General note

Access to the S6 cell sorter is restricted to Power Users who have completed the sorter training at the Cytometry Facility Sections highlighted in yellow indicate workflow deviations from Aria III sorters.

Questions / reporting technical problems:

Feedback form

Emergencies:

✆ **+41 79 152 20 69 (Mario)**
✆ **+41 79 518 26 49 (Manuel)**

Biosafety Note

The FACS lab is a BL2 laboratory. It is strictly forbidden to eat, drink or bring food into the lab. When using the sorter you are allowed to wear gloves all the time, however you ALWAYS have to spray them with 70% Ethanol before you touch the computer (mouse/keyboard). Instrument and computer must be left so they are safe to be touched without gloves by the next user!

Task for the 1st user of the day

The first user of the day is responsible to empty the waste tank of the previous day (check sink in Y44G3h). The liquid has been decontaminated with bleach overnight (check for label) and it is safe now to empty directly into the sink.

Starting up the sorter - Note: The order matters!

1. Start local instrument PC first ... *wait, 1-2 seconds, then...*
2. Start support PC ... *wait, 1-2 seconds, then...*
3. Set switch board to "Support PC"
4. Login with your core domain login on the remote computer.
   If you see an Admin Login screen, check the switch board is actually set to "Support PC"
5. The connection to the local computer will be established automatically.
   In case of a failure, establish the connection via the shortcut on the desktop "Connect to Instrument PC". Emergency solution: switch the switch board to "instrument" and use the "BDAdmin" account on the local computer, password "BDIS#2$$" to work locally on the instrument PC.
6. The Diva software starts automatically with the "DivaUser" account (password: MyDiva).
7. Turn on the air pressure (red switch of multi switch box on the right side of the sorter)
8. Turn on the main power switch (green button on the right side of the sorter) and if necessary the aerosol management system (use AMO for BL2 sorts only).
9. Lift the sheath tank to estimate the fill level of the sheath tank (an almost empty tank should be replaced before running the startup.
10. Select "Cytometer" →"Fluidics Startup" in the menu and follow the on screen instructions.

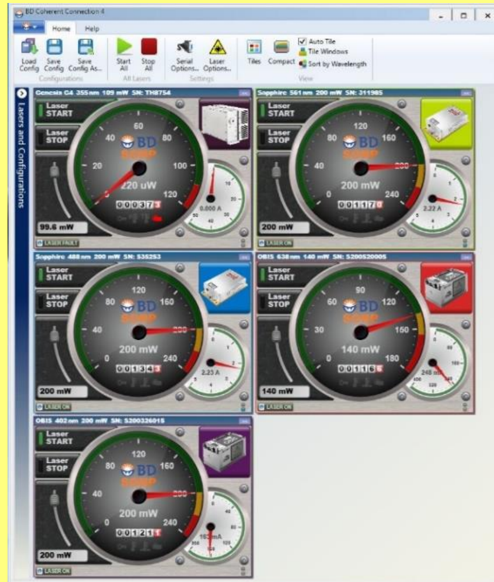
    Note: We only have a single filter attached to the fluidics line, which is used as both sheath filter and filter for the EtOH (regular filter incubation in EtOH reduces the chance of instrument contamination). The filter is ready to use after a single startup.
    Make sure that the lid of the sheath tank is properly closed while the tank gets pressurized (if the lid is not properly sealed pressurization and Fluidics Startup will fail).

Load and control laser powers

Before every experiment, check laser power settings via Coherent Connect Software.

Note: The coherent connect software starts automatically with the PC (autostart).
If it has been closed since then, restart it via the desktop shortcut (instrument PC) before your measurement to ensure laser settings are set correctly for your experiment.
See separate BD Coherent Connect SOP for further details on laser power controls and troubleshooting.

- Check that all 5 lasers appear in the Coherent connect software:



- Load a suitable configuration for your experiment via “Load configuration” in the BD Coherent Connect Software from the available standard configurations:

  “S6 default application settings”

All lasers full power for maintenance	10/30/2020 3:40 PM	File folder
UV off V200 B200 YG200 R140.cfg	10/30/2020 3:36 PM	CFG File
UV60 V200 B200 YG200 R140.cfg	10/30/2020 3:35 PM	CFG File

  a) Whenever UV 355 nm laser is not required chose “UV off V200 B200 YG200 R140”
  → UV laser output reduced to 0 mW to increase laser life, all other lasers full power.

  b) Whenever UV 355 nm laser is required chose “UV60 V200 B200 YG200 R140”
  → UV laser output 60 mW (instead of max. 100 mW) to avoid autofluorescence which may become too strong at 100 mW, all other lasers full power.

  Optional for Symphony comparisons: “Match Symphony analyzer laser power”
  Note: Violet laser power on Symphony is 100 mW instead of 200 mW at S6.

Symphony match UV off V100 B200 YG200 R140.cfg
Symphony match UV60 V100 B200 YG200 R140.cfg

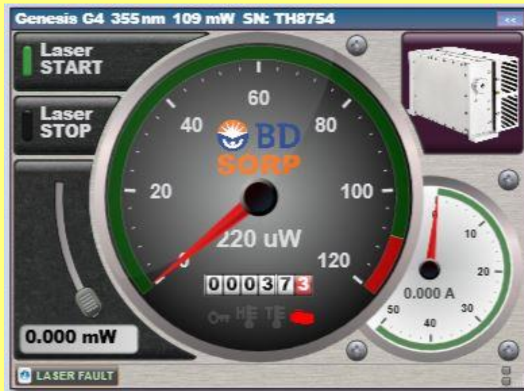
Note: You can create customized laser configurations if required. Please be careful to not overwrite existing configurations by clicking “Save configuration”. Please use the “Save as” function.

- **Important: Confirm all actual laser powers in the laser speedometers**

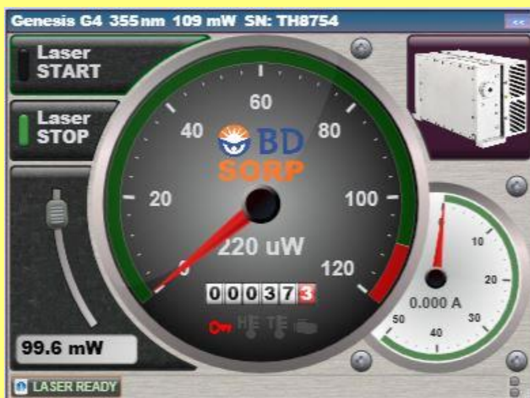
- **Starting of the UV laser (only if required for your experiment)**

  Important: After loading a laser power configuration, the UV laser has to be started manually to activate it (laser safety regulations).

  1. Laser is off → Press “Stop” and wait for red engine symbol on bottom right of the speedometer to switch to the red key symbol.



  2. Press “Start” to start the UV 355 nm laser



  3. Check that the needle of the laser speedometer is raised to the output specified in the configuration e.g. 60 or 100 mW after a short warm up period (this can take a few seconds).

The UV laser is now correctly activated, example shows 100 mW output ☺

Continue setting up the sorter

- 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 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.
  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 -> 67 kHz, 100µm -> 32-36 kHz, 130µm -> 14k 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.*

- For Biosafety rules and procedures see "SOP - Biosafety".

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 before 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 -> 15. 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 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 and contact the facility.
- 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".
- Load a tube filled with a dilute suspension of BD AccuDrop beads. Use the previously prepared tube if available (check fridge in the hallway, usable for about 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. It is described in the following paragraphs.

- 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).
- Open the Sort Layout within 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" 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.
- 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, position 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.

- 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 loading 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.

  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 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!*

  Note: If you use the 6-way sort feature of the S6 please adjust the two outermost side streams to a position where they just hit the outer tubes but no further out than needed. Targeting the outer side streams too far out may lead to deflected droplets hitting the deflection plates during the cell sorting. Please make sure to confirm that this does not occur throughout your sort (short circuit zapping noises and salt residues on the plates indicate this issue).
  Warning: This might only occur when sorting cells and might not show up during test sort or while sorting beads depending on your cells of interest creating a slightly different flightpath compared to PBS or beads.

The S6 is now ready for sorting once you have defined your experiment and the gating strategy.

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Starting and monitoring the sort

BIOSAFETY NOTE: *If you are sorting samples that need to be deactivated before disposal (such as virus-infected or human cells), add 200ml of 14% bleach to the waste container and indicate this for the next user (e.g. tape label => bleach added).*

- 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 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 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. 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, 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. 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 off 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.
- 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!
- Turn off the machine at the green button on the right side.
- Turn off the air pressure (switch off the plug connector on the right side of the sorter)
- Turn off the computer, the water bath and the AMO (if used).
- Follow the waste handling SOP and decontaminate the waste (waste SOP is also hanging next to the sink).
- 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 (Export → Experiments; can be reimported into Diva; e.g. to the “home” folder on the file sever = 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 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.