INSTITUTO DE MEDICINA MOLECULAR JOÃO LOBO ANTUNES
FLOW CYTOMETRY FACILITY
SORTING PROCEDURES
ADAPTED TO POWER USERS
(BD FACSARIA III & FACSARIA FUSION)

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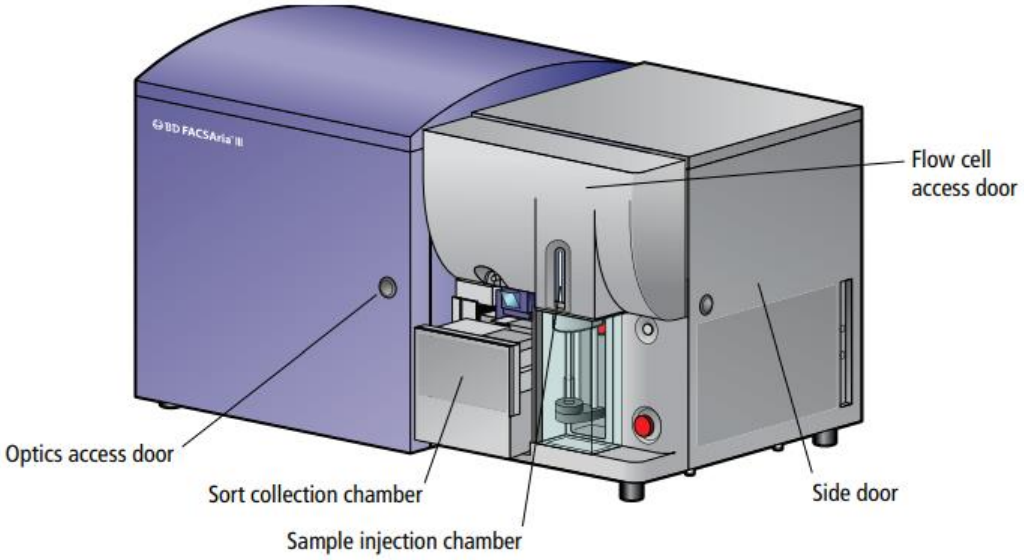
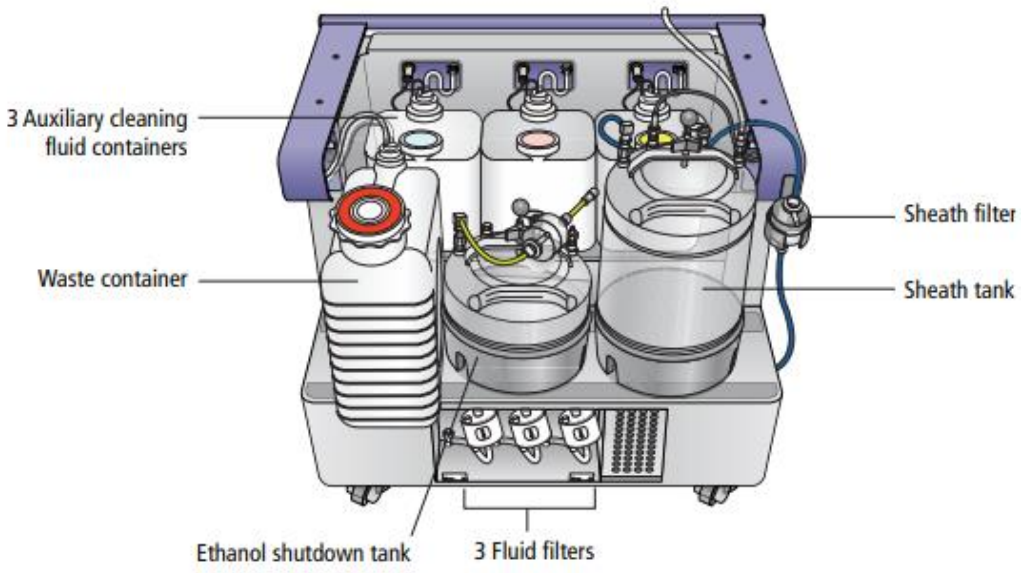
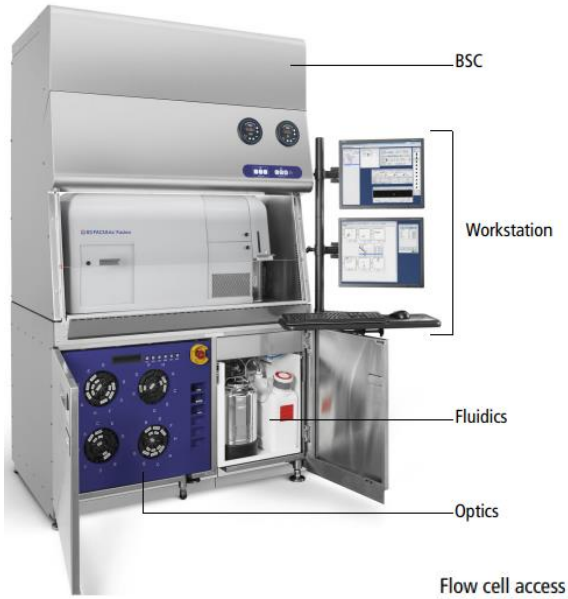
Equipments

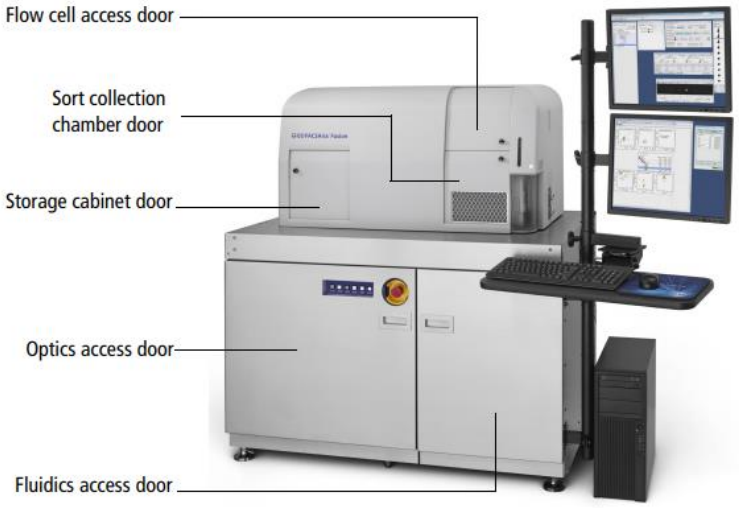
BD FACSAria III

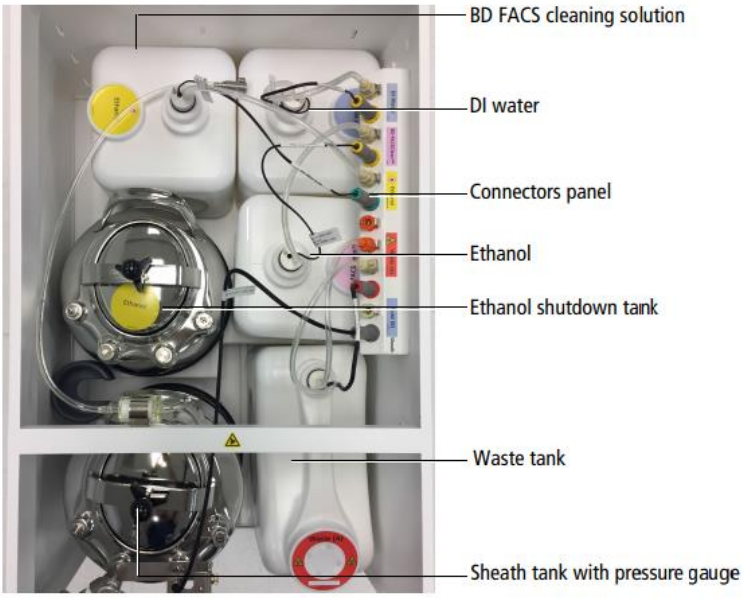




BD FACSAria Fusion







Sterile hood (BD FACSAria Fusion)

The hood has three buttons (Off – Low – High) on the left to control the AMS (Aerosol Management System) to exhaust the aerosols from the area around the collection tube holder. It is recommended to turn ON the AMS if human samples are being processed.

On the right, there are three "Cabinet Control" buttons: the first to switch ON the light, the second to turn ON the airflow of the sterile hood, and the third to pause the alarm (if the window is open).
The light should be switched OFF during acquisition.
The airflow should always be switched ON.
The AMS control should always be in LOW during acquisition (change to HIGH option if there is a clog issue).

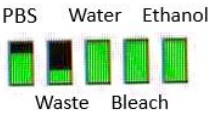
POWER USERS RULES

- The power users can only book their sortings on weekdays and only from 10am onwards.
- Users must have a valid reservation in a system before using it.
- If two cell sorters are already booked, the power user should send an email to the UCF staff to book the third sorter.
- The power users are responsible for performing their own sorting.
- The power users must know how to switch nozzles, calculate the drop delay, adjust for all types of collection tubes, perform the sorting, troubleshoot any issue that may arise and shutdown the sorter.
- The power user needs to show up to the sorter at the booked time for their sorting and insert the correct nozzle, calculate the drop delay, adjust for the correct collection tubes and perform the sorting.
- The power user needs to stop their sorting:
  - 15 minutes prior to the end of their booking to perform the cleaning if there is a user after (not accounting for the time for purity check);
  - 30 minutes prior to the end of their booking to perform the cleaning and shutting down the sorter if there is NO user after (not accounting for the time for purity check).
- Users must refill the sheath tank with PBS and the ethanol at the end of the booked session.
- If the waste container gets full, the container must be replace with a new one.

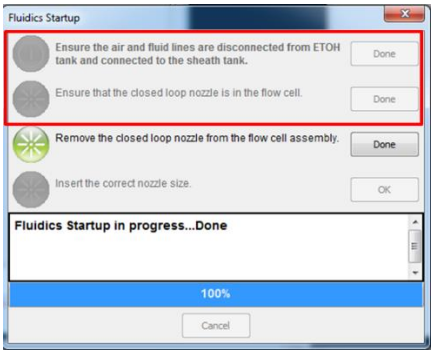
Fluidics Startup, Quality Control and Sorting Setup

- Only for Power Users Level I

1. Switch ON compressed air.
2. Switch ON the computer.
3. Wait a couple of minutes.
4. Switch on the instrument (BD FACSAria III – Green button on the left panel; BD FACS Aria Fusion - green button on the right side of the sterile hood). For FACSAria Fusion, turn ON the airflow of the sterile hood (by clicking on the second button on the right panel). If working with human samples, also turn ON the AMS system, selecting the LOW option (use the HIGH option only if there is a clog issue).
5.
6. Login on Windows. Username: Flow Cytometry User; Password: BDIS
7. Openflow cell access door. (This blocks laser emission, avoiding fixation of PBS crystals to the flow cell walls or burning the flow cell gel, which can be damaging)
8. Launch FACSDiva software.
9. Log in in the Administration account.
10. Wait a couple of minutes while the system connects to the cytometer and select “Use CST Settings”. If the system doesn’t connect, follow the troubleshooting 9 in the Appendix.



11. Check the reagents levels and fill any of the tanks if necessary.
12. Check that the Waste tank is not full. If it is full, change for a new one.
13. Perform Fluidics Startup: **Cytometer Menu > Fluidics Startup**, following the wizard instructions.
    a. Be sure that the sheath tank is filled and the waste tank is empty.
    b. Disconnect the blue fluid line and the transparent air line from the Ethanol tank.
    c. Connect air line to the sheath tank and the fluid line to the filter on the sheath tank
    d. After confirming each step in the software, startup procedure will start.
    (Leave the fluidics cart out until the end of the start up to make sure the tank is properly closed - no air escaping noises).



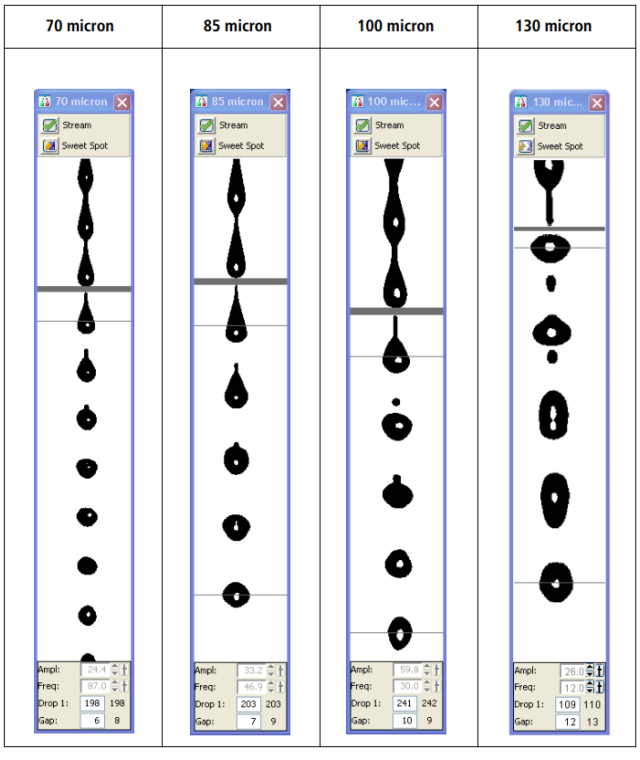
14. Remove the closed loop nozzle from the flow cell.
15. Insert the correct nozzle (red o-ring must point upwards), ensuring the cytometer pressure configuration is appropriate for the nozzle size:
    a. To change the configuration, go to the Instrument **Menu > Cytometer Configuration > Set configuration** to select the correct configuration.

Press OK **> OK** and close the window.

16. Wait for cytometer’s connection and click “Use CST Settings”.
17. Turn ON the stream.
    Open the sorting chamber door and ensure stream is falling in the center of the waste aspirator.If not, use a hexagonal screwdriver to adjust the sorting chamber position so that the stream is aligned with the waste.
18. Close the sorting chamber door.

19. Perform a quick clean cycle of 10 minutes FACS Clean (Flow Rate 6) and 10 minutes dH2O (Flow Rate 11).
20. Close the flow cell access door to open the laser shutter, only then can you see events on the FSC x SSC plot.
21. Check that there are events running while acquiring the FACS Clean to ensure the blue laser is ON and to ensure the machine is not clogged. If no events are showing, follow the Troubleshooting 7 in the Appendix.
22. Before running the quality control beads, ensure the lasers had enough time to warm up (ideally 30 minutes).
23. Evaluate the instrument optical alignment and detector sensitivity by running Quality Control beads – CS&T and/or Rainbow Beads.
24. Close the CS&T menu, wait for the cytometer's connection and click "Use CST Settings".
25. Establishing a stable drop pattern in the breakoff window is one of the most important steps in getting optimal results from the system. Adjust the amplitude until the drop breakoff is approximately in the top third of the Breakoff window.
26. Do not exceed 70 volts. If you cannot achieve a drop breakoff at <70 volts, do the following:
    • Check the flow cell for air bubbles. If you see bubbles, turn the stream off and back on.
    • Make sure the sheath pressure and drop drive frequency are appropriate. See *Table* below for starting values.
    • If the amplitude is <10 volts (except with the 70um nozzle), turn on Attenuation in the Side Stream window

27. Verify that the small satellite droplets are merging with the large droplets. The satellite drops should merge into the drops in 5 satellites or fewer. If the satellites are not merging, you might need to clean the nozzle by sonicating.
28. Enter the actual Drop 1 value as the target in the Drop 1 field.
29. Confirm that the stream is stable and has a suitable profile for sorting, as in the *picture* on the right. Turn on the Sweet Spot when the drop pattern is stable.
30. Open Accudrop Charge Delay Experiment.
31. Turn ON the deflecting plates + voltage + test sort.
32. Confirm that there is no fanning in the side streams. If there is, turn



OFF the stream, clean the voltage plates, the nozzle insertion spot and the sort block chamber.

33. Looking to the Side Stream window display, adjust the micrometer dial on the left side of the flow chamber to obtain the brightest bead spots.
34. If any fanning is observed even after performing step 32, adjust the correction factors for the drop charge applied to the 2nd, 3rd and 4th drops at the bottom of the Side Stream window.
35. Calculate the drop delay; see Appendix 5 "*Calibrating Drop Delay*".
36. Prepare the recipient holder for cell collection (2 falcons, 4 tubes, 96 wells…).
    a. If sorting to a plate, do not forget to install theACDU adapter.
37. Turn the deflecting plate's voltage ON and do a test sort.
38. Confirm that the stream is falling into the acquisition recipients.
    a. If using Eppendorfs, tubes, falcons: while doing the test sort, open the waste drawer, do a quick test sort and check if the drop is falling into the center of the acquisition recipients. Change the aperture values using the voltage sliders of the far left, left, right and far right side streams to make them fall at the center of the recipient.
    b. If using plates or slides, see section "*Align for plates or slides*".
39. Log in to the user's account.
40. Open the Sort Report Excel file and fill the fields accordingly.

Sorting Setup

- Power User Level II

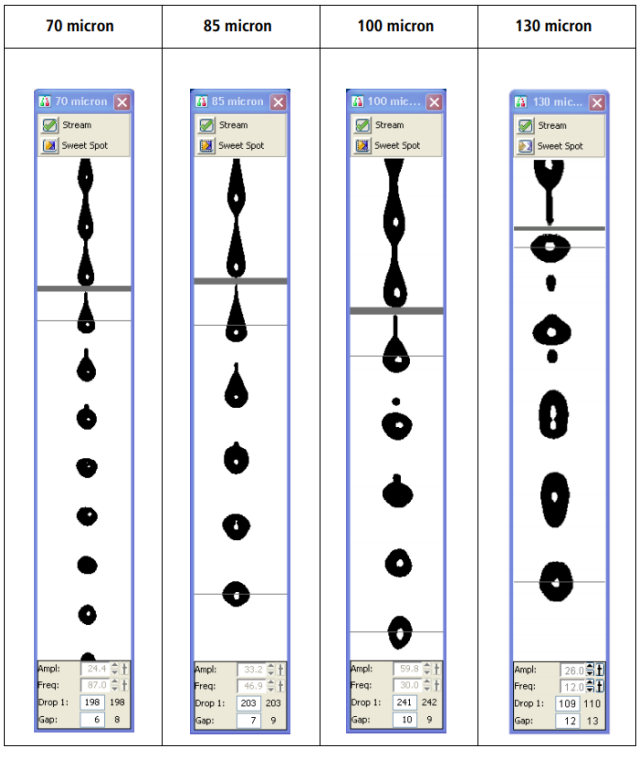
Log in in the Administration account.

1. Check that the Waste tank is not full. If it is full, change for a new one.
2. Insert the correct nozzle (red o-ring must point upwards), ensuring the cytometer pressure configuration is appropriate for the nozzle size:
    b. To change the configuration, go to the Instrument **Menu > Cytometer Configuration > Set configuration** to select the correct configuration.

Press OK **> OK** and close the window.

3. Wait for cytometer's connection and click "Use CST Settings".
4. Turn ON the stream.
   Open the sorting chamber door and ensure stream is falling in the center of the waste aspirator.If not, use a hexagonal screwdriver to adjust the sorting chamber position so that the stream is aligned with the waste.
5. Close the sorting chamber door.

41. Establishing a stable drop pattern in the breakoff window is one of the most important steps in getting optimal results from the system. Adjust the amplitude until the drop breakoff is approximately in the top third of the Breakoff window.
42. Do not exceed 70 volts. If you cannot achieve a drop breakoff at <70 volts, do the following:
    • Check the flow cell for air bubbles. If you see bubbles, turn the stream off and back on.
    • Make sure the sheath pressure and drop drive frequency are appropriate. See *Table* below for starting values.
    • If the amplitude is <10 volts (except with the 70um nozzle), turn on Attenuation in the Side Stream window
43. Verify that the small satellite droplets are merging with the large droplets. The satellite drops should merge into the drops in 5 satellites or fewer. If the satellites are not merging, you might need to clean the nozzle by sonicating.
44. Enter the actual Drop 1 value as the target in the Drop 1 field.
45. Confirm that the stream is stable and has a suitable profile for sorting, as in the *picture* on the right. Turn on the Sweet Spot when the drop pattern is stable.
46. Open Accudrop Charge Delay Experiment.
47. Turn ON the deflecting plates + voltage + test sort.
48. Confirm that there is no fanning in the side streams. If there is, turn



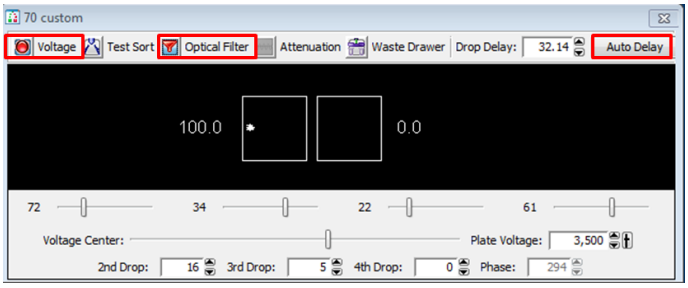
OFF the stream, clean the voltage plates, the nozzle insertion spot and the sort block chamber.

49. Looking to the Side Stream window display, adjust the micrometer dial on the left side of the flow chamber to obtain the brightest bead spots.
50. If any fanning is observed even after performing step 32, adjust the correction factors for the drop charge applied to the 2nd, 3rd and 4th drops at the bottom of the Side Stream window.
51. Calculate the drop delay; see Appendix 5 "*Calibrating Drop Delay*".
52. Prepare the recipient holder for cell collection (2 falcons, 4 tubes, 96 wells…).
    b. If sorting to a plate, do not forget to install theACDU adapter.
53. Turn the deflecting plate's voltage ON and do a test sort.
54. Confirm that the stream is falling into the acquisition recipients.
    c. If using Eppendorfs, tubes, falcons: while doing the test sort, open the waste drawer, do a quick test sort and check if the drop is falling into the center of the acquisition recipients. Change the aperture values using the voltage sliders of the far left, left, right and far right side streams to make them fall at the center of the recipient.
    d. If using plates or slides, see section "*Align for plates or slides*".
55. Log in to the user's account.
56. Open the Sort Report Excel file and fill the fields accordingly.

Calibrating Drop Delay

1. Open the "Accudrops Drop Delay" experiment in FACSDiva, and select the tube called "Drop Delay".
2. Prepare a tube of Accudrop beads (ideally 1 drop per 600 μL for the 70 μm nozzle; or 1 drop per 1 mL for the 100 μm nozzle) and install it on the loading port.
3. Open the Sort Layout named "Drop Delay".
4. Load the tube containing the Accudrop bead suspension.
5. Adjust the flow rate to achieve an event rate of 1000–3000 events per second for the 70 μm nozzle; 800–2000 events per second for the 85 μm nozzle; or 600–1500 events per second for the 100 μm nozzle.
6. Turn ON the voltage plates and the optical filter. Make sure the intensity value of the central stream is 100%.
7. Click to Sort on the Sort Layout window and choose the option Cancel to avoid the waste drawer to open
8. If needed, adjust the micrometer dial to obtain the brightest bead spot on the center stream.
9. If the left side stream is not completely contained in the left region, adjust the voltage slider to place the stream in the center of the region.
10. Confirm that the sum of the intensity values of the left and central stream is 100%.
    a. If not, confirm the event rate is within the recommended range (see point 5 above);
    b. Adjust the micrometer dialto obtain the brightest bead spots on the left and central streams.
11. Click the Auto Delay button on the Side Stream window.

12. Select Start Run in the Auto Drop Delay dialog.
    a. To calculate the Drop Delay manually, use the precision mode “Initial” for an initial estimate, and at the end use the “Fine Tune” mode to account for very small changes.
13. After the Auto Delay calculation, confirm the Drop Delay value is optimal by increasing and/or decreasing its value in 0.03-drop increments (click arrow control). Ideally, the intensity of the left stream should be as close to 100% as possible, and the central stream close to 0 (the sum of the two values should be 100%).
14. Change the Drop Delay value to 1 drop above and below the optimized value. This should make approximately 100% of the bead brightness to fall into the right region, leaving the left region with approximately 0. Choosing one unit above or below should provide similar results. If not, optimize the Drop Delay value to ensure this happens.
15. IMPORTANT: return to the optimized Drop Delay value and confirm that approximately 100% of the bead brightness is detected on the left region, and 0 on the right region.
16. Unload the tube and it will automatically turn OFF the deflection plates and the optical filter.



Align for plates or slides

1. Install the ACDU adapter under the waste drawer.
2. Open the *Sort Layout* window on the Accudrops Drop Delay Experiment.
3. Click on the *Access Stage* icon on the *Sort Layout* window. The stage will come forward.
4. Set the far left voltage slide to approximately 30, and set the remaining ones to zero. NOTE: Plate sorting is performed using only the far left stream, so the remaining ones should be set to zero.
5. Turn ON the Voltage, do a Test Sort, and open the Waste drawer.
6. Make sure the far left stream is falling within the hole of the ACDU adapter (ideally in the middle).
7. Turn OFF the Voltage and Test Sort, and close the Waste drawer.
8. Go to **Sort > Home Devices** (if the collection device is standard) or **Sort > Custom Devices** (if the collection device is customized).
    8.1. For **standard and custom devices**:
    8.1.1. Select the collection device that will be used.
    8.1.2. Click **Go to Home**. The stage will move to the Home position previously defined as the A1 well (if sorting to a device with wells) or the center of the slide (if sorting to slides).
    8.1.3. Turn ON the Voltage, and double click on the Test Sort icon on the Home Device menu, in order to place a small droplet on the A1 position.
    8.1.4. Make sure the drop is in the center of the well. If it is, click *Set Home* > *Close*. If it isn't, adjust the Home position by clicking on the appropriate arrow buttons to move the tray support as needed. Large arrows move the tray by five-steps. Small arrows move the tray by one-step. Keep adjusting and placing small droplets until the ideal position is reached. When you are done, click *Set Home* > *Close*.

    8.2. For **custom devices**:
    8.2.1. Adjust the position of the last well by clicking *Go To End*. Adjust the *End* position by clicking on the appropriate arrow buttons to move the tray support as needed. Large arrows move the tray by five-steps. Small arrows move the tray by one-step. Keep adjusting and placing small droplets until the ideal position is reached.
    8.2.2. *Click Set End* > Close
9. Turn OFF the Voltage.
10. Open the Sort Layout of the collection device to use. If there isn't one, you can open a Sort Layout and choose the collection device from the list on the top left corner.
11. Load Accudrop bead suspension.
12. Sort 100 beads to:
    12.1.The center of the slide, if sorting into slides
    12.2. Each well of the first and last columns of the plate (A1, B1, …, Z1 and An, Bn, … Zn), if sorting into plates.
13. Check if the bead drops fell in the center of the slide/wells. If not, repeat step 8-13 until it does.

Cell Sorting

1. Under the User's account in FACSDiva, create a new experiment and name it *"[YYYY_MM_DD] [User's name]"*.
2. In the "Cytometer" window, go to the "Parameters" tab and delete all parameters that will not be used in the experiment.
3. If the experiment needs compensation:
    a. Go to **Experiment > Compensation Setup > Create Compensation Controls**.
    b. Select/Unselect the box that asks whether you have an Unstained sample.
    c. Without recording, acquire all single staining controls and check if the positive peak is in scale and that there is good/maximal resolution between the negative and positive populations in the primary detectors.
    d. Adjust the voltage for each detector whenever it would improve resolution of positive from negative populations, avoiding excessive spread of negative populations.
    Note that the voltage of the fluorescence detectors cannot be changed after compensation setup.
    e. Record all single staining samples (and unstained if present).
    f. Make sure you have at least 300 events on the P2 (positive) gate of each single color. Ideally you should have 500 events in P2.
    g. Go to **Experiment > Compensation Setup > Calculate Compensation**

4. Create a new specimen and name it "*Sort [YYYYMMDD]*"
5. Create a new tube and name it "*Presort [sample name]*" (Name the sample tubes ensuring the type of tissue and the treatment or condition allowing the distinction between the total samples of the experiment)
6. Set up the experiment layout.
    a. IMPORTANT: select FSC-H, FSC-W, SSC-H and/or SSC-W to enable doublet exclusion.
7. Label all the channels (e.g. CD8-FITC).
8. If necessary, set up voltages for FSC-A and SSC-A.
9. If necessary, adjust the FSC-A scaling factor and replace the FSC Neutral Density (ND) filter (for larger cells use ND filters with larger numbers and vice-versa).
10. Load the sample to be sorted and record 10,000 events using the Normal Worksheet for each tube, instead of a Global Worksheet. (Eventually record more events if working with rare populations and/or if the researcher asks for it)
11. Confirm the compensation performed automatically, checking all the colors in the sample. Adjust manually if needed.
12. Establish the gating strategy based on the FMO controls, if they are available.
13. Confirm the gating strategy applies well to the sample and define the populations to be sorted.
14. Perform the back gating of all populations of interest through the entire gating hierarchy.
15. Create a sort layout and set it to acquire the desired populations.
    a. In the Sort Layout window, select the type of device from the Device menu.
    b. Select the sort precision mode from the Precision menu.
    c. Regarding the options from the Save Sort Reports menu, select **Save All**.

d. Enter the number of events to be sorted in the Target Events field or add limits to the number of cells to be sorted according to the type of collection tube:

Tubes	Precision mask	Collection volume	Max. number of cells		
			70 µm nozzle	85 µm nozzle	100 µm nozzle
1.5 mL tubes	4-Way Purity	250 µL	1 100 000	420 000	350 000
		500 µL	800 000	320 000	200 000
	0-16-0	250 µL	1 000 000		400 000
		500 µL	800 000		300 000
	Yield	500 µL	400 000		100 000
FACStubes	4-Way Purity	250 µL	3 700 000	1 700 000	1 200 000
		500 µL	3 500 000	1 400 000	1 000 000
		1000 µL	3 000 000	1 200 000	800 000
	0-16-0	250 µL	4 000 000	1 600 000	1 000 000
		500 µL	3 500 000		800 000
		1000 µL	3 000 000		600 000
	Yield	1000 µL	1 700 000		500 000
15 mL tubes	4-Way Purity	500 µL	12 000 000		4 100 000
		1000 µL	11 000 000	5 200 000	3 750 000
		2000 µL	10 000 000	4 800 000	3 500 000
		3000 µL	9 000 000	4 400 000	3 200 000
	0-16-0	500 µL	12 000 000	5 000 000	3 500 000
		2000 µL		4 200 000	3 200 000
		3000 µL			3 000 000
	Yield	1000 µL	6 000 000		2 000 000

e. Select the field(s) corresponding to the tube(s), well(s), or spot(s) where the population will be sorted and select a defined population from the Add menu.

16. Start sorting.
17. Throughout the sorting, ensure:
    a. There is a low number in the Events to Display tab, to quickly see any variation while acquiring the sample (e.g. variations in SSC-A and FSC-A pattern)
    b. The stream remains stable (look like at the beginning of the sort and the Drop 1 value is similar at the beginning). If not stop the sorting, adjust the stream and check the Drop Delay.
    c. That there is no fanning.
    d. The number of events is stable. If not unload the tube and agitate the sample.
    e. Sort efficiency (>90%). If not filter, dilute, vortex the sample or decrease flow rate.
    f. That the populations fall inside the gates that were set at the beginning.
    g. The collection and sorting temperatures are as desired
18. Write down the number of collected cells for each sample in the Sort Report file.
19. Check purity of the sorted samples (see section “Purity Check”) and write it down in the Sort Report.

20. Export the Experiment form the Software to the folder *D:\Backup por Unidade\year\semester\PI*
21. Complete all the information requested in the Sort Report file, save it and export as PDF to the folder *C:\Users\Admin\Desktop\AriaIII* or *C:\Users\Admin\Desktop\AriaFusion.*
22. Send an email with the Sort Report (and the Sort Layouts, if the researcher asks for it).

Purity Check

1. On the software, duplicate the sorting tube and name it "*Purity [tube name]_[population name]*"
2. Run FACSClean (flow rate 6) for 1 minute and Water (flow rate 11) for 1 minute before purity check.
3. Run the sorted sample at flow rate 1, and record 200 events inside the first gate (usually "cells").
    a. The percentage of events of the population of interest is expected to be higher than 95% when cell sorting was performed with a sort precision of Purity or higher.
4. Clean with water for 1 minute in between purity tubes.
5. In the sort report file, write all the purities in the purity check well for all the samples. (If the sorted population has <5000 cells, we only check the purity if the researcher asks to)

After Sorting

1. Run FACSClean for 10 minutes (flow rate 6.0).
2. Run water for 5 minutes (flow rate 11.0).
3. Switch off the stream.
4. Remove the Nozzle from the flow cell assembly.
5. Insert the "old" Nozzle into the flow cell assembly.
6. Clean flow cell with FACS Clean (3x) and water (5x), especially if using viability dyes.
7. Shutdown the Cytometer.
   (See next Section for the complete shutdown and other cleaning procedures)

Cleaning, and shutdown procedures

To ensure a proper performance of the instrument, and to establish a regular cleaning, the power users should do the recommended cleaning procedures below.

Cleaning the flow cell

Summary:

Cleans the sample path and the flow cell.

Frequency:

As a daily basis, it is recommended to run a clean flow cell with water in the beginning of the sorting/analysis and with FACSClean and water in the end, before the shutdown.

It can also be performed whenever the equipment shows poor optical performance – distorted scatter or high CVs.

Sample line backflush

Summary:

Flushes the sample line with sheath fluid.

Frequency:

After running samples with adherent cells or dyes.

When a clog happens

Fluidics Shutdown

Summary:

Removes sheath fluid from the lines and fills them with 70% Ethanol.

Frequency:

After finishing the sorting/analysis, in the end of the day.

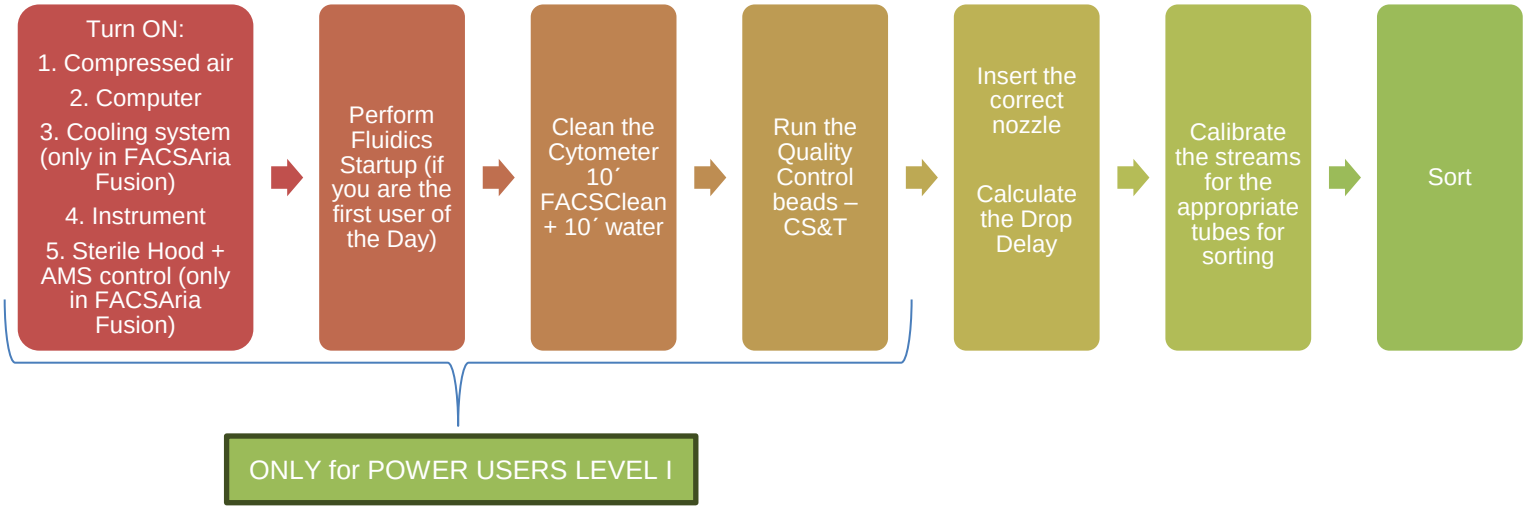
1. Unload sample tube, if one is loaded.
2. Open the flow cell access door.
3. On the FACSDiva breakoff window, turn the stream OFF.
4. Remove the nozzle from the flow cell assembly.
5. Insert the integrated closed-loop nozzle into the flow cell assembly
6. Clean flow cell with FACSClean (3x) and water (5x),especially if using viability dyes).
7. Go to the menu **Cytometer > Fluidics Shutdown** and follow the indications provided by the software.
8. Be sure that the ethanol tank is filled.
9. Disconnect the blue fluid line (without the filter) and the transparent air line from the sheath tank.
10. Connect both lines to the ethanol tank (respective ethanol filter).
11. After confirming each step in the software, shutdown procedure will start.
12. At the end, you are prompted to "*Install a tube with cleaning solution on the loading* port, and click OK". Prepare a new tube of RINSE, install it and select OK.
13. Exit FACSDiva Software and switch off the computer.
14. Switch OFF the instrument (green button).
15. Wipe down all cytometer surfaces that have been exposed to sheath fluid. Clean surfaces with a cloth dampened with 5% bleach solution or 70% ethanol, followed by

water. (The following surfaces should be inspected and cleaned when necessary: Sort chamber; Deflection plates; Sample loading port; Collection devices)

16. Switch OFF the hood controls.
17. Switch OFF cooling system (only in FACSAria Fusion)
18. Switch OFF compressed air.
19. Check if the waste tank is getting full. If so, swap it by an empty one.
20. Vent the air pressure from the sheath tank by pulling up on the ring on the pressure relief valve. Fill the sheath tank to the line with PBS (do not fill above the line).
21. The ethanol tank is refilled every second day. If it is a fill day, fill it.

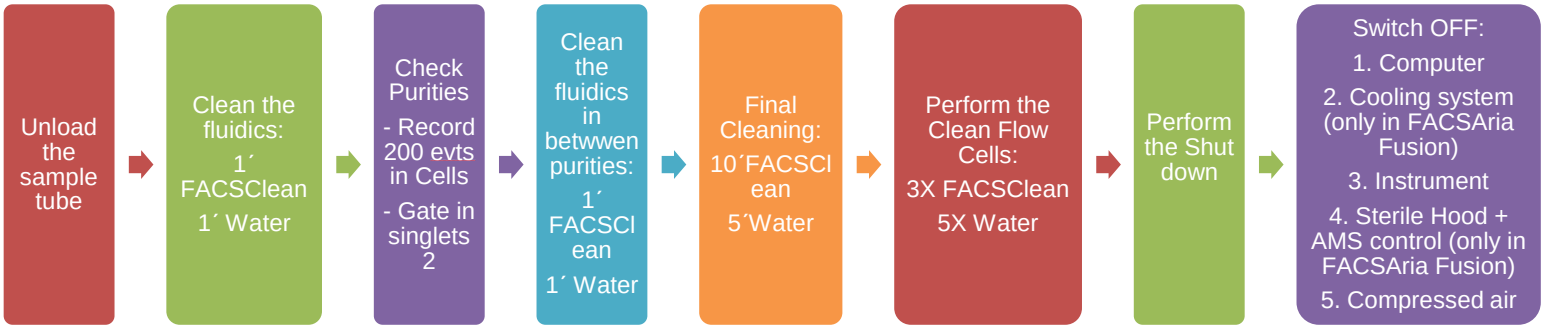
APPENDIX

1. How to Start a Sorting:

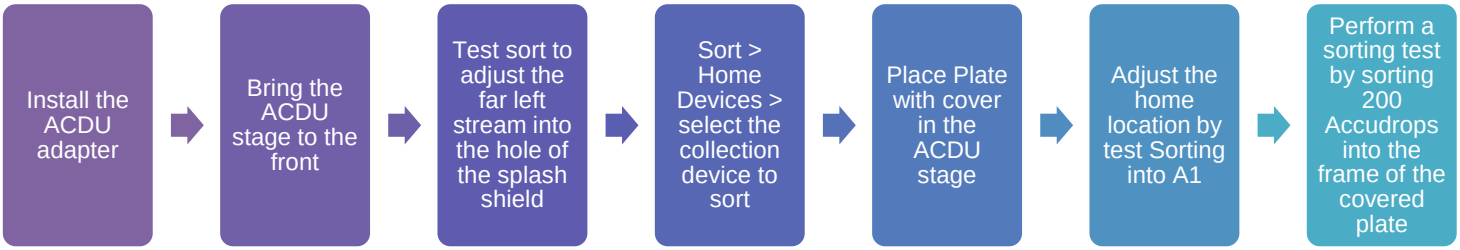


2. During sorting:

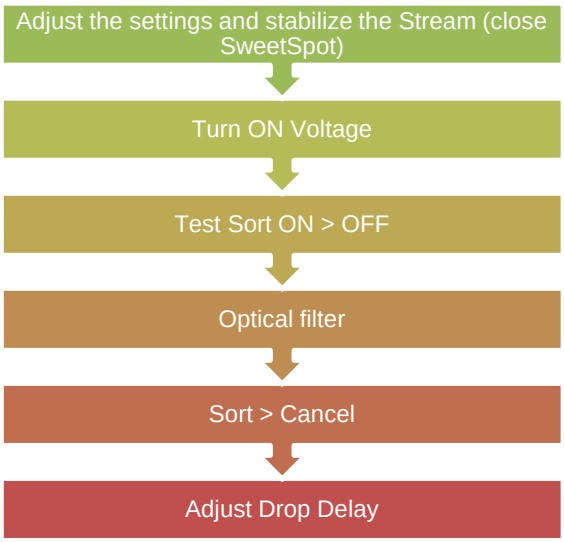
3. How to Finish a Sorting:



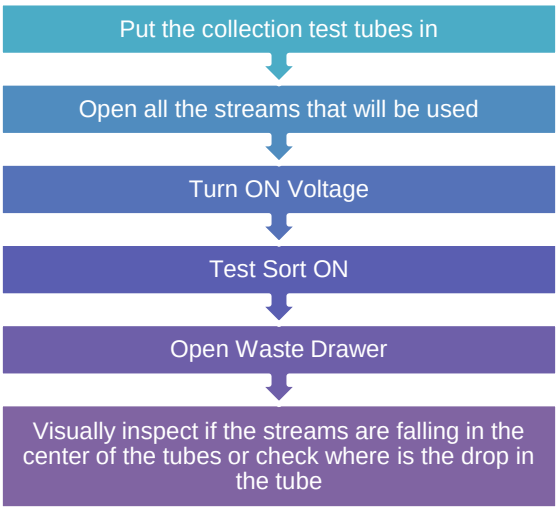
4. Plate Sorting:



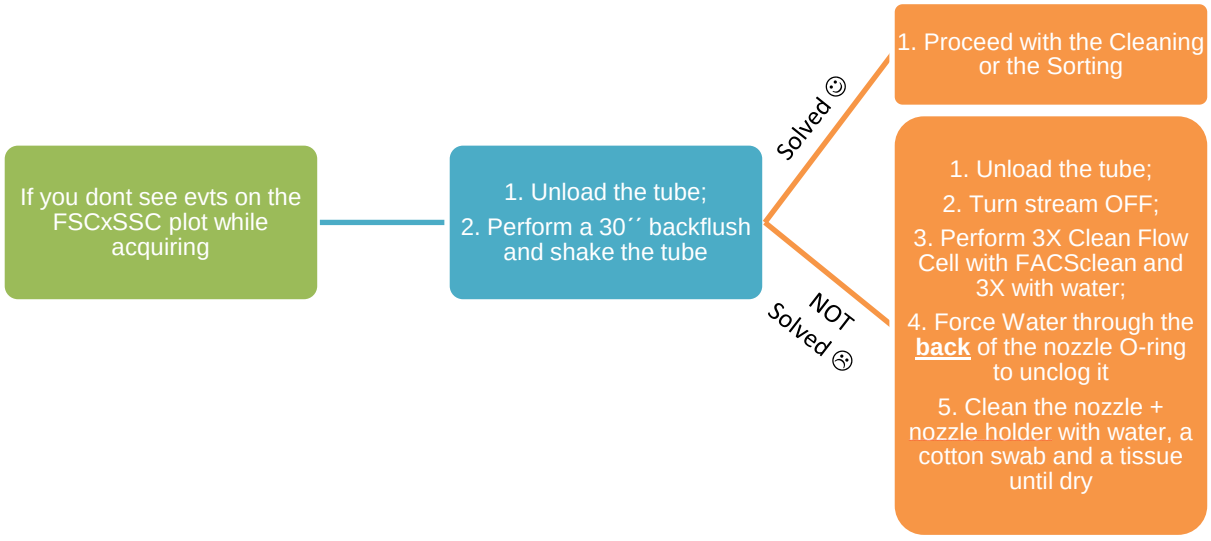
5. How to calibrate the Drop Delay:



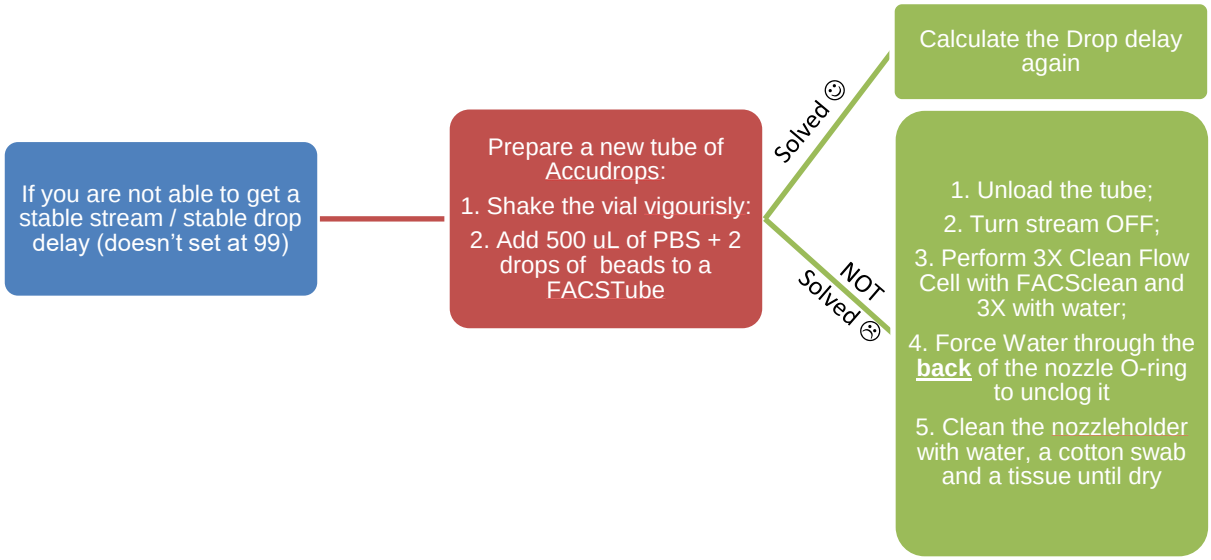
6. How to calibrate into Tubes:



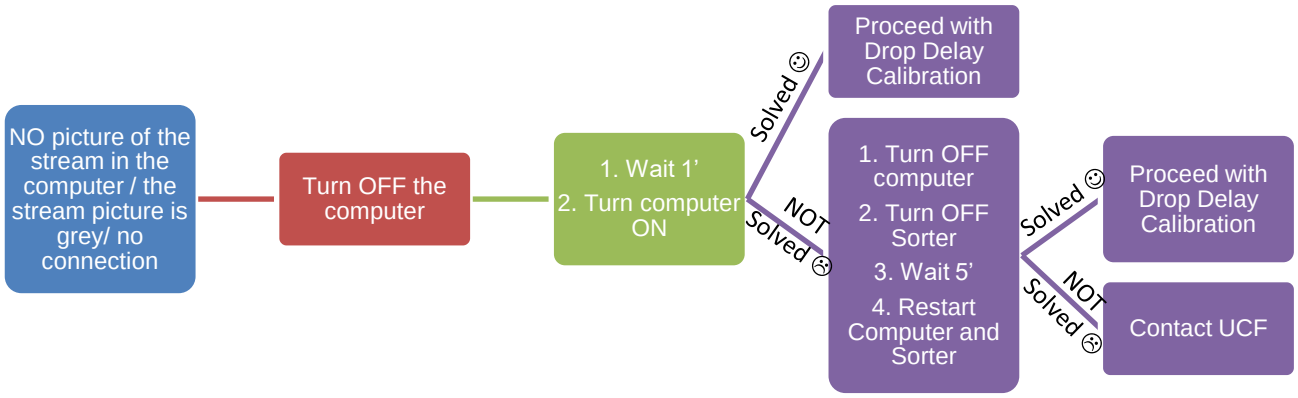
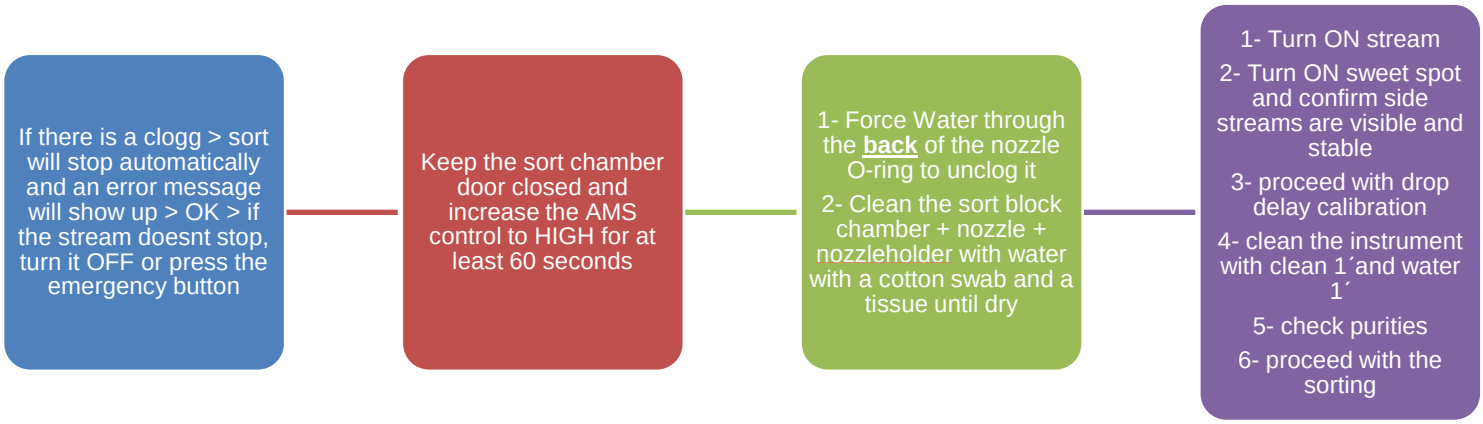
7. Troubleshooting: if you do not see events on the FSC x SSC plot while acquiring:



8. Troubleshooting: If you are not able to get a stable stream / stable drop delay (doesn't set at 99):



9. If you are not able to get a picture of the stream in the computer / the stream picture is grey/ cannot connect the cytometer:



10. Troubleshooting: If there is a clogg: