

# FlowJo

Data Analysis Software for Flow Cytometry

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PC User Documentation

**Kinetics Tutorial**



FlowJo was written by Adam Treister and Mario Roederer beginning in 1996, based on concepts developed at the Herzenberg laboratory at Stanford. We are indebted to our active and enthusiastic users worldwide for their ideas, discussions and tireless testing of new versions.

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• FlowJo Advanced Tutorial •  
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# FlowJo Kinetics Tutorial

FlowJo is a software application designed to be a comprehensive tool for analyzing flow cytometric data. Because kinetics analysis is a common task in cytometric analysis, FlowJo includes a platform for applying time dependant data analysis fully integrated into the software.

This tutorial is designed to instruct you on how to use the FlowJo kinetics platform to analyze kinetics data. A brief overview on the biology of kinetics is provided mainly for the purpose of defining the terms, but this manual does not include a comprehensive discussion. This tutorial is also written with the assumption that the user is familiar with the most basic operations in FlowJo, such as loading data, making simple gates, dragging analysis nodes to other samples groups, or the layout and table editors. If you are not familiar with these operations, a general tutorial of FlowJo is available at [www.Flowjo.com](http://www.Flowjo.com) as well.

This tutorial is designed to introduce you to the kinetics platform. Reading through it, you will learn how to operate FlowJo. Run the program as you perform the steps in the tutorial so that you can get the best feel for how the program works! Included with the demo data are a series of completed workspaces that will enable you to jump into the tutorial at any point and follow along.

As a note, we are pleased to be able to frequently update FlowJo to provide new features & analysis capabilities. Therefore, it is possible that the graphics shown in this tutorial may not exactly match the windows that you see when you run the most recent version of FlowJo. You can always download the most recent version of FlowJo from <http://www.flowjo.com/home/windows.html>.

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# Introduction

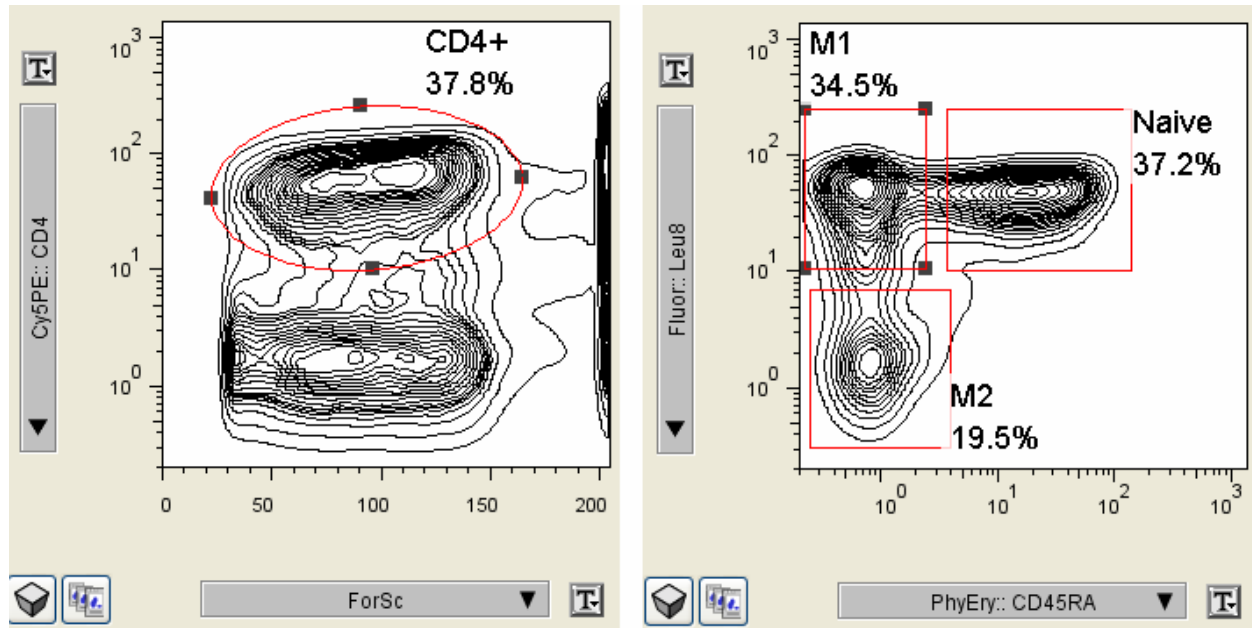
Often there is the need for researchers to evaluate cellular processes of molecule binding, uptake and/or efflux over time. The speed and throughput of flow cytometry is ideal to measure such responses at the scale of seconds or minutes. This tutorial will cover FlowJo's Kinetics Tool with the assumption that you are familiar with topics covered in the lessons of the basic *8-color PBMC Tutorial*. The intention is not to elaborate on the particulars of kinetics analysis, but rather how an end-user accesses the functionality of the Kinetics Tool in FlowJo. However, several references are provided at the end of this tutorial for further reading. Furthermore, the *Kinetics Technical Note* can be found on the FlowJo website to supplement this tutorial.

## *Kinetics Basics*

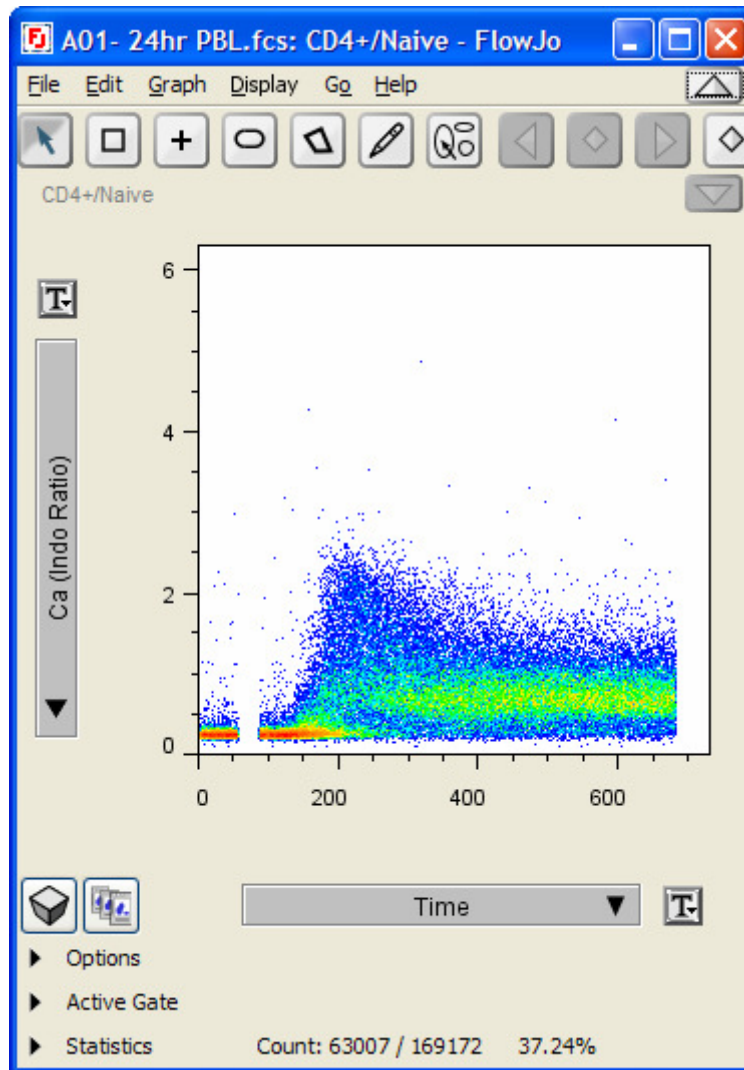
The most common application of kinetics studies is in evaluating changes to cytoplasmic calcium ion concentration.

## *The Experimental Data*

Our example file, as found in the Kinetic **Workspaces** of this tutorial, has primary blood lymphocytes (PBLs) loaded with Indo-1. Indo-1 is a selective calcium chelator, where the emission spectra of free dye and calcium chelate differ to allow for radiometric measurements of cytoplasmic calcium ions. The PBL sample was stained with CD4, CD62L (aka Leu-8) and CD45RA. Thus, subsets of CD4 can be identified: Naive (CD45RA+CD62L+), and at least three memory subsets (differentiated on the basis of expression of CD45RA and CD62L). In this collection, the side scatter parameter was not collected; rather, a hardware ratio of the two Indo-1 emissions was collected and named "Ca (Indo Ratio)". As per the figure below, the Kinetics **Workspaces** used a hierarchical gating strategy to define the CD4 subsets Naive, M1 and M2.



Collection for ~60 seconds was performed to establish the baseline calcium concentration (related to the ratio of the Indo-1 fluorescence); then anti-CD3 was added. The figure below demonstrates such a response for the Naive population of the sample. Within about 2 minutes cells begin to respond by fluxing calcium; the Indo-1 emission ratio changes substantially over time.



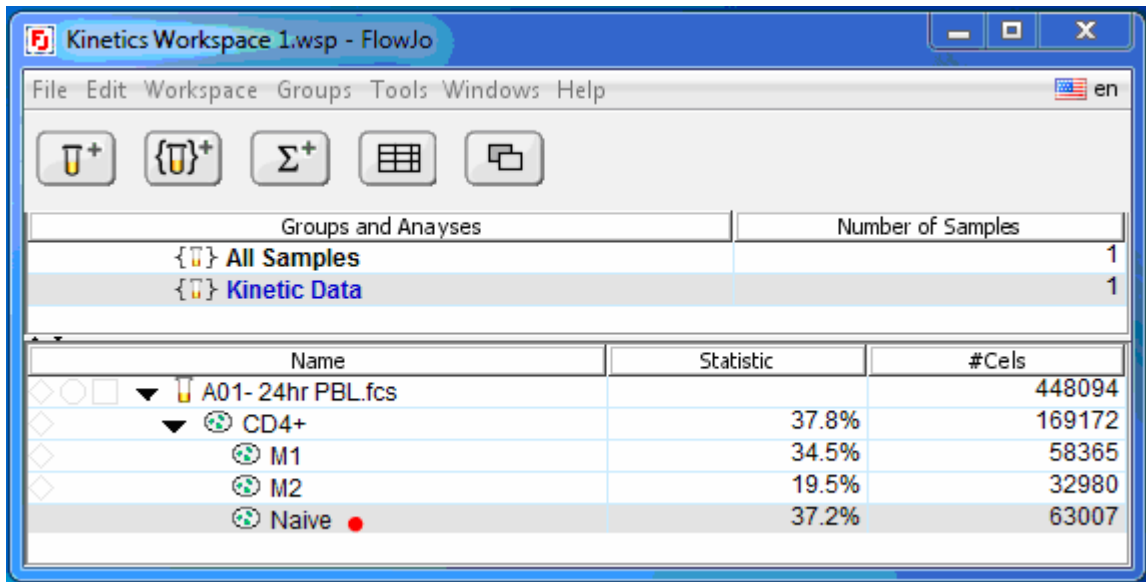
The pseudocolor plot in the figure above, while useful in demonstrating cell response over time, is not the most ideal view to easily compare differences between samples and/or populations within a sample. Furthermore, there are no statistics readily available for informative comparisons.

For this tutorial, we are interested in the response of the Naive cells and the two memory subsets, M1 and M2, in relation to calcium concentrations for this one sample.

# Lesson 1: The Kinetics Platform

## *Creating a Kinetics Node*

Open the workspace file Kinetics Workspace 1. Click on the “Naïve” gated population of the sample data file from the Workspace.

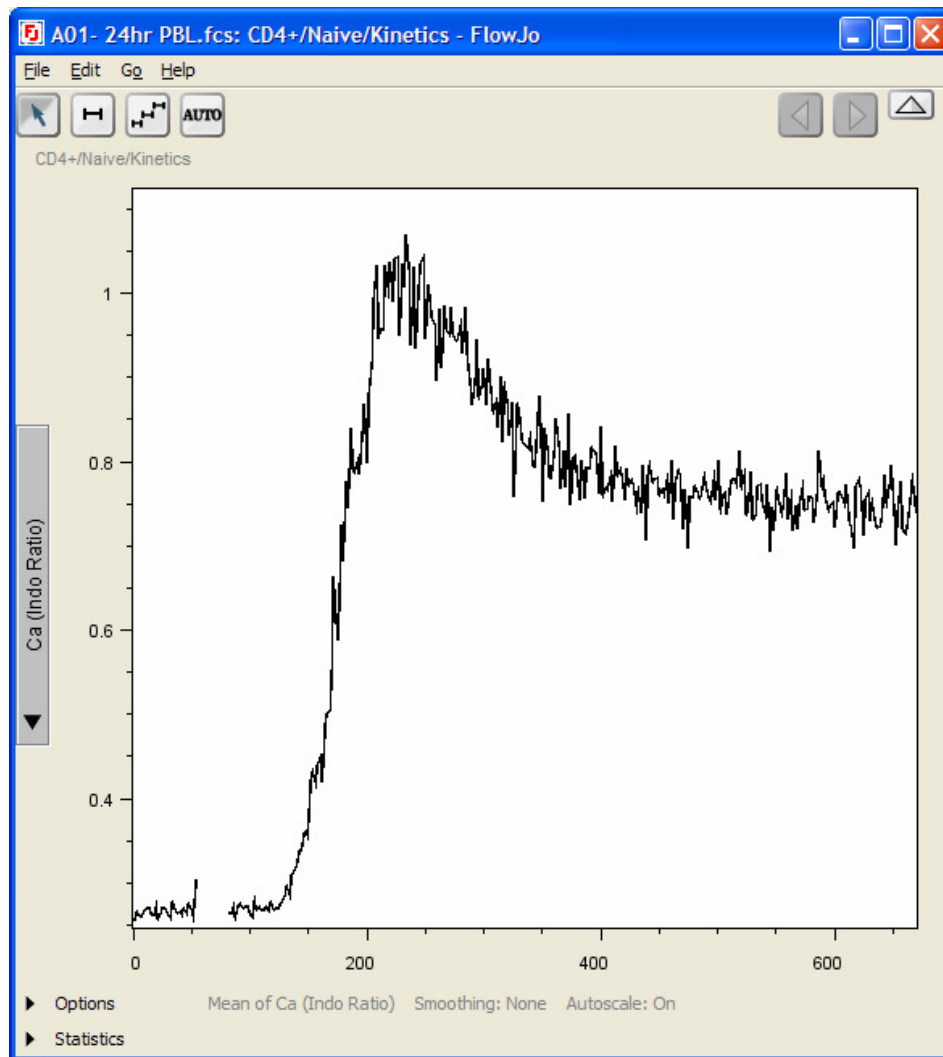


Go to the **Tools** tab in the Workspace menu and then pick the **Kinetics** option in the drop-down menu. A kinetics node, pictured in the illustration below, will appear in the workspace, and the kinetics platform will window pop up.

Name	Statistic
▼ A01- 24hr PBL.fcs	
▼ CD4+	37.8%
M1	34.5%
M2	19.5%
▼ Naive	37.2%
↗ Kinetics ●	

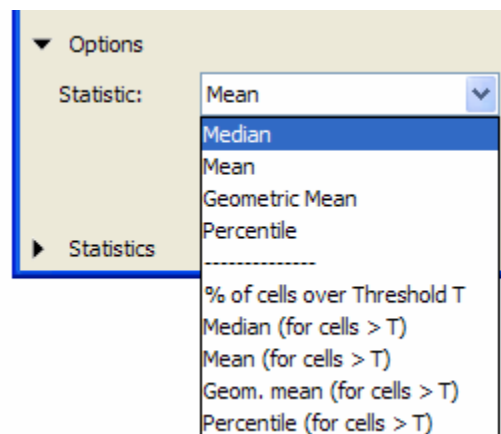
The **Kinetics Plot** view will always display time as the X Parameter, while FlowJo will normally select the ratiometric parameter for the Y axis. The Y axis parameter can be changed by simply pressing the arrow on the **Parameter** display and making a selection from the drop-down list of available parameters.



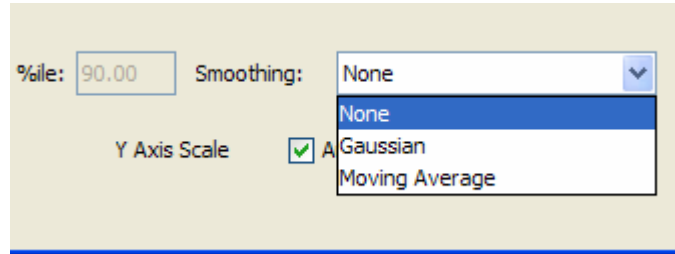


### Display Options

The kinetic plot summarises the pseudocolor-plot data by displaying an average fluorescence value of the cells at each time point. Clicking on the **Options** disclosure triangle, as shown in the illustration to the right, allows you to select which statistical format is used for averaging: **Median**, **Mean** or **Geometric Mean**. Alternatively, a fixed **Percentile** of your population can be displayed with ability to modify the actual percentage.




Two smoothing options are available; **Gaussian** or **Moving Average**. These functions are designed to produce a cleaner display of the data by averaging the measurements over small time

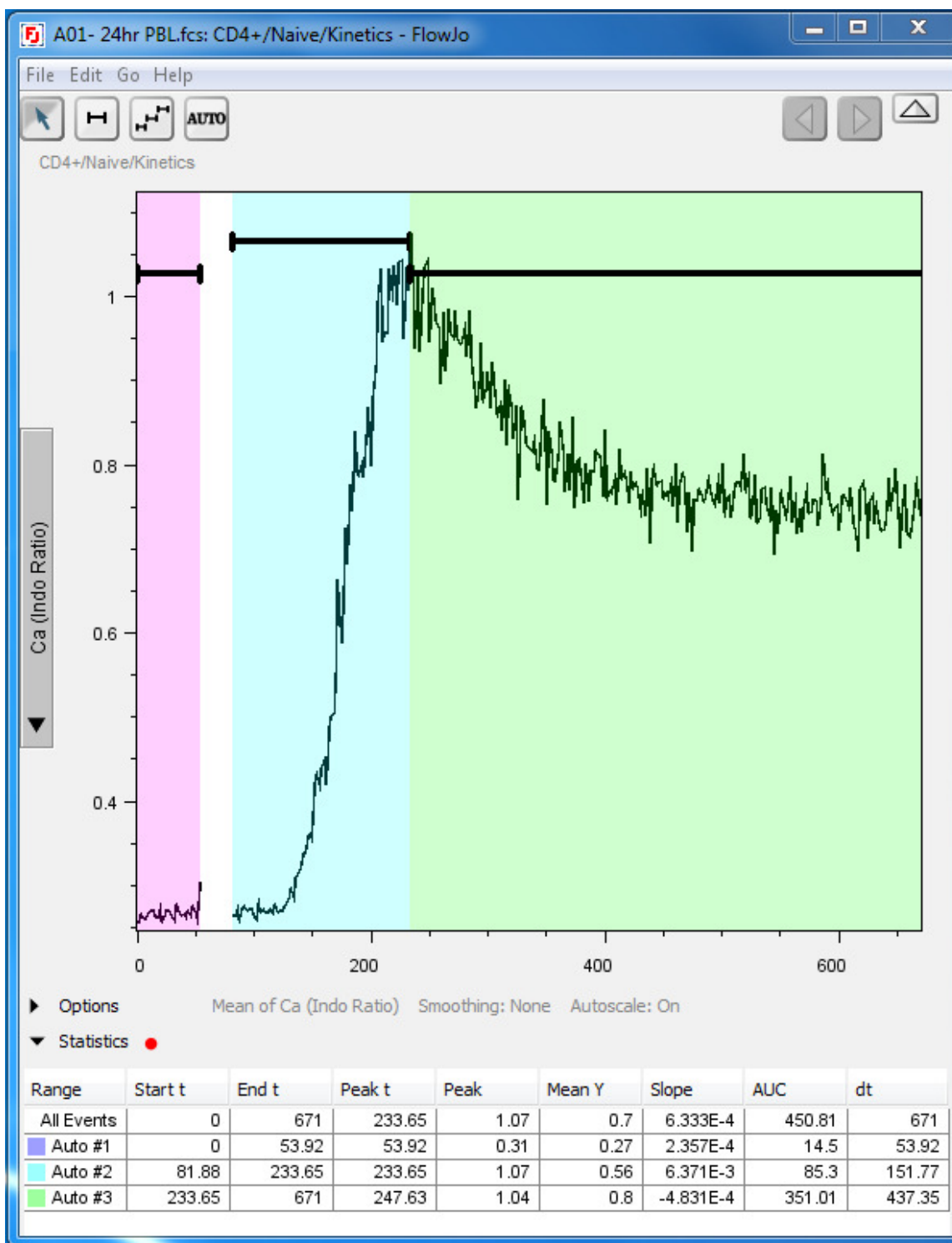


periods. The moving average tool takes the average of the time points within a three bin window. The window is then moved across the data one at a time, always calculating the average of the data within those bins. The Gaussian smoothing algorithm works similarly, except that it weights the average to reflect the data in the center of the window with more importance.

### *Creating Time Ranges*

The top-left corner of the Kinetic Plot view has three options to create time ranges. The icons are shown in the illustration  to the right. The arrow tool allows for editing of existing ranges. The first option, which looks like a single range gate, allows you to create time slices manually with the ability to name the slice as you see fit. The next button from the left allows you to create multiple equal time slices with the ability to define the number of total time slices and their length in relation to time. Finally, the **Auto** option automatically defines time slices based on what FlowJo distinguishes as baseline, response and resolution. With use of all three options, the individual time slices can be manually modified by clicking on the arrow button, clicking on a time range and dragging the gate (once the double-headed white arrow appears when you scroll over any time slice line).

Please select the **Auto** option. You will see three gates appear as shown in the figure below.



### *Kinetics Statistics*

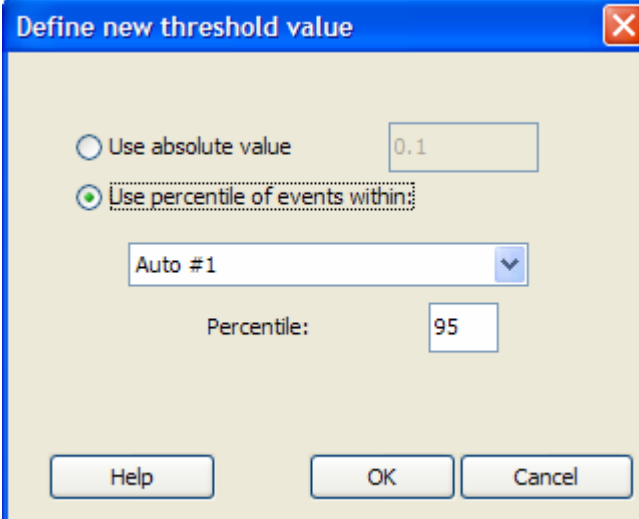
The statistics generated by the application of time slices can be viewed by clicking on the **Statistics** disclosure triangle in the Kinetics Plot view.

Statistics								
Range	Start t	End t	Peak t	Peak	Mean Y	Slope	AUC	dt
All Events	0	671	233.65	1.07	0.7	6.333E-4	450.81	671
Auto #1	0	51.92	33.95	0.28	0.27	8.877E-5	13.89	51.92
Auto #2	79.88	245.63	233.65	1.07	0.59	6.153E-3	97.24	165.75
Auto #3	245.63	671	247.63	1.04	0.8	-4.459E-4	339.07	425.37

**Start t**, **End t** and **Peak t** all refer to time points. **Peak** is the highest value observed within the given range. **Mean Y** is the mean of the parameter selected on the Y-axis within that time range. **Slope** is the change in the vertical dimension divided by the change in the horizontal dimension of a calculated (but not displayed) linear regression on the time range. A positive slope indicates that the y-axis parameter is generally increasing over the total time range, while a negative slope implies that it is decreasing. **AUC** refers to Area Under Curve, the integration of all the space under the kinetics display and another tool for comparison. **Dt** is the change in time for the range. Kaposi et al (2008) is an excellent reference that discusses interpretation of calcium flux data with use of statistics.

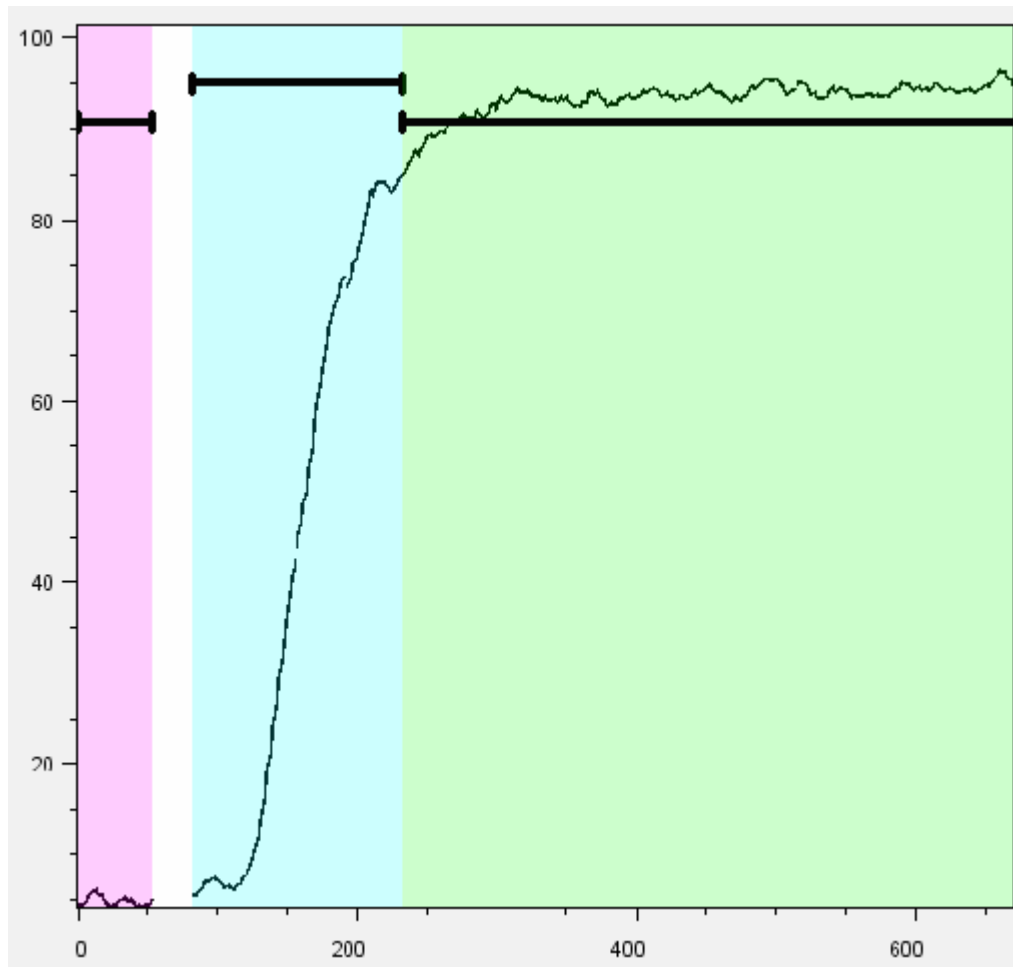
#### *Comparison to a Threshold*

With time slices introduced, there is another useful measure; the percentage of cells that are responding to the stimulus. This is calculated by setting a threshold value and computing the percentage of cells with a fluorescence value above this threshold. The baseline value can be either an absolute fluorescence value or a statistic computed on a time slice within the data. Generally, the baseline is derived from the events collected prior to stimulation. In addition, statistics such as median and mean can be calculated for these responding cells. Open the **Options** disclosure triangle again, and click on the **Statistic** drop down menu. Select **% of cells over Threshold T**. The **Define new threshold** window shown to the right will appear. Click on the circle next to **Use percentile of events within:** and select Auto #1 which represents our sample's baseline. Click **OK**. Notice that the display now shows the percent of cells exceeding the threshold.



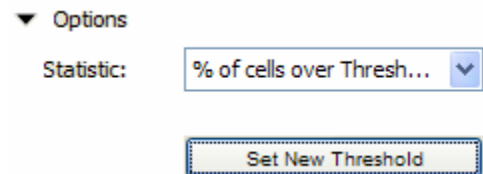
The dialog box titled "Define new threshold value" has a close button (X) in the top right corner. It contains two radio buttons: "Use absolute value" and "Use percentile of events within:". The "Use percentile of events within:" option is selected. To the right of the "Use absolute value" radio button is a text input field containing "0.1". Below the radio buttons is a dropdown menu currently showing "Auto #1". Below the dropdown menu is a text input field labeled "Percentile:" containing "95". At the bottom of the dialog box are three buttons: "Help", "OK", and "Cancel".

Now apply **Moving Average** smoothing to produce the plot shown below.



Note: You can always redefine the threshold via the **Set New Threshold** button, which has become active now below the **Statistics** drop down box in the **Options** disclosure triangle.

The model is now complete. You can close the Kinetics platform window by clicking on the red “X” in the upper right-hand corner. The model is maintained within the workspace and can be accessed and modified by double-clicking the Kinetics node from the workspace.



This ends Lesson 1. The steps covered in this tutorial to the point of completion are found in Kinetics Workspace 2.

## Lesson 2: Creating Kinetics Outputs

To begin the tutorial from this point, open Kinetics Workspace 2. Begin by adding a second file to the workspace, A04-PBL.fcs.

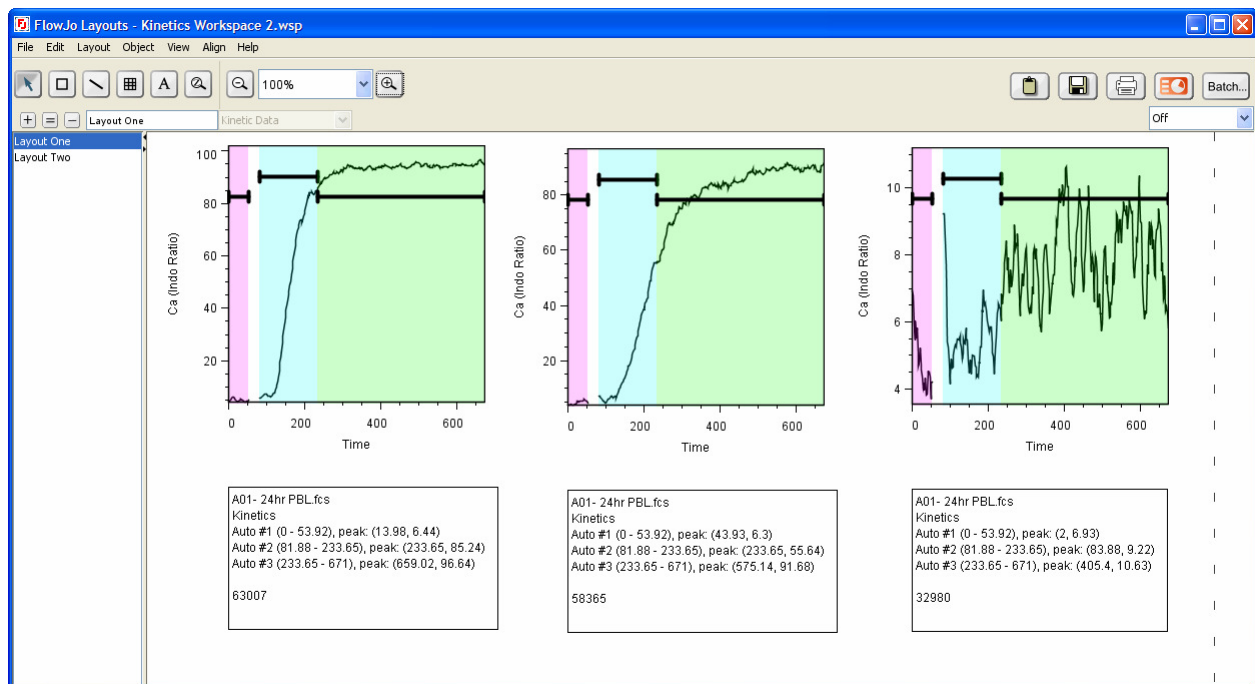
### *Applying the Model to other Samples*

Once we have created a **Kinetics Node** for a population, the node can be dragged and applied to other subsets or samples. Now drag and apply the **Kinetics Node** to the M1 and M2 populations.

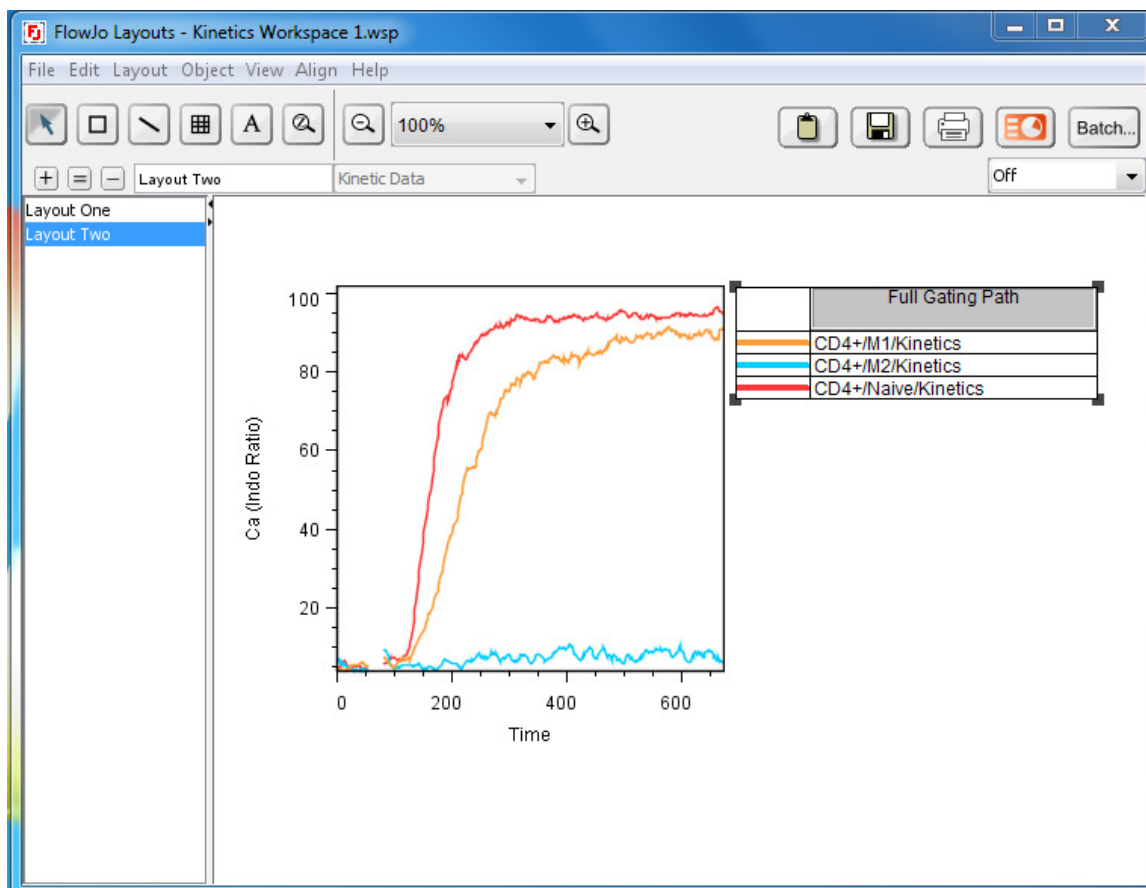
Now that there are multiple samples in the workspace, the Kinetics Node within the **Workspace** can be applied to **Workspace Groups** and one can take advantage of FlowJo's **Batching** tools described in other tutorials. Drag the gating hierarchy and kinetics node to the Kinetics Data Group.

### *Creating a Layout of Kinetics Graphics*

In order to display this data, each of the Kinetics Nodes for Naive, M1 and M2 can be dragged to the **Layout Editor**. Open the **Layout Editor**, title the new layout “Layout One” and drag in each of these nodes to create three individual plots. The outcome is shown in the illustration below.



One of the best approaches to visualising kinetics data is to overlay the plots of multiple populations. Click the “+” button to create a new layout, name it “Layout Two”, then create an overlay of the Naive, M1 and M2 populations. Here we can clearly see that not many M2 cells have responded to anti-CD3 stimulus as compared to the high percentage of cells that responded in Naive and M1. The outcome is shown in the illustration below.



### *Creating a Table of Kinetics Statistics*

To get all of the statistics from the Kinetics model into a format suitable for presentation, open the **Table Editor** and drag the kinetics node in from the naïve population of either file in the workspace. The complete list of statistics for each range will appear, as shown in the illustration below. As with any other set of statistics in the **Table Editor**, individual statistics can be removed by clicking on them and pressing delete on your keyboard, or reordered by dragging and dropping them up or down in the hierarchy.

FlowJo Tables - Kinetics Workspace 2.wsp: Kinetics

File Edit Output Help

+ = - Kinetics

Batch...

All Samples Sample

Column	Population	Statistic	Parameter	Name
Σ 1	CD4+/Naive/Kinetics	Start t	All Events	
Σ 2	CD4+/Naive/Kinetics	End t	All Events	
Σ 3	CD4+/Naive/Kinetics	Peak t	All Events	
Σ 4	CD4+/Naive/Kinetics	Peak	All Events	
Σ 5	CD4+/Naive/Kinetics	Mean Y	All Events	
Σ 6	CD4+/Naive/Kinetics	Slope	All Events	
Σ 7	CD4+/Naive/Kinetics	AUC	All Events	
Σ 8	CD4+/Naive/Kinetics	dt	All Events	
Σ 9	CD4+/Naive/Kinetics	Start t	Auto #1	
Σ 10	CD4+/Naive/Kinetics	End t	Auto #1	
Σ 11	CD4+/Naive/Kinetics	Peak t	Auto #1	
Σ 12	CD4+/Naive/Kinetics	Peak	Auto #1	

Press the Batch button and you will generate a table that includes all of these statistics for both files.

The workspaces completed to this point are saved as Kinetics Workspace 3.

This ends the tutorial. There is more documentation available in the reference web pages, which you'll reach from any of the *help* menu items within the program, or by looking at: <http://www.flowjo.com/>

If you have any questions, or ideas for improvement, please contact us at:  
flowjo@treestar.com

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# References

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Schepers E, Vanholder R, et al. (2009) **Flow cytometric calcium flux assay: Evaluation of cytoplasmic calcium kinetics in whole blood leukocytes.** *JImmunMeth.*

Kaposi et al. (2008). **Cytometry-acquired calcium-flux data analysis in activated lymphocytes.** *Cytometry A.*

Walshe C, Cragg M, et al. (2008). **Induction of cytosolic calcium flux by CD20 is dependent upon B Cell antigen receptor signaling.** *J. Biol. Chem.*