Analysis and sorting of cells with FACSAria II flow cytometer

Tiina Pessa-Morikawa / Revised 2018-10-23

The instruction refers to the operating manual (**FAUG, i.e. FACSAria II Users Guide** and page number) for additional information.

1. Check the fluid containers in the fludics cart

- Change the waste container if it is more than half full: First empty the spare container to the sink in the lab further on the right in the corridor if necessary. Add Virkon S powder to the container that you disconnect from the fluidics cart (100 g to full, 50 g to half full etc.) and leave it standing with date of Virkon addition noted on the paper on the top of the tank. When attaching the empty container to the cart, make sure that the fluid tubes do not get jammed.
- Fill the sheath container with FACSflow or PBS according to your need (max capacity 10 l, i.e. until the upper joining line). The ethanol container in front can be filled during instrument startup (capacity about 4 l, each shutdown requires approximately 1 l of 70% ethanol: 875 ml l Dilutus 80% + 125 ml milliQH_2O). (The DI, Bleach ja Ethanol containers in the back row are used for aseptic cleaning by instrument administrator).
- When the instrument is on, the fluid levels in the tanks can be seen in the squares at the bottom of the **Cytometer** window of the software, except for the front ethanol tank.

2. Turning the instrument on

- Turn on the computer.
- Turn on the instrument. The main switch is on the left side of the instrument in the upper corner. Make sure that the signal lights for the blue and red lasers are on and the third laser is switched on violet / near UV according to your need. If you do not specifically need the near UV for detection, select violet, which is the default laser.
- Log into the operating system of the computer with the username Administrator and password **BDIS#1**.
- Start the **FACSDiva** program. Select your account from the drop-down list and log in. If you do not have a username, ask the administrator to create one for you.

3. Selection of configuration, fluidics startup and starting the stream

- Always when starting, the Diva program opens a dialogue box about CST Mismatch. In the box, click the alternative **Use CST settings**.
- Choose **a nozzle** according to the size of particles in your sample (take into account eg. cell aggregates or other particles in the sample that may be bigger than the actual objects of your analysis). Instructions for selection of the

nozzle: Diameter of the largest cell in the sample should be no more than ¼ of the nozzle diameter. Blood cells and smaller nozzle 70 µm, cell lines 85 µm or 100 µm, especially large or fragile cells 130 µm.

- Check the current **configuration** of the instrument. It is visible in the upper bar of the program. If it is different from what you need, select **Cytometer > View configurations**.
  - ❖ From the list on the left, choose a **user configuration** that corresponds to the nozzle and optics you intend to use (eg. **user** configuration 85 µm violet), then click **Set configuration** from bottom left and **OK** from bottom right.
- Return to the Diva program and make sure that the configuration has been updated to the upper bar and to the Stream windows.

- Make sure that the fluid filters do not contain air bubbles. Any bigger bubbles should be removed. This is best done when the tank is pressurized, eg. during startup. Loosen the air valve slightly using the screw on the top of the filter. When doing this, use a paper towel to capture the liquid that will also seep out.

- Choose **Cytometer > Fluidics startup** and follow the instructions of the program step by step.

- Start the flow of the sheath fluid by clicking the icon in the upper left corner of the **Stream** window**.**
  - ❖ Wait for a few minutes until the instrument has reached the adequate pressure for drop formation.
  - ❖ Check the quality of the stream (reference pictures can be found in the quick reference guide and stream value table that are on the computer table).
    - o The stream can often locate slightly to the left of the midline of the window, but this is OK. However, by looking into the sort chamber of the instrument, make sure that it flows into the center of the middle waste drawer. If this is not the case, adjust the position of the sort chamber by loosening the screws slightly on both sides of the chamber and pushing the chamber carefully to either side.
    - o The satellite drop should merge with the main drop by the 3rd or 4th drop or earlier.
    - o If the stream is not OK, turn it off, remove the nozzle, clean it by gentle rinsing if needed, reinsert it and start again from the beginning.
    - o Black moving areas in the Stream window are an indication of fluid in the nozzle chamber. It can be dried off with swabs that are found on the computer table (See FAUG p. 227 for pictures).
  - ❖ When the droplet stream in the stream window appears to settle, increase or decrease the **Amplitude** value until the **Drop1** value (= position of the 1st single drop after break-off) approximately corresponds the value given

in the reference table which can be found on the computer table (eg. 150-180 with 70 µm nozzle), i.e. the break-off point of the first drop locates in the upper third of the stream window. Increasing the amplitude generally moves the break-off point upwards.
  - **Note.** The **frequency** value should be exactly as given in the table and should **not** be adjusted.

- When the right level for drop break-off is found, adjust the amplitude further until the **Gap** value (= gap between continuous stream and the 1st drop, shown in gray in the stream window) corresponds the reference value (eg. 6, upper limit 10, with 70 µm nozzle)
- When the stream is settled approximately to the target values, adjust the right (boxed) number to correspond the value given by the instrument (i.e. the number on the right)
- For sorting it is good to let the stream to stabilize further and fine tune if necessary right before beginning to sort.

4. CS&T

- Perform the quality control
  - Start the CS&T program by choosing **Cytometer > CST**
  - Wait for a moment for the CS&T program to form a contact with the instrument.
  - Get the CS&T beads from the refrigerator in room 4087 (third room along the corridor on the same side as the FACS lab) and dilute 1 drop of beads / 350 µl PBS in a 5 ml FACS tube.
  - Choose the current bead lot number (= the number printed on the bead bottle) from the list on the right in the window.
  - Insert the tube to the loading port of the instrument.
  - Begin the CS&T by clicking **Run**. The CS&T procedure takes about 5 min and the program saves the results automatically. You can study them in the **View report** and **performance tracking** sheets. In the users' guide for the CS&T program you can find information about the measurement values that the program generates and their limits.
  - Close CS&T and you get automatically back to the Diva program.

5. Creating an Experiment

- Create an experiment to run your samples in the **Browser** window either using the icons in the upper bar of the window or by choosing **Experiment > New experiment.** In a dialogue box you get a selection of templates, Blank experiment with a sample tube is a convenient alternative.
- Name the experiment as desired in the **Inspector window** sheet **Experiment** (or right click in the browser on experiment and choose **Rename** in the menu). If you begin the experiment names with date in form YYMMDD, the experiments will appear in the browser in chronological order.

- Create at least one **Specimen** under the experiment. Then create an adequate number of **tubes** under the specimen and name these as you like. You can add tubes into the specimen during the run. The latest will appear in the bottom, but it can be moved to another position by dragging. **Note.** The names for specimen and tube will be shown the plots on the worksheet.
- **Activate** one of the tubes by clicking the **icon** on the **left** side of the Browser window so that the triangle on it will become **green**. **Then and only then** the necessary sheets on the Cytometer window: **parameters**, threshold, **compensation** will become visible.
- In the **Parameters** sheet, remove all the irrelevant parameters by choosing the parameter/s to remove and clicking **delete** in the lower right corner of the sheet. This will prevent your files from becoming unnecessarily large. Instead of using the default parameter names, you can seek the names of the fluorochromes you are actually using from the lists, provided that they have been listed there.
- Necessary parameters are at least **FCS-A** and **SSC-A**, for sorting purposes also **FSC/SSC-H** (signal height) and **–W** (signal width), and fluorescence detectors for all of the fluorochromes you are using. If you do not know, which detectors correspond to your fluorochromes, there is a table attached to the side of the instrument, where you can check. More information about the emission wave lengths of different fluorochromes and of their compatibility with the detectors of the instrument can be found eg. at BD's web pages (www.bdbiosciences.com/external_files/media/spectrumviewer/index.jsp).
- If you want to collect data for other than the default number of events, 10 000, you can change the number from **Experiment > Experiment layout > acquisition.** Note that you have to choose the number for each event separately. You can also do this in the Acquisition Dashboard.
- For recording of the samples, draw the necessary plots on the **Worksheet.** Usually you need at least the Scatter plot **SSC-A vs FCS-A**, and the histogram plots for the wavelengths that you want to save. For multiple staining also fluorescence 1 vs fluorescence 2 scatter plots are needed. The data shown on the plots can be modified by changing the parameters shown on each axis (click the name of the axis and choose from the list).

6. Running the sample into the instrument, finding the right settings for the sample and recording the data

- Immediately before the run, FILTER all your samples either using separate Cellstrainers or through the blue Cellstrainer caps directly into the sample tubes. **Note**. To avoid clogging of the Nozzle you **MUST NOT run unfiltered samples**.
- Load first a control sample (unstained cells) into the loading port. **Activate** the corresponding tube in the **Browser**.

- Remember that the **front panel of the instrument must be CLOSED (down) when you are running the samples**. The flow from the sample will not start if the front panel is up.
- Click **Load** in the **Acquisition dashboard** window to lift the sample up into the sample chamber. The flow from the sample will start automatically. There will be a short delay during which all the icons remain gray. When the color returns, the events will start appearing in the plots of the worksheet.
- Check the **flow rate** (scale 1-11) in the **Acquisition dashboard** window and adjust it to a suitable level, so that you get a high enough event rate but do not run a risk to use all the sample for finding the right settings (eg. 500-2000 events/s).
- In the Cytometer window sheet **Parameters** adjust first the FCS ja SSC **Voltage values** so that you get your cells in as middle of the FCS-A – SSC-A plot as possible.
  - ❖ If the size of your cells is very small, you can change the scale from linear to logarithmic (In the Parameters sheet, column after the voltage numbers to the right).
  - ❖ It your cells are so big that you cannot get them to the plot by adjusting the FSC voltage, you can change the FCS ND filter 1,5 or 2,0 to the instrument (see FAUG p. 232 for more information. The other ND filters are in the same drawer as the nozzles). **Note.** If you change the filter, remember to replace the 1.0 ND back when you have finished your run.
- Adjust the voltage values for all the fluorescence detectors until the zero (unstained) peaks are at **10^2** on the scale.
- If you want to record the control sample, click **Record** in the **Acquisition dashboard** window. Note that the flow from the sample will stop after completed recording. Remove the sample from the instrument by clicking **unload**.
- It you use more than one fluorescence label, perform compensation if needed according to separate instructions (FAUG p. 147-8).
- Save the data from all the necessary samples: activate the right tube in the Browser (or use **Next tube** in the **Acquisition dashboard** window)**,** insert the sample, **Load, Record, Unload.**

7. Analysis and determining the sorting strategy

- For analysis and determination of the sorting strategy it is convenient to create a new global worksheet (Experiment > new global worksheet)
- Create the plots that you want and gate the populations of interest using the tools that can be found in the upper bar of the sheet. If you want to see only a subpopulation on a plot, right click the plot and choose **Show populations** > the population of interest.
- By choosing the population hierarchy to be shown on the worksheet **(Populations > Show population hierarchy)** you can determine and verify

the logic of the analysis or sorting strategy. In the same time you can see the relative sizes of your subpopulations.

- Note that you cannot plot a subpopulation defined by the histogram gating tool into a dot plot. For this reason, use the histogram in the lowest level of the hierarchy or use e.g. SSC or FSC versus fluorescence plot for gating instead.
- It is wise to check that the sorting strategy really works the way you think it should by making a short prerun (sort > analysis). This is especially important before you apply a new strategy for sorting of great numbers of cells.

8. Preparations for sorting

- In the Stream window make sure that Drop1 and Gap values are OK. Make adjustments if necessary and then start the **Sweet Spot** by clicking the icon in the upper left of the Stream window.

Adjusting the Drop delay

- Adust the D**rop delay** value (the previous value can be seen on upper right in the **Side stream window**).
    - ❖ For this purpose it is recommendable to use a separate experiment, i.e. close your experiment and open or create an **Accudop drop delay experiment** and open the sort layout in it (opens by double clicking the icon in Browser).
    - ❖ The Accudop drop delay experiment uses 2-way sort and has already defined a population, P1, and a sort layout to direct this to the left side stream. **Note.** 2-way sort in Accudrop drop delay experiment uses the **middle left stream**, which should be adjusted in the sorting window to be in the center of the optical filter (see below).

    - ❖ Dilute the **Accudrop beads** designed for this purpose according to the instruction sheet (1 drop in 500 µl PBS, but dilute more if necessary; see below).
    - ❖ Insert the tube with the beads into the instrument and click load.

    - ❖ Turn on the **Voltage** and the **Optical filter** from the same window.
    - ❖ Begin the sort by clicking **Sort** in the Sort layout window. When the program asks, if you want to draw in the waste drawer, answer **Cancel** (these beads will not be collected into a tube).
    - ❖ The events should now become visible in the square fields of the optical filter, partly in the main stream field, partly in the left. Adjust the flow rate so that you get 1000-3000 events/s with 70 µm or 800-2000 with 85 um nozzle. If it is difficult to see the side stream events, increase the brightness of the spot image of the middle stream by turning the micrometer dial until maximum brightness is achieved (see FAUG p. 169 and 38).

- Adjust the drop delay value so that 100% of the beads will go to the left steam. This can be done either manually (FAUG p. 168), or using the **Auto delay** function found in upper right corner of the side stream window (FAUG p. 171) (click Auto delay and Run, and the program will test different drop delay values and choose the best, which it will also set as the current value).

- Stop the sample flow, unload the bead tube, close the Accudrop drop delay experiment and return to your own experiment.

Creating a sort layout; sorting to tubes

- Begin a new sort by choosing **New sort layout** from the **Sort** menu.
- Define the necessary parameters in the Sort layout window (device, precision, target events, save sort reports).
- Select, which population you want to sort into which tube by clicking the corresponding box, choose ADD and the population you want. The best position for sorting is far left, the next best far right.
- Control that the side streams go into the collection tubes.
  - Place a collection device with tubes in it into the cytometer.
  - Turn on the voltage from the **Side stream window** (=the low broad window with streams showing as spots)
  - Click **Test sort** (this begins the side stream formation in the absence of a sample) and **Waste drawer** (removes the waste drawer from under the side streams).
  - **Carefully** open the sorting chamber on the instrument (**High voltage is on**, keep your fingers away from the instrument!!) and control the placement of the streams, adjust if needed with the voltage sliders in the Side stream window.
  - Return the waste drawer in place by clicking the Waste drawer icon, stop the test sort and turn the voltage off.

Creating a sort layout; sorting to microwell plates, slides etc.

- Begin a new sort by choosing **New sort layout** from the **Sort** menu.
- Define the necessary parameters in the Sort layout window (device, precision, target events, save sort reports)
- Select, which population you want to sort into which wells by activating the corresponding boxes, choose ADD and the population you want.
- Control that the side stream, which must be the **far left** one, hits the Home position in the microwell plate.
  - Check that the splash shield is in place in the cytometer.
  - Open **Sort > Home device** (or **Custom Device**, if you use other than standard plates). Pushing **Access Stage** from the Sort Layout window will bring the plate stage out.
  - Place a microwell plate with **lock on** onto the plate holder (plate well A1 matching home position = in the left corner towards you). Press the **Go to Home** button.

- ❖ Turn on the voltage from the **Side stream window**. Click **Test sort** (this begins the side stream formation in the absence of a sorting sample). Then click **Waste drawer** once and then again after a couple of seconds (removes the waste drawer and puts it back just to let a small drop through onto the microwell plate lock).
- ❖ Observe the result. If you cannot see any drop, or the drop is not in correct position, adjust the side steam position andor plate position with the arrows in the Home device window and try again.
- ❖ When the correct position has been reached, press the **Set Home** button.
- ❖ Stop the test sort and turn the voltage off.

9. Sorting and recording sorting control(s)

- (If needed, open the Sort layout by double clicking; the layouts can be found in the browser **under the worksheet**, on which you made the sorting strategy).
- Pipet suitable liquid (eg. cell culture medium) into the bottom of your collection tubes (200 – 500 ul) or wells (150-200 ul). Put the sample collection tubes into the sorting device OR remove the lid of your microwell plate.
- Insert the filtered sample to be sorted into the loading port (or just start the sample flow, if it is already there) and adjust the flow rate.

- Begin the sort by clicking **Sort** in the **Sort layout** window.
- When the program asks whether the waste drawer should be removed, click **OK.**

- Make sure that sorted events start to appear in the Sort layout and sample drops begin to appear in the collection tube OR that the microwell plate holder starts to move (do this from the outside, DO NOT open the instrument cover)
- You can pause the sorting e.g. for adding sample or changing sorting tubes by clicking **Pause** and restart by clicking **Resume.**
- If the sample tube accidentally runs dry, Sweet Spot and stream will close automatically. Wait a moment for the pressure to equalize then start and adjust the stream again. If Drop 1 and Gap values change, recheck the Drop delay value with beads.
- If you do continuous sort, stop the sort by clicking Sort again. Save the sort report.
- Stop the sample flow and remove the tube from the instrument.
- Control the sort by running a small sample of the sorted cells (1000-5000 is often enough) and record the data. For this purpose create and name new tubes. Note. If this control sample will stand for a while before the run, it is good to filter it. Dilute it also if needed, so that the tube will not accidentally become empty during data collection.

10. Shutdown of the instrument

- Turn off the stream.
- Choose **Cytometer > fluidics shutdown** and do as instructed.
- The **cleaning solution** needed into the loading port as the last stage of the protocol **= 70% ethanol**.
- **Release the pressure in the sheath tank** by first opening the pressure relief valve by pulling upwards using the attached ring. Then also open the lid. After the pressure is completely released, fasten the lid again.
- Close the instrument and Diva software plus computer if you do not want to go on with analysis. The program can be used even when the instrument is turned off.
- Change the waste container for an empty one if it is more than half full. Add Virkon to the removed waste container, mark the date of addition on a piece of paper for the next users to see. The waste should be left standing for at least overnight before discharging.