General note

Access to the Aria III cell sorter is restricted to Power Users who have completed the sorter training at the Cytometry Facility

Questions / reporting technical problems:

Feedback form

Emergencies:

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Task for the 1st user of the day

- The first user of the day must take care of the inactivated waste of the last usage of the device. A detailed SOP can be found next to the sink.

Starting up the sorter

- Start the instrument and support computers, set the switch board to "support" and login with your core domain login at the support computer. The connection to the instrument computer will be established automatically (in case of failure, establish the connection via the shortcut "Connect to Instrument Computer" on the desktop of the support computer or switch the switch board to "instrument" and use the "Admin" account on the local computer, password "BDIS#1").
- 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).
- Turn on the main power switch (green button on the left side of the machine) and if required the aerosol management system (use AMO for BL2 sorts only).
- Lift or tilt the sheath tank to estimate the fill level. An almost empty tank should be replaced before running the startup.
- Select "Cytometer" →"Fluidics Startup" in the menu and follow the on-screen instructions.

  Note: We only have a single filter attached to the fluidics line, which is used as both sheath filter and filter for the EtOH (regular filter incubation in EtOH reduces the chance of instrument contamination). The filter is ready to use after a single startup.
  Make sure that the lid of the sheath tank is properly closed while the tank gets pressurized (if the lid is not properly sealed pressurization 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 be at least 3 times the size of your cells (also consider potential clumps of cells!). Bigger nozzles (= lower pressure) might be beneficial for cell survival of 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 and in the title of the DIVA software in more detail.
  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 of this device are: 70µm -> 89 kHz, 85µm -> 67 kHz, 100µm -> 32 kHz, 130µm -> 16k Hz. Small variations between different instruments are likely. Keep the current frequency unless you cannot establish a stable stream. Please also check the status of the feedback form for updates.

Temperature Control Systems

- For keeping your collection tubes at a defined temperature do the following.
  Turn on the water bath, set the appropriate temperature for your samples and connect the collection tube holder to the ports for the recirculating water (default is 4°C, please set it back to 4°C immediately after your sort if you were using it at a different temperature).
- Check sample agitation and temperature settings under "Cytometer".
  This determines the conditions of your sample tube while sorting.

Setting up the stream

- Open the sort block access door and visually inspect the sort chamber. Make sure that the deflection plates are clean and dry. You can easily clean the plates with a water-soaked tissue followed by a dry tissue. Dry all surfaces which will get 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 and correct nozzle?
  - 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_2O for 15-30 seconds.
  - Is the sheath line still attached to the EtOH tank or does the sheath filter contain bubbles?
- Adjust the position of the breakoff point by adjusting the amplitude.
  *Check if the satellite drops 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 -> 12. 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 into the left field in the breakoff window.
- Activate the sweet spot function.
- Wait for the stream to stabilize (between 1-5 min depending on the used nozzle and the properties of the machine) and correct for deviations in the Drop 1 position.

Test the drop deflection

W A R N I N G : *A strong voltage potential exists between the deflection plates when they are turned on. Contact with the charged plates will result in serious electrical shock. Shocks of that magnitude can lead to atrial fibrillation.* ***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 side 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.
- 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 Cytometry Facility 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.
- If you observe fanning of the center/side streams or additional "ghost streams" this may indicate problems with the drop charging. Check for the following:
    - Deflection plates are clean (no salt precipitates) and dry
    - Remove nozzle and nozzle lock (black lever keeping nozzle in place) and remove any liquid or white salt precipitates (the latter with ddH_2O and dry everything well)
    - Try different Drop 1 position (higher position often works better!)
    - Use different frequency value for setting up the stream
    - In case the upper interventions did not succeed (repeated cleaning may be required to solve the underlying problem) you may try to adjust the 2nd, 3rd, and 4th Drop numbers. Make sure you write down the current numbers before changing. If you cannot improve the situation, put back the initial numbers. Contact the facility if you have changed the numbers or if you cannot solve the problem.
- Ensure that the **central waste** as well as the **inner left streams** are optimally illuminated before continuing with the drop delay.It is recommended to check drop deflection with all 4 side streams active, but at least with the side streams you want to use later.

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 wrong droplets which will ruin the sort results. 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. In case the experiment is not available you can create a new AccuDrop experiment using the menu "File -> Experiment".
- Open the Sort Layout within the Accudrop experiment (click the + in front of the tube) and select "2 tube" as device and "Initial" as a precision mode.
- Using flow rate 1 load a tube of diluted BD AccuDrop beads. Use the previously prepared tube if available (check fridge in the hallway, usable for minimum a month if kept in the dark at 4°C) or prepare a fresh tube with approximately 1 drop of beads in 300-500 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 typically more reliable and also gives you a good impression if and how the device performs. It is described in the following paragraphs.
- Click "Sort" to start charging the drops of the stream. 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. (Do not proceed if there is no side stream. Figure out why there is no side stream first.)
- Click on the "Optical Filter" button 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.
- Starting from sample flow rate 1, adjust the flow rate to achieve a threshold rate of 2,000–3,000 events/sec (dilute the beads more if too high at lowest flow rate).
- The sum of left and right box should add up to 100%.
  If 100% is not reached, check threshold rate is 2,000-3,000 evts/sec, positions of streams fall into the filter boxes and ensure correct focus of side stream illumination laser.
- Optimize the drop delay in the Precision mode Initial. To do so, slowly adjust the drop delay value in 1-step increments (Ctrl-click on arrows) to achieve close to 100% intensity in the left side stream.

  Wait a second after each click for a complete response to the delay change. If you go to fast you might miss it.
- Next, change the Precision mode to Fine Tune in the Sort Layout view.
- Continue optimizing the drop delay by small increments (click on arrows) until the left side stream intensity reaches a maximum (it should be >95%).

Under optimal conditions you can achieve >99% 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.*

- Update the position of Drop 1 in the stream window. This is your reference value.
- Unload the tube and store the remaining AccuDrop beads in the fridge for next users.
- If you require sterile sort conditions, it is recommended to run a tube with FACSClean for 1 min to decontaminate the sample line. Run a manual sample line backflash for ~30 seconds afterwards to remove all traces of clean of the sample line.

Setting up the side streams

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

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

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.

  WARNING: *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 waste 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.
- If you stopped the sort or it got interrupted, you can keep counting sorted cells when you restart sorting by clicking the "Resume" button. If you click "Start" it will start counting at 0 again.

Monitoring the sort: *Keep an eye on the stream window to check for abnormalities that may indicate a nozzle clogging. Observe the side 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 slow 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. (You need to run FACSClean for a min +manual Sample Line Backflash of 30 sec to remove the clean completely before loading your precious sorted cells. Without cleaning the sample line, the reanalysis might look dirty due to sample carryover, even though sort went well.)
- 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, e.g. the sample loading area and the collection tube area with 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. Always stop the stream, except the next user personally tells you to keep it running. (Users might be late or use a different nozzle size).
- Keep Diva running, close the remote desktop view and log out of your personal core domain account on the remote PC.

Last user of the day

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

- Turn off the stream.
- Ensure the EtOH tank is filled at least to the inner weld line. Refill if needed.
- 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 the H_2O tube!

  Depressurize the sheath tank. **DO NOT REFILL SHEATH** tank at the instrument.
  See "Exchange Tank SOP".
- Turn off the machine at the green button on the left side.
  Turn off the computers, the water bath and the AMO (if used).
- Follow the waste handling SOP and decontaminate the waste (SOP can be found next to the sink).
- Check PBS stock in the room. If there are less than 3 boxes with 10 L PBS send us a message via the Feedback Form.
- Clean the working area – do not leave (un)used tubes, gloves, etc. behind.

Data Export Policy & Diva Database

- After measuring and cleaning, export your data as *.fcs files* to the file server ("data" folder = storage for 3 months)
- If you wish to preserve experimental settings for future experiments, additionally save them as experiment file. Do copy "without data" and export the empty experiment containing your gating strategy and compensation (Export → Experiments; can be reimported into Diva; e.g. to the "home" folder on the file sever = 1GB permanent storage).
- **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 may be deleted without further notice.
- Every user is responsible for securing their data directly after their session.
- The Diva database will be regularly emptied by the Cytometry Facility 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.