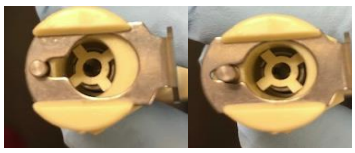
A3 Quick Start Guide

*__Download a copy of this instrument Help File__: salk.edu/fccfaccess > Help Files

**23 fluorescence channels (355nm, 405nm, 488nm, 561nm, 640nm lines) See chart on side for filter/detectors

GREEN: Start up, turning on
YELLOW: Running bead QC/calibration
ORANGE: Running Samples and HTS
PINK: Diva: Using the A3 Template and Application settings
BLUE: Cleaning up and Shutting down.
AQUA: Troubleshooting (broken up into different sections)

All FCCF instrument documents can be downloaded from our **Help Files** section on Salkland (salk.edu/fccfaccess, a shortcut has been placed on the workstation desktop). See also the A3 Troubleshooting Guide section, at the end of this document.

Fluidics Turn on Sheath cart Check Tanks	**FACSFlow cart:** Green power button, under the A3. **10L Sheath/Waste Tanks** (check before your run!): Each user must top off sheath/empty waste tank at the end of the session if there is less than 3L sheath / waste is at 7L. Although the tanks are equipped with audible alarms, perform tank maintenance if there's ANY chance you may not hear alarm beeping while using (e.g. using headphones, or using HTS/unattended!). **Sheath:** fill from "A3" labeled carboys containing 1x PBS with 1% 2-phenoxyethanol (surfactant). **Waste Tank:** unscrew/move probe over to second waste tank (*must contain 1L bleach). Empty full waste tank at STEM/FCCF sink then add 1L bleach.  A3/LSRII/Canto/Fusion all use quick release couplers; always check the female coupler if connecting couplers, to avoid o-ring damage. ***Left:*** wrong position, non-circular – this will damage male coupler o-ring. *Right:* Ready to connect, after repositioning by pressing metal side tab of female coupler (shows circular opening ready to connect with male coupler). Needs a small amount of o-ring lube periodically on the black o-ring of the male coupler, if difficult to connect.
Log into PC	**Use Salk Active Directory** credentials (contact IT Helpdesk for log in issues)**. Note:** must log into PC before starting the A3 (win10/Diva9); if restarting PC then restart A3 also (fluidics cart can stay on). **Last user:** restart PC before leaving (resets RAM)
A3 Power Button	**Green Button**, right side. Note: Turn off (1) A3, and (2) FACSFlow cart before leaving FCCF if you are the last user of the day.
Fluidics mode switch	**Black switch** is near A3 Power Button: Check it is set for running tubes (unless actively running a plate on the HTS)
Release trapped air	**0.2um sheath filter:** Next to A3, behind HTS; depress tip of valve to inside of beaker, empty the beaker into the waste tank.
Run bleach and H2O Flick waste line (loosens bubbles)	***Do NOT use PRIME button:** reserve PRIME for troubleshooting, to avoid unintentionally introducing air. Run fluids from a FACS tube while setting up, and gently flick orange waste line if stationary bubbles are seen in RUN (should move along to the waste tank when loosened by flicking the tube, while in RUN). RUN button must turn green, if yellow check the sheath cart is turned on: If cart is on and issue unresolved, refer to our Help Files (salk.edu/fccfaccess) for more detailed troubleshooting
Start UV laser (if first user) use the **CC desktop icon**	*Coherent Connect (CC) can be configured by FCCF for your AD log in to disable OBIS laser windows so they don't come up. **4 "OBIS" lasers auto-fire with no action needed (will not appear in CC). First user needs only to start UV (Genesis laser) in Coherent Connect** (slider plus START button). ***Close (X) CC window when not actively turning on/off UV:** never leave running in the background (RAM heavy, and can randomly turn lasers off!).
Log into Diva	**Calibration** (pw: bluecst) for QC, or your log in from user training (typically same as AD log in)
Bead QC ("before")	***First User:** check bead CVs in template THEN execute automated CS&T. Update mini notice board on LSRII if this was completed by you. **Optional for following users:** check bead CVs. New users: Make PDFs to send
Load Template/ Application settings Check CST bead MFI	• **If creating a new experiment, for convenience load the A3 Salk template:** This contains basic plots and an extra page with single color histograms for each channel. Experiment Menu > New Experiment > A3 Salk Template folder > select template. • **Cell optimized PMT voltages (OPV) are available to use as a starting point:** In the browser under the Experiment name, right click Cytometer Settings > Application Settings > Apply > use Starting OPVs. These were created using CD4+ lymphocytes, and represent a "sweet spot' for performance for fluors tested in the detectors (go to Help Files for info on the "voltration" characterization performed and list of fluors tested); adjustments may be needed for other cell types). • **Recommended:** Assess CST beads MFI in your template ahead of recording samples (uncheck the compensation box for the beads, turn back on afterwards). If changes are needed, adjust PMTv to put appropriate peaks within their histogram gates then re-save the Application Settings (typically reflects changes such as a service visit/laser alignment by engineer). This should be done at the start of every session to verify your Application Settings are up to date. Ask FCCF if you need help with establishing bead target channels in your template on the histogram worksheet (this can be done later if you remember to record a CST bead file at your first session after detector voltages for your panel have been established).
Prepare to run samples in tubes Log into Diva with your user account Intermittently clean (5min process) to ensure high quality data. Use the cleaning stylus as needed (eg weird scatter patterns)	• Use BD polystyrene FACS tubes, check tubes for cracks before running (otherwise tube will fill up). • **Filter samples: MUST be free from clumps or viscous material to prevent clogs** • **Fluidics control panel is on front of A3:** RUN button goes green when pressurized. If YELLOW: check the fluid cart is turned on. Also check the sample tube for cracks. Refer to Help File document (salk.edu/fccfaccess) if A3 was run completely dry. • Tube "support" is adjustable by rotating however it should not be necessary to "hold" the FACS tube on – if tubes are falling off, notify staff to replace bal-seal (holds tubes on). Never close the support arm with no tube, and never put in STANDBY if a sample is still on the SIP (retrieve the sample and install 1ml water before putting into STANDBY). • Refer to Diva user training guide located by the instrument to get started if needed • Never use DUPLICATE WITHOUT DATA, and make use of properly exported experiment templates (ask us!) • **Intermittent 5min cleaning:** (Optional, but recommended) Every 30-60min • **Running more than 50 tubes:** split into another specimen after 50 tubes to avoid Diva issues • **Running large files:** split into several files to avoid Diva issues (can concatenate in Flowjo later) • **Expedite recording:** set Diva to show less events while recording to allow faster processing. Delete any extra plots. ***For instrument issues: see troubleshooting section at the end of this guide.**
Automation with HTS	Set up by running in tubes before going to HTS: Refer to the HTS guide for the next steps (turn on/connect, prime, QC - run a few wells with beads, then samples). **Cleaning is done with HTS**, before changing back to tube mode for the post run bead QC.

Clean	*Skip to next step if using HTS*. See cleaning and tank maintenance instructions on **clipboard**, sign off at each step.
"Post-use" QC, Tank maintenance	**Optional:** Record bead profile in QC template. *New users: check beads in QC template and make PDF for first few sessions until signed off by FCCF. **Top off sheath/change and empty waste tank** if there is less than 3L sheath / waste is at 7L.
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 FCS3.1)> 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 "This PC" (icon on desktop); if need to map to Lab share: Right click This PC 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. Important! Prevents Diva getting buggy/slow: optimal is ≤ 4GB TOTAL user data in Diva!!
How to leave A3	**If you are not the last user:** leave lasers on, instrument in STANDBY (with less than 1ml water), do not restart the PC (unless also restarting A3 (otherwise A3/Diva connection issues). **Last user**: (1) Turn off lasers (basically the reverse of how you turned them on), (2) POWER OFF A3 green button and (3) POWER OFF FLUID CART green button, (4) RESTART PC (do not shut down).
Email photos/PDFs	Take photo of FLUIDICS PANEL (in OFF or STANDBY), with legible clipboard signed off > Send to fccf@salk.edu and next user for the day (check schedule). New users: send bead profiles for first sessions until signed off by FCCF.
TROUBLESHOOTING	
Nothing is coming on	Potential black out earlier tripping fuse on battery back up (UPS) and/or power conditioner units. Check back of these for a fuse switch, contact Salk Facilities if help needed (an FS watch engineer is also available out of hours, check Salkland for info).
Power Outage	UPS should come on with enough time to save your file and shut everything off for the outage.
RUN button yellow	Check that: (1) fluid cart is ON, (2) pressurized plenum tank (by the A3 side near sheath filter) shows 4.5PSI on the gauge and it has around 1 inch volume (i.e. not empty: should be maintained less than 2 inches by pump), (3) Check for air/bleed air: in sheath filter and at roll valve located between the plenum and A3 side panel. Refer to Help Files if more details are needed.
Waste alarm	If tank level is fine, sensor top near the "cap" is probably wet (forming salt bridge). Put A3 in standby, turn off fluid cart, remove probe from tank and dry the top with a kimwipe, turn cart back on. Refer to Help Files if more details are needed.
Sheath alarm	Refill tank (should hear pump working to maintain ~ 1inch in plenum on desk; if more than 1-2inch, notify FCCF: lower pump should not be heard continually). If pump dies out of hours and you can't wait: See Help File to manually fill the plenum to run
General PC issues.	**Anything that can be computer related**, email IT@salk.edu and provide the Anydesk info posted on monitor. This includes AD log in, Filer connection, Diva or Coherent Connect not opening (see below), computer freezing/crashing/blue screen etc.
Connection issues	Diva won't connect to A3: Turn off A3 and restart PC > wait a minute then log into the PC before turning on A3 > turn on UV laser > open Diva (FACSFlow can stay on). If persists, ask IT for help removing Ethernet (remove from network) and re-try.
Diva wont open	Reboot PC/A3 (follow the correct start up order). Can also ask IT to check Diva not getting blocked by network.
Missing laser signals	Restart A3/PC as for Connection Issues (above); if persists go to Help Files for troubleshooting before concluding laser is dead.
Tube pops off SIP	Ask staff for a new "BAL seal", in the mean time adjust (rotate) the black support knob under the tube
No plots, no detector list in Cytometer	(1) Make sure Worksheet checked (View menu), and Experiment is open (left click TWICE only to open). (2) Activate PMTs in **Cytometer** Window: Be sure to expand the specimen (click on the +) then click on the arrow to the left of a Tube.
Plot name missing	Software glitch: After activating a detector in Cytometer, if it does not appear in plot axis, close/reopen experiment
Not taking up sample Clogged Sample Injection Probe (SIP). *Avoid at all costs!*	***Check Fluidics Cart: Follow section on RUN button yellow (see relevant section) before troubleshooting as described below.*** Clog signs include: Event rate dropping, no events, NO BUBBLES WHEN PRIMING, or even simply "weird" or "lower than usual" scatter plot signals for cells/beads (usually for a partially obstructed SIP). Avoid by (1) **filtering** samples and **visually checking** samples before running, (2) **running bleach and water** periodically during your session if collecting sticky samples (eg tumor derived) and (3) **using DNAse (needs $MgCl_2$ to work)** in your protocol/buffers for samples with dead cells or tissue derived samples (tumor, intestine, brain etc). **Do not attempt to run the sample if mucous consistency material is seen.* If a clog occurs, try the **cleaning stylus** (remember to put a small kink in end to hold), and **Prime** (button on fluidics panel) function to try and clear blocked sample injection tube (SIT). Perform cleaning (see Usage Guide).
No signals at all in experiment plots	***Check Fluidics Cart: Follow section on RUN button yellow (covers checking for air in system) then ensure fluidic switch is set appropriately (tube vs HTS) before troubleshooting as described:*** (1) Install a tube of water > Hit PRIME > check for bubbles in the FACS tube: This is to check for a clogged SIP (go to section on clogged SIP if there are no bubbles). (2) Open the Bead QC template> Hit Acquire then PRIME button > check if ANY events show up in the FSC vs SSC: If there are no events at all while acquiring, but there is bubbling during PRIME there could be a connection/start up error (hardware board, 488nm laser, or Diva) > see earlier section on connection issues (i.e. restart A3/PC). (3) Check beads in QC template: If QC looks as expected but there are still missing laser signals in your experiment template, it may be corrupted: Start over fresh > Snip tool (or copy) the PMT voltages and type them into a brand new experiment.
Missing laser signal while running samples	***Check Fluidics Cart: Follow section on RUN button yellow (covers checking for air in system) first before proceeding: Confirm with beads in QC template if ANY signal vs a dim peak/dimmer than usual.*** No signal: Laser possibly did not start up right (try restarting A3/PC as described in Connection issues section) or laser dead (if no signal at all). Dim or unstable signal: laser delay needs calibrating by executing CST (unstable signal could also indicate end of life of laser). OK to use if laser is not needed, otherwise use another analyzer. See also Help File for procedure for troubleshooting laser using Coherent Connect.
Unstable signals, visually "drifting up and down" plots	***Check Fluidics Cart: Follow section on RUN button yellow, and flick the orange waste line if any stationary bubbles are seen while in RUN.*** Issue is most obvious with green laser or red laser (i.e. whichever are the last lasers in the system). Check for air in sheath filter, then as a precaution, perform air removal at Flow Cell > **Run an empty tube and watch for bubbles to come through to the waste line (clear orange tubing to waste tank), when bubbles appear switch to H_2O for 5min. Afterwards, execute automated CS&T to recalibrate the laser delay timing.** Refer to Help Files if needed for more detail.
Extra plots appeared for each tube	Accidentally clicked on the icon in the top left of Worksheet (toggles between Global/Normal View, indicated at the top of the Worksheet page). Click icon to return to Global View for Diva Worksheet.
HTS won't connect	**If (1) A3 is in RUN with SIP coupled to HTS, and (2) HTS is powered on:** Reboot Diva (close, re-open Diva and log in again). Should automatically connect (see status in corner of Cytometer window); otherwise select Re-initialize from the HTS menu.