

BD FACSDiva Data Analysis at CFC

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Reference

BD FACSDiva Software 6.0 Reference manual

Introduction

The final part of the flowcytometry experiment is the data analysis. There are multiple ways to analyse the data, using a variety of different programs. To facilitate correct data processing it is strongly recommended to follow the general analysis structure of the CFC. This method should prevent basic mistakes and helps the CFC staff when the researcher needs assistance with data analysis.

Material

Analysis computers equipped with DIVA software. Currently most computers are running DIVA6.1.2

Procedure

1. Starting up and Loading data
2. Worksheet organisation
3. Basic dot plots
4. Basic gates
5. Compensation
6. Layout design
7. Printing
8. Saving

1. Starting up and Loading data

Do make a reservation for a flowcytometry data analysis computer using Lotus Notes. Currently you can reserve for 4 different CFC Analysis stations. On your reserved time start a flowcytometry data analysis computer. This one must have DIVA software and a compatible dongle. Logon Windows as: cfcusers, password: cfcusers. If you use a different logon the windows configuration might not be compatible with DIVA software. The cfcusers configuration is adjusted for DIVA. At the DIVA startup screen select your username and type your password.

There are several data storage sides to upload an experiment from:

- Load a **Canto experiment**: <File/Import/Experiment/My Computer/CFC on CRPnas/Canto data backup/BDExport/Experiment/XxX/XXyymmdd blabla>
- Load an **Aria experiment**: <File/Import/Experiments/My Computer/CFC on CRPnas/Aria data backup/BD AriaExport/Experiment/XxX/XXyymmdd blabla>
- Load an **Analysed experiment**: <File/Import/Experiment/My computer/CFC on CRPnas/Analysed data/XxX/XXyymmdd blabla ANAyymmdd>
- Load an **experiment form USB stick or DVD**:
- File/Import/Experiments/My Computer/USB drive/XXyymmdd blabla
 - (XX stands for your initials)

The experiment appears in the browser window underneath the username with a small icon resembling a closed book, indicating a closed experiment. Open experiment with a double click on the experiment name. The closed book icon changes in an opened book. In the browser appears the Cytometry settings, Global worksheets and the Specimen. You can only analyse on an opened experiment.

2. Labelling and Worksheet set-up

After loading and opening the experiment, type in the used antibodies for all tubes in the **experimental layout**. Click on Experiment > Experimental Layout > Labels. Type the antibody or dye name at the correct fluorochrome column. Multiple identical entries can be made with assistance of the Shift or Control keys. Copy and past from an Excel or text file is not possible. Finish the entries with <OK>.

There are two types of worksheets in DIVA. In general the so called global worksheets are used for data display. The advantages of these are that the graphic setup once generated can be used for all tubes/samples. The alternative display is the tube specific worksheet (normal worksheet) where for every individual tube an individual worksheet is generated. Autocompensation uses tube specific worksheets because the gate P2, locating the positive population, is in general for every compensation control tube different. The left top icon on the worksheet window lets you toggle between global and tube specific.

In order to have header and footer, plus title displayed on the worksheet printout, place top and bottom margins at 15 mm or 1 inch. These values can be entered in File > Page Set-up. Make sure that you have selected A4. Correct the margins before making the page layout to prevent readjustments of the size of the plots and histograms.

Click on an uncovered part of the global worksheet and the **Inspector** window (with looking glass icon) will show the properties of the worksheet. Here you can ask for multiple pages, change the name, header, footer, page numbering and install an eventual grid on the worksheet. Most experiments can be handled with 2 pages vertical and 1 horizontal. Recommended is: tag Show and Print page numbers; tag Print headers and footers; take Experiment Name as worksheet title; tag Show worksheet grid and tag Snap to Worksheet Grid.

3. Basic dot plots on Global worksheet

Toggle to the **Worksheet** window and confirm the appearance of **Global Worksheet** in the title of this window. If it states: "**Normal Worksheet**", click on the first icon in the icon tool bar of the Worksheet window. The worksheet display should switch to the Global Worksheet.

You have loaded and opened the experiment of interest and activated a global worksheet. Create a dot plot by clicking on the 5th icon of the icon bar of the Global Worksheet. Place the mouse cursor on the left top of the worksheet. Push the left mouse button down and drag right downwards for 20 x 20 blocks. After release of the left mouse button a dot plot appears with FSC-A on the X-axis and FSC-H on the Y-axis. Click on FSC-H, select < SSC-A>, thus creating the 1st dot plot: **Forewards sideways scatter**. Display all events by a right click on this dotplot window <show population, all events>. Left click on the dot plot and within the **Inspector** window the dot plot properties become accessible. Here you can customize all names and displays of the dot plot.

Right click on the dot plot <duplicate>. Place adjacent right of the 1st dot plot. Change the parameters into SSC-A for the X-axis and SSC-H for the Y-axis. This is the 2nd dot plot: **Doublet discriminator**.

Right click on the dot plot <duplicate>. Place adjacent right of the 2nd dot plot Change the parameters into FSC-A for the X axis and time for the Y-axis. This is the 3rd dot plot: **Stability viewer**.

All 3 dot plots project events on a linear scale.

4. Basic gates

Enlarge the 1st dot plot (Forward Sideward scatter, FSC/SSC) by clicking on the 10th icon of the icon bar of the global worksheet followed by a right click on the 1st dot plot. The Forwards Sidewards scatter dot plot will enlarge. Create a polygon gate by clicking on the 14th icon of the icon bar of the global worksheet. Place the mouse cursor in the 1st dot plot underneath the population of interest. A right click marks the starting point of the polygon gate. Move the mouse clockwise around the population of interest right clicking every time a corner is needed, finishing the gate by clicking on the starting point. This is gate **P1**. Normalise the size of the 1st dot plot by clicking on the 11th icon of the icon bar of the global worksheet followed by a right click on the 1st dot plot. The **FSC/SSC** dot plot will resize to its original proportions.

Enlarge the 2nd dot plot, doublet discriminator, by clicking on the 10th icon of the icon bar of the global worksheet followed by a right click on the 2nd dot plot. The Doublet Discriminator dot plot will enlarge. Create a polygon gate by clicking on the 14th icon of the icon bar of the global worksheet. Place the mouse cursor in the 2nd dot plot a few cm below the right top. Start the drawing by a right mouse click. Move the mouse cursor diagonally downwards to the left bottom corner. When the line has passed all cells make a sharp bend with 3 or 4 edges and move the mouse cursor upwards making a final curve 2 cm to the left of right top corner. This almost triangular shaped gate should be moved over the population of interest, including events with proportional increased values for sideways scatter area and height, indicating single cells. The gate should exclude events with increased area but stabilized height signal, indicating doublets. This gate is **P2**. Normalise the 2nd dot plot by clicking on the 11th icon of the icon bar of the global worksheet followed by a right click on the 2nd dot plot. The **Doublet Discriminator** will resize to its original proportions.

Enlarge the 3rd dot plot, stability, by clicking on the 10th icon of the icon bar of the global worksheet followed by a right click on the 3rd dot plot. The stability viewer dot plot will enlarge. Create a rectangular gate by clicking on the 15th icon of the icon bar of the global worksheet. Place the mouse cursor in the 3rd dot plot on left top. Start the gate with a right click drag the mouse downwards to the right bottom. Include all events. This gate is **P3**. Normalise the 3rd dot plot by clicking on the 11th icon of the icon bar of the global worksheet followed by a right click on the 3rd dot plot. The **Stability Viewer** will resize to its original proportions.

In the

Combine the 3 gates to obtain a stable, single cell population of interest. Right click on the FSC/SSC window <Show Population Hierarchy>. In the window which has appeared left click on P1, shift down and click on P2 and P3. Release shift and right click in the dark grey area in the population hierarchy window <Intersect (AND) Gates>

Right click on new gate "P1 AND P2 AND P3", and in the Inspector window left click on color square. Select green.

Your population of interest appears with a green colour in all 3 windows

Colour the individual gates and all event light blue following the same procedure.

5. Compensation

In order to analyse the signal of a given fluorochrome on its assigned PMT, you should exclude the signals from other fluorochromes detected on this PMT. This problem is called **spectral overlap** and the solution is called **colour compensation**.

Within your experiment set-up you must have included an unstained control and single stained compensation controls for every fluorochrome used in this experiment. For each individual tandem fluorochromes within one experiment it is necessary to include additional single stained compensation control, due to the variation in tandem conjugation efficiencies.

Be aware that the compensation controls should have the highest fluorescence intensity of the antibodies used with this fluorochrome in this experiment. If not, the compensation might not be correct for the fluorescent signals above the compensation control. Especially **compensation beads** do sometimes suffer from this problem.

With DIVA two compensation methods can be used: Manual compensation and Automatic compensation. The later can only installed in acquisition mode, but once installed it could be modified during data analysis.

Manual compensation

On the Global Worksheet create 6 new dot plots in a 2nd and 3rd row, by duplicating the first row with 3 basic dot plots. Select the following parameters, with the first parameter on the x-axis and the 2nd on the y-axis. The quantity and naming are Canto specific. When handling data from Aria or other flowcytometer, different dot plots could be necessary.

- 4th dot plot log FITC-A vs. log FITC-A
- 5th dot plot log FITC-A vs. log PE
- 6th dot plot log FITC-A vs. log PerCP-Cy5.5
- 7th dot plot log FITC-A vs. logPE-Cy7
- 8th dot plot log FITC-A vs. log APC
- 9th dot plot log FITC-A vs. log APC-Cy7

All plots project events on a logarithmic scale. Within those 6 dot pots transform both axis to **Bi-exponential display**. This can be done by left click on the 4th dot plot, shift key down, left click on 5 other dot plots, and release shift key. The **Inspector window** shows the dot plot properties. Tag both the boxes for Biexponential display.

Only events that fall in the P1 AND P2 AND P3 gate (green ones) should be displayed. This can be done by left click on the 4th dot plot, shift key down, left click on the 5 other dot plots, release shift key. Right click on the 4th dot plot <show population> select P1 AND P2 AND P3. Load data by clicking in the **Browser window** on the histogram icon to the left of the tube with the unstained control. The icon should become purple. The events of this tube are displayed in the Global worksheet dot plots.

For calculating the compensation, the mean value of all fluorescent parameters of the unstained control should be noted down on a piece of paper. These mean values can be displayed in a **Statistics** window. Make a right click on the 4th dot plot <Create Statistics View>. Right click on the just created **Statistics View** window <Edit Statistics>. In menu **Population**: Tag the population P1 AND P2 AND P2, untag the rest. In menu **Statistics**: Tag in column "mean" all parameters -A used within this experiment. <OK>

Proceed to the sample with first compensation control, which will normally be FITC, by a left click on the histogram to the left on this tube in the **Browser Window**. Make a gate **P4** around the strongest FITC positive events/cells within the 5th dot plot (FITC vs. PE). The FITC positive population is moving up or down the PE axis during compensation adjustment. Be aware that the gate P4 should be large enough to contain the strong FITC positive events after compensation. Within the statistics view ask for the mean values of P4 of all the other fluorescent parameters. Compensate the FITC spectral overlap for all parameters until the mean value of all other fluorescent parameters are approximately identical to the mean value of the unstained compensation control. This is achieved by a right click on the tube name in the **Browser window**. The **Inspector window** shows information related to this tube. Click on compensation within the inspector window and afterwards click in the field **Spectral Overlap of Fluorochrome:** "PE", **-% Fluorochrome:** "FITC". Adjust the value in this field until the PE mean value of the strong FITC positive population is identical to the PE mean value of the unstained control. Observe within the FITC vs. PE dot plot the position of the strong positive FITC population. This population should run parallel to the X-axis and remain in gate P4.

After PE, continue with the FITC compensation in PerCP, the next line in the Inspector window. The gate around the strong FITC population in FITC vs. PE dot plot can be used for FITC compensations on all other fluorochromes. When the mean values of all parameters have been adjusted to the values of their negative control, right click on the tube name in the browser window > Copy Spectral Overlap. Highlight the next compensation control tube (normally PE) > right click > Paste Spectral Overlap with Zeros.

Create a gate around the strongest PE positive events/cells of the gated population of interest within a FITC vs. PE dot plot with both axis on bi-exponential display and follow the same procedure as for FITC, with PE in the fields **-% Fluorochrome** in the inspector window. When all mean values are compensated, right click on the tube name in the browser window > Copy Spectral Overlap. Highlight the next compensation control tube > right click > Paste Spectral Overlap with Zeros. Continue until all parameters have been compensated against each other.

Copy Spectral Overlap from the final compensation control and past Spectral Overlap with Zeros in all tubes of this experiment. To visually check the compensation overlap matrix, create dot plots with on the X-axis always FITC and on the Y-axis display all parameters used in this experiment. Within the **Browser** window select the tube with FITC compensation

control and check within all dot plots if the centre of the FITC strong positive population is placed on the same Y-value as the FITC negative population. This indicates correct colour compensation. In some cases the distribution of the FITC positive population over the Y-axis can be much broader than the negative control. This phenomenon is called spread. As long as the centres of positive and negative populations have the same Y-value and therefore the FITC population lies horizontally parallel to the X-axis, the compensation is correct. Check visually the parallelism of all the other fluorochromes. If there is no parallelism, the compensation for this combination should be readjusted, followed by a copy Spectral Overlap, Paste Spectral overlap with Zeros and the visual check of all colour combinations. If all fluorochrome combinations are correctly compensated, you can continue with the data analysis.

Autocompensation

Diva software has an autocompensation function. This can only be used whenever you have recorded the autocompensation controls. It was mend to record the compensation controls before acquisition of the test samples. The following samples could then be recorded already compensated, which allows direct result view. Because you can apply compensation post acquisition, we could alternatively calculate the compensation afterwards. The method has some strange twists, due to the alternative nature of this route. The autocompensation makes use of the system configuration. Be sure to perform your analysis on the analysis station, configured like the acquisition station, otherwise the autocompensation fails. Analysis station 1 has been configured like the Canto and Analysis station 2 like the Aria.

If you have recorded the autocompensation controls and you have booked for the correct analysis station, load data, generate basic plots and gates. Open specimen: compensation controls, tube: unstained control. You should assure that in the tube specific worksheets the position of gate 1 on the FSC/SSC is plotted around the population of interest. If gate 1 is placed correctly, right click on the gate <apply to all compensation controls>. Check the position of gate 2 in all other stained controls. Gate 2 should be placed over the 20% strongest positive cells of the population.

When all gates are placed in position goto experiment/compensation controls/calculate compensation. When prompted to give a name, type in the name of the experiment which you are working with. Link and save the compensation to the experiment. A yellow lock should appear on the cytometer settings underneath the experimental name. Right click on the cytometer settings and unlink from the set up.

Open the cytometer settings from tube 1. Right click/apply compensation. In the compensation setup list highlight the line with your experiment name/link. Click okay in all following dialogue windows. Right click on tube 1/copy spectral overlap. Highlight all the rest of the tubes; right click/paste spectral overlap with zeros.

In a global worksheet create dot plots for all colours used in the experiment. Plot on the X-axis always one fluorochrome (e.g. FITC) and plot on the Y-axis all the different colours used in this experiment. Load data from the tube with the compensation control for the first colour. Confirm in plots the horizontal position of the first colour positive cells. If the strong positive population is not on the same height as a weaker or negative population the autocompensation has failed. You should include a manual correction or check the position of the gates 1 and 2 in the autocompensation tube specific worksheets. When adjustments of the gate(s) have been necessary, you need to perform a new compensation calculation afterwards.

If the centre of the strong positive population has the same Y-axis value as the unstained control, the compensation is correct. Perform this test for all fluorochromes on the X-axis. After all fluorochromes have been confirmed to correctly compensated, you could continue with the data analysis.

6. Layout design

When the compensation is correctly finished, you could delete the 6 dot plots on the Global Worksheet used for visual check. The 3 basic dot plots with the 3 basic gates must remain. The Population hierarchy must always be visible.

The rest of the layout of the worksheet is experiment dependent. In general it's recommended to keep the same colour code for the gating hierarchy in every experiment and the same dot plot sequence. This facilitates the data viewing.

7. Printing

DIVA 611 has 2 printing possibilities: individual tubes and batch analysis. Individual tube printing is like MS office, File > print > OK. This gives a high quality print with sharp lines and true type text labels.

Batch analysis prints worksheets of the whole experiment, individual specimen or selected tubes. Right click on one the three possibilities > Batch analysis... Within the following window you could besides tag output to printer, choose the destination of an eventual generated PDF file and statistics csv file of the data selected. Start the processing with <Start>. The print is of a lower quality printout compared to the individual tube print. Currently we are in discussion with BD to find ways to improve the printing quality of the batch analysis.

8. Saving

When the experiment is analyzed, right click on the experiment name > rename. Add the extension ANAymmdd to the experiment name. Yy is year, mm is month and dd is day. Right click again on the experiment name > Export > Experiments.... Export the analyzed and renamed experiment to my computer/CFC on Crpnas/Analysis data/XxX. The analyzed experiment is saved on the server of the CRP.

In order to keep DIVA quick, please delete experiments from DIVA which have been analyzed and saved on the server. The more experiments remain in DIVA the more samples, the bigger the Data base, the slower the program, the higher the chances on crashes.