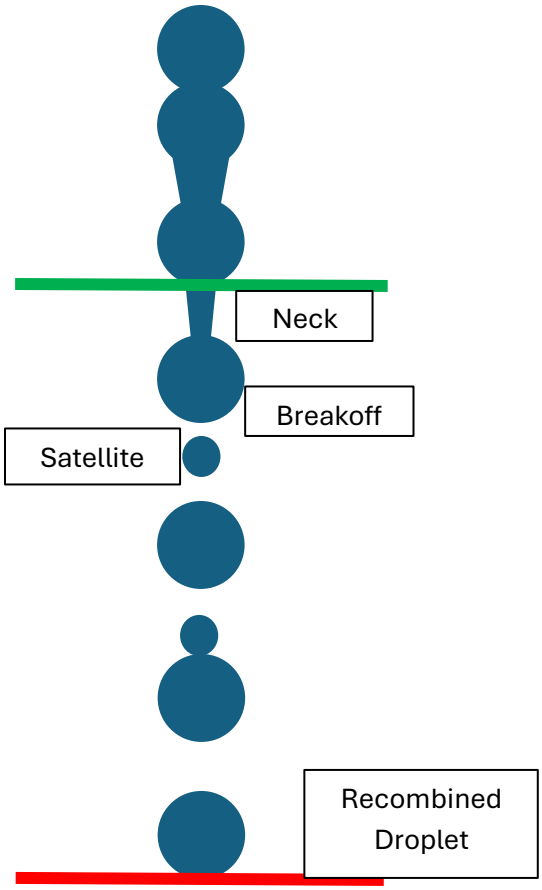
CytoFlex SRT User Guide

Startup

1. Check sheath fluid the day before to avoid bubbles in the lines.
    a. The waste should be emptied and filled with bleach until the bottom fill line. Ensure that the cap is turned (towards the left if the cap side is facing you) so there is enough slack for the tubes to be able to reach their ports. Also mind the wet connectors, as they have trace amounts of unbleached waste on them. No liquids should come into contact with the electrical wire connection, as this will disable the probe.
    b. The shutdown solution is located on the desk in the back left portion of the room. Fill if it is almost empty.
    c. Sheath recipe:
    4L sheath = 8mL HEPES + 200mL 20x PBS + 3792mL diH2O
2. Log into software, turn on biohood, turn on chiller, and check Wi-Fi connection.
    a. Un:
    b. Pw:
3. Turn on the chiller.
4. Spray the sort chamber and block with 70% ethanol to clean it and dry any excess ethanol. Inspect deflection plates and camera windows and clean if necessary.
5. Go to "*Cytometer*" in the menu at the top and select "*Turn On*".
    a. Do not turn on the machine unless the 'hat' is on the machine and the Biosafety cabinet is on, otherwise the machine will overheat.
6. In the bottom right corner, select "*Sorter Status*", then select "M*ore Details*" in the pop-up window.
7. In the "*Acquisitions*" panel or under the "*Cytometer*" menu, select "*System Start Up*". This should take about 15 minutes.
8. Insert the nozzle securely into the machine and hit "*Next*" in the dialog box
    a. Check the nozzle for salt build up and that the o-ring is undamaged and properly seated.
        i. If there is salt/a broken o-ring, see Troubleshooting below.
    b. Ensure the nozzle is firmly in place, it will 'click'.
9. Watch the machine to ensure pressurization/proper stream flow.
    a. If the machine fails to pressurize, check all connections on sheath tank. If they are tight, then reseat the lid of the tank and try again.
        i. You may have to reseat the lid more than once to retain pressurization. The lid should be firmly tightened with a roughly even view of the black o-ring around all sides of it.

b. The machine may leak if the stream becomes misaligned. This will almost always occur around minute 7-8 of the startup procedure. Always watch the sort chamber during this time and stop the stream immediately if you see sheath leaking off the illuminator. If the leak persists unchecked, you can flood the instrument. Clean (using 70% Ethanol) and dry the nozzle, nozzle lift, deflection plates, and sort chamber immediately after detecting a leak, then try startup again.
    i. NOTE: Use the can of compressed air to clear the loose flaps around the base of each deflection plate. Sheath has a tendency to get behind this area and will cause the voltage to fail if left alone.

10. The dialog box will ask if you want to do a QC standardization, select "*Yes*".
    a. Prepare beads.
        i. Shake the bottle of beads vigorously to resuspend them.
        ii. Put 3 drops of bead solution into a labeled FACS tube.
        iii. Check the lot number of the beads on the bottle and select the same lot from the screen dropdown menu (if the lot is expired, proceed anyway).
        iv. Select "*Start*".
11. If QC fails, see TROUBLESHOOTING below.
12. After QC is complete, you will be prompted to start a "Sort Calibration". You can select yes and do it now, or you can wait and unload the QC beads.
    a. If you select "*No*" you can find this again by going to "*Sorting*" in the menu at the top and selecting "*Sort Calibration*". This process is automated and takes about 5 minutes.
    b. If the calibration fails, see TROUBLESHOOTING below.
13. Once "*Sort Calibration*" passes, move the red guideline on the stream to first drop after the satellite drops reform.



a. If the stream becomes unstable, the machine will stop sorting. This can be caused by vibration, changes in temperature and pressure, and by air being sucked into the system.
    i. Keep the door to the lab firmly closed during sorting whenever possible and do not bump into the instrumentation, especially the wetcart.
b. You will need to re-run "*Sort Calibration*" during your experiment if it stops maintaining the stream. Make sure to readjust the red line after recalibrating.

14. Once QC and Sort Calibration have passed, the machine is ready to sort.

Experiment Setup

1. From the top left of the menu, select "*File*", then "*New Experiment*" or "*File*" > "*Open Experiment*" to copy a previous experiment file.
    a. Rename as: '[First Initial][Last Initial]_YYYYMMDD'
2. Under the "*Settings*" menu, select "*Select Channel*". Apply your labels to the appropriate channel.

3. Select the plot icon from the top menu. Click on it 3 times to make 3 plots, and set each plot as follows:
    i. SSC-A vs. FSC-A
    ii. SSC-H vs. SSC-A
    iii. FSC-H vs. FSC-A
    b. Create additional graphs based on the fluorophores/channels required by the panel. Make one additional graph per color and set the X-axis of each graph to be one color, so each fluorophore is represented.
        i. These graphs will be used for validating if your fluorophores are on scale, and later for compensation. They can then be repurposed or deleted once compensation has been completed.
    c. Add a population hierarchy graph to your page.
    d. Open "*Aquisition Settings*" from the leftmost panel.
        i. For new experiments: from "*Aquisition Settings*", select the "*Q/C Gains*" option. Your Gains will change to reflect the values calculated during QC.
4. "*Run*" a small amount of your fully stained sample.
    a. Do not "*Record*", only "*Run*". Recording will not allow you to adjust your Gains.
    b. Keep the Flow Rate to a speed of 10.
    c. "*Run*" for around 1,000-2,000 total events, or until you have an accurate idea of your samples fluorescent profile.
        i. If the fluorescence is off scale, raise/lower the corresponding Gain to ensure your data is within view. Remember that the SRT has a hidden 7^{th} log.
5. "*Stop*" running as soon as you are satisfied with your scale. The data from your run will be saved unless you manually overwrite it.
    a. Set up a gate on the target population, using the SSC-A vs. FSC-A graph as a guide, along with user input.
        i. Gates should have a margin around the target population; excess cells can be removed by further gating.
        ii. Adjust the graph area scaling by selecting the "*Hand*" tool. Use the hand tool with a line to adjust only the lower scale axis.
6. Select the FSC-H vs. FSC-A graph. This will be used for doublet discrimination.
    a. At the top of the graph, select "*Show P1*".
    b. Gate for doublet discrimination, ensuring you gate tightly on the FSC-A side, as the doublets will be present on the area-axis.
7. Select the SSC-H vs. SSC-A graph to refine your doublet discrimination.

a. At the top of the graph, select "*Show P2*".
b. Gate for doublets, hugging the SSC-A axis.

8. To Manually Compensate:
    a. Select "*Duplicate Without Data*" or "*New Tube*" to make another Tube.
        i. Load the first single color control into the SRT.
        ii. Name the new tube as desired by double clicking on the Tube name.
        iii. Select "*Record*".
        iv. Select "P3" for all compensation plots.
        v. Select the color of the currently running tube on the Y-axis for all compensation plots.
        vi. After recording sufficient data, manually adjust the data using either the Comp Window or Compensation Hand tool, so the positives align perfectly over the negatives.
    b. Repeat step a (i-vi) with each Single Color Control.
        i. When you have compensated all of your colors, make sure you copy your final compensation to all previous Tubes.
9. To Automatically Compensate:
    a. Create a compensation experiment by selecting "File", "*New Compensation*".
    b. Save the file to your folder.
    c. To Set up the experiment, select all the channels corresponding to the colors in your panel requiring the sample type (beads or cells). The unstained negative control tube will also be selected.
    d. Select OK. A compensation experiment is generated
    e. Import the gain settings from your experiment and confirm all tubes generated in the compensation experiment share the same gains.
    f. Record each single-color and negative control and review the plots for each control.
    g. Move the positive gate in the histogram plots so it captures the positive population. Repeat for all compensation tubes.
    h. Select "*Compensation Calculation*" in the Settings menu.
    i. Save and export your calculated matrix.
        i. The matrix will save as a .comp file. This .comp file will only be able to be imported into an experiment file that shares the same channel names.
    j. Open your previously created experiment (From the File menu, choose "*Open Experiment*." It should be listed as your "experiment name".xit.) or a new experiment.

k. To import compensation, select "*Compensation Matrix*" in the Settings menu and select "*Import Compensation Matrix*."
l. Select OK and then verify your Gains are identical to that of the comp experiment.
m. Always double-check the machine compensated correctly.

Sorting

1. To into tubes:
    a. Ensure your gates are finalized from a brief run of your experimental sample. You can always modify gates while sorting.
    b. Place an open collection tube in the attached arm of the instrument. The instrument can sort into epis, FACS tubes, and 15ccs.
    c. If you are using the far left and right positions with epis or FACS tubes, ensure the holder attachment is aligned to the center notch.
    d. Open the Sort window (icon with sample falling into tubes).
        i. Check each box for the position (R1, R2, L1, or L2) where you placed a collection tube. Assign which Gate you would like to sort from, and the initial volume in each tube.
            1. If you have a wide difference in the number of cells you collect in each sorted population, set the largest populations to collect in the centermost positions.
            2. Note: if you use an epi, add in 'dead volume' to the 5mL option, since there is no 1.5mL option. (Ex. if you have 200uL of buffer in a 1.5mL tube, set your volume as being 3.7mL.)
        ii. Select "0" as the target count to collect as many cells as possible. This will prompt a warning message later to remind you to watch the volume of the tubes. Alternatively, you can set a specific target number to be collected for each collection tube.
        iii. Make sure you are in the desired sorting mode: most likely purity mode.
    e. Double-check you are recording enough events. Shoot for at least 30,000 off one of the doublet discriminator gates or farther down the gate hierarchy. You will continue sorting after the recording has finished.
    f. Check Sort Calibration is functioning by looking to see if the circle in the Stream Window is green indicating Auto-maintain is on. If it is not, rerun Sort Cal.
    g. Set the speed to10.
    h. When you have verified the above, select "*Sort*".

    i. Sort Cal will go to “Monitor” as the sample loads. This is expected; it should return once the sample is pressurized.

i. “*Pause*” the sort to stop collecting data if needed.

j. You can increase the speed slowly by increments of 5 up to (but not more than) 60.
    i. Do not go above 8,000 events/second or have a recovery rate of less than 70%. As you increase your speed, you will decrease your recovery.

k. The collection tube will give an alert when it is 80% full. This can be silenced by selecting “*Mute Alert*” on the lower right of the screen.
    i. When the collection tube is around 90-95% full, “*Pause*” the sort, and manually swap the tube for an empty one.
    ii. Reset the tube volume in the Sort Window. This will not reset the number of collected cells, so be sure to note the number if you need to know how many cells are in each tube.

l. If the machine clogs, you will see either a complete or partial drop in the events/second that cannot be explained by the tube being empty. See Troubleshooting if this occurs.

m. If you lose Calibration, give it a minute to settle. If it doesn't automatically come back, you will have to stop the sort and rerun Sort Calibration.
    i. Sometimes, turning it back on manually will allow it to recover. Ensure that the new stream breakoff points are exact compared to the red and green lines you set earlier. If the stream has moved at all, DO NOT begin sorting. You will need to rerun the Calibration.

n. When you can no longer see the volume in the tube, begin to monitor the events/second.
    i. You will see a gradual drop in events, followed by a very sharp rise as the system begins to suck up air instead of the now-depleted volume. “*Pause*” and “*Unload*” the tube to visually confirm it is dry, then “*Stop*” the sort. If you are not fast enough, the automatic bubble detector will stop the sort for you.
    ii. After either seeing air yourself or having the detector stop the sort, “Backflush” at least three times to remove any air from the system. Debubbling the flow cell may also be necessary if the stream still appears fuzzy due to air.
    iii. Cap and collect your collection tubes, then write down how many cells you collected from the sort.

o. Make a new Tube and continue sorting your next sample.

Troubleshooting

1. If QC Fails:
    a. If DD1 fails, check the stream break off: the break off point should be visible in the top half of the status window.
        i. Run Sort Calibration just long enough for the machine to automatically reposition the stream, then cancel Sort Cal.
        ii. If that doesn't work, *gently* wiggle the nozzle to find good break-off height (usually in the second quarter from the top of the stream, but this can vary) and satellite formation and reformation.
    b. Check for air in the system.
        i. The stream droplets should be visibly sharp and not fuzzy.
        ii. Check if there is a bubble present in the filter; tap or vortex the filter to cause non-visible bubbles to rise to the surface.
        iii. If there are bubbles, under the "*Cytometer*" menu, run the following in this order:
            1. Sheath filter de-bubble > Run 3 times in a row (skip if there are no bubbles in the filter).
            2. Flow cell de-bubble > Run 3 times in a row.
            3. Backflush > Run 3 times in a row.
    c. If there is no bead peak, you may not have remembered to vortex the beads. If this is not the case, remake the bead tube and try again.
2. If Sort Calibration fails:
        i. *Gently* wiggle the nozzle or debubble and try again.
        ii. Put the machine in standby and remove the nozzle from the machine and reinsert it again.
            1. Select "*Initialization*" in the "*Acquisitions*" panel and wait to re-stabilize the stream. Run "*Sort Calibration*" again
            2. If the previous steps fail to resolve the issue, sonicate the nozzle, found below.
2. To Sonicate the nozzle:
    a. Put the SRT in "*Standby"* mode to turn off stream and remove the nozzle.
    b. Fill the sonicator with diH2O from the MilliQ to the fill line. Fill the provided beaker with diH2O as well.
    c. Place the beaker in its 'float' and then place the nozzle o-ring side down in the beaker. Gently rest the beaker in the sonicator bath using the float.
    d. Ensure the sonicator is plugged in, then set the timer for 5 minutes.

e. Once the timer is up, remove the nozzle from the bath and dry it gently with a KimWipe, before reinserting it into the machine.
f. "*Initialize*" to turn the stream back on and rerun Sort Callibration to continue sorting.
g. Once you have finished sonicating, drain the sonicator.

3. To clear a clog:
    a. "*Pause*" the sort and "*Unload*" the Sample.
    b. Perform "*Backflush*" three times.
    c. Reload the sample and see if your event rate has recovered. If it hasn't, proceed below.
    d. "*Unload*" the sample and "*Stop*" the sort.
    e. Under "*Cytometer*" select "*Daily Clean*." A dialog box will open.
        i. You may set the timer to be less than 5 minutes but depending on the severity of the clog you may need the full 5.
        ii. Mark where the volume on the tube. During the cleaning, ensure that the volume is actually going down. If it is not, notify staff and proceed with sonication.
4. To replace a broken o-ring:
    a. Carefully remove the old o-ring using tweezers. *Do not* damage or scratch the nozzle.
    b. Clean and dry the nozzle using 70% Ethanol.
    c. Gently hold the new o-ring upright. It has the appearance of a 'top hat', with the skinny side of the hat being the topside and the thicker side being the bottom.
    d. Gently place the new o-ring into the nozzle so it is upright and properly seated. Gently press it in with your finger, if needed.

Shutdown

1. Select the Printer Icon Drop Down and select "*Export to PDF*". Select which tubes to export and export them to the day's experiment folder. Select this folder from the Windows Files Explorer, and right click, then "*Send to*" > "*Zip*".
    a. Log into SharePoint and upload the zip file to your personal folder.
2. Under "*Cytometer*" select "*Daily Clean*".
    a. A dialog box will open. Make sure the cleaning time is set to 5 minutes for 10% bleach and for rinsing with diH2O.
        i. Ensure the level of bleach run is higher than the sample volume, and the level of diH2O run is higher than the bleach volume.
3. Under "*Cytometer*" select "*System Shutdown*".

    a. A dialog box will open. Follow the prompts on screen.
    b. Make sure to dry off the nozzle when you remove it to prevent salt crystals from forming. Inspect the o-ring once again.
    c. One of the final steps will ask you to remove and clean the following external components of the machine; the Nozzle lift, side stream illuminator, deflection plates, cuvette bottom, sort chamber, side stream detector windows, and sort output holder. Do so with 70% ethanol, Kimwipes, and cotton swabs. Dry and reassemble them.
4. Under "*Cytometer*" select "*Turn off*" to power off the machine.
5. Spray the sort chamber and bench with 70% Ethanol and wipe clean.
6. Close the sash on the Biosafety Cabinet and turn off the blower.
7. Turn off the chiller.
8. Log out of the software.