



FlowJo Basic Guide

FlowJo is an integrated environment for viewing and analysing flow cytometric data, presented in the form of a Workspace. The Workspace contains a list of all of the data samples that you load, the gates, statistics and other analyses that you apply, and the table and graphical layout that you design.

This document is designed to introduce you to the basics of the program, however we strongly recommend the attendance of the FlowJo courses organised by the facility. From the website is also possible to download tutorials containing data sets and PDF files, with a detailed description of the steps to follow in order to perform an analysis. We encourage our users to take the time and go through the tutorials, they are really useful!

1. Install FlowJo. The easiest way is to download the installer from the website: <http://www.FlowJo.com/download/index.html>

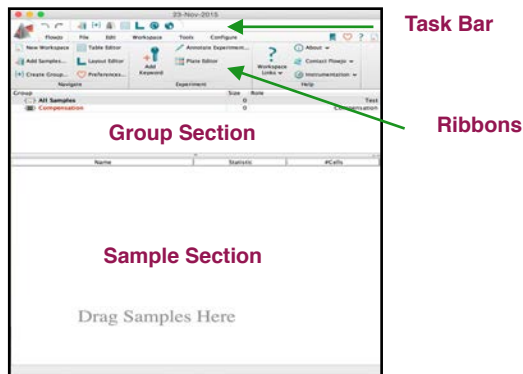


You can select your platform (Mac or PC), download the installer.zip file and double click it to extract the program. Once the zip file is extracted, double click on the icon to launch FlowJo. This version of the program will only allow to load demonstration data files. To obtain a trial 30-day serial number so that you can try FlowJo using your own data, simply fill out the form at: <http://www.FlowJo.com/FLS/registration/trial.html>.

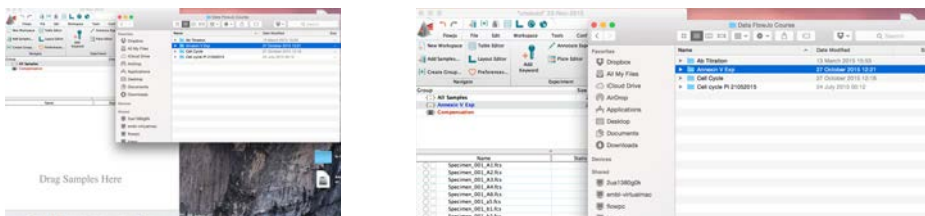
Once you finish the trial, come to the facility where FlowJo dongles are available, just book them through our ppms booking system.

Getting Started

2. After opening the program, you will visualise the workspace and its elements.



3. Creating a workspace: Drag the FCS files to the workspace one by one or the entire folder containing the files.

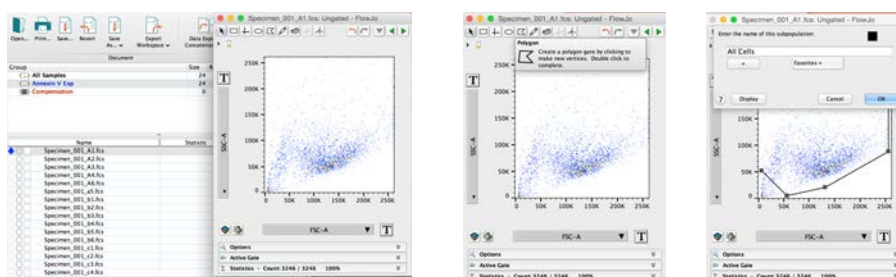


Files should now appear in the sample and group section of the workspace.

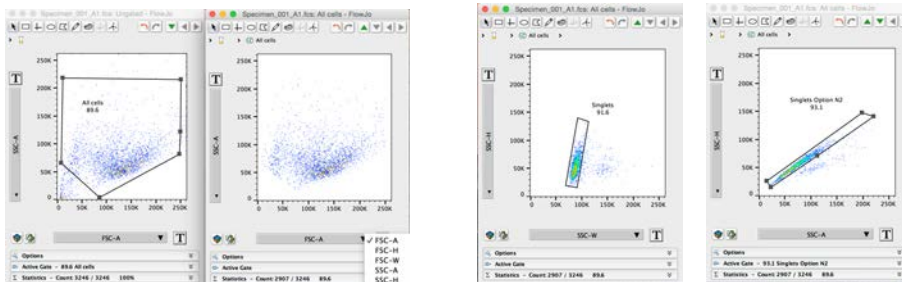
4. Save your workspace: Click on File, save as a workspace.



5. Analysing data: when double-click in the sample name, a graph window will appear. In the upper part of the graph window you will find a set of icons that correspond to several gating options. Select the icon for polygon gating: click the mouse to make a gate node and then continue clicking around the cell population located in the center of the dot-plot, double click to close the gate. FlowJo will ask you to name the gate: enter "All cells" and press ok.

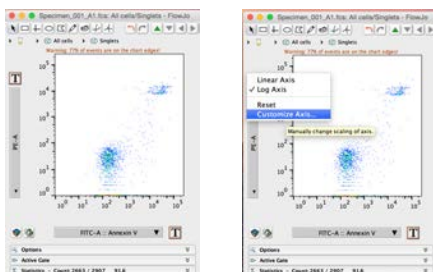


6. Double click within “All cells” gate to generate a new graph window showing ONLY the events contained in “All cells”. Change the axis of the new dot plot, by clicking in the black arrow (next to the axis name) and selecting the appropriate gates for doublet exclusion. Following, 2 options to exclude doublets:

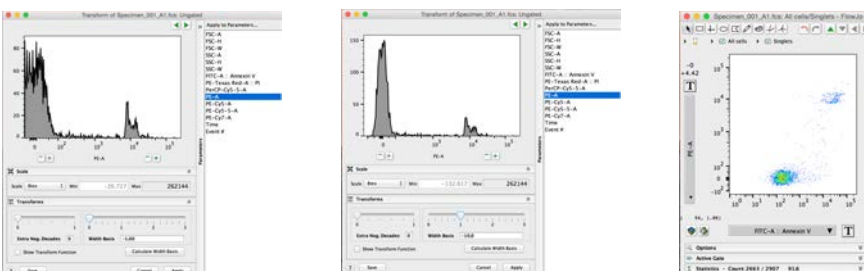


7. Make a third gate to eliminate dead cells. A dye for dead cell exclusion should have been added to the samples and considered for acquisition. In this analysis, dead cells have been included in the gates, since the aim of the experiment is to identify the frequency of apoptotic and dead cells by an Annexin V + PI staining.

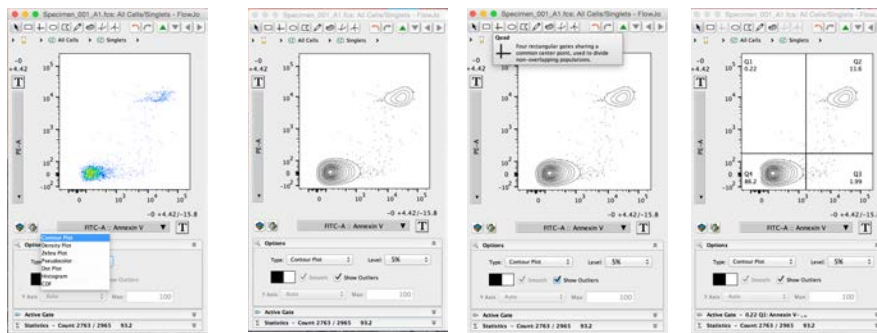
8. Open a new dot plot (double click on live cells or singlet population gate) and choose the parameters of your interest in the axis. If the population shows a profile with broken lines displayed close to zero values of the axis, a bi-exponential transformation should be applied in order to improve the resolution of the events with low signal intensity. Click on the **T** letter next to the y-axis and choose **Customise Axis** option.



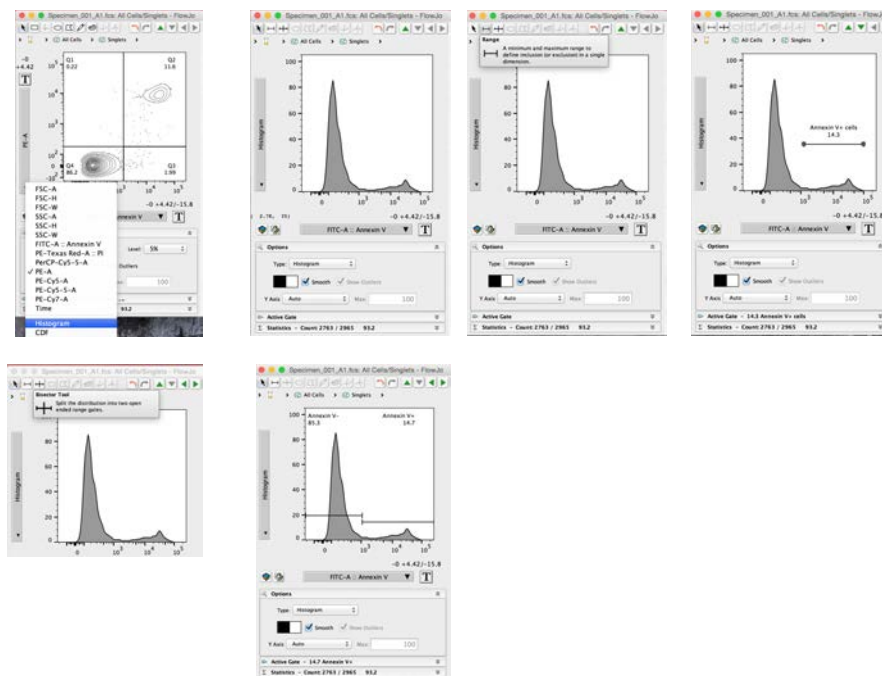
A new window will appear, where you could change the scale to Biexponential (Biex) and add negatives values to the axis by increasing the width basis. Click apply and check the new version of the dot plot. Apply the same transformation to the x-axis if required.



9. To visualise the data using another type of graph, open the **Options** tab just below the x-axis and select from the menu. Add gates to define target populations.



Data could be also visualise using histograms, a graph type that display a single parameter vs. cell number. Select the positive cells for Annexin V applying a range gate or define positive and negative population adding a bisector tool .



10. The sample section should now, shows the different populations that have been created and its hierarchy.

Accession EXP.wsp

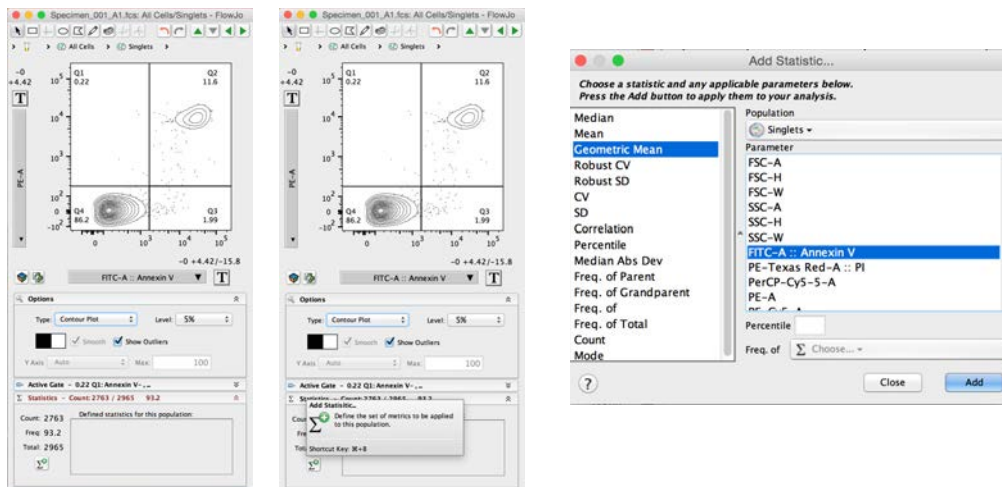
File Edit Workspace Tools Configure

Open... Print... Save... Revert... Save As... Export Workspace... Data Export / Concatenate... Find... Find

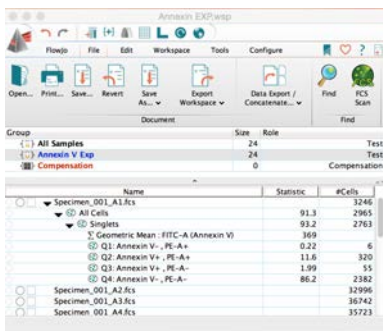
Group: All Samples, Annexin V Exp, Compensation

Name	Statistics	#Cells
Specimen_001_A1.Rs		3242
All Cells	91.3	2965
Singlets	93.2	2783
Q1: Annexin V ⁺ PE-A ⁺	93.2	6
Q2: Annexin V ⁺ PE-A ⁺	11.6	320
Q3: Annexin V ⁺ PE-A ⁺	1.99	35
Q4: Annexin V ⁺ PE-A ⁺	86.2	2382
Specimen_001_A2.Rs		32996
Specimen_001_A3.Rs		36742
Specimen_001_A4.Rs		35723
Specimen_001_A5.Rs		30766
Specimen_001_A5.Rs		5446
Specimen_001_B1.Rs		22537
Specimen_001_B2.Rs		20887
Specimen_001_B3.Rs		31030
Specimen_001_B4.Rs		25704
Specimen_001_B5.Rs		27004
Specimen_001_B6.Rs		22172
Specimen_001_C1.Rs		14584
Specimen_001_C2.Rs		17024
Specimen_001_C3.Rs		18417
Specimen_001_C4.Rs		19820
Specimen_001_C5.Rs		26029
Specimen_001_C6.Rs		14548

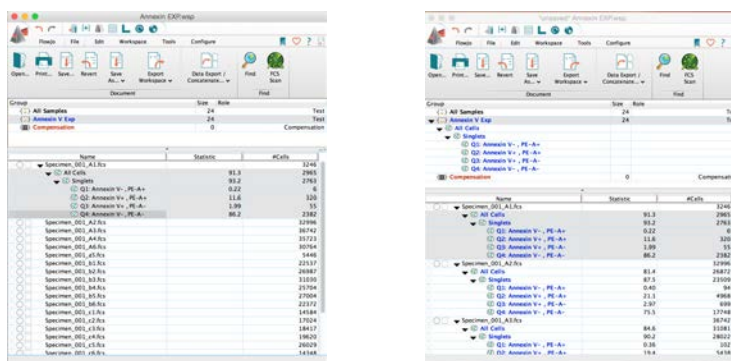
11. Do not forget to add statistical analyses if required. Double click on the population of interest, open the **Statistic Tab** and click on the statistic icon. In the statistic window you could select the function to apply and the parameter involved.



The applied function should appear in the gating strategy list. Apply the function to other populations, by dragging the Sigma icon into the population name.

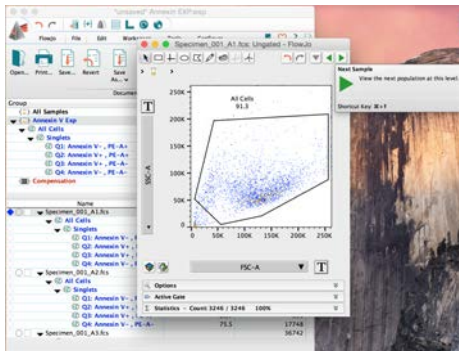


12. Now, select the gating strategy from the analysed sample and drag it into the Annexin V group in order to apply the analysis to all the samples.

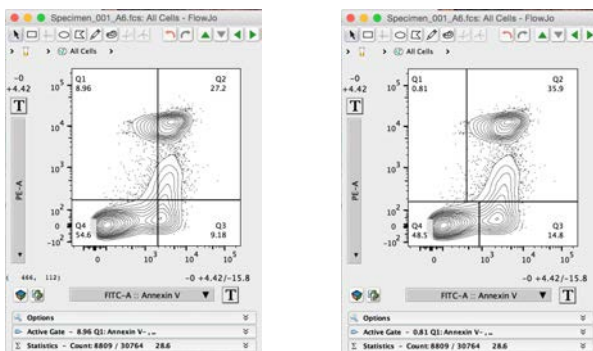


Is highly recommended to set-up the gates in the control samples to properly identify negative and positive populations, that will be then applied to the entire experiment.

11. Dedicated some time to check whether the applied gates fits properly every sample. For that, double click on the first sample and move through the sample group with the green arrow.



12. In some cases, gates must be modified. They are not rules to apply.... you will have to decided depending on your samples and the distribution of the populations. In this case I moved the gates (to the left) to avoid cutting the Annexin V+ population.

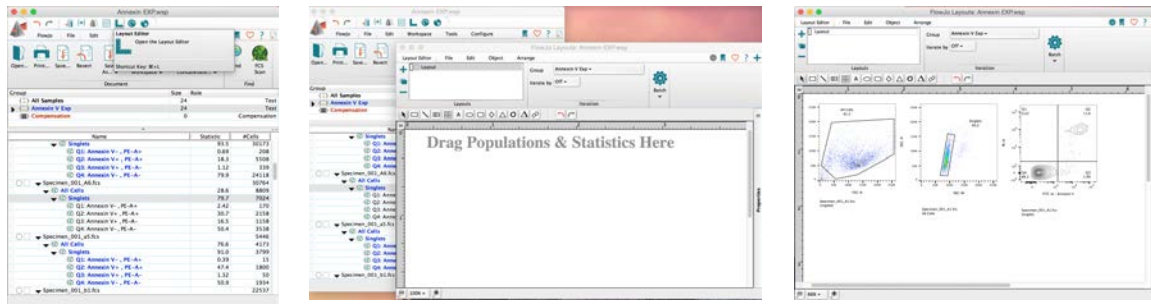


Samples where gates have been changed are easy to recognise, since the colour of the gates change to black, in the hierarchy list.

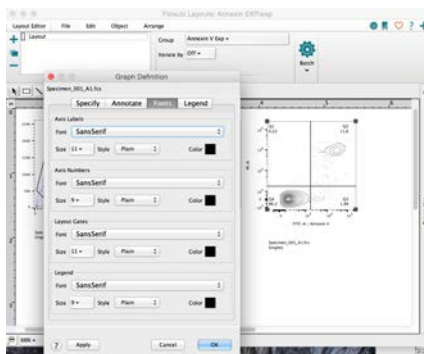
Group	Name	Statistic	#Cells
All Samples	Specimen_001_A1.Ris	24	Test
Compensation	Specimen_001_A1.Ris	0	Compensation
Specimen_001_A1.Ris	All Cells	91.3	30171
Specimen_001_A1.Ris	Q1: Annexin V+ PE-A+	0.81	296
Specimen_001_A1.Ris	Q2: Annexin V+ PE-A+	18.1	1508
Specimen_001_A1.Ris	Q3: Annexin V+ PE-A+	1.12	339
Specimen_001_A1.Ris	Q4: Annexin V+ PE-A+	78.9	24118
Specimen_001_A1.Ris	All Cells	28.6	30764
Specimen_001_A1.Ris	Q1: Annexin V+ PE-A+	79.7	7824
Specimen_001_A1.Ris	Q2: Annexin V+ PE-A+	2.42	170
Specimen_001_A1.Ris	Q3: Annexin V+ PE-A+	30.7	2159
Specimen_001_A1.Ris	Q4: Annexin V+ PE-A+	16.5	1158
Specimen_001_A1.Ris	All Cells	10.4	1538
Specimen_001_A1.Ris	Q1: Annexin V+ PE-A+	76.6	1446
Specimen_001_A1.Ris	Q2: Annexin V+ PE-A+	91.0	4171
Specimen_001_A1.Ris	Q3: Annexin V+ PE-A+	0.39	15
Specimen_001_A1.Ris	Q4: Annexin V+ PE-A+	47.4	1800
Specimen_001_A1.Ris	All Cells	1.32	50
Specimen_001_A1.Ris	Q1: Annexin V+ PE-A+	50.9	1994

13. Once, all the gates have been corrected in all the samples, layout and tables could be generated.

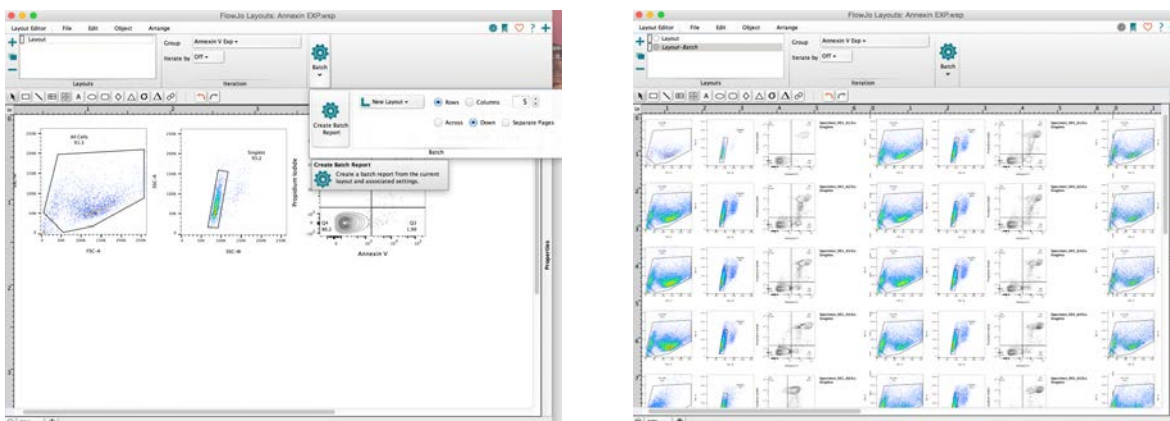
14. Open the layout section, by clicking in the **Layout editor** and start to drag populations in the indicated space.



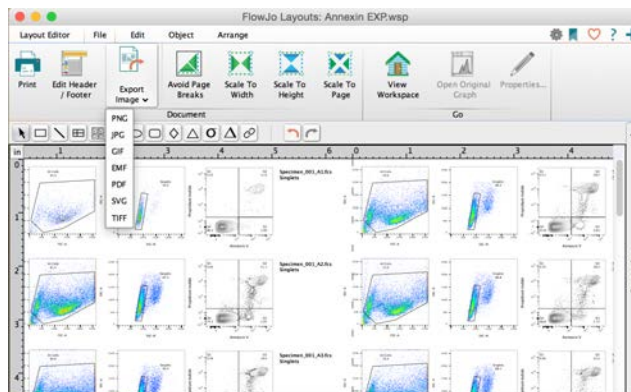
Graphs could be largely edited in order to change axis labels, fonts and legends, among other features. Double click in the dot-plot or histogram and explore the options in the four available tabs. Select and click OK.



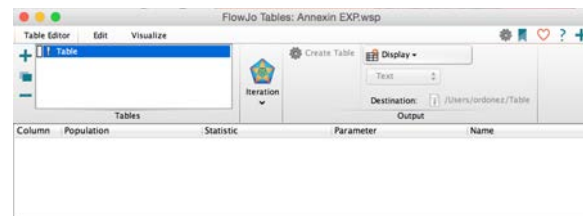
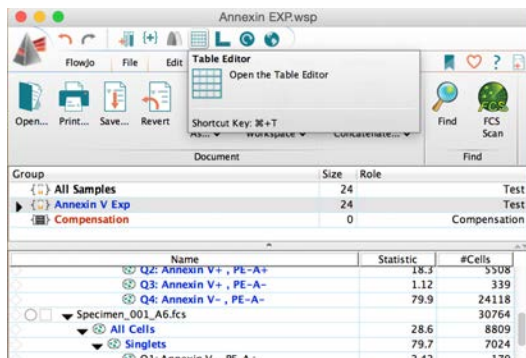
15. Once you have decided the graphs to be included in the display of the results, create a batch report as indicated. Batching will process all the samples of the group as you decided with the first sample, saving you a lot of time.



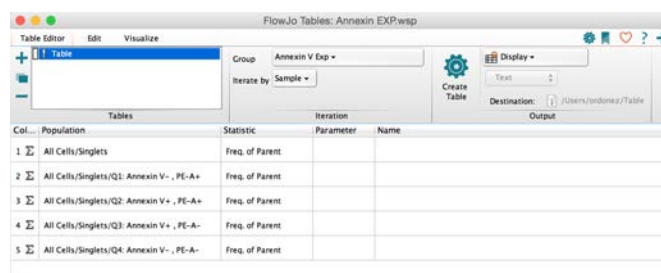
16. To export the layout, click on **File - Export Image** - Select the file format.



17. Tables could be created to summarise the information contained in your analysed samples. Open the **Table Editor** and drag from the hierarchy gating list, the populations and statistical functions that you want to include in the table.

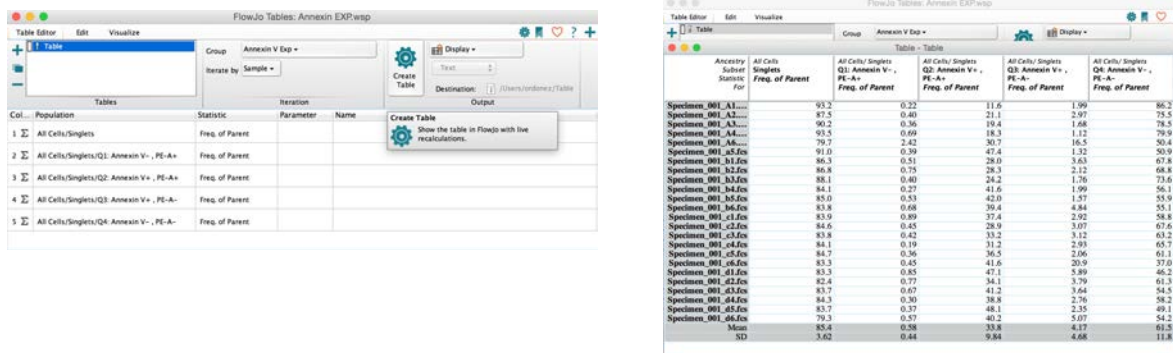


The table editor window should look like this:



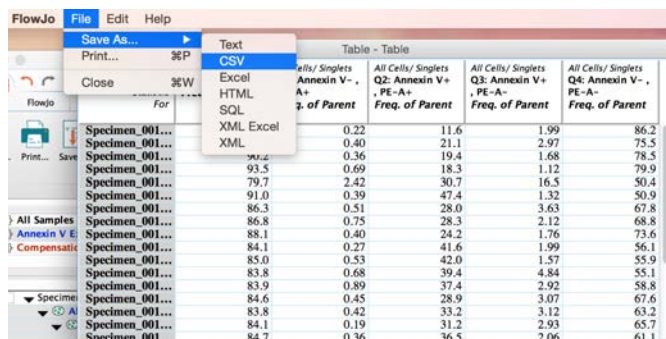
Information could be deleted from the editor by selecting the undesired row and pressing delete. To assign a name for the table click in the line highlight in blue and enter a new name related to the experiment or sample group.

18. When pressing **Create Table**, a window with the final version of the table will appear.



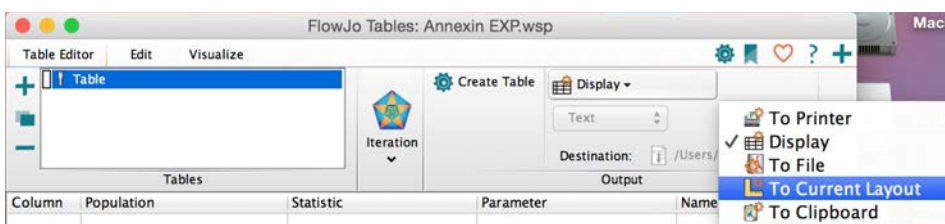
Specimen	All Cells/ Singlets	All Cells/ Singlets Q1: Annexin V-, PE-A+, Freq. of Parent	All Cells/ Singlets Q2: Annexin V+, PE-A+, Freq. of Parent	All Cells/ Singlets Q3: Annexin V-, PE-A-, Freq. of Parent	All Cells/ Singlets Q4: Annexin V-, PE-A-, Freq. of Parent
Specimen_001...	93.2	0.22	11.6	1.99	86.2
Specimen_001...	87.5	0.40	21.1	2.97	75.5
Specimen_001...	90.2	0.36	19.4	1.68	78.5
Specimen_001...	93.5	0.69	18.3	1.12	79.9
Specimen_001...	79.7	2.42	30.7	16.5	50.4
Specimen_001...	91.0	0.39	47.4	1.32	50.9
Specimen_001...	86.3	0.51	28.0	3.63	67.8
Specimen_001...	86.8	0.75	28.3	2.12	68.8
Specimen_001...	88.1	0.40	24.2	1.76	73.6
Specimen_001...	84.1	0.27	41.6	1.99	56.1
Specimen_001...	85.0	0.53	42.0	1.57	55.9
Specimen_001...	83.8	0.68	39.4	4.84	55.1
Specimen_001...	83.9	0.89	37.4	2.92	58.8
Specimen_001...	84.6	0.45	28.9	3.07	67.6
Specimen_001...	83.8	0.42	33.2	3.12	63.2
Specimen_001...	84.1	0.19	31.2	2.93	65.7
Specimen_001...	84.7	0.36	36.5	2.06	61.1
Specimen_001...	83.3	0.45	41.6	20.9	37.0
Specimen_001...	83.3	0.85	47.1	5.89	46.2
Specimen_001...	82.4	0.77	34.1	3.79	61.3
Specimen_001...	83.7	0.67	41.2	3.64	54.5
Specimen_001...	84.3	0.30	38.8	2.76	58.2
Specimen_001...	83.7	0.37	48.1	2.35	49.1
Specimen_001...	79.3	0.57	40.2	5.07	54.2
Mean	85.4	0.58	33.8	4.17	61.5
SD	3.62	0.44	9.84	4.68	11.8

The table could be exported in several formats, by selecting **File - Save As ...**



Specimen	All Cells/ Singlets	All Cells/ Singlets Q2: Annexin V+, PE-A+, Freq. of Parent	All Cells/ Singlets Q3: Annexin V-, PE-A-, Freq. of Parent	All Cells/ Singlets Q4: Annexin V-, PE-A-, Freq. of Parent
Specimen_001...	0.22	11.6	1.99	86.2
Specimen_001...	0.40	21.1	2.97	75.5
Specimen_001...	0.36	19.4	1.68	78.5
Specimen_001...	0.69	18.3	1.12	79.9
Specimen_001...	2.42	30.7	16.5	50.4
Specimen_001...	0.39	47.4	1.32	50.9
Specimen_001...	0.51	28.0	3.63	67.8
Specimen_001...	0.75	28.3	2.12	68.8
Specimen_001...	0.40	24.2	1.76	73.6
Specimen_001...	0.27	41.6	1.99	56.1
Specimen_001...	0.53	42.0	1.57	55.9
Specimen_001...	0.68	39.4	4.84	55.1
Specimen_001...	0.89	37.4	2.92	58.8
Specimen_001...	0.45	28.9	3.07	67.6
Specimen_001...	0.42	33.2	3.12	63.2
Specimen_001...	0.19	31.2	2.93	65.7
Specimen_001...	0.36	36.5	2.06	61.1
Specimen_001...	0.45	41.6	20.9	37.0
Specimen_001...	0.85	47.1	5.89	46.2
Specimen_001...	0.77	34.1	3.79	61.3
Specimen_001...	0.67	41.2	3.64	54.5
Specimen_001...	0.30	38.8	2.76	58.2
Specimen_001...	0.37	48.1	2.35	49.1
Specimen_001...	0.57	40.2	5.07	54.2
Mean	0.58	33.8	4.17	61.5
SD	0.44	9.84	4.68	11.8

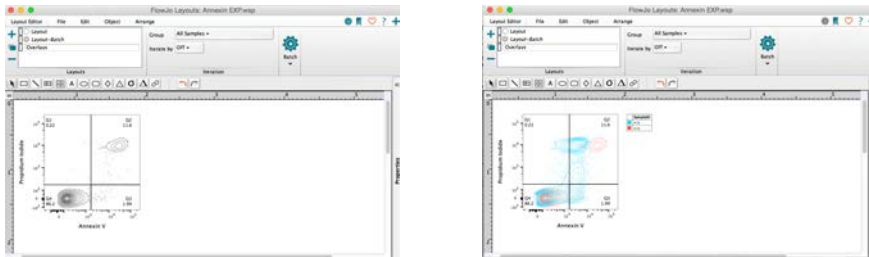
The table could be display in other location, by selecting one of the options in the drop-down menu, as shown:



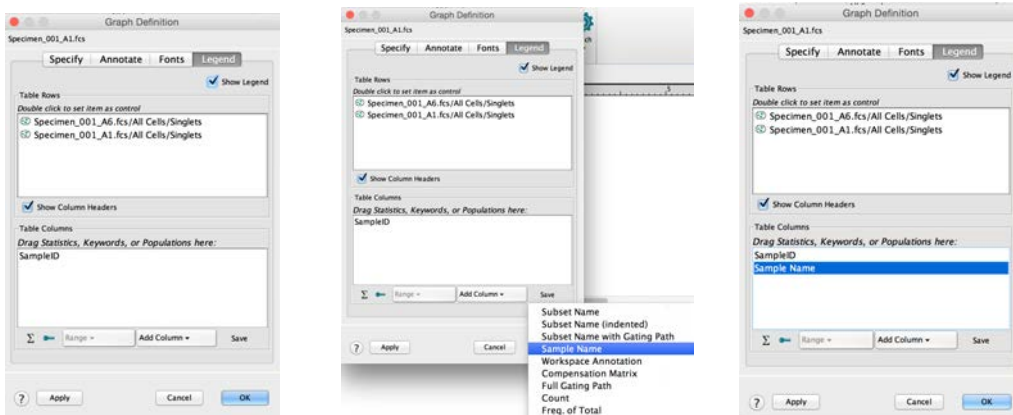
19. When finishing your analysis, press save and close the program. Note that the workspace (wsp) file contains all the gates, stats, layouts and tables created but it does not contain the FCS files of the samples by itself. I strongly recommend to save the workspace in the same folder where the FCS files of the experiment are kept. Like this, once you open the wsp file, FlowJo will quickly find back and load the data into the wsp file.

Tips for Data display in the layout

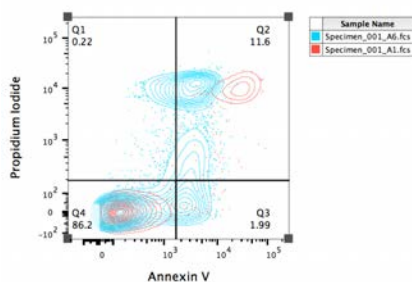
In some cases is very useful to visualise the data doing overlay of different samples as dot plots or histograms. To create an overlay, open the **Layout editor** and drag the target population (singlets) of the first sample that is usually a negative control or unstimulated condition. Edit the axis names and fonts as previously described, then just drag a second sample and drop it exactly over the dot plot or histogram initially drawn.



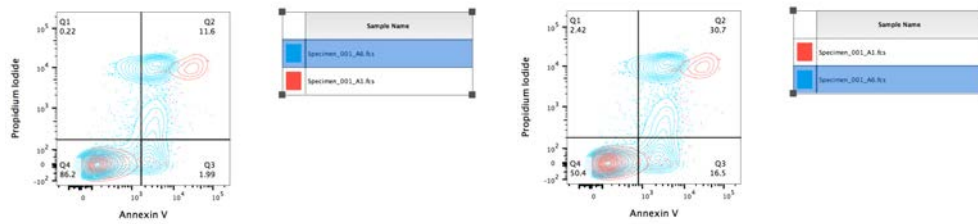
FlowJo will automatically display a legend that should be edited in order to show the name of the samples involved. Double click in the dot plot or histogram, to open the **Graph definition** window, select **Legend tab**, click on Add column and add Sample name to the legend as shown here:



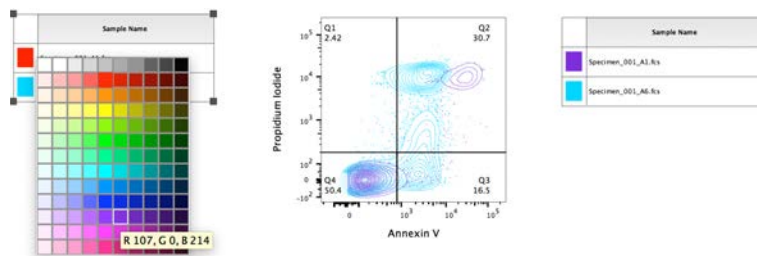
Remove Sample ID by selecting and pressing delete. The legend should look like this:



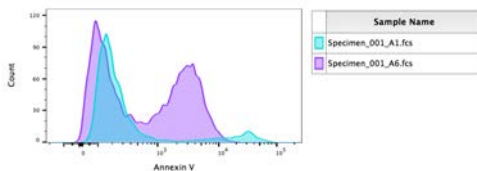
The order of the samples in the overlay and the colours of the populations could be also modified. Select the file name and move it down to invert the positions of the samples in the overlay graph.



Change the colours of the populations, by clicking in the coloured square next to the sample name and selecting a colour from the palette.



The overlay of the same samples show it as an histogram:



Histograms could be further modified; click on the right button of the mouse, select histograms and then select one of the three options:

