INSTITUTO DE MEDICINA MOLECULAR JOÃO LOBO ANTUNES

TRAINING CONTENT - FLOWJO

AUTHORS: Rute Gonçalves

APPROVAL: Mariana Fernandes

FLOWJO TRAINING

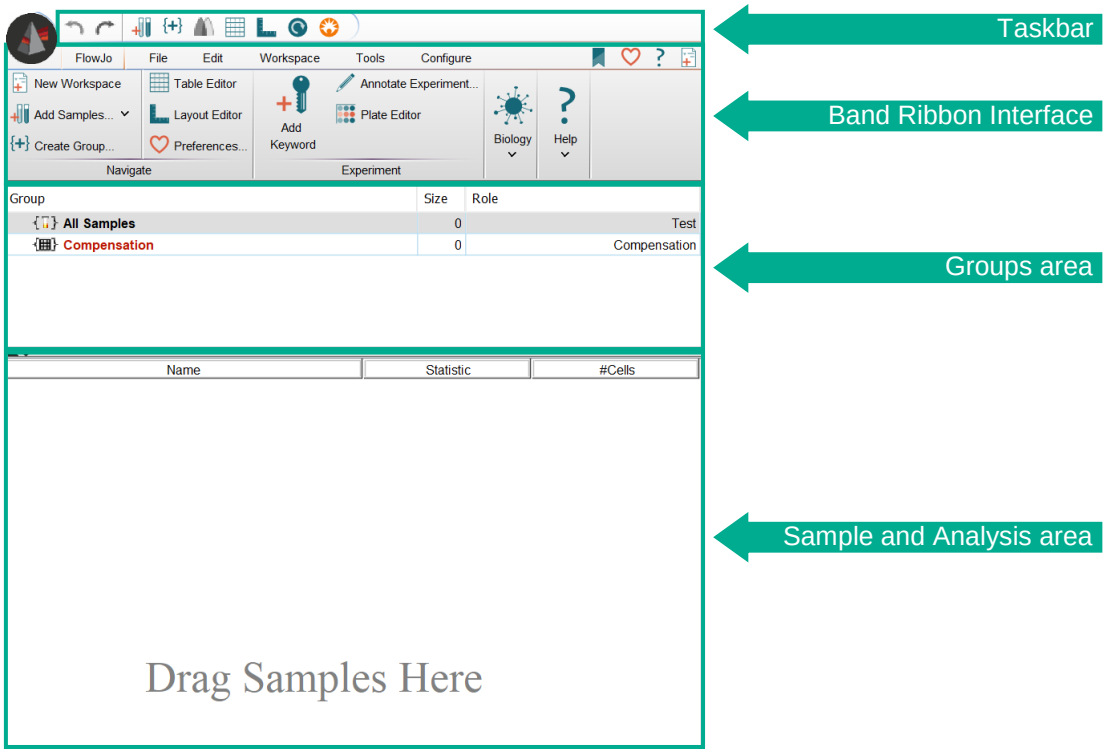
Explore the FlowJo Site: show where to install the program; the Overview; the versions to use; where to find older versions; where to check PC/Mac requirements.
To launch FlowJo you need a license. Explain all the options:

- Free 30-day Trial
- Purchasing FlowJo (*office@flowjo.com*) – Dongle vs FlowJo Portal
- iMM Licenses (FlowJo Portal) (Depending on the use and needs, some labs were allocated with some licenses)
- UCF FlowJo Workstations (show how to book in Agendo)

Show the Learn menu on the site (Webinar, FlowJo University and Tutorials)

Open the software

Show the name of the experiment and start by saving the workspace. Mention the importance of saving the workspace next to the .fcs files we are analyzing. Also, the importance of working with files from the computer and not from any server connection (avoid breaks and problems on the software).



Taskbar – Overview and open all the icons:

- FlowJo Icon (open, save, import, export workspaces)
- Undo - Redo;
- Add samples;
- Create groups (group samples according to specific criteria);
- Compensations (Wizard to do compensations from unstained and single color files);

- Table editor (create statistics that you can export into different files);
- Layout editor (create, edit and organize plots, tables and layouts of the overall analysis to export as images, PDFs and other formats);
- Refresh Workspace (whenever the workspace gets slower or blocked);
- BD research cloud (share data and panel design).

Band Ribbon Interface – Overview and open all the icons:

- Far-right icons:
  - Blue ribbon button (Customize the band ribbon interface) – Show all the bands that can be added, and how you can customize the workspace by drag and drop the icon into the interface.
  - Preferences (Adjust the settings of everything in the software – the "heart" of the software). First, explain the "Locate" and the "License" menus, and then explain how you can change many preferences on your layouts, table, fonts… etc. Suggest coming back here throughout the training.
  - Help tab (Redirects you to the FlowJo Library site (*https://docs.flowjo.com/*)). You can search everything there, in the menu, or by writing what you want to find out in the search bar.
  - New workspace button.

Open *FlowJo Training* folder (Desktop) and Drag samples to the Sample(s) and Analysis area.

Explain the Data – Show *FORM.UCF.086 - FlowJo Training - Users*. Reinforce the importance of defining the aims and goals of the analysis, even before launching the software. This will help you designing your strategy in the software and keep things as organized as possible.

Groups area – Where you can organize your samples based on specific criteria (e.g. conditions, time-points, sample type, strategy of analysis…). A key to saving time! It is populated by default with 2 groups: the "All samples" group, which contains all data files loaded into the workspace; and the "Compensation" group, which automatically includes samples that contain the word "comp" or "unstained" in the file name.

Start by creating more groups and organizing samples: Single colors on Compensation group – Samples on "LN", "SPL" and "All samples" (LN+SPL).

Explain the Group Interface, and how you can use keywords to match each sample to its own group. Also demonstrate the drag-and-drop way.

Sample(s) and Analysis area – By default, it shows your samples, the gating analysis, statistics and number of events. On the left of each sample names is a series of 3 icons known as sample badges: Population marker badge (Diamond), Sample quality badge (Circle) and Compensation matrix badge (Square).

Explain each one, and what kind of information they give by double clicking them to show the interfaces. Show the icon in the tool bar to check quality on all samples at the same time.

Sample name – Go to Preferences and explain how you can change the tube name. The "short file name" is the most commonly used (gives you the name you wrote on DIVA and/or the one you have in your disc). You can also use keywords for the name.

The columns on the sample panel area can be re-organized, added or deleted. You can choose to organize your fcs files based on the column info (e.g #cells, name (alphabetically)…).

Configure the columns (Configure → Settings Band → Edit columns). Click on keyword/info $FIL and then order data, based on that keyword. Also, show how to delete them.

You can adjust existing keywords from your .fcs file or you can add new keywords.

Adjust already existing keywords (PE-Stain – to change label name) and create a new keyword (Stimulation – Add new values)

Compensation wizard: Allows you to create your own compensation, by choosing specific single colors. You can try to create one matrix with beads and the other with cells as single colors, and assess which one fits better to your data.
In addition, the compensation wizard is the only way to create the SSM (Spillover Spreading matrix), very important to evaluate panel design.

Perform compensations on the wizard and then compare with the ones generated from DIVA.
Show how to apply different compensation matrixes to samples/groups.

Now that we have our samples organized, let us get started on the data analysis:

Right click on the first sample (LN1) and open the Graph Window.

Graph Window - Provides an interface to gate your populations and add statistics. It is the first step in creating your hierarchical tree in the workspace.

Explain:

- How to gate and all the types of gating;
- How to name populations;
- How to adjust the name position in the graph, and how in Preferences we can change these kind of options (e.g position, letter size and color, gate color…)
- Undo/redo icons;
- Going back and forward in the gating strategy and between samples;
- How to change parameters;
- How to adjust and transform axis (explain types of scales, the “Extra Neg” and the “Width Basis” options);
- How to duplicate the graph window;
- How to change the plot type and “active gate” options;
- How to add statistics (e.g. median of a specific population and a specific marker – show the new node on the workspace);
- How to close all the windows at the same time (Shift+command+W for Mac and Shift+Ctrl+W for PC).

Start creating gates on LN1 as in the following table, explaining why this strategy will help us solving our questions (Show *FORM.UCF.086 - FlowJo Training - Users*).

Gating strategy:

- Cells
  - Single cells
    - Live cells
      - CD3+CD4- (Non-CD4 T cells)
      - CD3+CD4+ (CD4 T cells)
        - CD4+CD25- (non Tregs)
          - CD62L+CD44low (Naïve CD4 T cells)

Show that we can drop gates and statistics on other samples or groups of samples (or use “copy analysis to group” options).
Do not forget to check the gates for all the samples, going back and forward between samples.

Gating Hierarchy: Explain the bold/color changing as we adjust a gate in one sample. Apply changes in all samples (show all the ways to do it). Show the synchronized option on the group mode (as you change the gate in one sample it will change in all the others from the same group). Show how to delete gates in groups.

Statistics: The default statistics parameter in the Sample area is the Frequency of Parent. However, we can also enumerate other statistics for gated populations using the Statistics Band of the workspace (some are there automatically as Median, CV and Freq. of; or we can click on the most versatile tool “Add statistics” option).

Ask for the median of CD62L and CD44 in both CD4 and non-CD4 T cells.

After gating our data and analyze all the statistics, we go further to show how to present our data. For that, we have the Layout Editor and the Table Editor.

Layout Editor: Where you can display multiple graph plots and statistics in a graphical report. The reports are exported as images or ´batched´ across samples to generate external reports.

Explain the layouts editor window:

- Ribbon tabs (5 menus);
- Add, duplicate and delete layouts;
- Drawing tools, grids and stats tools;
- Zoom functions.

Solving Question 1

Start dragging CD4+CD25- population into the layout to check CD62L x CD44 (Name the Layout “Question 1”).
Open the Graph Definition Properties.

On the Graph Properties, explain:

- 4 Tabs (Specify, Annotate, Fonts and Legend);
- How to change parameters, plot type and plot options;
- How to adjust fonts, letter color, size and style;
- Explain the annotation, the legend, and how to change the information on those;
- Explain how to adjust and settle most of the settings in the FlowJo Preferences.

Show and explain the following: Adjunct histograms, Ancestry, Backgating and Multigraph overlays.

Finish this first layout. Clarify the *Edit*, the *Object* and the *Arrange* tabs.
Be careful with the page orientation and size, to avoid cropping graphs.

Reviewing data using the iteration functions:
Iteration functions have 2 drop-down menus: Group and Iterate.

Explain the Iteration functions. Select the “All samples” group and choose an iterate option. Click up and down and see how we can scroll through files in a selected group.

Batch Reporting: Application of the current layout to the selected group of samples across the selected Iterate by criteria.

Clarify the batch suffix layouts and the batch orientation settings (try two different orientations).
Generate the batch report.

We want these %s represented into a table, to make it easy to visualize and to do the follow-on statistical analysis:
Table Editor: Where you can visualize and export the statistics enumerated from gated populations. Reports can be displayed for visual inspection, and exported as text and Excel.

Explain each Component:

- Ribbon;
- Table Editor;
- Edit;
- Visualize.

Highlight the CD62L+CD44low population, and Drag and drop to Table Editor.
Show how to rename a row, and how to change the statistic info (i.e to Freq. of total).
Create table (Display first, then into the Layout Editor and finally export as an Excel file).
Add heat mapping to the table editor on the Visualize menu, select “edit” and copy new table to the Layout Editor.

Create images and a PDF file from the Layout Editor (careful with the page breaks).

Creating overlays to compare multiple samples/populations in the same graph plot:

Solving Question 2

Create a new Layout (name the layout “Question 2”).
Drag and drop the “Live cells” population to check CD3 x CD4 plot. Drag and drop the CD4 and the non-CD4 population into the same graph. Show the legend, and how you can change sample order, colors and table information (try tube name). Show the Multigraph overlays again and how they can help to check differences in other channels. Batch the analysis.

We want the Median values of CD62L and CD44 for both populations, CD4 and non-CD4 T cells. We can represent them into a new table.

Highlight the median nodes for CD62L and CD44 on both populations, and Drag and drop to Table Editor. Rename the row.
Create table (Display first, then into the Layout Editor and finally export as an Excel file).
Add heat mapping to the table editor on the Visualize menu, select “edit” and copy new table to the Layout Editor.

Create images and a PDF file from the Layout Editor (careful with the page breaks).

Duplicate the Layout *Question 2* and swap the main graph into a histogram view (Properties – Specify – Type: histogram – Set Y axis to modal). Change the X parameter to CD62L. Duplicate this one and change X parameter to CD44.
Show all the different types of histogram overlays. Modify the histogram formatting (Coloring; Line style; Line weight). Pay attention to Legend: try changing the order of the samples and then add the median value for the X parameter.
Batch the analysis and save image and/or PDF.

At the end, do not forget to save your Analysis. Within the File tab of the Workspace window’s ribbon, we can find all the saving options:

- Workspace (analysis template and references to the data file)

Important: FlowJo workspace files do not store your data files (.fcs, .lmd, or .mqd files). Each time FlowJo runs, it accesses your data files and reads them into the workspace. This means that moving data files can cause a situation where FlowJo will need to be re-associated with them. To avoid this, we highly recommend storing your data files in the same folder where your workspace is stored.

- Archive Cytometry Standard (analysis template + data files on a Zip folder)
- Workspace template (analysis template)