Workflow for FACSCanto instruments

Condensed workflow

1. Turn on the cytometer.
2. Inspect fluid levels in sheath and waste tanks, replace/empty if needed.
3. Run the Cytometer>Fluidics Startup.
4. Confirm that the filter layout is correct.
5. Log in to your DiVa software account.
6. Duplicate an old experiment or create a new one.
7. Activate all the parameters you need.
8. Create plots to visualize all parameters.
9. Set optimal voltages using appropriate controls.
10. Record data for single color compensation controls for multicolor experiments.
11. Record data for the samples.
12. Clean the instrument (2 min each 10% Bleach, 2% Detergent, Sheath)
13. Run the Cytometer>Fluidics Shutdown.
14. Export the data and make sure to take it with you.
15. Log off Diva software.
16. Turn off the cytometer if last user for the day.
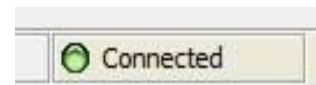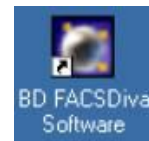
Detailed workflow

1. **Turn on the cytometer.**
   1.1. The instrument requires 10 minutes of 'warm-up time', which it will begin counting down once turned on and connected to FACSDiva. Fluidics startup, filter check and experiment creation can all be done during this warm-up.
   1.2. Turn on the cytometer - power button is on the left hand side.

2. **Inspect fluid levels in sheath and waste tanks, replace/empty if needed.**
   2.1. Check the weight of the sheath and waste tanks. If the waste is full and/or the sheath is empty, dump the contents of the waste tank into the sink, refill waste tank with bleach and refill the sheath tank with sheath fluid. Make sure the tubing is not kinked.

3. **Log in to your DiVa software account**
   3.1. Login to the Windows 7 computer (PW: BDIS)
   3.2. Launch FACSDiVa from desktop icon
   3.3. Log in to FACSDiVa using your Kerberos ID
   3.4. First time users: your password is the same as your Kerberos ID (Example KID: mdg250, password: mdg250). Please change it after you log in to FACSDiVa the first time (under Administration tab).
   3.5. Wait for the software to connect to the cytometer
      - It is connected when there is a green light in the lower right corner of Diva window beside the word "Connected."
      - Make sure that the instrument also shows as "Connected" in Inspector.
   3.6. When the "CST Mismatch" prompt comes up, choose "Use CST settings."

4. Run Fluidics Startup.

4.1. This is under the Cytometer file menu and will take about 6 minutes.
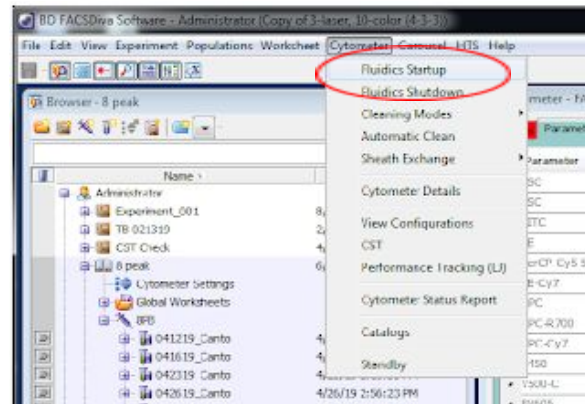
5. Confirm that the filter layout is correct

5.1. Use the printouts on optics cabinet and the labels on the detectors for reference

6. Experiments are either created from scratch (6.1) or duplicated and adapted from an old one (6.2)

6.1. Create a new experiment.
   - It will open automatically. Remember that an open experiment should have an open book icon 📖 beside its name as opposed to a closed book icon 📕 .
   - New experiments load the voltage settings that come from CS&T and are ideal for instrument operation. However, these are not related to your specific cells, and often need significant changes (especially to FSC).

6.2. Open and old experiment, right click on it and select 'Duplicate without Data.'
   - Duplicated old experiments have the voltage settings that were optimal on the day the original experiment was created. These will need adjusting.

6.3. Name (or rename) the experiment.

6.4. Click on 'Cytometer Settings' in your experiment and select your parameters in the Inspector window.

- You can change the parameters you need by clicking on a parameter name in the Parameters tab and selecting a different parameter from the drop down menu.

Parameter	Voltage	Log	A	H	W
FSC	442	☐	☑	☐	☑
SSC	325	☐	☑	☐	☑
FITC	387	☑	☑	☐	☐
PE	477	☑	☑	☐	☐
PE-Cy7	698	☑	☑	☐	☐
BV421	553	☑	☑	☐	☐

- Additional parameters can be added or deleted by selecting them and using the buttons at the bottom of the box. Keep in mind that the data for the deleted parameters is not recorded.
- For unlisted parameters, refer to the list of detectors located on the desk near the keyboard or on our website to determine which detector should be used for your fluorophore. Activate the parameter that belongs to that channel and is present in the parameter list (for example, activate the FITC parameter for GFP).
- Make sure the FSC and SSC parameters do not have "Log" checked unless you are sure you need one or both of them to be recorded in log.
- **Make sure to check W (width) for FSC & SSC to allow exclusion of doublets and clumps.**

7. **Create plots to visualize all parameters on global worksheet**
   7.1. Expand the global worksheet folder and click on the global worksheet.
   7.2. Create plots in the right window of the software (worksheet) by clicking

   or buttons and then clicking on the worksheet space. Change the parameters displayed in the plot by clicking the axis label in a plot and selecting a parameter from the drop down menu.

   - Plots created on global worksheets are displayed for all tubes in an experiment; plots created on normal worksheets are displayed only for one tube.

7.3. Always include a plot showing forward scatter area vs. side scatter area.
  - You don't have to create the entire gating strategy at this point. It is sufficient to represent each parameter on a plot so you can see that everything is on scale when you set voltages.

7.4. Below is an example of a global worksheet with plots sufficient for a 2-color experiment.
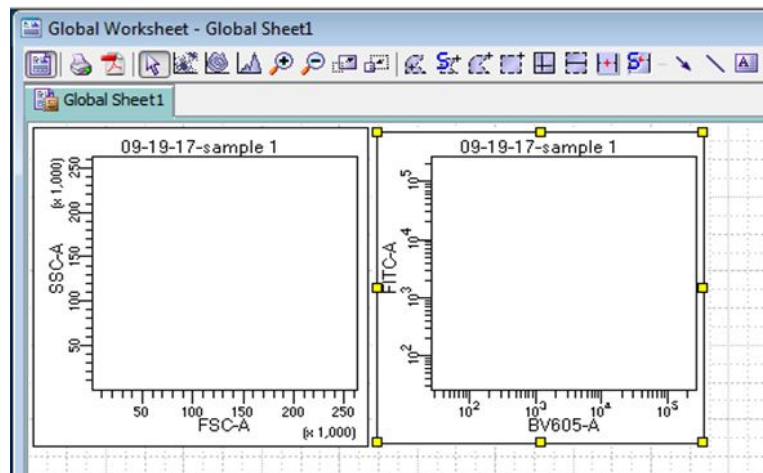
8. **Set up for automatic- or manual- compensation for multicolor experiments.**
  - Automatic compensation is recommended but only possible when you have a good set of single color controls. Manual compensation may be possible when you have non-ideal compensation controls and are assisted by a trained cytometrist.
  - You don't have to calculate compensation while at the instrument. Instead, you can just record data for single color controls and perform an automatic compensation calculation later in analysis software such as FlowJo or FCS Express. We recommend this approach because it is more time efficient.

8.1. If you choose to compensate at the instrument, you can use DiVa automatic compensation process or compensate manually. If you choose to use automatic compensation:

8.1.1. Select Experiment > Compensation Setup > Create Compensation Controls.

- This will generate a 'Compensation Controls Specimen,' which contains tubes for each parameter being assayed. These tubes are linked to Normal Worksheets that are tube-specific. These worksheets come pre-populated with plots for each parameter.

- The option to select an unstained control will generate another tube that is meant for your unstained cells. The worksheet generated has histograms for all parameters and is ideal for setting voltages. Voltages can be set using these tubes and worksheets or using the global worksheet and a non-'Compensation Control' tube.

- Instructions for completing auto-compensation are found below in section 10.3.

8.2. Create a specimen ⚗ and a tube 🧪 in the specimen, or you can choose to create an experiment with a specimen and a tube from a template.

- Duplicated experiments will already contain some specimens or tubes. These can be deleted and new ones made. If existing tubes or specimens are used it is very important that you confirm that these tubes do not contain any settings files, as these may not be overwritten by any changes above.

8.3. Name (or rename) the specimen and the tube so that exported data files have meaningful names (naming system for exported data is specimen_tube).

8.4. The browser panel for an experiment that is open and ready for use should look similar to the image below (auto-comp experiments will contain another specimen called 'Compensation Controls').
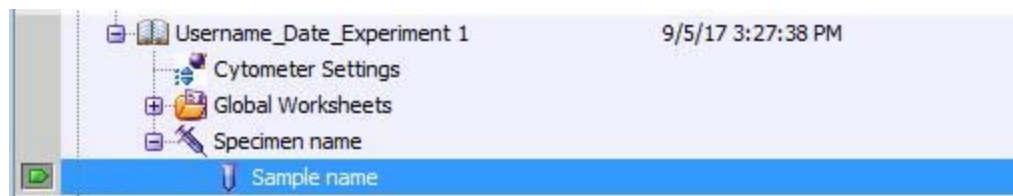
9. **Set optimal voltages using appropriate controls**
   9.1. Select the tube (this may be either a regular tube in your specimen, or one generated from the Compensation Controls specimen).
   - Make sure that tube pointer (the little arrow to the left of the tube) is green by clicking on the arrow.
   9.2. Check if the tube is a polystyrene 5 mL tube appropriate for the instrument and is not cracked.
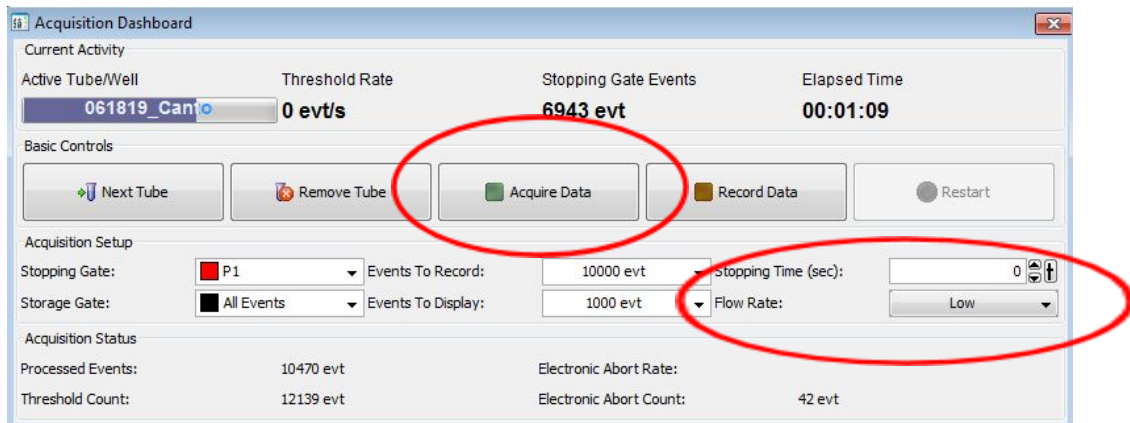

   9.3. Vortex (if able) and mount a fully stained sample, press Acquire Data in the Acquisition Dashboard. Acquisition should proceed with a low flow rate if possible.
   9.4. In the Cytometer panel, Parameters tab, adjust the voltages for each parameter so that all populations on scale.


NYU Langone DART Cytometry and Cell Sorting
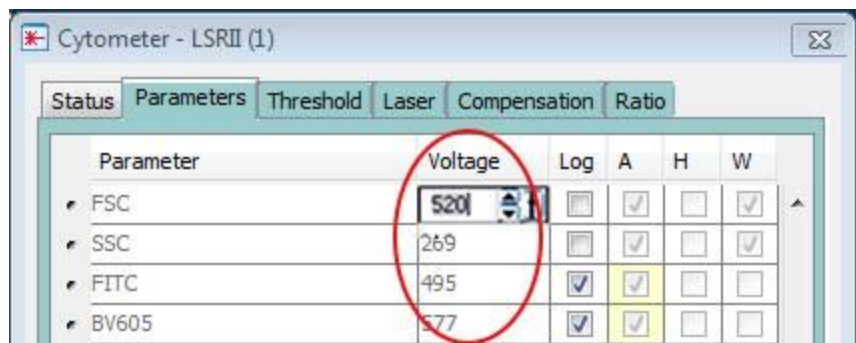
9.5. For forward and side scatter, adjust the volume until the main cell population is around the center of the plot area.
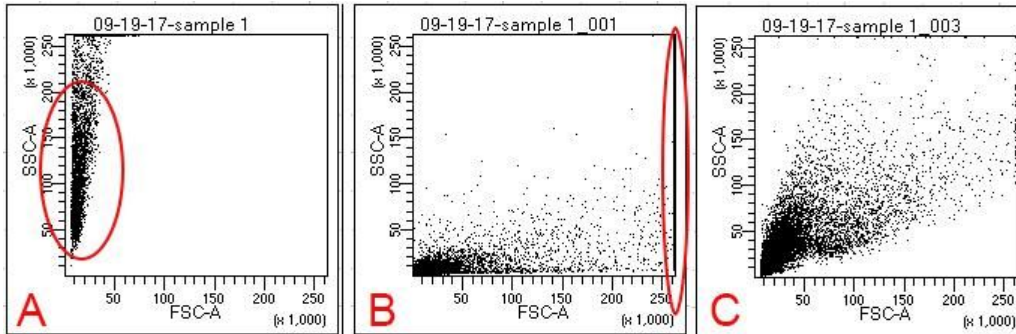
A. Forward scatter voltage too low, side scatter voltage too high
B. Forward scatter voltage too high, side scatter voltage too low
C. Forward and side scatter voltages optimal

9.6. For fluorescence parameters, adjust the volume until both the negative and the positive populations are on scale. Switching back and forth between a positive control and a negative control should help determine the voltages which supply the best signal to noise ratio.
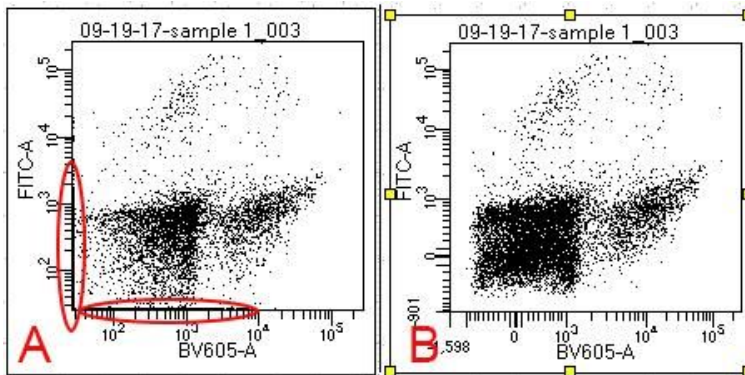
A. Parts of the negative population are off scale
B. Biexponential scale provides better visualization

9.6.1. If a positive population extends beyond the higher edge of the scale, reduce the voltage for the parameter until the positive population is fully on scale.

9.6.2. If a negative population extends beyond the lower edge of the scale, try increasing voltage to place it on scale, but make sure that the

positive population remains on scale. Use biexponential scaling for the corresponding axis to visualize the negative population if it still extends beyond the lower edge of the scale at the optimal voltage.

- Biexponential scaling can be toggled per plot by clicking on the plot and going to the Plot tab of the inspector. There are separate checkboxes for the X and Y axes.

10. **Record data for single color compensation controls for multicolor experiments (if you've used auto-comp)**

10.1. If you have compensation controls, record them after you have determined the optimal voltages for all colors.
- You should have compensation controls if you have more than one color.
- Do not change fluorescent parameter voltages that were set in the previous step (FSC and SSC can be changed).
- If you plan to do compensation in analysis software later, record 10-20K of events for each of the single color controls and a negative control.

10.2. To record data, press "Record" in the Acquisition Dashboard.

10.3. If you choose to use auto-compensation, you can complete the process once all tubes have been recorded.

10.3.1. View the data for each single color control, and gate the positive populations. You may need to gate negative populations as well if the 'Universal Negative' does not match the sample type in your positive control tube.
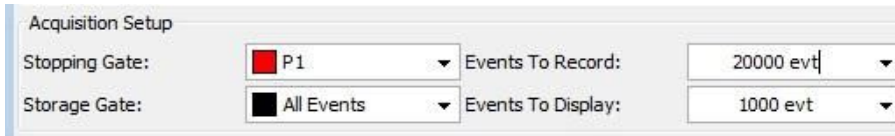
10.3.2. Select Experiment > Compensation Setup > Calculate Compensation.

10.3.3. Name the compensation setup and apply it to the experiment. Ensure that the voltage and compensation settings in the global 'Cytometer Settings' match the settings for your first tube.

10.3.4. Click out of the Normal Worksheet and back into the Global Worksheet. Staying in the normal worksheet will create a page for each tube which slows down instrument performance.

11. Record data for the samples

11.1. If you want to record a specific number of events for a specific population, create a stop gate in the acquisition dashboard panel. Specify the number of events you wish to record in the chosen gate.

11.2. If you are not using a stop gate, press Stop Recording once you have recorded a desired amount of data.

11.3. Using a storage gate limits the data that will be saved in your data file – this is NOT recommended.
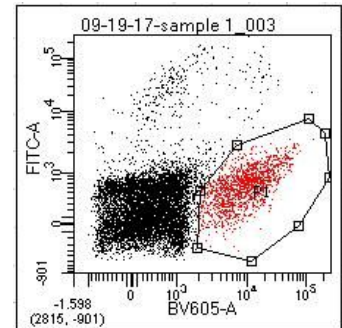
12. Clean the instrument

12.1. Using any tube, acquire 10% bleach for at least 2 minutes in "HI"

12.2. Acquire 5% Contrad detergent for at least 2 minutes in "HI"

12.3. Acquire sheath fluid for at least 2 minutes in "HI"

12.4. Remove tube and run Cytometer>Fluidics Shutdown, follow the instructions on screen.

13. Export the data and make sure to take it with you

13.1. Export your data to desktop while the instrument is being cleaned.

13.1.1. Select tubes to export.

13.1.2. Select File > Export > FCS Files, choose the 3.0 FCS version and a destination folder. FCS files can be opened and analyzed in FlowJo or other analysis software.

13.1.3. If you need pdfs of your plots, you can create them individually using a button on top of the worksheet, or you can use File > Batch processing to create a pdf of all selected tubes together.

13.2. You can export the experiment (File > Export > Experiment) if you wish. An exported experiment contains all of the settings and plots but no data. It can be imported into the Diva software if the original gets lost or corrupted.

13.3. Copy the data from desktop to a USB device or to a shared server (instructions above computer) or Google Drive/Dropbox.

Remember that data is not backed up and the core is not responsible for storing it - it can be removed at any time.

14. **Log off Diva software.**

15. **Turn off the cytometer if last user on the schedule for the day. Leave the computer on.**

Troubleshooting

99% of the time, cytometer issues are caused by a fluidics problem.
- Check that the sheath tank is not empty, the waste is not full, and the fluidics cart is on.
- Check that there are no visible bubbles in the cart's sheath filter.

- <u>Bubbles visible in sheath filter</u> have to be purged:
    - Sheath filter is located on the back of the fluidics cart
    - Holding a paper towel next to the air-bleed port, release the thumbscrew to let the air bubble out; tighten the thumbscrew afterwards.
    - Gently tap the sheath filter to release any trapped air. Repeat the thumbscrew release if needed.
    - It may be necessary to vortex the sheath filter if it has a lot of bubbles trapped in it.

- <u>If you don't see any events</u> and suspect a clog or air in the system, run Cytometer > Clean Flow Cell. Follow the on-screen instructions, noting that volume is being removed from the cleaning tube. Cytometer> Degas Flow Cell will help to more thoroughly remove air from the system.

- <u>If you don't see any events</u> when acquiring but there are no signs of a clog or bubbles, try restarting the software and/or the computer.

- <u>If the data looks unusual</u> while fluidics are functioning properly and events are displayed, check that the optical filters are in correct positions.