LSR II and LSR Fortessa User Guide

Ragon Institute of Massachusetts General Hospital, MIT and Harvard

Visit the Flow Facility website!
http://www2.massgeneral.org/aids/flow_cytometry.html
It contains information about our instruments, links to calendars and flow resources, and many core documents, as well as a flow cytometry syllabus of resources to expand your flow knowledge.

Please let me know if anything is not working properly or you have any problems with the instruments or the facility in general-- email mwaring@partners.org or parcfacs@partners.org

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Creating an Account

<u>To become a user:</u>

> --visit http://www2.massgeneral.org/aids/flow_cytometry.html and read the "Welcome New Users" document linked on the main page
>
> --go through the introductory material on the training page (linked in the list on the left side of the page)
>
> --attend the introductory class (dates are announced on the training page). There is a fee for attending the class, please provide information as requested on the website.
>
> --go through the "FACSDiva software quick start" on one of the LSR computers or PC analysis station (shortcut to the document can be found on the desktop), and go through the online DIVA tutorial (linked on the training page).
>
> --observe another person operate the instrument at least twice.
>
> --schedule time with core personnel for an orientation session and then to run at least your first experiment with you.
>
> -- Passing a proficiency test is required before you can run independently, and for the first few months limit your reservations to during working hours so that someone is in the lab in the event of some problem.

Usage rates are posted on the instrument pages on the website. For user-run analysis, billing is tracked by how long user is logged into the software for Diva. For data analysis, use the dedicated analysis workstations for free instead of getting charged for using the instrument computers.

Instrument Locations

The 3 Laser LSR II ("3L") is located in CNY149, room 5227. Take the central elevators to the 5th floor, go through the door to the left of the balcony, and then go through the door straight ahead to enter the lab, room 5228. Keep going straight and you will see the instrument against the window in the bay in front of you.

The 4 Laser LSR II ("4L") is located in CNY149, room 5556. Take the central elevators to the 5th floor, go through the door to the left of the balcony, and then go left down the hall. The flow lab is on the left, look for the card reader next to the entrance door. Authorized users must submit their MGH ID (either the 9 digit number on the back of your ID, or your blue card number) in order to activate their ID for access to the room. Please email the information to mwaring@partners.org.

The 4 Laser LSR II Rental ("4R") is located in CNY149 room 5104. Take the West Elevators to the 5th floor, and go through the door to the right of the balcony. Go to the right, past the break area, and enter the door to the lab space. Walk down the hallway, turn left, then left again and head towards the cold room. Go to the right, and the instrument is at the end of the hallway on the right.

The 5 Laser LSR Fortessa ("5L") is located in CNY149, room 5227. Take the central elevators to the 5th floor, go through the door to the left of the balcony, and then go through the door straight ahead to enter the lab, room 5228. Walk straight ahead and you will see the instrument in the bay in front of you. It is the blue instrument on the right.

Configurations

The cytometer detectors are arranged in either octagon or trigon arrays of filters, with a separate array for each excitation line. The entry point for the emitted light into the array is indicated by the square in the pictures below, and travels sequentially to each detector, first to "A", then "B", etc.
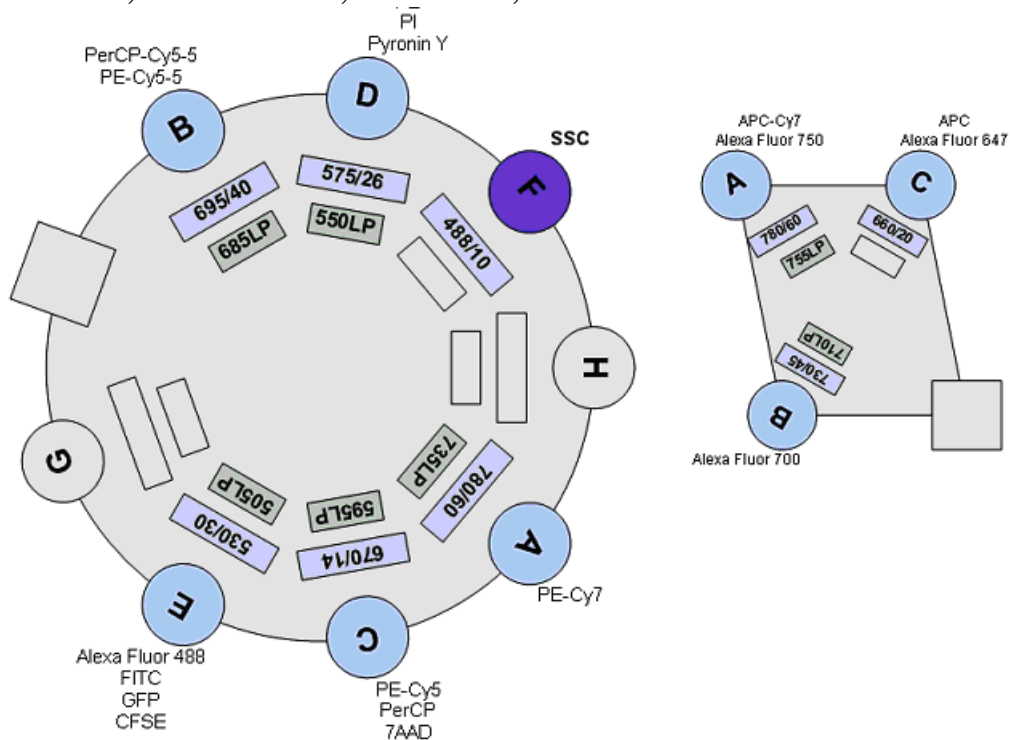
Filter choices for each detector are assigned in the Diva software as a "configuration", which links the fluorochrome name to the detector. It is up to the operator to make sure the filters match the configuration.

Each instrument has its own arrangement of filters, and some alternate configurations have been established for specific applications. These layouts are presented in table form on the following pages. Cells filled in yellow indicate filters that get changed for the alternate configuration. Instructions for changing configurations can be found elsewhere in this guide.

3L LSR II "Default" Configuration

	Fluorochrome	Detector	LP	BP
488 nm	PE/CY7	Blue A	735 LP	780/60
20 mW	PE/CY5-5, PerCP/CY5-5	Blue B	685 LP	695/40
	PE/CY5, PerCP, 7AAD	Blue C	595 LP	670/14
	PE, PI, Pyronin Y, DsRed	Blue D	550 LP	575/26
	FITC, GFP, Alexa 488, CFSE	Blue E	505 LP	530/30
	SSC	Blue F	blank	488/10
633 nm	APC/CY7, Alexa 750	Red A	755 LP	780/60
25mW	Alexa 700	Red B	710 LP	730/45
	APC, Alexa 647	Red C	blank	660/20
407 nm	Q800	Violet A	755 LP	780/60
50 mW	Q705	Violet B	670 LP	705/70
	Q655	Violet C	630 LP	660/40
	Q605	Violet D	595 LP	605/40
	Q585	Violet E	570 LP	585/42
	Pacific Orange, Alexa 430, Cascade Yellow	Violet F	535 LP	560/40
	AmCyan, CFP	Violet G	475 LP	490/40
	Pacific Blue, Alexa 405, Cascade Blue, Marina Blue	Violet H	blank	450/50

3L LSR II "APC Cy5.5"

	Fluorochrome	Detector	LP	BP
488 nm	PE/CY7	Blue A	735 LP	780/60
20 mW	PE/CY5-5, PerCP/CY5-5	Blue B	685 LP	695/40
	PE/CY5, PerCP, 7AAD	Blue C	595 LP	670/14
	PE, PI, Pyronin Y, DsRed	Blue D	550 LP	575/26
	FITC, GFP, Alexa 488, CFSE	Blue E	505 LP	530/30
	SSC	Blue F	blank	488/10
633 nm	APC/CY7, Alexa 750	Red A	755 LP	780/60
25mW	APC-Cy5-5, Cy5-5, VT680	Red B	685 LP	695/40
	APC, Alexa 647	Red C	blank	660/20
407 nm	Q800	Violet A	755 LP	780/60
50 mW	Q705	Violet B	670 LP	705/70
	Q655	Violet C	630 LP	660/40
	Q605	Violet D	595 LP	605/40
	Q585	Violet E	570 LP	585/42
	Pacific Orange, Alexa 430, Cascade Yellow	Violet F	535 LP	560/40
	AmCyan, CFP	Violet G	475 LP	490/40
	Pacific Blue, Alexa 405, Cascade Blue, Marina Blue	Violet H	blank	450/50

4L LSR II "Default" Configuration

	Fluorochrome	Detector	LP	BP
488 nm	PE-Cy7	Blue A	735	780/60
20 mW	PE-Cy5-5, PerCP-Cy5-5	Blue B	685	695/40
	PE-Cy5, PerCP	Blue C	600	670/14
	PE, PI, Pyronin Y, DsRed	Blue D	550	576/26
	FITC, GFP, CFSE, Alexa 488,	Blue E	505	530/30
	SSC	Blue F		488/10
633 nm	APC-Cy7, APC-Alexa 750	Red A	735	780/60
25 mW	Alexa 700	Red B	685	720/40
	APC, Alexa 647, Cy5	Red C		660/20
	Pacific Orange, Alexa 430, Cascade			
405 nm	Yellow, Q565	Violet A	535	560/40
25 mW	AmCyan, CFP, Horizon V500, Q525	Violet B	480	500/20
	Pacific Blue, Alexa 405, Cascade			
	Blue, Marina Blue, Horizon V450	Violet C		440/40
355 nm	indo-1(blue)	UV A	505	530/30
20mW	Alexa 350, Hoechst, Blue Viability			
	Dye, DAPI, indo-1(violet)	UV B		440/40

4L LSR II "Qdot" configuration

	Fluorochrome	Detector	LP	BP
488 nm	PE-Cy7	Blue A	735	780/60
20 mW	PE-Cy5-5, PerCP-Cy5-5	Blue B	685	695/40
	PE-Cy5, PerCP	Blue C	600	670/14
	PE, PI, Pyronin Y, DsRed	Blue D	550	576/26
	FITC, GFP, CFSE, Alexa 488,	Blue E	505	530/30
	SSC	Blue F		488/10
633 nm	APC-Cy7, APC-Alexa 750	Red A	735	780/60
25 mW	Alexa 700	Red B	685	720/40
	APC, Alexa 647, Cy5	Red C		660/20
405 nm	Qdot 655	Violet A	635	660/20
25 mW	Qdot 605	Violet B	595	610/20
	Pacific Blue, Alexa 405, Cascade Blue, Marina Blue	Violet C		440/40
355 nm	Qdot 705	UV A	685	710/40
20mW	Alexa 350, Hoechst, DAPI, Blue Viability Dye, indo-1(violet)	UV B		440/40

4L LSR II "APC Cy5.5" configuration

	Fluorochrome	Detector	LP	BP
488 nm	PE-Cy7	Blue A	735	780/60
20 mW	PE-Cy5-5, PerCP-Cy5-5	Blue B	685	695/40
	PE-Cy5, PerCP	Blue C	600	670/14
	PE, PI, Pyronin Y, DsRed	Blue D	550	576/26
	FITC, GFP, CFSE, Alexa 488,	Blue E	505	530/30
	SSC	Blue F		488/10
633 nm	APC-Cy7, APC-Alexa 750	Red A	735	780/60
25 mW	APC-Cy5-5, AT 680, Cy5-5	Red B	685	695/40
	APC, Alexa 647, Cy5	Red C		660/20
405 nm	Pacific Orange, Alexa 430, Cascade Yellow, Q565	Violet A	535	560/40
25 mW	AmCyan, CFP, Horizon V500, Q525	Violet B	480	500/20
	Pacific Blue, Alexa 405, Cascade Blue, Marina Blue, Horizon V450	Violet C		440/40
355 nm	indo-1(blue)	UV A	505	530/30
20mW	Alexa 350, Hoechst, Blue Viability Dye, DAPI, indo-1(violet)	UV B		440/40

4R LSR II "Default" Configuration

	Fluorochrome	Detector	LP	BP	
488 nm	PE-CY7	Blue A	755	780/60	
20 mW	PE-CY5-5, PerCP-CY5-5	Blue B	685	695/40	
	PE-CY5, PerCP	Blue C	655	660/40	*
	PE-Texas Red ,LiveDeadRed	Blue D	600	610/20	
	PE, PI, Pyronin Y, DsRed	Blue E	550	575/26	
	FITC, GFP, CFSE, Alexa 488, LiveDeadGreen	Blue F	505	530/30	
	SSC	Blue G		488/10	
640 nm	APC-CY7, APC-Alexa 750, LiveDeadNear-IR	Red A	755	780/60	
40 mW	APC-CY5-5, Alexa 700	Red B	710	730/45	
	Alexa 680	Red C	675	685/35	
	APC, Alexa 647, CY5, LiveDeadFarRed	Red D		670/30	*
405 nm	Qdot 655	Violet A	630	660/20	*
25 mW	Qdot 605	Violet B	595	605/40	*
	Qdot 585	Violet C	575	585/15	
	Qdot 565, Pacific Orange, Alexa 430, Cascade Yellow, LiveDeadYellow	Violet D	545	560/40	*
	AmCyan, Qdot 525, LiveDeadAqua	Violet E	475	525/50	
	Pacific Blue, Alexa 405, Cascade Blue, Marina Blue, LiveDeadViolet	Violet F		450/50	
355 nm	indo-1(blue)	UV A	505	530/30	
20 mW	Alexa 350, Hoechst, DAPI, Blue Viability Dye, indo-1(violet)	UV B		450/50	

4R LSR II "Factory" Configuration

	Fluorochrome	Detector	LP	BP
488 nm	PE-CY7	Blue A	755	780/60
20 mW	PE-CY5-5, PerCP-CY5-5	Blue B	685	695/40
	PE-CY5, PerCP	Blue C	655	660/20
	PE-Texas Red ,LiveDeadRed	Blue D	600	610/20
	PE, PI, Pyronin Y, DsRed	Blue E	550	575/26
	FITC, GFP, CFSE, Alexa 488, LiveDeadGreen	Blue F	505	530/30
	SSC	Blue G		488/10
640 nm	APC-CY7, APC-Alexa 750, LiveDeadNear-IR	Red A	755	780/60
40 mW	APC-CY5-5, Alexa 700	Red B	710	730/45
	Alexa 680	Red C	675	685/35
	APC, Alexa 647, CY5, LiveDeadFarRed	Red D		670/14
405 nm	Qdot 655	Violet A	630	655/8
25 mW	Qdot 605	Violet B	595	605/12
	Qdot 585	Violet C	575	585/15
	Qdot 565, Pacific Orange, Alexa 430, Cascade Yellow, LiveDeadYellow	Violet D	545	560/20
	AmCyan, Qdot 525, LiveDeadAqua	Violet E	475	525/50
	Pacific Blue, Alexa 405, Cascade Blue, Marina Blue, LiveDeadViolet	Violet F		450/50
355 nm	indo-1(blue)	UV A	505	530/30
20 mW	Alexa 350, Hoechst, DAPI, Blue Viability Dye, indo-1(violet)	UV B		450/50

5L LSR Fortessa "Default" Configuration

	Fluorochrome	Detector	LP	BP
488 nm	PerCP-Cy5-5	Blue A	685	710/50
50 mW	FITC, GFP, CFSE, Alexa 488, YFP, Chameleon Yellow, LiveDeadGreen, AO	Blue B	505	525/50
	SSC	Blue C		488/10
561 nm	PE-Cy7	YG A	750	780/60
50 mW	PE-Cy5-5	YG B	685	710/50
	PE-Cy5, 7AAD	YG C	635	670/30
	PE-Texas Red, mCherry, PI, LiveDeadRed, Alexa 568	YG D	600	610/20
	PE, DiI, DsRed	YG E		582/15
640 nm	APC-Cy7, APC-Alexa 750, LiveDeadNearIR, APC-H7, Alexa 750	Red A	750	780/60
40 mW	Alexa 700	Red B	685	730/45
	APC, Alexa 647, Cy5, LiveDeadFarRed, Sytox Red	Red C		670/30
405 nm	Q800	Violet A	750	780/60
100 mW	Q705	Violet B	685	710/50
	Q655	Violet C	635	670/30
	Q605	Violet D	600	610/20
	Q585	Violet E	570	585/42
	Q565, Pacific Orange, Alexa 430, Cascade Yellow, LiveDeadYellow	Violet F	545	560/40
	AmCyan, CFP	Violet G	505	525/50
	Pacific Blue, Alexa 405, Cascade Blue, Marina Blue, LiveDeadViolet, Cerulean	Violet H		450/50
355 nm	LiveDeadAqua, indo-1(blue), Q800*	UV A	505	525/50
60 mW	Alexa 350, Hoechst, Blue Viability Dye, DAPI, indo-1(violet), LiveDeadBlue	UV B		440/40

Schedulebook Calendar Instructions

The resources of the Ragon Institute Flow Cytometry Facility and Imaging Facility, as well as some other Ragon equipment (Luminex, RT-PCR machines, and Robosep—email separate administrators for rules for booking this equipment, in the "Notes" for the resource on the calendar) are reserved using the online calendar system "Schedulebook". New users must request a user name and password to be able to book time on the calendars. Email mwaring@partners.org with the instruments you wish to have access to.

There is a guest account available for viewing the calendars (but you may not reserve time using the guest account). Username is "flowguest" and password is "flow". You will NOT be able to book time with this account, only view the calendars.

I. Calendar/Reservation restrictions for cytometers
II. Usage instructions/tips
III. Ragon Institute Members---booking "Ragon" reserved time
IV. Standby Events
V. Administrators

I. CALENDAR/RESERVATION RESTRICTIONS FOR CYTOMETERS:

The following restrictions are in effect for booking time on the cytometers. For rules governing use of the other equipment, please contact the appropriate administrator as found in the "Resource Notes" for each device (the "N" button next to the device's name in the calendar).

1. Users that are not members of the Ragon Institute may not book time more than 1 week in advance.
2. Do NOT book time that already shows a reservation in "standby" (narrower dark blue column to the right of the reservation column). This time should be considered "booked" (see section IV below).
3. Reservations between 12 PM and 9 PM are limited to 2 hours in duration, and no more than 2 reservations per week can be made between 2 and 6 pm.
4. Tuesday, Wednesday, and Thursday afternoons from 3 to 7 are reserved for members of the Ragon Institute, and unreserved time is released to the rest of our users on the day before the time slot. For Ragon members, see Section III below for guidelines on booking time during these hours.
5. Sorting times on Tuesday and Thursday afternoons from 2:30 to 4:30 are reserved for members of Ragon Institute, and released on the day before the time slot. Grey areas of the calendar are NOT available for booking.
6. If you decide to not use your time or will not be using the entire time reserved, please update or delete your entry as soon as possible so that other researchers will know it is available for use.
7. If you fail to delete your unwanted reservation at least 1 hour before the start time and do not use your time, you may still be billed for 1 hour usage.
8. If you do not arrive for your reservation within 20 minutes of the start time, anyone else can use the instrument and you forfeit your reservation.

II. USING THE CALENDAR PAGE

- Once your new account is created, you can go to www.schedulebook.com and enter your login information in the fields on the right side of the page. If you are a sort only user, or don't yet have an account, you may log in with the Guest account, username "flowguest" and password "flow".

- There is a help menu, with a contents file that describes all of the features of Schedulebook. The basic controls have been summarized below, but this file is available for clarification.

- When you log in for the first time, all of the resources will be visible, but you will only have reservation privileges on the ones you requested. Only Administrators have reservation privileges on the sorting calendar—see the sorting guidelines for details.

- If you scroll over "tools", a dropdown menu will appear, with options for "New Event" ("sort only" or guest users will not see this option), "Search/Export", and "Personal Settings".

- Select "Personal Settings" and add an email account. Under "Visible resources", everything will be highlighted. Click on an item to select it, and then hold <control> and click to select multiple items at once (Mac users hold the <apple> key). These will be the resources you can see on your calendar page. This does NOT grant you privileges to reserve that resource, only to view it. For sorting and for Mike's calendar, you can select these items as visible, but you will NOT be able to make reservations—you must still email sort request forms to ragonfacs@gmail.com, or make arrangements to book training, instrument orientations, etc.

- The "Start time" and "end time" visible on this page refer to the displayed times on your calendars. If you will be booking time outside of the displayed window, you will need to adjust the times to view the other hours of the calendar. Modify the other preferences as you prefer. You can also change your password here.

- Under the "View" pulldown menu, you can change the calendar view. From the monthly view, you can switch to previous month, next month, or current month. By clicking on the number for a given day, you will switch to the daily view.

- When in the day view, you can go to previous date, next date, today, tomorrow, pick date (a popup calendar appears that you can click the desired day), and month view, which takes you back to the monthly view.

- There are also buttons on the top right to change the display date, the arrows advance day by day, or "pick date" will open a popup calendar to help you navigate to the date of interest.

- Selecting "Tools>New Event" or clicking on the "New Event" button on the top right will open a dialog window with a pull-down list of what resources you have access to and are set to "visible". If any instrument you need access to is missing, let me know and I will add it (make sure the desired resource is set to "visible" in "Tools>Personal Settings"). The date for the event will be whatever date was displayed when you chose "new event". You then choose a start time and end time, and type in any notes to create your event. Please include your telephone number as a "note".

- When you make a reservation, your display name will show on the calendar, and if there is an email address associated with it you can click on the name to send an email. If there was a note entered, it will display in a popup window if you click on the green "N". The "E" allows you to edit the reservation, and "D" will delete it. If you do not have those privileges (i.e. someone else's reservation) they will not show up as options.
- The wider column under each resource is the primary schedule, and the narrow column to the right is for events on "Standby" that overlap with another event (see images in section IV). Please do NOT book time that is already covered by a standby event, even if it is free on the main calendar, as this time should be considered reserved. I receive email notices of when each modification takes place, so I will know who actually booked the time first.
- Each resource can have a resource note associated with it, which appears at the top of the column next to the resource name as an "N" button. When it is red, it has been changed since you last viewed it. When it is green, you have already seen it. Resources also have Alerts, which will open a popup window when you attempt to make a reservation on that resource. This will contain messages about instrument down times, etc. The "!" next to the name of the instrument is a problem notification address, click on it to report a problem with the resource, and an email will be sent to the appropriate administrator.

III. Ragon Institute members

When booking time that is saved for the Ragon Institute, you can do it 3 ways:

1. Log in as the Ragon Institute (email Mike for the login info) and modify the reservation to free up the time you want to book. Then log in as yourself and book the time.
2. Log in as yourself, and when you book the time that overlaps with the Ragon Institute, be sure the check the button under the "notes" that says to book the reservation as a "Standby" event if there is a conflict. I then go through a few times a day and update the Ragon booking to free up the time and allow the standby event to take over the primary spot. Until your "standby" event becomes the primary event, it is possible for someone else to book the "primary" reservation—i.e. the Ragon event is from 3-7, if you book from 2-4 then your entire reservation will be put on standby, and it would be possible for someone to book 2-3 before I have had a chance to edit the Ragon event to make the standby reservation into the primary one (see section IV below). So, the 3rd option:
3. Book the time that overlaps with the Ragon reservation as standby, and book the open time as a normal reservation. In the above example, book 2-3 normally, and designate 3-4 as a "standby" event, so that no one tries to reserve your time.

IV. "Standby" Reservations

There is time reserved for the Ragon Institute from 3 to 7 on Tuesday, Wednesday and Thursday each week. Unreserved time is made available to the general public on the day before the event and can be booked by anyone.

Sometimes when this time is reserved by Ragon members, it is booked in "Standby" which appears as a darker column next to the main reservation column.

In the Schedulebook software, it is possible to still book time overlapping with the "standby" event. Please consider the "standby" time as taken, and do not encroach on this event—if there is a conflict, the core receives notifications of any changes to the calendar and I can determine who actually booked the time first—but I'd rather not have to play detective.

V. For Administrators:

--Please update the resource you are responsible for-- add notes with any contact info and any links etc that apply, and if there are outages etc please add a resource alert, if you have questions about these features check the "help" menu on Schedulebook.

--When adding a user, please use the full name for "display name", no nicknames and make sure last names are included.

--Before adding a new user, check to see if the user already has an account for another device.

--When adding access to a device, hold control (or apple on mac) so that you don't de-select resources that were given access previously.

--If you need to create a new device, use the "no users have access to this device" as the default, otherwise EVERYONE will end up with access to it. This defeats the purpose of having access restricted.

--Keep the names short (for devices and users).

Core Usage Rules and Penalties

Calendar usage:
--Calendar bookings are limited to 2 hours in length between 12 pm and 9 pm, and no more than 2 bookings per week between 2 and 6 pm. Non-Ragon users may not book time more than 1 week in advance, and Tues, Wed, and Thurs afternoons from 3 to 7 are reserved for Ragon users until the day before, when unclaimed time can be booked by all.

Violations of these limits will result in warnings for the first 2 occurrences, and a 1 week ban from reserving instrument time on a third offence.

Data Storage Limits:
--Data storage is limited to 2 experiments in Diva and nothing older than 2 weeks, and no more than 3 GB in the personal D drive folders. To avoid excess data in Diva, the core exports data as an experiment into users' D drive folders, into a new folder labeled "experiments mmddyy". Users are responsible for checking these folders to see if they need the data and storing or deleting as appropriate. These folders should be removed, and storage on the D drive should be minimized. You will be warned when your folder has exceeded the limits either by email or being listed on the dry erase board near the instrument.

Habitual abusers of storage space will be issued a violation.   A second violation will be issued if no action has been taken, and a 3rd violation will result in your access being blocked until it is reduced.

Instrument Usage:
--If you are the last user after 5PM ("after hours"), it is your responsibility to make sure the instrument is turned off at the end of the day. If you cancel on the day of your reservation and were the last user, you must make sure that the user before you knows that they need to turn it off, and all users should check the calendars when they are done to verify there is a reservation after them. The core receives notices when events are booked or cancelled so the timing of any changes can be verified.
--All users are responsible for making sure the tank levels are adequate for use before and after your time, and that instruments are kept in good condition (no broken parts, etc).

****Any misuse of the instruments will result in a warning for a first offense, a 1 week ban for a second offense, or a 1 MONTH ban for a third offense.****

Using the Instruments

--All human samples must be fixed, and no radioactive samples can be run at any time.
A protocol is in place so that noninfectious unfixed human cell lines may be run after proper instruction from core personnel.
--Gloves should be worn at all times while using the instruments and the computers connected to them.
--Start up the instrument by pressing the green button in the lower right corner. Make sure the computer is on. There is no special order that needs to be followed when turning on the instrument and workstation. Log into the computer with the username and password posted on the instrument, and open the Diva software by double clicking on the icon on the desktop. It takes several seconds to start, if you get impatient and click it again, it will crash and give an error message. Clear the error and then double click and WAIT. Each user or lab has their own login.

Checking the sheath tank

The sheath tank is kept on a digital scale to make it easier to determine the fluid levels. The scale has an auto shut off feature that can be disabled by holding the lb/kg button when you turn it on. If the scale reads anything besides 0 when the tank is removed from it, press "zero/hold" to reset it to 0. The different sized tanks have their capacities indicated on the scale readout. If the tank is less than half full, you should refill them.

To fill the sheath, disconnect the opaque green air line from the tank by pressing on the metal tab on the connector. Do not disconnect the clear sheath line as this will introduce air into the sheath path. Unscrew the black handle until it slides up and down, and then lift the round vent valve to release the pressure (the lid should drop into the tank), and remove lid. Make sure the black o-ring comes out with it—it sometimes sticks to the tank, and will sink if it falls into the sheath fluid. Remove the sheath filter from the holder attached to the leg of the table and lay it on the floor.

Carefully keeping the tubing/connector away from the sheath cube, fill tank with sheath fluid from white box up to weld seam inside tank (about 2 inches from top). Make sure black o-ring is still seated around lid, and cover tank. Reconnect air line to tank. Replace the tank on the scale.

Debubbling the sheath path should be done EVERY time you start up, in case the person before you did not do it or air has leaked into the lines, and requires the instrument to be turned on. Holding the body of the bubble filter, tap the BODY of the filter against the table and/or tank to get air bubbles to collect at the base of the bleed line then expel the air by releasing sheath fluid into the plastic bottle on the table. Do not hold by gray stem while tapping or filter will break off. Repeat as necessary. Bleed second roll clamp on side of instrument to remove air between filter and instrument—start stream flow, then pinch down to stop and start stream a few times to dislodge bubbles that may get caught on connectors. Then allow to drain for 8-10 seconds to ensure all air is removed. Observe the junction where tubing enters the instrument—if you see air there, contact Adam or Mike to purge the system.

Waste Handling

Biohazard box is for biohazardous materials ONLY (samples, gloves, etc.) ALL gloves and sample tubes go in the biohazard boxes. Empty glove boxes and other trash can go in regular trash can. There is a cardboard box for recycling white paper.

Due to the presence of paraformaldehyde in the fixed samples that are run on the instruments, all waste is collected for pickup, and does NOT go down the sinks.

Used sheath fluid and sample that has been run is deposited in the plastic carboy under the table. Bleach is added to the bottom of the tank to produce a 10% solution when the tank is full, inactivating any potentially infectious materials. A length of tubing is connected to the quick disconnect fitting on the cap to ensure waste is deposited directly into the bleach, and a filter is built into the cap to remove any aerosols from the air that is displaced from the tank as liquid is added. Be sure to verify that the filter is in place while the instrument is running.

When the liquid level in the tank reaches the "full" line drawn on the side, please swap the tank for the empty one nearby by doing the following:

Make sure the instrument is in "standby" mode with a tube of water loaded on the sample loading port and the arm centered. Disconnect the black electrical connector by turning 1/4 turn counterclockwise, and the orange waste line by pressing the metal tab. Unscrew the sealed cap from

the empty tank and transfer the waste cap to the empty tank, being careful not to drip waste from the sensors and tubing on the underside of the cap. Cover the full tank and set aside, and then reconnect the empty tank to the instrument.

Waste is collected in a 30 gallon drum that is kept in 5227. Core personnel will empty the tanks into the drum. If we are not around and both carboys are full, please wear a lab coat and use the funnel to transfer the waste to the drum, and replace the cap on the drum when you are done. Also remember to add bleach (kept on the floor next to the drum) to the empty waste container so there is about 1" of bleach in the bottom of the tank.
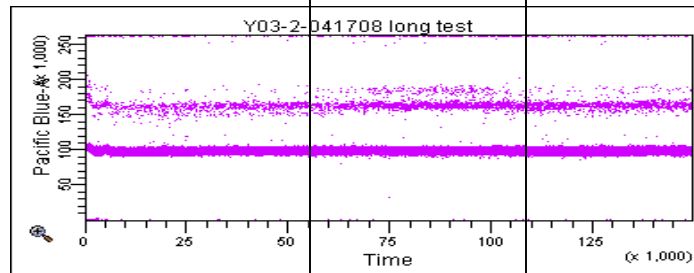
Laser warm up times

Below are plots of signal over time while running a rainbow bead, showing the warm up time required for each color laser. Samples should only be run when the laser power (and thus generated signal) is stable.
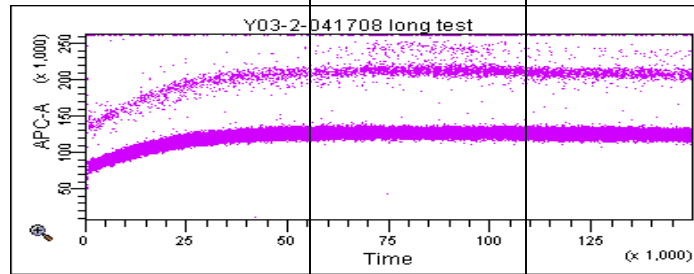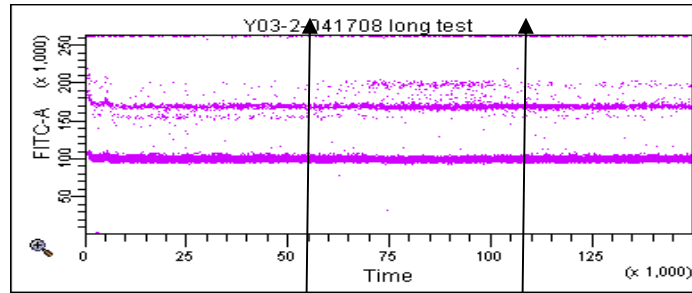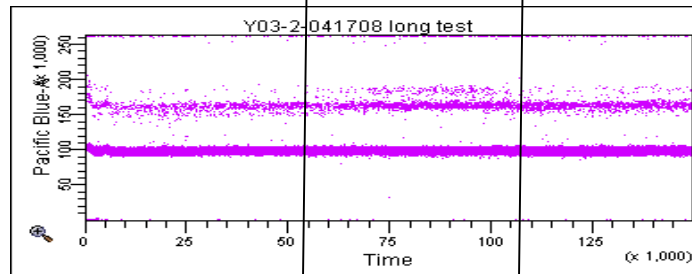
3 Laser LSR II

Blue
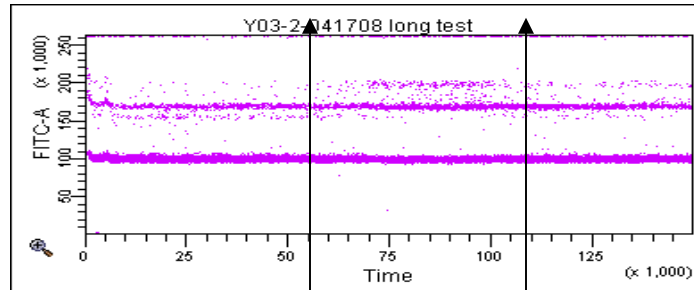
Violet

Red

10 mins   20 mins

4 Laser LSR IIs

Blue

Violet

Red
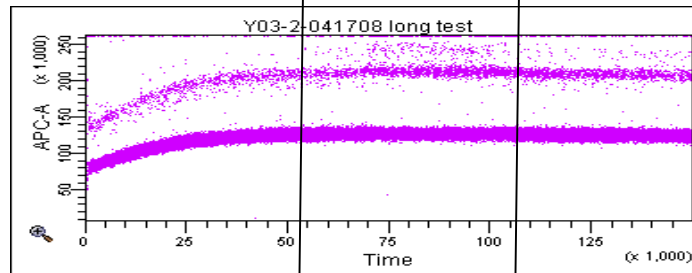
UV
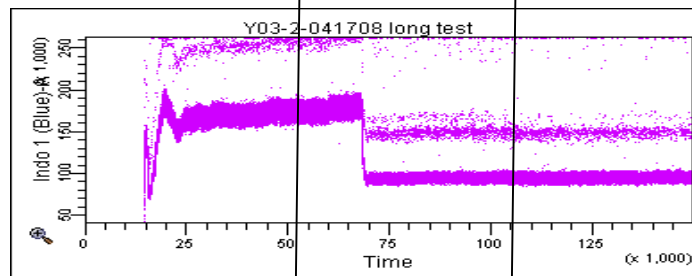
10 mins   20 mins

5 laser LSR Fortessa

The cube lasers used on the Fortessa warm up quickly, so that by the time you are able to log into the software they will be ready to go. UV is the only one that will only turn on when Coherent Connection is running, the others will turn on when the cytometer is on, but they can be turned off in CC. These plots were generated by turning the lasers off in CC, booting up Diva, and then turning on the lasers and starting acquisition.
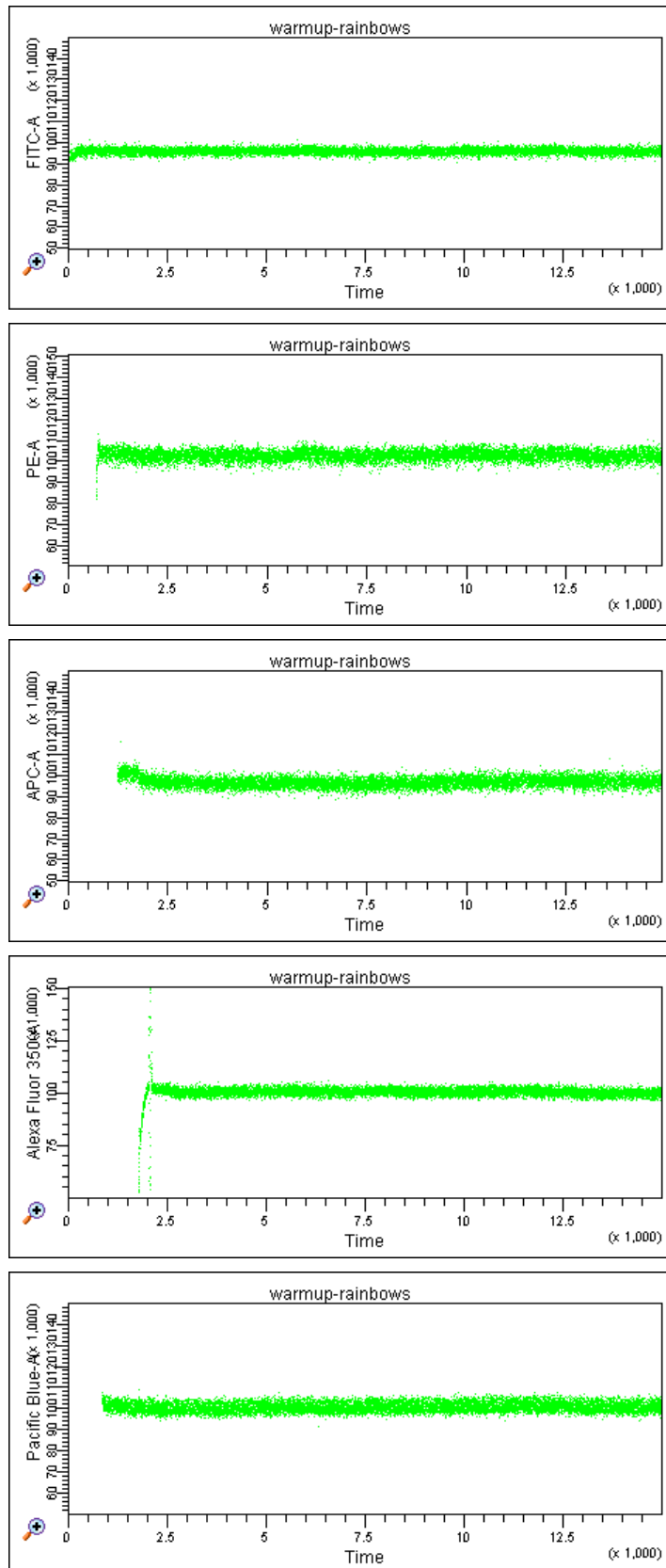
Sample Injection Probe (SIP) and Droplet Containment Module (DCM)

The port where the samples are loaded consists of a sample probe and a lever with a black knob which is called the "tube support arm". The metal tube that is visible is actually an outer sleeve covering a thinner tube that delivers your sample to the flow cell. The outer sleeve and tube support arm are part of the Droplet Containment Module (DCM).

In normal operation, the tube support arm is positioned to either side, and a tube is loaded on the sample port. There is a black ring around the top of the probe (BAL seal) that holds the tube in place, and when the arm is centered, the tube is pressurized. Tubes should be inserted or removed vertically without any pressure side-to-side so that the instrument is not knocked out of alignment. Push the tube all the way up on the probe: the top of the tube should touch the flat part above the BAL seal, and the metal probe should be in the very bottom of the tube. Center the arm to turn off the vacuum and pressurize the tube. If the seal is insufficient to hold the tube on the sample probe (see next paragraph), the arm is there to catch the tube before it falls on the benchtop and spills. It is NOT intended to hold the tube on, and the black knob should NOT touch the tube during operation.

If the tube does not stay on the sample probe on its own, the seal may be wet (wipe it off with a kimwipe), the tube may be cracked or worn (transfer sample to a new tube), or the seal may be worn (notify someone in the core to replace it).

While the instrument is on "run", sheath fluid is being supplied to the flow cell under pressure. If there is no sample loaded on the sample port, the sheath fluid would flow out through the sample probe and drip. The instrument is engineered with a DCM to prevent this from happening. When the Tube support arm is to the side, it turns on a vacuum that is connected to the outer sleeve of the sample probe, which aspirates the sheath fluid away. (see figure A on the next page--sheath backflushing through the probe in the center, being aspirated by the outer sleeve).

When a tube is loaded and the arm is centered, the vacuum is turned off and pressure is applied to the sample tube, driving sample up the sample probe and injecting it into the flow cell (see B--tube loaded on the probe, sample running up into the flow cell at the top). Note that if a tube is loaded and the arm is to the side, there is no pressure applied to the tube and the vacuum will aspirate your sample directly to the waste!

A   B   C   D
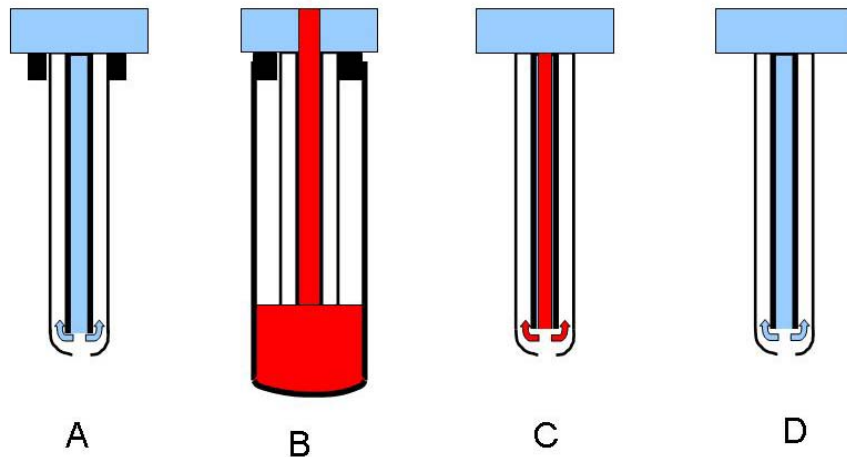
Immediately after removing your sample tube, there is still sample in the sample probe (C--sample probe with sample still in the tube), so it is necessary to wait 10-15 seconds to allow the sheath fluid to backflush through the sample probe and wash out the sample to avoid carryover to the next sample (D--clean sample probe). Cells usually don't stick to the outside of the probe, but beads tend to stick more so when running beads, or for extra precaution (if you just ran a positive control and you are looking for rare events), you could load a tube of water briefly or wipe gently with a kimwipe to make sure the outside of the probe is clean. This is also a good idea if you are swapping your controls repeatedly and rerunning them, or you will likely see events of the wrong color appearing in your control sample files when you finally record them.

Please pay attention and take note of anything unusual that happens while running your samples. If the SIP drips while the arm is to the side, the vacuum line is likely clogged and we will need to be notified so that it can be cleared. Likewise, if you are cleaning the instrument and notice the tube FILLING while the arm is to the side, this is another indication of the same issue.

Changing Configurations

Each laser on a cytometer has its own array of filters and detectors for measuring the signals generated. Which detector to use for each reagent is mapped out within the Diva software and is stored in a Configuration. Filters can be changed to match the requirements of your experiments, and new Configurations need to be generated to reflect these changes. Make sure your colors are represented on the configuration and the correct configuration is active.

At the front of this manual are the different configurations currently available on the LSRs. Determine which configuration (filter setup) is correct for your experiment and verify that the filters installed in the detector arrays match those in the Configuration diagram. Filters highlighted in yellow show which ones need to be changed from the default configuration in order to match the alternate configurations. Filters should be inserted with the bottom of the label facing the center of the array, and filters that are removed from the default configuration can be placed upside-down on top of the array to make it easier to locate them and identify what filters have been changed when switching back to default.

Just as important as verifying the filters is when you log into the software, it will display in the header which configuration is active: if this does NOT match the configuration you need to use, you must switch configurations. Under the "Cytometer" pulldown menu, select "View Configurations", and then choose the one from the list that you need to use, and click "Set configuration". Close the CST/Configurations windows and return to Diva. Verify the proper configuration is displayed in the header.

On the front of the instrument is a card that says "Default configuration" on one side and "configuration has been changed" on the other. Be sure to flip this card if you change the configuration. In the event that you forget to switch the filters back, this will notify the next user that the filters have been changed and they can correct the setup for their experiment.

"CST" or Cytometer Setup and Tracking, is a QC protocol that sets proper laser delays and area scaling, and tracks instrument performance to give early warning of problems arising. We only run setup on the default configuration, so that is the only configuration with the correct settings. When you change the configuration on the LSRs, it is important to take the following steps to make sure the proper "CST" settings are applied.:

a. When you first log into Diva, make sure the "default" configuration is the active configuration.

b. ONLY accept "use CST settings" when the Default configuration is active.

c. If using a different configuration, select "Keep current settings" AFTER accepting the settings from "default". If you select "keep current settings", the following errors will appear in the cytometer window. You can just 'clear' them.
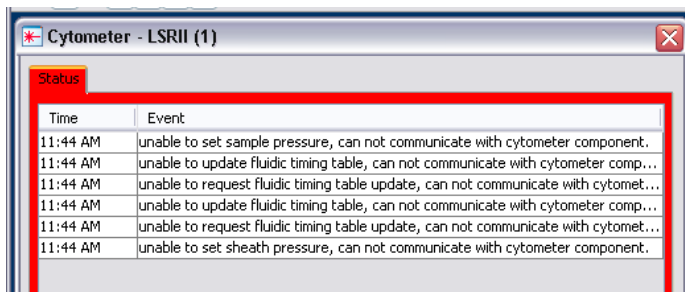

To make sure your settings are correct, make sure the "default" configuration is active, log out and then log in again and choose "Use CST settings" if the CST mismatch dialogue box appears. If it does not appear, then your settings match CST and you may proceed.

If you want to see how different the settings are, select "details" when you get the CST mismatch dialog window. Some of the out of date performance checks for the alternate configurations will be WAY off.

To change configurations:

1. From the cytometer pulldown menu, select CST. Verify that cytometer baseline and cytometer performance have passed and are up to date for your configuration.
2. To select a different configuration from the default, click Select Configuration, pick out the configuration you are going to use and click Set Configuration.
3. During your first time checking CST, it will say everything has failed until you select a bead lot. Select the appropriate bead lot from the Lot ID menu. Now verify the cytometer performance is passed and up to date.
4. If the performance is listed as out of date or failed, please see Mike or Adam.
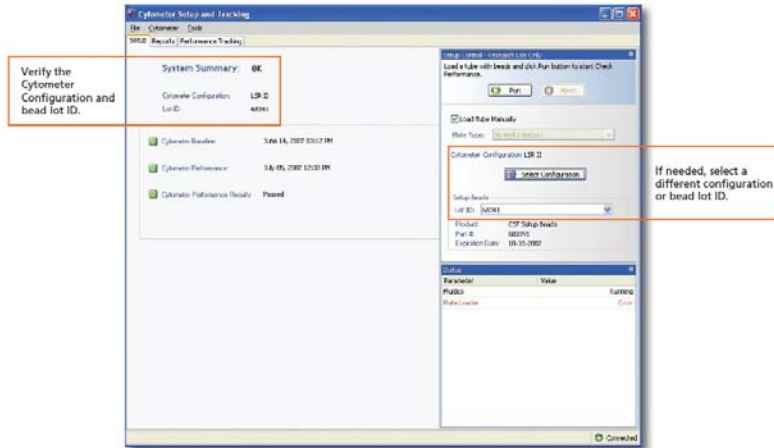
This is what a passed CST window should look like

Miscellaneous Tips and rules:

--No unlabeled tubes should be left on the desk. Please throw away any unlabeled tube you find.
--Fresh cleaning tubes should be made weekly.
--Label all tubes. Do not overfill (no more than 2/3 full)
--Don't let a tube run dry,
--Don't leave a sample tube on while tube support arm is to the side as your cells will be aspirated directly to waste
--Filter samples especially if there might be large clumps of cells.
--Recommend that you run water before you run your samples.
--There is a "sample fine adjust" knob, which allows the operator to set event rate to any level instead of just 3 fixed rates. (Lo flow rate with knob all the way clockwise is faster than Med flow rate with knob all the way counterclockwise).
--full standby (button), stops sheath fluid flow to conserve sheath. Use only when done, and always load water tube while instrument is in standby—while running samples, leave instrument in "run".
--PRIME: flushes the flow cell, refills with sheath--clears air bubbles (like when you let sample tube run out). May eliminate noise. Always remove tube before priming!

Tips and procedures specific to the 5 Laser LSR Fortessa

1.   "Coherent Connection" is a program that allows you to control the power output of the lasers on the system, and is started using the shortcut on the desktop before opening Diva. Please wait until the instrument connection is established before trying to open the software.
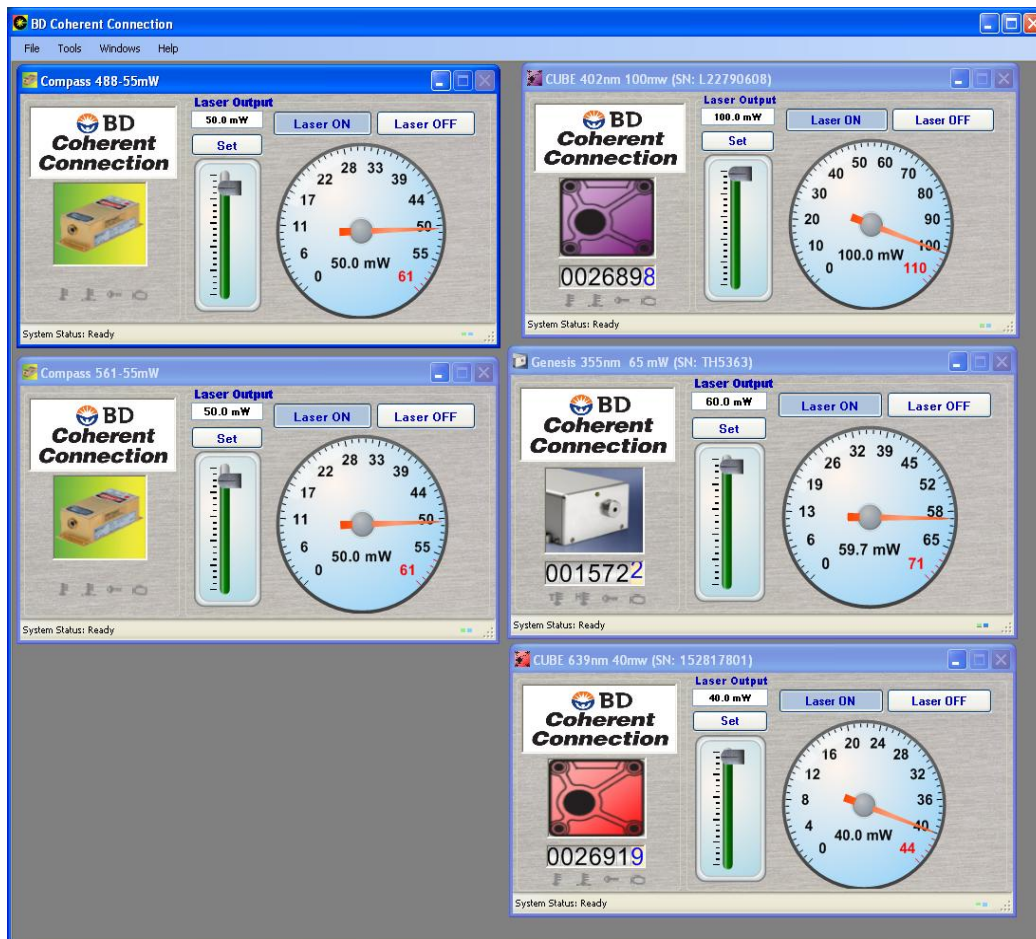
"Disconnected"   "Connecting"   "Connected"

All 5 lasers should have a window displaying the power setting and output as shown:

These should not be changed but can be used to see laser status for certain problems.

        At times, errors will occur when the program is loading, most often if you try to open CC before the instrument connection has been established—this is a known issue, and there is no other fix at this time other than the following:
--If either the blue (488) or green (561) lasers are not showing, use the "Tools" pulldown menu and select "serial settings" and then click the button to "scan ports", and the laser controls should appear again.
--If the UV controller is missing (it will sometimes disappear during the course of the day—this does not mean the laser is off, just that you cannot change the settings. You

likely will still get signals from the UV laser channels) it is necessary to restart the computer to get it back, just restarting the CC program does not restore it.
--If the software gives you an "unhandled exception" (I've only seen it for the red laser), the instrument most likely was not connected when the software was started. Quit and restart CC making sure the instrument is connected. If that does not restore the slider, then restart the computer, make sure the instrument connects, and then open CC again.

2.   Allow to run for at least 1 minute after priming before running samples—priming clears the waste lines of liquid, and the stream runs faster until it fills again (air offers less resistance passing through the connectors, once liquid is going through the stream slows down again from the back pressure). The system was aligned with the waste lines filled which is the normal operating mode, so when it runs fast the computer misses the green laser signals and all events will read as negative, and then once it fills again signals will come back to normal. This can be observed in any plots with signals from the green laser, all events will be negative and then gradually increase to a final high intensity, and if a time vs green parameter dot plot is created, the gradual increase can be observed. Make sure signals are stable before recording. The other lasers will also be affected, but to a lesser degree and will recover faster since they aer not as far from the blue trigger laser.

Name	Delay	A
Blue	0.00	
Red	103.87	
Violet	33.03	
UV	66.16	
YellowGreen	138.12	
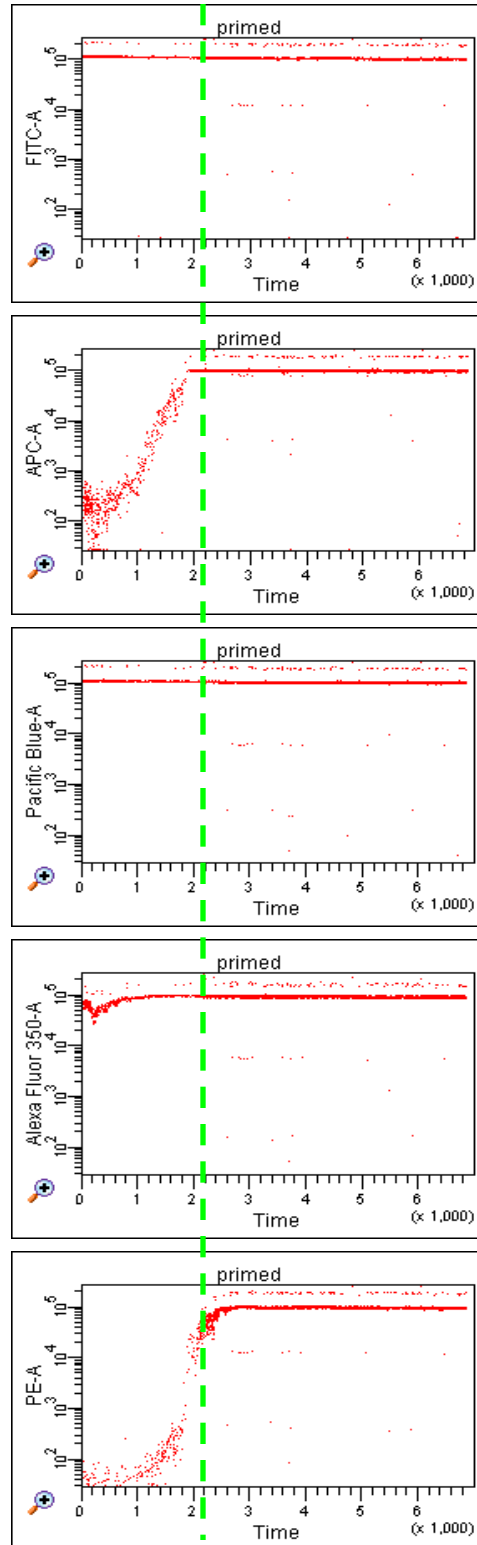
3. Like the 3 Laser LSR, Diva will take longer to start up as it looks for the HTS (High Throughput Sampler). It will give a "fluidics" error message, which can be ignored.

4. A few times in May/June 2010, the blue laser shut off during the day. The issue stopped but if you lose all events and have no signals, check Coherent Connect and make sure the blue laser is still "on". If not, turn it back on and everything should work.

5. The 5 laser LSR Fortessa runs Diva 6.1.3. Experiments created in this version cannot be opened in 6.1.2 so make sure you have the current version installed if you want to use Diva on a different workstation.

6. Keep in mind that PE and its tandems are detected off of the Green laser, but will still generate significant signals off of the blue laser. For this reason, it likely will NOT be possible to run PE-Cy5.5 and PerCP-Cy5.5 at the same time, even though they are measured in different channels. PE-Cy5.5 will still produce signals comparable to what is generated on the other LSRs on the Blue laser, so there will be so much signal in the PerCP-Cy5.5 detector that you likely will not be able to distinguish signals.

Running Unfixed Human Cells

Unfixed human cell lines (NOT primary tissue samples, even from "healthy donors") may be run on the 4 laser LSR2. A sign must be placed on the door, and the door locked to prevent entry while samples are run. Operators must wear a NIOSH N95 respirator, and wipe down the work area with ethanol after running samples.

--a particulate respirator must be worn at all times.
--Cover the card reader with the orange "biohazard--no entry" sign
--ALL TUBES MUST BE COVERED WHILE VORTEXING
--run 10% bleach when finished running your samples before doing the usual cleaning
--Make sure the acrodisk filter is in place on the waste tank.
--spray down work surfaces with ethanol when finished running samples
--a disposable lab gown must be worn and then discarded after completing the decontamination.

In order to wear a respirator, a form must be filled out ("Respirator authorization form") and faxed to OHS at 4-3944. In addition, a fitting must be scheduled with the Safety office by calling 6-2425. Contact Mike Waring for details and a copy of the form (mwaring@partners.org)
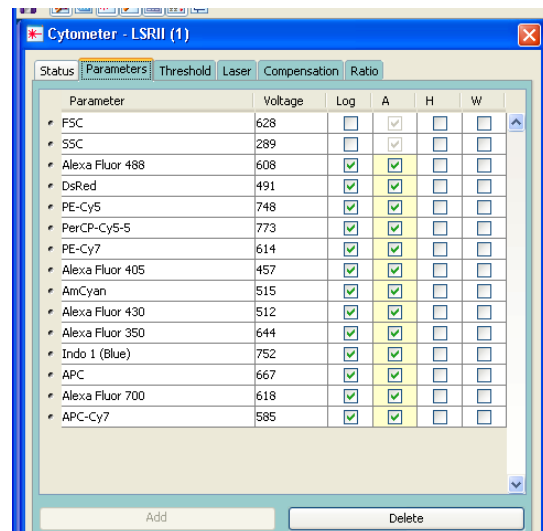
LSRII / Diva Startup Guide

If necessary to log into Windows, the username is "Adminstrator" and the password is "BDIS" on all core instruments.

1. Open FACSDiva software. Log-in to your profile.

5. If CST Mismatch dialogue appears, you should usually select "Use CST Settings". See "Changing Configurations" for the exceptions.
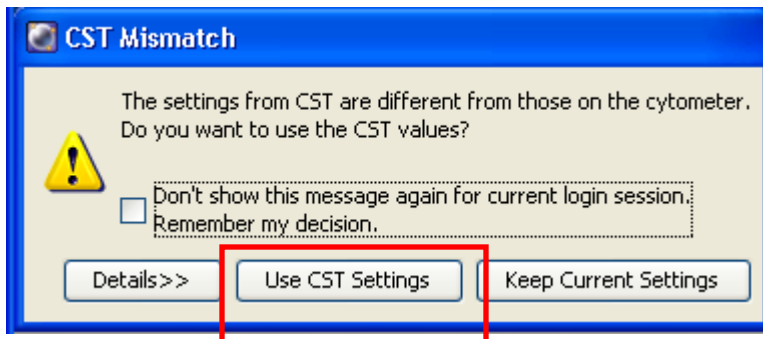

6. Click the "New Experiment" button to start a new experiment (brown square at top of Browser window). Rename experiment.

7. Highlight "Instrument Settings" and in the Inspector window you will see each detector listed, identified by the associated reagent that comes first alphabetically. Dyes occupying the same detector can be found in the configuration tables. New users may find it easier to delete all of the parameters and add them in one at a time, selecting their desired labels from the full list that will display for each added parameter. Delete unwanted parameters and/or switch default parameters to the labels in your experiment (i.e. Alexa 488 to GFP). If the reagent you wish to use is not listed, check the other configurations or talk to core personnel to have them added (if compatible).

8. If you wish to use optimized settings, verify that cytometer settings are linked to CST. The cytometer settings icon should have a ball in the upper right corner indicating that CST settings have been applied to the cytometer setup. → Cytometer Settings
- If not, right click Cytometer Settings and select Link To CST. These settings will give you the best resolution of dim signals but may not be appropriate for bright signals! This will be verified in 12 below.

9. Create Compensation Controls under Experiment > Compensation Setup.
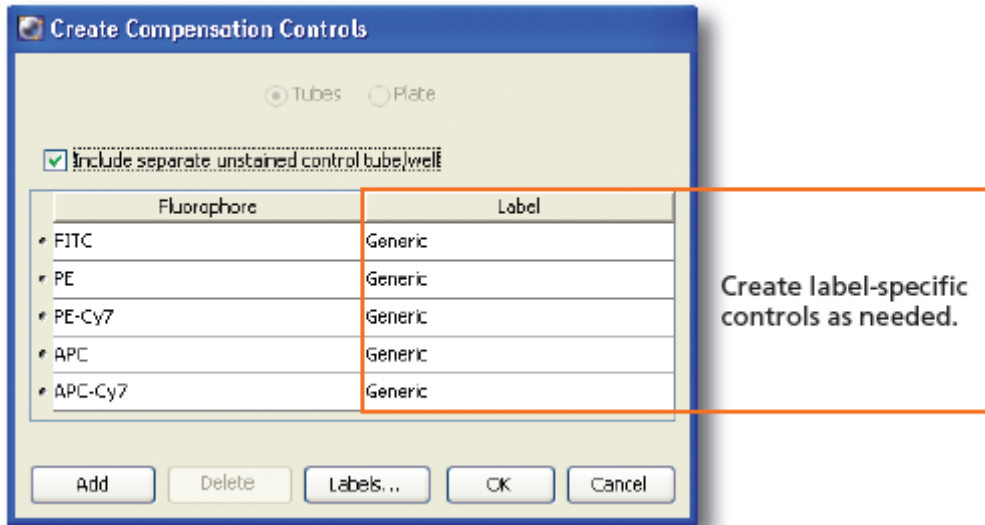
10. Load unstained control and set voltages so that your particles are on scale in FSC/SSC. Note CST settings are optimized for BEADS, and cells will usually require dropping FSC voltage 200-300 V.
11. It is a good idea to run each of your single stain controls on the unstained worksheet and verify that:
    1 – none of the signals are off-scale
    2 – signal in the primary detector is brighter than in all the others. (e.g. PE-Cy5 signal in PE-Cy5 is higher than in APC). Adjust voltages as necessary.

12. If not using CST settings, adjust fluorescence voltages so that the tail of the negative peak is at the end of the second decade on the log scale for all fluorescence parameters.
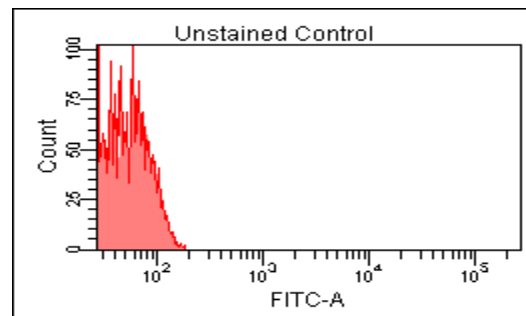
13. Do NOT change voltages for any parameter at any time after recording the unstained control or else you will have to go back and re-record every control again.

14. You can avoid moving the P1 gate for every sample by right-clicking the P1 gate and selecting Apply To All Compensation Controls after positioning it for the unstained control.
15. Record single stain controls. Make sure the positive peak is selected for each tube. Software should adjust the interval marker automatically but it is a good idea to double check and make sure the correct peak is selected.

16. Calculate Compensation by selecting Experiment > Compensation Setup > Calculate Compensation. You now have 2 options: "Apply" will copy the compensation to your experiment. "Link and Save" will link the compensation settings to the experiment and save the compensation setting in the compensation setup catalog. If you choose to Link and Save make sure you rename your settings (initials and date usually work best) before saving. A chain on the cytometer settings indicates that your compensation settings have been linked ( ). If you duplicate an experiment that has been linked to a compensation matrix, even if you re-run compensation it will be locked into the previous matrix--you must first unlink the experiment from the previous settings. To unlink, right click the Cytometer Settings below the experiment name and select Unlink.

17. Create a new Specimen by clicking the New Specimen button. ( ).

18. Create New Tubes as needed by clicking the New Tube button ( ) or clicking Next on the acquisition dashboard as needed. "New Tube" will generate an unlabeled tube defaulting to 10,000 events. "Next" will duplicate labels and options of the previous tube.

19. Click "Acquire" in the Acquisition Dashboard and verify that the signals are stable before clicking "Record" to record your data.

20. Perform shutdown procedure (see below, or back cover of hardcopy binder).

21. Leave the instrument in standby, with water tube loaded and approximately 1 cm of water in the tube. If no one is signed up after you please shut the instrument off. Please remove trash: leave it as you found it (or better!).

Summary for using LSRII

1. Turn on cytometer before using according to the warm up times.
2. Check sheath and waste, fill or change as necessary
3. 5 Laser only: when cytometer has connected to computer (icon in taskbar at bottom of screen), start Coherent Connection software.
4. Verify configuration (filter setup).
5. Log in. Create new experiment or duplicate existing template or import template
6. Rename experiment. If template, skip to 9.
7. For New Experiment, choose parameters (colors).
   a. select "instrument settings", and view list in "inspector" window
   b. highlight unwanted parameters, and click "delete" button in window to remove
8. go to Experiment>set up> create compensation tubes
9. run unstained cells in order to set FSC, SSC, and negatives for all parameters
   a. click acquire but not record
   b. adjust voltages as necessary
   c. depending on your panel, additional voltage adjustments may be required, see "Optimizing Setup of LSR" below.
10. Run unstained beads
    a. Click acquire
    b. Move FSC/SSC gate to where beads are, or adjust voltages if necessary (ONLY for FSC and SSC).
    c. Right-click on gate and "apply to all compensation tubes"
    d. Click record
11. click NEXT
12. Instrument will display what tube it wants to record next (follows order of parameters in the list)
    a. Do NOT change voltage after unstained tube has been acquired. If it is necessary to do so, you will have to re-run all samples.
    b. Click record.
13. When recording is finished, repeat 11 and 12 for each color.
14. set gates for positive populations (do not need to set gates for negatives)
    a. If you are using a mixture of sample types for compensation controls (eg. GFP cells and beads for other colors) you must define a negative population that matches the particle for the positive. If you run beads for the negative, you must draw a negative gate in the GFP cells control sample (and have negative cells in the sample!).
15. go to Experiment>Set up> calculate compensation. Choose "Apply only".
16. create new specimen. Will contain new tube. Rename tube
17. Click on acquisition pointer to left of tube.
18. Go to Experiment>layout and name the colors (i.e. CD3-FITC)
19. click on button in upper left of worksheet window to change to global sheet. Whatever tube is active (green acquisition pointer) will be displayed in plots created on this worksheet. Create desired plots.

a. Draw in gates
b. Right click on margin of graph, choose SHOW POPULATION HIERARCHY to see how populations are related
c. on subsequent plots, right click margin of graph and select "SHOW POPULATION" and select gate/region you wish to display

20. change EVENTS RECORDED in acquisition window.
21. Load tube, click acquire. If all looks ok, click "Record"
    a. Will stop automatically when target # of events is reached.
    b. To stop recording before target number, click record button again- will save data acquired so far.
22. click NEXT TUBE to go to or create next tube, rename it, and return to #21.
23. When last tube is finished, turn off acquisition by clicking "Acquire" (will stop automatically if target # is reached).
24. Clean instrument (see "Shutdown Procedure" on p 41 or back cover of binder):
25. right click on experiment name to "CLOSE EXPERIMENT"
26. Export FCS files—highlight experiment name, File>export>FCS. Be sure to save to your personal folder on the D drive and NOT to the BDExport folder.
27. Exit Diva.
28. Burn files or transfer over network.

Doublet Discrimination

1. Turn on FSC Height and Width and SSC Height and Width when selecting your parameters.
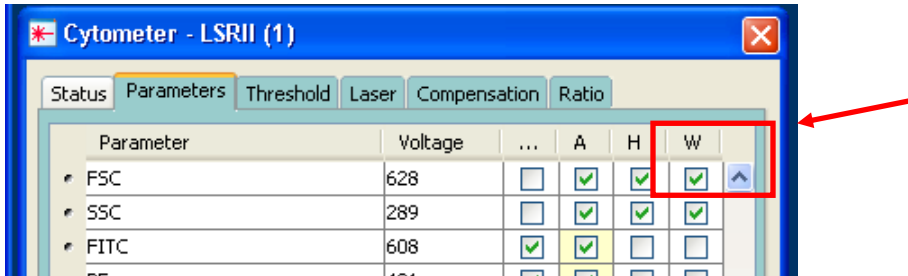
2. Gate on your population of interest in the FSC-A vs. SSC-A plot like normal. In this example we are gating on lymphocytes.
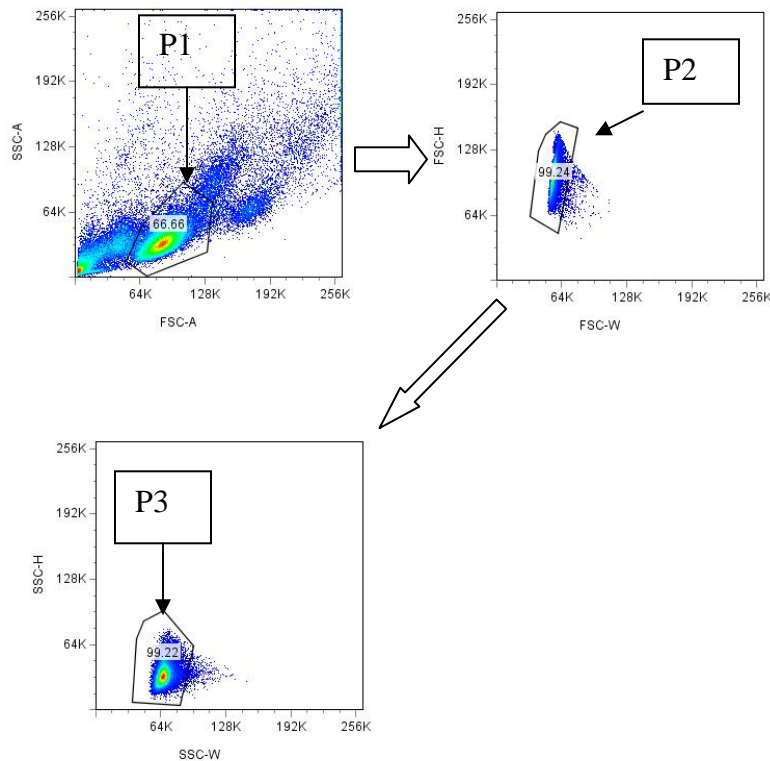
3. View the lymphocyte population (P1) in the FSC-W vs. FSC-H plot. Gate on the bulk of the population, ignoring the doublets tailing off to the right that have higher width signal.

4. View the FSC discriminated doublets (P2) in the SSC-W vs. SSC-H plot. Once again, gate on the bulk of the population, ignoring the doublets with high width signal off to the right.

5. P3 will be your population of interest with the doublets gated out.

It is also possible to use a FSC-A vs FSC-H plot, and gate on the events along the diagonal for your single events. On the LSR, usually gating on FSC is sufficient to remove doublets (on the sorter, both FSC and SSC should be used).

"Wersto et al. 2001. Doublet Discrimination in DNA Cell Cycle Analysis. Cytometry 46:296"

From the Purdue Message Board:
"You should always use DNA area for the DNA histogram because total fluorescence is what you want to measure. You can then use either peak (height on the DIVA) or width as the other parameter to give doublet discrimination. I give a short explanation in my book in Chapter 6, DNA Analysis, available for no charge at http://flowbook.denovosoftware.com/.

Regards,

Michael Ormerod,"

Backgating

Sometimes it is a good strategy to make sure that your cells of interest appear where you expect them to on plots earlier in the gating tree, to make sure that you are not accidentally clipping the population (for example CD8 cells typically have slightly lower CD3 signal, so if you gate on the top portion of the CD3+, you will lose many CD8+). Set up a plot showing "all events" on your plot of interest (CD4 vs CD8), and gate on your population of interest (CD8+). Then create a FSC/SSC plot (or whatever plot you wish to backgate onto) and "show populations>CD8+". You will then be able to see where all of the events that are producing signal in the CD8 channel are localized, and possibly adjust your gates accordingly.

Cytometer Shutdown Procedure

1. Remove your last sample
2. Fill a tube 2/3 full with 0.1N NaOH. Mark fluid level with sharpie.
3. Load tube on sample port, run with lever ("tube support arm") to side for 1 minute--this will clear the tubing of the "Droplet containment module". Verify that the fluid level drops 1 cm or more --if not, please notify me.
4. Move lever to center and "RUN" on "HIGH" for 2 minutes. Fluid level should drop another 1 cm.
5. Unload tube, and replace with a tube of Coulter Clenz (blue liquid) and repeat 2-4.
6. Unload tube, and repeat with a tube of <u>water.</u>
7. Leave water tube on sample port with less than 1 ml of water in tube. Do not leave too much liquid in tube, as some backflow will occur, and if it fills completely then fluid will be forced into the sample pressurization holes, into the air line, and clog the hydrophobic filter.
8. Leave machine in "STANDBY", or turn off if no one is using it after you.

Before you leave, please check the sheath and waste tanks to make sure they are ready for the next user. If the waste tank is up to the "full" line, then swap in the empty one. If the scale for the sheath tank is below the "fill" weight, please refill it.   Check the reservation calendar to see if anyone is signed up after you. If not, please turn the instrument off.

Optimizing setup of the LSR2 system (voltages and compensation)

The goal of optimizing your voltage settings on a digital cytometer is to have good separation between your positive and negative populations while minimizing the overlap between your parameters. A new method of instrument setup and tracking has been developed that will be recommended for all users of the flow facility. For a more in-depth description of the processes involved, the following documents will be useful:

> -- BD Application note "Establishing Optimum Baseline PMT Gains to Maximize Resolution on BD Biosciences Digital Flow Cytometers"
> http://www.bdbiosciences.com/documents/Establishing_Optimum_Baseline_PMT.pdf#search=%2823-8389-00%29
> --"Flow Cytometry Controls, Instrument Setup, and the Determination of Positivity", Cytometry Part A, 69A, 1037-1042 (2006).

The first step in this process is to determine the amount of electrical noise in the system. The documents linked above will explain how this is done, but this part of the setup will be performed as part of facility QC and the results posted by the instruments. In short, the operator runs a dimly fluorescent bead and records data files where voltages are set in 50 volt increments (all parameters at 250V, all parameters at 300V, etc, up to 800). A plot of CV vs mean will allow you to derive the Standard Deviation (SD) of the electronic noise in the system. The target mean value for your negative population should be 8-10 times this value to minimize the contribution of noise to your signal, and resolution of dim and negative particles will be optimum. (Note that beads and cells, and even different types of cells, will have different amounts of fluorescence in each channel, but the *target mean signal* for each is still the same to optimize resolution).

For most parameters with clearly defined populations, it is sufficient to set your voltages so that your unstained cells are in the first decade (mean of around 50, tail ending at around 200), but if you have a dim signal and wish to optimize the separation, set the voltage so the mean signal is 8-10 times the SD of the noise (typically resulting in a mean signal from 100-300 depending on the channel). As you increase above this setting, the negative population will also gain signal and your separation will not improve. In addition, higher voltages will mean that you will get a higher response from overlapping signal, making compensation more difficult, so it is preferable to use the lowest voltage that gives the result you want.

In the event that your positive population goes off scale at these settings, you have 2 options. Since you have separation of 3 logs or more, you may reduce the voltage to bring your bright population on scale. Alternatively, you may titer down your antibody, which would allow you to save on reagents while still getting enough separation to identify your population of interest. Another important consideration is that if your antibody is that bright in its own channel, it will also be bright in any channel it overlaps into, so titering the antibody will also aid in compensation, whereas lowering the voltage

will make it harder to compensate. With something like GFP, which cannot be titered, you will have to lower the voltage or use a neutral density filter in that detector (uniformly decreases signal reaching that detector) and deal with any compensation issues that this causes.

<u>Compensation</u>

Using these voltages you have determined, you can generate a compensation matrix and see if any problems arise. When calculating compensation, it is critical to match the positive and negative particles—if you are using beads labeled with antibody, the positive population must be compared to unlabeled BEADS, while labeled cells must be compared to unlabeled cells, so that you are correcting only for the fluorescence contribution of the fluorochrome and not any difference in signal from cell to bead (or from cell to cell—Lymphocytes and monocytes have very different autofluorescence). Note that when SETTING the voltages, you want to optimize for the experimental particle you are measuring (usually cells), and then collect the compensation particles at these settings, regardless of what type they are.

It is preferable to use antibody capture beads, especially if you have a label that is dim or low frequency (<1% of the population). Beads will give you a bright, uniform signal for each reagent. If you are using antibody capture beads for all of your colors, you can simply record unlabeled beads as the unstained control, and then single stained samples for each color, and the software will automatically use the unstained control as the negative for all tubes.

For labels such as PI, CFSE, or viability dyes, which will only stain cells, you may just use cells for ALL of your controls (unless one or more of your labels is dim or low frequency—see below), and collect a sample of unlabeled cells as your unstained control. Be sure to consistently gate on the same cell population (e.g. only lymphocytes), as different cells have different levels of autofluorescence.

For viability dyes, you are typically gating out the positive events, so that the spillover of the viable cells is minimal since they have minimal signal—if you have been using labeled cells as the control and comparing them to unlabeled beads, the effect should be minimal but it is not the best way to do it.

Invitrogen has a line of Live/Dead fixable stains that are amine-reactive dyes that will bind to any available amine groups. To compensate these dyes, they also produce Arc beads (A10346), which are amine-coated beads that will bind the dye and produce positive signal. The live cells do have some signal so it is important to correctly compensate these dyes, as opposed to other viability dyes where they are excluded by the live cells and thus live cells are truly negative. Live/Dead Red is PE-Texas Red,

With CFSE, where you are interested in the POSITIVE events, it becomes more problematic—for instance, there should be minimal overlap of CFSE into the Pacific Blue channel, but if you are comparing CFSE labeled cells to unlabeled beads, you may in fact see the signal in the unstained beads is higher than the CFSE stained cells in Pac

Blue because the autofluorescence of beads in Pac Blue is higher than the cells. In this case it is much more important to compare to unlabeled cells to determine overlap.

If one or more of your antibodies is dim or very rare (<1% of the population) AND you have a reagent that must use cells (GFP, CFSE, PI, or an antibody that does not have a compatible capture bead), you may need to use a mix of beads and cells for compensation. If this is the case, each compensation control tube will need an "internal" negative population for comparison. Compensation requires comparison of labeled and unlabeled particles of the same autofluorescence, otherwise you will be correcting for the difference in autofluorescence in addition to the spectral overlap of the fluorochrome. In many cases, the difference is minimal, but as an example the autofluorescence of most beads is a log higher than that of cells when using the violet laser, which will skew your values.

When preparing your beads, add a drop of the "negative" beads that don't bind antibody. For cells, either mix unstained cells into your single stained sample, unless the "unstained" will pick up the label (such as with PI), in which case you can bring a tube of unstained cells and just "append" them to your data file of stained particles. You will then need to go through and draw a new interval gate on the negative population in each of your single color histograms, and calculate compensation as usual. The software now uses the negative population defined in each tube for the comparison rather than the unstained sample. Alternatively, you could collect unstained cells as the unstained control, and do not draw a negative interval marker for colors using cells as the control. The software will use the negative populations you defined for the bead samples for those colors, and the unstained sample as the reference for the cell samples that do not have negative populations defined.

Remember that your particles will be giving off the same amount of light (unless you change the laser power, which is fixed), and adjusting the voltage changes the sensitivity of the *detector*. Compensation is determining what percentage of the "primary" signal is being detected in the other channels—if you have 30% overlap of FITC into PE and you **increase** the voltage in the FITC detector, you will increase the primary signal while the overlap signal stays the same—thus, the % of overlap will decrease. If you decrease the FITC voltage, the measured FITC signal will be lower while the overlap into PE will be the same, so you will have a higher % of overlap. Likewise, decreasing the PE voltage will decrease the amount of overlap signal detected, also resulting in lower % of overlap. However, if you decrease the PE voltage in this case, then your measured PE signal for your PE control will be lower, and your % overlap into PE-Cy5 (for example) will be greater.

If any parameters require >100% compensation (which means that the "overlapping" signal is brighter than the "measured" signal), it may be necessary to further adjust your settings. One quick trick you can use is to run (without recording) each of your single stained samples with the "unstained control" tube active—you will be able to see how much signal is generated by your particles in all of the channels, and just make sure that the brightest signal is in the histogram for the color that control is labeled with--your APC single stain control should have the brightest signal in the APC

histogram, but you will see some signal in APC-Cy5.5—just make sure it is not brighter than signal in APC.

In this case, if APC-CY5.5 is brighter, you can either increase the voltage to the APC channel, or decrease the voltage in the APC-CY5.5 channel (or both), so that the APC signal is brighter. Remember that this will impact the other colors as well, so if you choose to decrease the voltage to APC-Cy5.5, then the APC-Cy5.5 sample will have lower "primary" signal, and the spillover into APC-Cy7 could be brighter—a balance must be struck to get everything to agree. Adjustments of more than 50 V in either direction from your initial settings should be made with great caution—please see me if you have any problems.

Once you have determined the "optimal settings" for your samples (good separation and minimal overlap), it is then possible to run a mid-range bead (see below) at these voltage settings and determine the mean signal levels for these beads in each of the channels. By running these same beads and matching these values on subsequent days or on a different instrument (by adjusting the voltages for each parameter to match the signal levels), you will ensure that the instrument is detecting the same signal levels from day to day (but compensation should still be calculated with each experiment, as tandem dyes may vary). The Application Note referenced above recommends a "mid-range bead" (BDBiosciences cat # 556298) but the signal on the UV-B (440/40) channel at typical voltages is off scale, so a different particle needs to be used for these channels. Spherotech Midrange Rainbow Fluorescent particles, RFP-30-5A, seem to work well in all channels. This procedure is detailed more fully in "Tracking Settings with Rainbow Beads" available on the website.

LSR2 Optimization Summary

I. Determine control samples required
    a. You will need one tube of unstained cells (for the initial run at least).
    b. You will need one single-stained control per color in the experiment.
    c. Antibody capture beads should be used for your single stained controls unless any of the following apply:
        i. If you are using PI, CFSE, or Invitrogen amine-reactive viability dyes*, these reagents will ONLY stain cells, so cells must be used as control for this color. Note: *Bangs Labs make an amine-tagged bead that may be used with the amine reactive viability dyes instead of cells—but unlabeled bead from Bangs Labs may be different from BD comp beads!
        ii. If you are using an antibody that is NOT from mouse, rat, or hamster, BD's antibody capture beads will not bind them and you will need to use cells as your controls for at least those colors.
    d. For single fluorochrome labels (FITC, PE, APC, Pacific Blue, etc), if the signal for your antibody is dim (no defined population can be resolved) or frequency is low (<1% of population) and you wish to use cells, you may substitute another antibody of the same color with a more robust response (CD8, CD4, etc) for the purposes of compensation. If using beads, they will give a bright uniform signal so it doesn't matter how the antibody stains cells.
    e. If you are using a tandem dye (PE-Cy5, APC-Cy7, etc), you MUST use the same exact reagent as in the experiment—if it is dim or low %, capture beads should be used.
    f. If you are using mixed controls (some cells, some beads), each sample tube must have a negative population in it. Compensation must be done comparing the same particle so that differences in autofluorescence are not skewing the calculation. If you have mixed particles, you cannot collect a "universal negative" for all of them to be compared to. This means for beads, you must include a drop of the "negative" beads (that do not bind antibody) in your tube. For cells, if 100% of the cells get labeled and if the staining won't bleed over, then you can add some unstained cells to the stained cells (as long as the stained cells have been washed, etc). Otherwise (like with PI) you can use the tube of unstained cells (will explain below).

II. Initial setup
   a. You will need unstained cells, unstained beads (only if all comp tubes are labeled beads), single stained compensation tubes, FMOs if needed, and midrange rainbow beads (Speherotech RFP-30-5A).
   b. Create new experiment. Select "instrument settings" and select desired colors in the "inspector" window (magnifying glass icon).
   c. Go to Experiment pulldown menu>instrument setup>create compensation controls.
      i. Activate "unstained control" tube and load unstained cells.
      ii. Adjust voltages for FSC/SSC to get events on scale, and for fluorescent channels so that:
         1. for clearly defined populations, the edge of your negative population should be around 200 (tick after 10^2, mean~50)
         2. If low signal is expected/experienced in that channel, then mean of negatives should be between 100 and 300.
      iii. If using only cells, record unstained cells as unstained control.
      iv. If using only beads, record unstained beads as unstained control.
      v. If using mixed beads and cells, go to Instrument pulldown menu, "modify compensation controls", and turn off "Unstained control tube" checkbox at the top. Click OK. This will remove the "unstained control" tube from the Comp Control Specimen.
      vi. Continue on recording each of your single stained controls. If comp controls are mixed particles, each tube should have a negative population in it. If necessary, to create a "negative" population in a cell sample, you can collect the stained sample and then load the unstained cells and "append" to the file (click Record again, and choose "append").
      vii. Go through each of your worksheets and make sure the positive population is properly gated. If you do not have an unstained control tube, you must also draw new interval gates on the negative population in each histogram.
      viii. Instrument pulldown menu>Intrument setup>calculate compensation.
   d. With experiment name highlighted, click on syringe button to create a new specimen.
   e. Expand specimen to view tubes.
   f. Activate first tube, rename as "rainbow beads"
      i. Load and run midrange rainbow beads, record.
      ii. Expand rainbow beads tube, and select "instrument settings" that is revealed. These will show the settings used for collecting that tube in the inspector. Click on the "compensation" tab, and click on the checkbox to disable compensation.

iii. Select the green "worksheet" button in the browser to create a new global worksheet. If it is greyed out, click on an element in your experiment and it should become activated.

iv. Draw FSC/SSC plot on worksheet, and histogram for each color. Draw a region around your single bead population. This should be P1.

v. Right-click on border of any plot, and select "create statistics view". Right click on statistics view and select "edit statistics view"

vi. In the "header" tab, turn off all checkmarks. In the Populations tab, turn off all checkmarks except for the one under "Show Population" that is next to your FSC/SSC population (P1). In "Statistics" tab, make sure only "mean" for all fluorescence parameters are checked. Click OK.

vii. The values displayed indicate the fluorescence levels of the beads for each of the channels you measured. Make note of these values.

g. Return to the main global sheet, draw your plots for gating, and record your sample tubes. To add tubes to your specimen, you may either click the tube button at the top of the browser, or click "next" in the acquisition controls.

III. Future experiments

a. If duplicating your experiment layout, go to the Global Sheet 2 with the rainbow bead plots. Otherwise, re-create it.

b. Load a tube of your Mid Range Rainbow beads (same lot number as before).

c. Adjust the voltages so that the mean signals detected for each parameter match the values detected during the initial setup (within reasonable limits, i.e. +/- 5% or so). This should be done with compensation turned OFF, and will result in your detectors having the same response to the same fluorescence as in your initial setup experiment.

Run compensation tubes as usual, calculate compensation, and run sample tubes.

Tracking settings with Rainbow Beads

Purpose: to ensure consistent signals over the course of a project, and verify proper function of the cytometers.

Rationale: The components of a cytometer can display variability from day to day. Fluctuations in laser power, changes in temperature and humidity affecting the PMT's, and changes in alignment on the instrument are just some examples of variables that can impact the output. In order to minimize these variations, a consistent fluorescence standard should be run with each experiment to make sure that the signal output of the instrument for a given level of fluorescence is kept consistent. To do this, we use a stable source of fluorescence (rainbow bead with a matched lot #), and then adjust the user controlled settings (voltages) to match the output.

Unless there have been some major adjustments made to the instrument, settings should not vary by more than 20V or so. An added benefit of monitoring the instrument settings with beads is that it is readily apparent when something has happened to the instrument, such as a laser dying etc.

Procedure: After establishing settings for a given panel of reagents (explained elsewhere) such that all signals are on scale, compensation between channels is minimized, and separation of populations is either optimal (for dim labels) or sufficient (for bright ones), the operator collects a data file of a rainbow bead or other particle that gives positive signal for the established settings. For the next experiment, the first step would be to run the rainbow beads, and match the UNCOMPENSATED MFI from the initial run, to make sure the output of the instrument for the same level of fluorescence is the same.

Step by step

On the initial day of your experiment:

1. Establish your panel settings (discussed elsewhere) and run your experiment (calculate compensation, collect your data files).
2. "Duplicate without Data" to create a copy of your experiment you just ran. Right click on the "Cytometer settings" directly beneath the experiment name, and "Unlink from setup" if that is an option. (Only happens if you link your compensation matrix instead of "apply only"). With "Cytometer settings" still selected, view the Compensation tab in the inspector. Select the first line, scroll to the bottom of the list, hold shift and select the last line, so that all compensation pairings are selected, and click "clear". This will set all of your values to 0.
3. Create a new global worksheet (button shown below), and label it "setup"
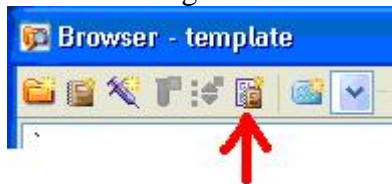
4. Draw a FSC/SSC dot plot, and a histogram for each color. See image at the end of this document.
5. Create a new tube, call it "baseline" and collect a data file of mid-range rainbow beads (Spherotech, RFP-30-5A) or another appropriate control particle. It may be necessary to change the FSC or SSC settings to be able to visualize the beads, and this is fine, and will not affect your compensation results.
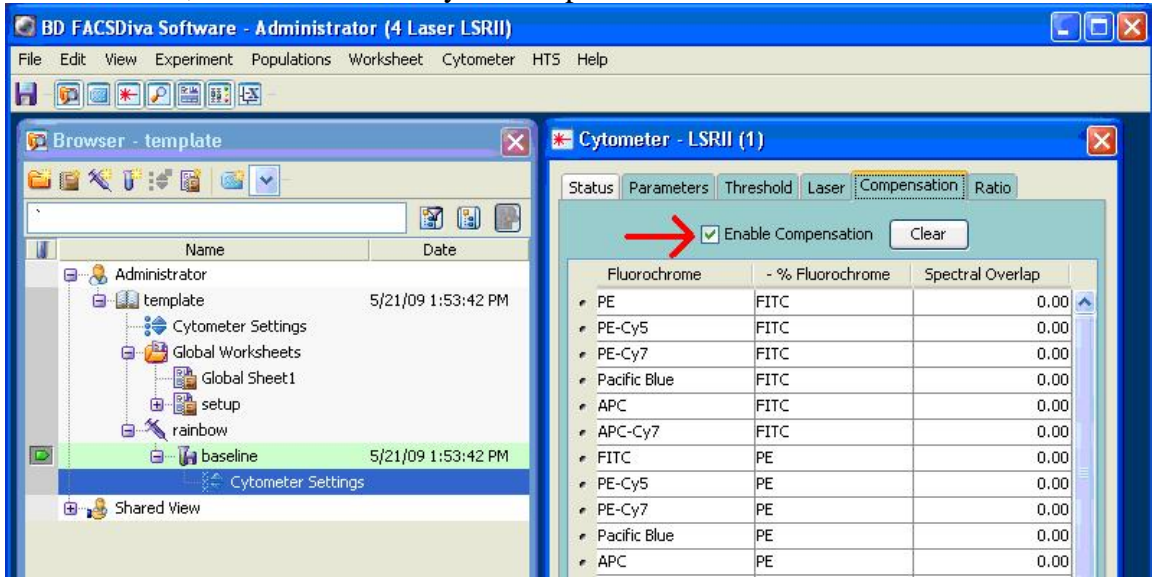

6. On the "setup" worksheet, draw interval gates on the peak in each histogram. To accurately locate the peak, you can use the "+" magnifying glass tool and draw a box around the area to zoom in on (as shown in FITC below).
7. Rename experiment "4 color template" or whatever name will identify this experiment type.
8. "File>Export>Experiment", and save the experiment to your D drive folder. This will create a template of your experiment with the baseline rainbow beads saved with it.

On subsequent experiments using the same layout:
1. "File>Import>Experiment" and import your template.
2. Change the name of the experiment to suit your method, and relabel any tubes as necessary.
3. Switch to the global sheet, and view the "setup" sheet. Activate the "Baseline" data file (specimen "Rainbows") and verify the interval markers closely bracket the peak of signal.
4. "Next Tube", label with the date, and run a tube of the SAME LOT of Rainbow beads. Check the instrument settings to make sure compensation is disabled ("Enable compensation" is de-selected or all values are 0).
5. Adjust voltages to center the peak in the interval marker (trying to match the MFI values in a stats view can be tricky as they refresh while the tube is running and can vary quite a bit).
6. Record a data file of the rainbows. Now you can go back and run your compensation tubes as usual, and no further voltage adjustments should be

necessary (other than FSC/SSC for your cells). Double check that compensation is "enabled" after calculating compensation.
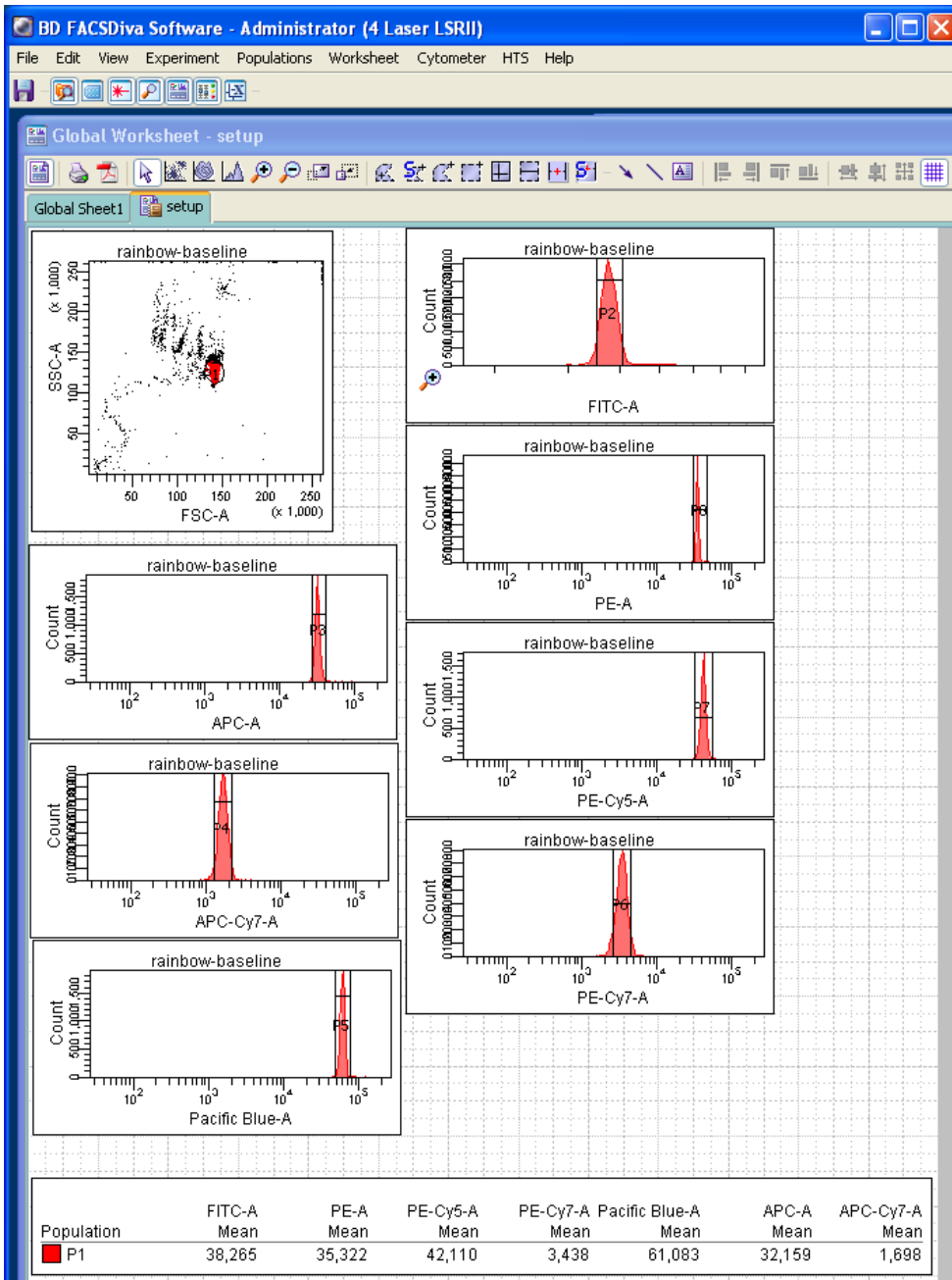
DIVA Software tricks and tips and traps

-In the "Time" scale, 1 second= 100, so "6" on the bottom scale (x1000) is 1 minute.
-Do not use "/" in experiment name, when exported it becomes ,2f, (so that 1/15/05 would be saved as "1,2f,15,2f,05"). Use _ or -.
-Double click on worksheet buttons to make them "sticky"—action will be performed each time you click the mouse button. Escape or click another button to turn off.
-Stopping acquisition before reaching your target number of events can slow down response time.
-Please report any software crashes or any other issues to me by email or in person.
-You can open Task Manager by pressing control/alt/delete and then choosing "task manager". Under the "processes" tab, find diva.exe, and the number on the right indicates how much memory you are using. As this number increases, response time will also increase. Over 1,000,000 and the program is likely to crash/freeze. The more events you collect, and the more tubes in the experiment, and the more dot plots and statistics views you create, the more memory that is used and the higher this number is. To reset it you have to quit the software and reopen. Sometimes minimizing the FACSDiva window will also clear the memory. If it jumps back up to 1,000,000 again, you will have to either logout or quit and reopen.
--Do not export directly to external storage devices. It will take much longer, and some files may be omitted or truncated. You should export to the "D" drive and then copy.
--If you export as an experiment and then open those files in Flowjo, H and W parameters will be switched, so that selecting FSC-H in Flowjo will display FSC-W.

Edit>user preferences, in the General Tab, you should de-select "Save Analysis after recording through Global worksheet" and note the option for "Load Data After Recording" – if you collect many events, you can turn off this option to avoid the delay between tubes while the software processes the data for display.

Data Management

- A 9-color, 500,000 event data file will be roughly 30 MB, 1 million events 60 MB, so 16 tubes (data files) of 1 million events will be 1 GB!
- The Diva "database" within the software (everyone's experiments that appear in the "browser" window while the program is running) can only handle 15 GB total data. Users are limited to 2 experiments each within Diva, and nothing older than 2 weeks.
-User folder/file storage—each user has a folder on the D drive for storing experiments and files. Please minimize data storage on core instruments. No more than 3 GB in any personal folder. To check folder size, right click the folder and select "Properties".
- Please keep all files and folders in your user folder- any unidentifiable folders or files not in user folders will be removed.
- Every Monday excess data is exported from the Diva software so that no one has more than 2 experiments in their profile and nothing is older than 2 weeks. Please maintain your own folders and do not rely on us to do it for you! If excess data is found in your diva profile, it will be exported as an experiment to your folder on the D drive, in a new folder labeled "experiments MMDDYY". If you need the experiment in Diva for duplicating or to export as FCS, it can be imported back in and it will appear exactly as it was when we exported it. If you do import something back in, please delete it again when you are done! If you make changes to an experiment you have imported in and wish to save them, you must re-export the experiment. If you need the files in the "experiments MMDDYY" folder, please move them out of that folder. Please delete any experiments you already have backed up elsewhere, and delete the "experiments MMDDYY" folder once you have made sure you don't need any of the contents.

Exporting

There are 3 ways to export your experiments: "experiment", "fcs", or "template"—Different information is saved depending on your choice.

Exporting as an experiment creates a folder with the same name as the experiment, and an XML file with the same name that has all of your experiment information (parameters, settings, compensation matrix, dot plots, tubes and specimens, etc), and saves each tube as an fcs file which is given just a number as a file name. This format is mainly for use with Diva.

It is possible to use these FCS files in Flowjo, but you will have to edit the columns to show the "tube name" to identify the different tubes, and if you record H and W parameters (for doublets, cell cycle, etc) these 2 labels are switched (so selecting FSC-H in Flowjo will show you FSC-W measurements).

Once exported in this format you can delete the experiment in the Diva software, and then import it back in at a later date and it will appear exactly as it was when you exported it. The folder name must match the name on the XML file, so if you change the name of the folder, you will not be able to import it as Diva will not recognize it.

Exporting as FCS files will also generate a folder with the same name as the experiment, and then one file per tube in the experiment, labeled as Specimen_tube. Files in this format can be loaded into Flowjo or other third party software for analysis. However, they do NOT save the analysis objects from the experiment in Diva, and if you wish to import them back into Diva, you will have to generate an experiment layout to import them into.

One strategy of data management is to export the data tubes as fcs files, and then duplicate your experiment without data, and export the empty experiment as an experiment. When you wish to view your data files in Diva again, import the empty experiment file, and then import the fcs files into that empty experiment.

Exporting an Experiment template: one of the export options for an experiment is to export it as a template, and it will be saved in the list of Diva templates that can be accessed by using the pulldown menu to create a new experiment. DO NOT USE THIS OPTION.

--Do NOT export directly to an external drive, as it will take twice as long, and sometimes truncates very large files or does not export all files. It is faster to export to your folder on the d drive and then copy/transfer to your external drive or burn a cd.

Experiment Templates

If you are using the same panel on multiple days, there are a few strategies to avoid having to re-create an experiment layout each time. Some are described below.

If you opted to "Link and Save" your compensation settings on your experiment (a yellow chain will appear near your instrument settings), they will carry over to the new experiment, and even if you calculate a NEW matrix, it will be locked into the old one. Unlinking is essential to make sure your compensation is correctly applied. Right click on the instrument settings icon (right under the experiment name) and select "unlink from ####". This can be avoided by choosing "apply only" when compensation is calculated.

If you have an experiment in your browser that you wish to reuse, you can simply right click the experiment and select "Duplicate without data" (experiment must be open). This will make a copy of the framework of the experiment but will not include any data files, so you can just record new files for each sample tube. Again, make sure experiment is not "linked" to a compensation matrix. The best way to reuse experiment layouts is outlined in the "Track Settings with Rainbow Beads" document.

The preferred method would be to "duplicate without data", and then rename the experiment to "MW 6 color template" or something you'll recognize. If the original experiment used the "Link and save" option for compensation, right click the instrument settings and select "unlink". Click on the "instrument settings" and view "compensation" in the Inspector, select all lines, and "clear" to set all values to 0. Then choose File>Export>Experiment to export your EMPTY experiment as an experiment file and store it in your D drive folder--the folder created which matches the experiment name will only have the xml file in it. For future experiments with this layout, simply Import the template experiment and it will be ready to go, and you won't need to keep a FULL experiment in your browser for duplicating.

Transferring Files

There are several options for transferring files from instrument workstations.

Flashdrives and external storage may be used, but please ensure they are virus free, and do NOT export directly to the drive—export to the D drive first, and then copy them to your external storage. Diva will take longer to export to a usb drive, and will sometimes truncate or leave out some of the data files. Partners is now requiring all portable storage devices to be encrypted. CDs or DVDs may be burned as well. Other options using the network are described below:

File transfer:
Using Internet Explorer, go to portal.partners.org and type partners\abc12 (your ID code) and your partners password.
The drives mapped to your profile will appear in either "Network Places" or in "My computer". You can drag and drop, but be aware of storage limits on the server.

Email:
Research computing offers a large file transfer service that zips the folder or files for you and emails a link to download them. Go to https://transfer.research.partners.org, you will need to create an account and it is pretty self-explanatory.

MAD Server:
We have an account on a MAD storage server which is web-based, you upload your files over the web to the server and then download them to a different computer on the web. Contact Mike for details.

There are other means to connect over the network that require passwords etc. See Mike if you want to try other transfer methods.

BD High Throughput Sampler User's Guide
For the
## 3 Laser BD LSRII and 5 Laser BD LSR Fortessa

I. Introduction

The BD High Throughput Sampler (HTS) is a high-speed sample loading device used on the 3 Laser BD LSRII and the 5 Laser LSRFortessa located in Rm. 5227 of the Ragon Institute Flow Cytometry Core Facility. The HTS provides users the ability to acquire samples from 96-well or 384-well plates for efficient analysis with minimal carryover. This guide will serve as a step-by-step process of installation, acquisition, shutdown and removal of the HTS system. (Note: All users must be trained on installation/removal prior to their first use of the HTS.)
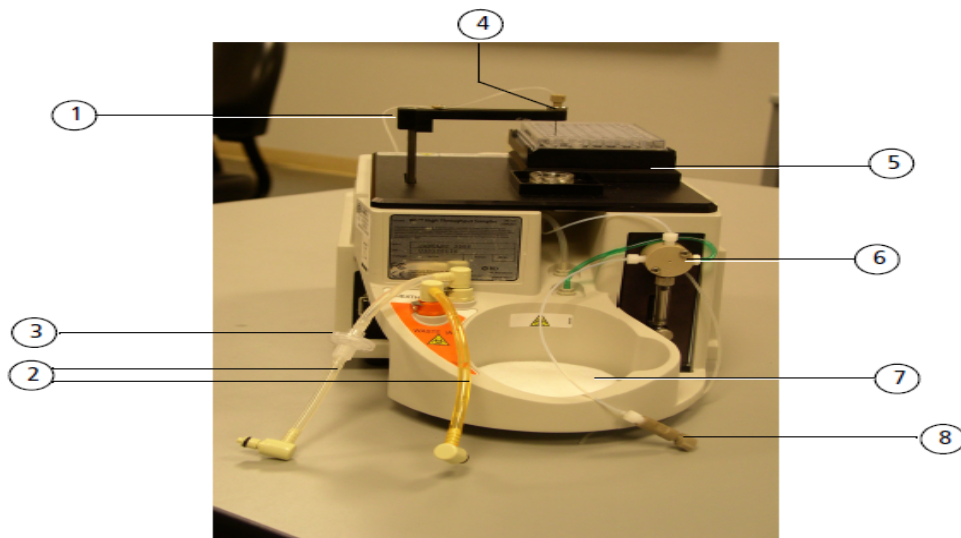
1	Probe assembly—moves front to back and up and down to transfer sample between plate holder and injection port/wash station
2	Fluidics tubing: sheath (clear) and waste (orange)
3	Sheath filter—filters incoming sheath fluid to HTS unit
4	Injection port/wash station—provides interface for sample injection and probe washing
5	Plate holder—moves left to right and front to back to position plate so the probe can pick up sample
6	Secondary pump and valve—delivers sample to flow cell in high-throughput mode
7	Absorbent pad—collects potential overflow from the injection port/ wash station or drips from the cytometer sample injection tube (SIT)
8	Sample coupler—connector between HTS unit to cytometer SIT

II. Installation of HTS for Plate-Based Acquisition. (Note: The devices are HEAVY and may require some assistance from Core Personnel)

There are two HTS devices located in Rm. 5227 on the shelves across from the 3 Laser BD LSR II and the 5 Laser BD LSRFortessa, each labeled accordingly.

1. Make sure sheath tank is full and waste is empty.
2. Prior to moving HTS devices: Switch acquisition control switch to Plate mode located to the right of the sample station with instrument in "standby". ⊌→⚏ (When Plate mode is on the aspirator pump is off, if instrument is in "run" the SIP will drip)
3. Move aspirator arm to the side and remove Droplet Containment Sleeve (DCM). Unscrew (clockwise) the tube retainer until you can remove the DCM sleeve freely. Store DCM sleeve in styrofoam rack.
4. Replace DCM with SIT protector which is kept in the drip tray on HTS unit (See Figures Below)

5. Move tubing and wires connected to LSR under the sample port for easy access.
6. Remove HTS safety cover and install HTS Unit onto the cytometer.
   3 Laser LSRII: HTS Unit has base plate attached and can be installed on to cytometer by inserting the metal rod in to the brace underneath the sample station. Leave the HTS Unit resting on the notch in the metal rod securing the HTS Unit away from LSR to allow easy access when connecting cables and tubing.
   5 Laser LSRFortessa: HTS Unit base plate is permanently installed on the LSR. HTS Unit is placed on the sliding base plate to the right of the sample station. It

will be easier to attach power and communication cables with HTS unit facing the user.

7. NOTE: DO NOT GET THE POWER CORD WET. IT WILL SHORT CIRCUIT THE ENTIRE INSTRUMENT. Connect the power and communication cables to ports on the rear right side of the unit. (See Figure Below)
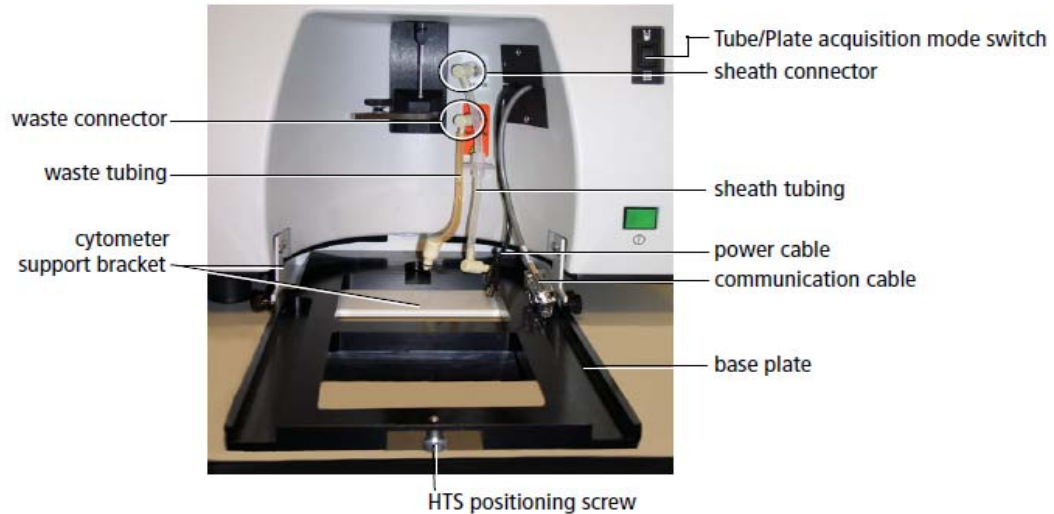

8. 5 Laser LSRFortessa: Once power and communication cords are connected, slide the HTS Unit in to place with absorbent pad underneath the SIP

9. 3 Laser LSRII: Disconnect sheath tubing from HTS unit and connect to LSR, as seen above.

   5 Laser LSRFortessa: Sheath tubing is permanently connected to the LSR.

10. Locate tool box and find the "purging assembly line" rubber tubing with one open-end and one female connector attached. Setup a beaker to catch sheath bled from instrument. Connect the female socket to the sheath tubing connected to the LSR and allow sheath to bleed for a few seconds. (See figure below)

11. Disconnect "purging assembly line" and place a few drops of sheath fluid from sheath dropper in the empty sheath connector on HTS prior to reconnecting lines.

12. Connect the sheath and waste line from the LSR to the HTS unit.

13. CAREFULLY, attach the sample coupler to the SIP (Sample Injection Probe). Push sample coupler onto the SIP until you feel a stop. Hold the sample coupler in place and tighten the top nut. (See Figure Below)
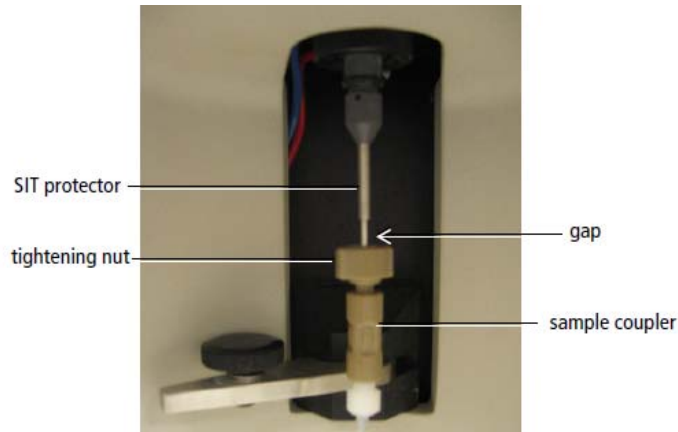
14. 3 Laser LSR II: Slide HTS unit all the way back toward the cytometer.

15. Place safety cover back on HTS Unit and turn on the HTS power switch.

16. Set Instrument to "Run" and open BD FACSDiva software.

17. In BD FACSDiva software, select HTS > Prime, 2-3x to remove air bubbles caught in tubing. (Note: Safety cover must be on for software to execute Prime function. Inspect blue/green tubing connected to the syringes/pumps on the front and back of the HTS unit where air is usually seen. Tap tubing in between primes to loosen any air bubbles caught in tubes. It will be necessary to remove the safety cover to access the tubing on the front syringe/pump, tap tubing then replace cover before priming. Many Primes may be necessary in order to completely remove all air)

18. NOTE: If Diva is already running, install HTS device and select "ReInitialize" from HTS drop-down menu. Communication between HTS unit and software will now be connected.

III. HTS Acquisition

Setting up Plate: (Note: If running <u>multiple plates</u> under the same setup and compensation conditions, one set of wells for each is necessary on the first plate only and subsequent plates may contain specimen only wells.)

1. Row A will be used for Setup Controls (Note: Ideally, two wells will be used. One will contain unstained experimental cells for determining FSC vs. SSC voltages and the other will contain mid-range rainbow beads used for determining voltages for parameters)
2. Row B will be used for single-stained Compensation Controls
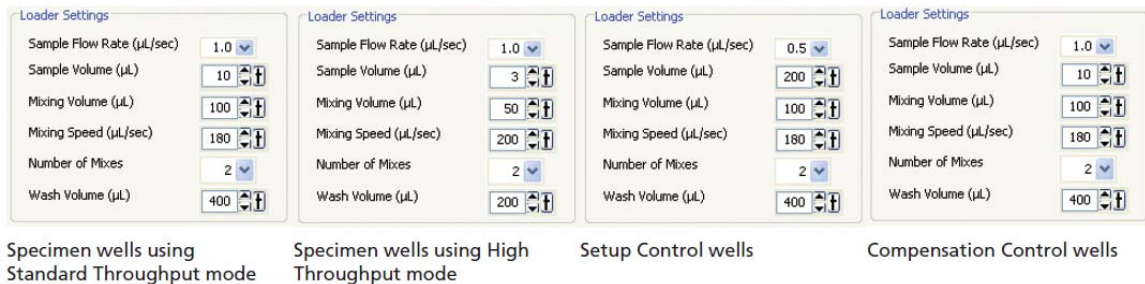3. Remaining rows will be used for Experimental Specimens.

Setting up Experimental Plate Editor and Worksheet: (Note: If <u>multiple plates</u> are to be run with the first containing setup and compensation wells and any subsequent plates are specimen only wells, multiple plate layouts must be created to accommodate designs.)

1. Create a new experiment in the browser window and select the parameters used in the experiment.
2. In the browser toolbar select the type of plate used in the pull down menu next to the plate icon. Plate layout should appear. If not, double-click created plate icon in browser window to view layout editor. (See Figure Below)
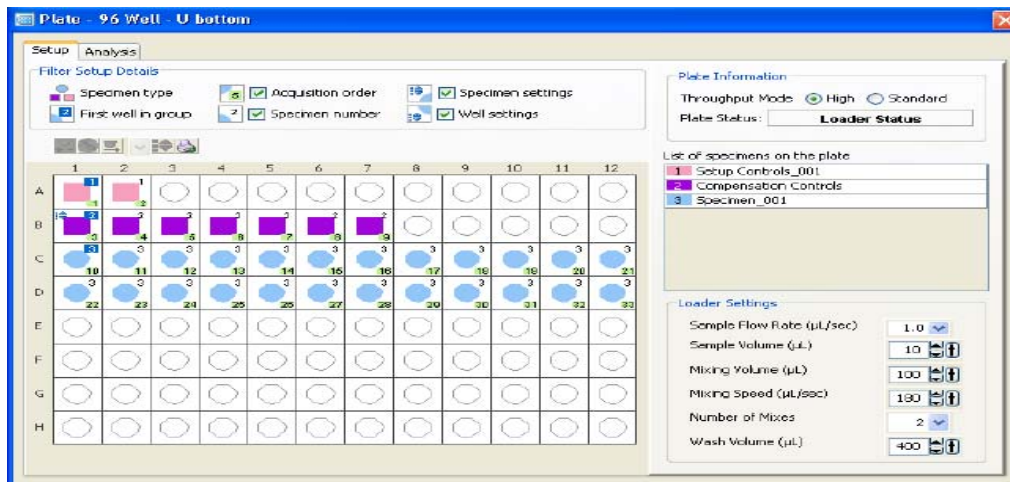
3. In the plate layout window, select the first two wells (A1/A2) and click the setup well icon in the plate toolbar. This will be used to determine FSC vs. SSC voltages for cells as well as parameter voltages and applies default settings for acquisition accordingly. These wells are collected in Standard Mode allowing time to adjust voltages. The remaining wells for compensation and specimens can be run at either Standard or High Throughput speeds.

4. In the plate layout window, select the first well in row B (B1) and right-click the highlighted well. In the menu, select Setup > Create Compensation Controls… This will assign each well in row B to a single control and applies default settings for acquisition accordingly. (Note: Make sure plate layout matches the order listed in "Create Compensation Controls". If necessary, reorder fluorophores/parameters by dragging bullet points up/down in list)

5. In the plate layout window, select the remaining wells that have experimental samples by either holding the Shift key to select continuous wells or the Control key to select individual wells. Select the Specimen icon to apply specimen wells. This will assign each selected well with a specimen and applies default settings for acquisition accordingly.

6. Select the Throughput Speed for Specimen tubes. Default settings are applied with respect to Standard/High Throughput speeds. (Note: These settings can be modified depending on sample volume and concentration.) (See Figure Below)

Specimen wells using Standard Throughput mode   Specimen wells using High Throughput mode   Setup Control wells   Compensation Control wells

7. Note: Plate acquisition can only be recorded using a Global Worksheet. View the Unstained Normal Worksheet, and choose Edit > Select All > Copy. Then view the global worksheet, right-click and choose Paste.

8. Layout setup is complete and samples are ready to be acquired. (Note: If running multiple plates under the same setup and compensation conditions, create one layout for each plate that includes the proper setup/compensation/specimen wells)

Running Plate:

1. Remove safety cover from the HTS unit and place the plate with well A1 at the back-right corner of the stage, closest to the sample probe. (See Figure Below)

well A1

front of HTS
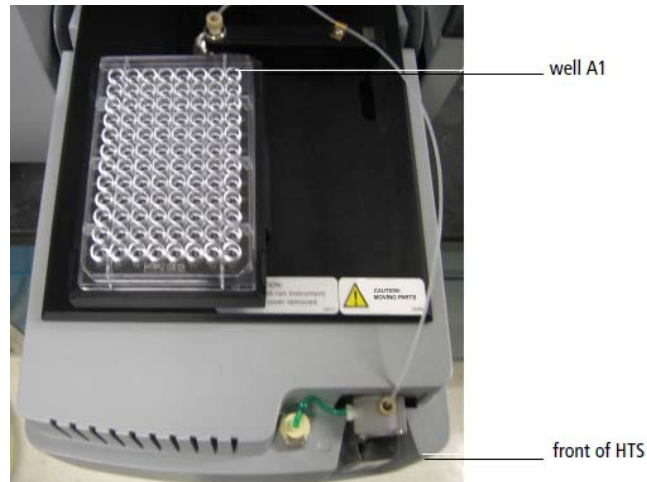
2. Replace HTS safety cover.

3. In the Acquisition Dashboard select Show Plate Controls.

4. Select the two Setup Control wells in the plate layout of the Setup view. They will appear outlined in green.

5. Select Run Wells on the Dashboard. The setup wells will be injected in to the cytometer and adjustment of voltages can take place at this time.

6. Once voltages have been set, Compensation tubes can be acquired. Select all compensation tubes and click Run Wells on the Dashboard.

7. Once Compensation tubes have been recorded go to the normal worksheet and individually click through each of the tabs labeled with single controls. (Note: Since, the HTS system only runs using the Global Worksheet, the software does not apply gates to the individual compensation wells until each tab has been viewed by the user and positive gates have been determined.)

8. Experiment > Compensation Setup > Calculate Compensation

9. View the Global worksheet

10. IMPORTANT: Setting Events to Record: Either click each well separately and select desired events, or go to Experiment > Experiment Layout. Under the Acquisition tab, select wells and assign number of events to record. (Note: In the plate editor window only the green highlighted well is changed when selecting events to record even if multiple wells are selected. If sample is loaded and not collected, it is LOST)

11. Select specimen well and click Run Wells on the Acquisition Dashboard

12. Once plate has finished running, a second plate can be loaded on to HTS with the correct plate layout selected or move on to the cleaning/shutdown procedure.

IV. Cleaning/Shutdown Procedures
1. A separate plate must be created with 8 wells: (See Figure Below)
   a.   A1-A4 = 4 wells Coulter Clenz
    b.   B1-B4 = 4 wells DI Water
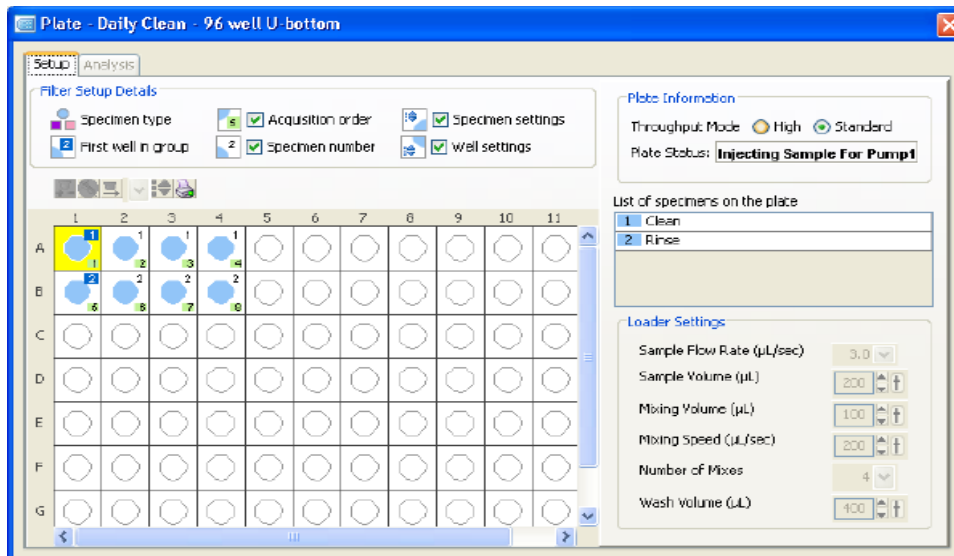
2. Remove Cover on HTS and place Cleaning Plate in same orientation as previous experimental plates.
3. HTS > Clean, select Daily Clean
4. Replace cover and click OK
5. Once finished daily cleaning procedure, move on to Removing HTS

V. Removing HTS Unit and Set Up for Tube-Based Acquisitions (Note: The devices are HEAVY and may require some assistance from Core Personnel. Reference images found under "Installation of HTS for Plate-Based Acquisition")

1. 3 Laser LSR: Hold both sides of the HTS unit and slide it away from the cytometer until it stops in the base plate.

2. Disconnect sheath line from the HTS and connect the purging assembly line (female connector and open end) from step 10 of installation. Place the open end of tube in a beaker of H$_2$O. (See figure below)
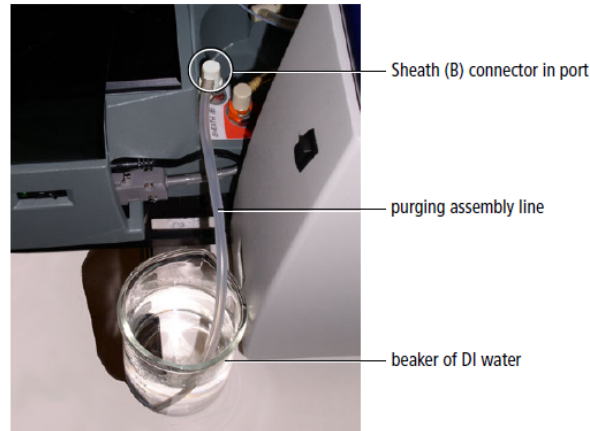
3. HTS > Prime 3x
4. Place cytometer in "Standby"
5. Disconnect H$_2$O priming tubing from HTS.

   3 Laser LSRII: Reconnect sheath tubing to HTS and disconnect other end from LSR

   5 Laser LSRFortessa: Disconnect sheath tubing from HTS Unit
6. Disconnect the waste line from the cytometer
7. Detach the sample coupler from the SIP
8. Disconnect the power and communication cables from the unit. Cover power cord adapter with a clean, dry tube.
9. Carefully lift and remove the unit.
10. Remove SIT and place in absorbent tray on HTS Unit
11. Replace the DCM sleeve
12. Tuck all cords attached to the cytometer off to the side out of the way of the sample chamber
13. Switch acquisition control switch to tube mode.

IMPORTANT!

VI. Additional Procedural Notes and limitations:

1. Cytometer must be placed in "Run" and cover installed on HTS prior to opening BD FACSDiva software in order for it to be recognized by the computer.

2. If Diva is already running, install HTS device and select "ReInitialize" from HTS drop-down menu. Communication between HTS unit and software will now be connected.

3. Cytometer can be placed in "Standby" when not running plates, primes, cleans, etc.

4. Cell concentration should not exceed 6×10^6 cells/ml in 250µl volume.

5. Software will stop acquisition (recording of a well) and proceed to the next well when either the specified number of events collected is reached or the stopping time is reached. Stop time (sec) = sample volume (µl) divided by flow rate (µl/sec). Set number of events high if you want to record up to the stop time.

6. Plate should be mixed prior to HTS setup. Avoid creating any air bubbles in well.

7. Use only plates made by either BD Falcon or Costar. Only these plates are validated for the HTS.

8. Do **not exceed** a 1M cell count per well in the acquisition field of the Diva program (location in the acquisition layout). This will result in a plate memory error.

Troubleshooting

--Missing experiments in your profile: Read section on "Data Management"

--Tube Fills while cleaning or running samples:
  -check to see if tube is cracked
  -if during cleaning, fills while arm is to side, DCM is clogged- notify core personnel..

--clogged sample probe/ no sample flow:

--High pitched alarm--waste has an alarm built in. check tank to see if it is full.

No events; press prime while acquiring, this should trigger hundreds of events per second for a brief time—if you see no events, restart the computer. If you do see events, then the computer and instrument are communicating and the sample probe may be clogged. With the instrument on run, center the arm without a tube loaded. If it drips, that is normal. If it does not drip, the sample probe is clogged. You will have to notify core personnel.

Baseline Correction and potential issues

In order to remove background signals due to unbound fluorophores, dark current, and ambient light, the Diva Software averages signals between events and subtracts this from the total signal.

If there is no signal for that parameter, this can result in a negative value for that event.
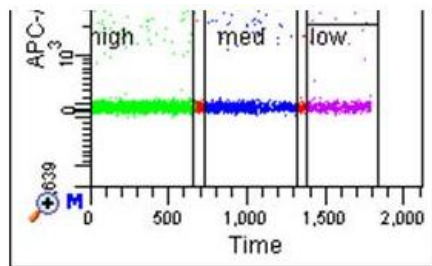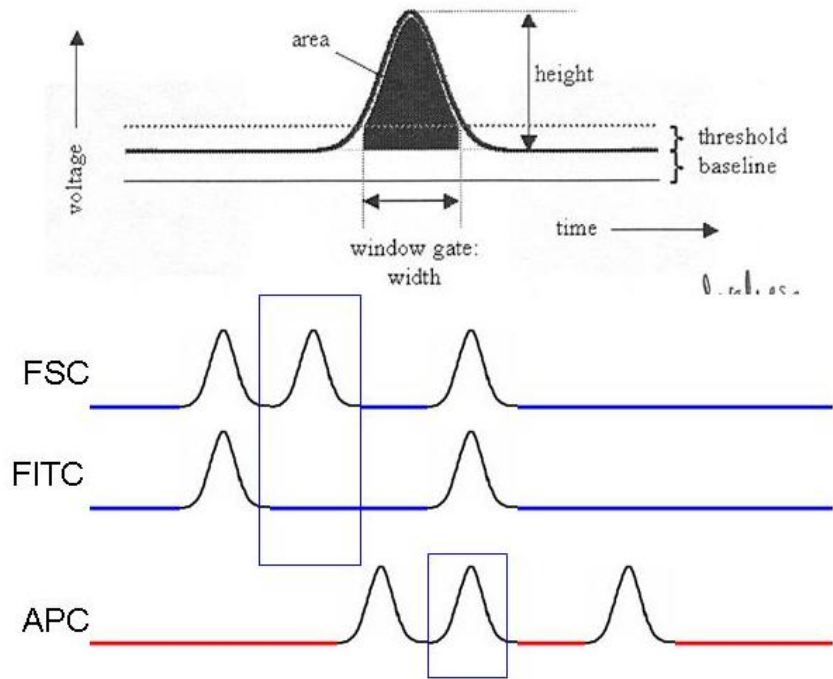
Population	APC-A Mean
high	37
med	36
low	38

Most of the time, signals are corrected appropriately and this results in a population mean that is consistent regardless of flow rate. (You may possibly see a higher CV/more spread at higher flow rate due to greater measurement error).

Population labels to the left refer to the flow rate used for acquisition of the events in that gate.

Where it can go wrong

Usually threshold is set on FSC– in cases where particles are very different in size, one may be below threshold while the other is on scale (such as cells and beads or debris).

Because it is below threshold and is not an "event", the signal of the small particle is averaged into the baseline and subtracted from the signals.

In this example, negative cells and fluorescent beads are in the same sample. The beads are so small they fall below FSC threshold and are ignored. Their bright fluorescent signal is averaged into the "baseline" which is subtracted from the signal measurement

This can lead to your negative populations shifting artificially far into negative values and can lead to problems with your data files.
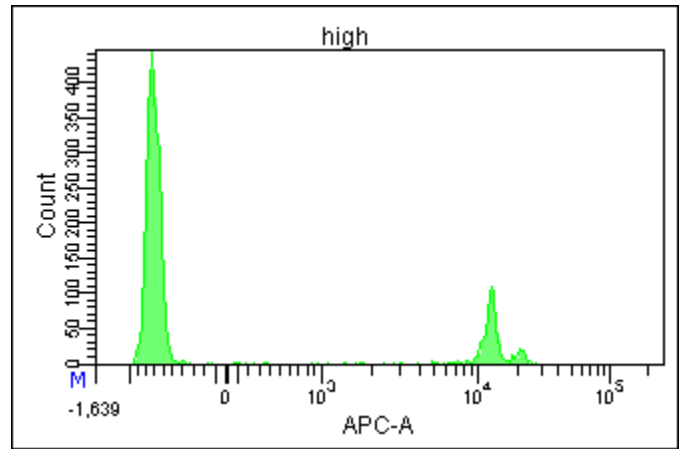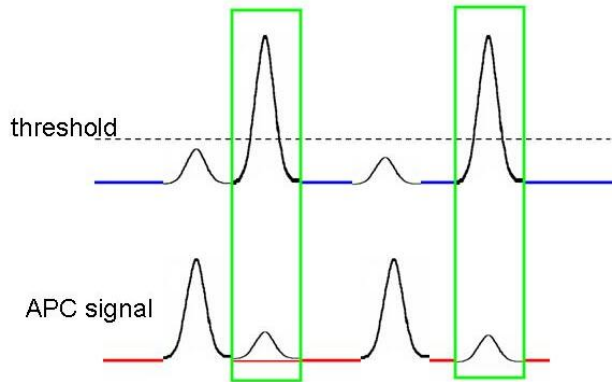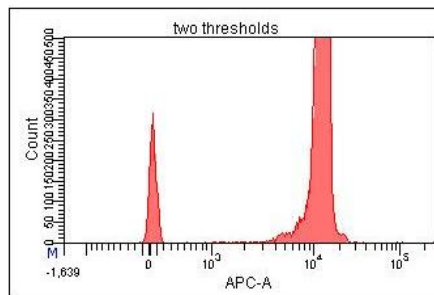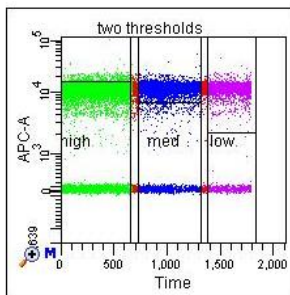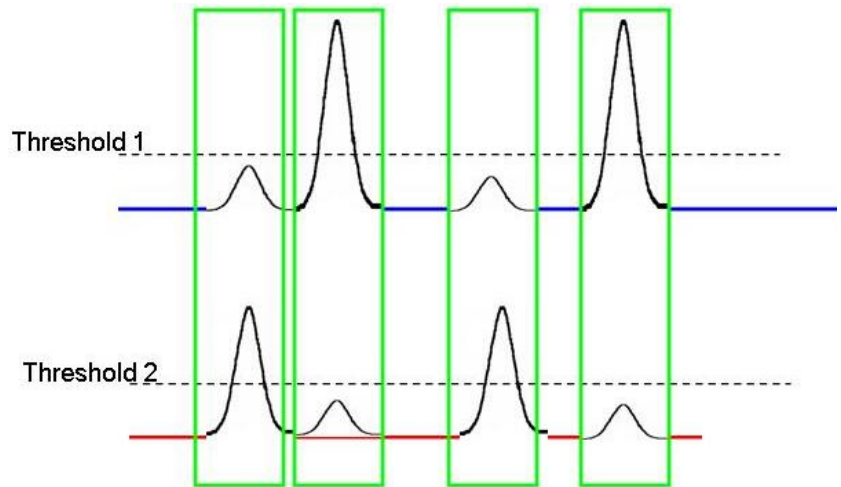

Confirming issue:
- One way this issue can be illustrated is to run at different flow rates. The higher the flow rate, the more "undetected" particles that will be present, and the higher the baseline error
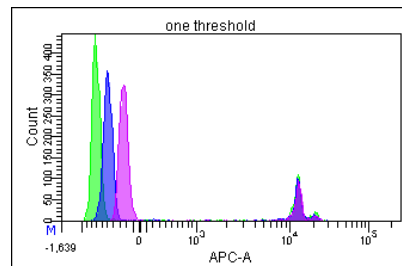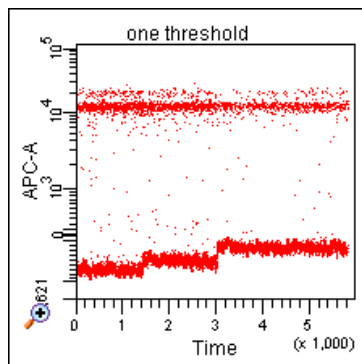
Tube Name: one threshold	
	APC-A
Population	Mean
high	-717
med	-500
low	-251

Solution:

Apply a second threshold: Applying a second threshold that identifies the small particle makes sure all events are detected and the baseline is not erroneously high—events above T1 OR T2 will be recognized and recorded. Alternatively, you can decrease your FSC threshold to include the debris, but you may not be able to set it low enough to detect all of them.

From the BD whitepages:

2.5 Baseline Restoration

PMT signals can contain a high level of background from a variety of sources: light from unbound fluorophores, PMT dark current, and ambient light. Background signal is eliminated in two stages. The first, gross adjustment is made during the initial conversion of the signal from current to pulse. (Photomultiplier tubes are a source of current.) After pulse conversion in the preamp, the output signal falls in the range of 0 to 5 V. This initial gross adjustment preserves the dynamic range of the A/D converters for signals of interest, and maintains a safety margin of 100 mV.

The second, final adjustment is made just before area and height are measured. The FPGAs (Figure 2) calculate a running average of the data outside any window gate to remove the last 100 mV (Figure 3).

From the Purdue message board, Marty Bigos (Gladstone Institute of Virology and Immunology) answered a question from someone who asked about a drop in signals when running on high with large amounts of debris blocked by threshold:

       I will post a hypothesis on why the SSC decreases with increased flow rate when there are a significant number of particles below threshold (FSC). The DiVa systems (Aria, Canto, VantageDiVa) have a baseline subtraction calculation whereby each measurement channel is sampled between threshold events and an average value calculated which is then subtracted from the measurements on that channel (when measuring Area). So if you had s lot of RBCs in your sample below threshold, the faster you ran your sample, the higher your average background baseline on SSC will be. To see if this is the case, you could look at SSC-H. This does not have an integrated baseline subtracted, so it should remain constant regardless of flow rate.

On Jun 18, 2009
Dear flow cytometry experts,
Several of our researchers are having the same problem. Voltages are set based on an unstained sample. Then a CD150 sample is run. The negative population appears MORE NEGATIVE than anything in the unstained sample.
We have seen this on FACSAriaI, AriaII, and LSRII. We have seen this with CD150-PE as well as CD150-PECy7. We are hopelessly confused. How is this even possible? Has anyone out there seen anything like this? I'm attaching a picture (tif file) of what I'm talking about.
Thank you,
Kat Folz-Donahue
Flow Cytometry Core
Harvard Stem Cell Institute | Center for Regenerative Medicine
Massachusetts General Hospital

From: Mario Roederer <roederer@drmr.com>
Date: Sun, Jun 21, 2009 at 4:01 PM
Subject: Re: [Cytometry] CD150 negative population shifts down! How is this possible?
To: Cytometry list <cytometry@flowcyt.cyto.purdue.edu>
Hi Kat,
I haven't seen your attached picture (it wasn't attached).... but I have a pretty good idea of what is going on. We first noted this behavior some years ago, in the very early days of BD's digital electronics, and it hasn't gone away. We've come to affectionately refer to it as the "baseline restore issue"... While we are not positive that we understand what's going on, we have explored this issue a great deal, discussed it with BD and others, and think we have an explanation. And we have at least 2 partial solutions:

(1) Use additional washing steps of the cells after staining; and
(2) Ignore the problem

Details below!

We originally saw this issue with QD655 staining (which is very bright, like PE). However, we also began to see it occasionally with other reagents, but usually only those that are very bright. We explored this issue quite a bit with a CD8 reagent (which is bright on cells). We found, first of all, that it didn't happen with all samples -- it seemed to be sample specific. (More on this later).

Second, the extent to which the negatives crept down (towards more negative values) depended on the brightness of the reagent (use lower concentrations to lower the positive values, and the effect diminishes) as well as the frequency of the brightly-positive events(mix unstained cells with stained cells to varying extents, and the effect diminishes as well).

Ultimately, I believe that what is going on is that the "baseline restore" on the instrument will sometimes over-estimate the background fluorescence. Baseline restore is one process by which the instrument calculate the background of the stream as cells go through, in order to determine the actual fluorescence of the cell when it goes through. Thus, as the background in the stream increases, the instrument subtracts more from the cell's fluorescence in order to get the actual cell-associated fluorescence.

The background is estimated as the average fluorescence outside of any trigger events, over some recent time frame before the cell entered the laser.

The problem with this approach is that if you have very bright clumps of fluorescence that go through the system without a trigger happening (because they don't have enough scatter signal, i.e., they are debris), this can artefactually increase the baseline dramatically. The next cell that comes through will have WAY too much fluorescence subtracted from its measurement, and will go way negative.

This hypothesis is supported by our observations: diluting the sample with unstained cells will dilute the debris significantly, lowering the rate at which this happens. Using less-brightly-staining reagents will to a large extent lower the fluorescence of any such debris/clumps, also diminishing the effect. And finally, the problem tends to occur more with "dirty" reagents, for example, our early home-conjugated Quantum Dot reagents that have a lot of aggregates.

We hypothesized that if this was an issue associated with debris, then additional cell-washing steps following staining would reduce the problem -- and this is in fact the case. So while there is no simple solution to avoid this problem entirely, if you add additional washing steps (particularly, low-spin steps that separate cells from debris), you will significantly reduce the incidence of this problem. (That's partial solution number 1).

Practically speaking, though, you can try to ignore it. This issue never results in increased background -- so the background gates you establish on FMO's (or whatever control you have) are still valid, no matter how far below this gate the "negative" cells wander. And while theoretically this problem can diminish the measured fluorescence of positive cells (and in fact we've seen that it does so), the diminution is fairly small and

would only affect some dim cells – and given that the staining is very bright to see this problem at all, it probably isn't a real issue. (That's partial solution number 2).

Unfortunately, this does lead to some ugly displays, where the "negative" cells are far more negative than they should be. There's nothing you can do about that, except to repeat the mantra that "all cells below measurable fluorescence are the same" -- i.e., there are no subpopulations of negative cells!
regards,
mr

Repair Log

Version Log:

Version 2, released 9/1/11