

STANDARD OPERATING PROCEDURE FOR THE LSR II

All users must follow the SOP & fill in the logbook.

If you are unsure about anything please do not hesitate to ask for help.

Before running samples you should complete the FACS Analysis safety form, have it signed off by your PI, and submit it to the Facility manager for approval.

If you are running potentially biohazardous samples jump to the end of the SOP for details of how to alter the working procedure.

Start up Procedure

1. Check that there is enough sheath fluid and that the waste tank is empty. If you are running potentially bio hazardous samples add bleach to the waste tank.
2. The machine has been rewired such that when you switch on the LSR II the Facs Flow Supply System (FFSS) automatically switches on. This is to protect the LSR II from being run dry. For this to work the Green power button on the FFSS needs to be always left in the on position. I have taped it down. Please do not touch it.
3. Switch on the LSR II (green rectangular button). Make sure the cytometer is in standby mode. Prime the FFSS system by pressing and holding down the green square button for 10 seconds (labeled Pump 1 on the FFSS the system)
4. Make sure there is a tube containing 3ml dH₂O on the SIP
5. Now bleed any air out of the sheath filter. Place a small beaker or blue tissue paper under the valve to catch facsflow. Roll the wheel on the pinchcock back and forth. Air bubbles plus facsflow will bleed out into a small beaker.
6. The LSR II must be primed by depressing the PRIME button. Do this twice.

Allow 30 minutes for the lasers to warm up if you are the first user of the day. I recommend running distilled water through the system for 20 minutes during this warm up period.

FLUIDICS CONTROL

Like the Calibur the machine has three flow rates:

Low 12ul/minute
Medium 30ul/minute
High 60ul/minute

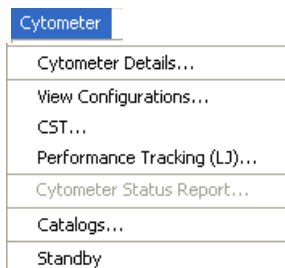


In addition there is a fine control knob.

Usually it is not necessary to move it. If you wish to use it start by return it to the off position turn it anticlockwise as far as it will go. Now if you make five rotations clockwise this doubles the flow rate – for example:

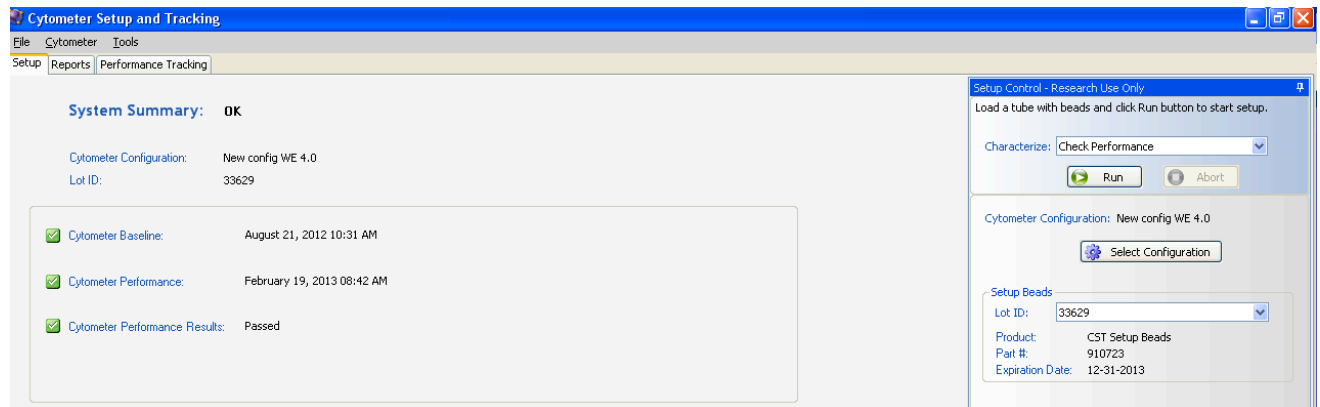
Low 24ul/minute
Medium 60ul/minute
High 120ul/minute

7. Switch on the PC.
8. The initial password to get into windows is BDIS. Open Diva -click on the icon on the desktop. Each user has an account. Enter your password to log on. Wait for DIVA to connect. Only then open CS&T (from the cytometer menu)



CS & T Daily performance check

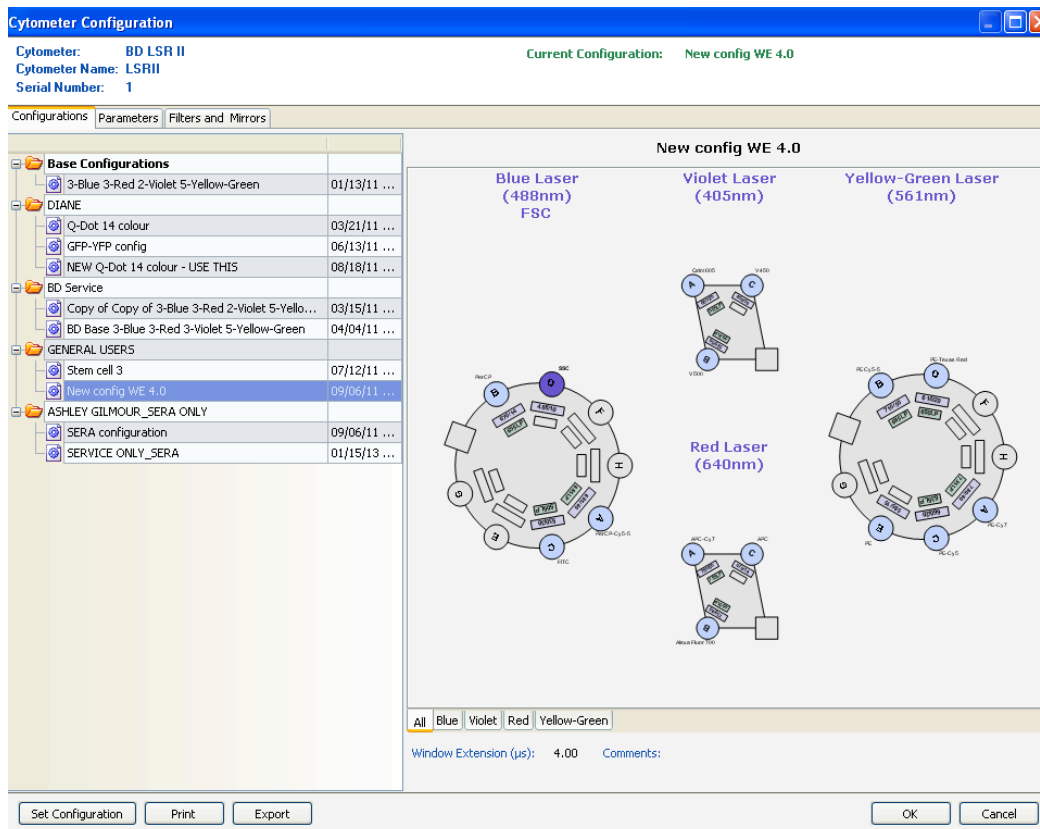
The facility manager, or first user should run this on a daily basis. When the CS&T software opens the FACSDiva programme disconnects from the cytometer this takes a few minutes. Check the configuration. Everybody except Pfizer study staff should work on the default config NEW CONFIG WE 4.0, and run CS&T on this.



Select

Characterize:


When you come to the machine if the current configuration is the SERA config. Click *Select Configuration*. This screen opens up:



Go to the folder entitled General Users and highlight New Config WE4.0) Then click Set Configuration, then OK

Check the current bead lot we are using and ensure you take the correct beads from the fridge. *The lot number of the beads is NOT the number on the box – but is on the side of the bead bottle.*

Prepare Beads: 1 drop of CS&T beads in 350ul of fresh sheath fluid.
Critical Step – Run the CST beads on LOW flow rate.

If the machine passes the CS&T with warnings  Let the FACS manager know ASAP. You may proceed with your experiment.

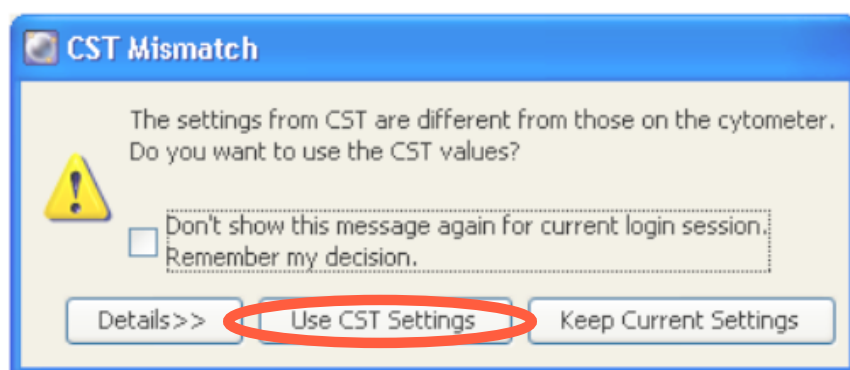
If the machine fails the CS&T - **X**, inform the FACS manager. Don't run your samples until the issue is resolved.

CS&T only needs to be run once each day provided the machine passes. You do *not* need to run it again if you change from the SERA config to New Config WE4.0

Put a cap on the beads, label and date them. Store in the fridge for next day.

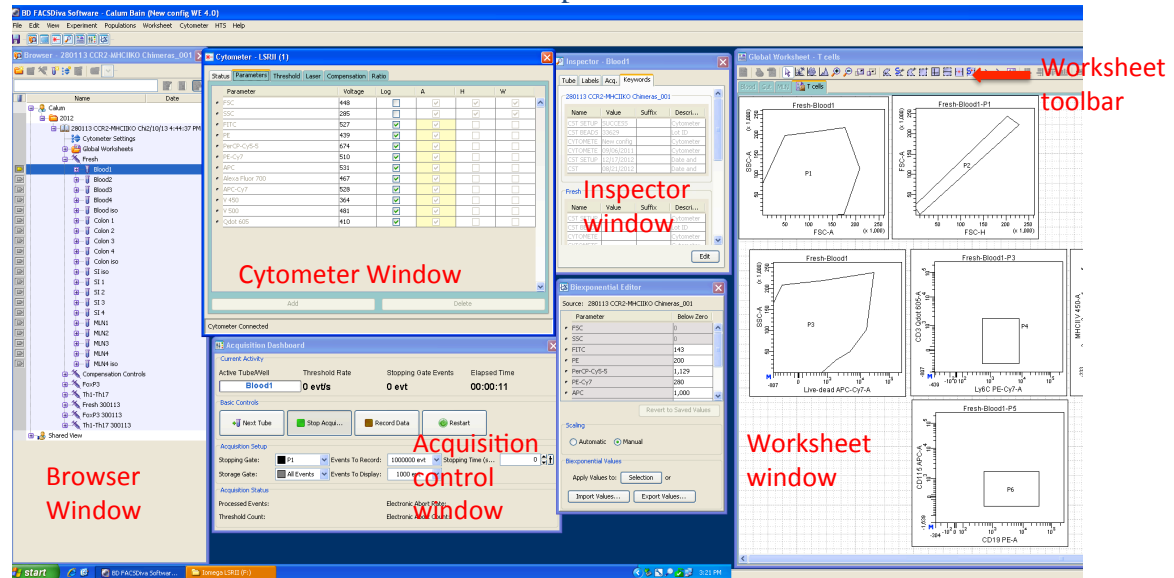
To exit the CS&T programme **DO NOT** Click on the cross in the red box but go to File on the top menu and select exit.

CS&T closes and Diva opens. When this box pops up click “Use CS&T”



SETTING UP TO RUN YOUR SAMPLES

This is an overview of the Diva workspace:



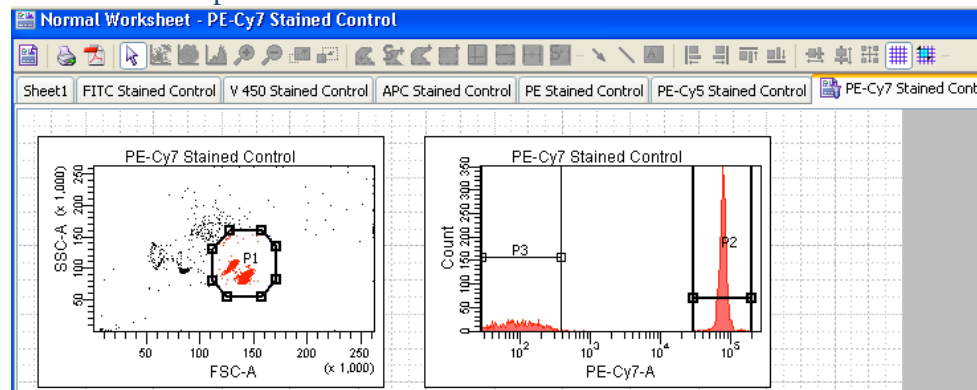
Worksheet Window

This is where you create the plots and stats for your experiment.

There are two types of worksheet in Diva:

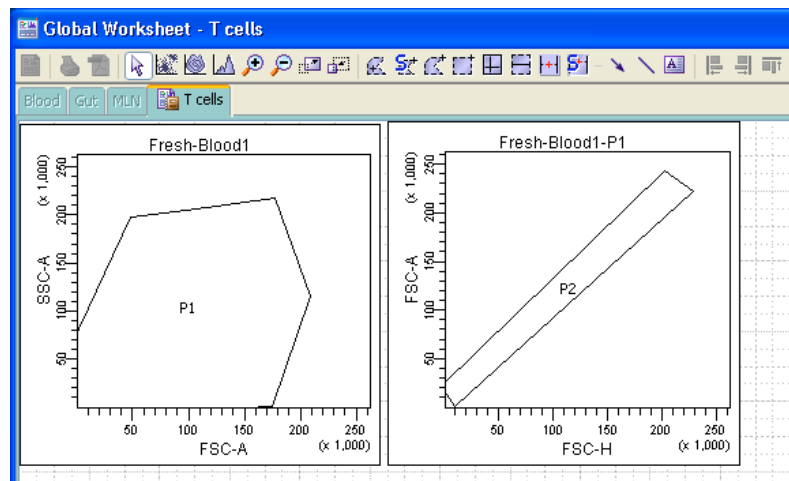
Normal worksheet: grey tabs – each normal worksheet is specific for one tube.

Used for compensation controls.



Global worksheet: turquoise tabs - allow you to apply the same analysis

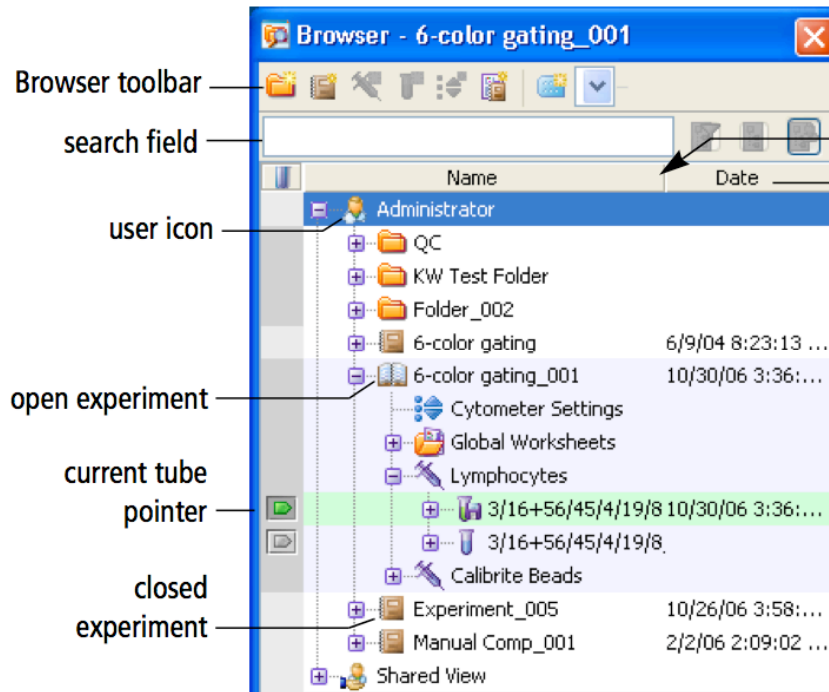
gates/stats successively to many sample tubes.





If you alter the gates on the global worksheet when looking at a particular sample these new gates are then applied to all samples. Get around this by creating more than one global one worksheet and copying your plots across, then work with the new gates.

The **Browser Window** lists all the experiments in the database:



Either create a new experiment, or use a previously saved template, or open an existing experiment and “duplicate without data”. This option carries across your template and all instrument settings into the new experiment.

To create a new Folder click this icon:  Right click to rename.

To create a new experiment in the folder click this icon:  Right click to rename.

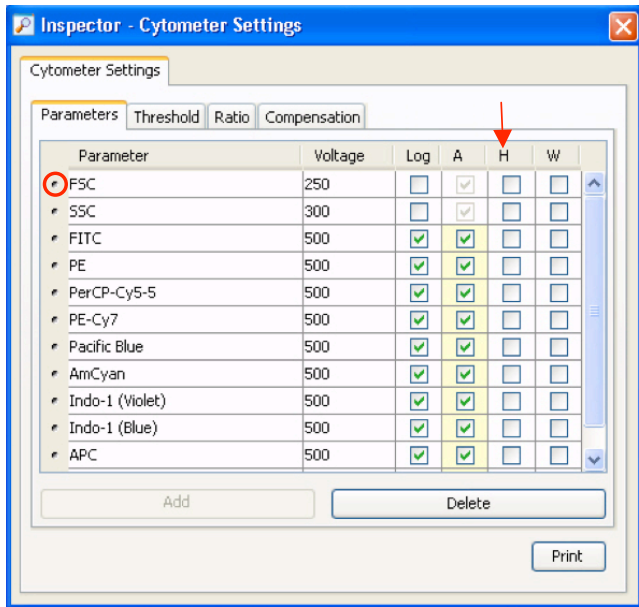
Double clicking on the experiment icon opens (book open) or closes it.

Cytometer Window

There are 6 Tabs in this window

Click on the **Parameters Tab**:

- Delete unwanted fluorochrome parameters.
- Check that Lin mode is selected for FSC & SSC and Log mode for all fluorescence detectors.
- Tick FSC-A, FSC-H, FSC-W to allow doublet discrimination plots to be drawn.

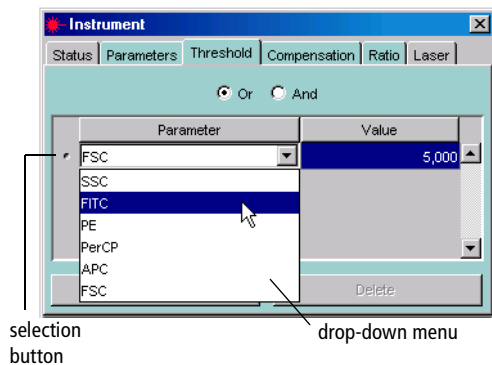


Click on the **Threshold Tab**:

Threshold values can be adjusted from 200 - 250,00 on the LSR II.

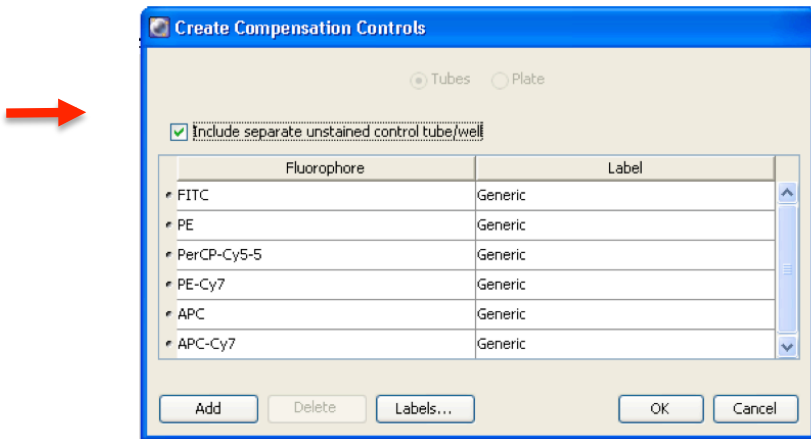
The typical default is a threshold of 5,000 on FSC.

Set the threshold on SSC if working with bacteria, or the appropriate fluorescence channel e.g. Propidium Iodide, if running cell cycle analysis.



Click on the **Compensation tab** to set up **auto compensation**:

Experiment (top menu bar) > Compensation Setup > Create compensation controls.



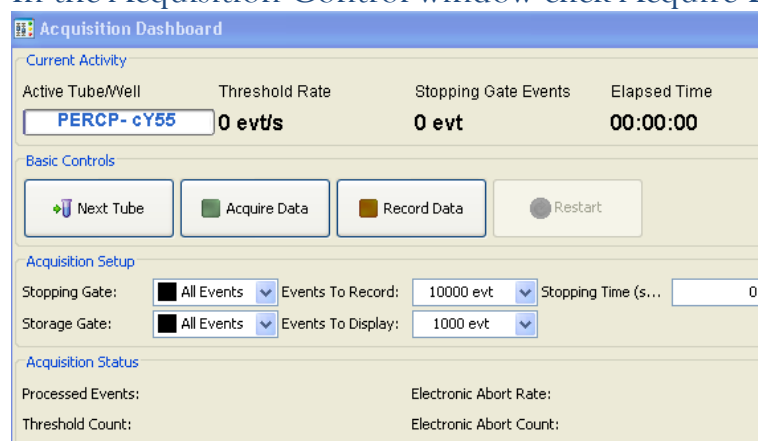
In the pop up window tick include unstained sample, and check that you have included all the fluorochromes you will use. Click OK

Compensation Controls will be inserted in your Experiment. These come with pre-defined plots on a *normal* worksheet.

Highlight the unstained Control tube in the browser (current pointer arrow must be green)

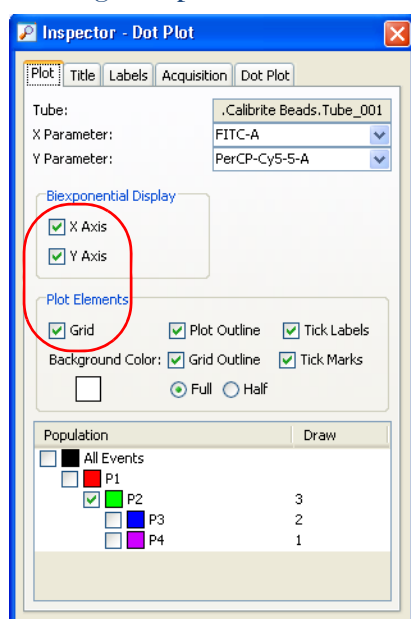
Load the unstained LSR control sample onto the SIP and press the run button on front of the LSRII.

In the Acquisition Control window click Acquire Data:

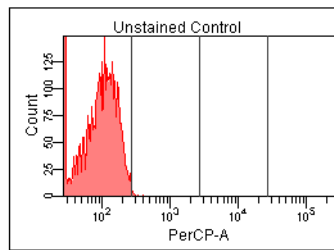
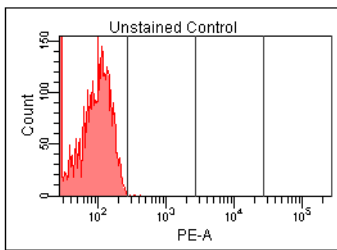


Adjust FSC and SSC voltages in the Parameters Tab to bring the population of interest onto scale.

Enable Biexponential display on the x-axis of the histograms by right clicking the plot and checking the boxes in the INSPECTOR WINDOW.

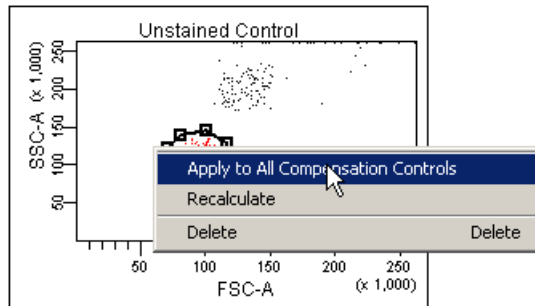


To adjust for auto fluorescence place the unstained peaks in the first decade of the histogram by adjusting the fluorescence channel voltages if necessary.



Move gate P1 onto the cells of interest.



Right click on this gate and select “apply to all compensation controls”



NOW run all single controls on the *unstained* worksheet without recording – to check that all the events are on scale, and that each stain is brightest in its own channel. If you need to adjust the voltages DO SO NOW.

You are strongly recommended to add negative cells to each single colour control tube – this allows you to check that you have both your negative & positive signals on scale, and well separated.

Acquire and record 5,000 events for the unstained sample. You must click

 for data to be saved. A floppy disc appears next to the tube in the browser:  when data has been recorded.

After recording, click  the worksheet for the next single pops up. Load your tube, acquire and record.

Repeat this for all control tubes. Run *all* the single samples and compensate in the same manner – remembering to alter the axes on the dot plots accordingly.

NOTE: If you change the voltage of any fluorescence detector during compensation you must check the compensation on ALL previously run single stained tubes again and recompensate as necessary.

When all controls have been recorded click:

Experiment (top menu bar) > Compensation > Calculate Compensation.

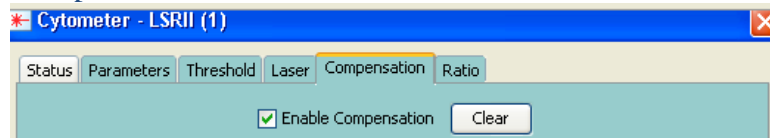
The experiment will be compensated. In the pop up box click Apply Only.

Do not alter any fluorescence detector voltages now or your compensation will be invalid. (you may alter FSC/SSC)

In the CYTOMETER window click the *compensation* tab and check that “Enable Compensation” is ticked.

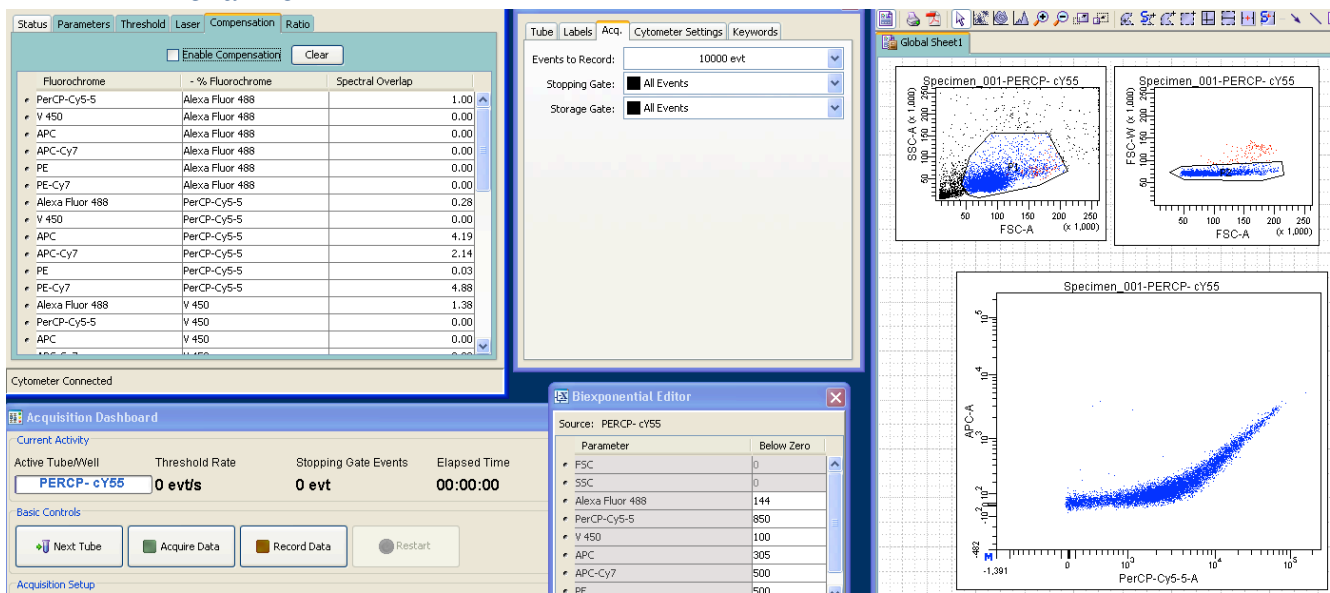
Manually Tweaking Auto Compensation

Open a global worksheet. Acquire and record each of your single controls. Now click on the compensation tab. Make sure ‘enable compensation’ is ticked

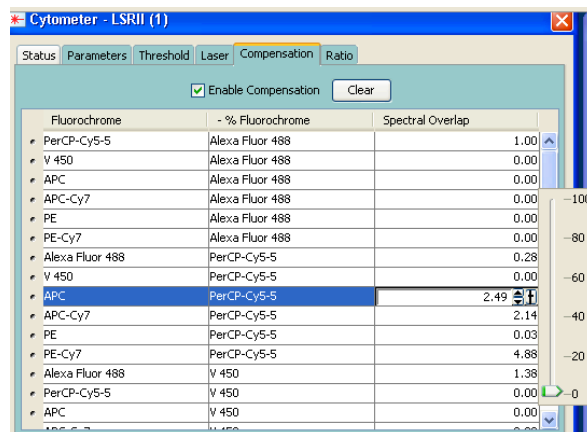


Draw a dot plot and view each single control (plotted on the X-axis) against every other colour sequentially on the y-axis.

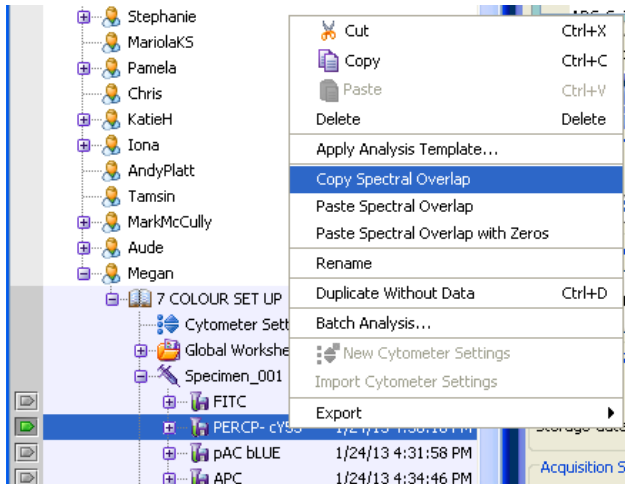
In this case you can clearly see that more compensation has to be applied manually to take the PerCP-Cy5.5 signal out of the APC channel



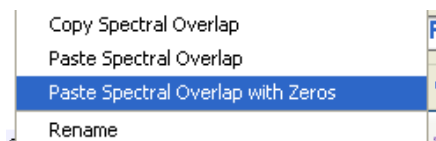
Apply compensation by clicking on the arrows or the slider:



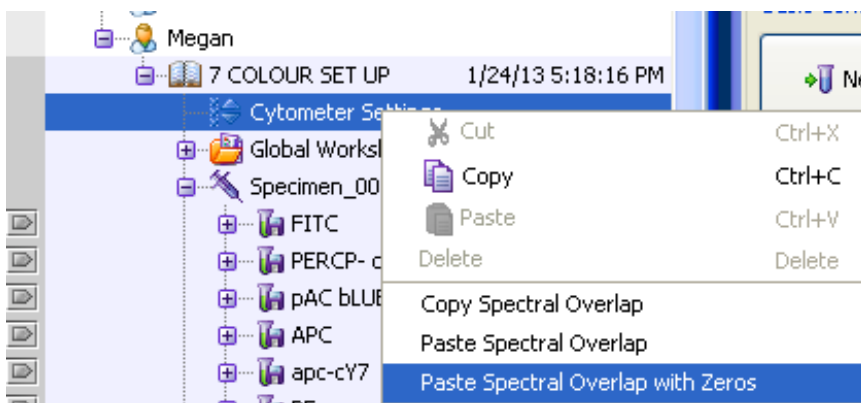
When the sample has been compensated in all the required parameters it is necessary to copy the compensation for this tube onto the next single control tube. Right click on the highlighted tube and select copy spectral overlap:



Highlight the next tube and paste the compensation values onto it by pasting spectral overlap **with zeros**



Repeat this process for every compensation tube tweaking the compensation if needed and copying the spectral overlap across to each subsequent tube. When the final tube has been compensated copy the spectral overlap from this and paste with zeros into the *cytometer settings* for the whole experiment.



You are now ready to run your experimental samples.

In the Browser:

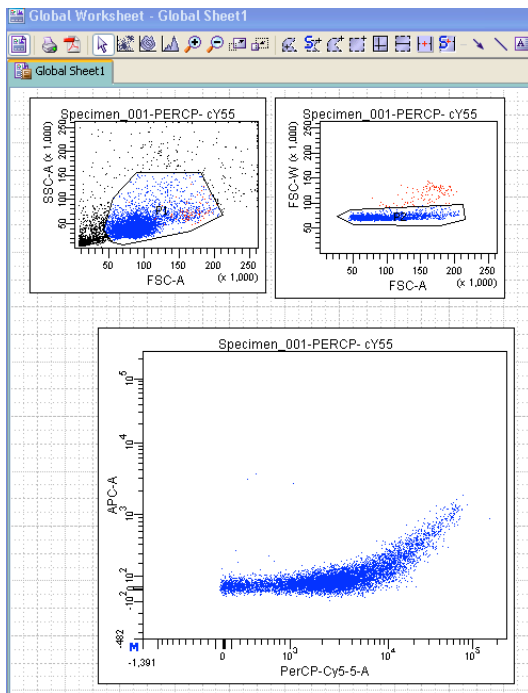
Open new Specimen:

Click  or Experiment > New Specimen

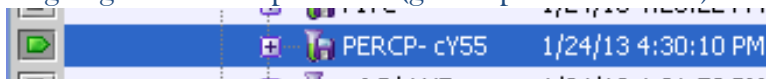
Rename Specimen

Open tube 001(double click) and rename.

On the **Worksheet window** click this icon  to change to a global worksheet.



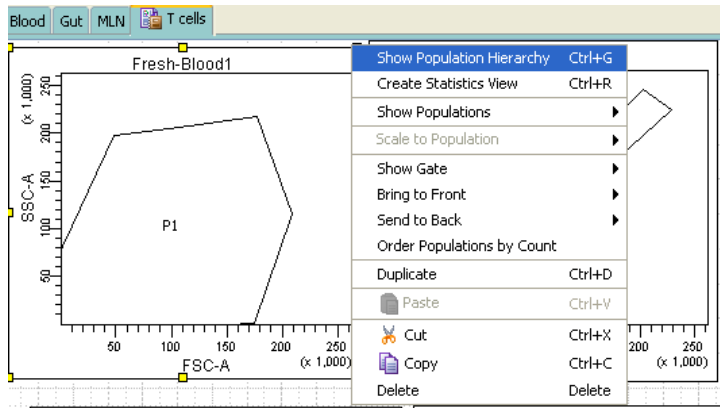
Highlight the sample tube (green pointer arrow)



Now create your Plots – Typically FSC vs SSC, then FSC-H vs FSC-A plot for doublet discrimination. Live/Dead discrimination. Create further plots as required.

Right click on the first plot and from the drop down menu select

Show Population Hierarchy:



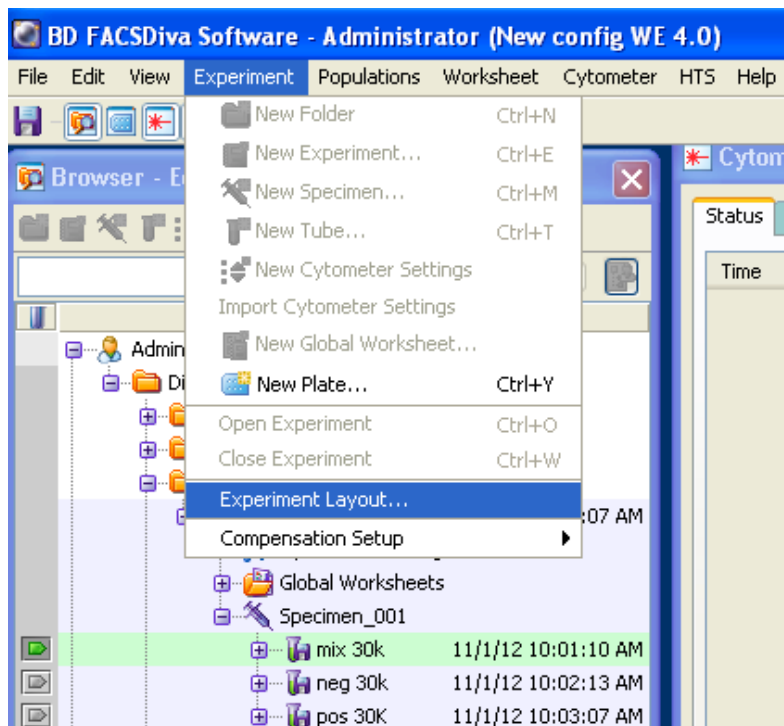
The population hierarchy box shows the gating logic applied and some basic stats.

Tube: Vuk CCL19-PE

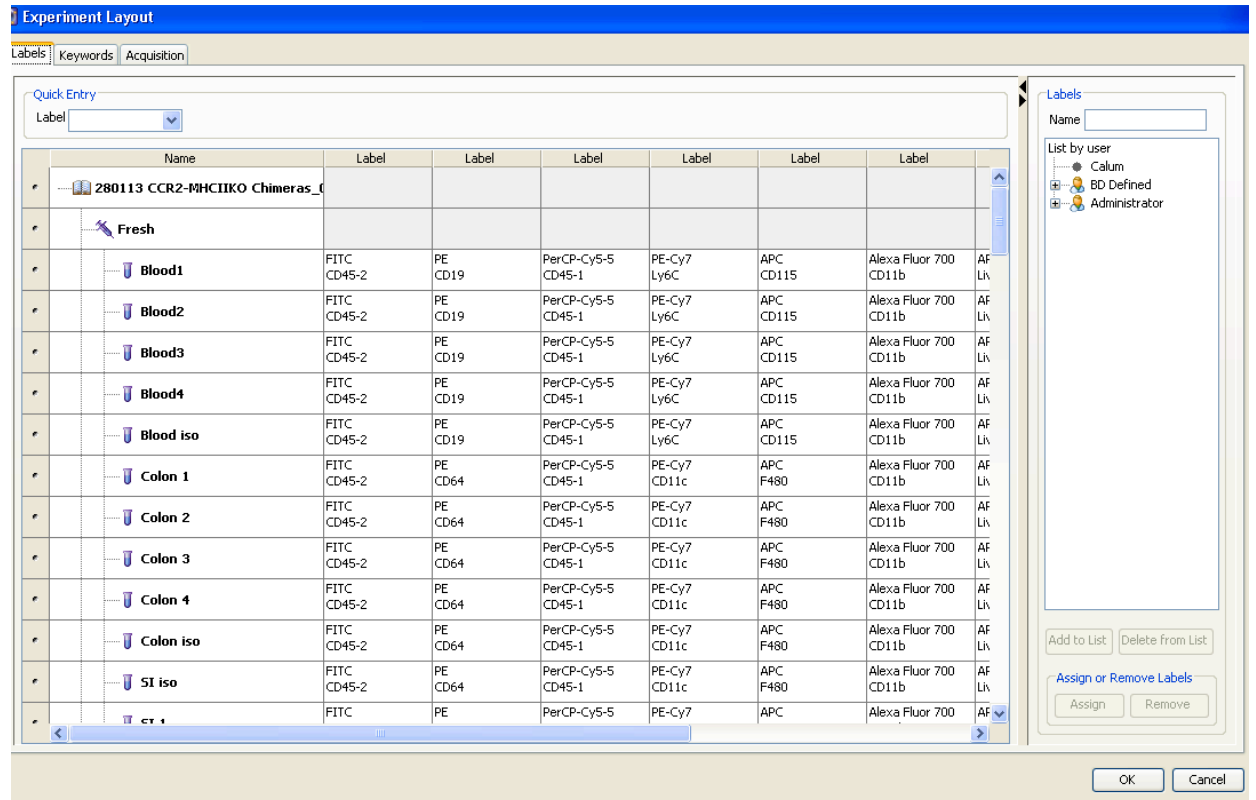
Population	#Events	%Parent	%Total
All Events	1,003,447	###	100.0
■ P1	609,717	60.8	60.8
■ P2	9,830	1.6	1.0
■ P3	2,076	21.1	0.2
■ P4	310	3.2	0.0

If you wish to alter the labels on your plots from generic to specific use the experiment layout feature:

Experiment (top menu bar) > Experiment layout.



The Experiment Layout window opens up:



Click on each box and type in the marker name.

Right clicking on the box allows you to copy and paste labels onto other samples.

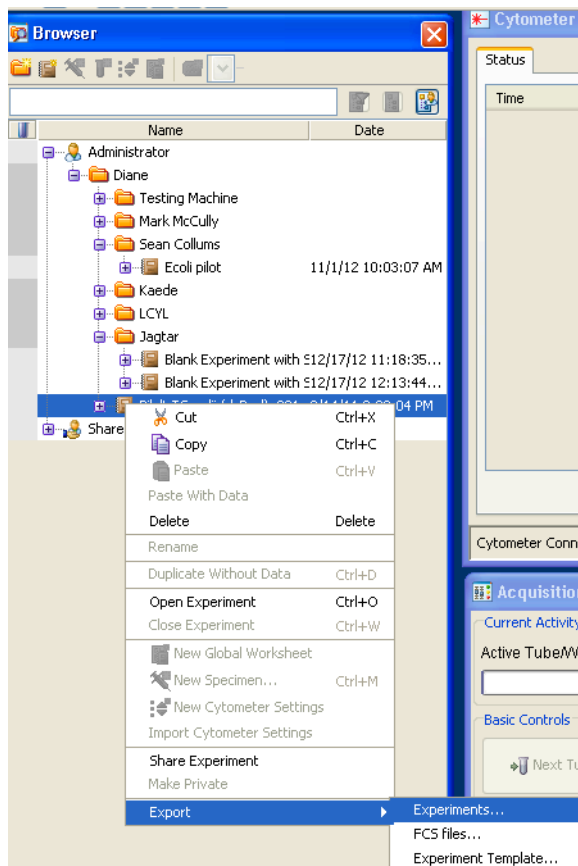
In the Acquisition Dashboard set the number of events to record, stopping gates etc.

Now run your samples & remember to record them.

When you have finished close the experiment export your FCS files.

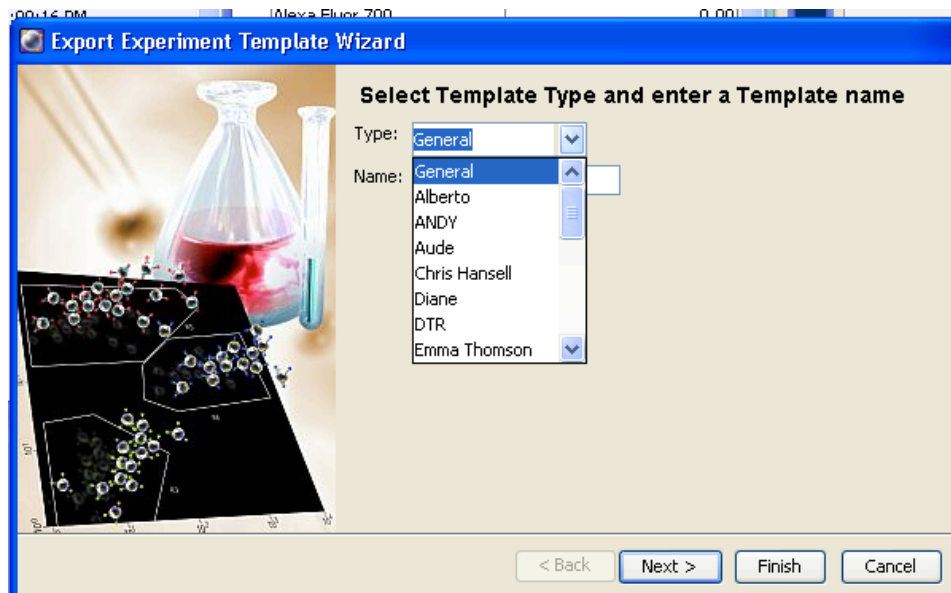
To Export Your Data

Right click on the experiment in the browser and click Export.
There are 3 options:



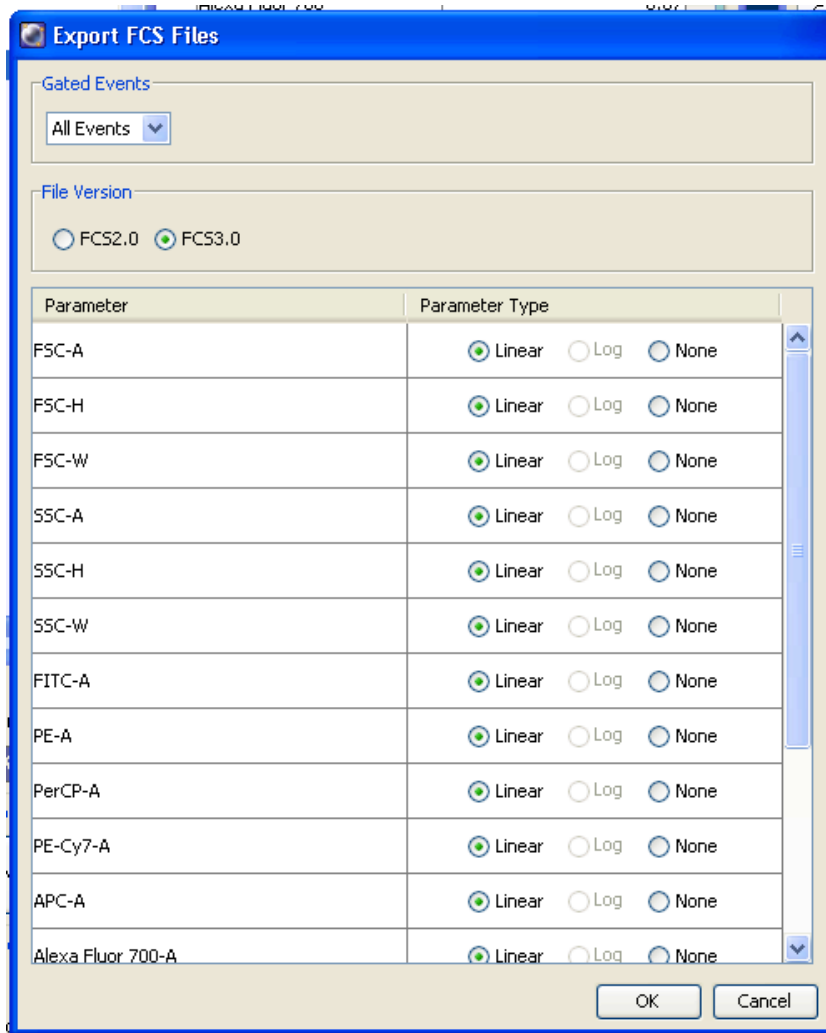
- 1) Export > Experiment: Lets you save the entire experiment with all settings. This allows you to reload the whole experiment into the database if needed. Click on the pull down menu arrow to specify which external drive to save it to.

2) Export > Templates



3) Export > FCS data

When the following window opens up select FCS 3.0 and then Linear for each parameter. Click OK .You will be asked where you wish to save the data. Click browse, and select the appropriate location.



Experimental Setup & Data Storage

⚠ Do not keep opening the same experiment & adding specimens to it. This results in enormous experiments, which hog space on the hard drive.

All users should create a new experiment each time. You have 3 options.

- i) Make a completely new experiment from scratch.
- ii) You may use your optimized instrument settings by duplicating an existing experiment

Open the old experiment in the browser window. Right click and select Duplicate without data.

- iii) You can open a pre saved experiment template.

Data Management Policy



Because the LSRII acquires many parameters, and is heavily used the hard drive fills up fast. When the hard drive is full samples cannot be acquired.

Within 48 hours after acquisition all users should back up and check their data files have transferred correctly, then return and delete the FCS files from the hard drive.

You may leave the FCS files on the machine for up to two weeks.

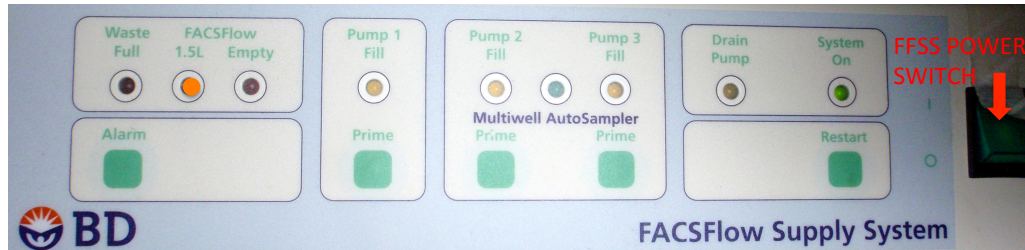
Since it can takes ages to export large data files the machine will be available on Monday mornings from 8am-12 noon to allow users to backup and delete data not backed up immediately after acquisition.


I shall delete ALL files, which are older than two weeks, or more frequently if the hard drive is full. You have been warned!

Changing The Tanks Mid Experiment

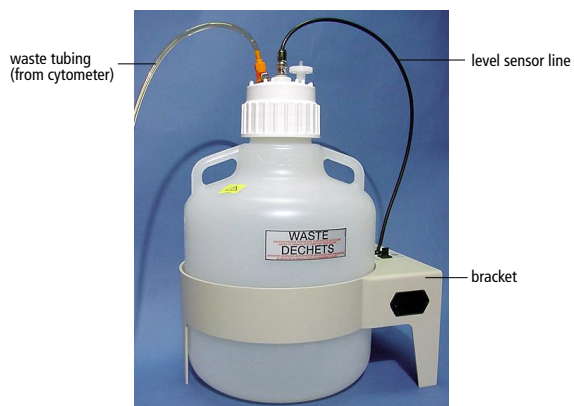
Sheath Tank

When there is only 1.5litres of sheath fluid left the orange warning light flashes on the FFSS - the problem is that this is not easily visible under the table.




The sheath alarm sounds when the sheath tank is dry. Best to always check there is enough sheath fluid before commencing work. To refill the sheath tank make sure the LSR II is in  mode. To mute the alarm, press the green square button on the FFSS.

Quickly replace the sheath fluid. The FFSS still registers that the tank is empty. In order to reset it switch off the FFSS power switch for a couple of seconds. After switching it back on immediately press the prime Pump 1 button.



An alarm will sound if you need to empty the waste tank during an experiment.

To empty the waste, disconnect both the lines going into the lid before unscrewing the lid. After emptying the tank in the sink replace the lid and reconnect both the lines.

 All users must wear the safety specs provided in the FACS lab when emptying the waste tank.

Cleaning between users



Large Experiments: If you are running many samples, after every 30th sample pause and clean to avoid blockages before resuming your experiment.

If you are running unfixed human cells you must decontaminate the machine by running FACSClean through it during the cleaning procedure.

1. Set the fluid control to RUN HI, install a tube containing 3ml neat Coulter Clenz on the SIP with the support arm to the side (vacuum is on aspirating to waste), and let it run until half has been aspirated.
2. Move the support arm under the tube (vacuum is off). Allow the Coulter Clenz to RUN on HI for 4 minutes.
3. If you have been running unfixed human cells repeat steps 1 & 2 using FACSClean to decontaminate the machine - otherwise go to step 4.
4. Install a tube containing 3ml of distilled water on the SIP with the support arm to the side (Vacuum is on). Let it RUN on HI until about half the water is left.
5. Move the support arm under the tube (vacuum is off). Allow the water to RUN on HI for 4 minutes.
6. Set the fluid control to STANDBY LOW
7. Leave the SIP sitting in the distilled water tube to prevent salt deposits forming and blocking the injection tube.
8. If you were *not* running human unfixed cells empty the waste. Disconnect the orange waste line, and fluid sensor from the waste.
9. If you were running human cells - add 2 bleach tablets to the waste and leave overnight to decontaminate before pouring down the designated sink.

Leave a note so subsequent users know not to empty the tank.
10. Leave the machine switched on in STANDBY mode if another user has booked to use it within the next two hours.
11. Fill in the log to indicate you have cleaned the machine and left it in STANDBY mode and sign it.

If your booking is not the last one of the day, it may become so if other users cancel. Check the online diary before leaving, to ensure have not become the last user of the day.

LSR II Shutdown Procedure (last user of the day)

1. Install a tube of Coulter Clenz on the SIP (with support arm to the side) until half has been aspirated.
2. Run Coulter Clenz on HI flow rate for 4 minutes, with support arm under the tube.
3. Install a tube of neat FACS Clean on the SIP (with support arm to the side) until half has been aspirated.
4. Run neat FACS Clean on HI flow rate for 4 minutes, with support arm under the tube.
5. Run FACS Rinse on HI flow rate for 3 minutes
6. Run distilled water on HI flow rate for 3 minutes. Place a tube containing 1ml of distilled water on the SIP
7. Leave the LSR II in STANDBY for 10 min to let the lasers cool down.
8. Switch off the LSR II. When you switch off the LSR II the FFSS will automatically switch off.
9. If no one has run biohazardous samples empty the waste.
10. Replace the box of FACSFlow if nearly empty and sign off the log book.

Additional steps required if running biohazardous samples

- Wear gloves in addition to the usual lab coat.
- If you spill any sample on the bench immediately clean with 70% ethanol or Virkon.
- Ensure that your samples are filtered good quality single cell suspensions which will not clog the machine in the first place. blockages can potentially generate aerosols.
- Priming a blocked machine can create aerosols.
- A dripping SIP could present an infection risk.
- Add bleach tablets to the waste tank and leave to decontaminate overnight before emptying the waste. Leave a note on the tank to alert subsequent users.



Remove all your rubbish

Dispose of all waste appropriately in accordance with the Facility safety rules i.e.

Return to the Flow Lab to empty decontaminated waste down the designated sink in the Flow Cytometry Facility next morning.

All pipette tips should be placed in the metal tin for autoclaving or in the disposafe sweetie jar provided.

Gloves and plates should be placed in the autoclave waste bin.

Used FACS tubes should be tidied up, and put into the autoclave waste bin.

If you drop any tips/pastettes/nitex tubes pick them up and bin them

Wipe down the bench with Virkon or 70% ethanol.