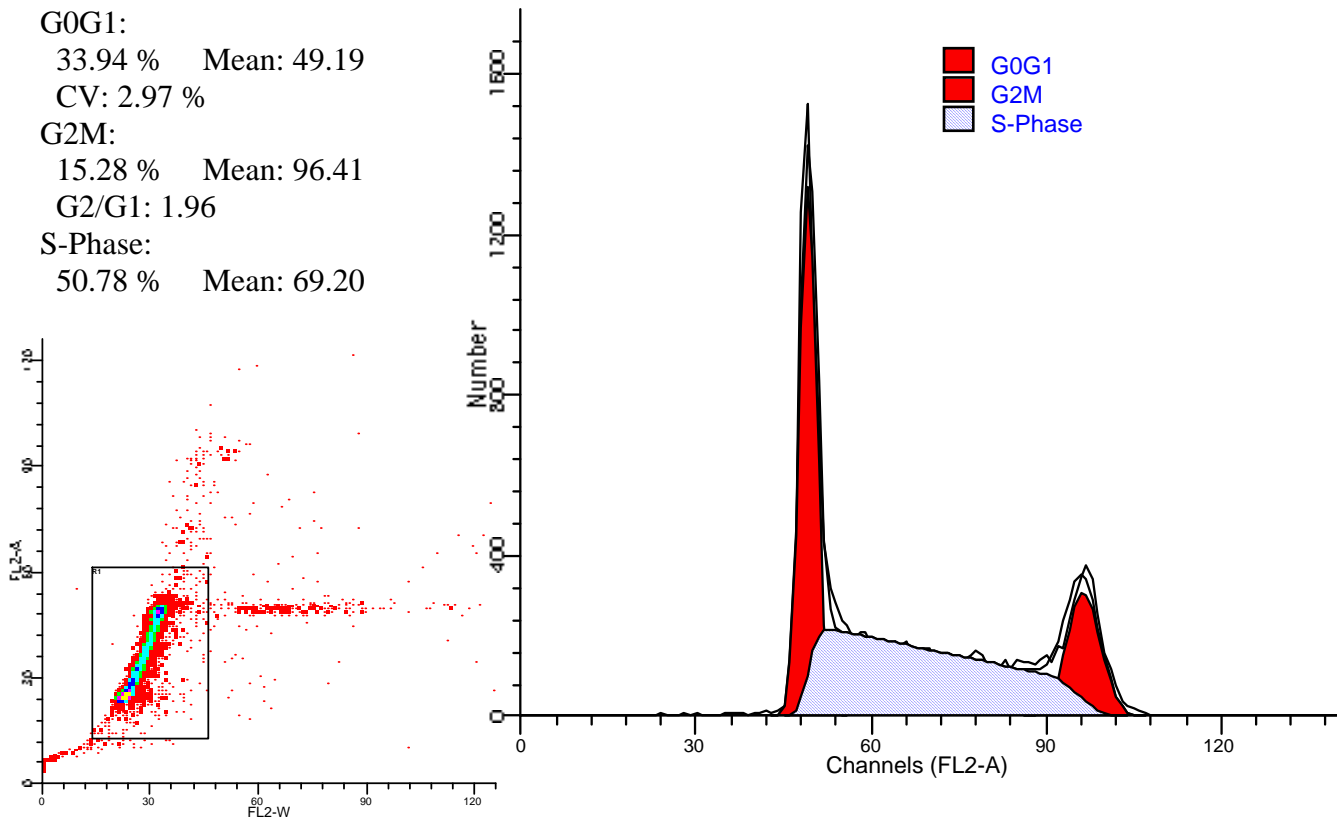
CELL CYCLE BASICS

Analysis of a population of cells' replication state can be achieved by fluorescence labeling of the nuclei of cells in suspension and then analyzing the fluorescence properties of each cell in the population. Quiescent and G1 cells will have one copy of DNA and will therefore have 1X fluorescence intensity. Cells in G2/M phase of the cell cycle will have two copies of DNA and accordingly will have 2X intensity. Since the cells in S phase are synthesizing DNA they will have fluorescence values between the 1X and 2X populations.



The resulting histogram consists of three populations: two Gaussian curves (1X and 2X peaks) and the S-phase population. Adjacent populations overlap each other. Because of this, a modeling program is required to de-convolute the populations and assign percentage values to each population. Expert and subjective review of the modeling software's cell cycle phase percentage assignment is the final stage of cell cycle analysis prior to reporting the results.

G0G1:
 33.94 % Mean: 49.19
 CV: 2.97 %
 G2M:
 15.28 % Mean: 96.41
 G2/G1: 1.96
 S-Phase:
 50.78 % Mean: 69.20



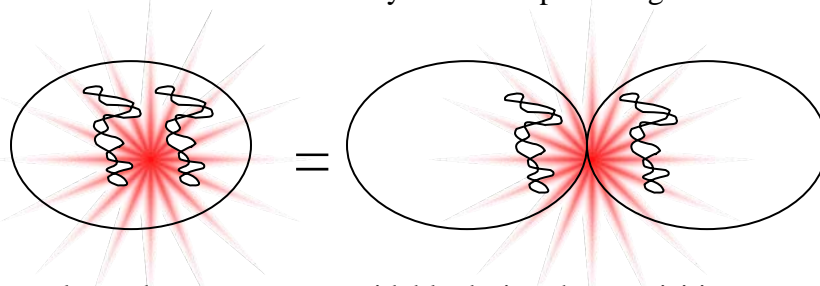
FLOW CYTOMETRY CELL CYCLE DATA: DUE DILIGENCE

Special considerations must be taken when optimizing a flow cytometer for cell cycle analysis. Because the DNA histogram is the final product of the flow cytometer that is subjected to curve de-convolution analysis, the integrity of the data must be high and the fidelity of individual cell's fluorescence must be maintained. This is mentioned because of the simple nature of cell cycle analysis: looking at cells with distinct fluorescence levels. As mentioned before, cells in G₀/1 have 1X fluorescence and G₂/M cells have 2X fluorescence. What happens if 2 G₀/1 cells pass through the beam (whether stuck together or not) at the same time?

AGGREGATES: A STICKY SITUATION

Cells can stick to on another. Also, despite the hydrodynamic focusing that occurs at the flow cytometer's laser intercept, cells can pass through the laser at the same time even if they're not stuck together.

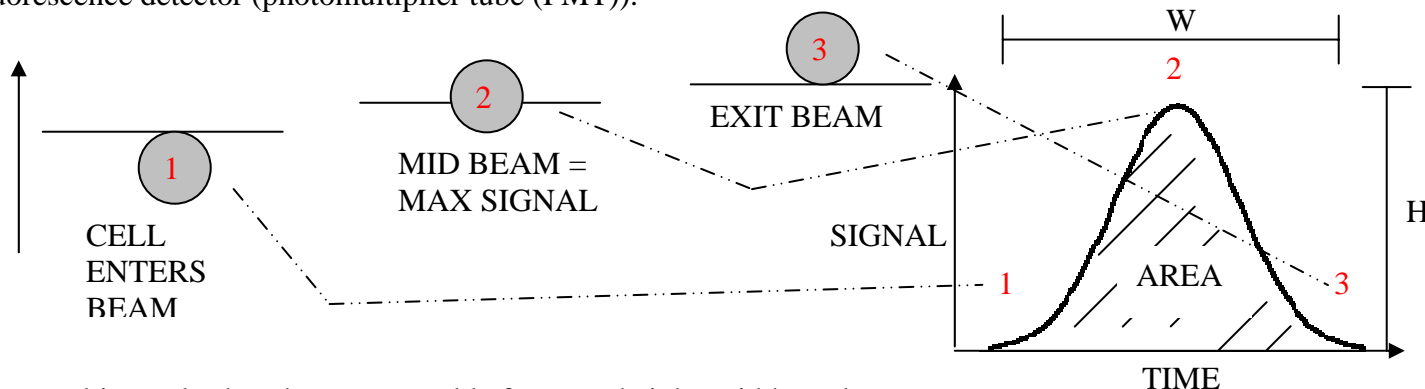
What results is the recording of one type of event as another type entirely. Because you are examining the nuclear fluorescence of each cell, two cells in G₀/1 that are stuck together will have as much nuclear fluorescence as one G₂/M cell. This will result in analyzed G₂/M percentages that are erroneously high.



Aggregates and simultaneously read events are unavoidable during the acquisition part of cell cycle analysis. The way to maintain the fidelity of the data is to exclude these non-single cells from later analysis. This is accomplished through the use of signal pulse processing by the cytometer and careful optimization by the operator. This must be performed during data acquisition.

PULSE PROCESSING

As cells transit through the laser beam, their fluorescence signal ultimately generates a voltage pulse by the fluorescence detector (photomultiplier tube (PMT)).



The resulting pulse has three measurable features: height, width, and area.

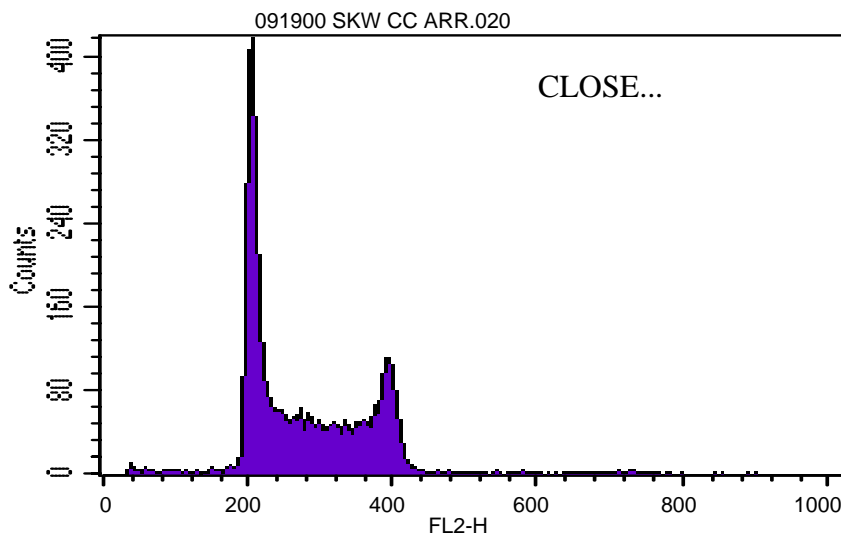
Height = maximum fluorescence intensity. Width = transit time. Area = total fluorescence of particle.

VIEWING PULSE PROCESSING PARAMETERS

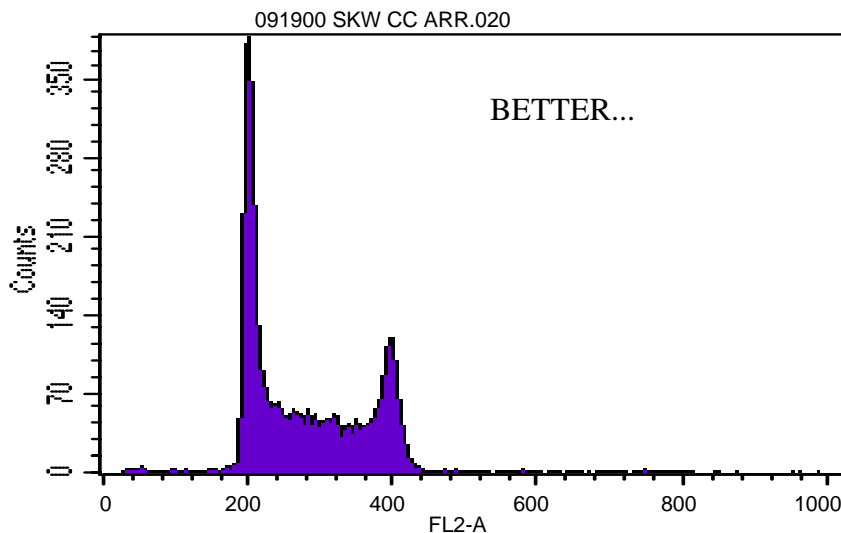
Usually, only the height (maximum fluorescence emission) signal is recorded (so THAT'S what the H in FL2-H means!) In cell cycle analysis, however, all three parameters must be considered and visualized.

Pulse width is indicative of particle transit time. Single cells will have a smaller pulse width compared to cells that have aggregated.

Below is a histogram of the FL2 peak emission values (FL2-H). Alone, it can act as the subject of a deconvolution program. However, since FL2-H alone gives no information about total fluorescence or singularity, it cannot be representative of the sample and thus cannot permit accurate cell cycle analysis.



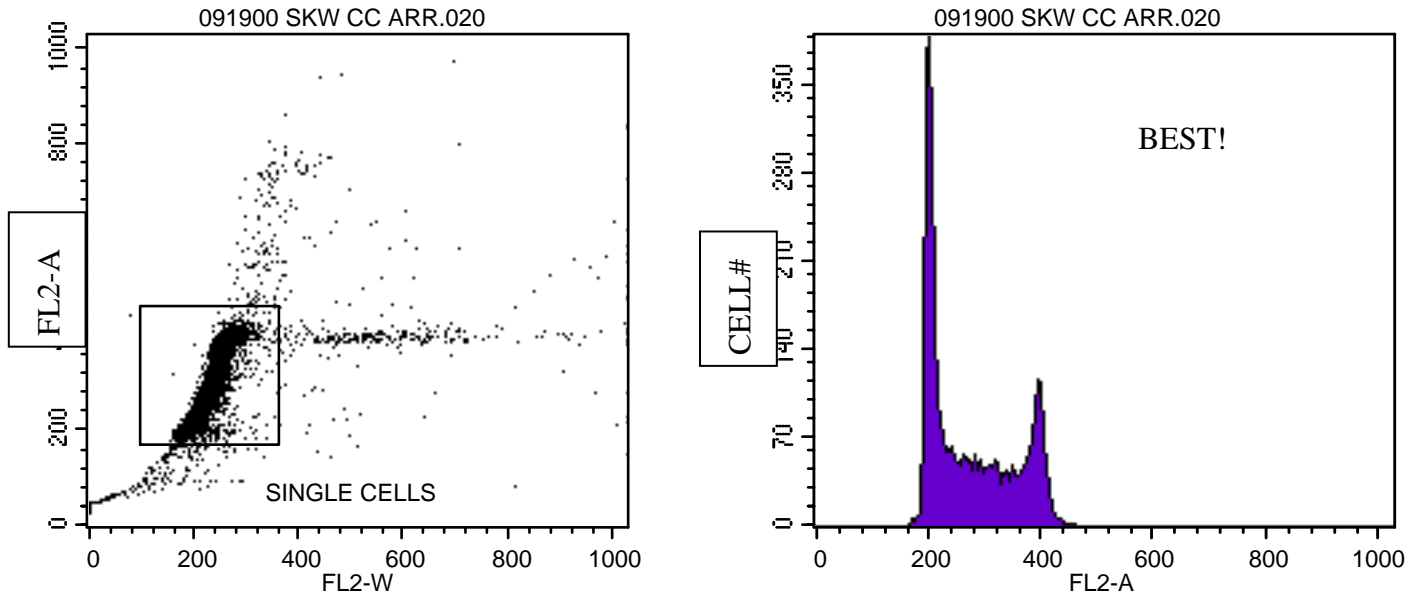
This is a histogram of FL2-Area (total cell fluorescence). This graph looks VERY similar to the FL2-H histogram. Although it is showing us the correct parameter for DNA analysis, it still does not allow for aggregate discrimination.



THE RIGHT WAY TO EXCLUDE AGGREGATES: FL2-W vs. A

The left plot (below) shows pulse width versus area, and this is the plot used to distinguish between single cells and aggregates. Single cells (G0/1 or G2/M) will have similar pulse width (transit time) values. Aggregates will have larger width values and can be easily seen on the plot to the right of the single cell region.

Single cells have been gated (left plot) and an FL2-Area histogram has been drawn and formatted to show only the events inside of the single cell region.

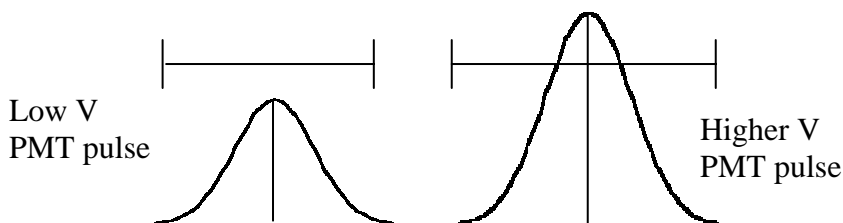


These are the plots you will use in the analysis program. But before you get to that point, you must first optimize the flow cytometer to generate data that looks like this. How is this done?

A REVIEW OF CYTO-ANATOMY: PMT VOLTAGE AND AMPLIFIER

When a cell passes through the laser, a pulse is generated (see above). Assuming proper threshold adjustment, the characteristics of this raw pulse will be determined by one thing: the cell and the PMT voltage.

The PMT is like a mousetrap for photons. When the photons from a fluorescent cell strike the PMT, it begins snapping. When a bright cell fluoresces, the PMT receives a big signal and therefore produces a correspondingly large voltage pulse. Because the PMT operates by way of its applied voltage, the sensitivity (how hard it snaps at a cell) can be increased or decreased by increasing or decreasing the PMT voltage.



By increasing the PMT voltage, you can change a pulse's height, width and area values

WHAT AN AMP GAIN DOES

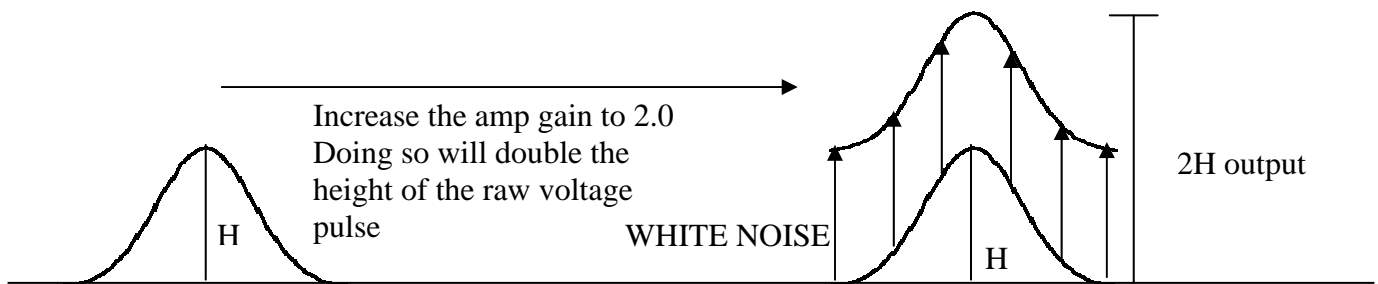
Once the PMT is generating a usable voltage pulse, the time has come to carefully modify that pulse to suit your needs. That's right, YOU get to shape your data the way you want to! How is this done? Let's just say it's like putting icing on a cake.

Sometimes you need to move things around on a parameter. You do it all the time with Forward Scatter. Ever notice that the FSC **Voltage** is adjustable only in large increments of 10 (E -01, E00, E01, etc)? In order to carefully move a population on Forward scatter, you need to take that rough voltage pulse from the FSC detector and change it delicately. This is done with the FSC Amp gain.

Note that the forward scatter parameter is labeled FSC-H. The H stands for Height. The forward scatter parameter is showing only the height of the voltage pulse. What the amp gain does is this: it takes the raw voltage pulse from the detector and pre-amp (more machine guts) and adds a carefully measured amount of white noise on top of it...kind of like adding icing to a cake!

No Amp Gain: Raw pulse from PMT

Amplified 2x original (amp readout 2.0)



The amp gain on a flow cytometer ranges from 1.00 (no alteration of raw voltage pulse) to 9.99 (nearly 10 times the amplification). On FSC, if you need to amplify more than 9.99, you adjust the rough voltage setting to the next step (a ten-fold increase) and bring your FSC Amp gain setting back down to 1.00 – and then adjust your amp gain as necessary.

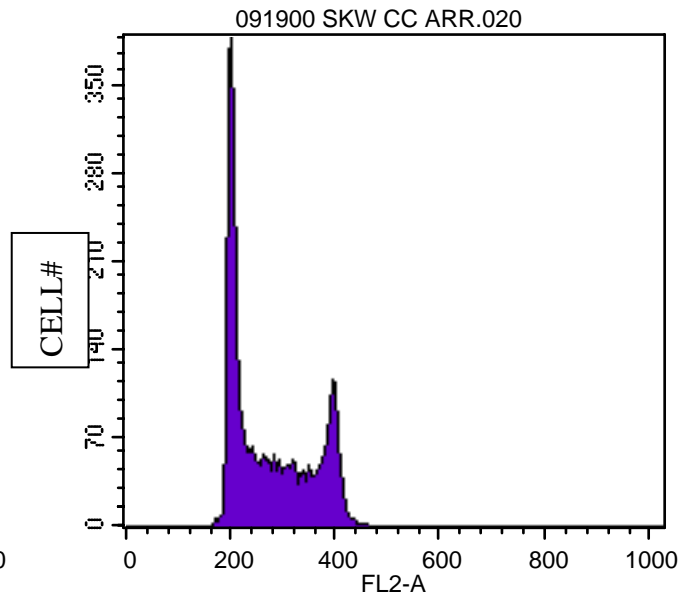
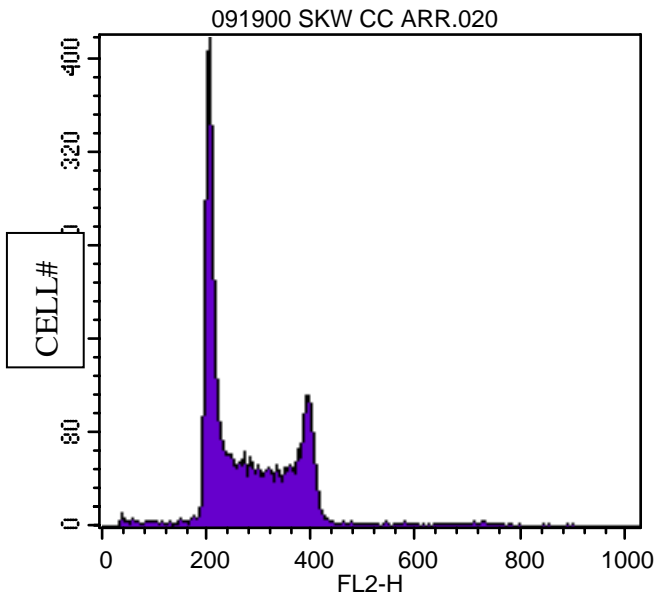
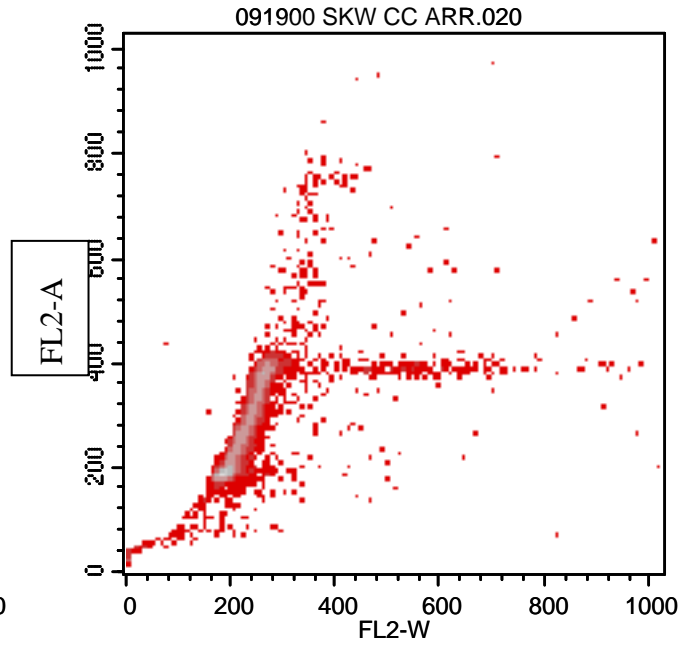
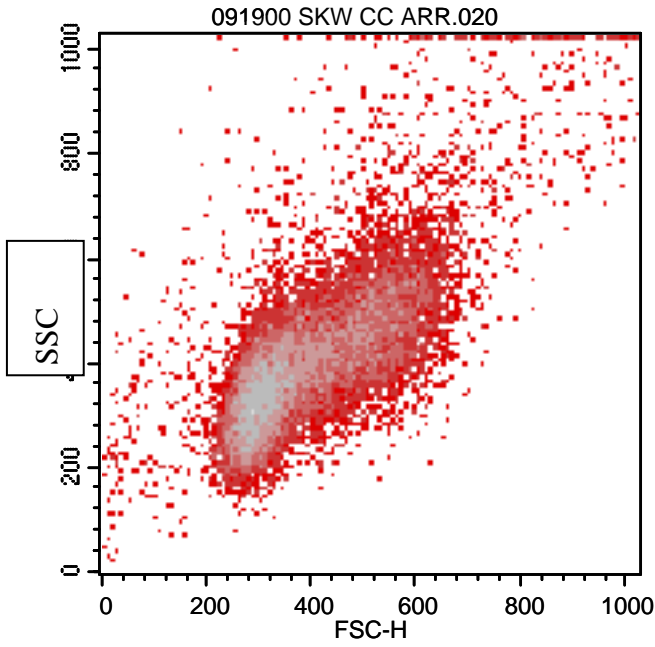
An illustration of amplifier function involves electronic music playback. A CD player reads the media and has line-level (low voltage) outputs in the back. Why can't you simply connect speakers directly to a CD player? Although the signal is present in high fidelity, the amplitude of the signal is very small. So small, in fact, that the speakers' magnets won't be energized with enough voltage to vibrate and produce sound. What you need to do is plug the CD player into a stereo amplifier. The amplifier takes the original signal (read, PMT voltage pulse) and carefully adds a layer of white noise at every point of the signal waveform (read, amp gain). The resultant signal will be exactly the same (the pulses look the same) but because of the amplification it will now have enough amplitude to power a loudspeaker. In flow cytometry, we don't need to add so much amp gain to our PMT voltage pulse that we can power a loudspeaker, but we do add enough amp gain to inch it up on a parameter to make it look good.

SO WHAT DO I ADJUST TO OPTIMIZE FOR CELL CYCLE ANALYSIS?

Step by step optimization –be sure to ask for expert assistance:

- Draw the following plots: Two parameter: FSC/SSC, FLW/FL2A; Histograms: FL2-H, FL2-A
- Set your threshold on FL2 at 20. In the art of flow cytometric cell cycle analysis, it is standard to set the threshold on the DNA fluorescence parameter at a value of 10% of the location of the G0/1 peak. The G0/1 peak will therefore be placed at a value of 200 on the FL2-H parameter (see below).
- Select FL2 as the DDM parameter (lower right portion of the Detectors/Amp Gain window).
- Put FL2 in LIN amplification. Linear amplification is necessary to resolve the 1x and 2x fluorescence peaks of the G0/1 and G2/M cells (respectively) on the FL2 parameter. If the amplification were logarithmic, the peaks would be smashed together.
- Place the Amp gains for FL2, FL2-A and FL2-W at 1.00.
- Place a sample on the machine and run in LOW speed (low speed ensures a narrow core stream which results in less cell to cell variability in measurement and thus higher fidelity)
- Because the threshold is set on FL2, no events will be seen unless the FL2 PMT has enough voltage to generate pulses above threshold. Increase the FL2 voltage if no/few events are registering in the machine.
- Once the events are above threshold, adjust FSC and SSC as usual.
- The G0/1 peak will be discernable from the G2/M peak. With a diploid control, you will see two distinct peaks on the FL2-H histogram.
- Adjust the FL2 voltage so that the G0/1 peak has a value of about 100 on FL2-H.
- There should be a similar profile at this point on the FL2-A histogram.
- Increase the FL2 amp gain (not voltage) to move the peaks up on the FL2-H histogram. Since the amp gain only adds height to a raw voltage pulse, the population will only move on FL2-H. The other FL2 derived parameters (W and A) will be unaffected.
- Increase the FL2-A amp gain until the G0/1 peak has a value of 200 on FL2-A
- Increase the FL2-W amp gain until the single cell population has a value between 200 and 600 on FL2-W.
- Adjust the FL2 voltage, amp gain and the FL2-W and –A amp gains until the data appears similar to the data below:

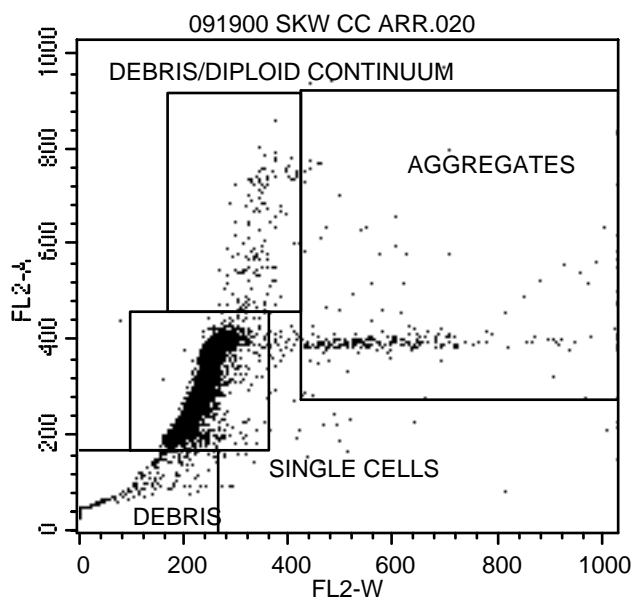
WHAT GOOD DATA LOOKS LIKE



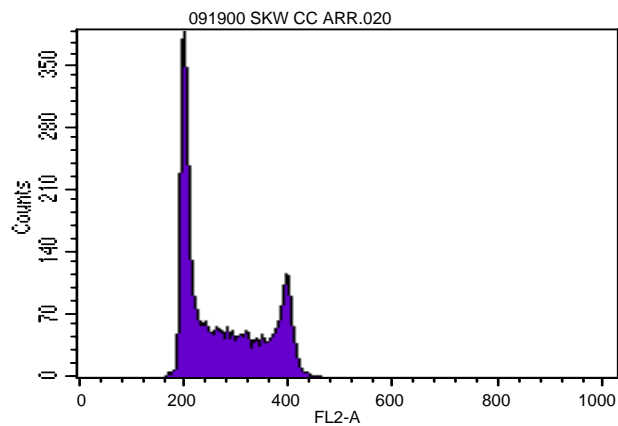
Generating data like this involves careful adjustments and readjustments of PMT voltage and Amp gain settings.

ACQUISITION DETAILS

Accurate de-convolution of the DNA histogram requires that at least 10,000 events are available for analysis. Because the single cells are the subject of analysis, it's important that, during acquisition setup, a collection gate is used to ensure that enough single cells will be stored. The best plot for aggregate/debris discrimination is the FL2-W vs. FL2-A plot. Use this plot to draw a gate around your single cells. The following plot shows the single cell gate as well as the other populations on the plot.



Set Acquisition and Storage so that 10,000 events are counted in the single cell gate. Be sure accept and store all events.



When analyzing the data in the cell cycle analysis program, use the single cell gate to exclude debris and aggregates -- so long as they are not continuous with the single cell population. In the above plots, the aggregates and debris can be easily resolved. In the following plots, however, the aggregates, debris and single cells blend together. In this case, software debris/aggregate modeling is required to accurately de-convolute these populations in addition to the G0/1, S and G2/M populations. If high levels of debris and aggregates are present in your sample, accurate data analysis might not be possible.

