

Tutorial: Kinetics Tool

Often there is the need for researchers to evaluate cellular process 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 *FlowJo Data Analysis Tutorial* (additionally, the *Derive Parameters* Tool can be useful when using Kinetics, which is also covered in a tutorial). The intention is not to elaborate on the particulars of kinetics analysis, but rather limit its scope to 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.

The most common application of kinetics studies is in evaluating changes to cytoplasmic calcium ion concentration. 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 Figure 1, the Kinetics **Workspaces** used a hierarchical gating strategy to define the CD4 subsets Naive, M1 and M2.

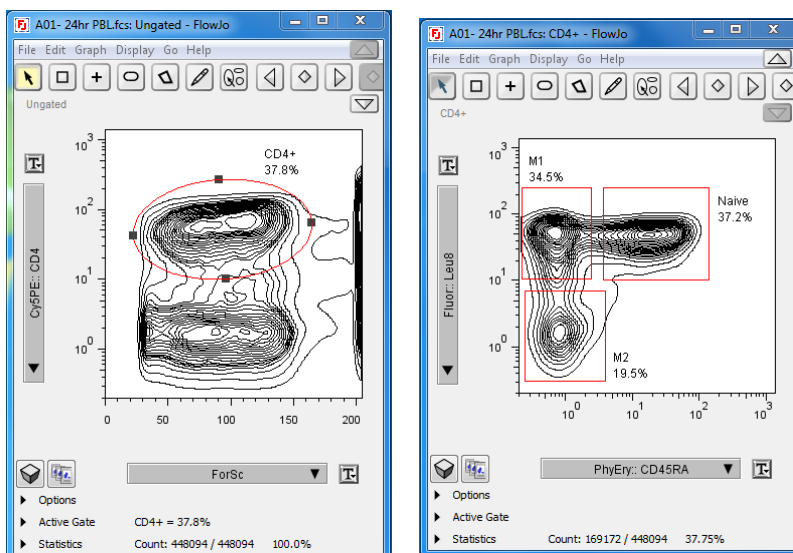


Figure 1: Gating Strategy used for the PBL Sample loaded with Indo-1.

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. Figure 2 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.

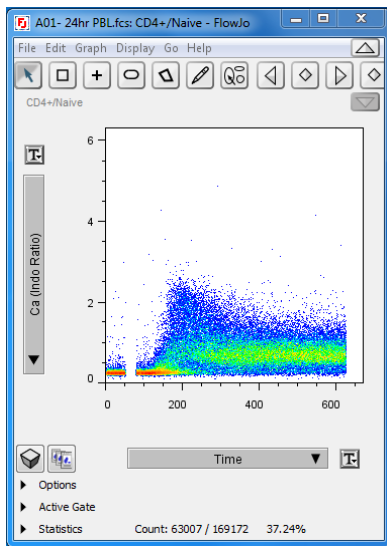


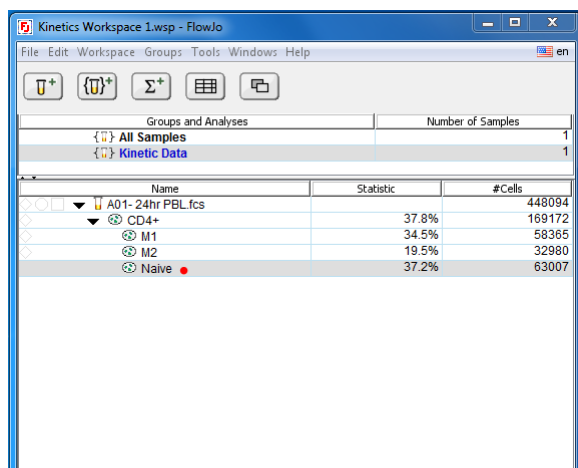
Figure 2: The Calcium Efflux Response of Naive Population with stimulus of anti-CD3.

The pseudocolour-plot in Figure 2 while useful in demonstrating cell response over time, it 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.

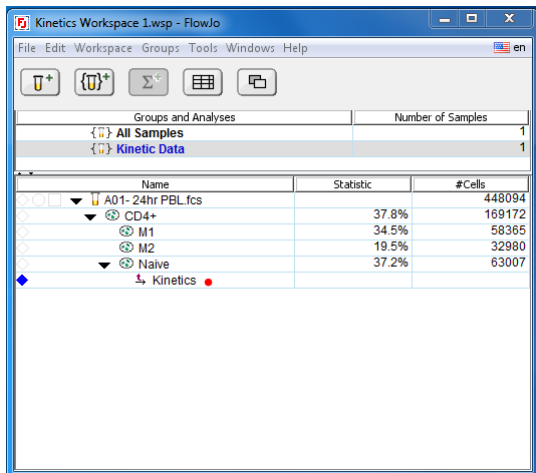
For this tutorial, we are interested in the response of the Naive cells and the two memory subsets, M1 and M2, in relation to calcium concentrations for this one sample.

Steps for Kinetic Analysis:

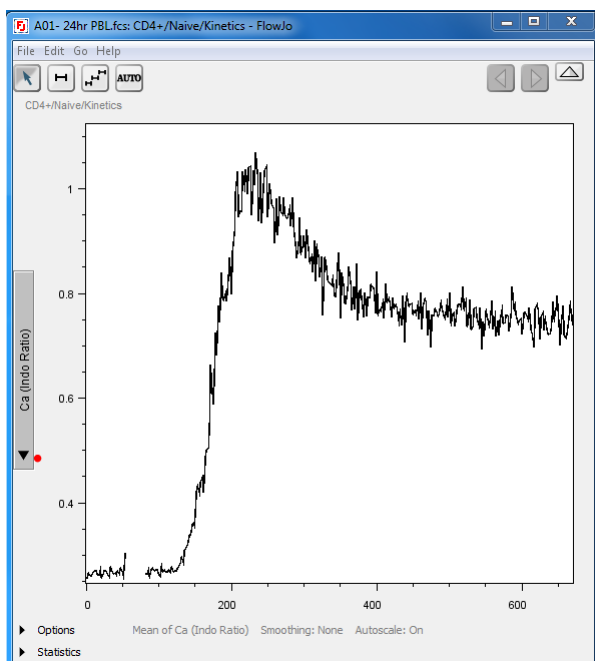
- 1) Open *Kinetics Workspace 1*. Select the Naive gated population from the **Workspace**.



- 2) Then go to the **Tools** tab in the **Workspace** menu and then pick the **Kinetics** option in the drop-down menu. In the **Workspace** you will now see a **Kinetics Node**.



- 3) 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 selection from the drop-down list of available parameters.

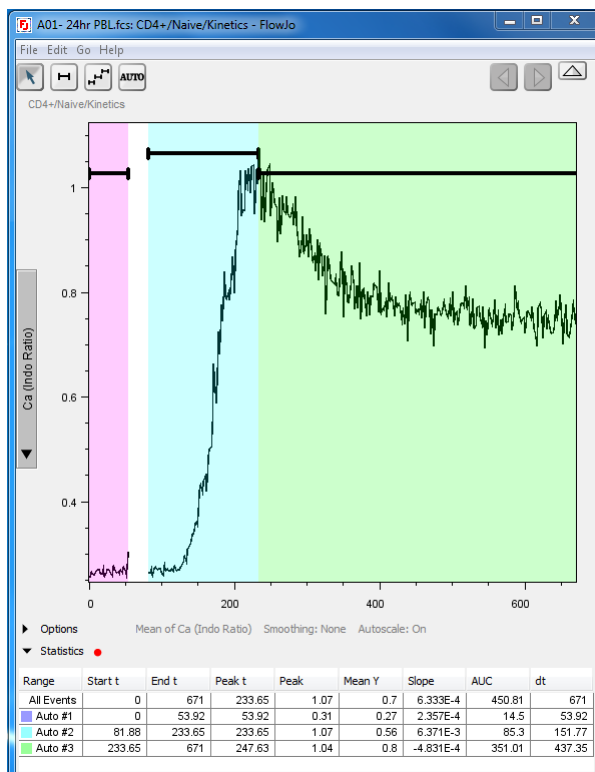


- 4) The **Kinetic Plot** summarises the pseudocolour-plot data by displaying an average fluorescence value of the cells at each time point. Pressing the **Options** arrow allows you to select which statistical format is used for

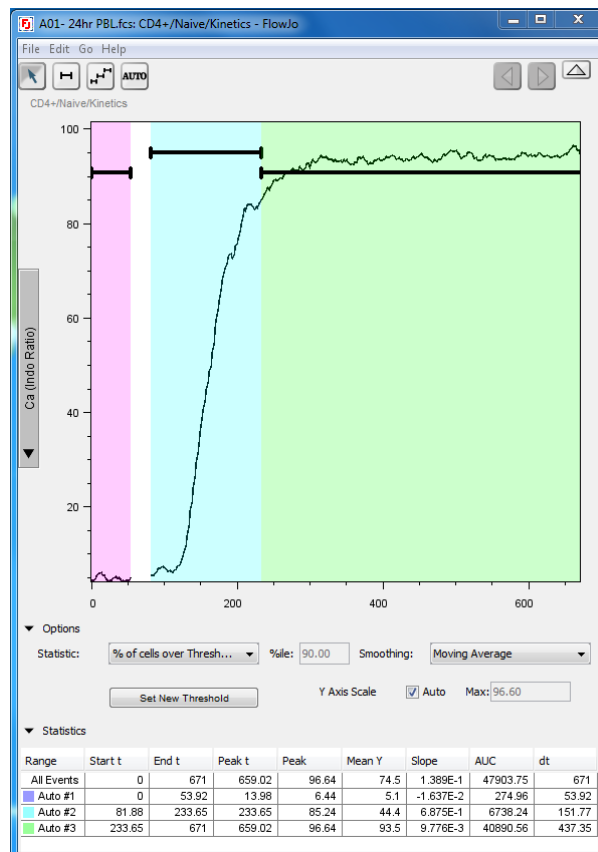
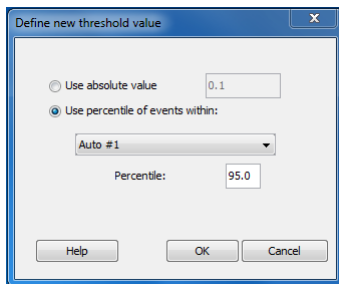
averaging: **Median**, **Mean** or **Geometric Mean**. Alternatively, a fixed **Percentile** of your population can be displayed with ability to modify the actual percentage. Furthermore, two **Smoothing** options are available; **Gaussian** or **Moving Average**. For this tutorial, leave these settings as per default.

- 5) The top-left corner of the **Kinetic Plot** view has three options to create **Time Slices**. The first option allows you to create time slices manually with the ability to name the slice as you see fit. The second button from the left allows you to create multiple time slices with the ability to define the number of total time slices and their length in relation to time. And finally, the **Auto** option automatically defines time slices based what FlowJo distinguishes as baseline, response and resolution. With use of all three options, the individual time slices can be manually modified by dragging a time slice line (once the double-headed white arrow appears when you scroll over any time slice line). Please select the **Auto** option.

- 6) The statistics generated by the application of time slices can be viewed by selecting the arrow next to the **Statistics** label in the **Kinetics Plot** view. Note AUC refers to Area Under Curve. Kaposi et al (2008) is an excellent reference that discusses interpretation of calcium flux data with use of statistics. These statistics can also be exported to a table by dragging the **Kinetics Node** from the **Workspace** into the **Table Editor**.

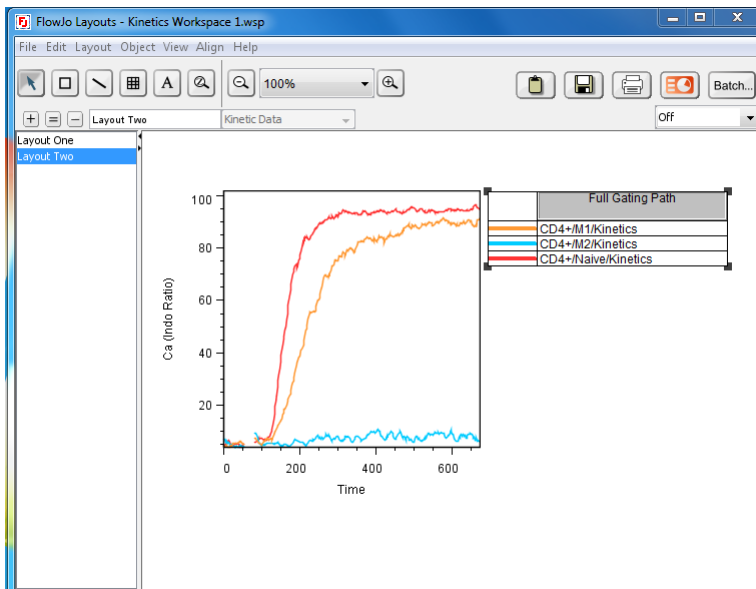
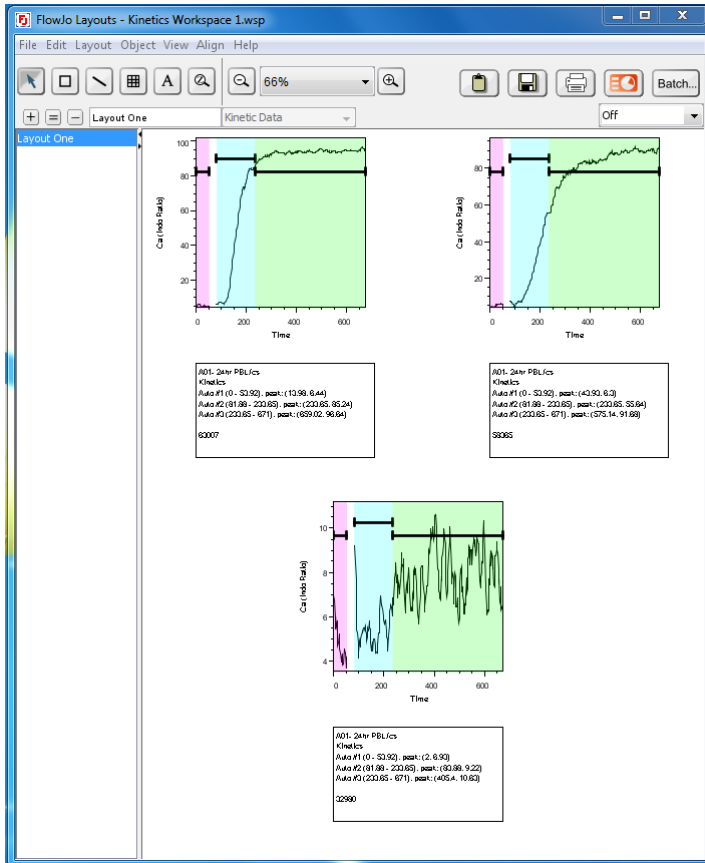


- 7) With time slices introduced, there is another useful measure; the percentage of cells that are responding to the stimulus. This is calculated by setting a threshold value and computing the percentage of cells with a fluorescence value above this threshold. The baseline value can be either an absolute 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. Select the **Options** drop down menu and within **Statistic** select **% of cells over Threshold T**. Within the **Define new threshold value** box select **Use percentile of events within:** and select **Auto #1** which represents our sample's baseline. You will also note that you can always redefine the threshold via the **Set New Threshold** button. Now also apply some **Moving Average Smoothing**.



- 8) Once we have created a **Kinetics Node** for a population, the node can be dragged and applied to others. Now drag and apply the **Kinetics Node** to the M1 and M2 populations.
- 9) In order to display this data, each of the Kinetics Nodes for Naive, M1 and M2 can be dragged to the **Layout Editor (or Table Editor)**, as seen in

Layout One. One of the best approaches to visualising kinetics data is to overlay the plots of multiple populations. In our case overlay the plots of Naive, M1 and M2 as seen in Layout Two. 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 steps covered in this tutorial to the point of completion is found in *Kinetics Workspace 2*.



10) *Kinetics Workspace 3* has two files. This **Workspace** illustrates the ability to now compare the populations of two separate samples as oppose to populations within one sample. Regardless, the aforementioned steps of FlowJo **Kinetics Tool** analysis still apply. Now, however, the Kinetics Node within the **Workspace** can be applied to **Workspace Groups** and one can take advantage of FlowJo's **Batching** tools described elsewhere in FlowJo tutorial documentation.

References:

Shapiro H (2003). **Practical Flow Cytometry** (4th Edition) New Jersey: John Wiley & Sons.

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.*