



## Abstract

Complete evaluation of a cell population requires more elaboration than just phenotypic characterization. It is necessary to describe dynamic properties of the biological system in order to fully appreciate its complexity. Measurements of cellular proliferation provide a powerful tool to ascertain functional properties of cell types as they divide.

Proliferation was traditionally measured using a radioactive probe, tritiated thymidine. However, with the use of modern flow cytometry and the advent of fluorescent dyes such as Carboxyfluorescein diacetate Succinimidyl Ester (CFSE) and PKH (a proprietary, lipid-binding dye from Sigma-Aldrich), proliferation assays have become more robust and safer. FlowJo's proliferation platform provides the appropriate and necessary tool to model and quantitatively analyze complex proliferation data.

## General Information

CFSE and PKH fluorescent probes bind to lipids within the cell membrane. Upon cell division, the relative fluorescence intensity of the daughter population is approximately half that of the parent. Therefore, a series of distributions or peaks is generated. Fluorescence intensity decreases from the parent (highest) to the final generation (lowest). FlowJo's curve fitting algorithm will locate, identify, and model a Gaussian distribution at the highest fluorescence intensity. By halving the fluorescence value for each successive generation, FlowJo's proliferation platform can model every Gaussian distribution that results from the progeny. Numerous statistics are generated, including the division index, the proliferation index, and the percent divided.

FlowJo uses non-linear curve-fitting techniques. It examines the data, makes a guess as to the values for the parameters, and then begins making small changes to the parameters that determine the fit. It iterates through the fit until it can make no more significant improvement.

Based on the algorithm that FlowJo uses, the generated report provides division statistics in terms of number of divisions rather than number of cells that exist as a result of the proliferation. The number of divisions is more meaningful, biologically, since the number of cells grows exponentially with divisions and therefore does not lend itself well to the linear averaging used to compute the index.



# Proliferation

## Statistics Reported in the Proliferation Platform

**Number of Peaks:** The number of generations fit in the model (publications cite eight as a reasonable maximum resolvable peaks)

**Peak Coefficient of Variance:** All peaks stem from the same population measured under the same conditions. This assumes that division does not affect the variance of the population.

**Peak Ratio:** Normally we would expect the dye concentration to be cut in half with each division, but the data may reflect slightly different conditions.

**Undivided Mean:** This shows the fluorescence intensity of the original populations before division.

**Division Index:** The average number of divisions undergone by a responding cell.

**Proliferation Index:** The average number of divisions undergone by all cells, including the undivided peak.

**Percent Divided (Precursor Frequency):** The percentage of cells from the original sample, which have divided.

*Note: Division Index = (Proliferation Index) X (Percent Divided)*

**RMS:** Root Mean Square is the measure of the quality of the fit.

FlowJo's calculations of the Proliferation Index differ from others in the field. Historically, proliferation studies were performed with tritiated thymidine uptake experiments (a measurement that increases exponentially with cell division). This led to a statistic based on a bulk measurement, which is exponentially related to divisions. With the fluorescence measurements available in cytometry, it is more reasonable to speak of the number of divisions than of the number of cells resulting from the divisions.

The two measures are not inconsistent, but the relationship between the two is exponential, so it's not straightforward to see the relationship between two statistics returned by different software. Make sure to define proliferation index explicitly in your methods.

*Be sure to click the Calculate button after each change to Options.*

*Click the Create Gates button to add children to this population for each generation.*

*Constraining the Fixed Ratio will adjust the ratio of fluorescence between adjacent peaks. Ideally, this value should be just less than 0.5.*

*Increasing or decreasing Peaks will adjust the total number of proliferations expected or previously established.*

*Fixed Coefficient of Variance (CV) will ensure the width of each peak is constant.*

*Setting a Fixed Background will adjust for the known autofluorescence level of the cells.*

*Constrain Fix Peak 0 to make the undivided population start at a specified value. This is useful when few cells are left undivided.*

*Peak Coefficient of Variance*

*The number of events in each generation.*

FlowJo Statistics:  
 RMS: 3.87  
 #Peaks: 8.00  
 Peak cv: 4.06  
 Peak ratio: 0.514  
 Undiv. Mean: 274  
 Div. Index: 0.985  
 Prol. Index: 2.24  
 %Divided: 43.0  
 Background: 1.16  
 mRMS: 3.87

Cell Counts:  
 Generation 0: 2511  
 Generation 1: 1621  
 Generation 2: 1469  
 Generation 3: 2679  
 Generation 4: 3985  
 Generation 5: 3503  
 Generation 6: 1727  
 Generation 7: 1

Options:  
 # Peaks: 8  
 Fixed Ratio = 0.514  
 Fixed CV = 4.06  
 Fixed Backgd = 1.16  
 Fix Peak 0 = 274  
 Draw Model Sum  
 Fill Components



## Hints

- Refining your raw data with gates helps the fit. Gate out debris and cells not pertinent to the analysis.
- If there is a substantial undivided peak and a completely divided generation, gate the cells in the middle of those two peaks and try applying the proliferation platform to those cells.
- Use control samples to set the undivided peak and its coefficient of variance. Then use constraints to ensure that stimulated samples will reflect the same analysis conditions.

## Links and References

<http://www.flowjo.com/vX/en/proliferation.html>

Daily Dongle: [http://flowjo.typepad.com/the\\_daily\\_dongle/2007/05/dongleoids\\_inde.html](http://flowjo.typepad.com/the_daily_dongle/2007/05/dongleoids_inde.html)

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2. Muul LM, Silvin C, James SP, Candotti F. **Measurements of proliferative responses of cultured lymphocytes.** Current Protocols in Immunology. Coligan JE (ed). J. Wiley and Sons: 7.10.1-7.10.24 (2008).
3. Allsopp CE, Nicholls SJ, Langhorne J. **A flow cytometric method to assess antigen-specific proliferative responses of different subpopulations of fresh and cryopreserved human peripheral blood mononuclear cells.** J. Immunol. Methods 214(1-2):175-186 (1998).
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5. Givan AL, Fisher JL, Waugh MG, Bercovici N, Wallace PK. **Use of cell-tracking dyes to determine proliferation precursor frequencies of antigen-specific T cells.** Flow Cytometry Protocols: Methods in Mol. Biology, 2nd ed. Hawley TS, Totowa NJ (ed)., Humana Press Inc.: 109-124 (2004).
6. Parish CR. **Fluorescent dyes for lymphocyte migration and proliferation studies.** Immunol. Cell Biol. 77(6):499-508 (1999)
7. Wells AD, Gudmundsdottir H, Turka LA. **Following the fate of individual T cells throughout activation and clonal expansion.** J. Clin. Invest. 100(12):3173-3183 (1997).

\* Technical resources regarding the use and properties of the various fluorescent probes can be found on the manufacturers' websites.

