LSR Operational Notes

Initial Set-up:

- Turn on the LSR (green button) allowing a 30 minute warm-up time for the blue and red lasers, 45 minutes for the violet laser and 60 minutes if using the UV laser.

  Special Note: If you are using either the **Behemoth** or the **Octopus** you need to open the cytometer lid in order to improve air circulation for the UV laser. Raise the lid **GENTLY** so as not to knock the lasers out of alignment.

- Empty the waste can if needed.
    - Twist off lid, dump contents into sink, add about 1/3 of a bottle of bleach to waste container and replace lid.

- Fill sheath fluid tin.
    - Loosen the bracket on the tin and depressurize by pulling up on the pressure relief valve.
    - Remove the lid and fill to the top weld line with sheath fluid.
    - Replace lid making sure large "O" ring is in position.

- CAREFULLY look for air bubbles in the sheath filter and throughout the sheath fluid tubing and bleed off before they get into the cytometer. Start by bleeding sheath from the filter followed by the down-stream bleeder valve.
- Log in to Windows using your standard hospital login.
- Go to the FACS DIVA Software icon and double click to open.

Quality Control: (Make sure lasers and detectors are working properly.)

- Log on as "Quality Control" at the users "Name" drop-down menu. Password=qc.
- Open QC folder.
- Scroll to last QC dated experiment and double click.
- If you get a warning window that says "CST Mismatch" check the "Don't show this message again for the current login session" and then choose "Keep BD FACSDiva Settings."
- Right click> "Duplicate without data".
- Right click> "Rename" (rename experiment with current date and delete the numerical suffix).
- Check to make sure that the cytometer sample flow rate adjuster knob on the cytometer (SAMPLE FINE ADJ) is 5 turns in the middle.
- Add 1 drop of Spherotech Rainbow beads (vortexed thoroughly) to approx. 400uL FACS flow sheath fluid in a 12x75 tube (BD #352054 or like).
- Return the stock vial of Rainbow beads to the refrigerator right away.
- Click the sample activation flag to the left of the sample tube icon such that it turns green.
- At the "Acquisition Control" window click "Acquire".
- Set cytometer to "RUN" beads on "LOW".
- Vortex and load tube with beads on SIP and observe histograms, looking for stable, sharp, symmetrical peaks.
- Click "Record" once peaks are sharp and stable.
- When finished, put water back on SIP.
- File>Log out.

Note: If peaks do not look good remove beads from the SIP, replace with water and "Prime" the cytometer at least several times. Check beads again and repeat if necessary until QC histograms are acceptable.

Experiment set-up:

To set up an experiment you can either set one up from scratch or set one up by duplicating a previous experiment as in using a template.

Set Up an Experiment from Scratch

- Log in under lab PI name.
- Find personal folder.
- Create and name a new experiment (click lab notebook icon at the top of the browser).
- Add a needle icon and rename it "Voltages".
- Click on the "+" sign next to the needle icon to reveal the first sample tube.
- Highlight the first tube under the needle, right click and rename it "Unstained Cells".
- Be sure the "Unstained Cells" sample tube activation flag is highlighted green.
- Go to the "Instrument" window and click on "Parameters".
- Delete detectors that you won't be using for your experiment.
- Click the green flag under the "worksheet" heading on the graphics screen to go from a "Global" to a "Standard" worksheet.
- Create a dot plot with FSC (forward scatter) on the X axis and SSC (side scatter) on the Y axis.
- Draw a polygon gate on the scatter graph (dot plot) you just made, estimating where your cells will be.
- Create a histogram for each color you will be using.
- Activate each histogram to open its "Inspector".
- In the "Inspector" window for each histogram, put a check in the P1 box (gate) to designate the source of the data for the histogram.
- In addition check the "Mark Grid" checkbox in the Inspector for each histogram.
- Proceed to **Set Voltages** in these instructions.

-OR-

Set Up an Experiment via "Duplicate without Data"

- Log in under lab PI name.
- Find personal folder.
- Open the previous experiment that you wish to duplicate.
- Highlight the experiment name>right click> "Duplicate without Data".
- **If there was a compensation panel from the previous experiment you need to delete it from the new experiment in two steps.**
  - ➢ First, highlight the "Compensation Samples" needle icon>right click>delete.
  - ➢ Then highlight the "InstrSettings" line that is just below the experiment title in the browser.
  - ➢ Right click the "InstrSetting" line and "Unlink" from the previous experiment.
- Go to the needle icon that you created and named previously for your samples and delete all individual sample tubes except for the first one.
- Go to the "Voltages" needle icon and activate the "Unstained Cells" sample by clicking on the flag to the left of the sample tube icon so that it turns green.
- Open the "Standard Worksheet" on the graphics screen by clicking the green flag under the "Worksheet" heading at the top of the graphics screen to reveal the voltage settings graphs.
- Proceed to **Set Voltages** in these instructions.

Note: By "Duplicating without Data" you will already have voltages set in addition to all the graphs that you created previously. **However, you still need to check "Voltages" and make background auto fluorescence adjustments just as if you were doing the experiment from scratch.**

Set Voltages: (Set background levels based on cell auto fluorescence.)

- Click on the "Parameters" tab in the "Instrument" window to reveal the voltage adjusters for each detector.
- Run unstained sample and adjust voltages using the voltage adjusters.
- Place the peaks in the first quadrant of the grid or at the very least below 10^3 on the X-axis while still being able to see the entire peak.
- Once settings are complete set "Events to Record" to 10,000 and click "Record".

Compensation: (If you are using more than one color)

Whether you have set up an experiment from "Scratch" or "Duplicate without Data" you must set up and calculate a new compensation panel if you are using more than one fluorescent marker.

- Go to "Experiment" at the main menu bar >Compensation Set-up>Create Compensation Controls (click OK in the pop-up window to confirm compensation sample colors).
- The software automatically creates tubes with names under a new "Needle" icon labeled "Compensation" as well as graphs on separate worksheets for each single color stain on the graphics screen under the standard worksheet.
- Go to the first sample under the "Compensation" sample group labeled "Unstained Control" and activate the sample by clicking on the sample activation flag to the left of the sample tube icon such that it turns green.
- Place your unstained sample on the SIP running on high and "Record".
- Repeat for each of the individual single stain compensation samples listed on the compensation panel by clicking on "Next" on the acquisition controls window to advance to the next sample.
- Put DI water on the SIP.
- After recording each of the compensation samples go to the "Unstained Control" sample worksheet on the graphics screen and adjust the P1 gate on the scatter graph around the population of interest.
- Click on the P1 gate to activate it, right click and click "Apply to all Compensation Controls" so that you have the same P1 gate on each compensation sample.
- Using the auto fluorescence maximum intensity signal on the X axis from the unstained sample, set the gates for each of the single color samples in order to define positive staining. Do this by looking at each detector (color) on the "unstained" worksheet and assessing the max signal for that particular detector (color). Having determined the max value, switch to the worksheet for that particular colors single stained sample.
- Set the left edge of the interval gate at the point on the x-axis which was the max signal for the "unstained" sample and then pull the right edge of the gate all the way to the right in order to define the area of positive staining.
- After setting all gates go to "Experiment" at the main menu bar>Calculate Compensation.
- If the compensation is successful a file pop-up window will appear asking you to name and save the compensation file.
- Choose Link + Save and name your compensation file the same as your experiment name and save.

Running Samples:

Run Samples from an Experiment Set Up from Scratch

- Create a new needle icon (experiment group) for your samples and name it.
- Add antigen and fluorophore labels to each detector in the "Inspector" window. (Refer to attached sheet on Deleting Unused Detectors and Adding Detector labels.)
- Switch to and create a "Global" worksheet on the graphics screen via the green flag under the "Worksheet" heading on the graphics screen.
- Create a dot plot for FSC/SSC.
- Add a polygon gate to the scatter graph to gate on the cell population of interest.
- Add dot plots and/or histograms specific to your experiment paying attention to the structure of the population hierarchy you want by using the "Inspector" for each graph to designate the source of the data.
- In order to set up the hierarchy properly it is important that you **create graphs in the following order**. First, create the graph and choose the parameter(s) you wish to see on the graph. Second, mark the checkbox for the source data in the graphs "Inspector". Finally, place any gates desired on the graph.
- Click on the sample activation flag for the first sample tube in order to activate the acquisition controls window.
- Set "events to record" at the acquisition control window to the number desired.
- Highlight the first sample tube, right click and "Rename" if necessary.
- Start running first sample.
- Click "Acquire" to make sure events are <10,000events/sec and then click "Record".
- After 1st sample, click "Next" on the acquisition control window or "New Tube" at the top of the browser to create the next sample tube.
- Highlight and right click on the new sample tube and "Rename" the sample.
- Run the second sample making sure that its sample activation flag is green prior to recording and so on.

-OR-

Run Samples from an Experiment Set Up Via "Duplicate without Data"

- Click on the needle icon that you named previously for your samples and rename it if necessary.
- On the graphics screen switch to the "Global" worksheet via the green flag under the "Worksheet" heading revealing the graphs that were made in the previous experiment.
- Click on the sample activation flag for the first sample tube in order to activate the acquisition controls window.
- Start running first sample.
- Click "Acquire" to make sure events are <10,000 events/sec and then click "Record".
- After the first sample, click "Next" on the acquisition control window or "New Tube" at the top of the browser to create the next sample tube.
- Highlight and right click on the new sample tube and "Rename" the sample.
- Run the second sample making sure that its sample activation flag is green prior to recording and so on.

Print Your Data (if needed)

- Click on the PDF icon at the top of the graphics window.
- Save the PDF to your network drive.
- Print from your local printer.

Export Your Data: (Do this immediately after each experiment before you leave.)

- While your data is stored on the C: drive of the computer in your experiment files it is incumbent on you to transfer your data via FCS files to a network drive after each experimental run so that you have a saved copy that is not dependent on the cytometer computers. **Your Data is Your Responsibility**.
- Highlight the experiment name that is open (brown lab notebook icon that looks like an open book).
- File>Export>FCS files.
- Export as FCS 3.0 files.
- Save your files on your U drive for example by using "Browse" and "Choose Directory" to set the path and destination folder such that your files are sent over the network to your personal storage location..
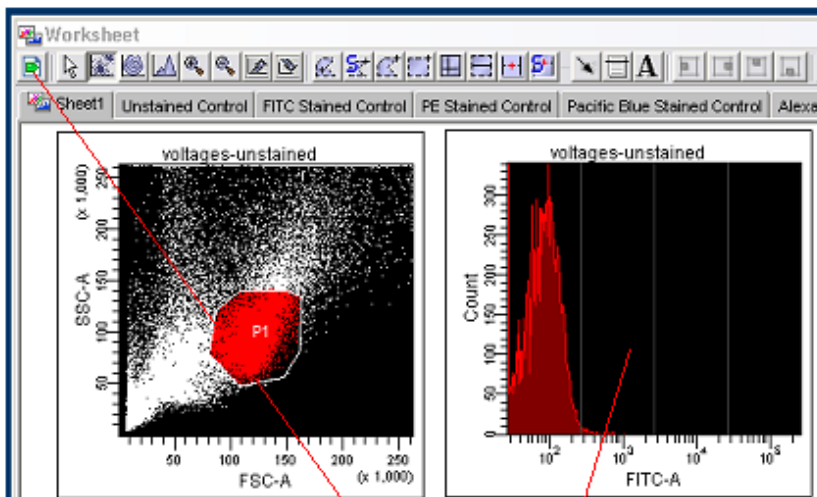
Shut down:

- After exporting data clean the machine.
- Create a new "needle" icon and rename "Cleaning". Rename the first tube "Bleach".
- Put 10% bleach on the SIP with the arm in the open position for 1 minute after pressing record.
- After 1 minute close the arm on the SIP and run on "high" for an additional 5 minutes and then stop recording
- Create a second sample tube and rename it "Water" and start recording.
- Repeat with DI water with the arm in the open position for 1 minute followed by 5 minutes with the arm closed, running on "high" and stop recording when done.
- Close experiment and log out of DIVA.
- Check the schedule to see if anyone is on the machine after you on that day.
- If someone is to follow leave the computer and the cytometer on, having put the cytometer in "Standby" and logging off of the computer. Make sure you log completely out so that you are off the network.
- Otherwise <u>leave the computer on</u> but shut down the cytometer by pressing the green power button .

Special Notes:

If you are using either the Behemoth or the Octopus we open the cytometer lids in order to improve air circulation for the UV laser. When turning the machine off, the lids need to be closed **VERY GENTLY** so as not to knock the lasers out of alignment.

The cytometer computers are for the purpose of running the cytometers. **ABSOLUTELY NO USE OF THE INTERNET OR E-MAIL IS PERMITTED WHILE ON THESE COMPUTERS.** DIVA is a finicky program and the running of other programs or surfing the web can cause problems. Failure to follow this policy can result in a suspension of your cytometer usage privileges. **Please abide by this policy.**

BD FACSDiva Software   QualityControl (Copy of Copy of 4 Blue 2 Violet 2 325UV 2 Red)

File Edit View Experiment Populations Worksheet Cytometer HTS Help

New Folder   Ctrl+N
New Experiment   Ctrl+E
New Specimen   Ctrl+M
New Tube   Ctrl+I
New Cytometer Settings
Import Cytometer Settings
New Global Worksheet
New Plate   Ctrl+K

Open Experiment   Ctrl+O
Close Experiment   Ctrl+W

Experiment Layout

Compensation Setup   ▶   Create Compensation Controls
                           Modify Compensation Controls
                           Calculate Compensation

Browser - QC

Date

Qua...

Global Worksheets
Rainbow Beads
QC   9/9/16 3:13:18 PM

16 11:55:10 AM
16 12:55:35 PM
16 11:37:35 AM
16 1:57:41 PM

Create compensation controls and calculate compensation thru the Experiment tag on the main menu bar. Go to "Compensation Setup" to "Create Compensation Controls" and then go to "Calculate Compensation once you have run the necessary compensation control samples.

The "Parameters" tab in the "Instrument" window lets you relabel and choose detectors. The voltage adjusters next to each detector are where you set voltages based on the level of autofluorescence (background) of your unstained cells. You can also use the Height (H) and Width (W) checkboxes on FSC (forward scatter) in order to discriminate between singlets and doublets for applications such as cell cycle analysis.

Acquisition Dashboard

Current Activity
Active Tube/Well   Threshold Rate   Stopping Gate Events   Elapsed Time
QC   0 evt/s   0 evt   00:00:00

Basic Controls
Next T...   Acquire...   Record...   Restart

Acquisition Setup
Stopping Gate: All Events   Events To Rec...   5000 evt   Stopping Time ...
Storage Gate: All Events   Events To Disp... 1000 evt

Cytometer - LSRII (1)

Status Parameters Threshold Laser Compensation Ratio

Parameter	Voltage	Log	A	H	W
FSC	533		✓		
SSC	312		✓		
(488) 505LP-530/30	434		✓		
(488) 555LP-575/26	408		✓		
(488) 635LP 695/10	444		✓		
(488) 735LP 800/65	591		✓		
(405) 450/50	341		✓		
(405) 505LP 525/50	443		✓		
(633) 660/20	587		✓		
(633) 680LP 730/30	493		✓		

Add   Delete

Control of the acquisition and recording of data is done thru the "Acquisition Controls" window. This is where you also set the number of events that you want to record for each sample. The "Next" button in the "Acquisition Controls" window is where you navigate thru your sample list in the browser either by creating a new tube or moving thru existing tubes.

The sample activation flag must be "on" by clicking it such that it turns green in order to activate the various windows necessary for control of the cytometer, such as the "Acquisition Controls" and the voltage adjusters under "Parameters" in the "Instrument" window. The location of the sample activation flag also designates into which tube icon data is being recorded.

COMPENSATE

When using multiple colors, calculate compensation for each detector by using the single stained controls for each fluorophore. Set the initial P1 gate in the FSC/SSC dot plot and "Apply to All Compensation Controls". After that, set the P2 gates for each color in order to define positive staining based on the negative staining from the "Unstained Control" sample

SET VOLTAGES

Use the standard worksheet by clicking on the green flag under the "Worksheet" heading on the graphics page. Create histograms with the "Show Grid" checked in the "Inspector" for the histogram in order to set voltages using the unstained cells. Adjust voltages so that peaks in the histograms are withing the first quadrant if possible.
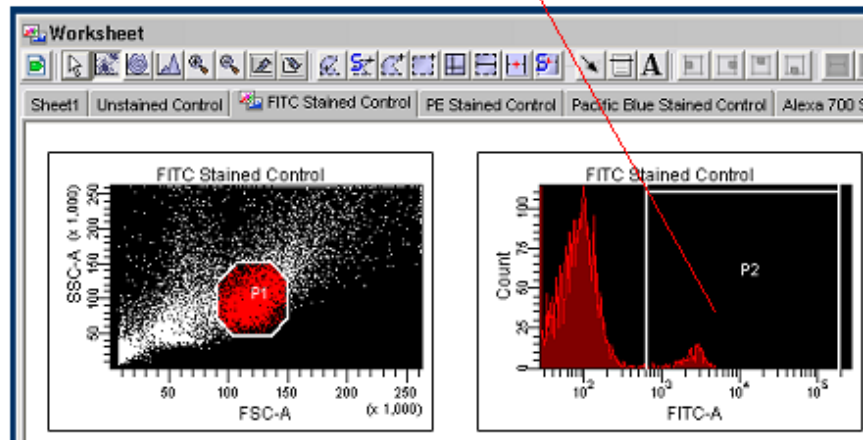
SHOW POPULATION HIERARCHY

You can view the population hierarchy you have created with its parent/daughter relationships by activating any graph, right clicking and clicking on "Show Population Hierarchy.
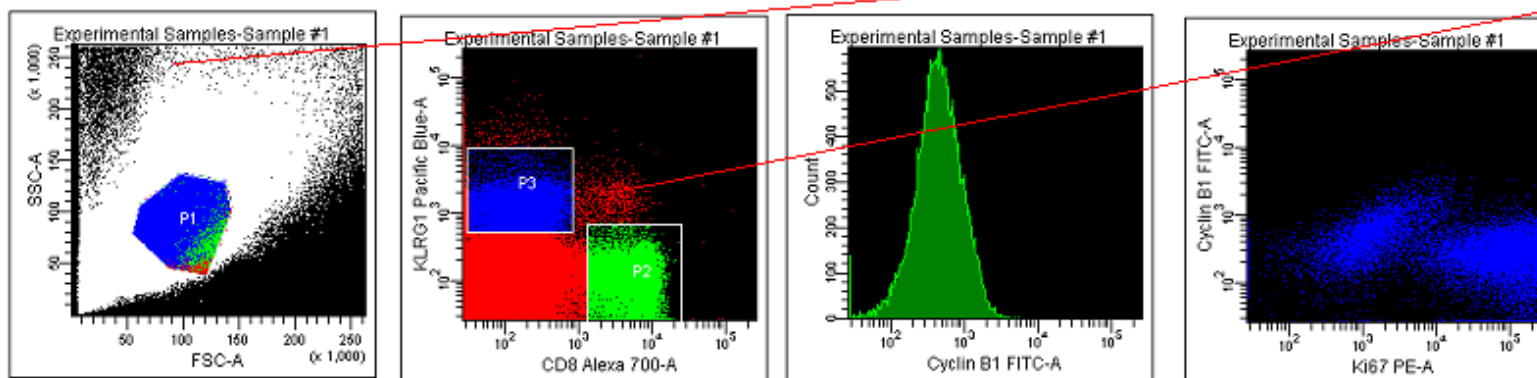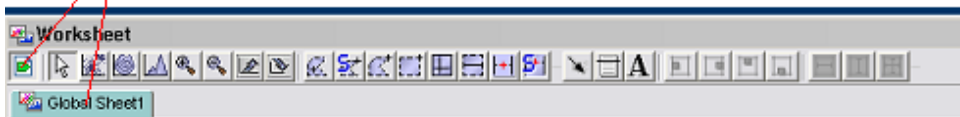
RUN YOUR SAMPLES

Once you have all of your settings and compensation taken care of switch to the "Global Sheet" via the green flag under the "Worksheet" heading in order to set up graphs for your actual experimental samples.

CREATE YOUR EXPERIMENT GRAPHS AND POPULATION HIERARCHY

Create a forward scatter/side scatter dot plot to initially gate on the cell population of interest. Follow this up with additional dot plots and/or histograms containing gates in order to create a population hierarchy via the checkbox in the "Inspector" for each graph that is germane to your experiment. Remember the order.......

Graph>Parameter(s)>Data Source via the Inspector>Gates