Caltech Flow Cytometry Facility
User Guide for CytoFLEX S Analyzer

Revised 12/2022

Pre-check before switching the instrument on

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1) Check the sheath fluid level before starting your cytometer run. We recommend topping off the sheath fluid (DI water) before starting your experiment to avoid running out while analyzing your samples. To refill or top-off the tank:
   a) Unscrew and remove the sheath probe from the sheath tank. Place it in the metal holder.
   b) Remove the cap from the second full sheath tank on the shelf and insert the probe.
   c) Empty the first tank into the sink and re-fill it to its upper level (3500 mL) using the ELGA DI water dispenser
   d) Replace the cap and place the now full tank onto the shelf above the cytometer

2) Check and empty the waste container
   a) Unscrew and remove the waste probe from the waste tank. Place it in the metal holder.
   b) Empty the waste into the sink. Use eye protection and pour close to the drain to avoid splashing
   c) Add a small amount of bleach into the bottom of the tank
   d) Place the tank back into the tank rack and re-insert the waste probe

3) Wipe down the area using 70% ethanol or CaviWipes. This is a shared facility and this is good practice to clean the area when using shared equipment in any laboratory.

4) Fill a fresh tube of DI water and a fresh tube of FlowClean (blue solution). There is a box of tubes available to you for all cleaning procedures.

5) There are small biohazard bags that you may use on the shelf above the instrument to help you keep your area clear during your experiment

Starting Up

1) Turn on the cytometer by flipping the red switch on the surge protector next to the computer.

2) Turn on the computer. There is no password for the computer.

3) Open the CytExpert software using the shortcut on the desktop or taskbar, and log in.

4) Check the bottom left of the screen to verify that the Cytometer is connected.

5) Check to see if the instrument is set up in tube or plate mode.
   a) First, check the manual switch located above the tube loader; P should be facing forward (toward the biosafety cabinet) for plate loader. P should be facing the right (toward the door) for semi-automatic/tube mode.
   b) Next check in the software (Cytometer → Sample Injection Mode) that you are in the correct mode for your run.
      i) Semi-Automatic is for 5mL FACS Tubes or 1.5mL Eppendorf tubes
      ii) Plate Loader is for 96-well plates (U, V, or Flat-bottom)
   c) If you change the mode, you will see a dialog box appear prompting you to change the appropriate probe. Close the dialog box, then exit the CytExpert Software.
   d) Then turn off the cytometer using the red switch on the power strip. Allow it to sit for 1 or 2 seconds.
   e) Switch the cytometer back on, then log back into the CytExpert Software.

Tube Mode Plate Mode

System Startup Program

1. From the Cytometer Menu, select "System Startup Program." The system startup program takes 10 minutes.
    a. **Tube Mode**: Click "Initialize" and place a freshly filled tube of 2mL deionized water in the sample loader. Clean tubes are available in a box on the shelf above the instrument. Click Start. System will initialize and prime.
    b. **Plate Mode**: Dispense ~250 µl of deionized water into the wells highlighted blue; the default wells are A1, A2, and A3. Load the well plate onto the loading platform and select start.

2. Close dialog box to quit when start-up is finished.

Checking Cytometer Performance

1) From the QC/Standardization menu, select "Start QC/Standardization."

2) Retrieve the box of RTU QC Calibration Spheres from the refrigerator.

3) Select the correct bead Lot ID from the menu (bead lot is printed on each bead vial and on the box of beads)

    a) **Tube Mode**:
        i) Use the aliquot of beads in the labeled tube in the box. If the aliquot is empty, review the SOP for preparing a fresh aliquot. These beads do not require dilution, they are "Ready to Use" (RTU). If you dilute the beads, QC will fail.
        ii) If loader arm is not out, click "Initialize" to move the loader arm out, or you may gently manually pull the arm out.
        iii) Install tube onto the cytometer loading port.

    b) **Plate Mode**:
        i) Place 2 drops of RTU QC Calibration Spheres in well A1. These beads do not require dilution, they are "Ready to Use" (RTU). If you dilute the beads, QC will fail.
        ii) Then, select Plate Settings and choose the well with QC beads in it; the chosen well will be highlighted in grey.
        iii) Confirm the correct plate type is designated (U, V, or Flat bottom)

4) Click Start. The performance check takes approximately 2 minutes to complete.

5) Once the performance check is complete, view the report.

6) Verify that the performance passed. There will be a green checkbox next to each passed parameter. It also says "QC passed" at the bottom of the report.

7) If **QC FAILS** (you see a red X instead of a green checkbox in the report):
   a) Troubleshooting Step 1:
      (a) Prepare fresh RTU QC Calibration Spheres (see separate SOP to prepare the beads) and ensure that you have chosen the correct bead lot in the dropdown menu. Then run the QC again. This rules out that the QC failure is related to dilute beads, beads that have been exposed for too long to UV light from outdoors, or incorrect target values.

   b) Troubleshooting Step 2:
      (a) **If the QC continues to fail on Gains or CVs:** perform the prime function two times (Cytometer menu → Prime) and try again.
         (i) WARNING: when the instrument fails QC, it will ask you if you want to set the new gain settings it just generated as new target value. Click NO in the dialog box. You do not want to set your gains for the day using bad settings.
         (ii) You will need to restart the CytExpert software for the prime function to be available in the menu. This is a known glitch in the software that has not been corrected in software updates.

      (b) **If the QC continues to fail on laser delays or low event rate:** perform the above procedure (Priming 2x) then Run QC again. Notify facility staff to help track the issue.

      (c) **If the QC continues to fail on laser power**: perform the above procedure (Priming 2x), then wait an additional 5 minutes to allow the laser more time to warm up. Then attempt to run QC again. If you see the value move closer to the target range, but still fail, wait another 5 minutes then try again. Notify facility staff to help track the issue.

   c) Troubleshooting Step 3:
      i) If the QC fails again, you can repeat troubleshooting step 2 one more time.

   d) Troubleshooting Step 4:
      i) In the unlikely event of a 3rd failure, perform the "Deep Clean" Function (Cytometer menu → Deep Clean). You will need to restart the CytExpert software for the prime function to be available in the menu. This is a known glitch in the software that has not been corrected in software updates.

         (a) If you receive an error message saying the deep clean solution is empty, continue with the deep clean anyway and let facility staff know so they can top-off the solution and reset the software. The software notifies on a timed basis. It does not weigh the bottle to track the actual volume.

         (b) Once the process is complete, attempt to run the QC beads again. Notify facility staff that you needed to complete the Deep Clean procedure, regardless of whether the QC passes to help track the issue, provide troubleshooting assistance, and ensure that you are not billed for troubleshooting time.

8) Once the instrument passes QC, select “Close QC/Standardization” from the File menu.
   a) If it does not pass, notify facility staff. They can help you determine if you can continue to run your experiment or if you should move to another instrument.

Creating your Experiment

1) View the Detector Configuration in the Cytometer home screen menu and ensure the CalTech Flow configuration is selected.
   a) You should have verified instrument configuration compatibility with your experiment before purchasing reagents and preparing samples. In the unlikely event that the software is not in the correct configuration, switch to the correct one then select “Set Configuration.” Then notify facility staff.

2) Select OK to close the Detector Configuration screen.

3) Select the File menu and click on “New Experiment” to create a new experiment.

4) A popup window prompts creating a new folder for data storage. Rename the experiment as you see fit and save it to your user folder.

Creating New Experiment Settings and Determining Gains

1) Select “Set Channel” in the Settings menu

2) Uncheck all parameters you will not be using. Type in labels if you choose.
   a) Unselected parameters will not be saved into the data files.
   b) Select Apply to all empty tubes, and OK. Select Close.

3) Insert graphs onto the worksheet using the available icons on the toolbar to view your cells as they are acquiring and recording. Next to the plots, use the Population Hierarchy and Statistics icons to bring up each type of table.

4) There are many types of plots to use: 1-D histograms and 4 types of 2-D plots
   a) Right click on plots, or select the grey/white gear box to change properties. Axis ranges can be adjusted here.
   b) Click the title of the plot to display gated events or make combo gates using the commands and, or, and not.

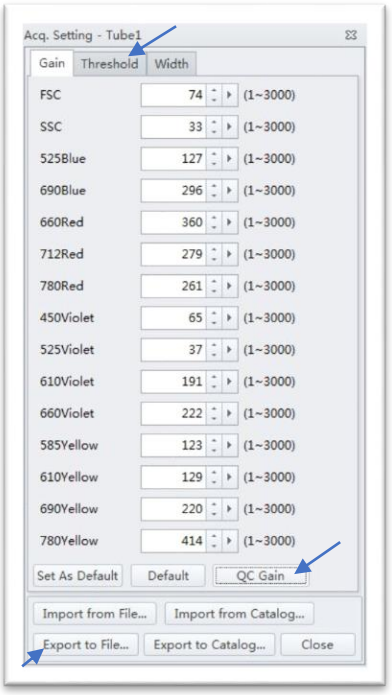
c) You can set the modes to linear or log by clicking in the plot, then right clicking and selecting property. Most cells are viewed in linear. Bacteria, small cells, and other submicron samples are viewed in log. Fluorescence is also viewed in log by default.
d) You can set the maximum and minimum value for each parameter from this drop-down menu. Default is 1,000,000 max and 100 min. Save the document in your folder.

5) Adding tubes or wells:

a) **Tube Mode**: Add a tube to the experiment using the tube with a + in the Tube Window
   i) The name of the tube can be changed by double clicking the name, or right clicking and selecting "Edit Name" in the dropdown menu that appears

b) **Plate Mode**: Open the Plate Menu by selecting the square icon with four dark circles in it. In the plate menu add a plate using the icon with a + at the top left of the menu. Plate type and sampling order can be specified at this point. You can also create plate templates for future use.
   i) The plate number, type and sampling sequence can all be checked at the top of the menu
   ii) When designating a well select its location and set it as a sample well
      (1) The name, mixing time, backflush time, and other parameters can be customized
      (2) Set channel names and labels. When exporting and importing settings files, the channel names must be the same. Labels can differ.
      (3) The gains can be set now or by using the Acq. Settings menu
   iii) Designate a well for each color control including the unstained control
   iv) Eject the plate loading platform, and load the plate that matches the designated wells

6) Select "Acq. Settings" button in the Acquisition Window or by Cytometer →Acq. Settings. The gain adjustment window will appear.

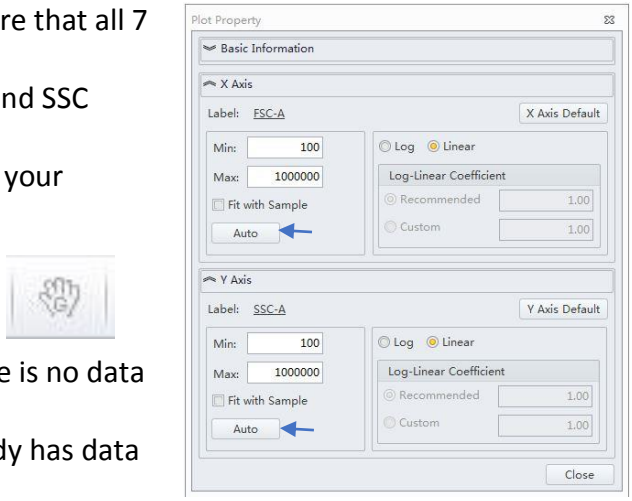


   a) Click the button entitled "QC Gains" to use the gain settings that were determined when you ran QC.
   b) Threshold can also be adjusted to remove signal noise. If you are running small particles, lowering the threshold settings is absolutely necessary in order to see your cells.
      i) The default setting of 10,000 typically works well for mammalian cells, but is too high for bacteria, platelets, and other types of smaller particles.
      ii) For bacteria/submicron samples, 2000-5000 is usually low enough. However, setting the threshold too low will allow too much noise and cause your events and populations of interest to be lost within it.
      iii) You may also adjust the threshold by clicking the icon in the toolbar and dragging across the area that you would like to threshold out.

7) Sample flow rate, the number of events displayed, and the events and time to record can be controlled. During setup, it is recommended to run samples “Slow” 10 uL/min.

8) Begin adjusting settings with the unstained tube or well first.

9) Optimize the FSC and SSC gains to place the population of interest on scale.
   a) On the plot for FSC/SSC, click on the grey/white gear icon to open the “Plot Property” dialog box
   b) Add a zero to the MAX parameter of 1,000,000 to ensure that all 7 logs of dynamic range are visible on the plots
   c) As the sample is running click “Auto” on each the FSC and SSC parameters to help zoom to the population.
   d) Adjust gains in the Acq. settings for FSC-A and SSC-S so your population of interest is close to the center of the plot.
   e) You can also use the hand tool with the letter “G” (for gain) and use the mouse to drag the populations to the middle of the dot plot.



   f) Gains can only be adjusted while running or when there is no data in the tube
   g) Run and overwrite to change gains in a tube that already has data

10) Inspect the single-color control tubes or wells one at a time.

11) Verify that the positive populations are on scale.
   a) If a positive population is off-scale, first try adjusting the number of decades shown on the plot.
   b) If a population is still off-scale, lower the gain for that parameter until the positive population is entirely on scale. However, on a future run, if you are staining your samples, it is best to titrate your antibodies to optimize your staining, as opposed to adjusting the gains. This is because the gains are optimized in a standardized manner during QC, using the lowest gain at which the brightest signal can be achieved with the beads.
      i) If you are running samples that are smaller than the RTU QC Calibration Spheres (3 microns), you may consider using your own validation beads after QC that fluoresce in the channels that you will be using and are similar size to your samples. The gains required may need to be raised in a more standardized manner.

12) Make sure that your gains are being applied to each tube as you look at the different controls
   a) Gains can be applied to empty tubes by right clicking the tube and selecting “Apply Acq. Setting to…”

13) You now have your gain and threshold settings. Choose “Export” in
      i) the Acq. Settings window to save.
   b) This gain and threshold setting file will be used for compensation
   c) This .acq file will only be able to be imported into experiments or compensation files that share the same channel names with it.
   d) It is recommended to repeat this process every time you run your experiment.

Calculating the Compensation Matrix

1) Create a compensation experiment by selecting “New Compensation” in the File menu.

2) Use the default file path and select Save.

3) Setting up the experiment**:**
   a) **Tube Mode**: Select the channels requiring compensation and the sample type. The unstained negative control tube can be selected if needed.

   b) **Plate Mode**: Select the plate type, sampling sequence, and the channels requiring compensation, including an unstained well. A well location must be chosen for each channel.

4) Select OK. A compensation control experiment is generated.

5) Import the gain settings of your experiment and check that they applied to all tubes/wells.
   a) Confirm that all tubes or wells share the same gains
   b) This will ensure that the compensation samples are run with the same settings as your experiment.

6) Install the first control onto the cytometer. Select Run.

7) As the sample is running click “Auto” on each the FSC and SSC parameters to help zoom to the population of interest. Move and re-size the FSC/SSC gate so that it encloses the population of interest. If you have a small number of cells in your controls, you can adjust the stopping rules to record as few as 5000 events to calculate reliable compensation values. Once these setup steps are done, select “Record.”

8) Once the sample is finished recording, right click the FSC/SSC gate and select “Apply to all Compensation Controls”

9) Install the next tube onto the cytometer, or move to the next well in the plate. Select Record.

10) Once all tubes or wells are recorded, review the plots for each control. Move the positive gate in the histogram plots so that it encloses the positive population. Repeat for all compensation tubes or wells.

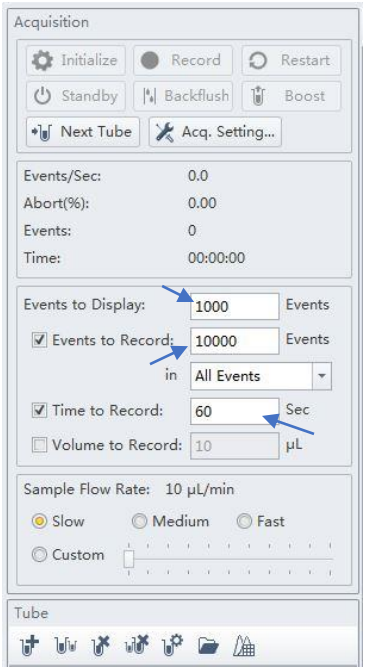
11) Select “Compensation Calculation” in the Settings menu.

12) The matrix displays. Save as to export the matrix and specify where to save it.
   a) The matrix will save as a .comp file. This .comp file will only be able to be imported into experiment files that share the same channel names.

1) Open your previously created experiment (From the File menu, choose "Open Experiment." It should be listed as your "experiment name".xit.)

2) If compensation was performed, import it using the compensation icon in the tube window and apply to all tubes (Import → Compensation). Select "Compensation Matrix" in the Settings menu. Select one of the following:
   a) Import compensation matrix and transform it with current gains.
   b) Import compensation matrix.
   c) Import compensation matrix and gain.
      (1) Select "Import Compensation Matrix." (Note: If you adjust your gain after compensation is preformed, you can import compensation and transform it with current gains.) Select OK.

3) Adjust your stopping rules to display at least 10,000 events per second – you will need to change this since the software defaults to 1000 (as shown). Record a minimum of 10,000 events in your FCS files, and lower the stopping time to something more reasonable - 60 seconds (as shown) is a good place to start because the cytometer does not have a sensor to detect when a tube or well has been run dry. The instrument default is 10 minutes (600 seconds).



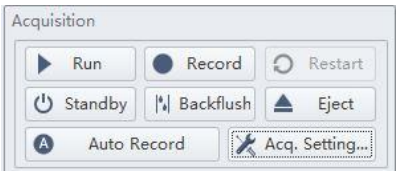
4) Adding tubes or wells to the experiment**:**
   a) **Tube Mode**: Add as many tubes to the experiment as necessary. Rename them by right clicking on them.
   b) **Plate Mode**: if you completed step 6B under "Creating New Experiment Settings and Determining Gains," wells should have been created. If you have a separate plate for your experimental samples and controls, you may need to create a second plate and repeat this step as applicable.

   NOTE: You can have multiple plates in a single experiment. You can also run in tube mode and plate mode within the same experiment. Recommendation is to run setup and optimization steps in tube mode, then switch to plate mode for experimental samples if you are running in plate mode.

5) Adjust the sample flow rate in the Experiment Window as needed. For your first sample, you should start at 10 ul/min (slow), then move up to 60 uL/min (fast) while monitoring the event rate to ensure that it is not too high and that the abort rate is not too high.

6) Recording**:**
   a) **Tube Mode**: Click the first tube. Ensure that the sample has at least 100 uL of volume. You may need to initialize to bring the tube holder out again, or gently pull it forward manually. Install the tube, load it, then click either "Run" or "Record." If you click Run, the sample will record events until you click STOP. If you click Record, it will stop according to the stopping rules set in the Acquisition dashboard.

b) **Plate Mode**: Click the first well of the plate in the menu on the left where the placeholder files are listed. Click the eject button. Carefully load the plate into the plate loader and ensure it is seated properly. Ensure that you have at least 45 uL of volume in each well. Set your stopping rules if you did not do so when you set up the plate initially. Then hit "Auto Record." If you notice you have hit a bad well, use the SKIP function that appears once Auto Record is in progress. The Pause button will also appear after you click Auto Record, but it does not work, this is a known glitch in the software, so do not use it. If you need to stop the plate early, use the STOP button which appears after you click "Auto Record."



7) Continue for all tubes or wells you wish to run.

Data Export

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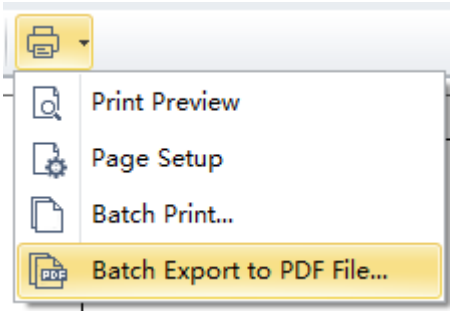
1. Users will be responsible for exporting their data to the Sharepoint or a safe location.

2. The facility will not be saving all data files indefinitely. In any shared resource laboratory, data is not guaranteed.

3. You may also export individual PDFs or your data:
   a. Create a folder entitled PDFs in the folder you made for the experiment you are running
   b. Make sure that all plots, population hierarchies, and statistics windows are within the page breaks of the experiment layout.
   c. Click the arrow next to the printer icon.
   d. A drop-down menu will appear and you will select "Batch export to PDF."
   e. Check the box next to each tube or well for which you would like to create a PDF and select the destination as the new PDF folder you created within your experiment.
   f. Click OK. A separate PDF will be generated for each FCS file.



1) Select Daily Clean from the Cytometer menu.
   a) **Tube Mode**: Fill two fresh tubes each with 2mL of DI water and FlowClean. First place the FlowClean on the instrument for 5 minutes (7 minutes if you were running bacterial or viral samples). Then when prompted by the software, switch to the DI water tube and continue running it for 5 minutes (7 minutes if you were running bacterial or viral samples). Do not reduce the cleaning time below 5 minutes for each step.
   b) **Plate Mode**: Load FlowClean solution into the orange wells, and load DI water into the blue wells as indicated on screen. Select Run. Default cleaning time is 12 minutes.

2) After the Daily Clean procedure is completed, close the Daily Clean window.

3) Log out of the CytExpert software.

4) Turn off the cytometer and computer when finished.