Canto II: Start up and QC, and using the instrument Version2

The following procedures are described in this document for Canto II usage: (1) start up, (2) checking cleanliness (in Diva using QC template), (3) calibration (executing CS&T program), (4) recording mandatory cleaning files every 30min following the cleaning procedure described in this document, and (5) Files/photo proof to be sent to fccf@salk.edu

Before starting, refer to "**Where Stuff Is Kept**" (map) for location of MQ water carboys, beads, red biohaz collection, sink, etc. To help keep things running and limit damage to instruments accidentally left ON, we have implemented a few changes:

- All user protocols must be reviewed prior to access (if possible, DNase I treatment must be included to help keep the instrument cleaner). In addition, all samples must be filtered before running on an analyzer.
- Instrument is running in MQ H_2O instead of PBS for sheath. *Note: Cracked FACS Tubes will not hold pressure (and will fill up with water!). Check tubes carefully before installing onto Canto!*
- Return empty carboys to the appropriate pick up location: Place empty carboys *outside* the FCCF main door. Autoclaved water carboys can also be collected from here if needed (check also in STEM by the LSRII-1).
- Help maintain the Canto II:
  - REFILL **sheath tank and shutdown solution tanks**: Autoclaved MQ water is available in round carboys (either nearby or outside FCCF). Spray off the dedicated funnel (usually kept by the fluidics cart) with 70% ethanol and use to refill 20L square tanks if these are in use (we are working on a new cap adaptor, when in use, it will allow the 10L autoclaved water carboys to be directly installed onto the cart): *It is OK to fill with less if you have difficulties with the ergonomics.* Due to the container neck size, the Shutdown Solution is easiest to fill directly from the MQ water gun by the sink in STEM.
  - EMPTY the waste tank in the STEM core sink after use (add bleach before reinstalling onto fluidics cart).
  - REFILL the MQ H_2O syringe when empty and replacing the filter as needed (e.g. H_2O has high event rate)
  - EMPTY the red trash bag when full
  - RINSE and RECYCLE empty bleach containers (blue recycle bins)
  - RESTART the computer before leaving the area
- QC instructions are included in this guide. Follow instructions and send the required files as necessary. The **first user of the day** must additionally calibrate laser delays by using the automated bead QC in Diva as described.
- Perform the 5min cleaning procedure as described in this guide **every 30min of use** and record FCS files
- As a condition of access, you must send the following items to fccf@salk.edu for review:
  1. TAKE A PICTURE of the GREEN POWER BUTTON to show standby or OFF. *Please send this as soon as possible after use so we can confirm the state of the machine.*
  2. TAKE A PICTURE of the CLIPBOARD (legible, signed off by you) in front of the LOGGED OUT MONITOR SCREEN
  3. RECORD FCS FILES AND SCREENSHOT THE BROWSER when performing the 5min cleaning procedure described below every 30 mins
  4. ANY REQUIRED QC FILES: (1) Before- and (2) After- bead profiles, (3) CS&T report if it was run

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1. **Check Tanks** (see also Quick Start guide on the last page of this set of instructions – check sheath, waste, shutdown solution tanks) *Note that instead of PBS, this system is now running MQ H2O. Refill with autoclaved water (look for the round carboys nearby or outside FCCF main door)

2. **Power up Canto at Green button, log on to PC and log into workstation**. *(Optional but recommended → double click on the Tera Term icon to launch a communication window. Leave this running in the background; cytometer code will populate the Cytometer Status window (e.g. VX Works logo; monitor codes when troubleshooting instrument issues – copy and send to FCCF@salk.edu.* *SEE NEXT PAGE FOR WORKSTATION UPDATES AS OF 1/4/20*

3. **Open Diva and log in and log in under Calibration (password orangecst)**. *(Cytometer Status window should be displaying the Cytometer IP address at this point; once Diva is opened, it should start loading various hardware boards as it connects to Diva)*

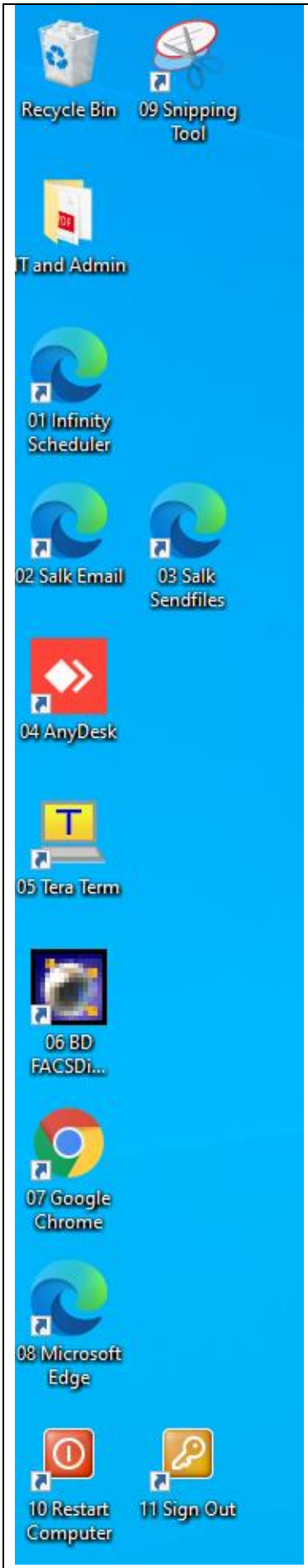


Figure 1. Screenshot of Desktop

To the left is a screenshot of helpful desktop icons for easy use:

1. **Infinity Scheduler to access Canto calendar:** To make another booking or check if there is another user after you. If there is, leave on standby and email user that Canto is on. If not, turn off machine at green power button.
2. **Salk Email:** To send us the required files and pictures.
3. **Salk Sendfiles**
4. **AnyDesk:** If you need another user or a FCCF member to help you during your session remotely
5. **Tera Term:** Same as Cytometer Status
6. **BD FACSDiva Software**
7. **Google Chrome**
8. **Microsoft Edge**
9. **Snipping Tool:** To snip any error codes in Tera Term or to snip cleaning files
10. **Restart:** Double click to restart
11. **Log Off:** Double click to log off

4. **Check for Fluidics Start Up then restock/refill etc. if needed**: Fluidics Start Up and Shutdown are now done only ONCE A WEEK. Bottom right corner in Figure 2 should say Fluidics Start Up done, if not you will need to do a Fluidics Start Up by going to Cytometer Menu > Fluidics Start Up. While this is running, (i) check there is bleach available for later (make sure bottles are not empty: empty containers should be rinsed and put into recycling, (ii) refill water syringe, sheath and shutdown solution carboys if needed, (iii) grab CS&T beads from fridge if needed.

Fig. 2 (Above). Fluidics Startup Check

Obtain from the appropriate Fridge (more in FCCF sorter room if needed): Prepare 1 drop of CS&T beads (**orange** vial, not black label vial) into a FACS tube. Labeled: date, "ORANGE CS&T" and the Lot ID from vial. Dilute in 300ul filtered DI water from a syringe at LSRII. Parker Room: Fridge is located outside door by the corridor entryway. *Main stocks are in FCCF.*

**Beads are expensive, so please protect from light, label and cover with parafilm before returning to the fridge for the next use (please make your best effort to try and re-use beads, however make up new beads if needed to check that CVs are not wide due to using old beads left out in the light. Light will also photobleach far-red channel signals). If significant "non bead" scatter events are observed, rule out instrument cleanliness issues by checking that a newly filtered tube of water ran the same amount of time has minimal event rate.*

5. **Open the QC bead experiment located in the Browser.** Make a new specimen for every session and re-label e.g. DATE-NAME-LAB-Lot# (e.g. 092820Caz-FCCF-70757). Re-label any tubes you run appropriately e.g. BEFORE (pre-usage record) or AFTER (post-usage record) or AFTER EXTENDED CLEANING (if you were troubleshooting) etc. If there are issues with the template (e.g. another user deleted gates by accident, or changed the settings), simply load another template to use: ***Experiment Menu → New Experiment → Select DAILYQC template from QC tab***. Run filtered water in LO and ensure event rates are mostly 0-20 events/second (reflecting cleanliness) before proceeding with QC; change the syringe filter if there appears to be significant debris with freshly filtered water (these should be kept nearby the computer, email FCCF@salk.edu if more are needed). Run diluted beads in LO and start acquiring. Should see 3 peaks in each histogram, the scatter plot should not have a lot of debris in addition to the beads; see example in Figure 1. Record a file of the CS&T beads then on the scatter plot, adjust P1 and P2 around the 2 sizes of singlet bead populations. Make a PDF showing the plots and send to fccf@salk.edu. Continue on to the automated CS&T calibration if you are the first user of the day.

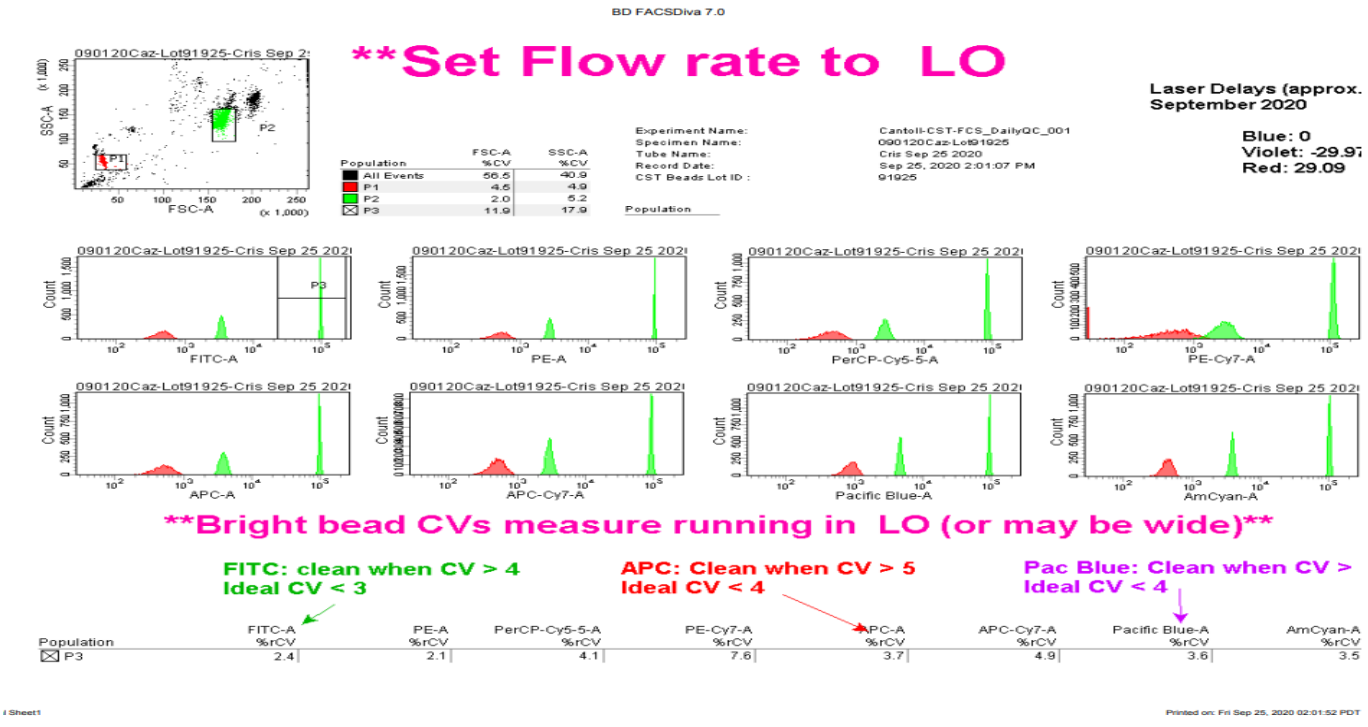
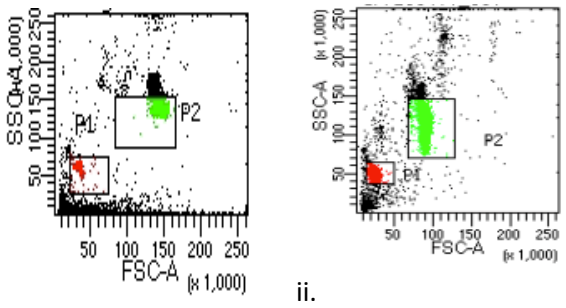


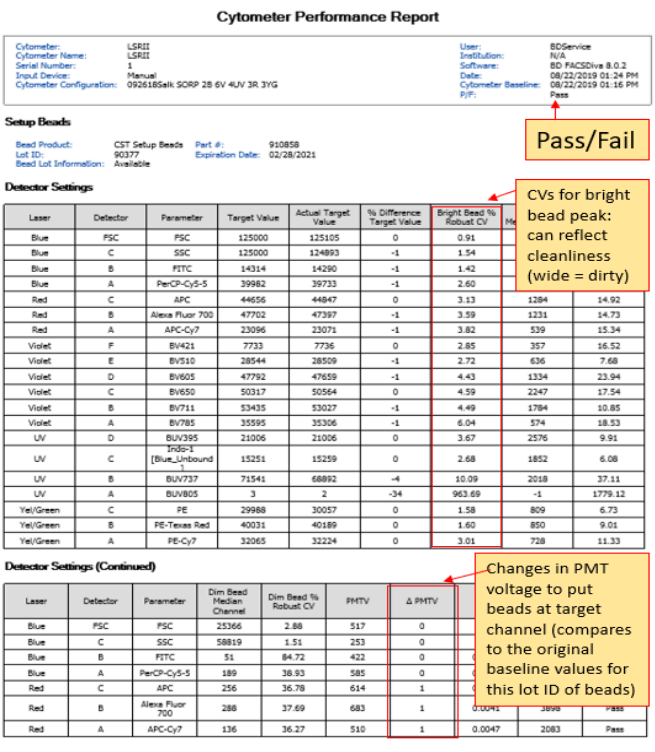
Fig. 3a *(Above)*. Example of CS&T bead profile from LSRII-2. Beads are 2 sizes on the scatter plot and produce 3 peaks of intensity in most channels. Cleanliness can be noted from the scatter plot (no excessive extra events besides beads).

Fig. 3b *(Right)*. (i) Example of excessive "non bead" events that are debris and (ii) elongated/ "strange" side scatter pattern compared to before running samples, indicating possible debris stuck to flow cell: instrument is likely dirty and needing longer cleaning (if instrument has been cleaned and beads still look dirty, check with new beads). Consult with core staff for the next corrective steps needed



6. IF YOU ARE THE FIRST USER OF THE DAY, Execute the CS&T program: In Diva, go to the Cytometer Menu → CS&T (wait for it to Connect - check status in lower right of window)→ On the right side of the CS&T window, Select Bead Lot ID# from the middle pane drop down menu (if not already automatically highlighted). If your bead lot is not available, proceed any way; choose one to use as you can still successfully calibrate the laser timing (notify staff later) → select Performance Check from the "Characterize" drop down menu. Follow prompt to load beads etc. Note that while the CS&T mode is open, Diva interface is disconnected (will be indicated in bottom left of Diva screen that it is in CS&T mode). Example of a report is shown in Figure 2. Check the report when completed (e.g. scroll through both pages) if it passes with warnings, or fails, try more cleaning cycles (especially if instrument hasn't been used in a while) and repeating CS&T. If this does not resolve the issue, close CS&T and open the QC template and check again how beads look, if peaks are well resolved it is probably good to use cautiously (check with core staff if needed) as imperfections with beads may not show up when running biological samples. Typical issues on the report are wider than acceptable CVs, meaning that the instrument needs cleaning or running water longer. Reports are organized on the left side of the CS&T Screen (in the Reports Tab). Export a PDF (locate the report and right click on it) to send to fccf@salk.edu. Close CS&T when done. Log out of Diva Calibration username and log back in with your own Diva account to prepare for running beads. Choose "**Use Cytometer Setting**s" any time the message pops up.

Fig. 4 *(Below, left and right)*. 2 page CS&T report generated in Diva. Pass/Fail is indicated in the header of page one; if CS&T fails then report will highlight in red the out-of-specification value. Wide CVs or large positive gains in PMT voltage are indicators that instrument may be dirty. Calibrated Laser Delays are indicated at the end of the second report.



Cytometer Performance Report

Cytometer: LSRII
Cytometer Name: LSRII
Serial Number: 1
Input Device: Manual
Cytometer Configuration: 092618Salk SORP 2B 6V 4UV 3R 3YG
User: BDService
Institution: N/A
Software: BD FACSDiva 8.0.2
Date: 08/22/2019 01:24 PM
Cytometer Baseline: 08/22/2019 01:16 PM
P/F: Pass

Pass/Fail

Setup Beads

Bead Product: CST Setup Beads Part #: 910858
Lot ID: 90377 Expiration Date: 02/28/2021
Bead Lot Information: Available

Detector Settings

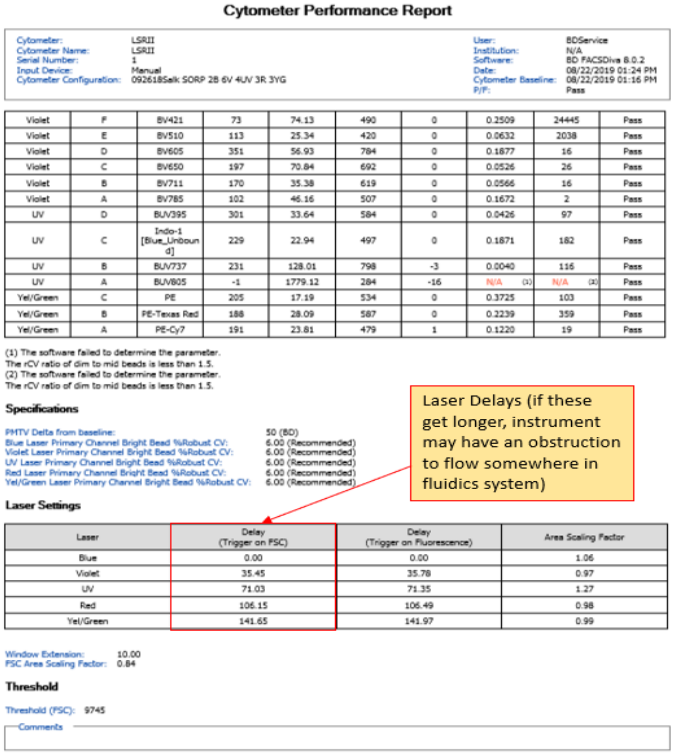
CVs for bright bead peak: can reflect cleanliness (wide = dirty)

Laser	Detector	Parameter	Target Value	Actual Target Value	% Difference Target Value	Bright Bead % Robust CV		
Blue	FSC	FSC	125000	125105	0	0.91		
Blue	C	SSC	125000	124893	-1	1.54		
Blue	B	FITC	14314	14290	-1	1.42		
Blue	A	PerCP-Cy5-5	39982	39733	-1	2.60		
Red	C	APC	44656	44847	0	3.13	1284	14.92
Red	B	Alexa Fluor 700	47702	47397	-1	3.59	1231	14.73
Red	A	APC-Cy7	23096	23071	-1	3.82	539	15.34
Violet	F	BV421	7733	7736	0	2.85	357	16.52
Violet	E	BV510	28544	28509	-1	2.72	636	7.68
Violet	D	BV605	47792	47659	-1	4.43	1334	23.94
Violet	C	BV650	50317	50564	0	4.59	2247	17.54
Violet	B	BV711	53435	53027	-1	4.49	1784	10.85
Violet	A	BV785	35595	35306	-1	6.04	574	18.53
UV	D	BUV395	21006	21006	0	3.67	2576	9.91
UV	C	Indo-1 [Blue_Unbound]	15251	15259	0	2.68	1852	6.08
UV	B	BUV737	71541	68892	-4	10.09	2018	37.11
UV	A	BUVB05	3	2	-34	963.69	-1	1779.12
Yel/Green	C	PE	29988	30057	0	1.58	809	6.73
Yel/Green	B	PE-Texas Red	40031	40189	0	1.60	850	9.01
Yel/Green	A	PE-Cy7	32065	32224	0	3.01	728	11.33

Detector Settings (Continued)

Changes in PMT voltage to put beads at target channel (compares to the original baseline values for this lot ID of beads)

Laser	Detector	Parameter	Dim Bead Median Channel	Dim Bead % Robust CV	PMTV	Δ PMTV			
Blue	FSC	FSC	25366	2.88	517	0			
Blue	C	SSC	58819	1.51	253	0			
Blue	B	FITC	51	84.72	422	0			
Blue	A	PerCP-Cy5-5	189	38.93	585	0			
Red	C	APC	256	36.78	614	1			
Red	B	Alexa Fluor 700	288	37.69	683	1	0.0041	3898	Pass
Red	A	APC-Cy7	136	36.27	510	1	0.0047	2083	Pass



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User: BDService
Institution: N/A
Software: BD FACSDiva 8.0.2
Date: 08/22/2019 01:24 PM
Cytometer Baseline: 08/22/2019 01:16 PM
P/F: Pass

Violet	F	BV421	73	74.13	490	0	0.2509	24445	Pass
Violet	E	BV510	113	25.34	420	0	0.0632	2038	Pass
Violet	D	BV605	351	56.93	784	0	0.1877	16	Pass
Violet	C	BV650	197	70.84	692	0	0.0526	26	Pass
Violet	B	BV711	170	35.38	619	0	0.0566	16	Pass
Violet	A	BV785	102	46.16	507	0	0.1672	2	Pass
UV	D	BUV395	301	33.64	584	0	0.0426	97	Pass
UV	C	Indo-1 [Blue_Unbound]	229	22.94	497	0	0.1871	182	Pass
UV	B	BUV737	231	128.01	798	-3	0.0040	116	Pass
UV	A	BUVB05	-1	1779.12	284	-16	N/A (1)	N/A (2)	Pass
Yel/Green	C	PE	205	17.19	534	0	0.3725	103	Pass
Yel/Green	B	PE-Texas Red	188	28.09	587	0	0.2239	359	Pass
Yel/Green	A	PE-Cy7	191	23.81	479	1	0.1220	19	Pass

(1) The software failed to determine the parameter.
The rCV ratio of dim to mid beads is less than 1.5.
(2) The software failed to determine the parameter.
The rCV ratio of dim to mid beads is less than 1.5.

Specifications

PMTV Delta from baseline: 50 (BD)
Blue Laser Primary Channel Bright Bead %Robust CV: 6.00 (Recommended)
Violet Laser Primary Channel Bright Bead %Robust CV: 6.00 (Recommended)
UV Laser Primary Channel Bright Bead %Robust CV: 6.00 (Recommended)
Red Laser Primary Channel Bright Bead %Robust CV: 6.00 (Recommended)
Yel/Green Laser Primary Channel Bright Bead %Robust CV: 6.00 (Recommended)

Laser Delays (if these get longer, instrument may have an obstruction to flow somewhere in fluidics system)

Laser Settings

Laser	Delay (Trigger on FSC)	Delay (Trigger on Fluorescence)	Area Scaling Factor
Blue	0.00	0.00	1.06
Violet	35.45	35.78	0.97
UV	71.03	71.35	1.27
Red	106.15	106.49	0.98
Yel/Green	141.65	141.97	0.99

Window Extension: 10.00
FSC Area Scaling Factor: 0.84

Threshold

Threshold (FSC): 9745

Comments

7. Log out of Diva Calibration username and log back in with your own Diva account to prepare for running samples.

- If you are having issues running samples, open the QC template and check how beads look (compare with example below)
- If you have issues seeing signals after QC passed, we have seen template (experiment) corruptions where laser delays are not up to date (so specific laser signals may appear to be absent when you run samples). Timing values for each laser can be verified as follows: (1) the most recent CS&T calibration report contains the correct values on pg2, compare these timing values to (2) laser delays being used in an open Experiment – check Experiment associated delay values by looking in the **Laser** tab of the **Cytometer** Window. If an experiment does not display the calibrated laser delay values, it is best to start over fresh: Copy your detector voltages from that experiment (eg screen grab), make a new experiment (to update the laser delays) and type the detector voltages in.

8. Remove beads and run water while getting set up for your experiment. Record cleaning files ≤30min as detailed below and take a photo of Diva browser showing time stamps when finished using Canto II (send to fccf@salk.edu):

Every 30min (or less!), clean and record files of each cleaning reagent to use the timer in Diva:

When using CLEAN FLOW CELL (Cytometer Menu> Cleaning Modes) function add at least 2ml liquid to FACS tube and check uptake. Refill tube as needed:

2min CLEAN (10% Bleach) with 3-5 CLEAN FLOW CELL interspersed:
After each CLEAN FLOW CELL cycle, install tube with bleach and keep running.

2min RINSE (5% Contrad detergent) with 3-5 CLEAN FLOW CELL interspersed:
After each CLEAN FLOW CELL cycle, install tube with RINSE and keep running.

1min H_20 with 2 CLEAN FLOW CELL: do the CLEAN mode at the start only, then run for 1min

The event rate running water must be low 0-20evt/s in Diva (try making new filtered H2O if event rate is high).

9. Final Cleaning and shutdown/standby: After running samples, follow clipboard checklist (i.e. perform thorough post-use cleaning while recording cleaning files to use the timer). Under the Calibration log in: Record/create an "after" use bead PDF (Calibration log in) to demonstrate cleanliness before finishing up at the machine. Take a photo of the Power button showing status (e.g. OFF = no green light) before leaving. Clean longer if instrument has unacceptable CV values or scatter indicates instrument is dirty, email the core if you have issues with getting the instrument clean (you may be requested to assist with execution of extended cleaning procedures the following day if needed, if the instrument is dirty following your usage).

Submit files and photos to fccf@salk.edu:

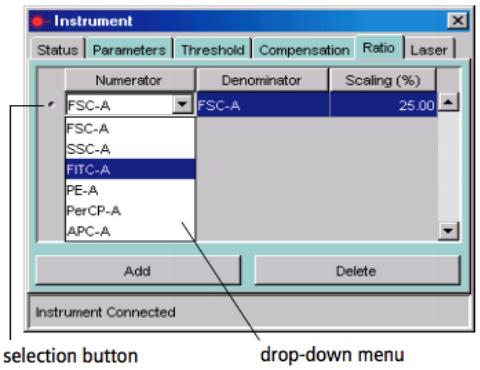
1. PICTURE of the GREEN POWER BUTTON *Send ASAP!
2. PICTURE of the CLIPBOARD signed off by you
3. RECORD FCS FILES AND SCREENSHOT THE BROWSER when performing the 5min cleaning procedure described below every 30 mins
4. CS&T beads after
5. CS&T beads before *if the machine was dirty before use*
6. CS&T Program Report *if you are the first user of the day*

10. Restart the PC as the last step before you leave (do not shut down, only RESTART): to reset/free up computer RAM. Double click restart icon to restart. Double click log off icon to log off.

11. Empty/change the red biohaz trash bin if full: Double bag, do not twist bag neck (steam access will be limited) – scrunch it in your fist and secure with autoclave tape. Deposit in large round red biohaz collection tub. Install 2 new bags (See Help File "Where Stuff is Kept" for map for info for FCCF and STEM if needed).

Canto II Quick Start Guide

*Similar to LSRII but 8 colors only, no UV laser or 561nm laser (488nm, 640nm, 405nm lines)
e.g. FITC/PE/PerCP/PECy7/APC/APCCy7-/DAPI/BV510

Check Fluid Cart	**Sheath Tank:** Refill at end of session (uses about 1.2L per hour). Sheath is MQ H2O. Refill from autoclaved water carboys (spray down funnel with 70% ethanol before using) or MQ gun in STEM if no carboys are available.
	Waste Tank: previous user empties and added 1L bleach ready to use
	Shutdown Solution: switch to full bladder if needed (it is MQ water, refill from sink)
Power Button	**Green Button**, left side. DO NOT FORGET TO TURN OFF (you will be charged time)
Log into PC, open Cytometer Status	**Salk Active Directory** credentials (contact IT Helpdesk for log in issues) DO NOT FORGET TO LOG OUT. **Cytometer Status** icon on desktop > IP address loads when ready
Log into Diva	**Calibration** (pw: orangecst) for QC, or your log in from user training (typically same as AD log in)
Fluidics Start Up	IF NEEDED: From **Cytometer** menu
Bead QC ("before")	Check bead CVs in QC template, execute CS&T in Diva if first user (if bead CVs are OK). Make PDFs
Run samples	Use BD polystyrene FACS tubes (NO mini tubes) Check tubes for cracks before running (otherwise tube will fill up). **Check samples are free from clumps or viscous material to prevent clogs** System loads/unloads tubes when **Acquire** button is clicked (Acquisition Dashboard) Ensure that SIT flush box is checked (Acquisition Dashboard): Remove tube, allow to rinse completely; arm will release when cycle is complete ready for next tube *If Canto does not release tube, force quit Diva and re-open
	<u>*Tip: SSC-W for doublet discrimination on Canto:</u> Manually derive SSC-Width if cells are off scale on plot. (1) In the **Instrument** Window of Diva under **Parameters** tab, make sure Area (A) and Height (H) are checked. (2) Then go to the **Ratio** tab and create a parameter of SSC-A (numerator) and SSC-H (denominator). *Cells are off scale as default of 25% does not fit all cell types.* Manually change the scaling factor (eg try 20%, or 15%) in the right column while using this new parameter in place of "SSC-W" on a plot to visualize as cells move down from off scale to on scale.
	
Clogs	Avoid clogs by (1) <u>filtering</u> samples and <u>visually checking</u> samples before running, (2) running bleach and water periodically during your session if collecting sticky samples (eg tumor derived) and (3) <u>using DNAse</u> in your buffers for samples with dead cells or tissue derived samples. Use **cleaning stylus** (remember to put a small kink in end to hold on to), and **Clean Flow Cell** function (Cytometer menu) to try and clear blocked sample injection tube (SIT). Run 10% bleach on HI for 5min once cleared followed by water 2min before resuming collection.
Export Data	Right click on the experiment in the browser→ recommend to (1) "**Export Experiment**" first (browse, locate to Desktop) then repeat a second time choosing (2) "**Export FCS Files**" (choose FCS3) browse, locate to Desktop)→ "replace all" FCS files. This gives you a folder with your template as well as correctly names data files.
Connect to Filer	Go to "Computer". If need to map to Lab share: Right click Computer and select map network drive. Type in path and lab, e.g. \\filer.ad.salk.edu\IMPL-K (*your lab)
Delete old data	In the browser: Right Click → Delete
Clean Canto	Run cleaning (record files). Make a screengrab showing cleaning files in Browser. Cleaning and tank maintenance instructions in Usage Guide and on **clipboard**, sign off at each step.
Bead QC ("after")	Check bead CVs in QC template. Make PDF of "after" bead file.
Email PDFs/photos	NO FLUIDICS SHUTDOWN. NO tube of water needed on SIT afterwards. POWER OFF at green button on side/ RESTART PC. Take photo of green button status with legible clipboard signed off > Send PDFs and photos to fccf@salk.edu