Cell Cycle

FLOWJO
Flow Cytometry
Analysis Software

Abstract

Fluorescent dyes, such as Propidium Iodide and the Hoechst dyes, bind to DNA, giving cytometers the ability to quantify the DNA in individual cells. It is possible to classify cells into phases of cell cycle based on the amount of DNA they contain.
Using curve-fitting algorithms, FlowJo fits the DNA histogram into three mathematical distributions, representing the populations of cells in each of the phases. Because of the variability inherent in biological data and staining techniques, the DNA histogram can elude the fitting algorithms, and user intervention is required to constrain aspects of the fit. FlowJo allows you to view three different models, constrain different fitting parameters, and automatically calculate the percentage of cells in G1/G0, S, and G2/M.
As with its other analysis platforms, FlowJo supports copying cell cycle analyses to other samples or experiments. Before generating the combined output, you can easily navigate between successive graphs to check for sample-specific adjustments.

General Information

FlowJo fits cell cycle data using one of two mathematical models: the Watson Pragmatic model [1] or the Dean-Jett-Fox (DJF) model. These models are used to define the G1, S, and G2 phases of the cell cycle. Both the DJF and the Watson model fit G1 and G2 with Gaussian curves. The Watson model makes no assumptions about the shape of the S-Phase distribution; it fits the region between the identified G0/G1 populations exactly by first subtracting the G0/G1 and G2/M portions of the data and then building a function that fits what remains. The DJF model fits the S-phase with a second-degree polynomial. A synchronized peak within the S-phase can also be chosen when using this model.

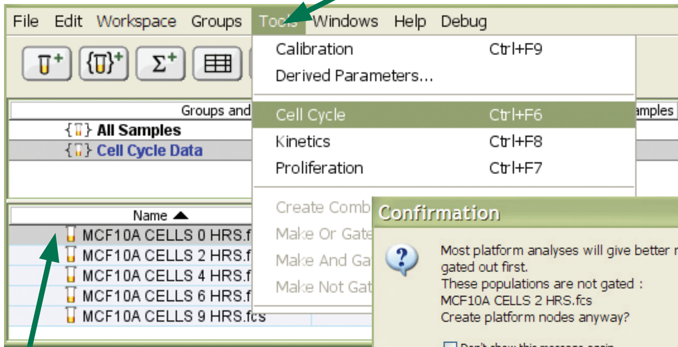
If the distribution is not fit adequately by the unconstrained model, characteristics of the peaks can be constrained. The models are initialized based on the most common DNA distribution, specifically an identifiable G0/G1 and G2/M peak, with the G2/M peak located at roughly twice the intensity of the G0/G1 peak. Specific knowledge of the system biology involved in a particular experiment and the specific experimental conditions may suggest constraints to use. Each Gaussian peak (G1, G2, or the synchronous population) can be constrained by relative position to another peak or by manually dragging the location of the mean by clicking on a distribution and dragging the cursor horizontally. In addition, the peak widths of each Gaussian can be constrained to an absolute value, in relation to each other, or manually by clicking on a distribution or dragging the cursor vertically. The model is immediately recalculated and displayed making the constraining process fully interactive. By judicious use of ranges to constrain peak positions, and relative values for the peak widths, you should be able to fit most distributions.

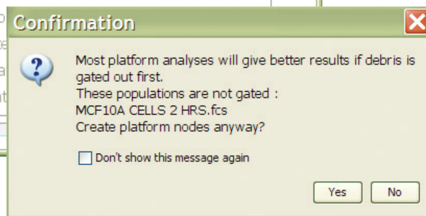
This Tech Note focuses on the mechanics of analyzing cell cycle data using FlowJo. Other sources are referenced to provide an overview of the entire process [3], choosing the appropriate DNA staining dyes for your experiment [4], and factors that affect the quality of DNA histograms [5].
Demo cell cycle data and workspace can be found at: *www. flowjo.com/home/demo_data.html*

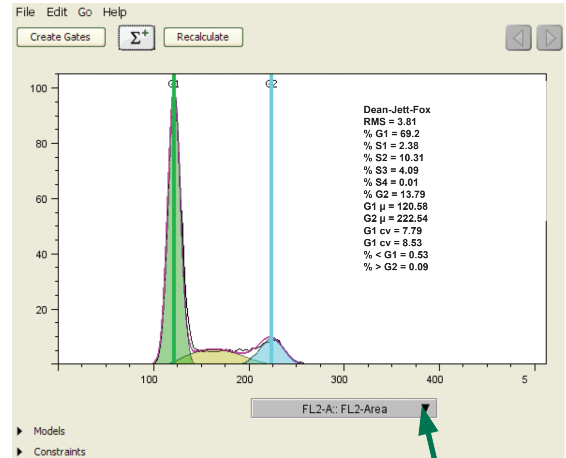
Procedure

1 Launch the Cell Cycle platform.

Click once to select the sample or subpopulation in the workspace you would like to analyze. Under the Tools menu, select Cell Cycle.







Cell Cycle analysis is performed on this population... any gated subset can also be selected and analyzed.

If you have not yet gated out debris, FlowJo will suggest that you do so.

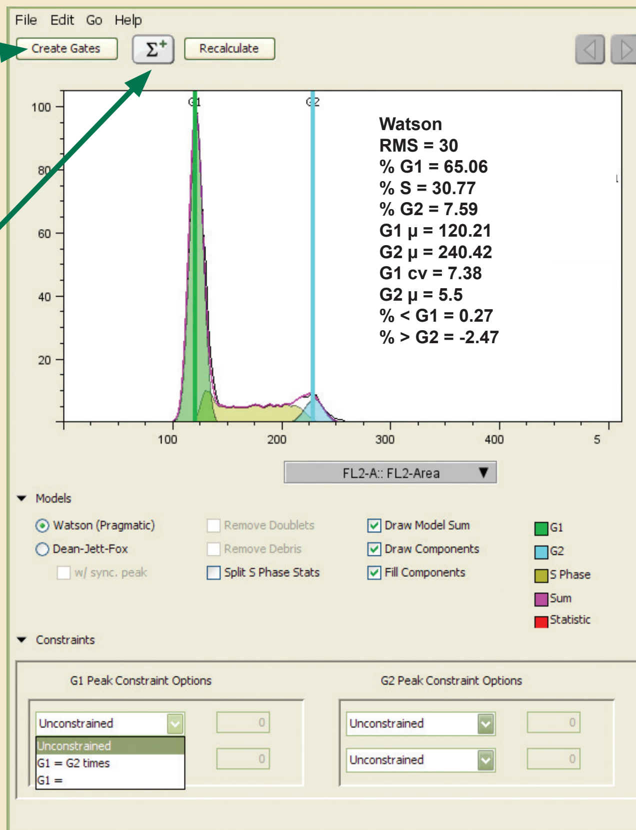
Select the DNA quantitation parameter for the X-axis.

2 Choose a model to fit your data.

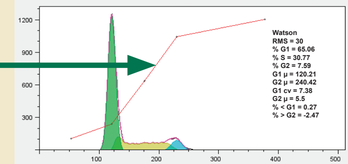
Each model attempts to fit curves to the stages of the cell cycle. The Watson-Pragmatic and the Dean-Jett-Fox models both fit Gaussian curves to the G1 and G2 phases. However, the Dean-Jett-Fox model fits the S phase with a mathematical function, but the Watson model does not.

Create gates corresponding to each cell cycle phase.

Add another statistic (calculated on another parameter) overlaid on the DNA histogram.



Median of FL2



File Edit Workspace Groups Tools Windows Help Debug

Groups and Analyses	Number of Samples
All Samples	5
Cell Cycle Data	5

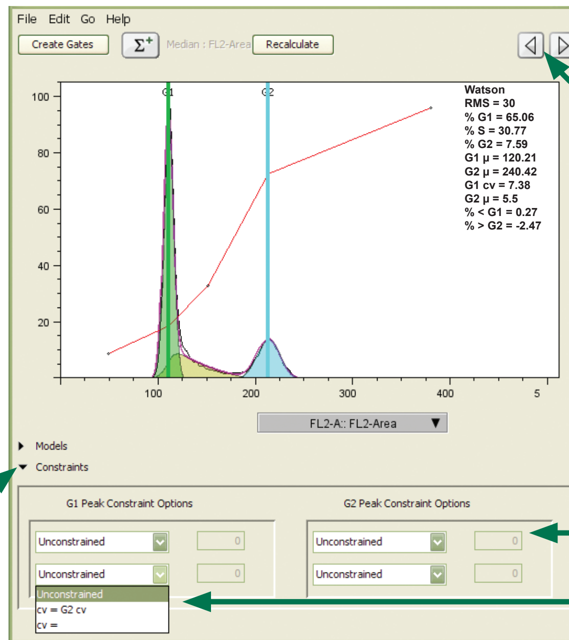
Name	Statistic	#Cells
MCF10A CELLS 0 HRS.fcs		14884
StageG1	71.67	10667
StageG2	17.63	2624
StageS	10.31	1534
Cell Cycle-1		
MCF10A CELLS 2 HRS.fcs		14622
MCF10A CELLS 4 HRS.fcs		14444
MCF10A CELLS 6 HRS.fcs		14619
MCF10A CELLS 9 HRS.fcs		14799

Watson
RMS = 30 — *Root Mean Squared*
% G1 = 65.06
% S = 30.77
% G2 = 7.59 — *Fraction of cells in G1, S, and G2*
G1 μ = 120.21
G2 μ = 240.42
G1 cv = 7.38
G2 μ = 5.5 — *Distribution stats of G1 and G2 peaks*
% < G1 = 0.27
% > G2 = -2.47 — *Fraction of cells below G1 and above G2*

3 Adjust the model.

The fit of the model can be assessed by comparing the model to the DNA histogram and by a low Root Mean Squared (RMS) score. If the model does not fit, you can either change models or constrain one of the model parameters to assist fitting.

Edit the position of the G1 or G2 peak by dragging the center line horizontally with the mouse; edit the CV by dragging the peak vertically.



Arrows cycle through samples in the Workspace with Cell Cycle analysis.

*Click the **Constraints** disclosure triangle to edit the G1/G2 curves. Return curves to their original shape with **Restore Curves**.*

Constrain the position of either the G1 or G2 peak in relation to the other.

4 Apply the Cell Cycle node to other samples.

Copy the Cell Cycle platform node to a sample or group to apply the models and constraints.

To copy exact gates, copy the subset names (Stage G1, S, and G2) to a sample or group.

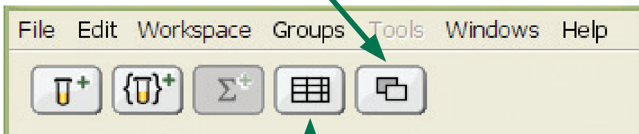
Drag the Cell Cycle node onto a group to place these analyses onto all samples in that group.

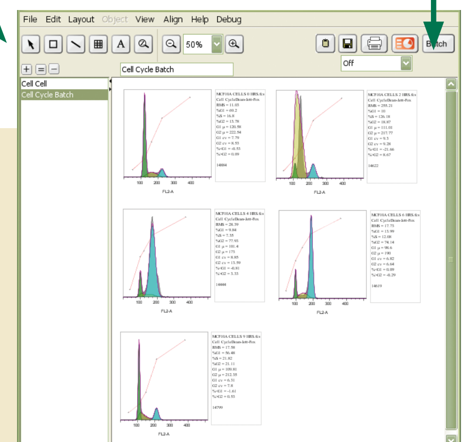
5 Generate a graphical report in the Layout Editor.

Open Layout Editor.

Drag the Cell Cycle node to the Layout Editor.

Generate a batch layout to display graphs of all Cell Cycle analyses.





6 Generate a table of statistics in the Table Editor.

Open Table Editor.

Drag the Cell Cycle node into the table editor.

Generate a table of statistics.

File Edit FlowJo Help

Samples	Model	RMS	%G1	%S	%G2	G1 μ (FL2-	G2 μ (FL2-	G1 cv	G2 cv	%<G1	%>G2
MCF10A CELLS 0 HRS.fcs	Dean-Jett-Fox	11.03	69.20	16.80	13.78	120.58	222.54	7.79	8.53	-0.53	0.09
MCF10A CELLS 2 HRS.fcs	Dean-Jett-Fox	255.21	10.00	126.18	18.87	111.01	217.77	9.30	9.28	-21.66	8.67
MCF10A CELLS 4 HRS.fcs	Dean-Jett-Fox	28.39	9.84	7.35	77.93	101.40	173.00	8.85	13.59	-0.81	3.33
MCF10A CELLS 9 HRS.fcs	Dean-Jett-Fox	17.58	56.48	21.82	21.11	109.81	212.35	6.31	7.80	-1.61	0.53
MCF10A CELLS 6 HRS.fcs	Dean-Jett-Fox	17.73	13.99	12.08	74.14	98.60	190.00	6.82	6.64	0.09	-0.29
Mean	-∞	65.99	31.90	36.85	41.17	108.28	203.13	7.81	9.17	-4.90	2.47
Standard Deviation	-∞	105.96	28.65	50.23	31.97	8.69	20.95	1.28	2.66	9.39	3.75

FLOWJO

Using Models

- Cell doublets will report twice the amount of DNA signal as individual cells, and this doubling can confuse cell classification. To remove doublets prior to analysis of a cell cycle distribution, graph the Area vs. Height (or Width) of the dye that measures DNA. Doublets will have a higher ratio of area to height.
- Apply the models of choice to the data without any constraints. For most distributions, FlowJo will be able to locate the G1 and G2 peaks and thereby accurately fit the distribution with the model.
- The fit of a model can be assessed by eye or by the Root Mean Squared (RMS) value. Lower RMS values indicate a better model fit. Because the RMS calculation depends on the model and constraints, a range of RMS values that indicate a good fit cannot be predefined.
- If FlowJo fails to fit the model(s) to the data, then it will display "Invalid" in the RMS error field. In this case, you will want to help FlowJo fit the data by constraining different parameters or use an alternative model.
- Start with the more distinguishable peak position and constrain the complement to be centered relative to the known peak.
- The Dean-Jett-Fox model has an option to fit a synchronous peak. A synchronous peak usually results after a population is released from a cell cycle arrest, which causes a large fraction of cells to travel through the phases of the cell cycle in a synchronized manner.

Constraining the Fit

- It may be useful to analyze a control sample that has a good distribution and to set constraining ranges for the peak(s) based on that sample. For the difficult sample acquired in the same experiment, constrain values such as the G1 and G2 coefficient of variance (CV). Or constrain the position of the G1 peak in the well-fit population. This range will automatically be applied when the Cell Cycle node is copied to the difficult sample in the Workspace list by drag-and-drop.
- Also consider constraining the peak positions relative to each other. For example, the G2 peak position can be defined to be 2x the G1 peak, or the G1 peak to be 0.5x the G2 peak. However, caution must be used in this case. Some dyes, such as PI, do not always identically stain cells at each stage of the cell cycle.
- In general, the CV for the G2 peak should be the same as the CV for the G1 peak. This is because cells within a single phase should theoretically all have the same amount of DNA and should produce the same signal intensity. The width of either G0/G1 or G2/M is predominately a product of the cytometer's measurement error. This means that setting the G2 CV equal to the G1 CV is generally a useful constraint. Alternatively, force both CVs to be equal to the calculated value for a well-defined distribution collected in the same experiment.

Links and References

1. Watson, Chambers, & Smith. **A pragmatic approach to the analysis of DNA histograms with a definable G1 peak.** Cytometry 8:1-8 (1987).
2. Fox. **A model for the computer analysis of synchronous DNA distributions obtained by flow cytometry.** Cytometry 1:71-80 (1980).
3. Givan A. **Flow cytometric analyses of the DNA content of cells: An overview.** Bowdoin Cytometry Course (2002).
4. Poot M. **Nucleic Acid Probes**. Current Protocols in Cytometry J.P. Robinson, Eds. New York, J. Wiley: 4.3.1 - 4.3.10 (1997).
5. Rabinowitch PS. **Practical considerations for DNA content and cell cycle analysis**.
6. Bagwell, et al. **Optimizing flow cytometric DNA ploidy and S-phase fraction as independent prognostic markers for node negative breast cancer specimens**. Cytometry 46(3):121-35 (2001).
7. Bauer KD, et al. eds., **Clinical flow cytometry: Principles and applications**, Baltimore, Williams and Wilkins (1993).

FlowJo is a product of Tree Star, Inc.
www.flowjo.com
1- 541-201-0022
email: flowjo@treestar.com

TN.CellCycle.20100423