Flow Data Analysis with Flowjo (based on version 7.6)

SOP015
02/03/2010
Wim Ammerlaan

Reference

http://www.flowjo.com/

Introduction

Flowjo is a flowcytometer data analysis program, which has supplementary features when compared to the BD FACS Diva software.
Flowjo can be, for example, used for:

- Overlay histograms
- Calcium flux
- Proliferation (CFSE)
- Population comparison

There are numerous other functions in Flowjo and the program frequently updates, which makes it impossible to explain everything. To get more familiar with all the options, the program must be explored by the user. The extensive help functions within the program, facilitates easy learning. For a start, here follow some procedures for the current most common functions.

Installation

The CRP-S CFC has one licence for a Flowjo Windows version, which means there is one dongle. Flowjo can be installed on every analysis computer but needs this dongle to function. Installation can be done via the Flowjo website http://www.flowjo.com/ Download/Windows.
Please note there are 2 main versions, one for Apple and one for Windows. We have the Windows version. Download the latest final version, not a beta version.
The dongle is a USB key with a big label. Standard this one is plugged in Ana1. Due to low performance of this computer it will not run all the functions. Ana2 can be used as alternative. For example the 3D view cannot be used on Ana1, but works fine on Ana2. Just remove the Flowjo USB dongle from Ana1 and plug it in any USB port of Ana2. Start Flowjo. After use, do return the dongle into Ana1. This is to prevent the dongle from getting lost.
Check regular on the Flowjo website for updates.

Load Data (Export out of Diva)

Flowjo can read flowcytometer data (the listmode file, lmd) in FCS2.0 and 3.0 formats. These are standard Flowcytometer data formats. Diva produces FCS3.0 lmds. Data acquired with Diva software is normally stored in experiments and should be exported out of Diva in order to see the sample labels in Flowjo. This procedure goes as follows

1. Import the experiment in Diva
2. Right click on the experiment name
3. Export <FCS files>
   a. Gated Events < All events>
   b. File version <FCS3.0>
   c. Parameter type <Linear>

   d. <OK>

4. Browse < D/BDExport/FCS/Flowjo Export > <OK>. At this location will be created a directory with the experiment name as directory name and the sample names as names for the LMD files.
5. Start Flowjo, the **Workspace** window opens
6. Click on the left top icon “Add Samples”
7. The directory D/BDExport/FCS/Flowjo, should appear, otherwise search for it.
8. Click on the experiment of interest <Open>
9. In the top part of the **Workspace** the experiment name appears
10. In the lower part, the individual samples

Gating Towards the Population of Interest

1. Double Click on a tube of interest in the **Workspace**
2. A **Graph** window pops up with a FSC-A vs. SSC-A Pseudo Colour Density plot
3. In the **Graph** window under Options the Graph Type can be changed if desired
4. The parameters can be changed by a left click on the either the X or Y axis label and select the parameter of interest.
5. At the top of the **Graph** window are various icons, representing various gating possibilities. Select the one of choice, e.g. the forth <Create an elliptical gate>
6. Pull the gate over the population of interest and adjust size and orientation by clicking on one of four black squares and move as desired.
7. In the **Workspace** underneath the tube of interest appeared a new line with a gate pictogram and the gate name. Right click on the gate name opens various possibilities including a rename function for this population.
8. In the **Graph** Window, double click inside the gate and a new **Graph** window appears, displaying only the events lying in the first gate.
9. Again the appearance and parameters can be changed, and a new gate drawn, e.g. FSC-A vs. APC Cy-7 for eventual dead cell discrimination. Double click in the new gate will generate a third **Graph** window only displaying events lying in Gate 1 AND 2. Continue this process with the desired gating strategy in order to view the population of interest.
10. In the **Workspace** appears under the tube of interest the gating hierarchy.
11. The complete gating hierarchy or only individual gates can be copied and paste in individual other tubes of the experiment. When the gating hierarchy should be applied to the whole experiment copy and paste this into the Experiment name in the top panel of the **Workspace**. All samples in the lower panel will show the gating hierarchy.

Compensation

1. The Diva compensation is imported into Flowjo with the FCS files. In the Flowjo **Workspace** on the left of the sample name a small pictogram of a grid is shown, indicating the presence of a compensation matrix.
2. If a new compensation matrix is needed, right click on the experiment name in the **Workspace** top panel <Use Current Group for Compensation>. The **Compensation Editor** window pops up.
3. Create a gating strategy for the population necessary for compensation for all compensation tubes (see previous chapter “Gating towards the population of interest”)
4. In the **Compensation Editor**/ Matrices and Transforms/ Select the at the parameter line in the Sample column the tube containing this parameter specific compensation control.
5. In the Universal negative line select tube 1 the negative control.
6. In the positive column select the gating of preference. Flowjo adds a line gate in your gating hierarchy for selection of only the strongest positive events in this sample.
7. In the **Compensation Editor** Windows / Wizard Graphs adjust the gates by double clicking on the histogram of interest. A **Graph** window pops up. Adjust the line gate to select only the strongest positive events. Close the **Graph** window

8. In the **Compensation Editor** window change the name of the matrix in the Experiment name.
9. Drag the compensation matrix name out of the **Compensation Editor** over the experiment name in the top panel of the **Worksheet.** In the lower panel, left of the sample name a small pictogram of a grid appears in the same colour as the compensation matrix name, indicating this compensation is used for this sample.
10. Compensations can be changed by opening the **Compensation Editor** and manually entering values or adjusting the gates in the wizard

Exporting and Printing of Graphs

1. From the **Graph** window all pictures can be printed or copied and pasted directly in PowerPoint
2. In Flowjo a print page can be generated by clicking the **Workspace** on the fifth icon "Open layout Editor", which opens the layout window.
3. From the **Workspace** window any line can be dragged into the **Layout** Window for printing in a personal designed report form report form
4. Multiple Layouts can be generated by clicking on <+> in the 2nd icon line of the **Layout.** Names of the layouts can be changed in the field right of the 2nd icon line.
5. Graphs be can adjusted to the desired appearance on the layout via multiple methods which have to be explored by the user.
6. Direct Printing, or pdf formation or powerpoint export can be performed from here.

Saving

1. In the **Workspace** file menu < Save As>
2. Save the workspace under an appropriate name at a known destination. (e.g. My Documents/ user XX/ Flowjo
3. Layouts, gating hierarchy and compensations are saved.

Overlay Histograms

1. After the samples are correctly gated and compensated, histograms of negative and positive samples can be overlaid in the layout window.
2. In the **Workspace** lower panel double click on the population line of the negative control for the overlay histogram. In the **Graph** window check the X-axis is either on bi-exponential display or without transforms. Make sure the overlays are at the same X-axis format.
3. Drag the negative control histogram from the **Workspace** into the **Layout.**
4. Drag the sample for overlay from the **Workspace** into the **Layout** over the negative control histogram. The black lines will change colour. Drop the overlay and within the plot two histograms with different colours become visible.
5. Multiple samples can be entered in one overlay histogram by repeating step 4 with various samples.
6. Within the legend, which appears next to the overlay histogram, the appearance of the overlay histograms entries can be adjusted. Right click on the sample name in the legend shows the possibilities.
7. Double click on the overlay histogram will show its properties and various possibilities to adjust to personal liking.
8. The overlay histogram can be copied into PowerPoint or printed directly.

Calcium Flux

1. For analysis of Calcium Flux Experiments all parameters should be linear and a new derived parameter should be made of Fluo4/Fura Red plotted against time.
2. Load experiment of interest
3. Perform Gating Strategy

4. In **Workspace** Right click on the preferred population line
5. Select <Derived Parameters…>. The **Derived Parameters Definition** window opens
    a. Type in the field Derived Parameters at the top of the of this window the new parameter name: “Ratio”
    b. Insert reference select <Comp FITC-A>
    c. Click on <division icon>
    d. Insert reference select <Comp PerCP-A>
    e. Scale <Linear>
    f. Minimum < 0 >
    g. Maximum < 2 >
    h. < OK >
6. In **Workspace** right click on the same preferred population line, select ,<Kinetics>
7. The **Kinetics** window opens
8. Select on Y-Axis ratio
9. Various options are possible to present data. Most recommended is to overlay various treatments with the positive and negative control following the same method as **Overlay Histograms.** Printing and export to PowerPoint goes with copy and past or dragging into the **Layout**

Proliferation (CFSE)

1. For analysis of CFSE fluorescence reduction, indicating the number of proliferation steps of the sample, perform the necessary gating strategy and compensation.
2. Make sure the biexponential display function is off for the Comp FITC-A Parameter.
3. Right click on the preferred population line in the **Workspace**, select <Proliferation>
4. The **Proliferation** window pops up
5. Select for the X-Axis < Comp FITC-A CFSE >
6. Flowjo performs an automated de-convolution of the CFSE stained population
7. Tag both < Draw model sum > and < Fill components >
8. When the RMS value is higher than 2 and the proposed distribution looks wrong, move the orange population to the higest point of the most strong FITC peak
9. Add 2 additional peaks in the field # Peaks in the options menu
10. Calculate again.
11. For proliferation steps percentages, click < Create Gates > button. Give a name for the subpopulations < OK >
12. In the **Workspace** appear the newly generated generations underneath the preferred population line
13. In the **Workspace** click on the fourth icon of the icon bar “Open Table Editor”.
14. The **Table Editor** window appears.
15. Select all the generation created underneath the sample of interest in the **Workspace** and drag those into the Table Editor right panel
16. In the field next to the + = - icons type the name of this table
17. At the statistic column the “**frequency of parents**” should be mentioned.
18. In the column name, the names of the sub populations can be typed in.
19. In the menu <Output> select < To File>
    a. File format < Excel >
    b. File name < as you like >
    c. Tag box <Open file after creation>
    d. <OK>
20. The percentages are exported into an Excel file.

Population comparison

1. For analysis of differences between various samples Flowjo has developed a Population Comparison.
2. After compensation and gating make a right click on the population of interest in the **Workspace**, select <Population comparison> , The **Population Comparison** Windows pops up

3. Drag the negative control sample in the top part of the **Population Comparison** Window, on the “drop control population here”
4. Overlay histograms appear from all parameters and 7 different methods of population comparison results are calculated.
5. In order to make sense out of all these numbers, you should look at the number with caution. The numbers are only indicative and the biological relevance should become clear from checking the controls. What is the use of this then? You can get numbers, not related to biased gating but purely mathematical. The numbers can used in publications without hesitation, with more relevance then the % pos based on the line gate in a histogram, but do use a few negative controls to estimate how much % positive can still be false positive.