LSR II Users Guide
Stanford Shared FACS Facility

PPE must be worn at all times in our BSL-2 environment
*Lab coat, gloves, and closed toed shoes are mandatory; respirator and goggles are available*

An Instrument Configuration is located on the website in the Instrument Overview area
*The configuration lists the lasers and filters for each machine*

Do I need to run beads when I collect data using ‘Diva Digital’?
For each data collection, you need to first collect data on 197 calibration beads. This is to ensure that the instrument is running properly.

What samples do I need to bring?
- All users **ALWAYS** need to bring single stained compensation controls
- If you have a 7 color panel you should bring: 8 compensation controls
  - One tube stained with each individual antibody
  - One unstained tube
  - No PI or viability dye in the single color control

Don’t have enough cells for compensation?
User can purchase compensation beads from many different suppliers
- eBioscience UltraComp Beads 01-2222-42 *(for panels including UV and Violet dyes)*
- eBioscience OneComp Beads 01-1111-42 *(for panels that do NOT include any UV or Violet dyes)*
- BD CompBeads 552845 ‘Anti-Rat/Hamster’ *(for most anti-mouse antibodies)*
- BD CompBeads 552843 ‘Anti-Mouse’ *(for most anti-human antibodies)*
- ThermoFisher Amine Reactive Compensation Beads A10628 *(for LIVE/DEAD Fixable stains)*
Starting Up the System
*Allow 30 minutes for the lasers to warm up*

1. Turn on the Cytometer.
   a. The power switch is located on the lower right corner on the Cytometer.
   b. The fluid control button lights are **GREEN** and the **STANDBY** button light is amber.

2. SSFF computers are left on at all times.
   a. If computer is turned off, log into Windows with User Name: FACS User
      i. There is no password

LSR-II 20L Pump System Instructions (Cytek)
Fluid managers are in use on all our LSR IIs. Using the pump offers the following advantages:
- No need to open and fill a metal tank.
- Stabilizing of sheath flow rates allow use of a 6 uSec window extension.

**To use:**
1. The pump system turns on automatically with the LSR.
2. Verify that the pump is on. The LSR will not work properly otherwise.
   *Its switch is located on the blue box labeled “Cytek” under the LSR with the black lever switch.*
3. Check the sheath tank. Fill the sheath tank if there is not enough for your run.
4. Check the waste tank. Empty the waste tank if full.
5. Please email **facs-issues@stanford.edu** with any problems or questions

**Changing the tanks:**
1. The cube is on a scale, so you can check the weight of the cube (are there guidelines for what the readout should be? Instrument use is ~1L/h or 2.2lbs).
2. If the sheath cube is empty replace it with a new one (it is heavy so lift carefully) New cubes are located in either the hall or the rear of the room.
   *Note: The cube closure just screws on – there is no pressurization of the cube. Be careful not to kink the tubing. Turn the outer ring only so you do not stress the wires for the sensors.*
3. Empty waste in sink in hallway when full, using the protective face shield, gloves and lab coat.
   *Remember: Add bleach to line marked on carboy.*
4. Alarm sensors are connected to the cube and the waste, indicating when they need attention if you forget. The LSR won’t run samples properly if the pump is turned off.
   *The pump should **NEVER** be turned off manually!!*

Diva Software Instructions
1. Log into the Diva software using BDFACSDiva software icon on the desktop
   *Username is Sunet ID/ password starts with a capital letter*
2. Watch for the “Instrument connected” status in bottom of the Cytometer window
3. If CST mismatch dialog box appears select use **CST settings**
Setting Up Your Experiment
1. Create an Experiment: Select Experiment → New Experiment → Select the SSFF tab → choose the newest date available → select experiment Click → OK
2. To Rename -- right-click → Rename → Use descriptive name and date
3. Edit menu → User Preferences → Select desired defaults.

You can deselect save analysis and load data after acquisition to stop instrument from reloading data after acquisition. This helps if you are recording large data files.

Calibration
1. Remove the water tube from the SIP
2. Push the RUN button on fluidics panel and note the drop of saline back flushing from the SIP
3. Push the LO button on Cytometer panel
4. Place the standard 197 calibration beads (located in rack or freezer) on the SIP
5. Go to ‘Beads Specimen (syringe icon)’
6. CLICK on ‘+’ sign
7. Click on ‘197 Beads’ tube (The green arrow should be pointing at the tube) This makes acquisition controls active

8. In Acquisition Dashboard CLICK → Acquire Data

9. Set Events to Display to 50-100 for calibration
10. Set flow rate to LO
11. After beads appear on plots, use fine tune (black knob) to adjust flow rate to approx. 200 beads/sec
12. Check that the “delay window extension” global worksheet is selected.
13. Follow the on-screen instructions and check the instrument laser delays, and area scaling.
   a. Check the laser delays by closing the window extension to 0.
   b. Confirm the Forward scatter threshold is set to 50% of the forward scatter signal.
   c. The area signal for the Green, Red and Violet lasers should drop no more than 50%.
      (You can use the height value for a quick reference to 100 % signal.)
   d. If the delays are incorrect first check the CST values. Use the latest performance shortcut on the desktop for the most recent CST run.
   e. Open the window extension back to 6.00
   f. Check that Area Scaling values are correct. Area should be greater than or equal to the height within 10%. If relationship is off, make adjustments in the area scaling section in the laser tab.
   Acquisition must be stopped in order to change the worksheets
15. Switch to the “standards” global worksheet.
16. Adjust the PMT voltages (*cytometer window → parameters tab*) so the measured values are at or around displayed target values in bold (within 10%).

17. Press **RECORD DATA** in tube labeled ‘197 beads’

**Experiment Definition**

1. Select the next specimen in the experiment **Specimen** Click the ‘+’ to see **Tube_001**
2. Highlight **Tube_001** in new specimen area → click on arrow on left
3. In the Cytometer pane, delete parameters you will not be using.
   a. In Parameters tab, click on small button to left of parameter name.
   b. Click delete button (*use shift key and highlight for multiple deletions*)
   c. Repeat for each parameter you’re not using
   d. Do not delete Parameters that may be of interest.
   e. If you add back a deleted parameter to the experiment it will come in with different voltages, not specified PMT settings by facility. This may cause problems for resolution. Check cytometer settings for the 197 bead tube to see correct PMT voltage to assign when adding back a parameter.
4. Keep forward and side scatter height and width selected
5. The data may be viewed compensated or not, but the uncompensated data is written to the database along with the matrix so that other compensations may be used later in FlowJo.

*Note: For each tube collected in Diva software, uncompensated data is recorded and the compensation matrix is applied as a transform, it does not affect your data.*

All samples must be filtered for sorters. The analysis instruments have nylon mesh squares on the desktop if you need them

**Compensation**

- Corrects for the emission of one fluor into the detector used to measure another.
- Flow Cytometer use filter sets that are optimized for very specific wavelengths of light.
- Dye/Fluorochrome emissions often span from one filter into another.
- **COMPENSATION IS VERY IMPORTANT** - without it, our data cannot be interpreted properly.
- To calculate compensation, every experiment needs an unstained control as well as each fluorochrome singularly.
- Antibody capture beads can be substituted for cell controls.
1. Create Compensation Controls. Select Experiment → Compensation Setup → Create Compensation Controls.
2. In the Create Compensation Controls window, ensure that all of your colors are labeled “Generic”. Then select “OK”. *Do not compare negative cells to positive beads or vice versa.*

3. Diva will create a new specimen called “Compensation Controls”. Expand the specimen and activate the first tube.

4. Set Events to display to 5000.

5. Run unstained cells or beads first.

6. Adjust the FSC and SSC voltages to place the population of interest on scale.

7. Adjust the FSC threshold value to exclude most of the debris without excluding the population of interest.
   *Setting it to 50% of the smallest cell of interest works well.*

8. Move the P1 gate on the FSC/SSC plot to include your cells of interest.

9. Put your fully stained sample on. Make sure the positives are on scale. If your positives are above $10^5$ you will need to decrease the voltage.

   *Remember, if you significantly lower the PMT voltage below the baseline settings in order to bring the positive population on scale, then the dim populations might not be easily resolved from the negative populations for that parameter. It is acceptable to have the negative peak shift above the first log decade in some detectors.*

10. Check each single color in the unstained cell tube area so we can define that all events are on scale.

11. Run and record each of your single-stained controls. Use the P2 gate to select only your positive peak. Use an interval gate to create a P3 gate around your negative peak.
12. Select Experiment ➔ Compensation Setup ➔ Calculate Compensation. When the Single Stained Setup box appears, select "Apply Only" to apply the calculated values to your experiment.

*Error messages warn you of compensation values that are >100%. This can indicate one of two things, the voltage settings used are such that one of your dyes is brighter in an off-channel than it is in the channel selected and a channel receiving less total photons has been over amplified, or that the color combination at the settings you have chosen is not optimal. If you are still having problems, it may indicate that you do not have a good color combination.*

13. Return to your original Global Worksheet by clicking on the Global Worksheet icon in the Worksheet Window.

**Running Samples**

1. Create a FSC-A by SSC-A plot on the global worksheet and create a polygon gate to select your cells.

2. Create a population hierarchy by right-clicking on any dot plot and selecting “Show Population Hierarchy”. Create other dot plots or histograms as needed for your experiment.
3. Make sure the machine is in **RUN**.
4. Load your first sample tube onto the instrument and click “**ACQUIRE DATA**”.
5. Adjust your FCS and SSC voltages if needed.
6. Select the number of events to record in the Stopping Gate settings on the Acquisition Dashboard and click “**RECORD DATA**”.

![Image of Acquisition Dashboard with buttons for Next Tube, Acquire Data, and Record Data highlighted.](image)

7. Save your analysis template for future use. Click on the analysis global worksheet, right click on analysis, export template, rename, and export to general.

**Clean-up**
1. Run **BLEACH** on **HIGH** for 5 minutes
2. Run **WATER** on **HIGH** for 5 minutes
3. Leave the machine in **STANDBY** and **LOW** with a tube of **WATER** on the SIP.

**IF you ran BSL-2 samples follow the additional cleaning steps below:**
4. Spray cavicide on all surfaces. Contact time for cavicide is 5 minutes unless your APB requires a longer period. **Please let staff know if different contact times are needed.**
5. Fill-out the biosafety clipboard next to the machine. List the biohazard, user, lab and clean up procedure.
6. Empty and flush the tupperware catch basin under the sample tube with bleach, then water, and dry with paper towels; dispose of the paper towels in the biohazard waste container.

**SAVING YOUR DATA (this can be done during Cleanup, to save time)**
1. Click on the name of your experiment.
2. Select Export→Experiment
3. Make sure your data is being exported to **C:\Export**
4. Click **OK**
5. On the Desktop, click on **FACS Data CHECKIN**
6. The appropriate experiment should be displayed, but if not select it.
7. Click **OK**
8. Find your name and Click **OK**

**Check the Schedule—Is someone running less than 2 hours after you today?**
   If **Yes**, leave the machine as is, in **STANDBY**.
   If **No**, follow the **shutdown** instructions.
**Shutdown** (Only do this if you are the last person of the day!)
1. Make sure you have followed the cleaning instructions
2. Turn off the flow cytometer.
3. Clean up your work area.

**Basic Troubleshooting**

*Always tell the FACS Facility if there are ever any problems with the analyzer, even if these steps resolve it.*

**If the Cytometer and Computer are not connecting**
1. Turn off the computer and cytometer.
2. Turn on the cytometer and wait for 5 minutes then turn on the computer.

**The RUN button stays Yellow**
1. Check that the FACSFlow Supply System is on.
2. Make sure the sheath is full. Make sure the waste tank is empty.
3. Check to see if your tube is cracked.

**I don’t see any events!**
1. Check your settings.
   a. Turn off all gates.
   b. Set your Threshold on FCS at no more than 5000.
   c. Could also be a software burp. May have to log out of DiVa and log back in
   d. Make sure you’re in the proper worksheet

**If you still see no events the LSRII may be clogged**
1. Remove any tubes from the sample probe.
2. Press PRIME. Wait for STANDBY to turn orange.
3. Press PRIME again. Wait for STANDBY to turn orange.
4. Try rerunning a (freshly filtered) sample. If that still doesn’t work:
5. Put a NEW tube of **BLEACH** on the instrument and **RUN** on **HIGH** for 5’. Mark the level on the tube and watch the volume - it should decrease.
6. If the volume level decreases, put a NEW tube of **WATER** on the instrument and **RUN** on **HIGH** for 5’.
7. Try the 197 beads again using the bead settings
8. If that still does not work, ask for help!

**Rules**

**Data Rules**
1. Do NOT use USB sticks, thumb drives, external hard drivers or any other form of USB with the computer.
2. Do NOT connect to the internet from this computer.
3. You MUST export your experiment.

**Running the Machine Rules**
4. You must FILTER your cells before running.
5. You MUST follow the cleaning procedure.
6. Leave the area neat and tidy.
Notes on Data

- Check in your data to protect it
- Data is deleted monthly. If it is not checked in it is gone.
- USB and personal back up devices are not allowed on our machines.
- Discs may be burned when alternatives are needed.
- A .jo file for MAC (legacy Flowjo 9.75 and older) and a .wsp (version 10 both Mac and PC). Will be sent to you through the data archive once your data is checked in
- Currently we are recommending FlowJo version 10.2
- Start FlowJo and from the FileMenu select Open and then open your .wsp file.
- You will be prompted for Username: ‘flowjo’ Password: ‘314159’.