

# Operation of the Pathology LSRII Research Flow Cytometer

Updated May 13, 2015 after FACSFlow and FACSDiva 8 upgrades

May 13 new text is blue

## I. Startup: Fluidics Maintenance, Warmup, and Priming

1. I will be turning on the instrument and checking sheath and waste levels first thing in the morning – **generally**. Exceptions:
  - a. If I'm on vacation
  - b. If you're running on the weekends
  - c. If you want to start your run before I get in.
2. If a, b, or c above is the case, then briefly check the sheath fluid level (lift the sheath cubitainer on the FACSFlow system & make sure it feels heavy) and make sure the waste container is not approaching full.
3. Note: The FACSFlow system will **alarm** if the sheath is getting low or empty or if the waste is full. However, it's preferable to not have this happen in the middle of your run. If an alarm goes off, first press "Alarm." Sound will stop. Once you've remedied the situation, press "Restart." Red alarm lights should go out and system should resume periodic pulse pumping of sheath.
4. You do not need to turn the FACSFlow system off and on.
5. To fill sheath:
  - a. Unscrew fill probe and carefully place in the black plastic holder at left side of FACSFlow system.
  - b. obtain clean funnel (not the one atop the liquid waste barrel) and fill cubitainer from a full 20 L sheath cube.
6. To empty the waste:
  - a. Put on a lab coat, safety glasses, and don a pair of gloves from elsewhere in the lab. Waste contains bleach which can ruin your clothes and it may contain noxious chemicals like formaldehyde and propidium iodide.
  - b. Unscrew waste emptying probe and carefully place in black plastic holder at right side of FACSFlow system.
  - c. Remove the large cap and set aside.
  - d. Using the waste funnel atop the waste barrel, carefully pour waste into the barrel from the SMALL hole. Pouring from the large hole may cause liquid to come out too fast and flood the area and spill on you.
  - e. Add enough bleach (under sink adjacent to LSRII) to cover the bottom of the waste jug.
  - f. Recap waste barrel, reattach waste jug to FACSFlow system.
7. Turn on the instruments:
  - a. Turn on the power to the LSR (square green switch on the lower right).
  - b. Make sure the selector switch above the power switch is set to plates (not tubes).

- c. Turn on the HTS (switch on the right side of the HTS).
  - d. Turn on the computer.
  - e. Press RUN on the cytometer.
8. Login to [Windows by clicking "Operator."](#)
9. Password is "flowcyto".
10. Double Click on "BD FACS Diva Software" icon
11. Sign in from drop down list.
12. Browser, Inspector, Instrument, Dashboard and HTS windows should come up.
13. Warmup time
  - a. 488 nm sapphire laser: 15 min
  - b. 405 nm violet laser: 30 min
  - c. 635 nm Helium-Neon (HeNe) laser: 15 min
14. [Push RUN on the LSR II. Let RUN for about ten minutes.](#)
15. [Prime the HTS.](#)
  - a. Click **HTS** at the top
  - b. Click **Prime**
  - c. When priming is complete, click OK in the popup dialog box.
  - d. [Repeat abc. \(Two primes total.\)](#)
  - e. Begin your run. **If** cell populations (particularly light scatter) don't look right, prime the LSR II flow cell:
    - i. On the LSR II, press the PRIME button. It will turn red.
    - ii. Wait for the STANDBY instrument to light up (amber).
    - iii. Press RUN. Wait about one minute.
    - iv. If your populations still don't look right, ask for assistance.

## II. Working with FACSDiva Software

1. If this is a new experiment, I recommend taking some time before the day of your actual sample run to sit at the computer and set up the experiment document and plate layout the way you want it.
2. You will have one or more **experiments** for your work. An experiment icon looks like a brown-covered notebook. You create experiments and access stored data in the Browser window.
  - a. An **experiment** will have a template with customized elements such as plots, gates, statistics views, and instrument settings.
  - b. Upon choosing New Experiment, you will be presented with a range of preexisting experiments as templates for your own work. You may import one of these or choose "Blank Experiment."
  - c. [You may also copy someone else's experiment by right-clicking and choosing "duplicate without data," then dragging it to your own folder.](#)
  - d. Select parameters, draw plots, and set up gates the way you want them. See section II.6, "Plots and Gates."

3. Each experiment will have one or more **plates**.
  - a. Each plate typically represents one day's experiment.
  - b. Once you have an experiment set up the way you want it (plots, gates, etc.)
  - c. You enter the following data for a plate (representative but not exhaustive):
    - i. Which wells have cells in them
    - ii. Which wells are setup wells (see below)
    - iii. Which wells are sample wells
    - iv. How much liquid to withdraw from each well
    - v. The speed (in microliters per second) of each sample through the well
4. Tell the instrument how many events you want to collect from each well.
  - a. You can do this by clicking each well at a time and setting the number of events individually. However...
  - b. Unfortunately the only way to do this *en masse* is to utilize the software's *Experiment Layout* window.
    - i. Click "Experiment," then "Experiment Layout" near the bottom.
    - ii. When the window appears, you will get an "exploded" tree diagram of EVERY plate in your experiment. If your plate of interest is new, it will be located at the BOTTOM of the window (scroll all the way to the end).
    - iii. You have three tabs to work with: Labels, Keywords, and Acquisition.
    - iv. Click "Acquisition."
    - v. Highlight the wells for which you wish to set a certain number of events to acquire.
    - vi. Type the desired number in the field at the TOP of the window. This will fill in all highlighted wells at once. Otherwise you have to go through each well individually.
    - vii. **Note:** Experiment Layout is where you can add information to plot axis labels. For example, if you're measuring GFP, you change the appropriate axis so that it reads "FITC GFP" instead of merely "FITC." See the Appendix entry "The Relationship Between Detector Names and Setting Up Your Experiment."
5. Tell the instrument whether you want the set number of events collected to satisfy a certain gate. This is useful if you need a set number of, for example, viable cells. This choice is set under "Stopping Gate."
6. **Plots and Gates**
  - a. Generally, a flow experiment will have one light-scatter plot (FSC/SSC) and enough subsequent plots to determine that voltages and compensations have been set correctly, and to answer the questions you wish to pose of your experiment.
  - b. **Gates** are a set of value limits (boundaries) that serve to isolate a specific group of [cytometric](#) events from a large set. Gates are made from one or more regions.
  - c. **Regions** are polygons drawn within the plots. One or more regions define gates.

- d. Draw plots by clicking the appropriate icon button in the top menu bar and drawing in the workspace. The majority of flow users employ bivariate dot plots and univariate histograms.
- e. Draw regions that are appropriate to your experimental goals.
- f. Pay attention to **population hierarchy**.
  - i. Regions are used to display subsets of events in subsequent plots.
  - ii. Light scatter (FSC vs SSC) is very frequently the top of the event hierarchy.
  - iii. A region drawn on, for example, putative live cells or putative lymphocytes within a light scatter plot can force subsequent plots to display information only from events falling within this region...but only if you set things up properly.
  - iv. Draw a region around putative live cells in a light scatter plot (FSC vs SSC). The region is automatically named P1.
  - v. Each plot in the experiment can be forced to display All Events or a subset of them (defined by regions, which are by convention named P1, P2, P3...)
    1. To do this right-click the borders of the plot and choose Show Populations>P1.
  - vi. When you define a population in a gated plot (a plot showing a subset of All Events), the new population is automatically a subset of the population in the plot.
  - vii. Make sure you can see the population hierarchy schematic window when you are setting up regions and gates. Always double-check your population hierarchy setup and double-check your plots so that you know exactly which population subsets are being displayed.

### III. Using the HTS Plate Sampling Unit on the LSR II Flow Cytometer

Purpose: The plate sampling system used by Pathology is different than many flow users are accustomed to (single tubes). There are important facets to keep in mind when using the HTS so you get solid flow results and don't introduce air into the machine, which would negatively affect your run and possibly the runs of those following you.

- The front pump is called the primary pump in the manual. It enables mixing and aspiration of sample; delivers sample to flow cell in standard (not high-throughput) mode.
- The back pump is called the secondary pump. It is only used in high-throughput mode.

#### Working with the defined-volume HTS sampling method

The HTS is a defined-volume sampler and you are limited to working with microtiter plates. You may not use the "classic" 12x75 mm tubes many of us are familiar with. You have to prepare well in advance and be aware of many parameters to assure success. The instrument can only aspirate certain volumes

at certain speeds, and you need to have enough cells to effectively evaluate your experimental results without exceeding an approximately 5000 events per second analysis rate.

I. Having enough cells

- “Enough” cells is defined as the quantity and concentration that gets you enough information to effectively complete your experiment. While this is ultimately up to you and your lab, we recommend 30,000 events per sample.
- In order to achieve this you have to have cells at a sufficient concentration. You can comfortably fit 250 ul of volume into a well of a 96-well plate.
- The HTS can aspirate between 10 and 200 ul at a time.
  - i. Therefore you need to think in advance how many cells in your sample are contained in what volume. If you have 500,000 cells in 250 ul in the well, then you have 2000 cells per ul and in theory you should aspirate at least 15 ul to obtain 30,000 events. In practice I recommend taking twice that to begin with, particularly if this is a new experiment with cell types with which you are unfamiliar.
- The HTS can move sample through the instrument at fixed rates measured in microliters per second. The available speeds are 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 microliters per second.
  - i. If you have a concentrated sample, use a speed that does not cause a measured event rate greater than 5000 events/sec.
  - ii. If you have a dilute sample, increase the speed to save time.
- The HTS will mix your sample for you. The HTS will ask you what volume you want to mix and how fast.
  - i. Do not ask the instrument to mix a volume that is greater than the volume in your well! Maintain a mixing volume that is half the “available” volume (see below).
    1. If you start with 250 ul in the well, use a mixing volume of 100 ul.
    2. As an example, if you aspirate 50 ul from the well running a sample and you have to re-run it for any reason, drop the mixing volume from 100 to 65.
  - ii. A common mixing speed is 180 ul/second.
- The HTS is “greedy.” **The HTS will always aspirate 20 ul MORE from your well than you ask it to.** This is very important to keep in mind. You THINK you aspirated 50 ul from your well, but the instrument ACTUALLY aspirated 70 ul.
- The HTS counts the ~50 ul in the bottom of your well as “dead” volume. This means that while you put 250 ul cell suspension in your well, **in practice you only have 200 ul.** The HTS calls the volume you put in the well minus dead volume as “available volume.”
- Therefore, be VERY VIGILANT about liquid volumes!

II. What kind of plate?

- We recommend using only 96-well V-bottom plates. Do not try to use flat-bottom plates.

III. How do I adjust detector voltages and run compensation controls?

- The HTS software requires that you tell the HTS whether you want a given well to be a setup well or a sample well.
- **Setup wells** (pink square in the display) can have detector voltages adjusted while sample is running.

- **Sample wells** (blue circle in the display) CANNOT have detector voltages adjusted while sample is running.
- Running a setup well will NOT result in any saved data.
- Running a sample well WILL result in saved data.
- Therefore if you need to save your setup (voltage) controls, you will need to delete them and set up them up again as sample wells (delete the pink square & change to blue circle).
- The HTS can do compensation for you. In practice we recommend setting single-color wells as sample wells and performing compensation for your experiment after-the-fact in a common flow cytometry program like FlowJo or Kaluza.
- You should plan your plate layout BEFORE you come to the flow cytometer.

#### IV. Collecting and Saving Data

#### V. Cleaning and Shutting Down

- After collecting and saving your data, clean the instrument.
- Always at LEAST run three water wells. If you have run intercalating dyes (propidium iodide), you must run at least the “Daily Clean – Short” plate. If you are the last user of the day, please run a “Daily Clean – Long” plate (see further descriptions below).
- If there is someone using the machine after you, ALWAYS make sure you walk away from the machine in STANDBY. **NEVER LEAVE THE MACHINE IN RUN MODE.** Doing this for an extended period will cause the instrument to fill up the waste jug, stopping the FACSFlow system, sending the system into alarm, and (if this happens after hours or on the weekends), potentially damaging valuable instrument components and necessitating a lengthy priming procedure on our part. Even if someone is using the machine shortly after your appointment, you don’t know if that user is going to be late, and leaving the machine in RUN wastes sheath. Finally, this point is emphasized because this step is easy to overlook, yet can have problematic consequences for both the Core staff and subsequent users.
- **How to set up and run Daily Clean plates**
  - i. Use the plate in front of the monitor. The lid is marked with a template for how you will fill the wells.
  - ii. You will use two commercial cleaning solutions and water in between each cleaning solution.
  - iii. The solutions are kept in 15 mL tubes in a rack next to the computer.
  - iv. The commercial solutions are called “FACSClean” and “FACSRinse.” “FACSClean” has bleach. Be careful of your clothes.
  - v. Load the plate onto the machine.
  - vi. Click “HTS” at top of screen
  - vii. Click “Clean.” This option will not be available if an experiment is not open.
  - viii. Choose one of three options.
    1. Daily Clean – Long: for the last user of the day. Takes 25 min.

2. Daily Clean – Short: in between runs during the day if you’ve used propidium iodide, DAPI, LIVE/DEAD dye, acridine orange, or any dye that may carry over into a subsequent user’s samples. Takes 8 min.
  3. BD Clean: FACSClean (bleach) and water only. Not used often.
- ix. Click “ok” when prompted for the “Plate template.”
    1. A plate schematic with wells to fill will appear.
    2. There will also be descriptions of what to fill the wells with in the Specimen Description fields.

Plate row and name of chemical	SHORT clean	LONG clean
Row A: FACSClean	3 wells (A1-A3)	4 wells (A1-A4)
Row B: Water (Milli-Q grade)	3 wells (B1-B3)	4 wells (B1-B4)
Row C: FACSrinse	3 wells (C1-C3)	4 wells (C1-C4)
Row D: Water (Milli-Q grade)	3 wells (D1-D3)	4 wells (D1-D4)

- x. Use these solutions and the marked droppers to fill plate wells up to the top:  
  
Click “confirm” when plate is loaded onto HTS.
  - xi. Note: if you did not have the instrument in RUN, an error will occur and you will have to repeat steps vii thru ix.
  - xii. When the plate finishes, remove it and rinse it out with water.
  - xiii. Return the plate to the paper towel, upside-down, in front of the monitor.
- Shut down
    - i. Exit FACSDiva software by clicking the “X” button at top right
    - ii. Click “Start” (lower left)
    - iii. Click “Shutdown”
    - iv. Click “ok”
    - v. Turn off the HTS unit via the rocker switch on the side.
    - vi. Turn off the LSR II by pressing the big green button
    - vii. You do NOT need to shut off the FACSFlow system.

# Appendix

## Sheath fluid for LSRII

Material needed:

BD Biosciences FACSFlow ready-to-use sheath fluid (\$\$), 20 L “cubitainer”, BD#342003, \$35.65 ea (order through Fisher)

Fisher “NERL” Diluent 2 (\$), 20 L “cubitainer”, Fisher #23-029-361, \$21.70 ea

Note: BD Biosciences “FACSFlow” cubitainers are made to fit in the slots on the FACSFlow apparatus. Other vendors’ cubitainers may not fit. OR, if they DO fit, the probe may not fit. Therefore don’t throw away old BD-brand sheath cubitainers.

1. To fill sheath:
  - a. Unscrew fill probe and carefully place in the black plastic holder at left side of FACSFlow system.
  - b. obtain clean funnel (not the one atop the liquid waste barrel) and fill cubitainer from a full 20 L sheath cube.
2. To empty the waste:
  - a. Put on a lab coat, safety glasses, and don a pair of gloves from elsewhere in the lab. Waste contains bleach which can ruin your clothes and it may contain noxious chemicals like formaldehyde and propidium iodide.
  - b. Unscrew waste emptying probe and carefully place in black plastic holder at right side of FACSFlow system.
  - c. Remove the large cap and set aside.
  - d. Using the waste funnel atop the waste barrel, carefully pour waste into the barrel from the SMALL hole. Pouring from the large hole may cause liquid to come out too fast and flood the area and spill on you.
  - e. Add enough bleach (under sink adjacent to LSRII) to cover the bottom of the waste jug.
  - f. Recap waste barrel, reattach waste jug to FACSFlow system.

## The Relationship Between Detector (Parameter) Names and Setting Up Your Experiment.

Sometimes users are confused about parameter names (they are all named for a fluorochrome) and the fluorochromes or dyes they will use in their experiment.



Within the cytometer there are multiple fluorescence signal detectors for each laser. Each detector is most accurately defined by a combination of its associated laser (488 nm sapphire, 406 nm violet, or 635 nm HeNe), a long-pass filter, and a bandpass filter of given wavelengths. To all of these detectors we have assigned fluorochrome names. Generally speaking, we name the detectors according to the most commonly employed fluorochrome whose emission spectrum is detected most efficiently/strongly by that detector.

However, it is important to keep in mind that several different fluorochromes can be detected in the detector named "FITC," for example. If you wish to test the expression level of GFP in a transduced cell line, you employ the detector named "FITC," because its characteristics (receiving signal from the 488 laser, having a 505 nm longpass filter, and a 530/30 bandpass filter) permit the detection of signal emitted from FITC, an extremely common fluorochrome. You will have to either relabel your experimental plots as "GFP" using the Experiment Layout feature or simply remember that you're analyzing for GFP and not FITC in subsequent days.

Some dyes have very broad emission spectra (propidium iodide) and their signal can be detected in more than one detector. Users will often assign the PE-Texas Red detector for observing PI emission, but PI signals can also be observed using the detector named "PE."

An example of being able to observe signal from a variety of fluorochromes in one detector is the 406 nm laser detector that lacks a longpass filter and has a 450/50 bandpass filter. It is named "Pacific Blue." It can detect signal from the fluorochromes Pacific Blue, Cascade Blue, DAPI, Hoechst, Alexa Fluor 405, and the commercial LIVE/DEAD (Violet) stain (Invitrogen/Life Technologies)

**I don't see my fluorochrome/dye of interest on the list. How do I know which detector to use on the LSRII?**

A helpful resource is BD Biosciences' Spectra Analyzer, a JAVA-based web tool found at

[http://www.bdbiosciences.com/ca/research/multicolor/spectrum\\_viewer/index.jsp](http://www.bdbiosciences.com/ca/research/multicolor/spectrum_viewer/index.jsp)

Remember to maintain the distinction between *excitation* and *emission* spectra when using the tool.