# Introduction to FlowJo

Flow Cytometry Core Facility

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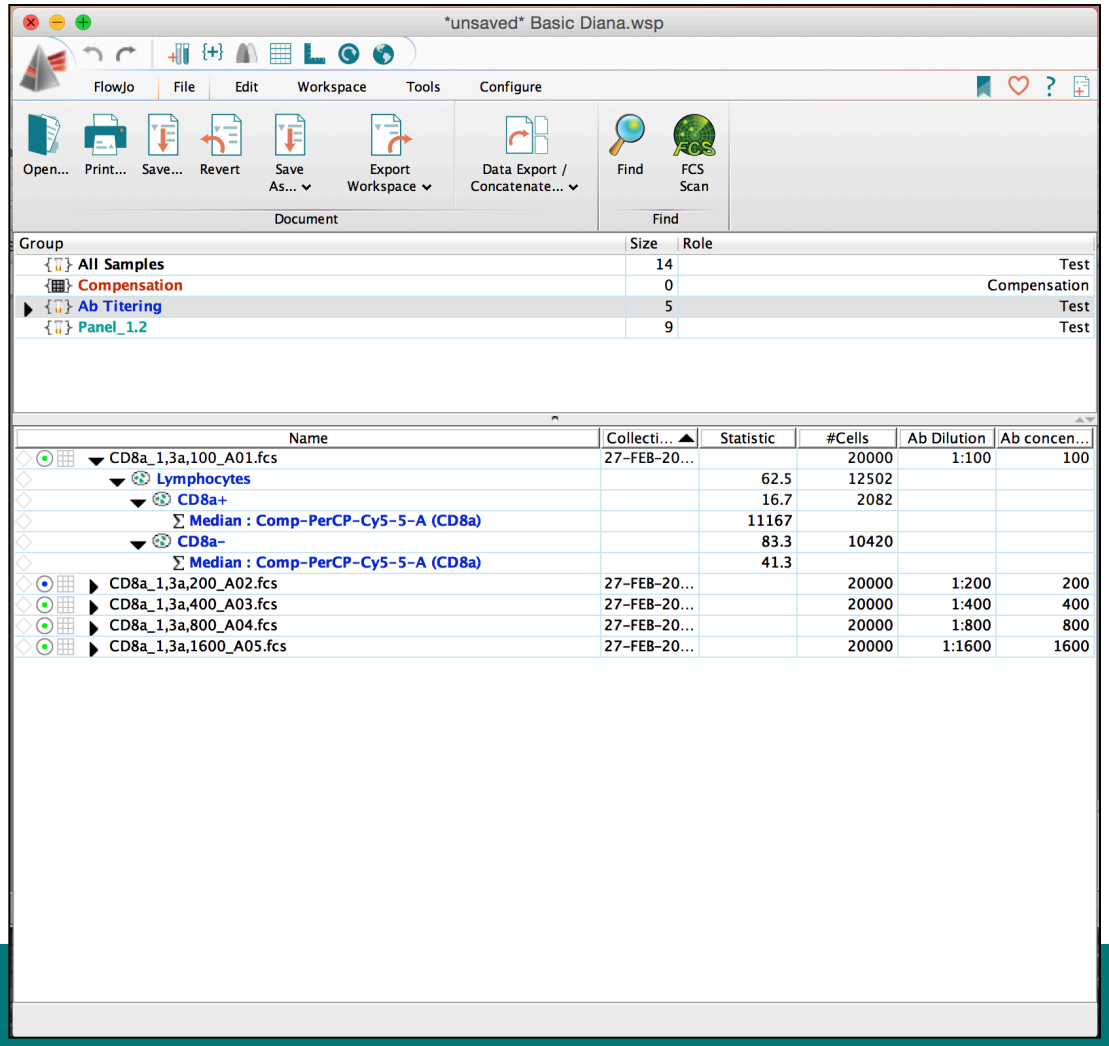
# FlowJo software

FlowJo is an integrated environment for viewing and analyzing flow cytometric data, presented in the form of a Workspace.



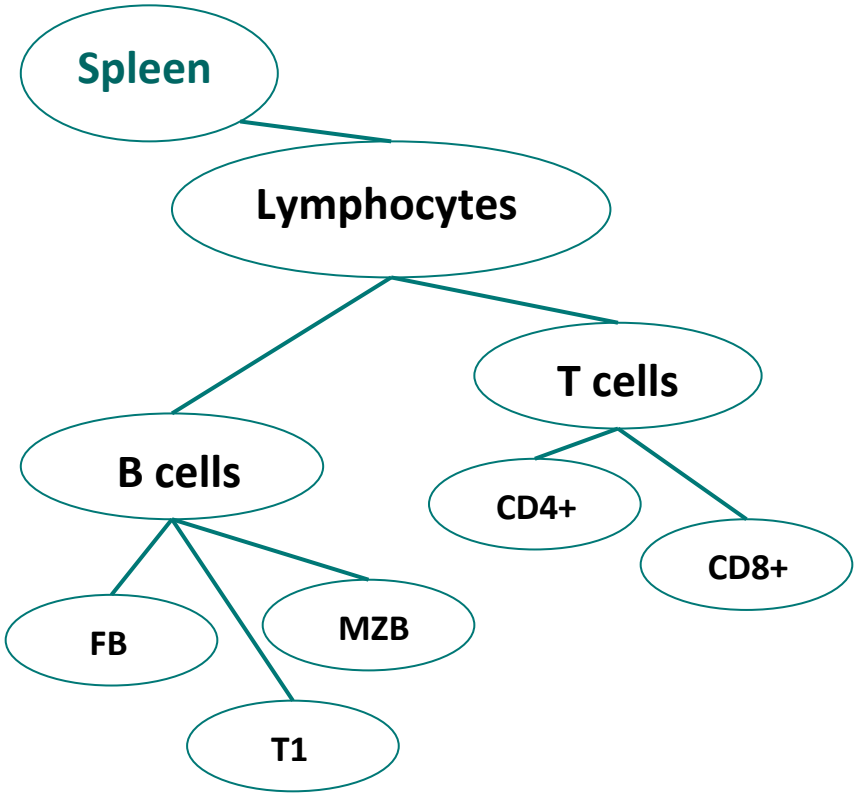

**Task Bar**

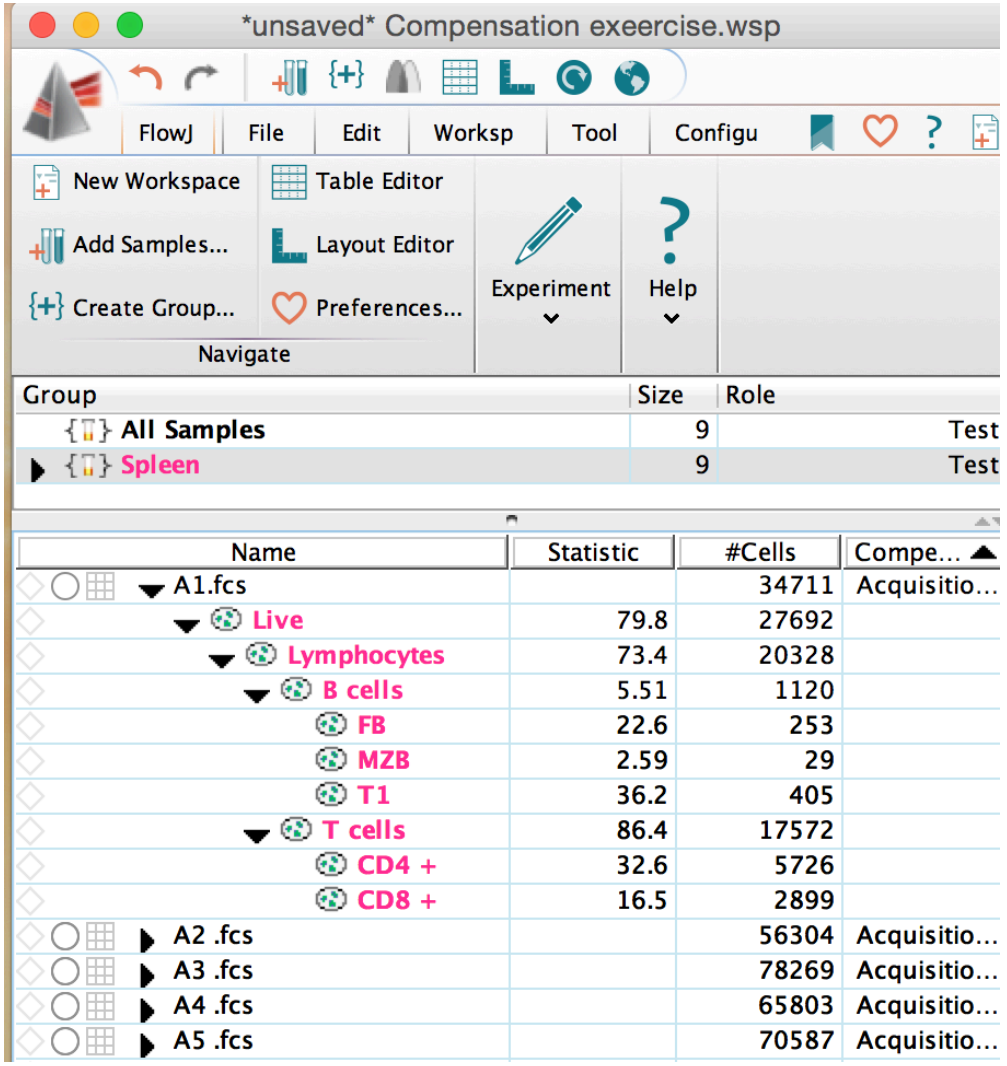
**Ribbons**

**Group section**

**Sample section**

# Population Hierarchy



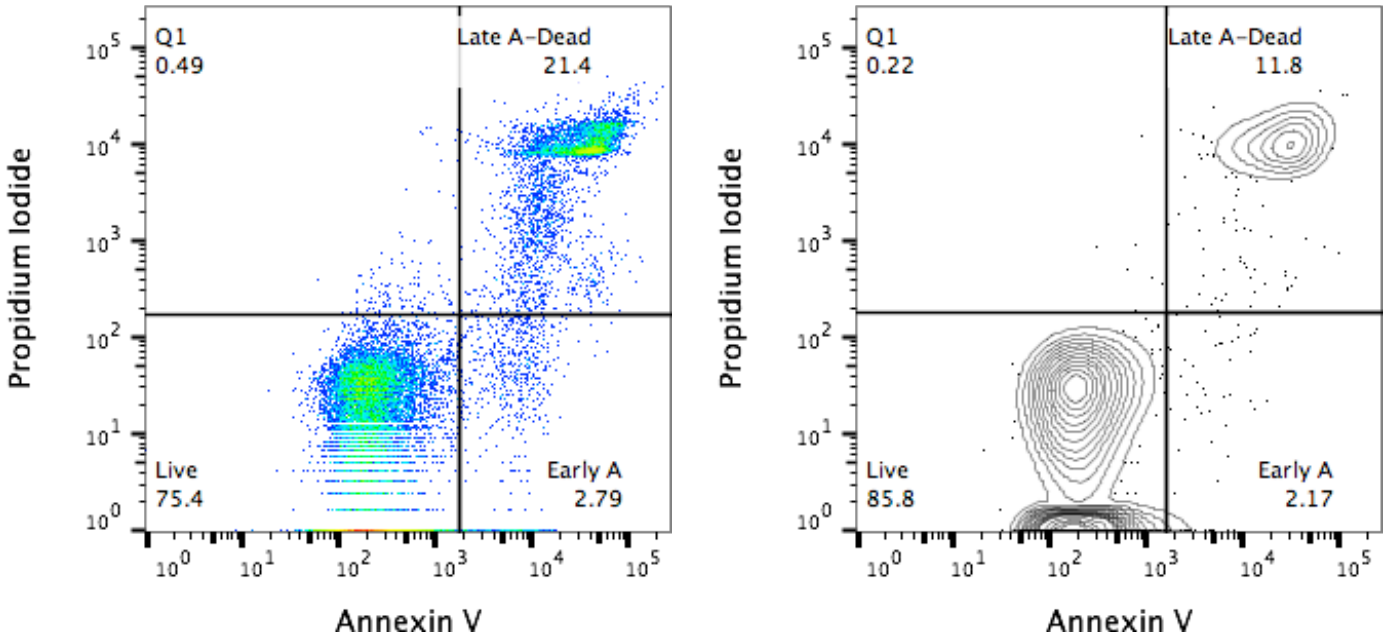




Allow the visualization of the populations defined for a sample and the relationship between gated populations

# Data display

Flow cytometric data is usually display using logarithmic scale, due to the high dynamic range between negative and positive populations.

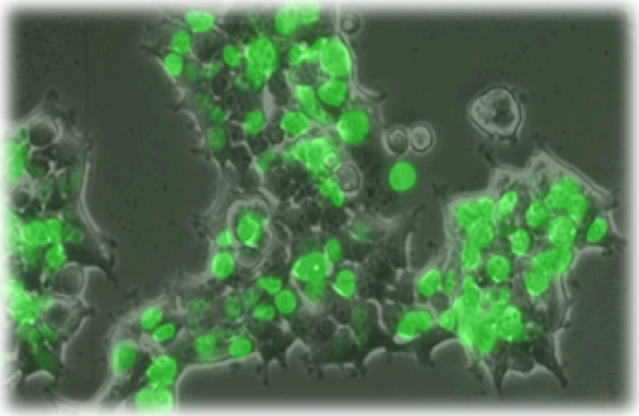
Logarithmic displays allows the visualization of subset of cells with medium to high amount of fluorescence. However, its might offer a truncated view of subsets with little, not cell-associated fluorescence or fluorescence values below zero.

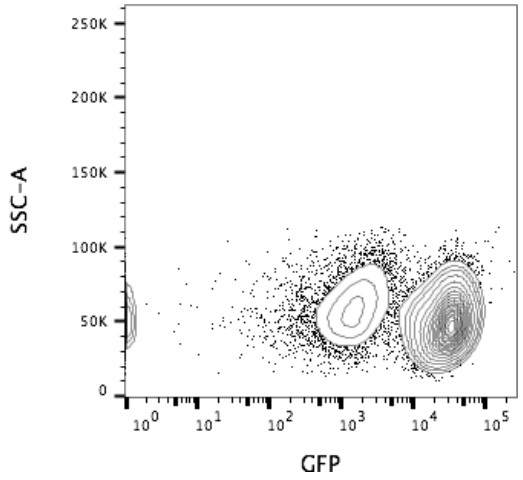


# Fluorescence signals below zero

Flow cytometers  measure cell-associated fluorescence that can be essentially zero but not negative.

**Negative – Intermediate - High**






**The image displayed in the graphs is no longer “pure” fluorescence , it is a corrected measurement derived from a fluorescence signal.**

Negatives values are a consequence of 2 different operations related to the way the instruments collets and corrects data:

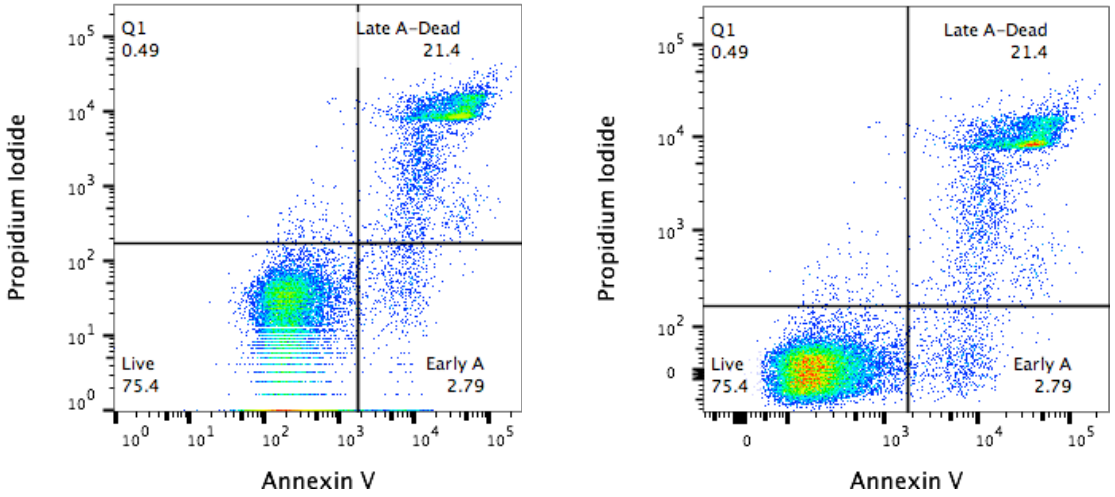
- Baseline correction
- Compensation

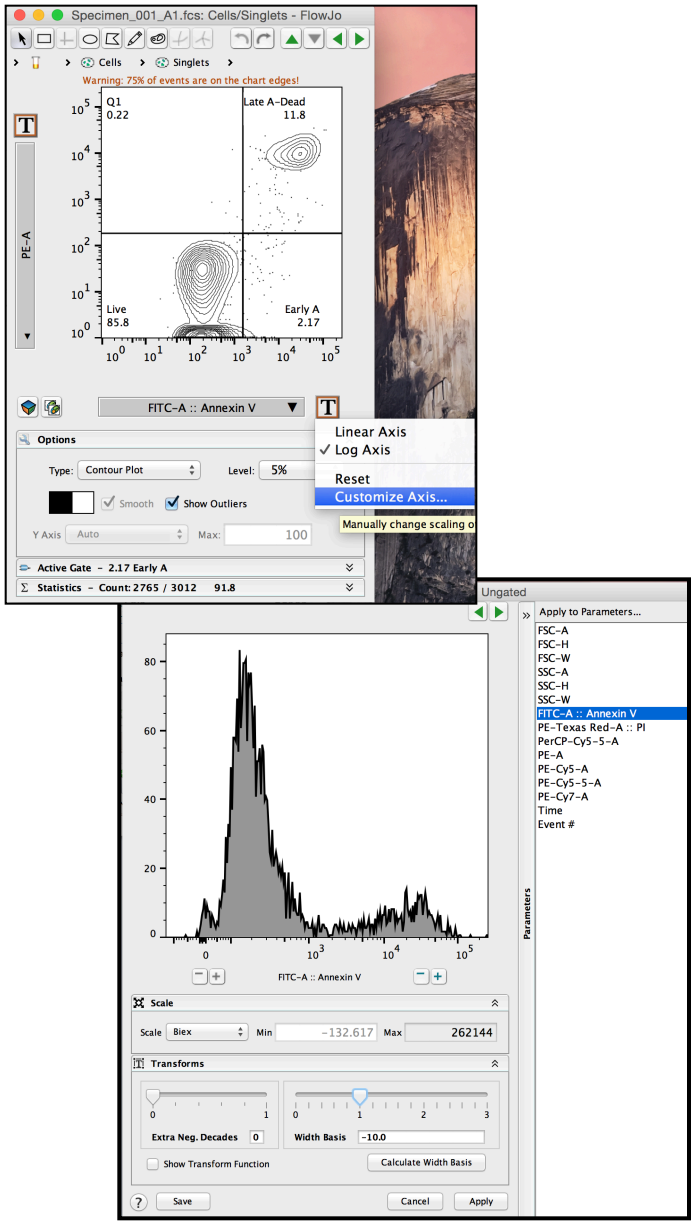
# Bi-exponential Transformation

Transformation do not modify your actual recorded fluorescence data, only the amount of visual space that is allotted to a specific region of the data.

The biexponential scale is a hybrid of a linear and logarithmic scale. The low end of the data is displayed with a linear scale allowing negative numbers and zero to be displayed, while the high end of the data is displayed using a logarithmic scale.

Bi-exponential display helps to visualize data that is compressed against the lower x and y axis, improving it resolution.






**Extra Negative Decades:** Amount of negative space is shown.

The **width basis** is the amount of space shown on a linear scale, on both sides of zero. For example, if you set the width basis to -10 then the space from -10 to +10 will be shown linearly and the space above and below that will be scaled logarithmically.

**Positive Decade Range (+/- buttons)** - This setting allows you to control how many decades the data is displayed over.

**Calculate width basis:** Automatic calculation made by FlowJo.



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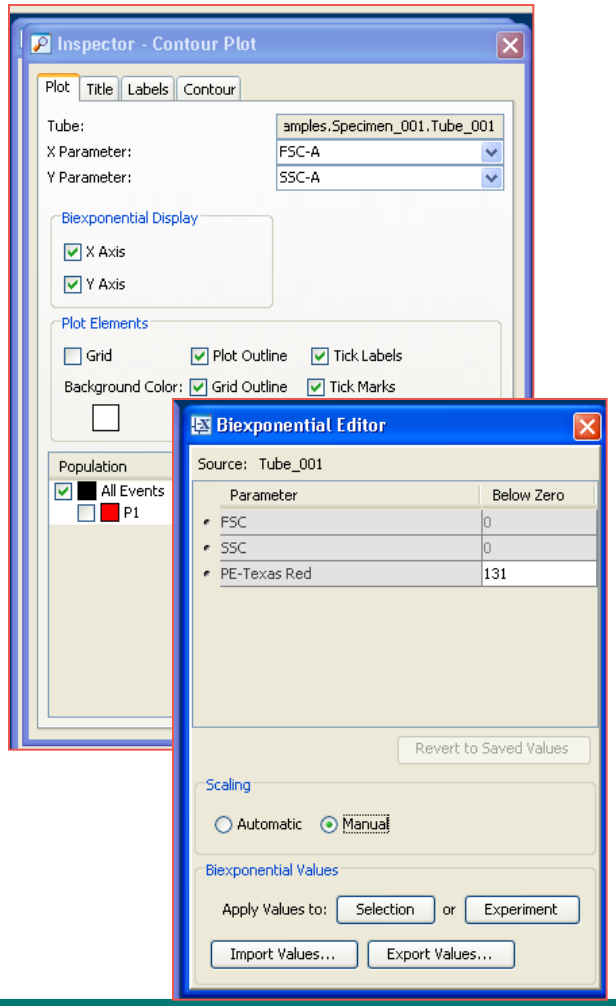
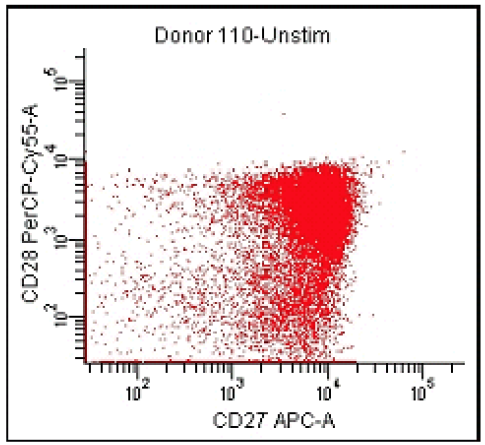
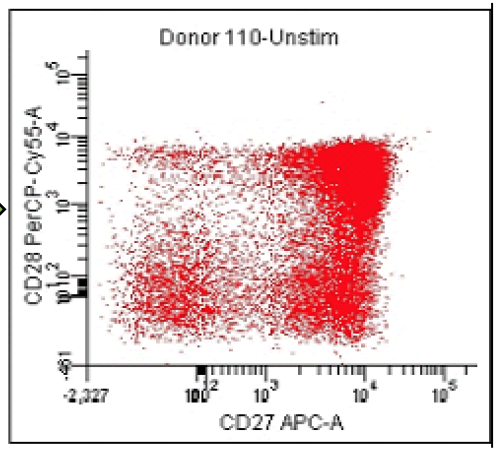
# Bi-exponential scale could be applied in the acquisition software (FacsDIVA).




no biexponential scaling




biexponential scaling (both axes)

**Bi-exponential display reveals the hidden double-negative population that is not visible in the standard log scale!!!**



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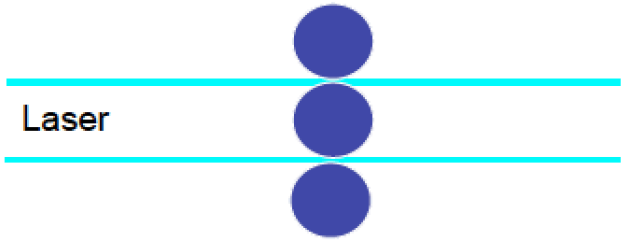
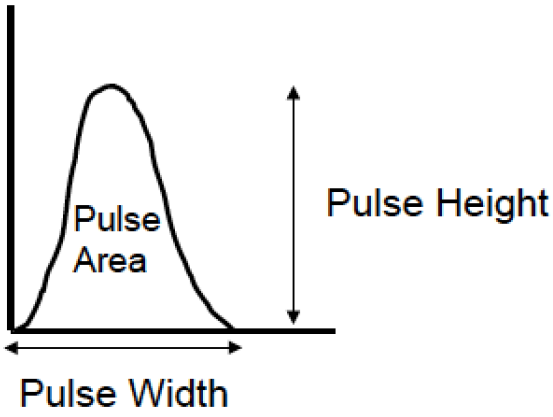
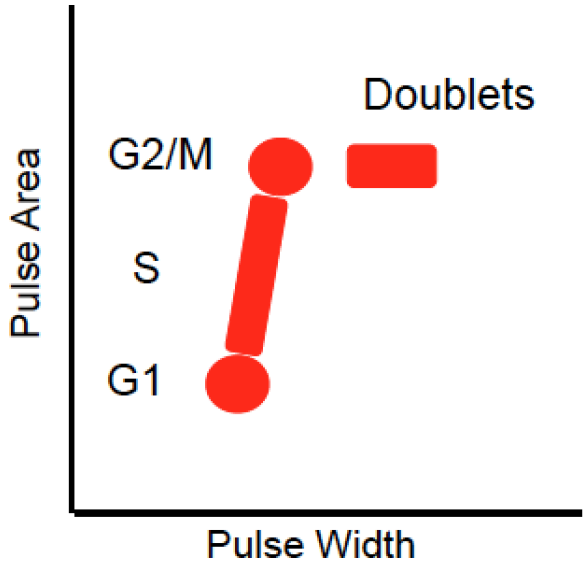
# The beginner's guide to pulse processing



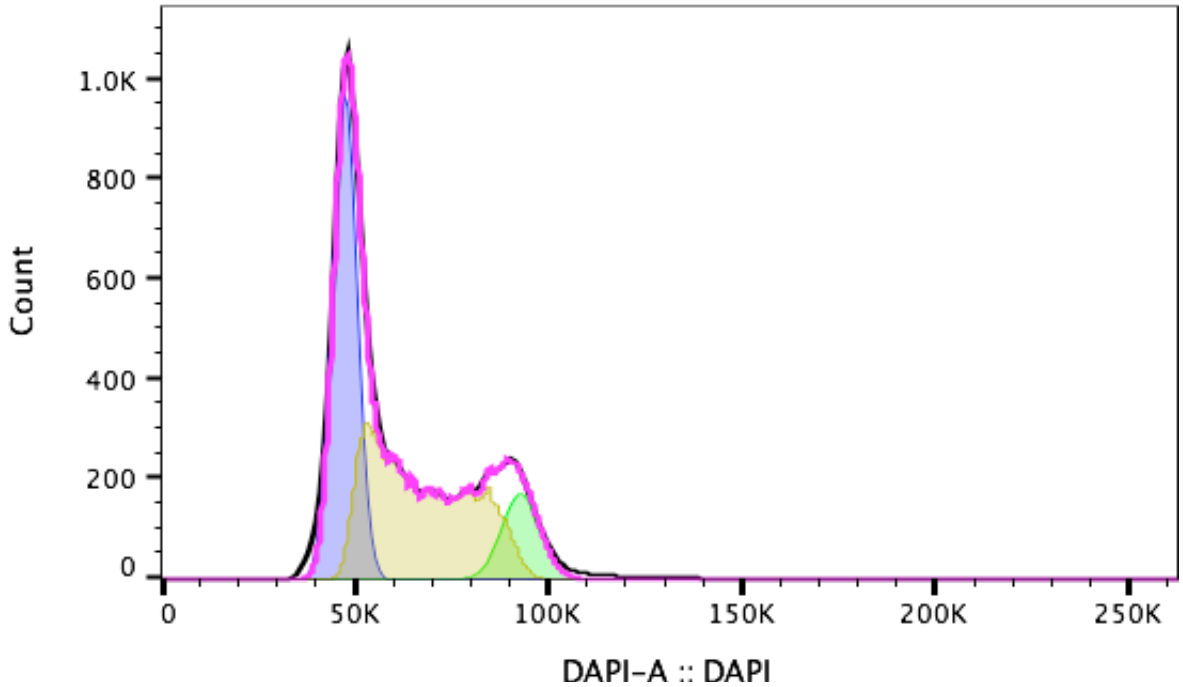




**Slide from Derek Davis, The Francis Crick Institute London UK.**

# Data Acquisition

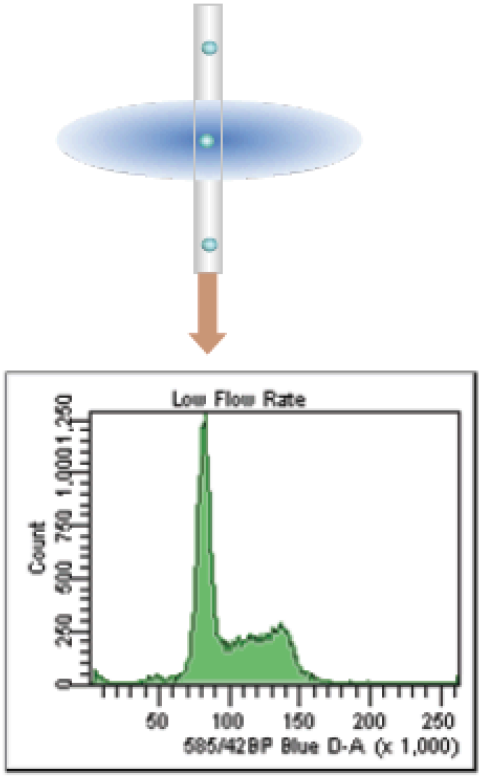
Because fluorescent intensity is a relative measure, the position of the data in the DNA histogram can be controlled. It is convention to place the G0/G1 peak at the 50 or 100 intensity unit mark.

PMT voltage could be slightly modify during acquisition in order to place G1 peak at the same position in every sample ….. If possible.

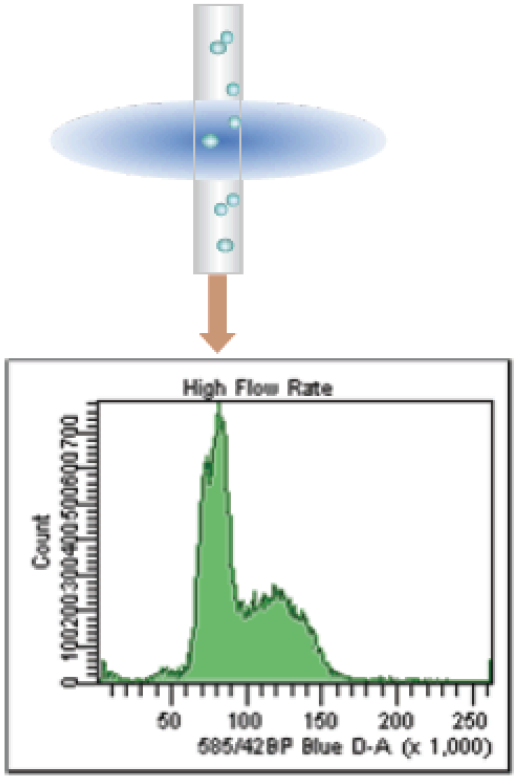


# Flow Rate

Narrow Sample Stream:
Low Flow Rate



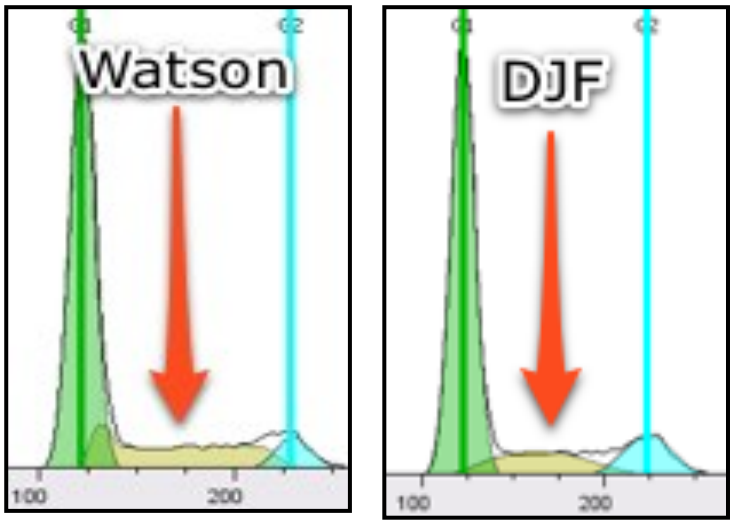

Wide Sample Stream:
High Flow Rate



# Cell Cycle Models

FlowJo uses two model options to fit your data: Watson (Pragmatic) and the Dean-Jett-Fox models.

**Comparison of the S-Phase Fit**

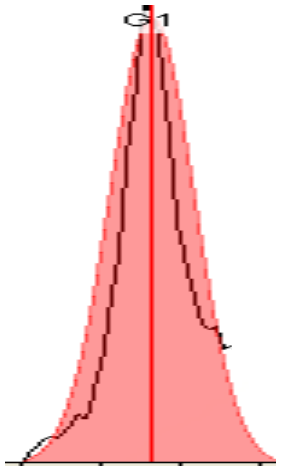



**Watson (Pragmatic) model**: makes no assumptions about the shape of the S-Phase distribution.

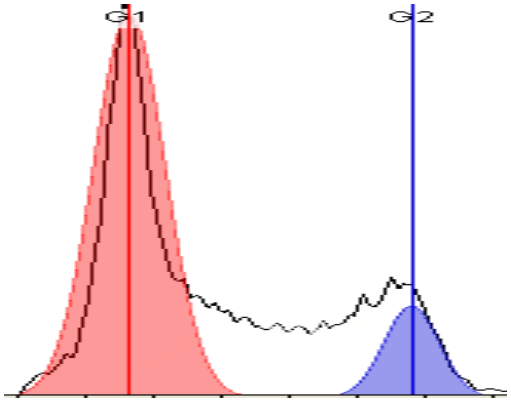
**Dean-Jet-Fox** model assumes that the S-phase can be modeled by a second degree polynomial (that is convoluted with gaussian distributions of varying width throughout S-phase).

# Watson (Pragmatic) Model

The Watson model assumes that only the data within the G0/G1 and G2/M peaks are normally distributed and that one of those two peaks is identifiable.




**G0/G1 peaks** are approximated to a Gaussian distribution
**G1 mean** is guess by finding the channel with the most cell in the left portion of the data.
The **standard deviation** (SD) or width of the population is approximated by finding the width of the distribution at 60% of the maximum height.
**Fit improvement:** A minimization process is executed over a range of -3 to 1 standard deviations about the first guess mean




**G2 peak:**

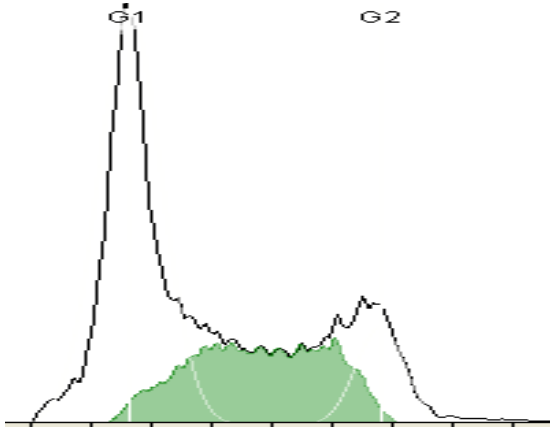
G2/M mean is placed at 1.75 x the intensity of the G0/G1 mean.

The **standard deviation** (SD) is estimated in the same manner and a second Gaussian distribution is fit to the data using the same minimization process.

**S phase:**

Gaussian distributions of G0/G1 and G2/M are subtracted from the data to leave only the cells that will be considered to be S phase.

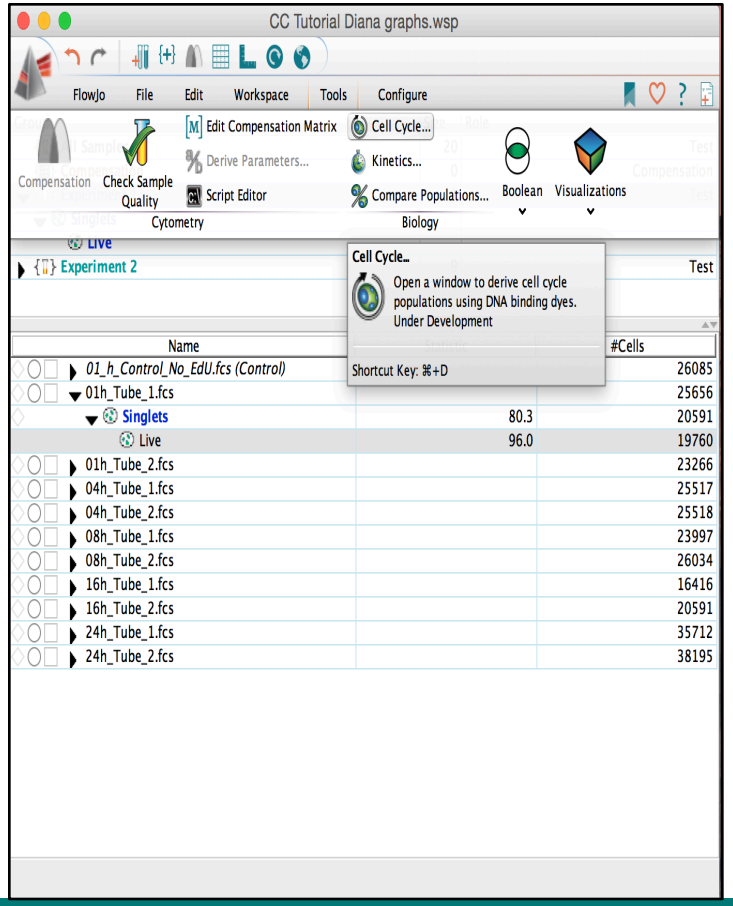
Once only S phase cells remain, a curve is fitting by an integrating function and the edges are modified in order to reflect the G0/G1 and G2/M edges.

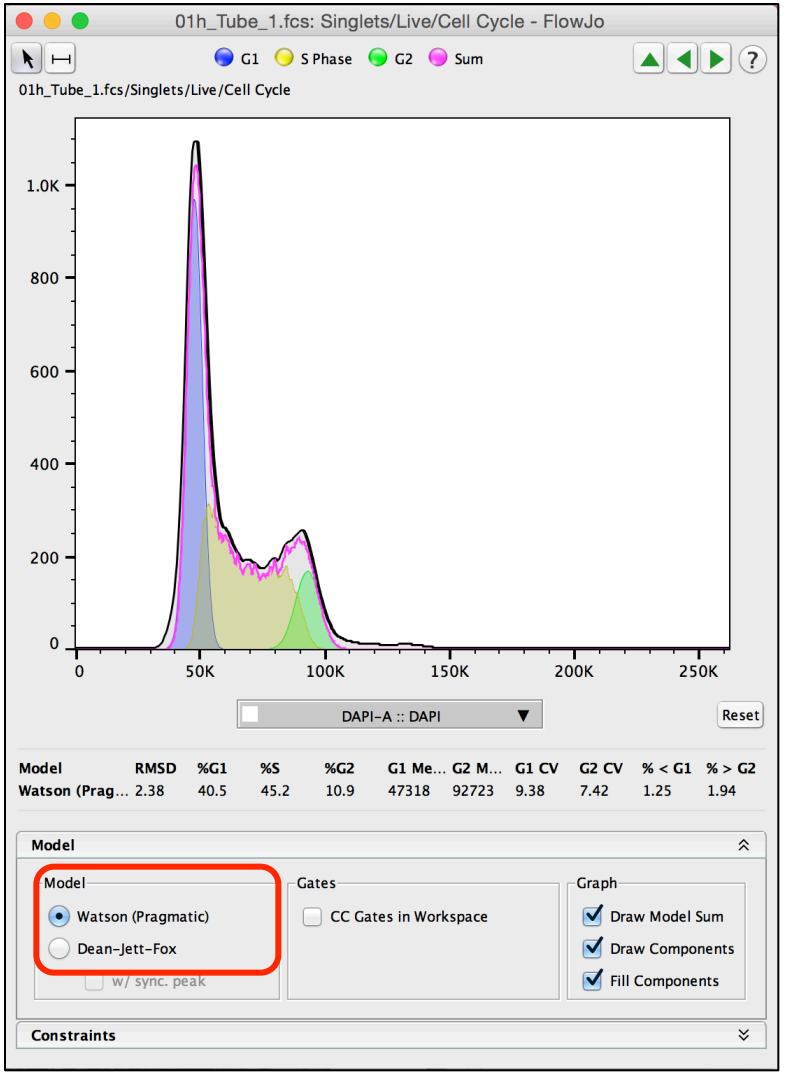



**The complete model is then the sum of the three parts.**

# Cell Cycle Platform

Drag the samples and start the analysis of the control sample.



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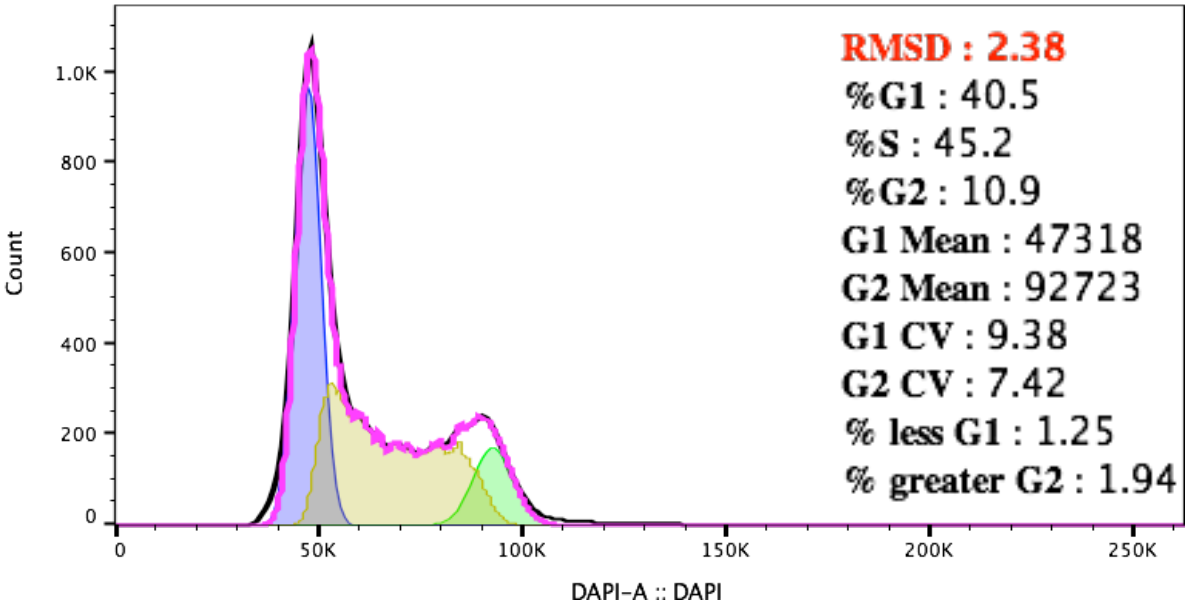
# Result Display




Reflects the quality of the fit.

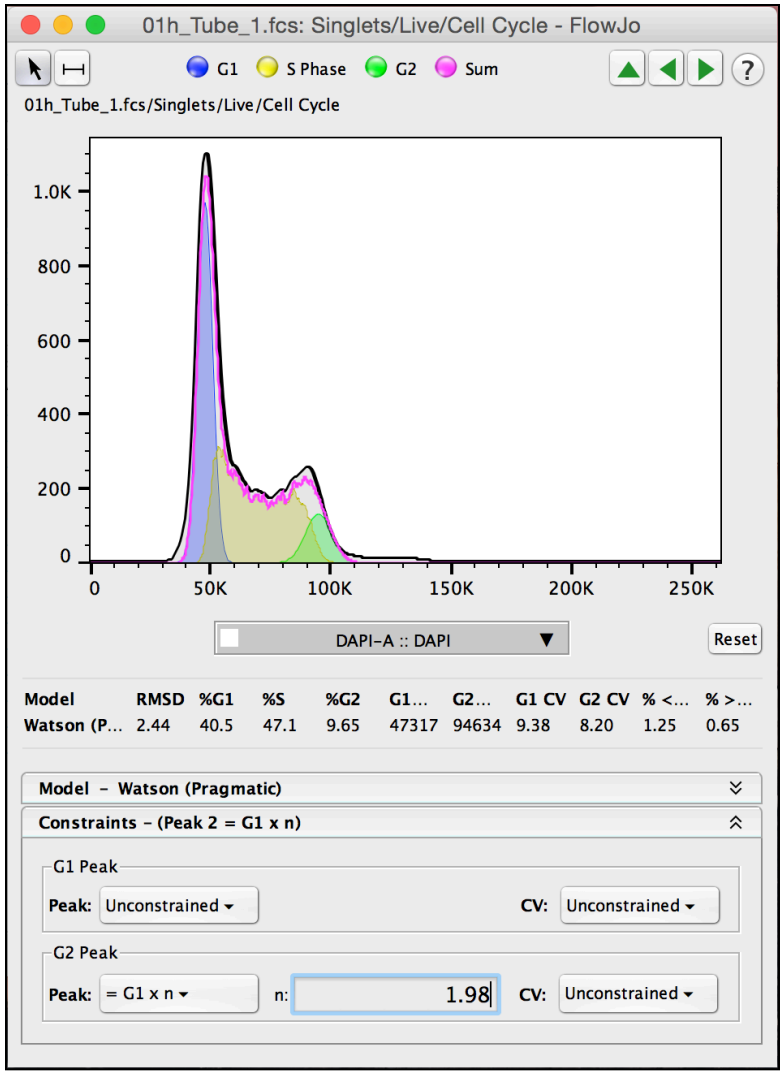
% of cells ascribed to a given population

Gaussian distributions can be completely described by a mean (μ) and a CV.

The **Root-mean-square error (RMSE)** is a frequently used measure of the differences between values predicted by a model and the values actually observed.

**Since RMS is a measure of the distance from the model to the data, a smaller RMS indicates a better fit. Usually….. RMS value of**

# Improving the cell cycle model




Constraint settings:

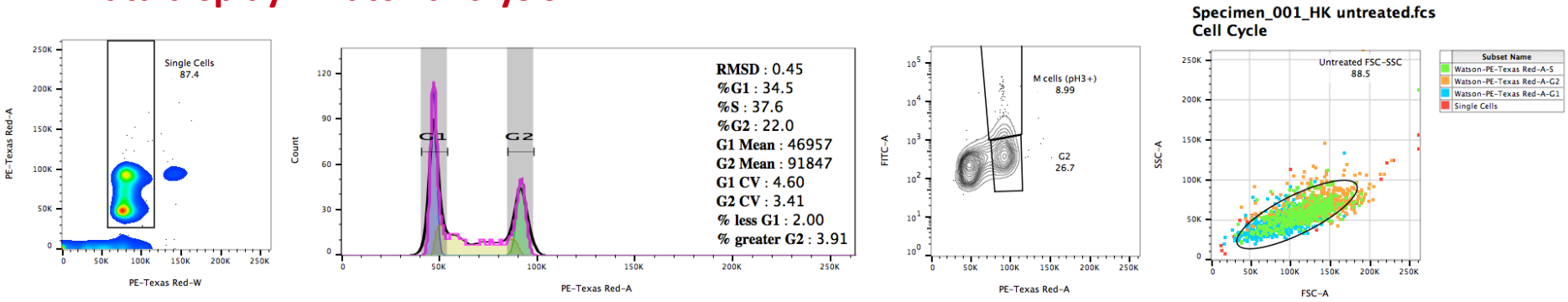
**Peak location**: Set G1 or G2 peak positions based on a control sample.

**Mean constraint**: Constraint the peak positions relative to each other.
The biology of the system tells us that G2/M cells should have twice as much DNA as G1 cells. You could define the G2 peak position to be around 2X the G1 peak or the G1 peak to be close to 0.5X the G2 peak.

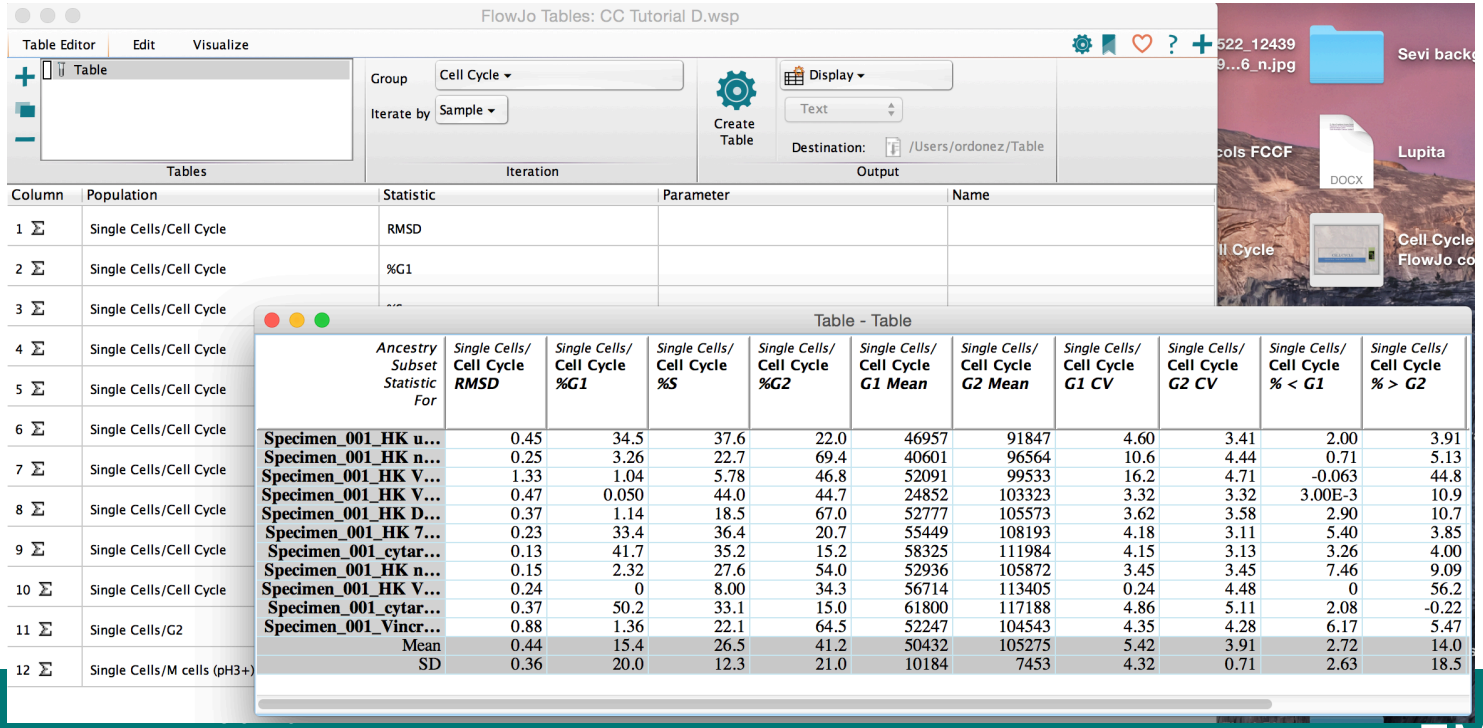
**CV constraint**: Set the same CV values for G1 and G2 peaks.
The two population are subject to the same illumination error derived from the analyzer.

**RMS can be used to determine whether a constraint improved the fit or not.**

# Data display – Batch analysis




# Summary table




| Ancestry Subset Statistic For | Single Cells/ Cell Cycle RMSD | Single Cells/ Cell Cycle %G1 | Single Cells/ Cell Cycle %S | Single Cells/ Cell Cycle %G2 | Single Cells/ Cell Cycle G1 Mean | Single Cells/ Cell Cycle G2 Mean | Single Cells/ Cell Cycle G1 CV | Single Cells/ Cell Cycle G2 CV | Single Cells/ Cell Cycle % < G1 | Single Cells/ Cell Cycle % > G2 |
|---|---|---|---|---|---|---|---|---|---|---|
| Specimen_001_HK u… | 0.45 | 34.5 | 37.6 | 22.0 | 46957 | 91847 | 4.60 | 3.41 | 2.00 | 3.91 |
| Specimen_001_HK n… | 0.25 | 3.26 | 22.7 | 69.4 | 40601 | 96564 | 10.6 | 4.44 | 0.71 | 5.13 |
| Specimen_001_HK V… | 1.33 | 1.04 | 5.78 | 46.8 | 52091 | 99533 | 16.2 | 4.71 | -0.063 | 44.8 |
| Specimen_001_HK V… | 0.47 | 0.050 | 44.0 | 44.7 | 24852 | 103323 | 3.32 | 3.32 | 3.00E-3 | 10.9 |
| Specimen_001_HK D… | 0.37 | 1.14 | 18.5 | 67.0 | 52777 | 105573 | 3.62 | 3.58 | 2.90 | 10.7 |
| Specimen_001_HK 7… | 0.23 | 33.4 | 36.4 | 20.7 | 55449 | 108193 | 4.18 | 3.11 | 5.40 | 3.85 |
| Specimen_001_cytar… | 0.13 | 41.7 | 35.2 | 15.2 | 58325 | 111984 | 4.15 | 3.13 | 3.26 | 4.00 |
| Specimen_001_HK n… | 0.15 | 2.32 | 27.6 | 54.0 | 52936 | 105872 | 3.45 | 3.45 | 7.46 | 9.09 |
| Specimen_001_HK V… | 0.24 | 0 | 8.00 | 34.3 | 56714 | 113405 | 0.24 | 4.48 | 0 | 56.2 |
| Specimen_001_cytar… | 0.37 | 50.2 | 33.1 | 15.0 | 61800 | 117188 | 4.86 | 5.11 | 2.08 | -0.22 |
| Specimen_001_Vincr… | 0.88 | 1.36 | 22.1 | 64.5 | 52247 | 104543 | 4.35 | 4.28 | 6.17 | 5.47 |
| Mean | 0.44 | 15.4 | 26.5 | 41.2 | 50432 | 105275 | 5.42 | 3.91 | 2.72 | 14.0 |
| SD | 0.36 | 20.0 | 12.3 | 21.0 | 10184 | 7453 | 4.32 | 0.71 | 2.63 | 18.5 |



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# END