General remarks

Only power users having passed the sorter training at the FCF are allowed to operate the Aria III cell sorter.

Questions / reporting technical problems: Emergencies:

⌨ **Feedback form**   ✆ **044 635 53 36**
                                            ✆ **044 635 02 17**

Starting up the sorter

➢ Start the local and remote computers, set the switch board to "remote" and login with your core domain login on the remote computer. The connection to the local computer will be established automatically (in case of failure establish the connection via the shortcut for the remote desktop on the desktop or switch the switch board to "local" and use the "Admin" account on the local computer, password "BDIS").

➢ Start the Diva software and log into FACSDiva with the "Diva" or "DivaUser" account and password "MyDiva".

➢ Ensure that all lasers required for your experiment are set to "ON" (controllers on the left hand side of the machine). By default, the 405nm laser is switched off unless needed, to extend the lifespan of the corresponding fiber optics.

➢ Turn on the main power switch (green button on the left side of the machine) and if necessary the aerosol management system (Use AMO for BL-2 sorts only).

➢ Select "Cytometer" →"Fluidics Startup" in the menu and follow the on screen instructions.

Note: Spray sheath lines connectors with 70% EtOH and make sure that they are properly connected (Don't remove the EtOH and sheath filters from the corresponding tanks).
Make sure that the lid of the sheath tank is properly closed and the tank pressurized (if the lid is not properly sealed air will leak out and Fluidics Startup will fail).

➢ Insert the correct nozzle size for your samples.

Available nozzle sizes: 70µm, 85µm, 100µm (and 130µm). As a rule of thumb, the nozzle orifice should at least be 3 times the size of your cells (also consider potential clumps of cells!). Bigger nozzles (= lower pressure) might be beneficial for sorting fragile cells.

➢ Choose the correct configuration for your nozzle via "Cytometer" → "View Configurations" and accept "CST settings" when prompted. The current configuration is visualized over the stream window.

Each instrument configuration is optimized to a preset sheath pressure:
70µm -> 70psi, 85µm -> 45psi, 100µm -> 20psi, 130µm -> 10psi.
Selecting the appropriate configuration will also change the related values for drop drive frequency, drop charge levels, laser delay and area scaling.

Default drop drive frequencies are: 70µm -> 87 kHz, 85µm -> 45-47 kHz, 100µm -> 30-32 kHz, 130µm -> 14k Hz. Small variations between different instruments are likely. Keep the current frequency unless you cannot establish a stable stream.

Temperature Control Systems

➢ Turn on the water bath, set the appropriate temperature for your samples (by default 4°C) and connect the collection tube holder to the ports for the recirculating water.
   This controls the temperature of your collection tubes.

➢ Check sample agitation and temperature settings under "Cytometer".
   This determines the conditions of your sample tube before sorting.

Stream set up

➢ Open the sort block access door and visually inspect the sort chamber. Make sure that the deflection plates are clean and dry. If necessary clean with a water-soaked tissue followed by an EtOH-soaked tissue. Dry all surfaces which will be charged.

➢ Start the stream by clicking on the "stream" button on top of the drop breakoff camera window. When the "stream" button changes to a green tick mark (after some seconds), you should see the formed stream in the corresponding window.

➢ Check that the stream hits the center of the waste aspirator. If not use the Allen key for readjusting the sort block and center the stream into the waste.

➢ If the stream does not form at all or appears unsteady, check the following:

   • Did you use the correct configuration for your nozzle size?

   • Make sure the nozzle is properly inserted and not clogged (visual inspection with the stereo microscope). If clogged, sonicate it in a tube filled with ddH$_2$O for 15-30 seconds.

   • Is the sheath supply still attached to the EtOH tank?

   • In case you suspect air in the flow cell, perform a "clean flow cell" with 70% EtOH by choosing the menu "Cytometer -> Clean Flow Cell". When completed, try restarting the stream again.

➢ Adjust the position of the breakoff point by adjusting the amplitude.
   *Ideally, the drops before the breakoff point will appear pear-shaped ("birnenförmig"). Satellite drops should merge with the leading drop (exception 130 nozzle, trailing drop)! Use the default gap values as a guideline for optimal setup: 70µm -> gap 5-6, 85µm -> gap 8-9, 100µm -> gap 10-12, 130µm -> 11-13. More information can be found in the BD user manual on page 105. Note: Generally, the preset frequency values provide good starting values for these settings. Please adjust the values **only** if the stream requires corrections.*

➢ Transfer the current value for drop 1 and gap into the left field in the breakoff window

➢ Activate the sweet spot function.

➢ Wait for the stream to stabilize (between 1-20 min depending on the used nozzle and the properties of the machine). Recheck the drop 1 value and correct if necessary.

Test the drop deflection

W A R N I N G : *A great voltage potential exists between the deflection plates when they are on. Contact with the charged plates will result in serious electrical shock.* ***Do not touch the deflection plates when the plate voltage is on.*** *The plates remain energized even when the sort block door is opened. Red light on the left of the sort block indicates whether the plates are charged or not.*

➢ Adjust the stream illumination laser position on the left hand side of the sort block to ensure maximal illumination of the central stream.

- ➢ Click the voltage control button in the side stream window. The voltage warning light will turn on, indicating that the plates are now charged.

- ➢ Make sure the center stream image does not move after the plates are turned on. If you notice this please contact the FCF staff immediately.

- ➢ Click on "Test Sort" and check if drops are deflected for the activated positions. In case you cannot observe any deflection recheck the position of the stream illumination laser and the deflection plates for liquids or salt precipitates.

Adjusting the drop delay

Since particle analysis and droplet formation do not happen simultaneously, it is essential to calibrate the instrument for the time delay between the two processes. An incorrect drop delay will result in charging of the wrong droplets and will ruin the sort experiment. The correct timing is defined empirically using AccuDrop calibration particles.

- ➢ Open the existing AccuDrop experiment template at the bottom of the Diva software browser list or create an AccuDrop experiment using the menu "File -> Experiment".

- ➢ Load a tube filled with a dilute suspension of BD AccuDrop beads. Use the previously prepared tube if available (check fridge next to the sorter, usable for ~1 week if kept in the dark at 4°C) or prepare a fresh tube with approximately 1 drop of beads in 500 µl PBS).

  There are two ways for adjusting the drop delay: manual or automatic. We strongly recommend the manual procedure because it is faster and gives you a more robust setup. It is described in the following paragraphs.

- ➢ Starting from flow rate 1, adjust the flow rate to achieve a threshold rate of 2,000–3,000 events/sec.

- ➢ Open the Sort Layout of the Accudrop experiment (click the + on the tube) and select "2 tube" as device and "Initial" as a precision mode. Click "Sort" to start deflecting the beads.

- ➢ When asked whether to open the aspirator drawer, select "Cancel" (there is no need to collect the beads, "Cancel" will result in sorting the beads to the waste).

- ➢ Turn on the voltage in the side stream camera window and ensure that the sorter deflects a left side stream.

- ➢ Click on the "Optical Filter" control in the Side Stream window.

  This control moves the emission filter that allows you to detect the AccuDrop beads in front of the lower camera. When the filter is activated, the image switches from a raw image to a processed (digitized) image. The two boxes indicate the regions of the image in which the left and center stream intensities are quantified and shown as percentages of total intensity.

- ➢ If the left side stream is not fully targeted in the left box, adjust the voltage slider and place the stream in the center of the region. Ensure that the central stream is visible in the right box.

- ➢ Ensure that the sum of left and right box add up to 100%.
  If 100% is not reached, check threshold rate is 2,000-3,000 evts/sec, position of streams correlates to the filter boxes and ensure correct focus of side stream illumination laser.

- ➢ Optimize the drop delay in the initial mode. To do so, adjust the drop delay value in 1-drop increments (Ctrl-click on arrows) to achieve close to 100% intensity in the left side stream.

  Wait a few seconds after each click for a complete response to the Delay change.

- ➢ In the Sort Layout view, change the Precision mode to Fine Tune.

- ➢ Continue optimizing the drop delay by small increments (click on arrows) until the left side stream intensity is >95%.

In optimal conditions you can achieve a close to 100% deflection using the FineTune Precision mode. You will notice that the maximum deflection efficiency will be maintained through a certain range of drop delay values ("plateau"). The most robust setup is achieved by defining the edges of the plateau (e.g. 90% deflection) and to calculate the corresponding drop delay for the middle of this plateau.

➢ Unload the tube and store remaining AccuDrop beads in the fridge for next users.

➢ If you require sterile sort conditions, it is recommended loading a tube with FACSClean for 1 min to decontaminate the sample line.

Setting up the side streams

➢ Turn on the voltage & "Test Sort" and optimize the position of the side streams using the voltage sliders.

Note: Plate sorts require to target the far left voltage slider in the inner left 4 way position.

We recommend using the provided "side stream setup card" to target the side streams for 2way or 4way sorts.

Alternatively you may start a test sort and open the aspirator drawer to aim the side stream(s) into the bottom of each collection tube. *Be careful not to touch the deflection plates while they are charged!*

➢ If you observe fanning of the center/side streams this may indicate incorrect decharging. In this case write down the current 2nd, 3rd, and 4th Drop numbers and try to "tighten" the streams by modifying these values. If you do not succeed please put back the initial numbers and contact our FCF staff.

*Note: Generally, the sort setup mode provides good starting values for these settings. Please adjust the values **only** if the streams require corrections.*

The Aria is now ready for sorting once you have defined your experiment and the gating strategy.

Starting and monitoring the sort

➢ Open the sort collection chamber door and install the collection tubes, plate, or slide.

➢ Set the flow rate to 1, install the sample tube on the loading port and click "Load". Adjust the flow rate for the desired event rate.

➢ Record some data and verify your gating strategy and hierarchy is set correctly.

➢ Define the populations to be sorted in the Sort Layout and choose a "Precision Mode".

➢ Start acquisition and click on "Sort" in the Sort Layout. Click "OK" when prompted which will open the aspirator drawer and turn on the deflection plates.

W A R N I N G : If you click "Cancel" at the message prompt, sorting will start with the deflection plates off and the drawer closed. Populations will be counted in the software but no deflection (actual sorting into tubes) will occur. If you sort with the drawer closed, events will be sorted into the waste even if you had the voltage manually switched on and side streams are observed in the camera window.

➢ Sorting continues until the required number of cells has been sorted or you run out of sample. The drawer will close automatically once the sort is completed or becomes interrupted. If the number of target events is set to "Continuous", sorting continues until you manually stop sorting

by clicking the Stop Acquiring or Sort button.

➢ To pause the sorting, click the "Pause" button in the Sort Layout. The counting of sorted cells is retained when you restart sorting by clicking the "Resume" button.

Monitoring the sort: Keep an eye on the stream window to check for abnormalities that may indicate a nozzle clogging. Observe the stream camera window for deflected drops and compare to counts in the Sort Layout. Regularly control for movements of the Drop 1 position and deviations in the Gap values. A slowly but steady reduction in the event rate is often a sign of a clogging sample line.

Note: When the Sweet Spot is on, sorting will pause automatically if the Drop 1 or Gap values move out of tolerance range. However, the sorter will continue consuming your sample hoping that the values move back into tolerance range at some point. This ensures that sorting occurs only under the proper breakoff conditions but you may lose significant amounts of sample if you do not notice this! If a more severe problem like a clog is detected, the stream shuts off automatically and the sorting stops: the deflection plates are switched off, the aspirator drawer closes, and the sample tube is unloaded.

After the sort

➢ Stop the sort and unload the sample tube.

➢ If desired perform a reanalysis and check the sorted populations for purity. Go to "Sort→ Sort report" and check the report for that particular sort layout (can be exported to PDF if necessary).

➢ Print sort gates to PDF and export your experiment to preserve settings and FCS files and delete it from the database (see data policy below).

➢ Clean the instrument, i.e. the sample loading area and the collection tube area with an EtOH.

➢ Load a tube of **FACSClean** and run it for **5 min at sample rate 11**.

➢ Load a tube of **FACSRinse** and run it for **5 min at sample rate 11**.

➢ Load a tube of fresh **DI water** and run it for **5 min at sample rate 11**.

➢ Check whether somebody else is scheduled after you in the instrument booking calendar. The stream should be switched off when the time between you and the next user is above 30 min. Keep Diva running, close the remote desktop view and log out of your personal core domain account on the remote PC. Do not switch of the stream if there is another user right after you!

Last user of the day

If you are the last user of the day, proceed to the fluidics shutdown

➢ Turn off the stream.

➢ Go to "Cytometer →Fluidics shutdown" and follow the on-screen instructions. At the end of the shutdown procedure, the system will ask for a tube with cleaning solution. Load a tube with DI water.

➢ Check PBS stock in the room. If there are less than 3 boxes with 10l PBS send us a message via the **Feedback Form**.

➢ Turn off the machine at the green button on the left side.
Turn off the computer, the water bath and the AMO (if used).

➢ Refill the Ethanol tank.

➢ Follow the waste handling SOP and decontaminate the waste (waste SOP is also hanging next to the sink).

- Clean the working area – don't leave (un)used tubes, gloves, etc. behind.
  - Refill the PBS tank if necessary (see SOP "Aria tank exchange - AFS")

Data Export Policy & Diva Database

- After measuring and cleaning, export your data as *.fcs files* to your fcf-files folder on our server (V-drive, data storage for 3 months).
- If you wish to preserve experimental settings for future experiments, additionally save them as experiment file (Export → Experiments; can be reimported into Diva; e.g. to fcf-home, H-drive, 1GB permanent storage).
- **After exporting your data delete them from the Diva database.**
  A large Diva database will slow down the software significantly.
- **Please note: Only data stored on the server are secured by a backup.**
  The local computer and Diva database have no backup.
  Locally saved data will be deleted without further notice.
- Every user is responsible for securing their data directly after their session.
- The Diva database will be emptied by FCF Staff without further notice to ensure the stability of the software.
- For your convenience, you may keep experiments without data marked as "template" in the Diva software. ("Duplicate without data" and rename to template_xxx)
- Do not use subfolder structures in Diva, this information will be lost upon export.
- Always rename your experiment files with e.g. date and your initials.
- For safety reasons the use of USB sticks is disabled. Check "Data Management & Access" under "IT Infrastructure" on our website for more information.