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CytoFLEX Training Guide

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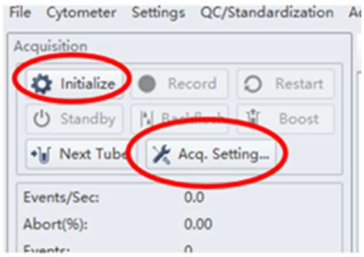
Initial setup

- Log in Windows, open CytExpert software.
- **Cytometer → Sample Injection Mode →** choose the desired mode:
  - **Plate Loader** = 96 well plate
  - **Semi Automatic** = Tubes
    (*Do not choose **Manual!***)
- Set the knob above the Sample Injection Port (SIP) on **TUBE or PLATE** mode, as needed.
- You will be prompted to **restart the cytometer** after switching modes:
  Click **Cytometer → Turn OFF**. Wait a few seconds. Click **Cytometer → Turn ON.**
- If you are the first user of the day, you may be asked to do a **System Startup** (see the **Troubleshooting section**).
- **You may start your compensation or the actual experiment, as necessary.**

COMPENSATION with single color control in TUBES

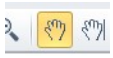
A. Create *New Compensation* with samples in TUBES

- Perform **INITIAL SETUP** (see page 1) to set the cytometer in **Semi-Automatic** (tube) mode. Depending on the machine, sometimes it is necessary to turn off/on the cytometer to make it recognize the Tube or Plate mode.
- Click **File → New Compensation**
    - Navigate to the desired file path, edit the default name if desired and click **Save.** We highly recommend creating one subfolder in the …*Documents\CytExpert Data* folder for the data.
    - **Check the boxes** for each channel you will be using. Choose **Beads** or **Cells** from the column on the right.
    - Unselect the boxes for unstained cells and unstained beads.
    - When choosing your colors, if you do not see your fluorophore of interest in the list, make sure you select the drop-down arrow to see more options.
    - Set parameters to **area** not height.
- In the **Acquisition** panel:
    - Click **Initialize** to bring out the sample arm

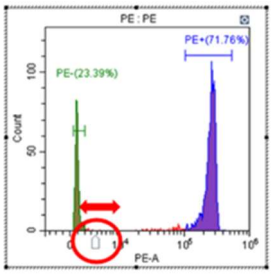


    - Open **Acq. Setting…** before running compensation and choose **QC Gain** to change the gains to values optimized by the daily QC routine.
- Put the tube in the holder, highlight corresponding tube in the **Tube** panel.
- Click **Run**.

B. Adjust the acquisition settings then record all single-color controls

- On the first plot, drag the gate over the main population of cells or beads.
    - Use the **Pan** tool  to move the population into view by clicking and dragging on the plot. Alternatively, click the gear at the top right of the plot to open up **Plot Property** and click **Auto** for both the X and Y axes, then zoom in  if necessary.

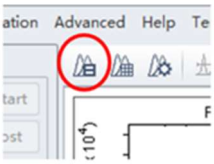
    - If not using an unstained control: click and drag the tab on the x axis to see both +/- populations (some populations may be off scale).
      Note: It is possible to use the universal unstained for some tubes and the negative population in other tubes.



- Click **Record**
    - Adjust the gates to make sure they are over the correct populations.
    - If you adjust the gain or change voltages: Click **Acq. Settings…** → **Set As Default**. When moving to the next tube, click **Acq. Settings…** → **Default** to apply previous changes to the new tube.
    - Finish recording all single-color controls.

C. Calculate compensation

- Click the left-most icon on the top toolbar.



- **Inspect the matrix**.
  The compensation values above 100% will be shown in yellow or in red.
  It is desirable to have compensation values as low as possible. However, compensation values around 100% are normal for some combinations of fluorochromes.

D. Save the compensation matrix

- Click **File** → **Save**

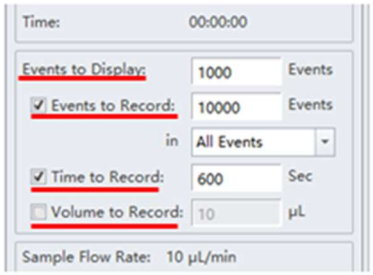
Now you may begin your actual experiment!

(If you do not continue with running an experiment, you must clean the cytometer: click **Cytometer** → **Daily clean** and follow the prompts.)

EXPERIMENT with samples in TUBES

. Create a new experiment with samples in tubes

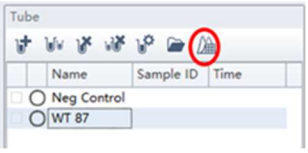
- Click **Cytometer → Sample Injection Mode**.
  If the cytometer is not set in **Semi-Automatic** mode perform **INITIAL SETUP** (see page 1).
- Click **File → New Experiment**.
- Specify the **file path** and **Save** the experiment.
- Click **Settings → Set Channel**… and **Uncheck** the fluorochromes you do not need.
- Click **Settings → Label**… to add antigen names or other information.
- Click **Cytometer → Acquisition Settings** and then click QC gain**.** *(Do not click Default!)* This step will adjust the gains for each detector as recommended by the vendor.
- In the **Acquisition** panel, choose your stopping and recording criteria (**Events to Record**, **Time to Record**, **Volume to Record**).
  - The cytometer will stop recording at whichever criteria is met first.
  - The default **Events to Display** is 1,000 and can be adjusted in this panel as well. Many users prefer to see more events.



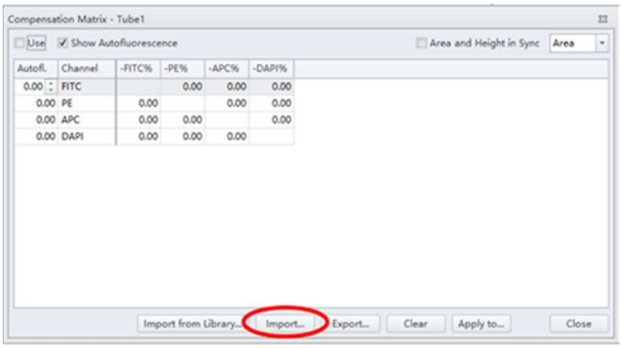
- In the **Tube** panel, rename the tube by double clicking.
  - Add tubes by clicking the **New Tube** icon. If necessary, delete tubes by clicking the **Delete Tube** icon.

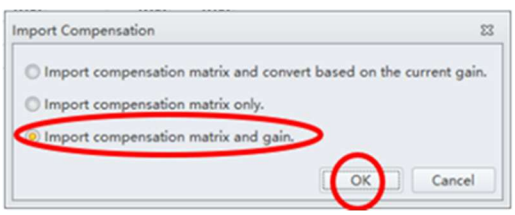
A. Apply the compensation matrix

- In the **Tube** panel, import compensation by clicking on the **Compensation Matrix** icon.



  - Click **Import** in the window that pops up. Navigate to the compensation file and click **Open**.
  - Select **Import compensation matrix and gain**. Click **OK**.

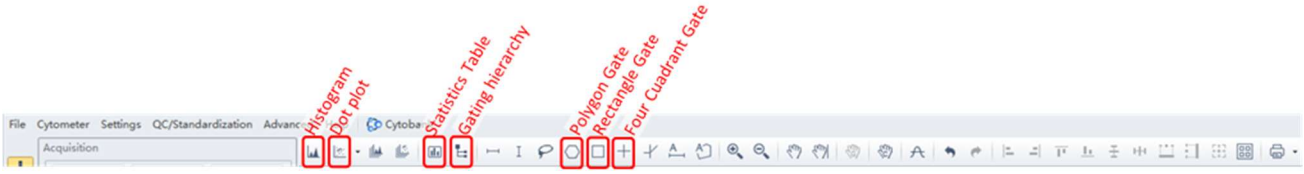




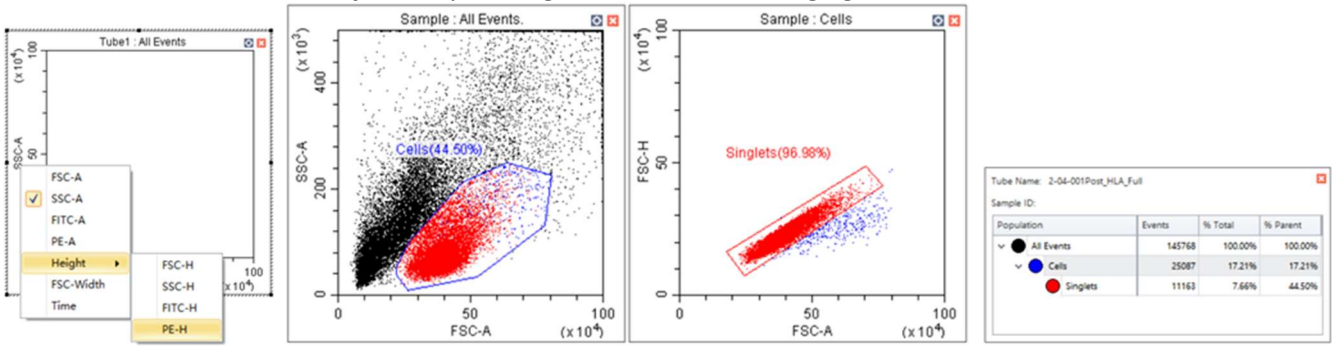
B. Set up the plots, histograms and the gating strategy

The icons at the top of the screen should be used for adding plots, histograms, gating hierarchy view and statistics tables.

Note: Use the mouse to hover over any icon in the software to determine its function.



- Choose **histogram** and **plot** parameters by clicking on the axis name and choosing from the dropdown.
- It is common to start with a **FSC / SSC dot plot** to identify the main population(s) of cells.
- **Singlets** are typically identified using a SSC-A / SSC-H and/or FSC-A / FSC-H plot.
- Create a **Hierarchy** view by clicking the icon . Change gate names and/or colors if desired.



- Consult the literature or ask the staff for suggestions on setting up plots and gates for your analysis.

C. Adjust the acquisition settings and record all samples

- Click **Initialize** and **Run** your sample until cell populations are visible on the plot. It may be necessary to adjust the gains for FSC and SSC and/or the range of data displayed on each plot.
- Create gates around populations of interest. Clicking the title of the plot to choose the correct population.
- For displaying fluorescence intensities, volume of sample run etc. add a **Statistics** view by clicking the icon .
- Once your plots and gates are set up, **Run** and **Record** each of your samples.
  - *Note:* Before recording your actual samples. it is recommended to load a tube with clean deionized (DI) water and run it as it would be a sample to confirm that the number events detected in water is low. This will confirm that the sample line is clean there is no abnormal noise generated on the cytometer.
  - Please plan on transferring your data to your One Drive, Dropbox etc. as soon as your experiment is completed in order to avoid data loss. The computers connected to equipment are not on the CHOP network and data is not backed up automatically.

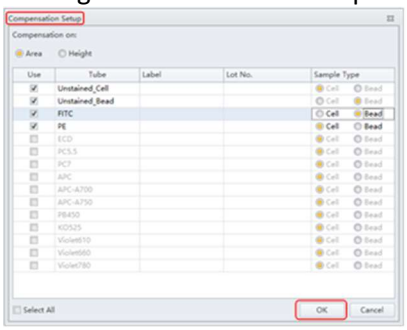
D. Clean the cytometer and Log out of Windows

- When you are finished recording your samples, clean the cytometer:
  - Click **Cytometer** → **Daily Clean** and follow the instructions on screen (run a tube of Coulter Clenz and a tube of DI water for 2 minutes, each).
- Log out of Windows.

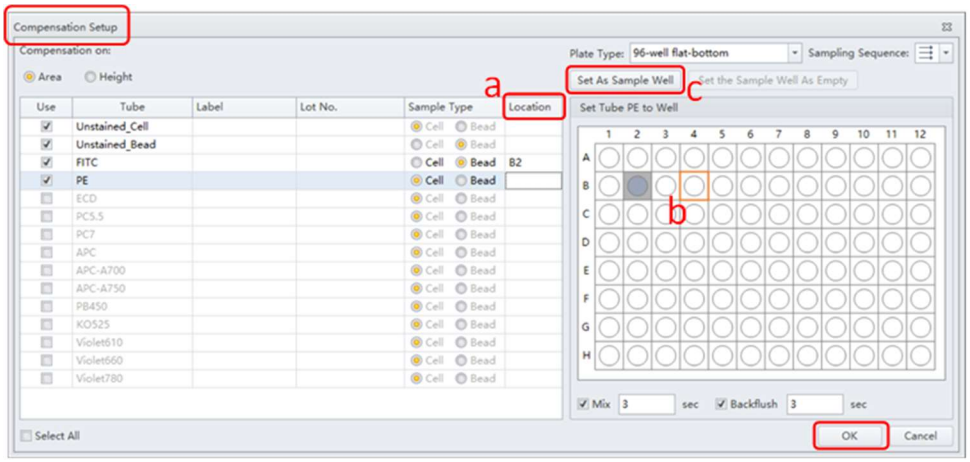
COMPENSATION with single color control in 96 WELL PLATE

. **Create *New Compensation* with samples in a 96 WELL PLATE**

- Perform **INITIAL SETUP** (see page 1) to set the cytometer in plate mode.
- Click **File → New Compensation.**
- Navigate to the desired file path and select **Save**. The **Compensation Setup** window will appear.



- Select the fluorochromes in your panel and click OK.
- Select the **Plate Type** and **Sampling Sequence**.
- Select the channels requiring compensation calculation, and the sample type (cells/beads).
- Assign the well locations.
    a. Highlight the fluorochrome in the **Location** column.
    b. Select the desired sample well for the fluorochrome.
    c. Select **Set As Sample Well.** The well location will populate in the **Location** column.
- Click **OK**.

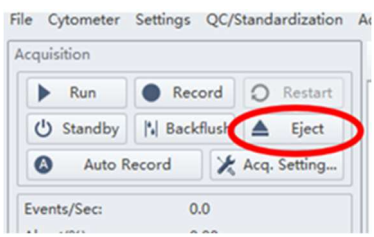


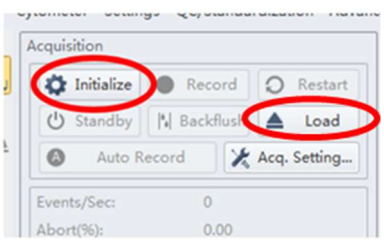
In the example to the left, the well **B2** has already been assigned to the **FITC** control.

The well **B3** is about to be assigned to **PE** by clicking **Set As Sample Well** button.

- Plots for all fluorochromes will be created, including for the fluorochromes you do not choose.
- Open **Acq. Setting…** before running compensation and choose **Recommended** to change the gains to values optimized by the daily QC routine. (*Do not select Default!*)

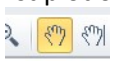
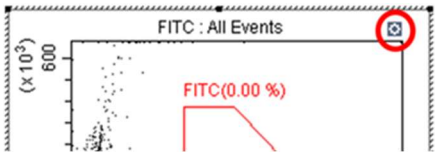
- Hit **Eject**. Place the plate on the stage and click **Load**, then click **Initialize**.

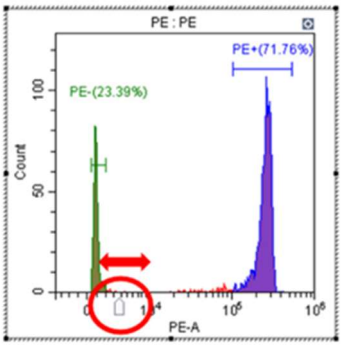




- Highlight the desired well in the **Tube** panel.

A. Adjust the acquisition settings and record all single-color controls

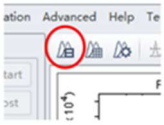
- After clicking **Initialize**, click **Run**.
- Position the gate on the first plot over your main bead/cell population.
    - Use the **Pan** tool  to move the population into view by clicking and dragging on plot. Or, click the gear at the top right of the plot to open up **Plot Property** and hit **Auto** for both the X and Y axis, then zoom in  if necessary. It is also possible to customize each axis manually.
    - 

    - If not using an unstained control: click and drag the tab on the x axis to see both +/- populations (some populations may be off scale).
      Note: It is possible to use the universal unstained for some tubes and negative populations for others.
      

    - Adjust the gains on the detectors if necessary.
- Click **Record**
- If you changed voltages: Click **Acq. Settings… → Set As Default.** When moving to the next tube, click **Acq. Settings… → Default** to apply previous changes to new tube.
- You can wait to record until you have looked at all the fluorochromes and adjusted voltages, and then record all at once.
- Repeat to record all single-color controls.
- Adjust the gates to make sure they are over the correct populations.

B. Calculate Compensation

- Click the left-most icon on the top toolbar to **Calculate Compensation**.



- Inspect the values in the matrix.

C. Save the Compensation Matrix

- Click **File → Save.**

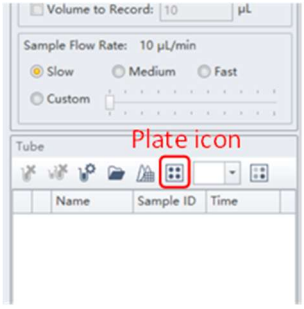
You are ready now to begin your experiment!

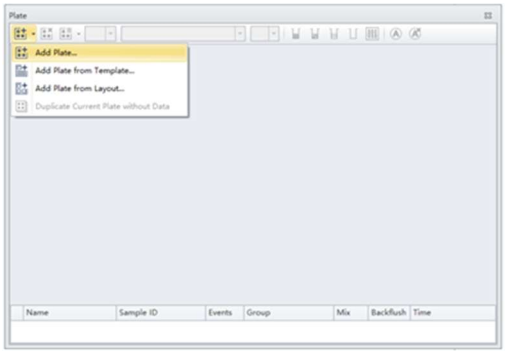
(If you do not continue with running an experiment, you must clean the cytometer: click **Cytometer → Daily clean** and follow the prompts.)

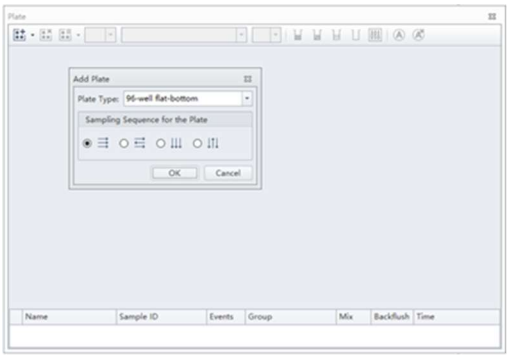
EXPERIMENT with samples in PLATES

. Create a new experiment with samples in 96 WELL PLATE and apply the compensation matrix

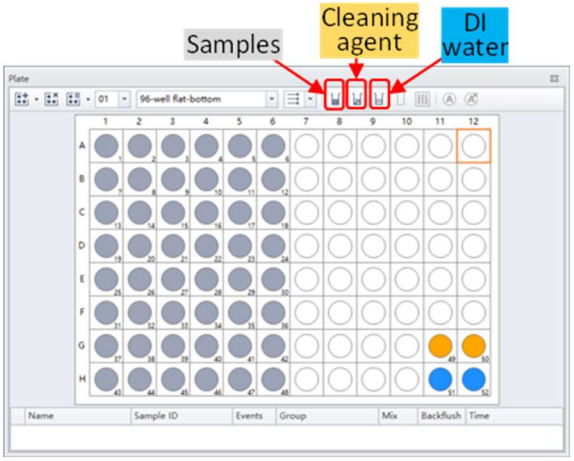
- Open **CytExpert** software. If the instrument is off, click **Cytometer → Turn on.**
- Click **Cytometer → Sample Injection Mode → Plate Loader.** Perform the **INITIAL SETUP** (see page 1) if necessary.
- **File → New Experiment…** → navigate to the desired file path and click **Save.**
- In the **Tube** control panel, open the **Plate** window by clicking on the icon that looks like a plate. In the **Plate** window, click the top left icon to **Add Plate**. Choose the **Plate Type** and the **Sampling Sequence** that the cytometer will use to analyze the wells.



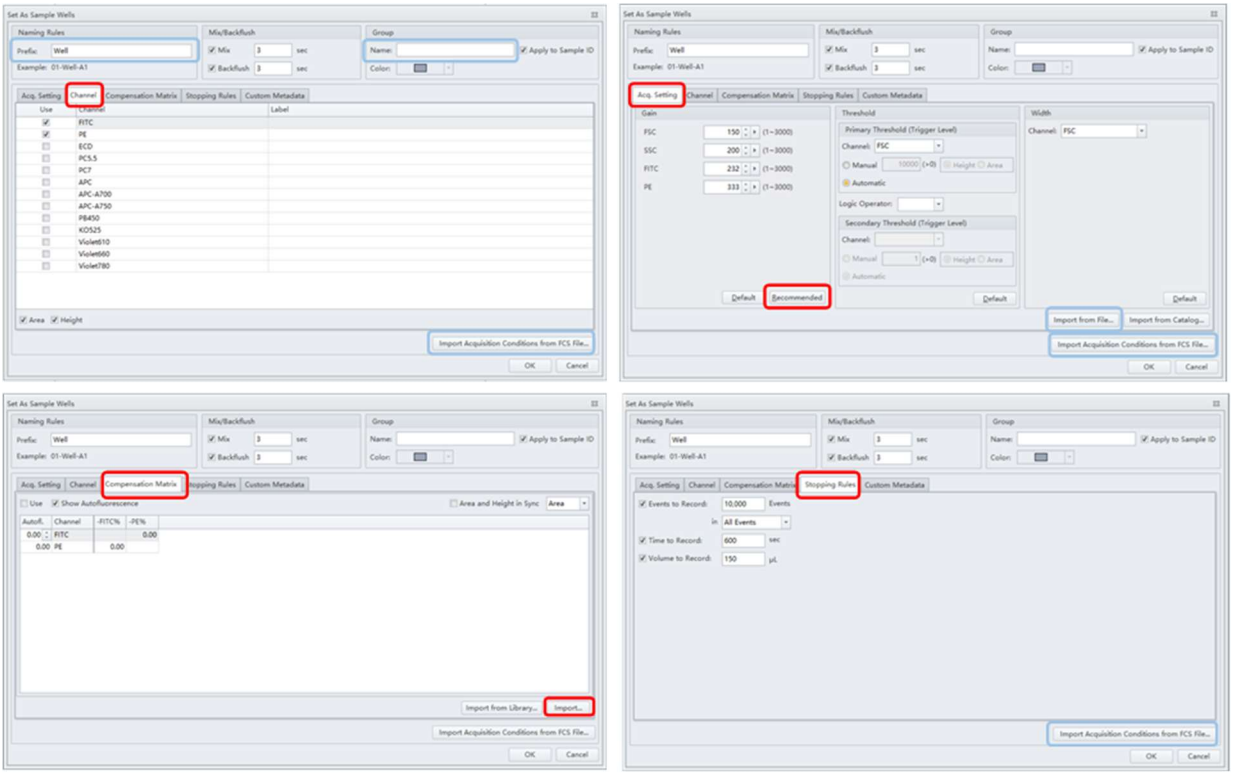




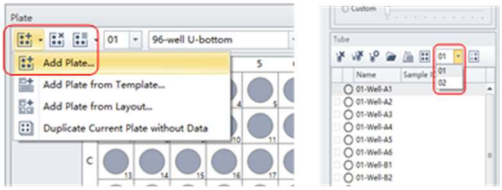
- Set up the plate layout:
  - Click and drag to highlight relevant wells. Click top icon to **Set As Sample Wells.**
    Highly recommended: add a cleaning sample and a water sample so the cytometer will auto-clean (use the tabs next to the **Set As Sample Wells** icon to set cleaning/water wells).



  - Optional: change the **Prefix** and set a name for the **Group**.
  - Select the desired **Mix** and **Backflush** settings from the **Mix/Backflush** section of the window.
  - We recommend starting with the **Channel** tab. Check off the fluorochromes you are not using. **Label** with antigen or any other necessary information. If desired, import the settings from an FCS file saved during a previous experiment. Leave both the **Area** and **Height** checked.
  - Go to the **Acquisition Settings** tab and click **Recommended**. (*Do not click Default!*)
  - Go to **Compensation Matrix** tab, click **Import** (not Import from Library). Navigate to the correct compensation file and click **Open**. Click **Import compensation matrix and gain.**
  - Go to **Stopping Rules** tab. Choose settings for **Events to Record, Time to Record**, and set a **Volume to Record.** *The recording will stop when at least one condition is met*.
  - Click **OK.**



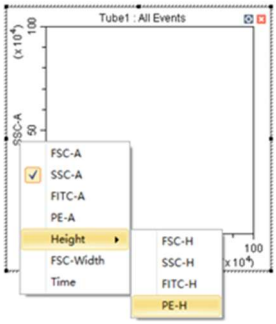
Optional: add multiple plates if necessary.

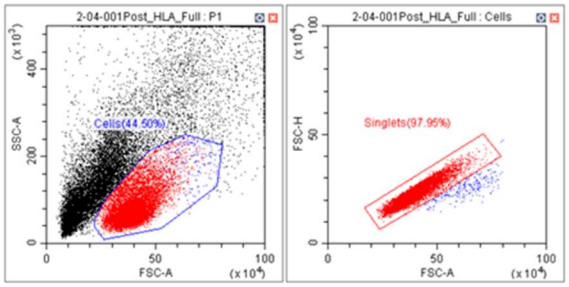


A. Set Up the Plots, Histograms and the Gating Strategy

Note: Use the mouse to hover over any icon in the software to determine its function.

- Choose plot parameters by clicking on the axis name and choosing from the dropdown.
- It is common to start with a FSC / SSC dot plot for identifying the main population(s) of cells.
- Doublet discrimination is typically achieved using a SSC-A / SSC-H and/or FSC-A / FSC-H.



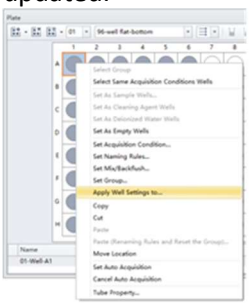


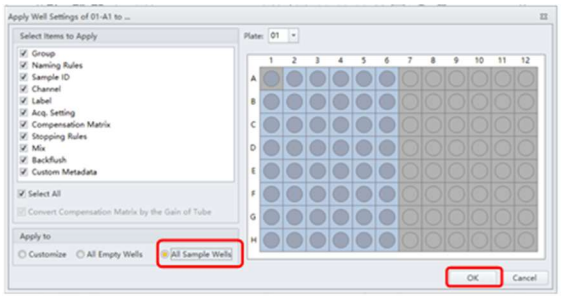
- Click **Eject**. Place the plate onto the stage and click **Load**.
- Select a well in the **Tube** panel**.** Click **Initialize** and **Run**.

- **Run** your sample until cell populations are visible on the plot. Create gates over populations of interest.
    i. Plots can show specific populations of interest by clicking the title of the plot and choosing the correct population.
    ii. Open the **Hierarchy** window  . If desired, change gate names and/or colors.

B. Adjust the acquisition settings then record all samples

- **Manually** select desired well in the **Tube** panel, or select the well on your **Plate** window. Click **Run** to begin acquiring your sample, make adjustments if necessary.
    - *Important:* If adjustments are made to the first well, the new settings will have to be applied manually to the rest of the wells: right-click on the well with adjusted settings and click **Apply well setting to…**
    - In the window that will appear, select **All Sample Wells**. *Optional*: uncheck the items that should not be updated.





- Click **Record** to record the sample in the selected well.
- Click **Auto Record** to record all the samples in the plate. We recommend recording at least one well manually and then use **Auto Record**.

C. Clean the cytometer and Log out of Windows

- If **you** did not set up cleaning and water wells on your plate you must manually clean the cytometer before you leave:
    b. Click **Cytometer → Daily Clean**
        i. Choose **Plate Type**.
        ii. Select one well for cleaning solution and two wells for water by highlighting the wells and choosing **Set As Cleaning Agent Well** or **Set As Deionized Water Well.**
        iii. Deselect any other wells by highlighting and choosing **Set As Empty Well**.
        iv. **Load** plate.
        v. Click **Initialize** and **Start**.
- **Save** data, **exit** CytExpert, and **log out** of Windows.
    - Please plan on transferring your data to One Drive, Dropbox etc. as soon as your experiment is completed in order to avoid data loss. The computers connected to equipment are not on the CHOP network and data is not backed up automatically.

Troubleshooting

. The System Startup Program

Typically, the staff runs the system startup on CytoFLEX cytometers. However, occasionally the software may ask a user to perform a system startup. The procedure can be done in Tube mode or in plate mode and it takes approximately 10 minutes.

1) System Startup in tube mode

- Open **CytExpert** software.
- Confirm that the cytometer is turned on and it is set to **Semi Automatic** mode (click **Cytometer → Sample Injection Mode**)
- Click **Cytometer → System Startup Program**.
- Follow the prompts. When the procedure is finished, close the window and begin your experiment.

- ***System Startup in plate mode*** can be done in a similar manner. Set up a plate with 3 wells of DI water. Click Cytometer → System Startup Program and follow the prompts.

A. An abnormally high number of events when running DI water

It is good practice to check at the beginning of the experiment that the cytometer does not have abnormal "noise". To do this, take a tube with ***clean*** DI water and run it on *High* using the "Recommended" settings. The cytometer should detect less than 30 events/second.

Important note: The water in the tube sitting by the cytometer is often contaminated with cells from the samples of the previous users. Discard the water and add to the empty tube fresh DI water from the bottle.

It should not take more than 2 minutes to clean a "dirty" cytometer. If you attempt to clean a cytometer with contaminated water, it is impossible to get the event rate lower than 30/second, not because the cytometer is dirty, but because the water in the tube contains debris.

B. Very few events when running samples

- The most common cause of "very few events" is **sample probe valve set incorrectly**.
  *For example, the sample valve is set in "**Plate**" mode when attempting to run a sample from **tube**.*
- If the event rate is still lower than expected AND the valve is in the correct position:
  - Check that the FSC gain is not lower than 150.
  - Check that the Threshold is Automatic
  - Confirm that there are sufficient cells/particle in the sample
  - Confirm that there are no visible cell aggregates in the sample

In case of a clogged sample line, it is also possible to observe "very few events" or "no events". Proceed with unclogging the sample line or contact the staff.

C. Clogged sample line

If the sample probe valve is correctly set and the event rate is very low, it is possible that the cytometer is clogged.

- Remove the sample and click Backflush twice.
- Load a tube with bleach 10% and run it for about 1 minute. When running bleach, it is typical to observe events that look like debris in the FSC/SSC plot. The event rate should be between 50 and 250/second, which is an indication that the cytometer is not clogged any longer.
- Run a tube with DI water for a minute or two to remove the bleach from the sample probe.
- Confirm that your cells do not contain any visible cell aggregates. Ideally, you should take **a droplet of sample on a microscope slide** and confirm that there are **sufficient cells** in your samples and **no large debris or cell aggregates are present**.