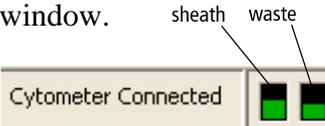


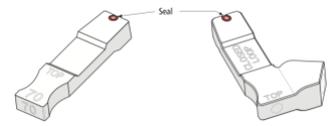
Stanford AriaII User Guide

Starting up

1. Turn on the computer. Wait for the computer to boot up.
2. Log in to the computer (user: facsuser, password: BDIS) and open BD FACSDiva software. Log in to Diva at the prompt with your Diva username and password.
 - Wait for Cytometer window to read “**Cytometer Disconnected**” (in red)
3. Turn on main power switch (the green button): left side for all FACS Arias, right side for Fusion.
 
4. Verify lasers are on (White button below the main power switch).
 - If using an instrument with a UV laser, open the OPSL software, clear the laser fault (turn off laser with button icon on right side of window, then turn back on) and set the laser power to the correct voltage.
 - Optimus Prime has a UV laser that auto starts with the power button. It is our only system with UV auto start function.
 - For Storm (FACS Fusion) open BD Coherent Connection 4 and make sure all 4 main lasers are on, and then turn on the UV laser (clear the fault, turn back on, set power). Wait 30 minutes for lasers to warm up before running samples.
5. Watch for “Instrument Connected” status at bottom of Cytometer window.
6. Replenish sheath if needed:
 - a) Turn off stream, if needed.
 - b) Disconnect the airline.
 - c) Pull up on the ring of the pressure relief valve to release pressure from the tank. Make sure tank is fully vented.
 - d) Unscrew the sheath tank cover knob and remove the cover.
 - e) Fill the tank with sheath fluid up to the upper weld line on the inside of the tank. Do not overfill.
 - f) Replace the cover and tighten the knob. Make sure the large O-ring on the inside lip of the cover is seated correctly and has not slipped out of position. The tank can leak if the cover is not secured properly.
 - g) The flow rate is calibrated with the sheath tank on the fluidics cart. If the location or elevation of the sheath tank is changed, it could affect the flow rate calibration.
 - h) Connect the airline. The system is ready to run again.
7. Check waste container, empty if necessary and add 10% of total volume in (1L) bleach.
8. Aerosol Management System must be used for sorting or analysis for maximum safety. Turn on the main power button on the back of the evacuator just above power cord. Press the POWER button on the membrane panel of the evacuator. Ensure that the suction control rate is set to 20%. (Do not set the suction control rate above 20%; higher rates could affect the stability of the side streams.) Verify that the filter flow gauge reads between 1 and 2.4 inches. (*Refer to pg. 14, Operating the BD Aerosol Management Option*)
 
9. Select the correct nozzle size for your experiment.

To choose which nozzle, take into consideration the following guidelines:

- Cells should not be more than 1/5 the size of the nozzle.
- There are 3 sizes currently available: 70µm, 100µm and 130µm.
- Fragile cells will do better with a larger nozzle.
- Sorting speed can be faster with the 70µm.
- Nothing but water should be used to clean these integrated o-ring nozzles for routine use. BSL-2 disinfection after an obstruction, Nozzle Obstruction Appendix page 16 and Aseptic Sort Set up page 17 are exceptions.



10. Check the nozzle under the microscope, clean by sonication in a 5ml tube of Di water if needed(30 sec-1 min). The integrated seal surface of the nozzle is very fragile, do not wipe it, gently dab it with the edge of a kimwipe to remove fluid.
11. Remove the closed loop nozzle by turning the lock key counterclockwise and place the nozzle carefully in the saddle above the forward scatter channel(avoiding any damage to the o-ring). Place the ceramic nozzle orange o ring facing up in the flow cell and turn the key clockwise to lock nozzle in position(12).
12. Clean sort chamber with a CaviCide soaked kimwipe. Make sure the deflection plates are clean and dry, use the flashlight to evaluate plates. Use the plate removal tool to remove and clean plates when preparing for an Aseptic sort.
13. Choose the correct configuration for your nozzle.

To choose your configuration, select the Cytometer menu > select View Configurations > choose the correct Stanford New configuration for your nozzle and pressure if it is not currently set at the correct one > click Set Configuration > click OK(*Choosing the configuration will change the sort setup automatically to the correct sort setup for your nozzle and pressure.*) > Exit CST > Upon returning to DIVA, choose “Use CST Settings” at the prompt.

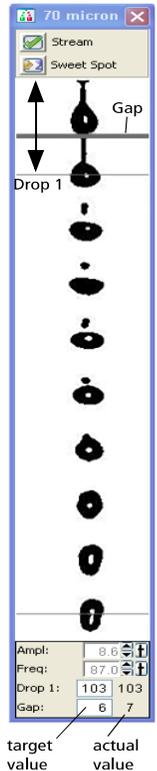


13. If preparing for an aseptic sort, follow the protocol “Prepare for Aseptic Sort” protocol (pg. 17).

Turning on the Stream

After you have chosen the correct configuration for your nozzle, click on the stream button at the top of the Breakoff window. See images above each machine for examples.

- Open sort block access door with thumb screw on left side and ensure that the stream starts up.
- The stream should flow smoothly from the nozzle into the center of the waste aspirator. If so, go to step e). If not, go through step c) to d).
- If the stream is flowing but is unsteady, check for bubbles in the flow cell. If you see bubbles, turn off the stream, wait until bubbles in the flow cell flow to the top, and turn on the stream again.
- If stream does not flow, or if you see any dripping or spraying, you will need to remove and reposition the nozzle. Cleaning the nozzle by sonication may also be necessary.
- Check that the stream location is in center of aspirator. You may need to use a hex tool to loosen the screws on each side of the sort block chamber to move the sort block so that the stream is centered.
- Verify that stream appears on the Breakoff window. If it does not you may need to restart the software.



Checking Cytometer performance

Note: CST will not run if the Sweet Spot program has been activated, be sure to de-activate.

- Close flow cell access door to unblock lasers.
- Select Cytometer > CST. The cytometer disconnects from the BD FACSDiva interface and connects to the CS&T interface.
- Verify that the cytometer configuration under the System Summary is the appropriate configuration for your nozzle.
- Run CST if:
 - you have turned the machine “on” from “off”;
 - if you are using a nozzle that has not had CST run within the last 24 hours.
 If CST is done, proceed to “Setting up your experiment”.
- Prepare the CST beads:
 - In a 12x75mm tube, add 0.35ml sheath fluid and 1 drop CST beads. Label tube with “CST”, the lot number, the date and the time.
 - Mix beads well by inverting the vial or gently vortex before running.
 - Store the bead suspension at 2°C to 25°C in the dark until you are ready to use them.

Note: Beads are stable at 2°C to 25°C for no more than 20 minutes in direct light, and up to 8 hours if protected from light.
- Select the correct bead Lot ID from the menu (bead lot is printed on each Bead vial).
- Remove the tube cap and install tube onto the cytometer loading port.
- Under Setup Control click Run to check the performance. This takes approximately 5 minutes to complete.

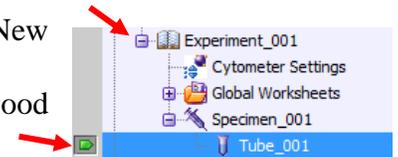
9. Once the performance check is complete, click View Report.
10. Verify that the performance passed- In the Setup tab, the Cytometer Performance Results should have a green checkbox displayed and the word Passed next to it.
 - a. WARNING(s) (CAN USE PARAMETER): A performance check will be flagged with a warning if the %CV is greater than 6% on the last detector off of a detector bank (meaning detectors other than the last can be greater than 6% without creating a warning).
 - b. FAILURE(s) (PARAMETER UNUSEABLE, FIND STAFF): A performance check will fail if the delta-PMTV is greater than +/- 50, indicating a possible issue with laser power as an example. Text or email staff if the delta-PMT is greater than 25 and make an entry on the incident log on the desktop so users are aware of the issue.
11. If any parameters did not pass, refer to the cytometry supervisor or staff and the BD Cytometer Setup and Tracking Application Guide for help troubleshooting.
12. Select File > Exit to close the CST window and connect back to the BD FACSDiva Interface. Click the Use CST Settings in the dialog box that appears.

Note: By selecting Use CST Settings, the laser delay, area scaling, and other cytometer settings will be updated to the latest optimized settings from the performance check.

Setting up your experiment

Note: All samples must be filtered.

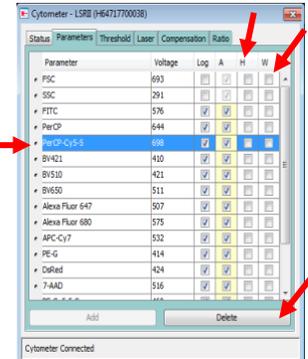
1. Create a new Blank Experiment. Experiment Menu > New Experiment > “Blank Experiment with Sample Tube” > “Ok”.
2. Rename the Experiment (right-click > Rename) using a good descriptive term and the date.
3. **(First time For Each Computer)** Go to “Edit > User Preferences”, select the user preferences that you would like. Generally, this is to *clear all preferences in the General tab except Load data after recording.*



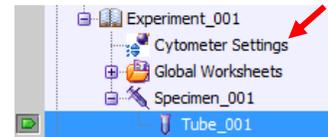
Creating New Application settings (First time you run an experiment)

Note: Application settings are associated with a cytometer configuration and include the parameters needed for the application, area scaling values, PMT voltages, and threshold values, but not compensation. Each time a performance check is run for a configuration, the application settings associated with that configuration are updated to the latest run. Using application settings provides an easy, consistent, and reproducible way to reuse cytometer settings for your commonly used applications.

1. Start with a new Blank experiment with sample tube.(See above).
2. Select Cytometer Settings in the Browser.
3. Delete all parameters you will not be using:
 - a) In Cytometer Window click on small button to left of parameter name that you want to delete.
 - b) Click delete button (use control key and highlight for multiple deletions)
 - c) Repeat for each parameter you are not using.
4. Click the H and W checkbox to select Height and Width for FSC and SSC to enable doublet discrimination.



5. Right-click Cytometer Settings in the Browser > select Application Settings > Create Worksheet. A second global sheet is added with the plots created according to your selections in the Parameters tab. You will use the gray boxes and crosshairs on this worksheet to guide your optimization.



6. To load any tube, ensure that the flow rate in the dashboard window is low (try 1 to start.) Install cells onto the cytometer by loading the tube onto the loading port > activate the tube in the browser window > click load in the dashboard window. The tube will be loaded and events will start acquiring automatically.
7. Load the unstained tube. Optimize the FSC and SSC voltages in the parameters tab of the “Cytometer” window to place the population of interest on scale. Do not change the Fluorescent channel PMT VOLTAGE unless the peaks go above 10/5th. The CST has defined the lowest voltage for resolution of the dim beads from the system noise. Changing the PMT values can have negative effects on the data, please see staff if you have any questions. You do not need to record a data file, unload the unstained cells.
8. Load a tube with all stains. Verify that the positive populations are on scale. If a positive population is off scale, lower the PMT voltage for that parameter until the positive population is entirely on scale. You do not need to record a data file. Unload the stained cells tube from the cytometer.
9. You now have your Application settings. To save, Right-click Cytometer Settings in the Browser > select Application Settings > Save > name the Application Settings appropriately > Click OK. The application settings are saved to the catalog.



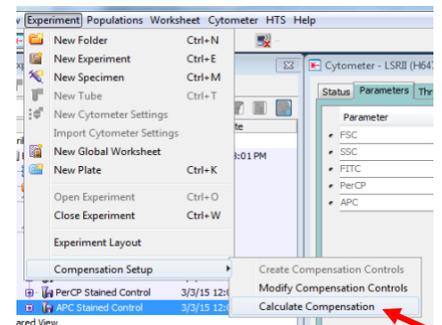
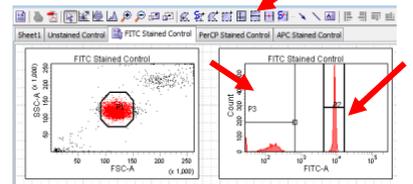
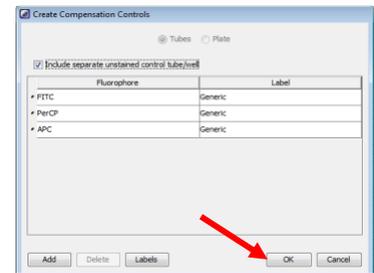
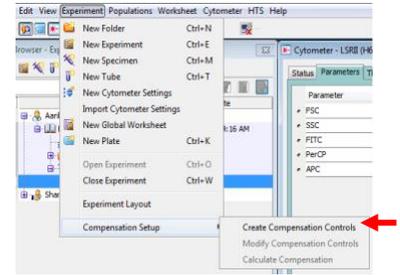
Note: Application settings do not include compensation settings.

Using previously created Application Settings (When you are doing the same experiment again)

1. In a newly created experiment, ensure that the current CST settings are applied. Then, right-click the Cytometer Settings icon in the Browser and select Application Settings > Apply.
2. Select your correct previously created Application Settings from the catalog.
3. Click Overwrite in the dialog that appears.
4. If a message appears about area scaling, click Yes to accept all changes to cytometer settings.
5. The parameter list and PMT voltages are updated to match the application settings you previously created.

Compensation

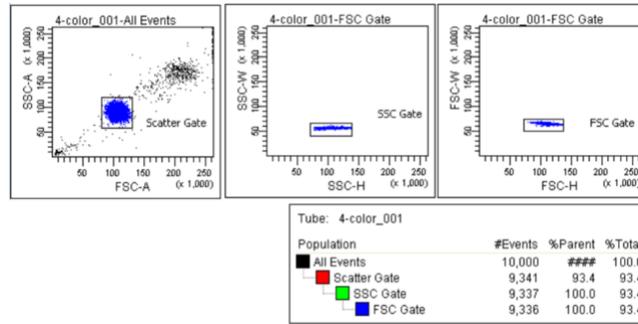
1. Make sure the H and W checkbox in the parameter tab are selected for FSC and SSC to enable doublet discrimination.
2. Ensure that you have the correct Application Settings applied, or if you are not using them, ensure that you have the correct parameters and PMT voltages for your experiment.
3. Select Experiment > Compensation Setup > Create Compensation Controls > click OK to close the Create Compensation Controls dialog. A compensation controls specimen is added to the experiment, along with an unstained control tube, and a stained control tube for each parameter. Worksheets containing the appropriate plots are added for each compensation tube.
4. Place the unstained control tube onto the loading port.
5. Set the current tube pointer to the unstained control tube in the Browser.
6. Click Load in the Dashboard.
7. Move the P1 gate to fully incorporate the singlet population.
8. Right-click the P1 gate and select Apply to all Compensation Tubes.
9. Click Record Data in the Dashboard to record the events from the unstained control tube.
10. Unload the unstained control tube. **Note: Do not change the PMT voltages after the first compensation tube has been recorded. To calculate compensation, all tubes must be recorded with the same PMT voltage settings.**
11. Click Next Tube in the Dashboard.
12. Acquire each compensation tube and record in this manner.
13. Verify that the snap-to interval gates encompass the positive populations
14. Select Experiment > Compensation Setup > Calculate Compensation > if the calculation is successful a dialog appears > name the compensation setup > click Link & Save to close the dialog box. Now you have saved the compensation setup and linked it to the experiment's cytometer settings.



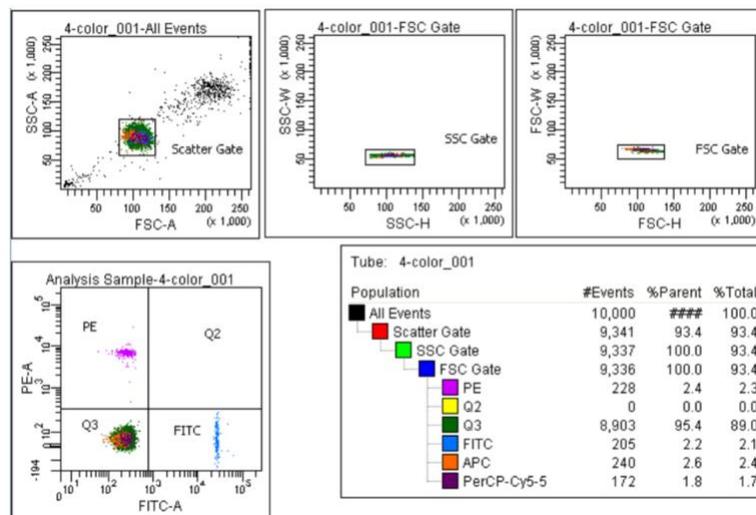
Recording Sample Data

1. After compensation, Click the Worksheets View button on the Worksheet toolbar.
2. Create worksheet elements (dot plots, hierarchy, statistics, etc.) you want to view for your experiment.
3. If necessary, create a new specimen and tubes for your samples. Activate the first tube, install the tube, load it and record it.
4. Continue for all tubes you wish to run.
5. Gate populations for sorting:
 - a) doublet discrimination (See figure below);





6. An example of gating strategy (See figure below). To show gating hierarchy, select a graph by clicking in it. Right click to show pulldown options, select **Show population hierarchy** displaying your gate nesting



Sorting

Setting up the Breakoff

- In the Stream window, keep the frequency the optimum value for your nozzle size.
Note: Ideally, you should not adjust this, but with the 100 or 130 nozzle especially there can be cases in which the optimum frequency needs to be changed.
 - For 70 μ m, optimum is 87.
 - For the 100 μ m, optimum is 30.
 - For the 130 μ m, optimum is about 15-18 (can vary).
- Adjust the amplitude (no less than 10 and no greater than 70) until the breakoff drop is in a good position.
 - The breakoff drop must be >100 and should ideally be within the top half of the image.
 - The satellite drops should merge with the drop ahead within 4 or 5 drops.
 - Cleaning the nozzle by sonication may also be necessary to achieve a good breakoff.

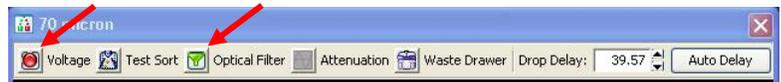
Setting the delay with Accudrop beads

- Pick the tube of Accudrop beads if there is one beside the Cytometer.

Otherwise make a new tube: add sheath fluid until 2/3 of the tube volume and add 1 drop of Accudrop beads, mix well.

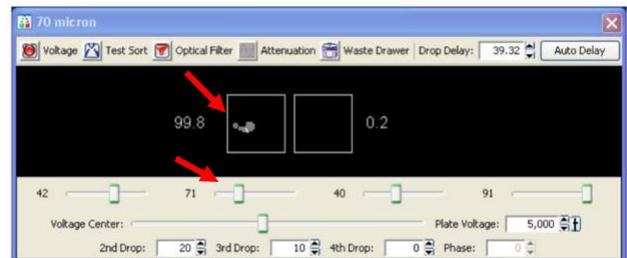
2. Verify that the stream is stable and enter by hand the actual Drop 1 value in the white Drop 1 target box. (The actual value appears next to the Drop 1 field.) Verify that the target Gap is set correctly for your nozzle. The gap for 70 should be 6, the gap for 100 should be 10, and the gap for the 130 should be between 12 and 21. Turn on the “Sweet Spot” this will automatically adjust the drop drive amplitude to maintain the stability of the breakoff point.
3. Open an Accudrop Experiment.
4. Click the “+” sign next to Specimen, activate a tube by double clicking the tube or hit the arrow on the left of the Tube.
5. Click the “+” sign next to Tube and double click the sort layout below the Tube.
6. Load the tube of Accudrop beads onto the Aria.
7. While acquiring, adjust the sample threshold rate appropriate for your nozzle (70 micron, 1000-3000; 100 micron 600-1500; 130 micron 400-1200 events/second).
Note: if this cannot be achieved using a flow rate setting between 1 and 5, adjust the bead concentration.
8. Verify that the accudrop laser is focused on the stream in the Side Stream window, use the adjustment knob to adjust.
9. Turn on the Voltage in the Side Stream window- Voltage turns red.

10. Click the Sort button at the lower left of the Sort Layout > click Cancel to the waste drawer



comment. Observe a left deflection stream in the stream window.

11. Click the Optical Filter button in the Side Stream window to allow only the Accudrop bead fluorescence to be seen.
12. Adjust the voltage slider in the Side Stream window so that the sorted Accudrop beads fall in the middle of the left optical filter box.
13. Click the Auto Delay button to the top right in the Side Stream window.



14. Click Start Run in the Auto Delay dialog.
15. Monitor the Auto Drop Delay window for progress. A message appears when the process is completed.
16. Once the drop delay is set, unload the Accudrop tube. There is no need to save the Sort report for this, so click cancel at the dialog box that appears.

Creating a New Sort Layout

1. Double click your own experiment to open.
2. Click the Sort Layout icon or select Sort Menu > New Sort Layout.
3. In the Sort Layout, select the Device and Precision you require. You can also select a specific number of cells to be sorted into



each tube if you like, otherwise, just leave it as “continuous”.

- Assign the sorting gates to be applied for each deflected stream.

Optimizing your Sort Streams – Sorting

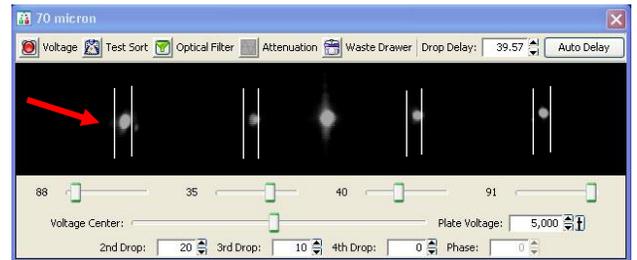
- Get some collection tubes, cover some parafilm or tape on the tubes. Load the collection tubes into holder, insert holder under sort block chamber.
- In Side Stream window, click Voltage button to turn on plate voltage - turns red.
- Turn on the Test Sort and use slider bars below the image in the stream window to aim the streams at the specific locations showing in the image.
- Demonstrate side stream overlay program, select desired device for targeting by clicking

the program icon .

- Click Waste Drawer button to open the drawer for about 1 second and close it. Check the parafilm or tape on collection tubes to see if the droplets are located at the sites that will make sure the side streams aim into the collection tubes. If not, adjust the slider bars, repeat steps 1-4 until optimized.

Note: You can adjust the sliders for the streams you are not using all the way toward the middle so they are not visible.

- When you have optimized your sort streams, close the Waste Drawer using the button on the Side Stream window, turn off the Test Sort, and turn off the Voltage.



Final Sort Setup

Note:

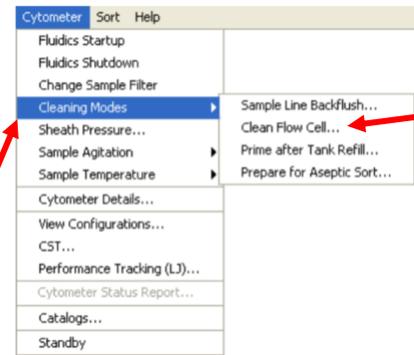
- Gates change in real time. If you adjust your sort gates in your template, they are immediately applied to the Aria.*
- When the Sweet Spot notices there is a problem with your stream, it will pause sorting, but not Acquisition. If there is a major problem with the stream, it may run through your whole sample without sorting any events. Be wary of this when the stream is unstable.*

- Load medium-containing collection tubes in to collection device.
- Insert collection device under sort block chamber.
- Make sure the instrument is sterile.
- Close the sort block chamber door, collection chamber door, the upper and outer door. Ensure aerosol containment is on. **Load** sample to start acquisition.
- To sort your sample, load your sample tube > click Voltage on > click Sort button in Sort Layout window > click OK in the dialogue that appears.
- Once complete sorting, unload sample tube > follow the AMO operation guide to clear aerosol > set unit on 100% for 2 minutes before opening the chamber, take out collection tubes.

Preparing for Next User/Shutting Down

- Load and run a tube of 10% bleach (FACSClean) for at least 10 minutes at a flow rate 5

2. Repeat with a tube of FACSRinse for at least 1 minute.
3. Repeat with a tube of DI water for at least 1 minute.
4. Turn stream off.
5. Remove the nozzle carefully.
6. Clean Flow cell with DI water as follows: Insert the closed loop nozzle. From the Cytometer menu, choose Cleaning modes, choose Clean Flow Cell. When prompted, install a tube filled with 3ml of DI water.
7. Clean your sort collection device, the lid and sample tube holder with caviside, wipe down the sort chamber, the sort drawer and the keyboard and mouse with caviside to decontaminate all surfaces.
8. **If there is a user after you, log out of the DIVA software, and leave instrument on.**
9. **If you are the last user of the day, exit the DIVA software, shut down computer, turn off the cytometer main power, and any additional components (water bath, AMO). Empty the waste container if it's over 50% of the volume.**



Data Export

- h. *Please backup your data by transferring it to the server immediately upon finishing your FACS experiment. This system is in place to ensure that FACS data for each user is backed up and is readily available for each user to access and download it from any location.*
- i. *Note that the Aria computers are not to be used to store your data. If too much data is left on the database, the DIVA software cannot function properly.*
- j. *Also, the database tends to be a fragile thing, and is prone to becoming corrupted. If this happens any data that was not backed up prior to the corruption may be inaccessible. Each user can only keep 2 experiments in the database to refer to, most should be deleted from the database after they are transferred to the Box account and make sure the data has been saved in your own devices. **Saving experimental templates will save gate structure and annotation, and will be preserved if Diva needs to be reloaded.***

1. Right-click your experiment title and go to "Export > FCS Files"
2. Browse to "C:\FACSDData"
3. Go to the Desktop
4. Open (double-click) the "DataUploader" program
5. A command terminal will open
6. Follow the on-screen instructions:
7. Leave the program to run until it closes on its own. **DO NOT CLOSE THE PROGRAM EARLY, OR TURN OFF THE COMPUTER.** Otherwise your upload will be cancelled.
8. You will receive an email from a member of the FACS Staff containing a link to your data folder on the SoM Box service. Follow the instructions to access and download your data. **This link will be used to access all of your future data.**

FACSria Scheduling Guidelines revised 20171201

Calendar URL: www.ssei.calendarhost.com

1. Please keep scheduled sorts to a maximum of 4 hours. Peak hours are Monday through Friday from 11am to 6pm. Approval must be obtained from the FACS Staff before scheduling a longer sort.
2. The calendars have a lockout system to prevent booking more than 2 weeks in advance. This is a rolling lockout, meaning that each day at 9am, the day 2 weeks in the future opens up for booking.
3. **Storm and Deadpool are reserved for emergency samples Tuesday through Friday from 1pm to 10pm.** This timeslot becomes available for booking at 9am the day before when sorting for primary tumor samples coming from surgery or emergency pup sorts. If the timeslot does not get taken for emergency sorts, it may be booked for regular sorts after 3pm the day before. *Storm is subject to guidelines 1 & 2 as well.*
4. Special requests for sorting outside of these guidelines must be cleared and booked through Cathy or Flow Staff. This applies both for those needing sorting slots farther than 2 weeks in advance and those needing more than 4 hours of peak hours.
5. Please update contact information when you sign up for time so that you can be contacted with instrument updates, schedule changes, etc. The system already has your email address, but it is helpful if you include the easiest way to reach you by phone (lab or cell phone).
6. Cancellations should be made on the calendar as soon as possible. Notify users by the mailing list (stemcell_facusers@lists.stanford.edu) so that the time can be used by another person, especially if the cancellation is less than 24 hours ahead of time. If necessary, please also contact Cathy Crumpton (crumpton@stanford.edu or 408-499-8620), so that other arrangements can be made for shutting down instruments or rescheduling.