## Table of Contents

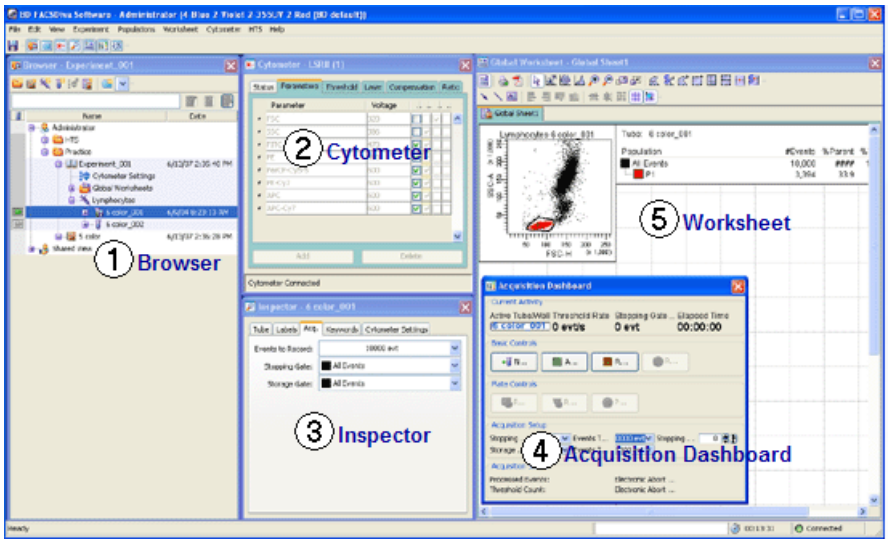
## 1. Startup

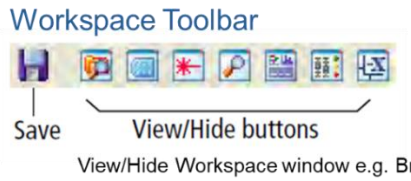
1. Check **Sheath and Waste** containers:
   - Empty Waste container in the sink – Add bleach to the mark (1L) (~10% final concentration).
   - Fill Sheath container with Sheath Solution (1X PBS).
   - **Note**: Full bottles of sheath are located in the hallway. When full the scale under the sheath tank should read approximately 0 lbs, and the tank needs to be re-filled before it reaches -16 lbs.
2. Turn on **Cytometer** – Green button on the right-hand side.
3. Check Sheath filter for air, bleed if necessary. Bleed side tubing.
4. Log in to Windows (Onyen + password).
5. Turn on necessary **lasers** using Coherent Connection 4, click 'Laser START'.
   - Set the UV laser to 100 mW, and other lasers at the top end of the Green region. See Optical Layout for appropriate laser powers (lsrfortessa-optical-layout.pdf (unc.edu))
   - **Note:** if you are the first user of the day pressing the Laser start button for the UV laser will not have an effect, you will need to manually set the power at 100 mW
6. Close Coherent Connection 4.
   - Note: Closing Coherent Connection 4 after starting the lasers will keep the lasers on, and help prevent a rare glitch where the software randomly shuts off the lasers. You will just need to remember to re-open Coherent Connection 4 at the end of your appointment to switch the lasers back off.
7. Launch **BD FACSDiva Software** (Username + password).

***Wait for the computer to establish a connection with the cytometer***
If the connection fails, restart the cytometer (green button) or alternatively power down the cytometer and the computer and then restart them both.

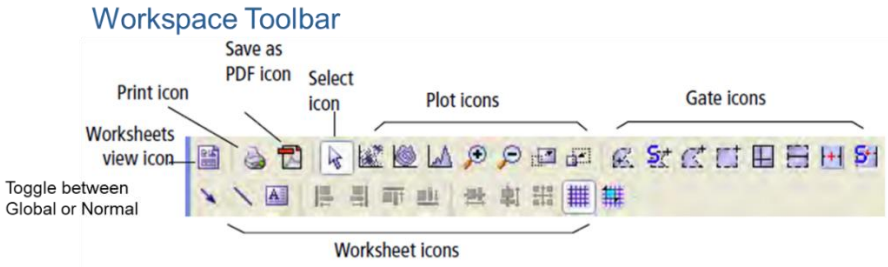
## 2. BD FACSDiva Software

### *Menus Overview*



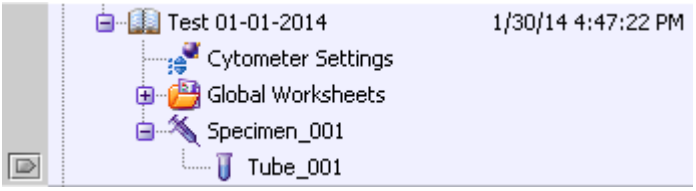

View/Hide Workspace window e.g. Browser, Inspector

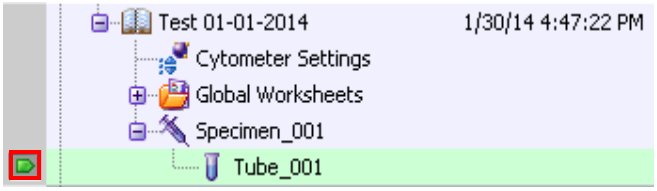



## 3. Setting Up Your Experiment

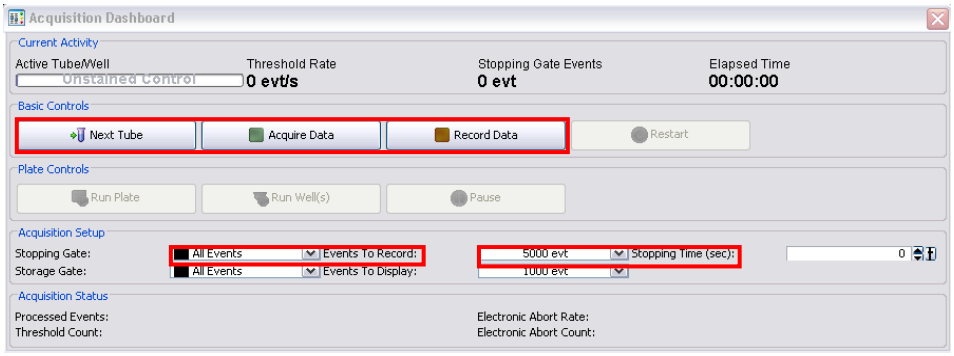
### *Adjusting Baseline Voltages*

- Click 'New Experiment' (2nd icon in the Browser toolbar) OR Recall a saved Experiment Template (Experiment menu>New Experiment…).
- Rename the Experiment (R-click>Rename).
- Click on Cytometer Settings and delete unused parameters in the Inspector window.
- Check the Height (H), Width (W), or both for FSC, SSC, or both to perform doublet discrimination.
- Check the Log box and the Area (A) box for all fluorescent parameters if running a phenotypic experiment. Height (H) and Width (W) are not typically used for phenotypic experiments, but can be helpful for more niche applications.
- Click on the Experiment (in Browser) and create a New Specimen (3rd icon in the Browser toolbar) then place the tube pointer on Tube_001 (Green arrow).



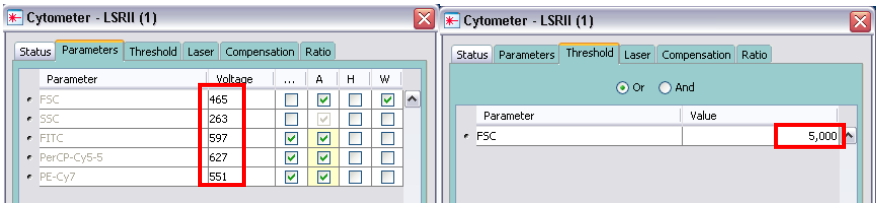




- In the worksheet add an SSC-A vs FSC-A dot plot, and appropriate plots (dot or histograms) for all of your parameters being used.
- The Acquisition Dashboard becomes available.




- **Filter your samples prior to running on the instrument**
- Load your unstained control on the cytometer.
- Click Acquire on the Acquisition Dashboard.
- Adjust FSC-A and SSC-A PMT voltages, as well as the threshold if needed.

**NOTE**: **Setting up a new blank experiment will set the PMT voltages based on the QC beads which may not be relevant for your experiment. The FCS PMT voltage will likely need to be significantly reduced when running cells because they are much larger than the QC beads. Similarly, the other PMT voltages may need to be adjusted.**




- Load each of your single color controls and adjust the PMT voltage to assure that the sample is brightest in the expected detector, on-scale (not piled on the edge of your plots), and that spill-over to other channels is minimal.

**NOTE: The brightest off-peak channel being >0.5 logs dimmer is a rule-of-thumb**

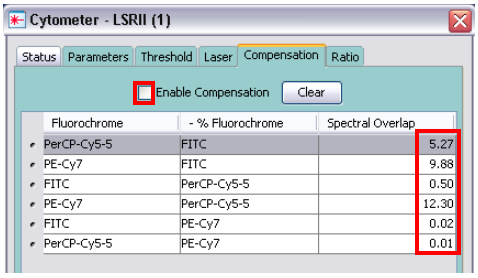
- Run a fully-stained sample to make sure all fluorescent parameters are on scale.

### *Compensation Set up*

- Go to Experiment menu>Compensation Setup>Create Compensation Controls.
- If using an unstained control, check the "Include separate unstained control tube/well" box.
- If you mix and match single color controls with different autofluorescence (e.g. by using a mix of cell and bead based controls) you can leave the unstained control box unchecked and instead select the internal negative populations with the P3 interval gate.
- Click OK.
- A Specimen called "Compensation Controls" is created. Open it by clicking on the "+"

### *Compensation*

- Place your tube pointer (Green arrow) on your unstained control
- Load your full-stained control or sample
- Click Acquire (adjust P1 if necessary – you can R-click on the P1 to "Apply to All Compensation Controls").
- Click Record (5000 events are automatically recorded though more may be needed depending on the type of control being used).
- Click Next Tube.
- Load then Acquire/Record your next single-stained control.
- Once all have been recorded, review/adjust the placement of the "snap-to gate" P2 for each control.
- If not using a universal unstained control, you will have to manually create a gate around the negative population of all your single-stained controls using the P3 interval gate.
- Go to Experiment menu> Compensation Setup>Calculate Compensation…
- Select '**Apply Only**' and click OK.
- Toggle back to the "Global Worksheet" (Worksheet View button) and select your Tube_001.
- Review and Enable Compensation




### *Running Samples*

- Place the tube pointer on Tube_001.
- While Tube_001 is selected, go to the Inspector window>label tab and input the names of your antibodies.

*Note*: If naming multiple tubes simultaneously, use the Experiment Layout (Experiment menu> Experiment Layout…).

- Rename Tube_001.
- Create Plots & Gates in the Global Worksheet.

*Note*: Apply a gate to a plot by R-clicking a plot and selecting Show Populations>"Name of the Gate" or by using the Inspector window (plot tab).

- Set the 'Events To Record' and the 'Stopping Gate' on the Acquisition Dashboard or in the Experiment Layout (Acquisition tab).

*Note*: To display the name of the applied gate on the header of a plot: click on a plot, go to the Inspector window (title tab) and check the 'Populations' box.

- R-click on any of your plot and select Show Population hierarchy to visualize simple statistics.

*Note*: To display data using Biexponential display: click on a plot, go to the Inspector window (plot tab) and check the Biexponential X and Y boxes.

- Load then Acquire/Record your samples (FMOs, biological controls, and experimental samples).

### *Exporting Data*

- Close your experiment (double-click on header)
- R-click Experiment>Export>FCS files… [FCS 3.1]
- Select Location (path)
- R-click Experiment>Export>Experiment Template… (if desired add information pertaining to your experiment)
- **R-click Experiment>Delete**

- The Core provides a server for temporary storage of data. An automated routine is run monthly to remove data that is >3 months old. Be sure to move your data off this temporary server onto a personal or lab owned location.
  J:drive (\\ad.unc.edu\med\microbiology\Groups\Flow Facility)

## 4. Shutdown

**Be considerate of others, start cleaning ~12 minutes before the end of your scheduled time**

1. Install a tube with 3mL of **1% Contrad** with support a**rm to the side** for **1 minute**
2. Put support **arm under** the tube and **RUN on HI** for **3 minutes**
3. Install a tube with 3mL of **FACSClean** with support a**rm to the side** for **1 minute**
4. Put support **arm under** the tube and **RUN on HI** for **3 minutes**
5. Install a tube with 3mL of **Deionized Water** (di$H_2O$) with support a**rm to the side** for **1 minute**
6. Put support **arm under** tube and **RUN on HI** for **3 minutes**
7. Leave a tube with ~**1ml** of di$H_2O$ with support **arm under**
8. Switch cytometer to **STANDBY** mode
9. Log off FACSDiva then Quit
10. Turn off lasers
11. Log out of Windows

**Last user turn off cytometer & FACSFlow Supply System**

## 5. Troubleshooting

- Coherent Connection not visible
  Alt + Space, Maximize
- Coherent Connection not displaying all lasers – [also occurs to first-time users]
  Tools > Serial Settings… > Scan Ports **or** turn the cytometer off then on
- Cytometer not connected
  Turn the cytometer off then on
- No cells being detected
  Cracked tube / Wrong tube (must be polystyrene)

## 6. References

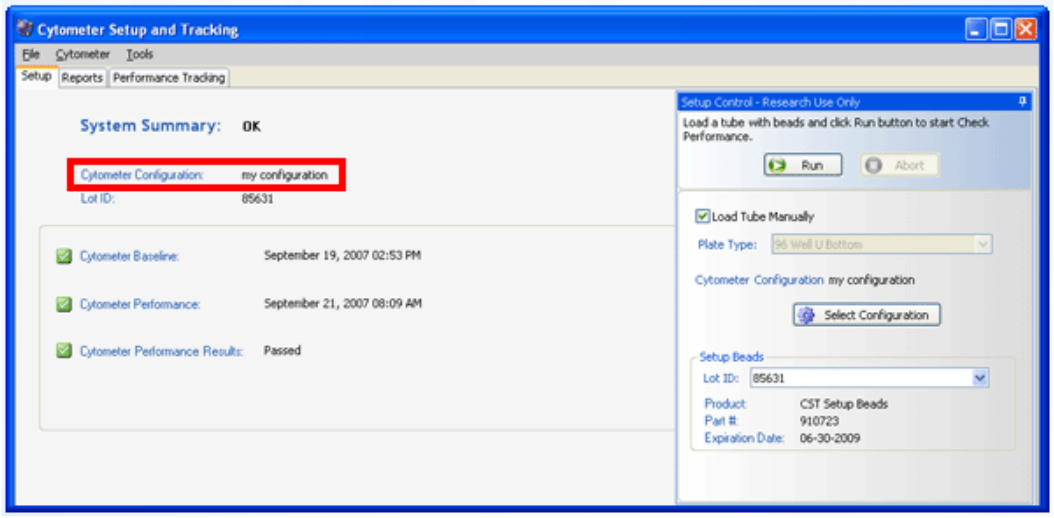
- BD FACSDiva 6 Online Course
- BD Training & eLearning

## 7. Quality Control – Cytometer Setup & Tracking (CST)

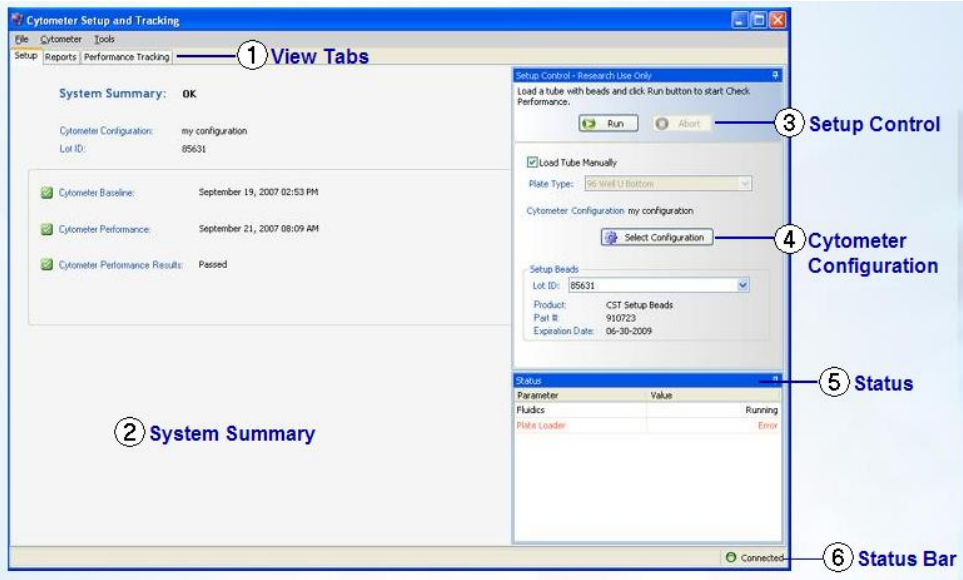
CST beads are used to optimize each PMT. This ensures that the ratio of signal to noise is the highest, and the CVs of the positive peak are the smallest.

### *Launching the CST Workspace*

Select Cytometer CST Menu >

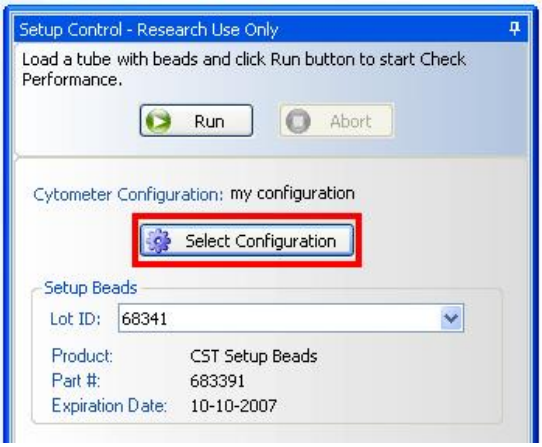


### *CST Workspace Overview*



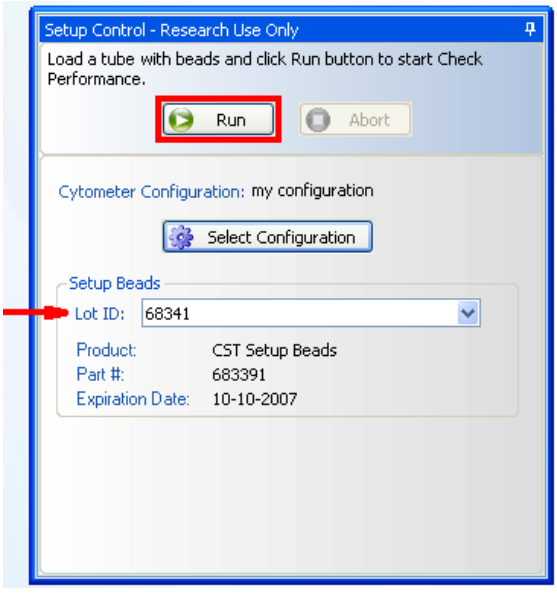
### *Instrument Configuration*

Select the Configuration that matches the optical bench layout (filters & mirror installed on the cytometer). Within the Cytometer Configuration windows, select a configuration from the left-hand side menu and click Set Configuration. Click OK to close the Window. Close CST Workspace.
*Note*: If necessary change filters & mirror to accommodate your experimental staining panel.



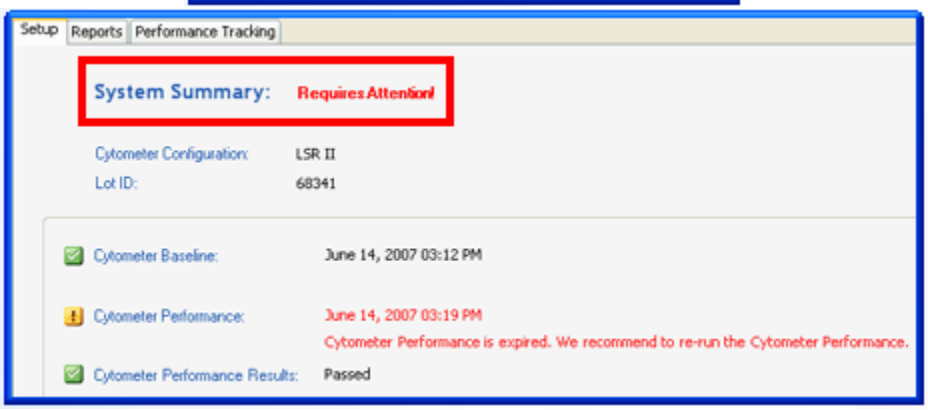

### *Running CST (Performance Check)*

- Prepare the CST beads (1 drop in 350µL of PBS)
- Verify the bead Lot ID is correct
- Select Run mode and Low on the fluidics control panel of the cytometer.
- Click Run in CST Workspace

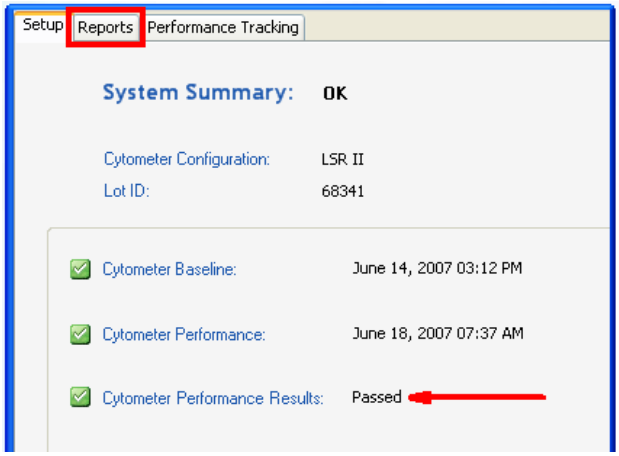


### *CST Results*

Under System Summary, check the system's status.
If the status is 'Requires Attention!', you must run a Performance Check for that configuration.
If the status is 'OK', proceed with setting up your experiment.

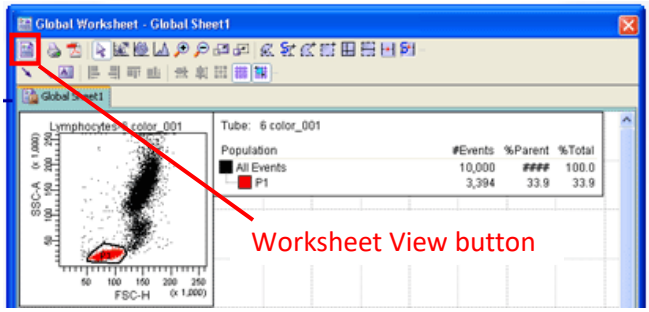




Click the **Report** and troubleshoot any warnings or failures.



## 8. Working with BD FACSDiva Software

Worksheets are where you create plots, gates, population hierarchies, statistic views, and custom text. There are 2 types of worksheets between which you can toggle (Worksheet View button):

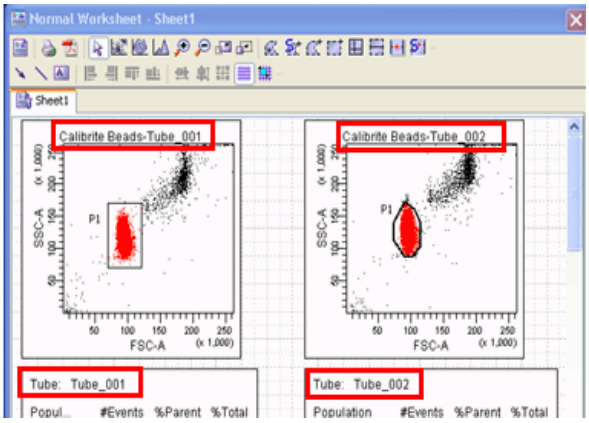


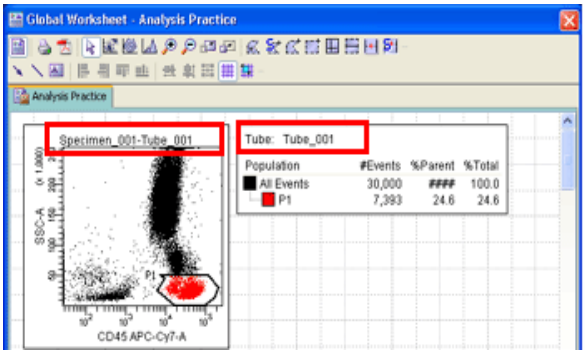

***Global***:

- Displays data from one tube at a time.
- Uses the same set of plots/gates to analyze any tube in an experiment.
- Used to record data using the Loader or HTS options.
- Used to perform batch data analysis.

***Normal***:

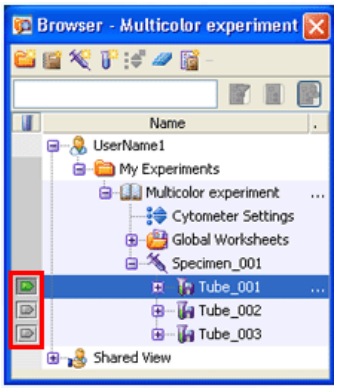
- Used to perform compensation.
- Displays data from several tubes simultaneously.
- Uses different gating strategies (on the same worksheet) for tubes within an experiment.

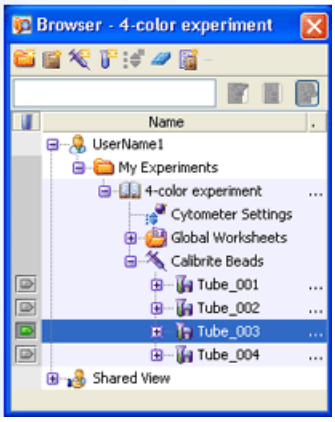


Data displayed in a **Global Worksheet** changes when the current tube pointer is moved to another tube.

Data displayed in a **Normal Worksheet** is not dependent on where the current tube pointer is set. For example, the plots in the graphics to the right display data from tubes 001 and 002. Additionally, you can use different gates and gating strategies for each tubes. Here, the P1 gates in the plots are different.




## 9. Revisions

| SOP version # | Date | Tracked Changes | Primary Reviewer | Secondary Reviewer |
|---|---|---|---|---|
| UT SOP 003.2 | 04.06.2023 | Rearranged set up for training purposes. Moved around pictures. Added additional steps for all around training. | Roman Bandy | |
| UT SOP 003.3 | 10/09/2024 | Added additional information and clarity | Bob Immormino | Ayrianna Hedgecock |
| UT SOP 003.4 | 11/20/2024 | Added a Notes page; Clarified J:drive data management | Bob Immormino | |

## Training Notes

**Start-up:** Check Sheath and Waste levels, Login to workstation with onyen, Start Lasers with BD Coherent Connection 4, Open instrument software; FACS DIVA

**CS&T performance / Quality-control test**: Performed weekdays by staff

**Create an Experiment:** Open a new Experiment or a previously saved Experiment Template (Experiment > New experiment). **Rename!** In the browser select cytometer settings and update for your experiment; Add FSC and SSC height and width and remove un-used fluorescent detectors.

**Create a Compensation Experiment:** Once parameters are set create a compensation experiment (Experiment > Compensation Setup > Create Compensation Controls). Briefly acquire all the controls and adjust PMT voltages to 1. Confirm that fluorophores are brightest in the expected detectors, 2. Put parameters on-scale, 3. Balance spill-over with spreading.

**Calculate Compensation:** After finalizing PMT voltages, collect the compensation controls (need >500 negative and >500 positive events; more is better). Calculate the compensation matrix (Experiment > Compensation Setup > Calculate Compensation); Apply Only.

**Generate a gating scheme and collect data:** Switch to the Global worksheets and add plots to set-up a gating scheme for your experiment. It is handy to take a picture of the PMT voltages with the snipping tool and keep digital notes in notepad. Doing this while on the cytometer will ensure that you have the notes if you need them later!

**Export Data and Templates:** Right-click on your experiment select Export > FCS files. Temporarily store files on the J:drive (\\ad.unc.edu\med\microbiology\Groups\Flow Facility). The J:drive will be cleared automatically every month

**Clean-up:** Contrad, FACSClean, di$H_2O$