BD FACS CantoII Analyzer
SOP

A - Pre-Inspection

1. Turn ON the air pressure by turning the blue valve towards the "+" symbol until the valve stops.
    a. *Do not tighten the valve as it can jam.*
    b. *Open fully the air pressure valve; partial opening can lead to incomplete/suboptimal system pressurization.*
2. <u>If you are planning to run a full 96 well plate</u>, inspect the fluidics cart:
    a. If the waste tank is almost full, change it with a new empty waste cubitainer box. Empty boxes are kept on the shelf at the C0311 entrance.
    b. If the sheath fluid tank is almost empty, replace it with a new full BD FACSFlow Sheath Tank. New Sheath cubitainer are on the bench on the right after the entrance of C0311.

    Check the **Appendix, 1 - Ordinary Maintenance,** for instructions on how to replace/refill solutions in the fluidics cart.

B – Instrument Startup

1. Turn the FACS CantoII ON by pressing the green power button on the left side of the analyzer (see Figure 1).

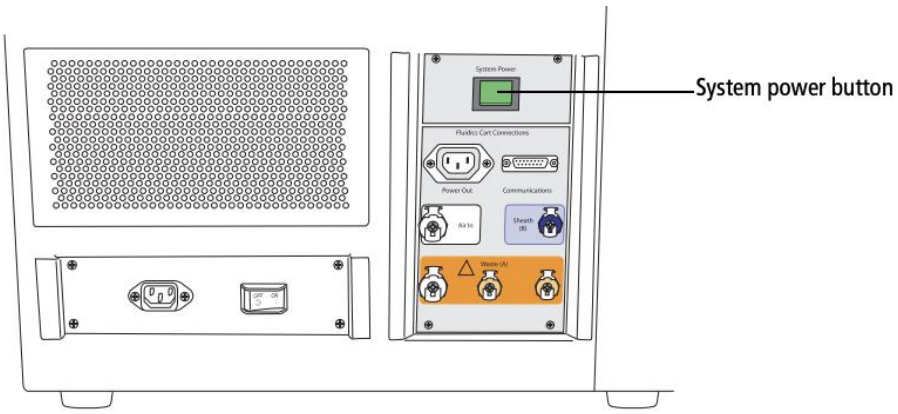

Figure 1 - FACSCantoII Power Panel

2. Turn ON the workstation by pressing the power button on the PC. PC login credentials are public and printed on a card attached to the PC monitor.
3. Launch the BD FACSDiva Software and log on with your private credentials. Allow the software to connect to the main unit.
    a. Whenever the software is connected to the main unit a dialogue box pops up asking which settings to use for the current session: please select "**Use CS&T Settings**".

   b. Once the software is connected to the main unit the **Cytometer Window** will display the status of the fluidics cart at the bottom. Replace/refill any solution if needed (see **Appendix, 1 - Ordinary Maintenance**).
4. After refill/replacement of **FACSFlow Sheath Fluid Tank**, **Shutdown Solution Tank** and/or **Cleaning Solution Tank**, please prime the tanks by selecting **Cytometer > Cleaning Modes > Prime after tank refill** and checking the tank to be primed.
5. Perform a fluidics startup by selecting **Cytometer > Fluidics Startup** and clicking **OK** at the confirmation dialogue box. When the startup finishes, select **OK** to close the dialogue box.
6. Open a Cleaning Template by selecting **Experiment > New Experiment > Cleaning with Tubes**. Use the Cleaning Template to perform a pre-clean:
   a. Run a tube of **BD FACSRinse** at Flow Rate = **High** for **5 minutes**;
   b. Run a tube of **MQ Water** at Flow Rate = **High** for **5 minutes**.

C - Daily QC (Cytometer Setup & Tracking System, CS&T)

1. Access the CS&T module by selecting **Cytometer > CST.** If performance check has already been run the same day, proceed to experiment setup (**Section D**), otherwise:
2. Prepare the CS&T beads by adding 1 drop of vortexed CS&T beads to 350 µl of PBS or FACSFlow Sheath Fluid. Vortex the resuspended beads. Once resuspended, if stored in the fridge **resuspended CS&T beads are stable and can be used for up to 5 days**.
3. In the left side of the CS&T module, select the **current lot ID** of the CS&T beads using the drop-down menu. The current lot ID is specified in the card attached to the PC monitor, and it is also written on the CS&T beads stock vial.
4. Under the task "Check Performance" select the button **Run** to perform the QC. A dialogue message will prompt the user to load the CS&T tube.
5. Load the CS&T tube and click **OK** to start the CST Performance Check.
6. Unload the CS&T tube at the end of the QC. There can be 3 outcomes to a Performance Check:
   a. **Passed**: it is OK to use the cytometer
   b. **Passed with Warnings**: it is OK to use the cytometer, but please always inform the BFC Staff of the CS&T result.
   c. **Failed**: it is not OK to use the cytometer. Please see **Appendix, 2.1 – Failure of Daily QC** for troubleshooting. If troubleshooting fails, please contact BFC staff.

7. Examine the CS&T report if needed, and close the CS&T module to return to FACSDiva. The instrument is ready to be used.

D - Setting Up and Running an Experiment on BD FACSDiva

1. To create a new experiment, select **Experiment > New Experiment**. Select an existing template from your lab tab, or open a **Blank Experiment** from the **General** tab.
2. In the **Browser Window**, click the Specimen icon to create a specimen within the new experiment (see Figure 2).

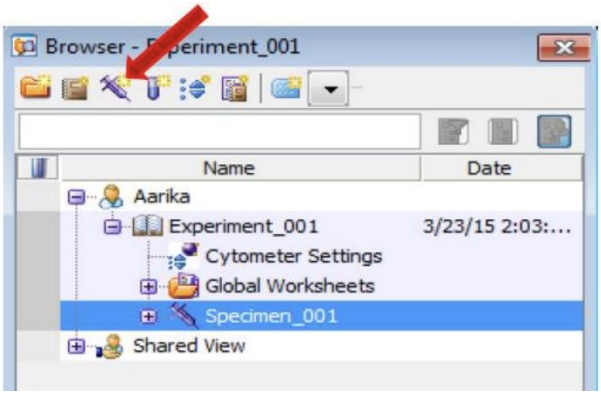

Figure 2 - Browser Window in FACSDiva

3. Expand the Specimen to find the tube, and make it active by clicking on the tube pointer (it turns green).
4. In the **Cytometer Window**, in the Parameters Tab, select all the fluorescence detectors and click **Delete**. Add back only the detectors you will use in your experiment (see Figure 3).

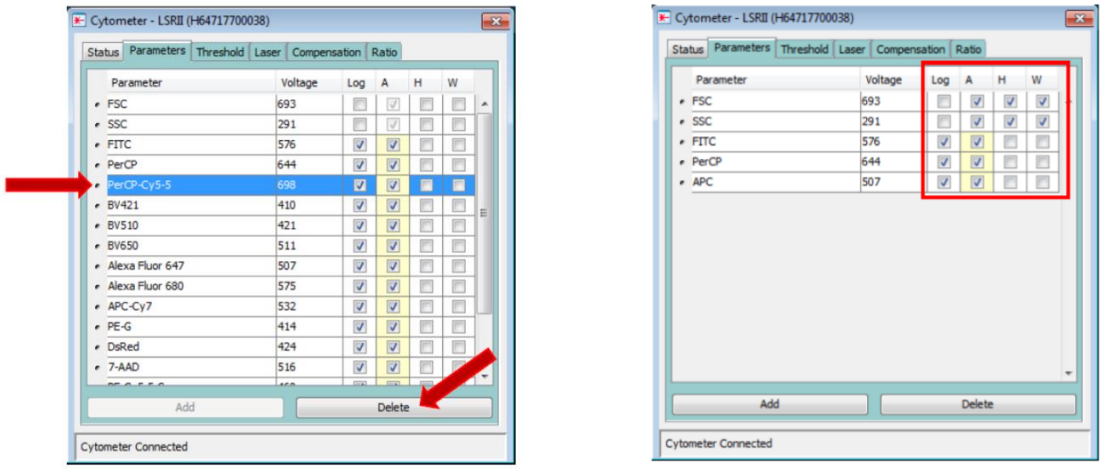

Figure 3 - Cytometer Window in FACSDiva

5. In the **Cytometer Window**, in the Parameters Tab, select to measure also H (Height) and W (Width) for the scattering parameters (FSC & SSC).
6. Optimise PMT voltages if needed (See **Section E – Optimising PMT voltages**)
7. Perform compensation if needed (See **Section F – Calculating compensation**)
8. In the **Worksheet Window**, select **Global Worksheet** and create plots and gates to analyse the samples (see Figure 4).

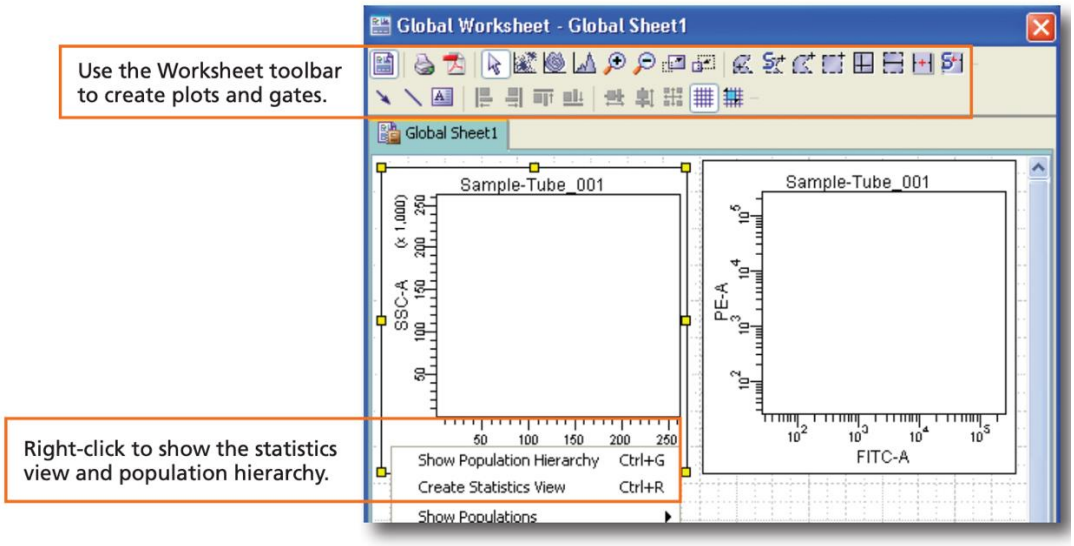

Figure 4 - Worksheet Window in FACSDiva

9. Create a table for the Population Hierarchy by right-clicking on a plot and selecting **Show Population Hierarchy** (see Figure 5).

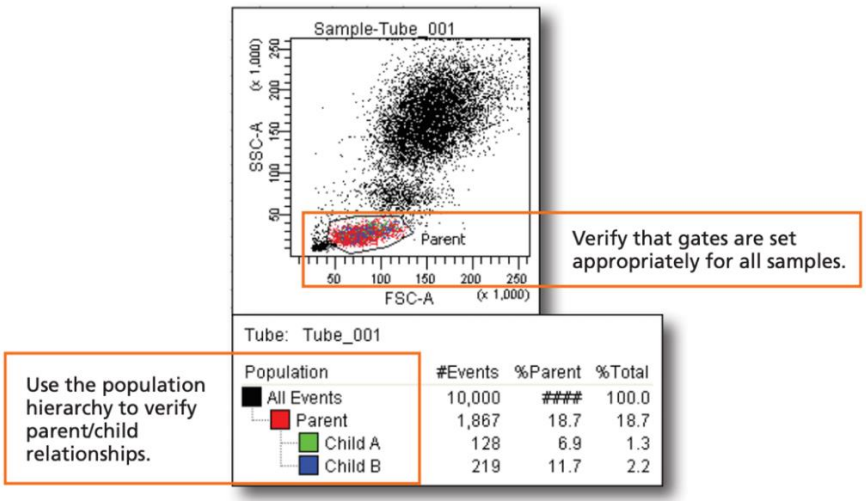

Figure 5 - Prepare plots, gates and population hierarchy in FACSDiva

10. Select **Experiment > Experiment Layout** to assign a reagent label to each fluorescence detector used (see Figure 6).

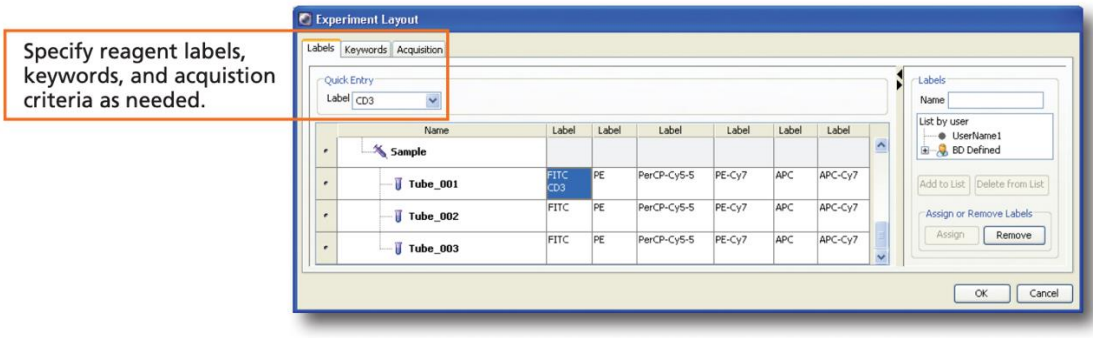

Figure 6 - Experiment Layout Window

11. Use the **Acquisition Dashboard** or the **Experiment Layout** to define the **Stopping Gate**, **Number of Events to Record**, **Storage Gate**. We strongly recommend setting the Storage Gate always to **All Events**, except for LegendPlex and CBA Assays.
12. Acquire & Record Data using the appropriate flow rate: please maintain the **Threshold Rate < 2,500 events/s**, to avoid clogging the instrument.

E - Optimising PMT Voltages

1. In the **Browser Window**, right-click on **Cytometer Settings** and select **Application Settings > Create Worksheet**.
2. Load the **Unstained Control** onto the SIT. Select **Acquire Data** on the **Acquisition Dashboard.**
3. Adjust the cytometer settings (FSC and SSC voltages) to gate the cell population (see Figure 7).

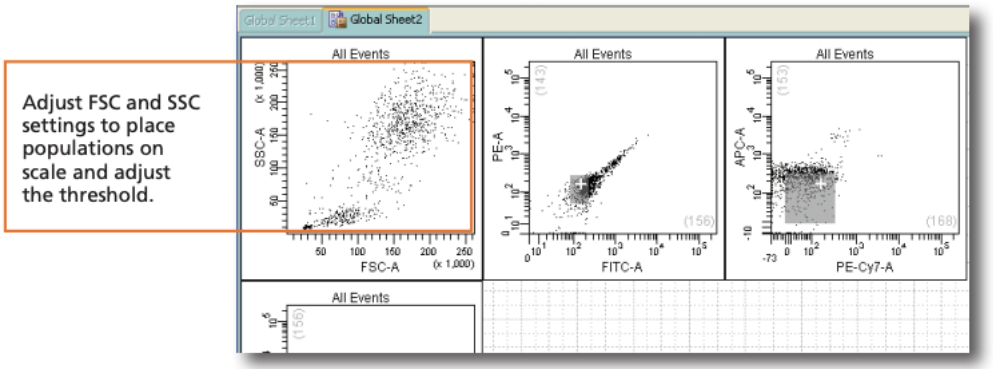

Figure 7 - Optimising PMT voltages for FSC and SSC

4. Adjust the cytometer settings (fluorescence detectors' voltages) so that the fluorescence signal in the Unstained Control is within the **grey box** for each fluorescence detector enabled.
5. Load and acquire **Single-Colour Controls**. Make sure you can see a positive population on scale. Adjust the PMTV for the relevant detector(s) (see Figure 8).

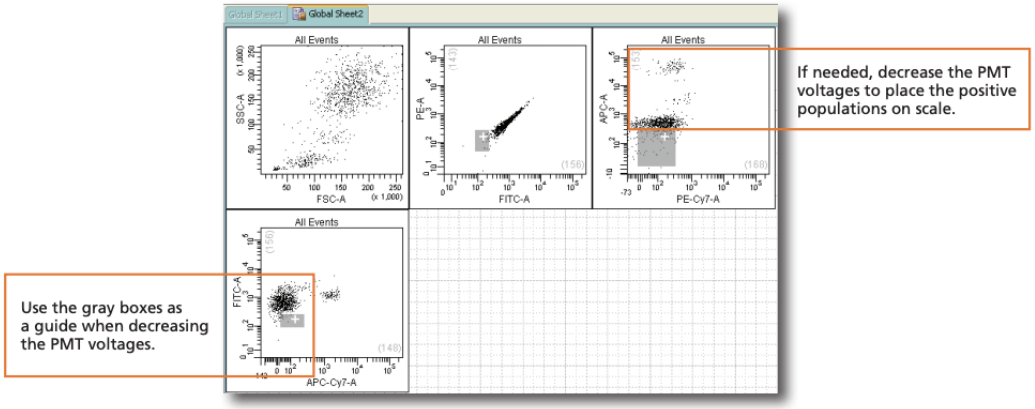

Figure 8 - Optimising PMT voltages for fluorescence detectors

F - Calculating Compensation

1. Compensation should be calculated if the experiment samples are stained with > 1 fluorescent dye.
2. To perform automatic compensation, select **Experiment > Compensation Setup > Create Compensation Controls**. FACSDiva will create a new specimen with tubes for each control (Unstained and Single-Colours) and a corresponding Normal Worksheet for each tube (see Figure 9).
3. Load the **Unstained Control** on the SIT and press **Acquire Data**. Inspect data on the plot and select **Record Data** to record the control.

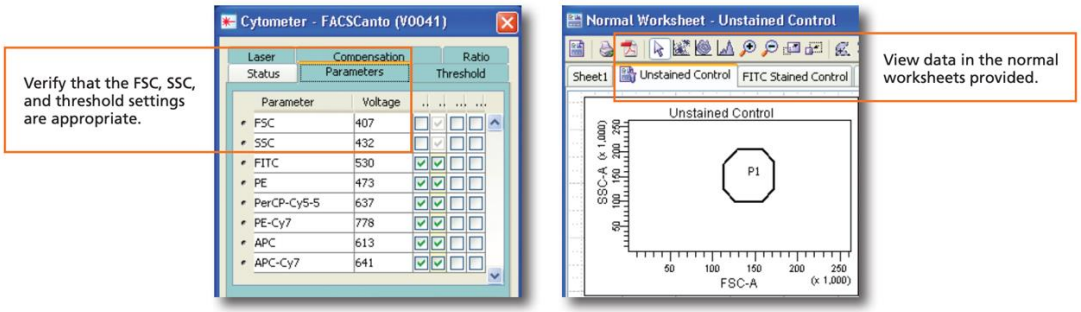

Figure 9 - Creating Compensation Controls in FACSDiva

4. Repeat the same operation as point 3 for the Single Colour Controls. Adjust the P2 gate so that it contains the positive population (see Figure 10).

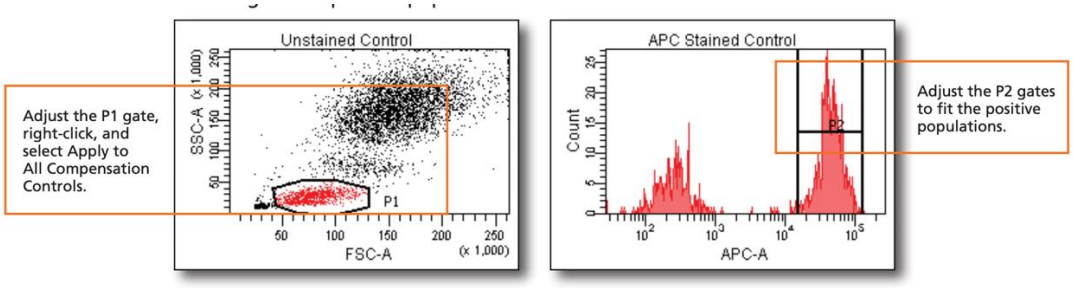

Figure 10 - Run Compensation Controls in FACSDiva

5. Select **Experiment > Compensation Setup > Calculate Compensation** to calculate the compensation matrix. In the dialogue box, rename the compensation if you wish, but choose **Apply Only**. The compensation matrix is applied to the experiment.
6. In the **Cytometer Window**, **Compensation Tab**, make sure the checkbox to enable compensation is applied for the experiment sample tube.

G - Exporting Data

1. To export the **Experiment** file, right-click on the Experiment name and select **Export > Experiment**. Choose whether to save as a folder or zip file, and select the destination folder, then click Save.
2. To export the **FCS files** of each sample, right-click on the Experiment name and select **Export > FCS files**. Select FCS format **3.1**, then the destination folder, and click Save.
3. To export an **Experiment Template**, right-click on the Experiment name and select **Export > Experiment Template**. Select your Lab in the **Type** drop-down menu or if no folder is available for your lab, create a new one by typing your Lab name in that field (we use the following nomenclature: [PI Family Name] Lab. Rename your Experiment Template if needed, and select **Finish**.

H - Post-Experiment Cleaning

1. Open a Cleaning Template by selecting **Experiment > New Experiment > Cleaning with Tubes.** Use the Cleaning Template to perform a post-experiment clean:

   a. Run & Record a tube with **2.5 mL** of **BD FACSClean** at Flow Rate = **High** for **10 minutes**;
   b. Run & Record a tube with **2.5 mL** of **BD FACSRinse** at Flow Rate = **High** for **10 minutes**;
   c. Run & Record a tube with **2.5 mL** of **MQ Water** at Flow Rate = **High** for **10 minutes**.

2. If another user is booked after you in the same day, log out from FACSDiva by selecting **File > Log Out** and choosing **Log out without fluidics shutdown** when prompted by the dialogue box.
   If no one else is booked after you, perform a Shutdown (See **Section I - Shutting Down the System**).

I – Instrument Shutdown

1. Perform a fluidics shutdown by selecting **Cytometer > Fluidics Shutdown.**
2. When the task is complete, click **OK** on the dialogue box.
3. Quit FACSDiva by selecting **File > Quit DIVA**
4. Turn off the PC.
5. Turn off the FACS CantoII by pressing the green power button on its left side.
6. Close the air pressure valve by turning it to the "-" icon until it stops, without tightening the valve.

Appendix

1 – Ordinary Maintenance

1.1 - Fluidics Cart

The fluidics cart contains 4 tanks (Figure 11):

- **BD FACSFlow Sheath Fluid Tank** (20L tank on the front right)
- **Waste Tank** (20L tank on the front left)
- **Cleaning Solution Tank** (5L tank on the back right)
- **Shutdown Solution Tank** (5L tank on the back left)

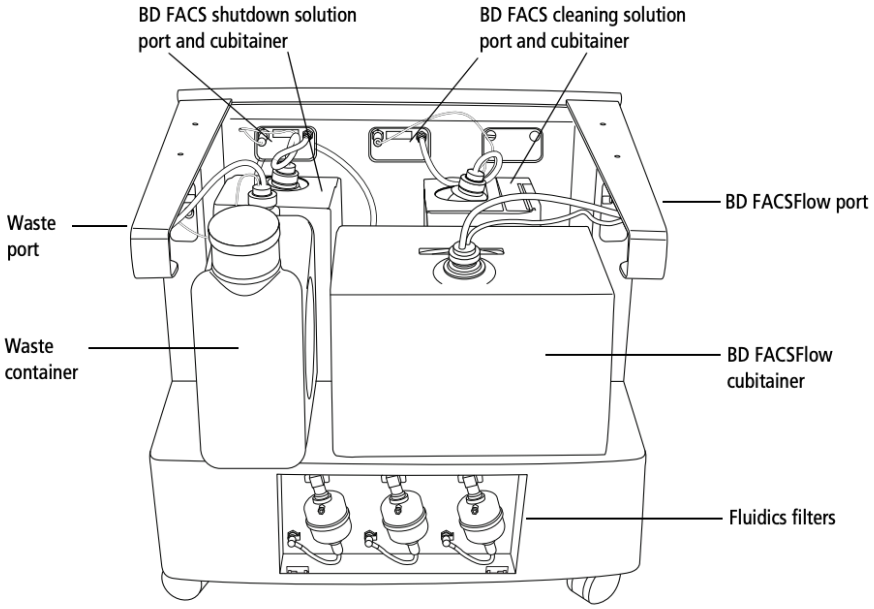

Figure 11- Fluidics Cart

The status of each tank in the fluidics cart can be monitored in FACS Diva, once the software is connected to the analyzer, and it appears as icons at the bottom of the **Cytometer Window** (Figure 12):

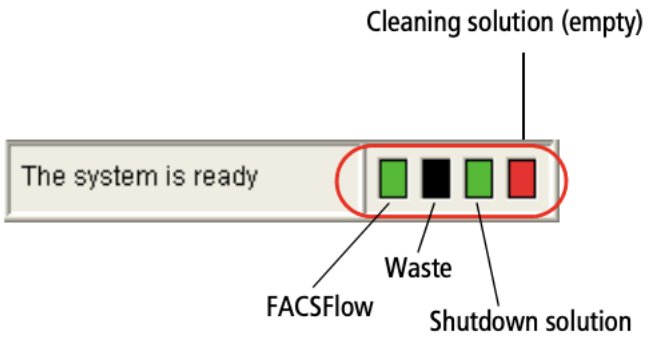

Figure 12 - Fluidics Cart Status in the Cytometer Window, FACSDiva

- A **green** colour of the icon indicates that the tank is OK
- A **red** colour of the icon indicates that the tank must be serviced:
    - Sheath Tank: replace with a new one
    - Waste Tank: replace with a new one
    - Shutdown Solution Tank: refill with MQ water
    - Cleaning Solution Tank: refill with BD FACS Clean

1.2 - Replacing the Waste Tank

When the Waste Tank icon becomes red and FACSDiva gives a dialogue box informing that the Waste Tank is full:

- Unscrew the cap of the Waste Tank and wait a few seconds for the pressure to dissipate
- Remove the cap and sensor assembly and set it aside (see Figure 13).

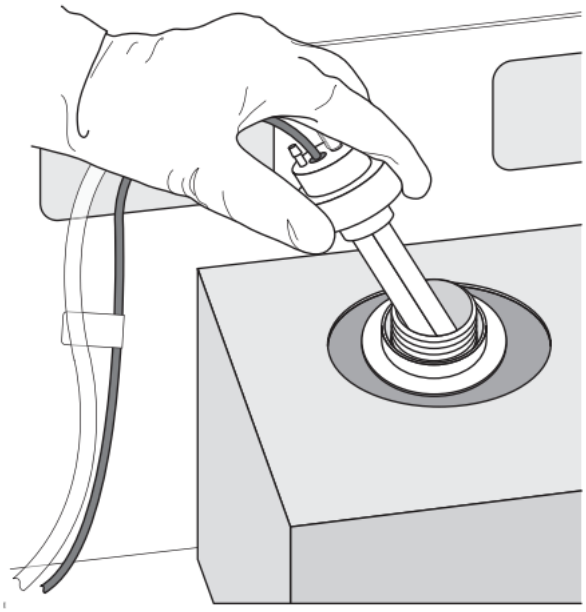

Figure 13 - Removing the cap and sensor assembly

- Close the Waste Tank using a plastic cap and replace it with a new empty cubitainer. *Empty cubitainers (with plastic caps) are stored on the first shelf near the sink and entrance of room C0311*.
- Close the cap and sensor assembly on the new Waste Tank.
- Place the full Waste Tank just removed on the Waste Bench in room C0311.

Do not disconnect the Waste sensor from the Cantoll, as it can lead to issues with its functioning.

1.3 - Replacing the Sheath Fluid Tank

When the Sheath Fluid Tank icon becomes red and FACSDiva gives a dialogue box informing that the Sheath Fluid Tank is empty:

- Unscrew the cap of the Sheath Fluid Tank and wait a few seconds for the pressure to dissipate
- Remove the cap and sensor assembly and set it aside (see Figure 13).
- Close the Sheath Fluid Tank using a plastic cap and replace it with a new cubitainer of BD FACSFlow Sheath Fluid. *New cubitainers are stored on bench beside the sink and entrance of room C0311*.
- Close the cap and sensor assembly on the new Sheath Fluid Tank.
- Place the removed Sheath Fluid Tank with leftover liquid in it on the bench with the full BD FACSFlow Sheath Fluid Tanks and label it as “leftover FACS Flow”.
- On FACSDiva software, prime the sheath line by selecting **Cytometer > Cleaning Modes > Prime After Tank Refill** and checking FACSFlow Sheath Fluid Tank. Click **OK** at the completion message.

Do not disconnect the Sheath Fluid sensor from the CantoII, as it can lead to issues with its functioning.

1.4 - Refilling the Shutdown Solution Tank and the Cleaning Solution Tank

When the Shutdown Solution Tank or the Cleaning Solution Tank icon becomes red and FACSDiva gives a dialogue box informing that the Tank is empty:

- Unscrew the cap of the Tank and wait a few seconds for the pressure to dissipate
- Remove the cap and sensor assembly and set it aside.
- Refill the Tank with the corresponding solution:
    - Autoclaved MQ Water for the Shutdown Solution Tank;
    - BD FACSClean Solution for the Cleaning Solution Tank.
- Close the cap and sensor assembly on the Tank.
- On FACSDiva software, prime the sheath line by selecting **Cytometer > Cleaning Modes > Prime After Tank Refill** and checking the tank(s) that was just refilled. Click **OK** at the completion message.

Do not disconnect the Sheath Fluid sensor from the CantoII, as it can lead to issues with its functioning.

2 – Troubleshooting

2.1 - Failure of Daily QC

When the Daily QC (CS&T Performance Check) fails:

- Check the FACS tube the date in which the CS&T Beads were prepared: if the beads are 4-5 days old, prepare a new FACS tube with CS&T beads and repeat the Daily QC.
- Repeat a pre-cleaning session (FACSRinse for 5 min at high flow rate, MQ Water for 5 min at high flow rate) and then repeat the Daily QC.

If none of the above steps leads to a QC pass, please contact the BFC Staff for further troubleshooting.

2.2 - The FACS tube does not load or fit to the SIT

If the FACS tube does not load into the SIT or does not fit and detaches when the sample is pressurized:

- **Damaged tube**: check the FACS tube for damages/cracks that can lead to failure in pressurization; transfer the sample to a new undamaged FACS tube if you observe any crack;
- **Moist tube**: wipe dry the internal surfaces of the FACS tube that make contact with the SIT at the top (first cm), removing moisture or drops of liquid that can affect the pressurization;
- **Improper tube:** only 12 x 75 mm polystyrene FACS tubes are compatible for use with the CantoII. Example of compatible tubes: https://www.sarstedt.com/en/products/laboratory/reagent-centrifuge-tubes/tubes/product/55.1579/

If none of these seems to be the cause of the issue, please contact BFC Staff for further troubleshooting.

2.3 - No events when acquiring data from a FACS tube

When you don't see any event in plots after loading a sample and selecting Acquire Data:

- **Incorrect Tube Pointer selected**: check that the correct tube pointer has been selected.
- **Damaged Tube**: check the FACS tube for damages/cracks; transfer the sample to a new undamaged FACS tube if you observe any crack.

- **SIT clogged**: prepare a FACS tube with 1 mL of BD FACSClean, load it into the SIT and wash the flow cell by selecting **Cytometer > Cleaning Mode > Clean Flow Cell**. Repeat the procedure with a FACS tube with 1 mL of MQ Water. If the issue persists, contact the BFC Staff for further troubleshooting.
- **Laser(s) switched off**: load and acquire CS&T beads as part of a normal experiment: if no events are detected and the instrument is not clogged, log out from FACSDiva and switch off & re-start the CantoII analyzer. Confirm that you can see events when acquiring CS&T Beads. If not, contact the BFC Staff for further troubleshooting.
- **Incorrect sample preparation**: all cells lost during sample preparation.

2.4 – FACS tube stuck into the SIT / Unable to unload FACS Tube

This issue happens when, after pausing/stopping an acquisition and/or recording, the user tries to remove the FACS tube once the aspirator arm is moving up again, resulting in the aspirator arm remaining "freezed" in the load position.

To avoid the issue: please unload the FACS tube immediately after the aspirator arm is lifted down after stopping an acquisition and/or recording.

To solve the issue: select **Cytometer > Cleaning Modes > SIT Flush**. This will induce unloading on the sample, but can also result in backflush of FACSFlow & sample in the tube.

2.5 – Float Switch Error

- **Low or no pressure in the system**: check that the air pressure valve is fully open. Check that the system is pressurized by reading the pressure gauge on the CantoII analyzer.
  If there is no air pressure, switch off the air pressure line and turn ON the CantoII

internal compressor by moving the auxiliary air supply switch to OFF position (see Figure 14). **Immediately inform the BFC staff.**

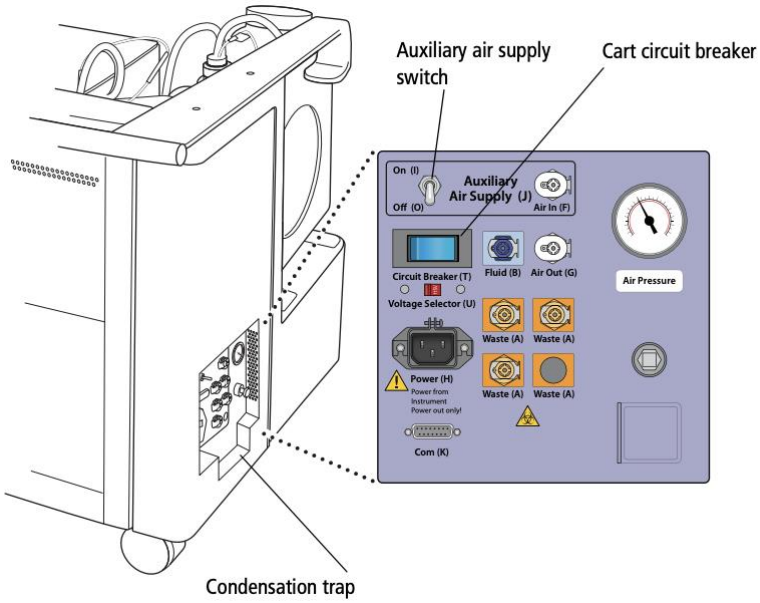

Figure 14 - Fluidics Cart Panel

- **Air in the fluidics lines**:
    a. Check the FACSFlow Sheath Fluid Tank and the Shutdown Solution Tank, and refill/replace if necessary.
    b. Prime the fluidics line by selecting **Cytometer > Cleaning Modes > Prime After Tank Refill** and tick the checkboxes for FACSFlow and Shutdown Solution.
    c. Loosen the **top bleeder valve** of the FACSFlow Filter (see Figure 15), located at the bottom of the fluidics cart, just below the FACSFlow Sheath Fluid Tank to release trapped air. Close the bleeder valve as soon as liquid starts bleeding out of the filter.

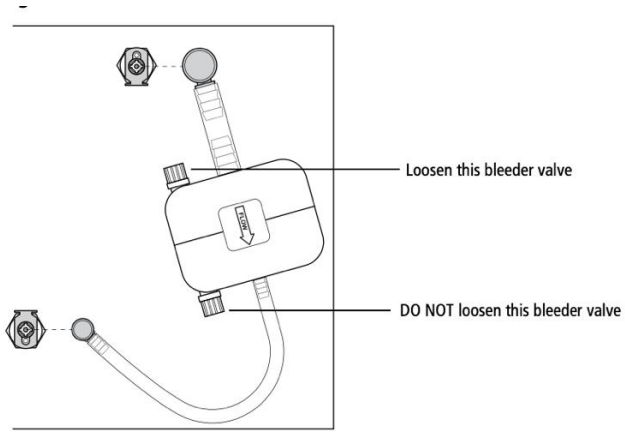

Figure 15 - Fluidics Filter

d. Perform a Fluidics Startup by selecting **Cytometer > Fluidics Startup**.
e. During the startup, monitor the FACSFlow Filter and the bleeder valve. Close the bleeder valve once you observe leakage of FACSFlow, that indicates successful removal of air from the filter.
   If you don't see any liquid leaking from the bleeder valve, contact **immediately** the BFC Staff.

If these procedures do not solve the issue, please contact the BFC Staff for further troubleshooting.