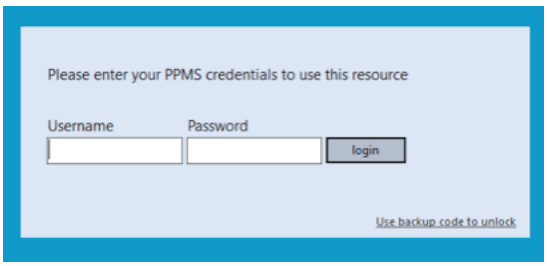
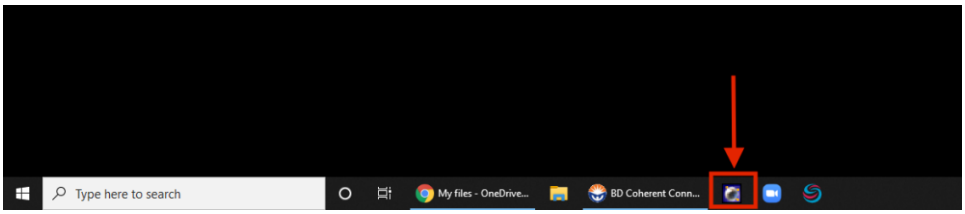
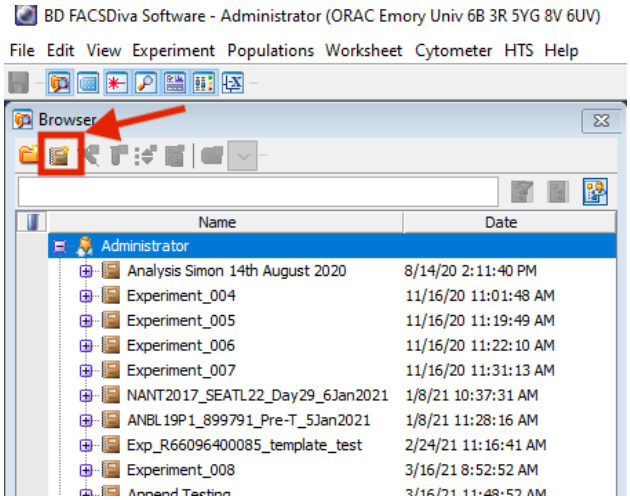
1. Log into PPMS (gray screen) with Emory credentials



2. Launch the Diva software from the icon in the Windows taskbar, select your lab from dropdown menu and hit **OK** to log in (no password)



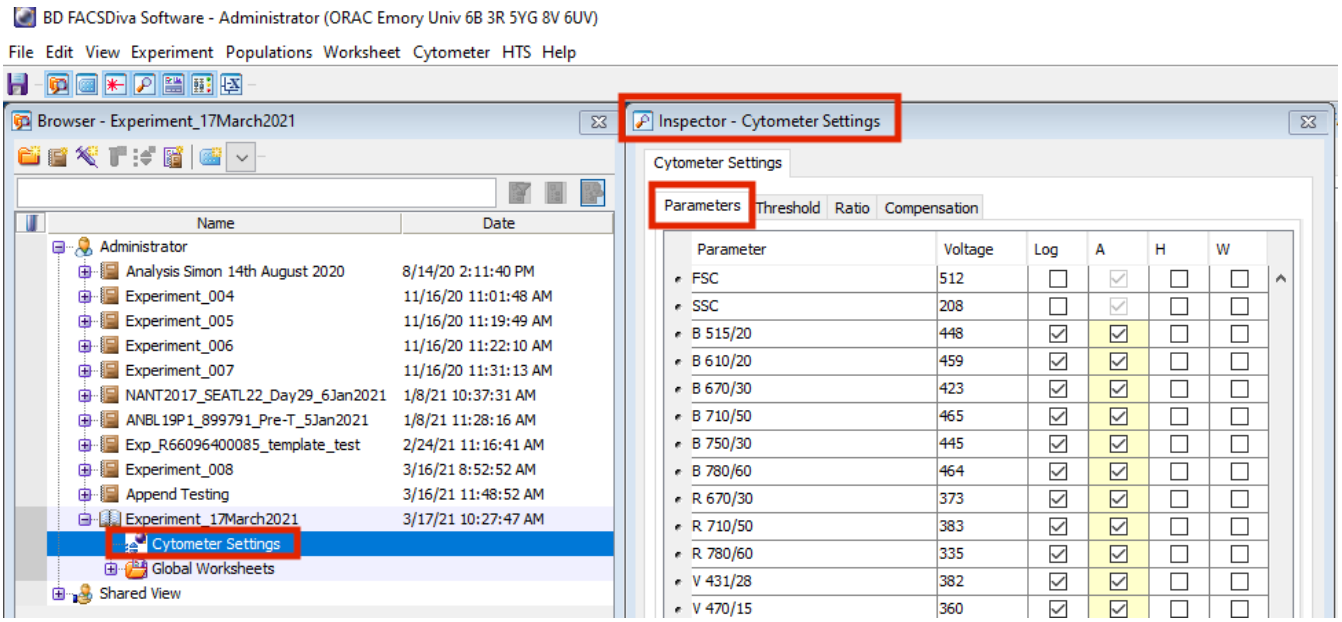
3. In the Diva Browser window, click the file icon to create a new Experiment.



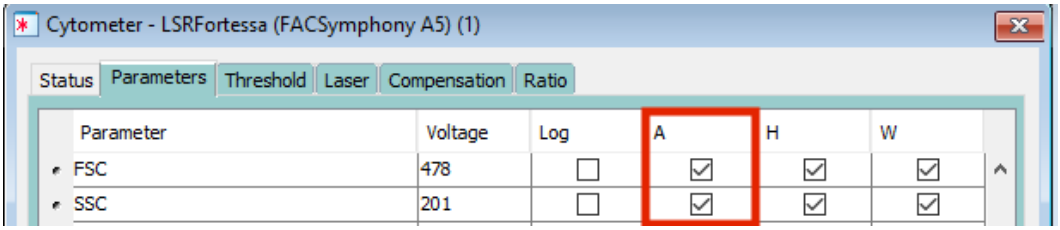
4. Rename the experiment by right-clicking on the experiment name in the Browser or edit the name in the Inspector window.
   Note: Include the date in the experiment name for data retrieval purposes

5. The open book icon to the left of the experiment name indicates the experiment is open.
   Note: to edit or acquire in any experiment in Diva, you must have the open book icon (double-click to open).

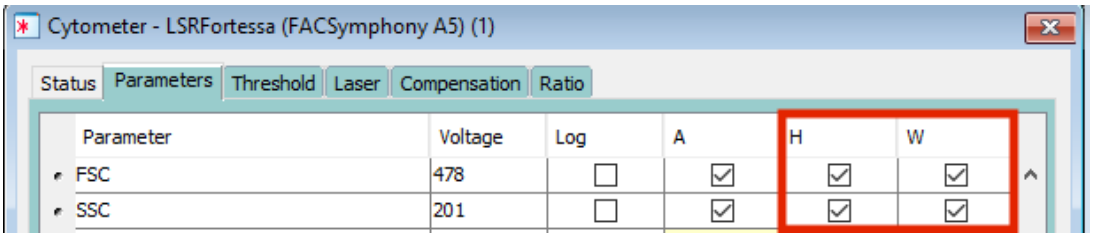
   Click **Cytometer Settings** beneath the experiment name to display the settings in the Inspector window.



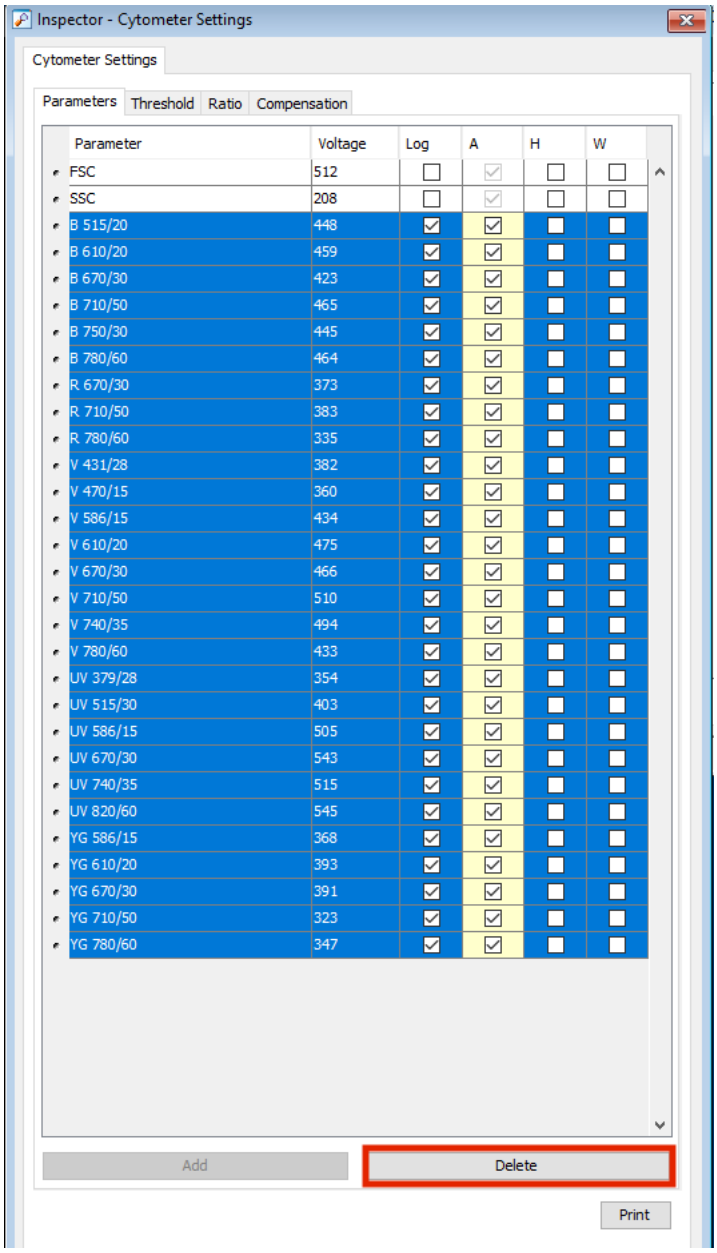
6. Select Parameters
   a. Go to the **Parameters** tab in the Inspector window and select appropriate detectors using check boxes:
      i. FSC and SSC: typically visualized looking at the Area **(A)** of the signal



      ii. Use Log **(L)** for FSC and SSC if acquiring bacteria or small particles
      iii. Collect Height **(H)** and/or Width **(W)** for scatter parameters in addition to Area **(A)** to enable doublet discrimination

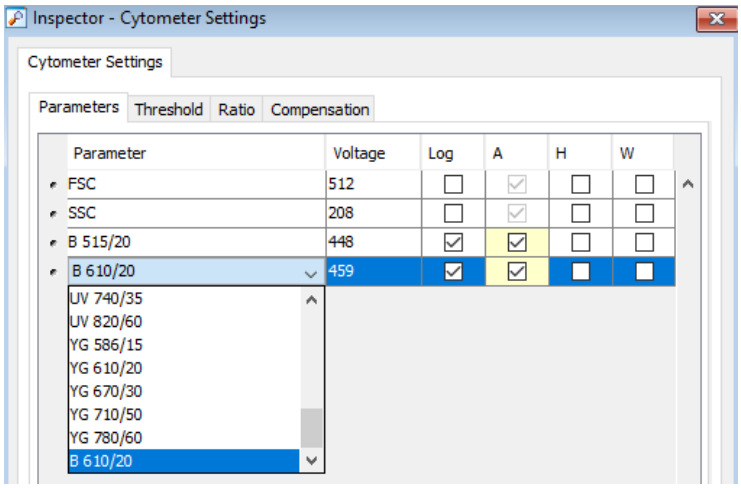


b. Select Fluorescent Parameters (same window as step 4)
    i. It is best practice to delete all fluorescent parameters first and then add back only the necessary parameters:
        1. Highlight all fluorescent detectors: click on dot to the left of the first fluorescent detector (the first detector listed underneath SSC), hold down the shift key, scroll down and click on the dot to the left of the last detector
        2. Select **delete** (bottom right-hand corner of browser window)

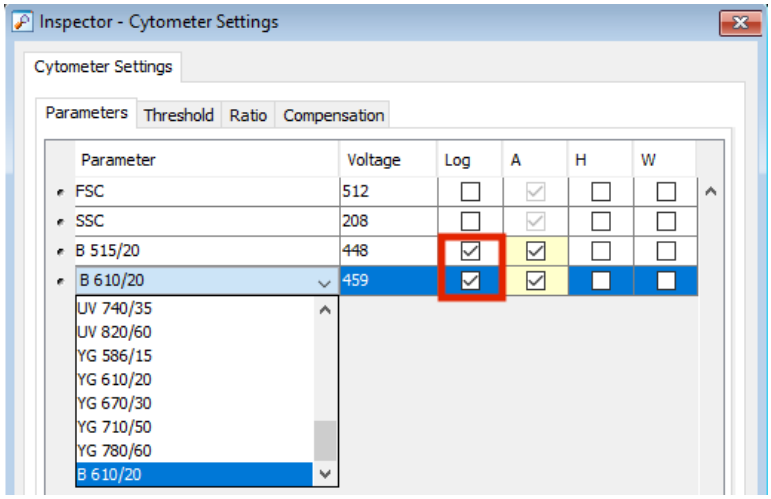


ii. Add back detectors for fluorochromes being used

1. Click the **Add** button (bottom left of window in above step)
2. Select the bandpass filter from the drop-down menu to add a fluorochrome to the list of parameters being collected. Consult the Instrument filter set attached to the SOP for commonly used fluorochromes and corresponding bandpass filters
   Important: The letter before the bandpass filter designates the laser excitation

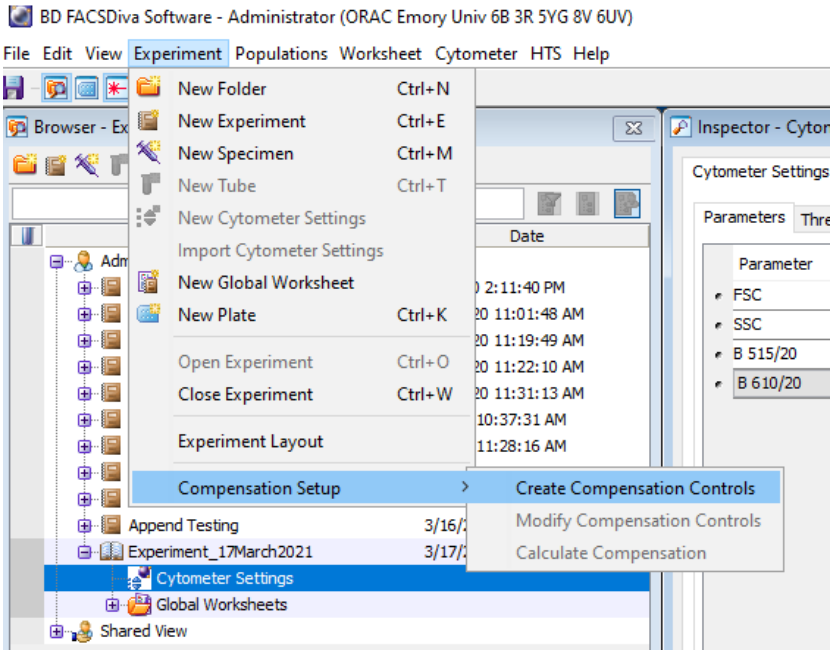


3. The number of fluorochromes in your experiment should match the number of fluorescent detectors in the Parameters tab of the Browser
   Exception: when using open detectors for autofluorescence detection or when acquiring data for spectral analysis
4. For most applications, fluorescent detectors should be set to a Log **(L)** scale (default). For DNA content/Cell Cycle analysis, a linear scale should be used.

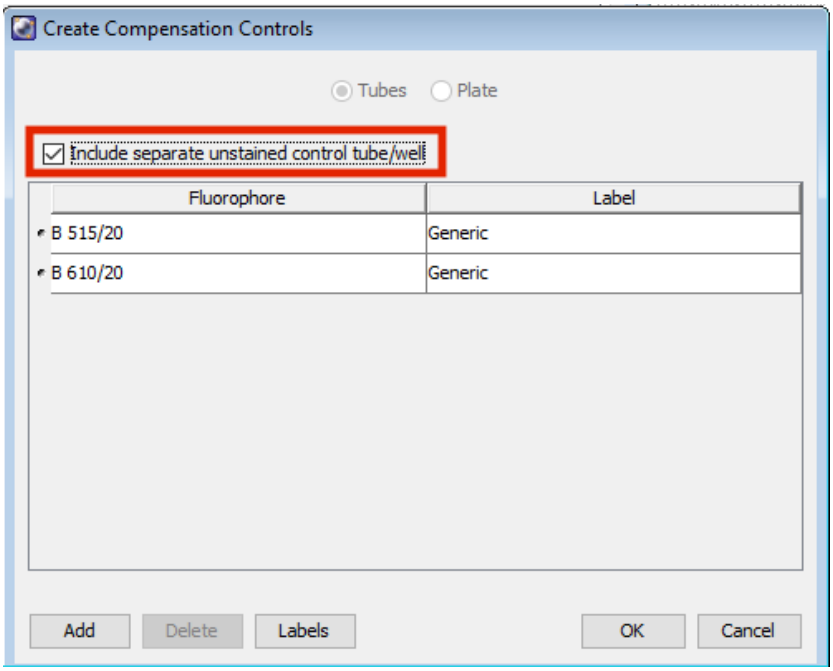


7. Optional - set fluorescent voltages based on bead target values if values have been previously established (see separate SOP: Standardization with bead target values)

8. Set up Compensation (experiments with > 1 color). For one color experiments, skip to step 10.
   a. Go to **Experiment** -> **Compensation** -> **Create Compensation Controls**



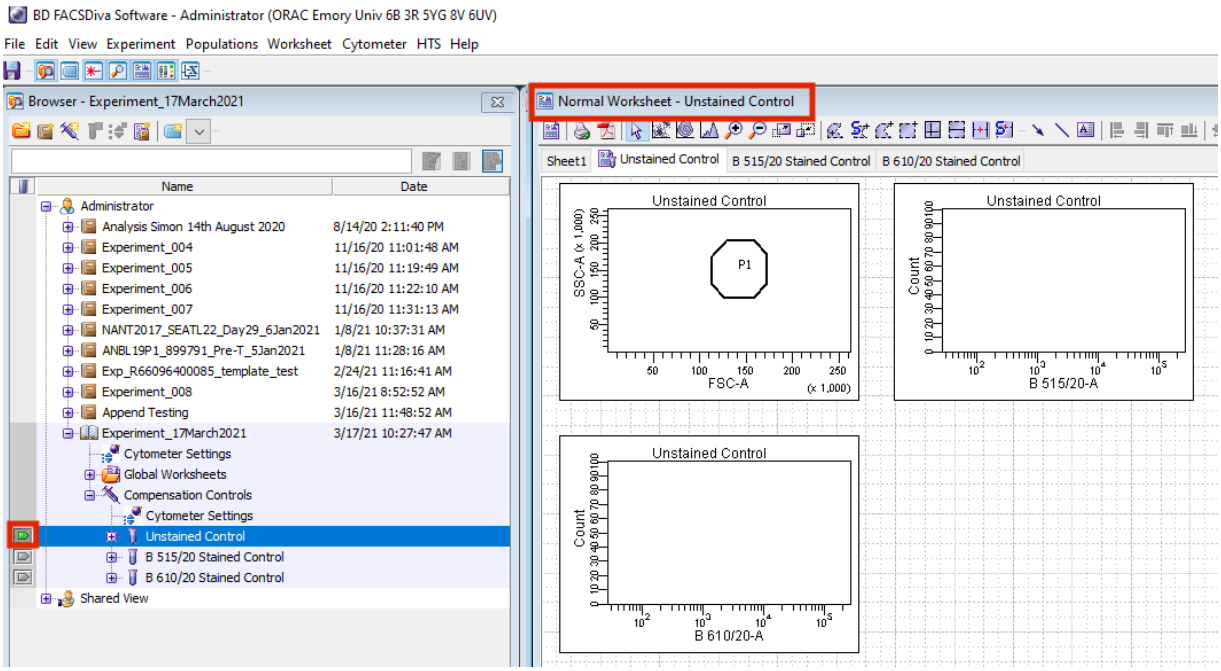
b. Select checkbox for **Unstained control** to use a universal negative



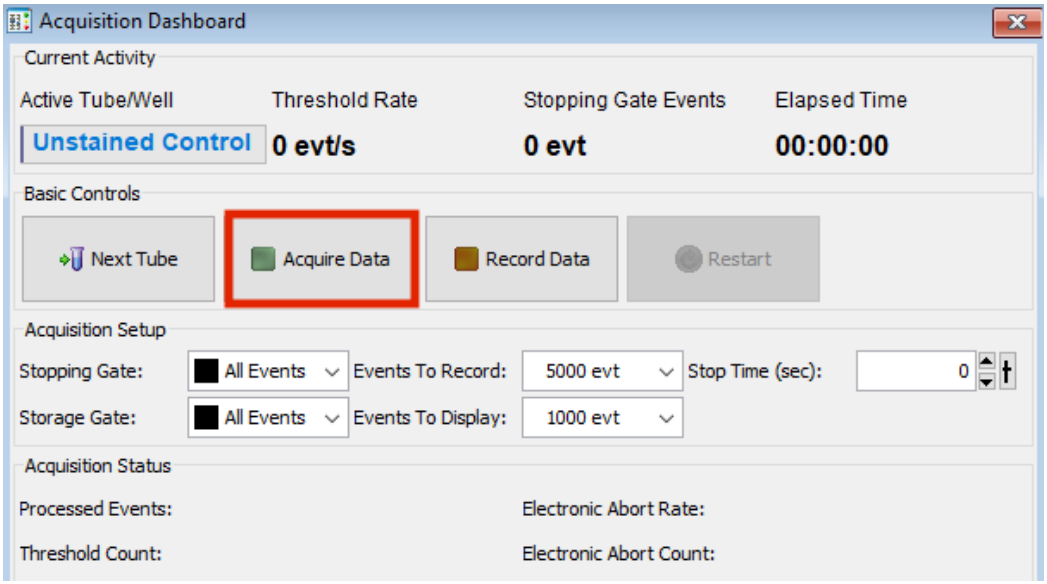
c. Confirm parameters in list match single stain controls to be recorded and click **OK**

9. Run Compensation Controls

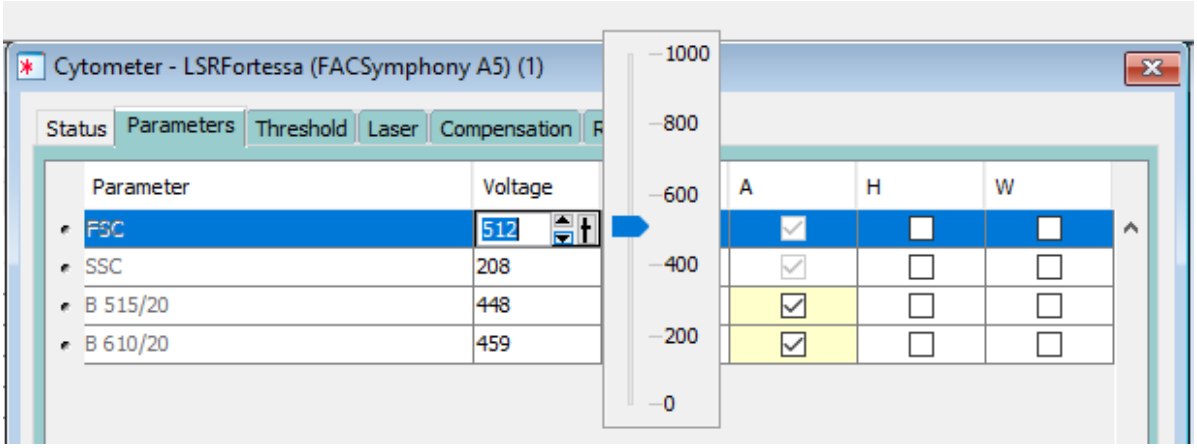
a. Expand the **Compensation Controls** Specimen and select the **Unstained** tube by clicking on the arrow to the left of the tube name (arrow should turn green). A Normal worksheet corresponding to the unstained control tube will appear in the worksheet window.



b. Set the instrument to **RUN** and flow rate to **LO** or **MED** (panel on front left of instrument)
c. Remove DI water tube on SIP by moving the support arm to either side and pulling the tube down
d. Install the unstained compensation control tube and move support arm under the tube
e. Click **Acquire** on the **Acquisition Dashboard** to preview the data without recording a file



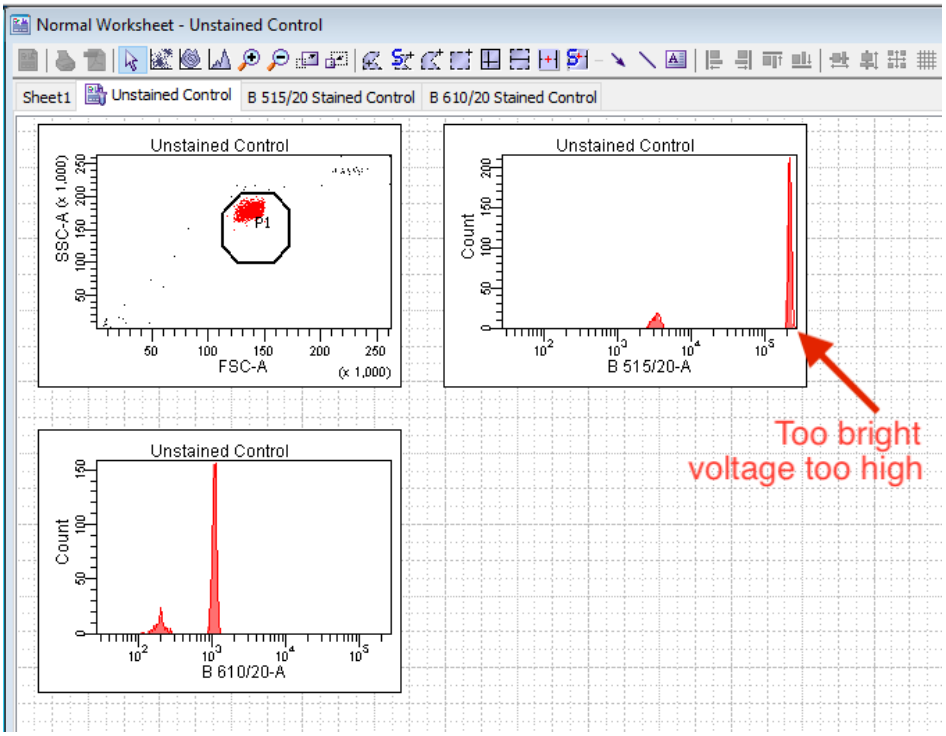
f. Adjust the FSC and SSC voltages to get cells or beads on scale in the **Cytometer** window -> **Parameters** tab

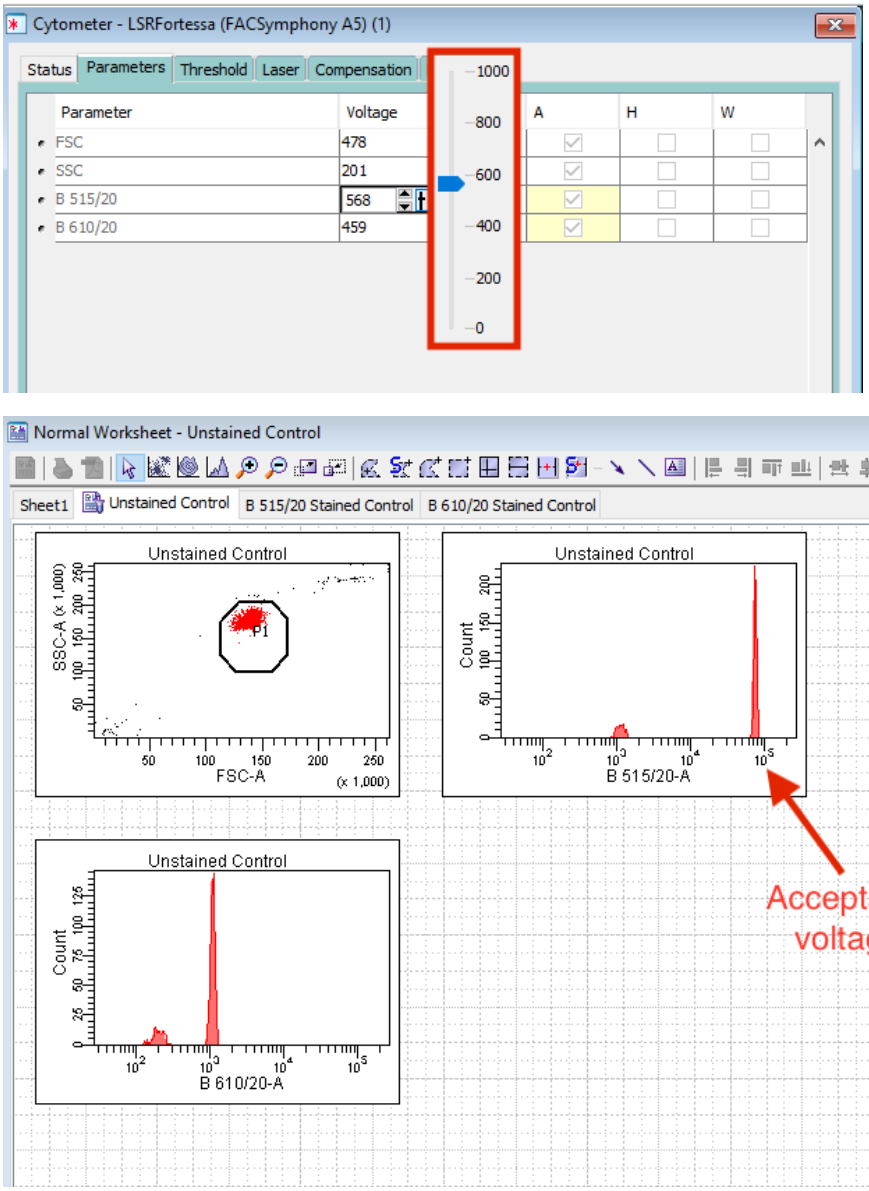


g. Stop acquisition and remove unstained tube from SIP.

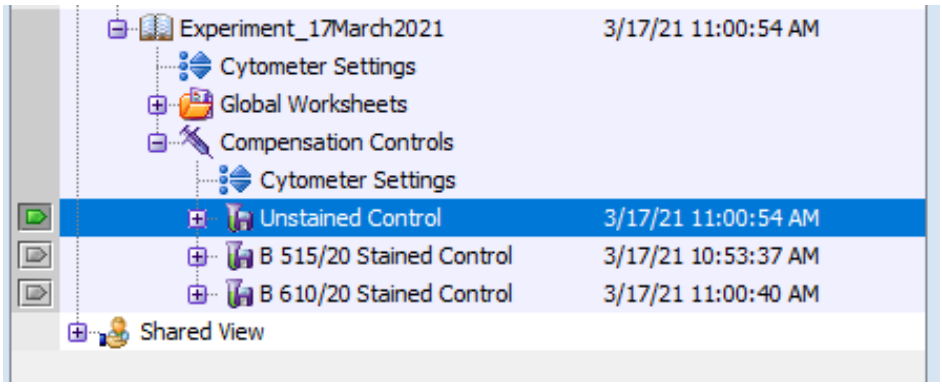
h. <u>While remaining on the unstained control</u> tube, preview each single color control and check that 1) a positive population exists and 2) all positive populations are not so bright that they appear off-scale (events will be visible to farthest point to the right of the histogram if off-scale).

i. Lower the appropriate fluorescent voltage in the **Cytometer** window -> **Parameters** tab while acquiring if population is too bright.

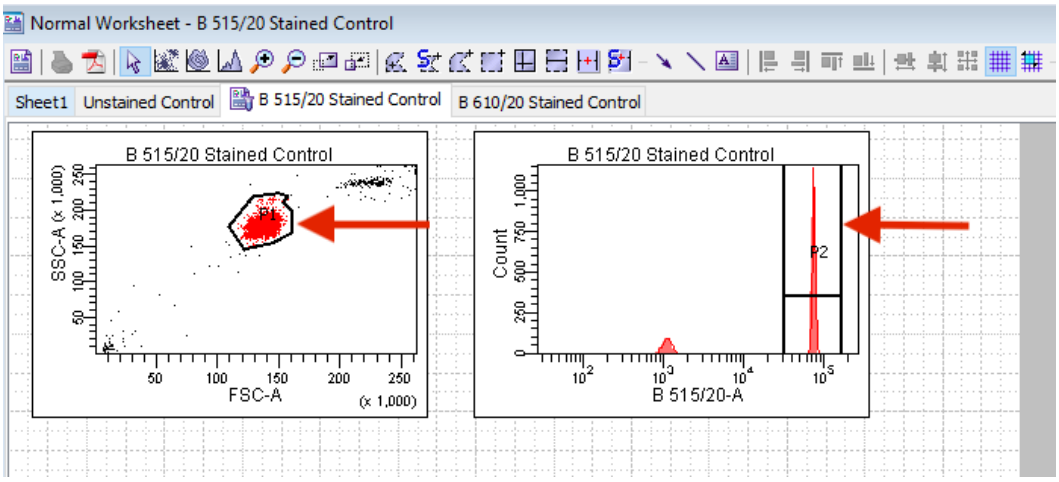




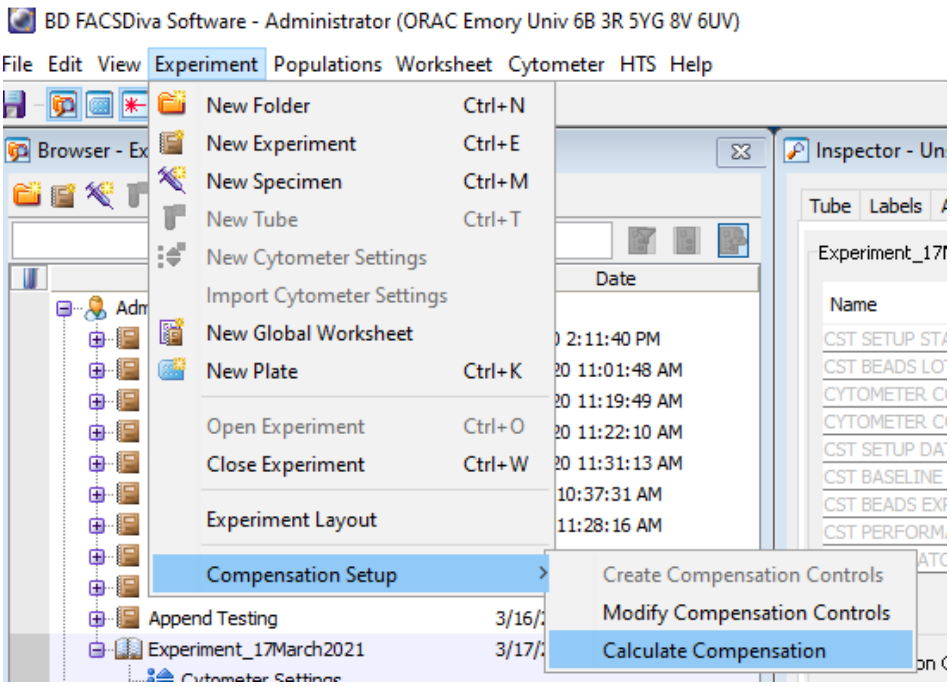
j. Once all compensation controls have been previewed and voltage adjustments made, acquire and record all compensation control tubes. There should be file icon beside each tube indicating it contains data.



i. For each tube, ensure the following:
ii. The P1 gate contains the beads or cells of interest while excluding debris, and
iii. The P2 (?) gate snaps into the correct place over the positive population after the file has been recorded. The P2 gate may be adjusted by manually dragging over the positive population if necessary.



k. Calculate compensation
   i. Go to **Experiment** -> **Compensation Setup** -> **Calculate Compensation**

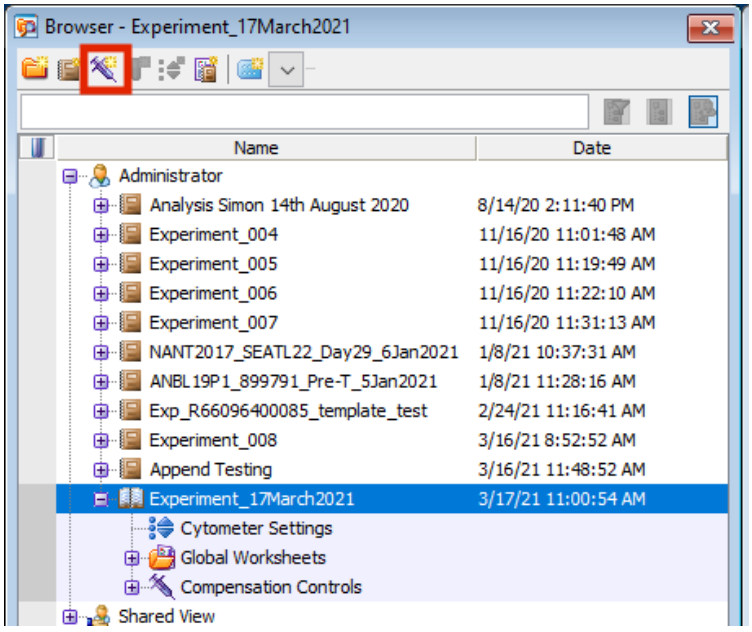


ii. Choose “Apply Only” option

10. Create Experimental Specimens and Tubes
    a. A specimen must first be added to an experiment before tubes can be added

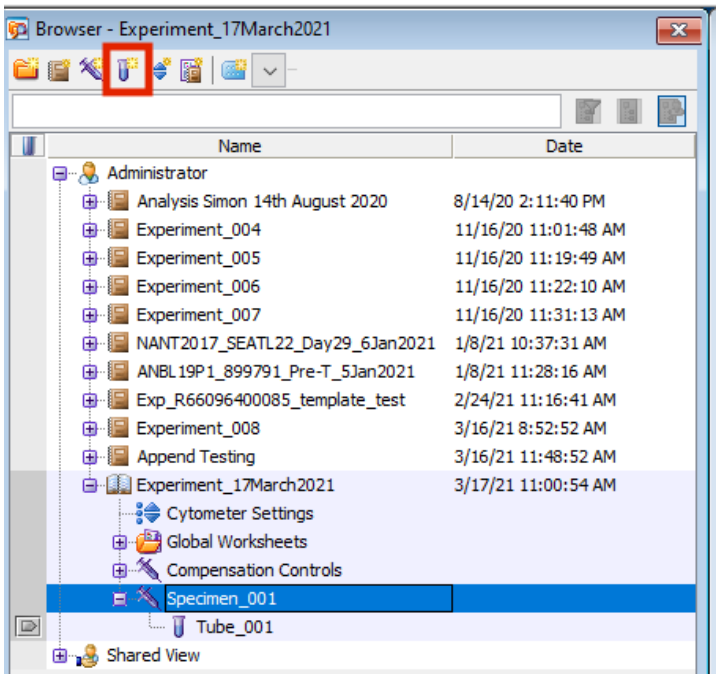
    Note: The filename will be a combination of the specimen and tube name, with the specimen name appearing first in the file name, followed by an underscore and the tube name

    i. Highlight the experiment name and select the syringe icon in the Browser window to add a specimen to an experiment

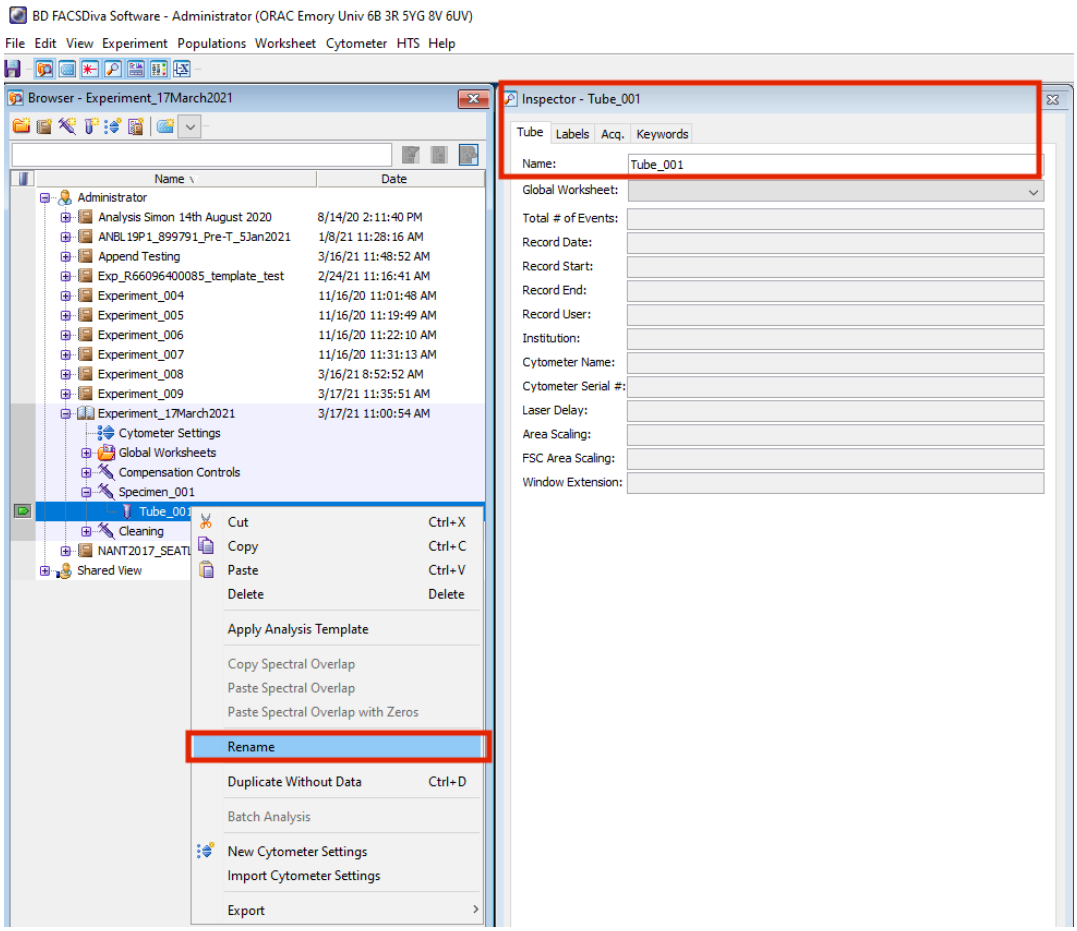


ii. Expand the specimen. By default, a single tube is automatically added to a specimen when created

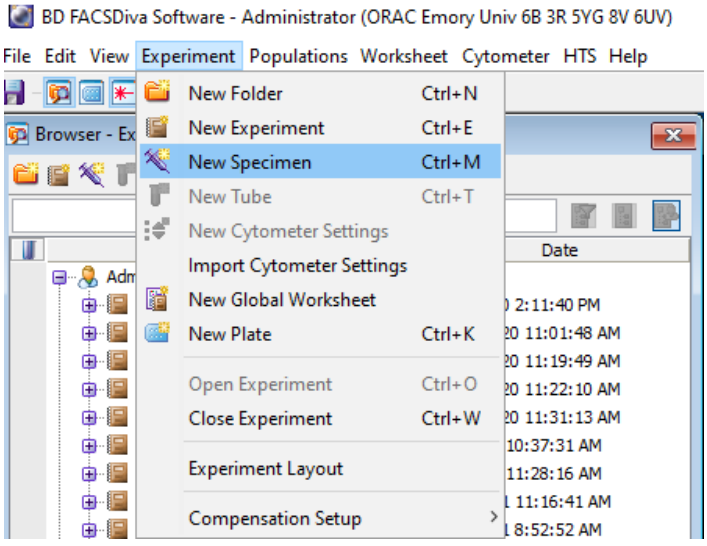
iii. To add more tubes to a specimen, highlight the specimen and click the tube icon in the upper left corner of the Browser window



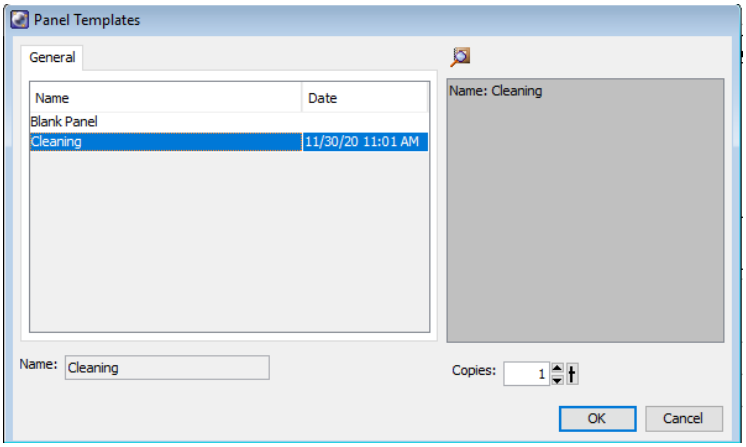
iv. Rename specimen and tubes if desired either in the Browser window or Inspector window



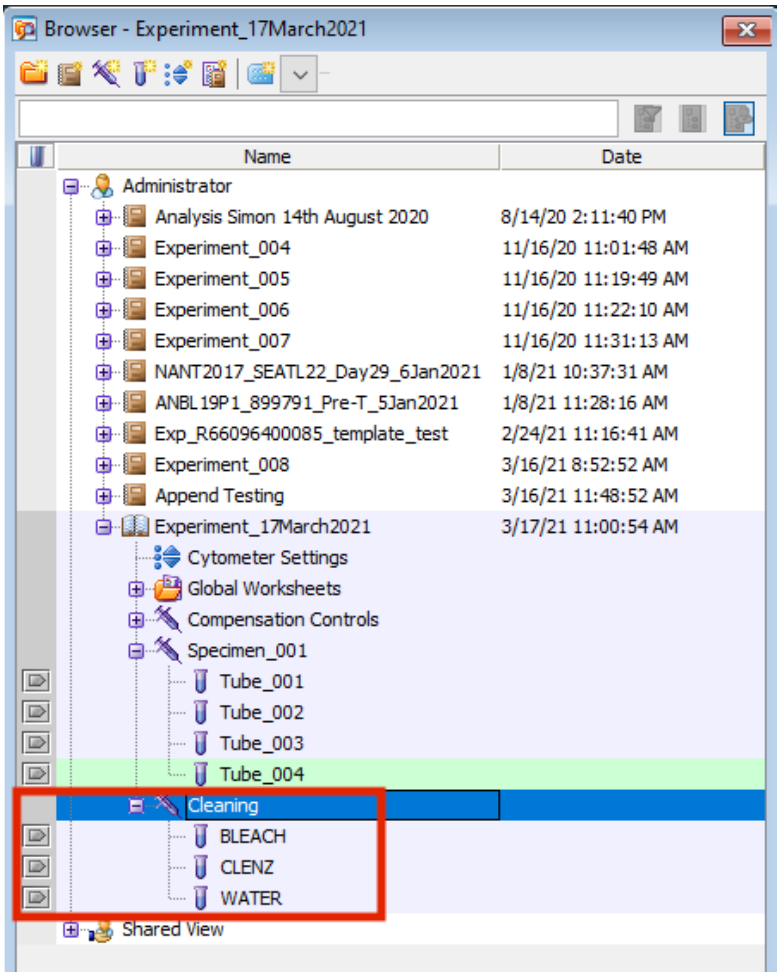
b. Add as many specimens and tubes as necessary for experiment
c. Add cleaning tubes
    i. Go to **Experiment** -> **New Specimen**



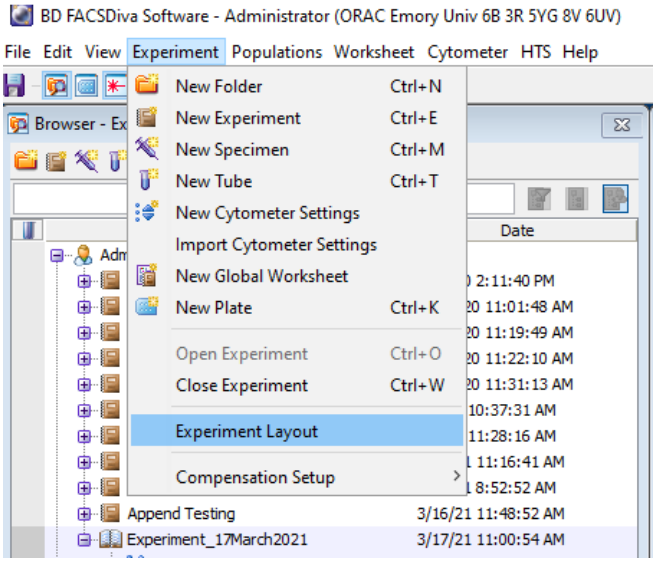
ii. Select **Cleaning** from the Specimen template menu



iii. A Cleaning specimen with 3 tubes is automatically added to the experiment:
   1. Bleach
   2. Clenz
   3. Water



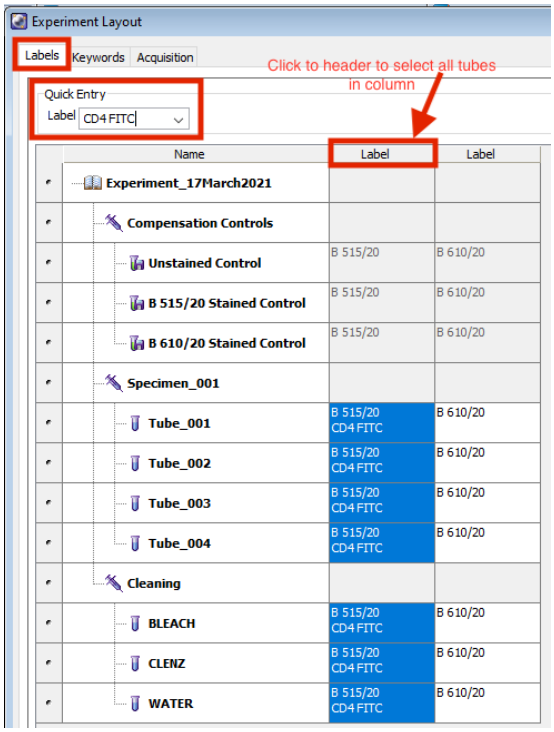
11. Edit the Experiment Layout (optional)
    a. Go to **Experiment** -> **Experiment Layout**



    b. Create labels for parameters

Creating labels adds additional information to the FCS file regarding the markers and fluorochromes in each panel and makes downstream analysis easier, for example “CD4 PE YG 586/15” is added to the detector label in the FCS file instead of just “YG 586/15”

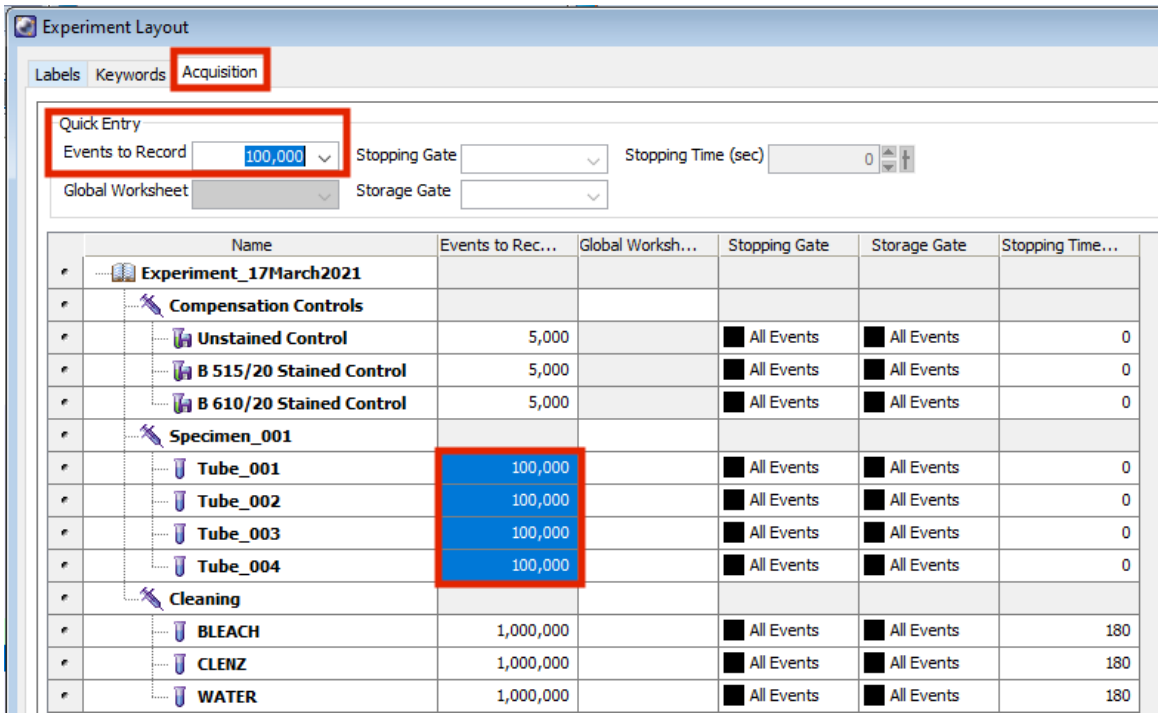
i. Go to **Labels** tab
ii. Click column header to add labels for all tubes at one time or alternatively click individual cells to rename labels for individual tubes
iii. Type the name of the desired label in **Label** box (top left-hand corner of **Experimental Layout** window while the cells are highlighted, e.g. CD4 PE, and hit enter



c. Set acquisition criteria

i. Go to the **Acquisition** tab in the **Experiment Layout** window
ii. Set events to record for each tube by clicking on column header to select all tubes at a time, clicking on individual cells, or holding down **Ctrl** on the keyboard and selecting multiple cells at a time
iii. With cells selected, type desired number of total events to record in the **Events to Record** box at the upper left-hand corner of the experimental layout under the words “**Quick Entry**”

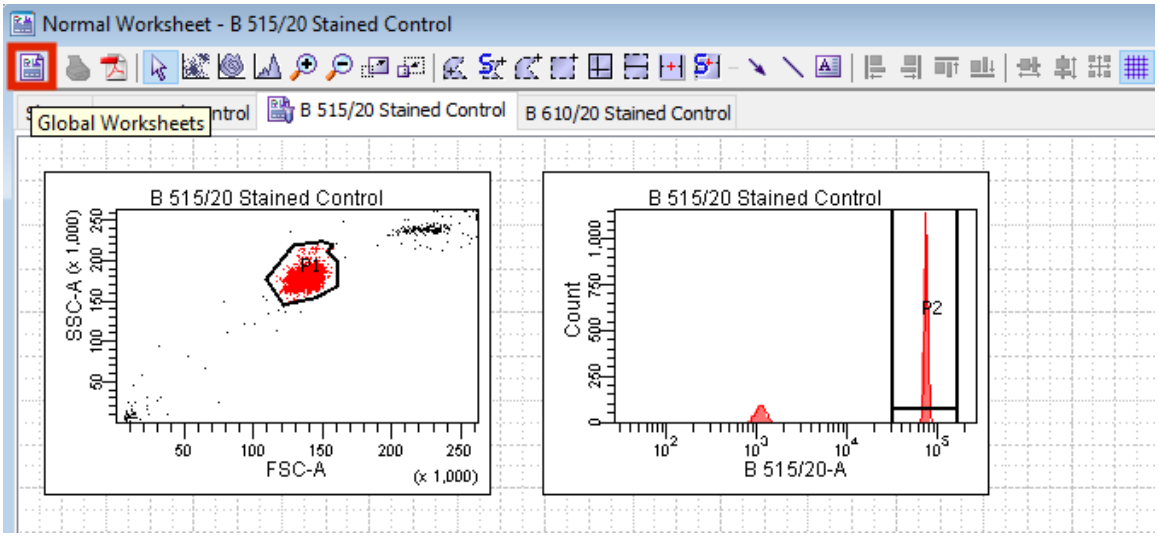
iv.



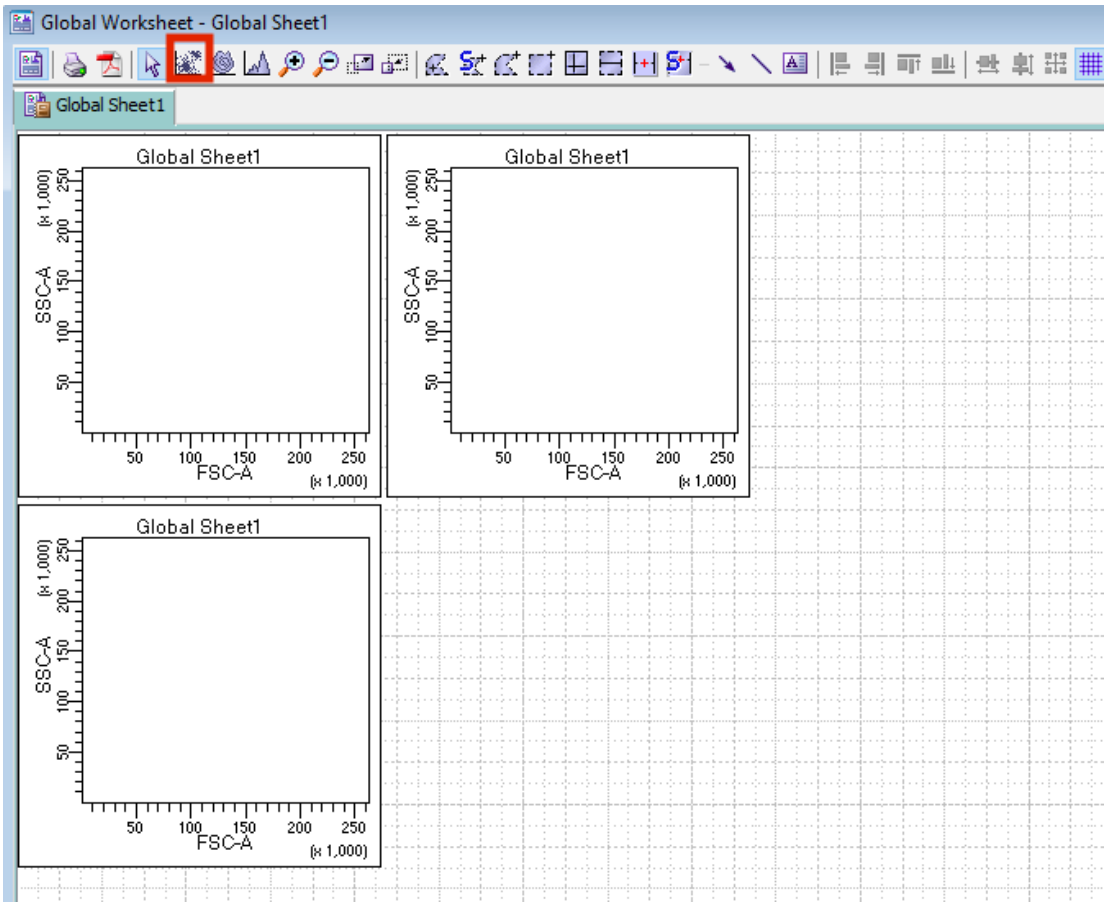
Name	Events to Rec...	Global Worksh...	Stopping Gate	Storage Gate	Stopping Time...
Experiment_17March2021					
Compensation Controls					
Unstained Control	5,000		All Events	All Events	0
B 515/20 Stained Control	5,000		All Events	All Events	0
B 610/20 Stained Control	5,000		All Events	All Events	0
Specimen_001					
Tube_001	100,000		All Events	All Events	0
Tube_002	100,000		All Events	All Events	0
Tube_003	100,000		All Events	All Events	0
Tube_004	100,000		All Events	All Events	0
Cleaning					
BLEACH	1,000,000		All Events	All Events	180
CLENZ	1,000,000		All Events	All Events	180
WATER	1,000,000		All Events	All Events	180

d. Select **OK** (bottom right-hand corner of window) to exit the Experiment Layout

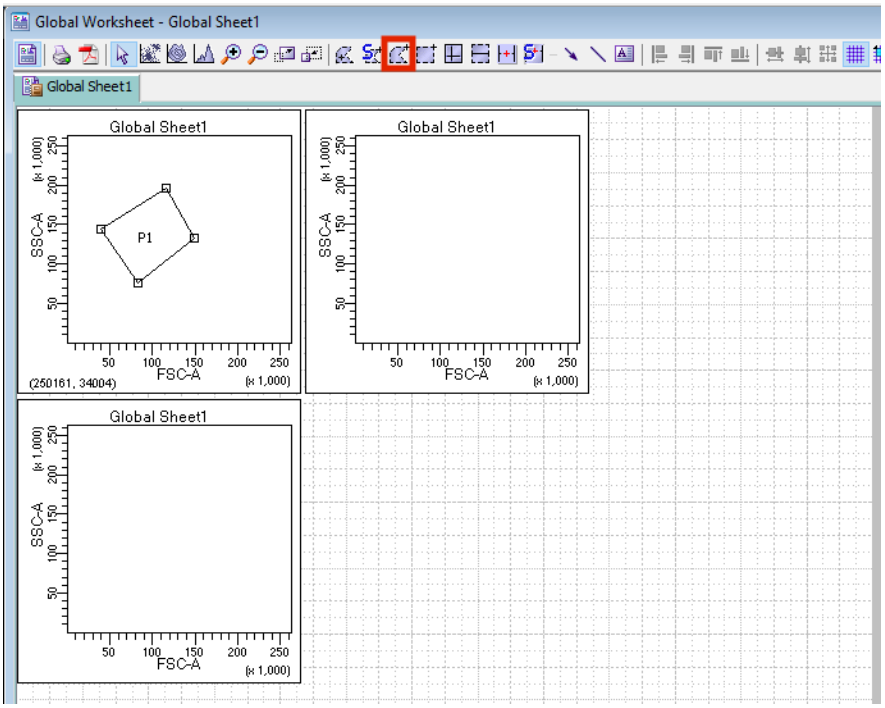
12. Set up the Global Worksheet to visualize data while acquiring
    a. Switch from the normal worksheet to the global worksheet by clicking the Worksheets view button in the Worksheet toolbar (top left-most button)
    Note: Plots in the *global worksheet* show data for the tube that is selected with the current tube pointer. Plots in the *normal worksheet* are tube-specific (only data recorded in the selected tube can be displayed.



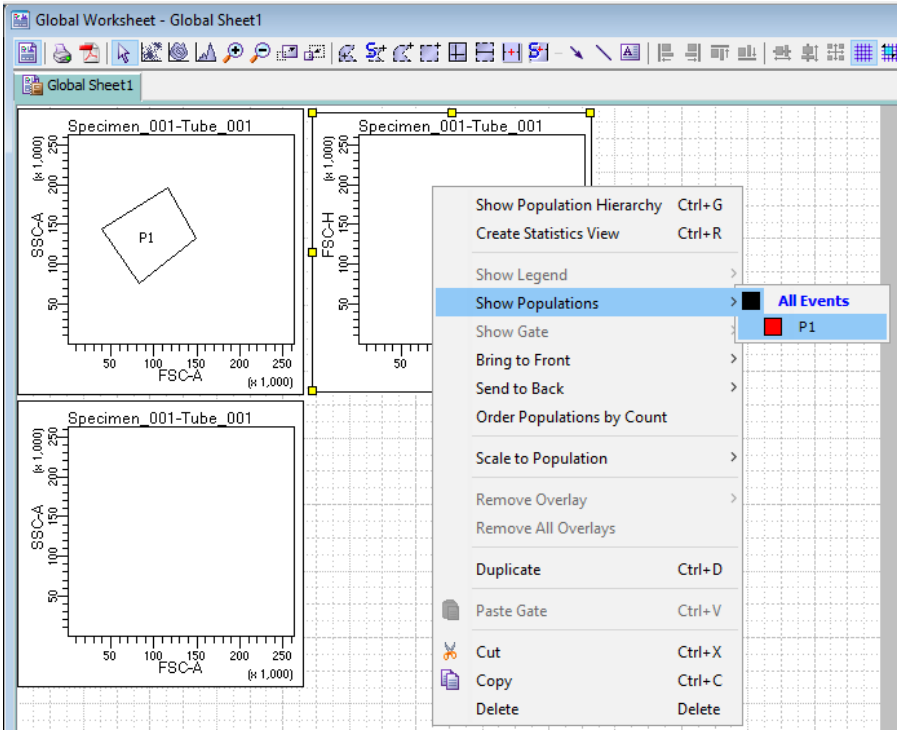
b. Create plots or histograms by selecting an option in the Worksheet toolbar and then clicking in the worksheet. Change parameters displayed on axes by right-clicking on each parameter name.



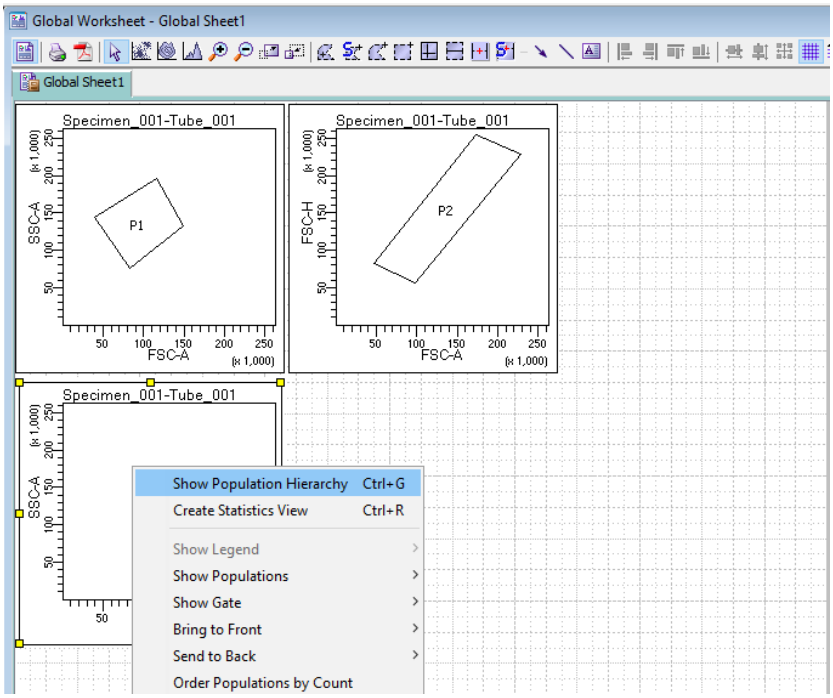
c. Create regions in plots and histograms using the tools in the Worksheet toolbar

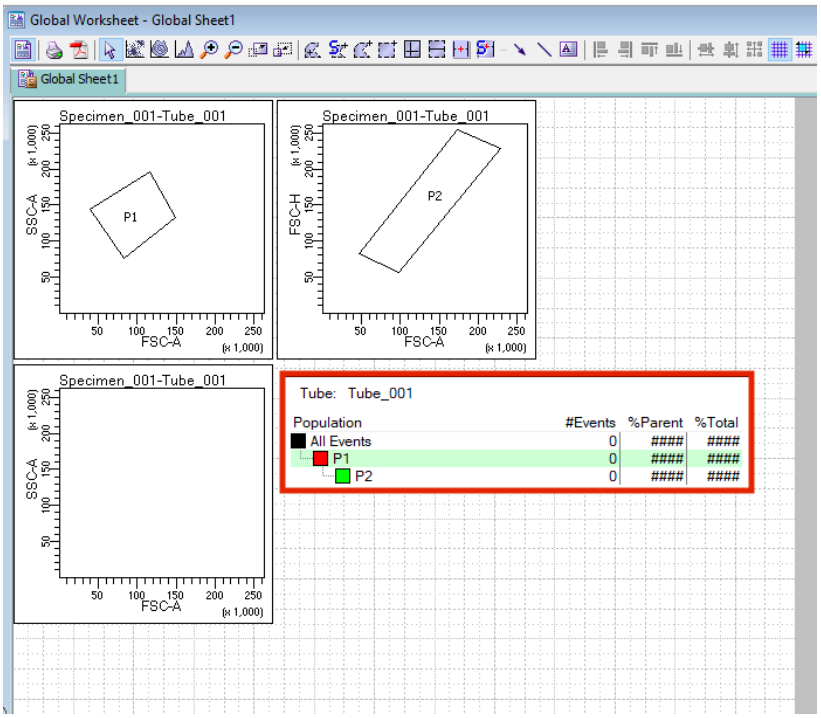


d. Gate histograms on populations of interest by right-clicking inside the white space on the desired plot to be gated and selecting the wanted region from **Show Populations**

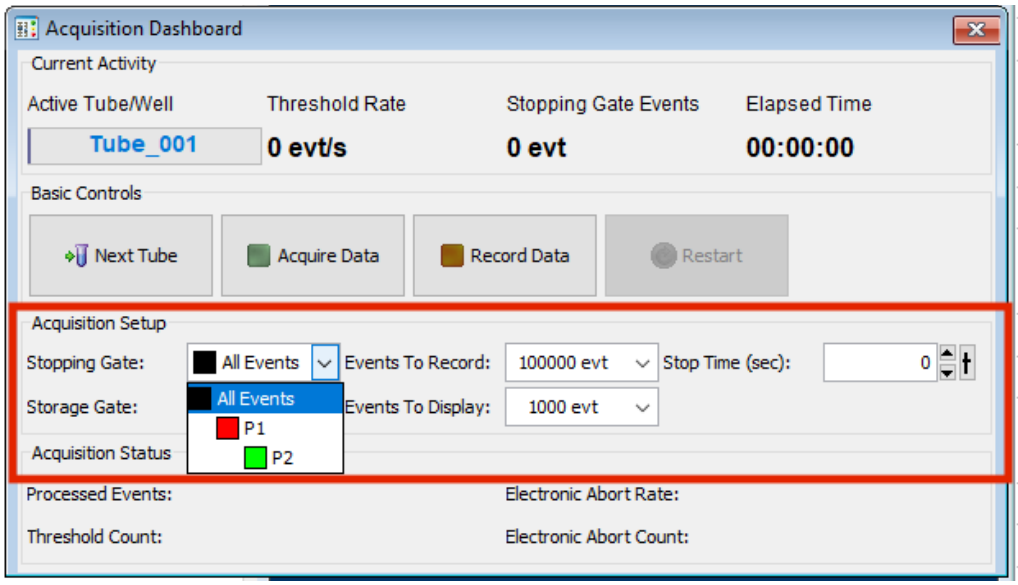


e. Create a population hierarchy by right-clicking on any plot and choosing **Population Hierarchy**

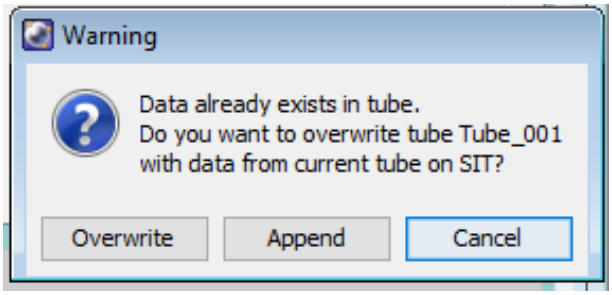




13. Acquire samples
    a. Set acquisition limits by either going to **Experiment** -> **Experiment Layout** -> **Acquisition** tab to set limits for all tubes at once or set the limits manually, before recording each tube, by editing values in the Acquisition Dashboard



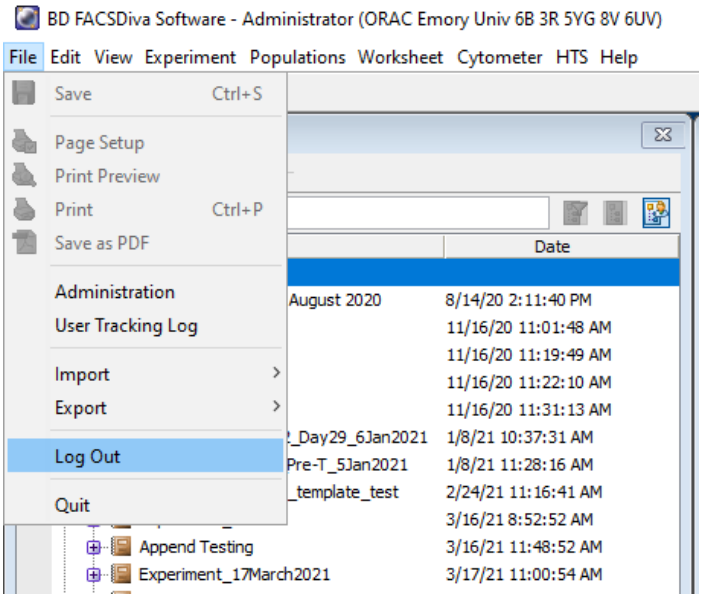
    b. To overwrite a previously recorded file/tube, start acquiring with the desired tube to be overwritten selected, select **Record** in the Acquisition Dashboard, and select **Overwrite** when prompted
    c. To append (add) to a previously recorded file/tube, start acquiring with the desired tube to be appended to selected, select **Record** in the Acquisition Dashboard, and select **Append** when prompted



14. Clean Instrument (transfer data while cleaning – see step 15)
    a. Use the cleaning specimen created in Step 10c and following the cleaning section in the SOP
    b. Put the instrument in **Standby** with tube of DI water on the SIP (arm under tube)
    c. Check the instrument schedule online and if you are the last user of the day, shut down the instrument by hitting the green power button on right-hand side of instrument.

15. Transfer data
    a. Go to File Explorer in the Windows toolbar and navigate to **Desktop** -> **FCSfiles shortcut** -> folder with today's date
    b. Open Chrome and log into OneDrive (bookmarked). Drag your experiment folder to OneDrive and ensure all files have transferred before logging out of OneDrive and closing the browser.

16. Log out of Diva



17. Log out of PPMS