FACS Canto II

System description

3 lasers, 8 (4-2-2) color configuration:

Blue laser (488 nm)
Channels for: **PerCP** (655LP, 670LP); **PE** (556LP, 585/42); **PE-Cy7** (735LP, 780/60); **FITC** (502LP, 530/30)

Red laser (633 nm)
Channels for: **APC-Cy7** (735LP, 780/60); **APC** (660/20)

Violet laser (405 nm)
Channels for: **Am Cyan** (502LP, 510/50); **Pacific Blue** (450/50)

- Ability to analyze tubes and well plates (HTS sampler)

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Startup procedure

Prior to startup, check fluid levels and waste container. Refill tanks if necessary and empty the waste tank if it's full. Report any problems observed, especially when the previous user didn't empty the waste or performed fluidics shutdown.

IMPORTANT

If you use the HTS unit, you have to do a fluidics shutdown at the end of your session with the HTS sampler connected

Startup

1. Open the pressure valve
2. If using HTS sampler, connect it to the sample line before startup
   If manually loading tubes, make sure that the HTS sampler line is disconnected
3. Turn on the computer and log in (password: BDIS)
4. Wait for HyperTerminal window to pop up
5. Turn on the instrument main power (green button)
6. When the system is booted up completely, open BD FACSDiva software
7. Log in with your username.
8. Whenever it's asking about CST settings, always select '*Use CST settings*'
9. Choose *Cytometer* → *Fluidics Startup* (it can take up to 7 min)
   IMPORTANT: During the Startup, check bubble filters – if there are any bubbles in there, degas them, otherwise you'll encounter instrument errors later on! To de-air the filters, open the upper vent caps until liquid is coming out. Repeat it for all filters.
10. Check if laser warmup has finished.
    When laser warmup is complete, a message at the bottom of the instrument frame appears "The system is ready".
11. Perform the CS&T check
    11.1. Press *Cytometer* → *CST* to exit Diva and enter the CS&T software
    11.2. Put one drop of CS&T beads (in the fridge, orange bottle) in 350 µl of PBS and vortex.
    11.3. Check that the Lot No. on the bottle matches the LOT No. in the software
    11.4. Select *Performance check* → *Run*
    11.5. CS&T check should pass without warnings. If not, please tell us immediately.
    11.6. Close CS&T software and reconnect to Diva

CS&T check guarantees that that machine is working properly and that the base voltages are adjusted to counterbalance machine ageing. Cytometry data are not reliable if CS&T check didn't pass or if it passed with warnings in the channels you intend to analyze.

If you want to compare fluorescence intensity results of different experiments and want to make sure they were acquired with the same settings, use application settings.

Exchanging/refilling fluid tanks during operation

If the instrument is running low on sheath fluid, you have to exchange the tank during your experiment. Do not refill sheath tank in middle of experiment because of possible air bubble formation. You can, however, refill the FACS Clean and Shutdown solution tanks – bubbles don't matter there too much.

1. Set the cytometer to standby mode first: *Cytometer → Standby*
2. Refill or exchange the empty tank, empty the waste tank if necessary
3. Reconnect the instrument with *Cytometer → Connect*
4. Prime the tank(s) with *Cytometer → Cleaning modes → Prime after tank refill*
   In the pop-up window, select the appropriate tanks. Don't forget to de-bubble the corresponding fluid filters!
5. Remove additional air bubbles with *Cytometer → Cleaning modes → Bubble filter purge and De-gas flow cell* (repeat this command 2-3 times)

Shutdown procedure

!!! If you analyze bacteria OR use live/dead staining (eg: DAPI, PI, 7-AAD, Zombie dyes), it's absolutely necessary to clean longer (3 x 10 min!!!) in order to prevent machine damage.

!!! If analyzing bacteria, it's necessary to do long clean after the 3 cleaning tubes:
Cytometer → Cleaning Modes → Long clean !!!
Long clean already includes the shutdown procedure, no need to do it afterwards.

Shutdown after acquiring manually loaded tubes

1. Remove sample tube if one is installed on the cytometer.
2. After finishing your samples and exporting your data, create 3 new tubes into your experiment folder, name them "clean", "rinse" and "water", set stopping gate to all events and events to record to 10'000'000. Set flow rate to HIGH and Stopping time to 5 minutes (300 sec).
3. Install a tube with 3ml BD FACS Clean on the SIT and click "RECORD Data" in the Acquisition Dashboard frame.
4. Repeat step 2 with FACS Rinse and then with MilliQ water, make sure to record all 3 cleaning tubes separately to prove that you actually performed the whole procedure.
5. When finished, choose *Cytometer →Fluidics Shutdown*, wait until it finishes
6. Click *OK* when a message informing you that the system can be turned off.
7. Turn off the system with the main switch.
8. Close the pressure valve.

9. Quit BD FACSDiva software and shut down the computer.
10. Empty the waste and refill all fluids tanks if empty.

Shutdown after using the HTS sampler

1. Standard HTS Clean: Prepare a wash plate: pipette 8 wells of 250µl FACS clean, 8 wells of 250 µl FACS Rinse and 8 wells of 250 µl MilliQ H_2O and install it on the HTS
2. Press *HTS →Clean*, a "Plate Template" window will pop up. Press *OK.*
3. A "*daily clean*" plate will be created in your Experiment folder, run the wash plate with the appropriate wells filled with the corresponding cleaning fluids. Make sure the cytometer lids are closed during operation, otherwise the cleaning procedure will fail.
4. Place a paper towel under the sampling coupler (see picture) before fluidics shutdown, which has to be removed once the shutdown procedure is finished. This is to prevent bleach from dripping into the system and also to monitor the tightness of the connector. If you observe any droplets on the towel afterwards, please inform a member of the BIF so that we can immediately act upon it to prevent further instrument damage.
5. Continue with the standard fluidics shutdown procedure: *Cytometer → Fluidics Shutdown*, leave the HTS connector attached

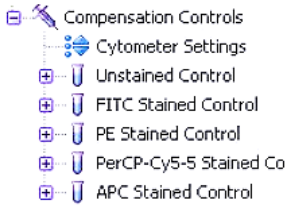
Please make sure to place the tissue in a way that it does not interfere with the sampling needle of the HTS

6. Turn off the software, instrument, PC as described above, empty the waste container and refill empty tanks.

Compensation

Select *Experiment* > *Compensation Setup* > *Create Compensation Controls*.

The Create Compensation Controls dialog opens, listing only those parameters previously defined in the cytometer settings. Click OK to add the specified controls.

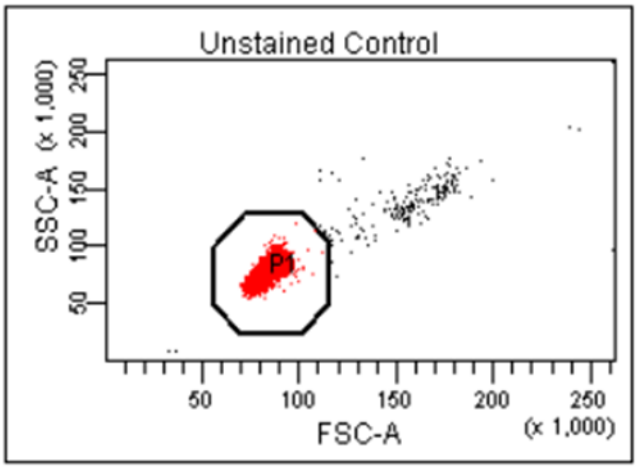


A new specimen with the compensation control tubes appears within the experiment in the browser window.

The unstained control will be used to verify the settings for FSC, SSC, and FSC threshold, and to gate the population of interest.

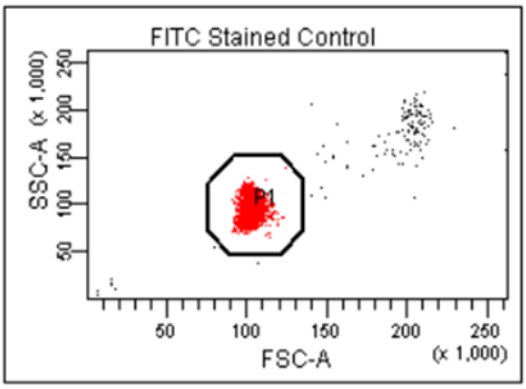
Compensation step-by-step

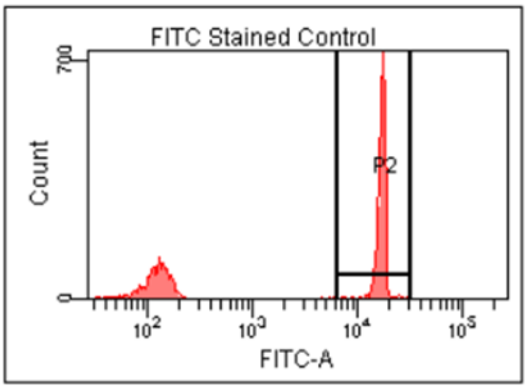
1. Install the unstained control tube onto the cytometer loading arm.
2. Expand the compensation specimen in the Browser.
3. Set the current tube pointer to the unstained control tube and click Load.
4. Verify that the population of interest is displayed appropriately on the FSC vs SSC plot. Adjust if needed. Click the Threshold tab and adjust the FSC threshold, if needed. Set the threshold to remove most of the debris without cutting off the singlet population.
5. Adjust the P1 gate to surround only the singlets.



6. Click *Record Data*. When recording is finished, click Unload and remove the unstained control tube from the cytometer.
7. Install the next tube onto the cytometer and repeat steps 5 and 6 until data for all stained control tubes has been recorded.

8. Once done with all tubes, double-click the single stained control tubes to display the corresponding worksheets.
9. Verify that the snap-to interval gate encompasses the positive population. Adjust the gates, if needed. Repeat for the remaining compensation tubes.





10. If all compensation controls look fine and the gates are set correctly, calculate the compensation matrix: Select *Experiment* > *Compensation Setup* > *Calculate Compensation*.
11. Enter the name of your experiment as the setup name, then click Link & Save.

IMPORTANT: Do not change the PMT voltages after the first compensation control has been recorded. In order to calculate compensation, all controls must be recorded with the same PMT voltage settings. If you need to adjust the PMT voltage for a subsequent compensation control, you will need to record all compensation controls again.

Doublet discrimination

This section describes how to adjust the gates to eliminate doublets and record singlet events.

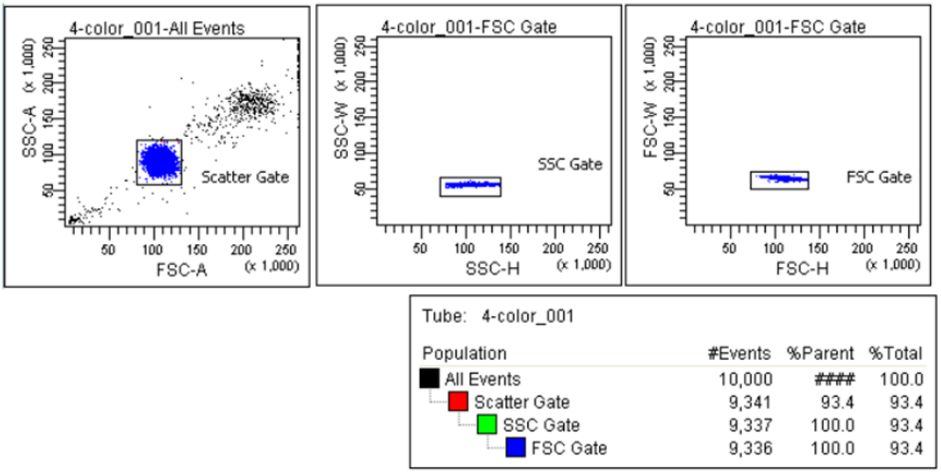
Always perform doublet discrimination before sorting!

If you had to compensate, make sure to switch back to the Global Worksheet before proceeding.

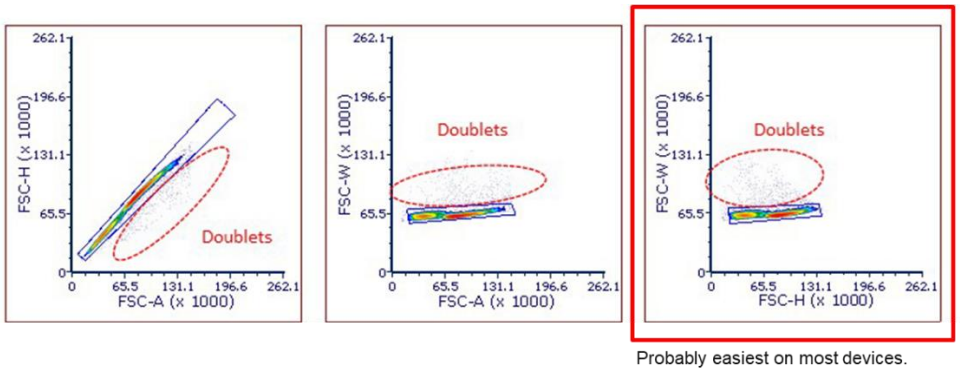
Use FSC based discrimination for eukaryotic cells, eventually combine with SSC based for complex samples.

Use SSC based discrimination for small particles, like bacteria.

1. Move the current tube pointer to the first sample.
2. Install a sample tube onto the loading port and click Load.
3. Change the Events to Display to 5,000 events.
4. Adjust the *Scatter gate* (or P1, whatever you call it) to encompass the singlet events.
5. Adjust the *FSC gate* to encompass the low FSC-W population. Don't forget population hierarchy! The *FSC gate* has to be a child of the Scatter gate.
6. Adjust the *SSC gate* to encompass the low SSC-W population. Don't forget population hierarchy! The *SSC gate* has to be a child of the *FSC gate* (or Scatter gate if you have no FSC gate).



There are different gating strategies to discriminate doublets from singlets:



Probably easiest on most devices.

Use the one that you feel most comfortable with.

Application settings

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 fora 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.

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

1. Start with a new blank experiment.
2. Select *Cytometer Settings* in the *Browser*.
3. Delete all parameters you will not be using:
    a) In Inspector 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, then 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. Adjust area scaling factors first, if necessary. This is a more advanced skill, see cytometry supervisor if you feel you need to adjust area scaling factors. For many (small) cells, using the CST Area scaling factors will work fine.
7. 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 and click load in the dashboard window. The tube will be loaded, and events will start acquiring automatically.
8. 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. You do not need to record a data file. Unload the unstained cells.
9. 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.
10. You now have your Application settings. To save, Right-click Cytometer Settings in the Browser, then select Application Settings>Save. Name the Application Settings appropriately and Click OK. The application settings are saved to the catalog. Application settings do not include compensation settings.

Using previously created Application Settings

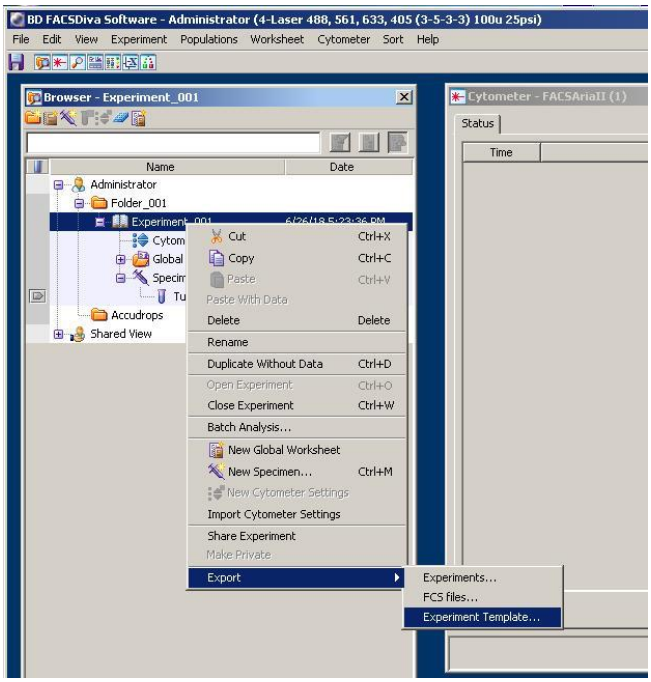
(When you are doing the same experiment again)

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.

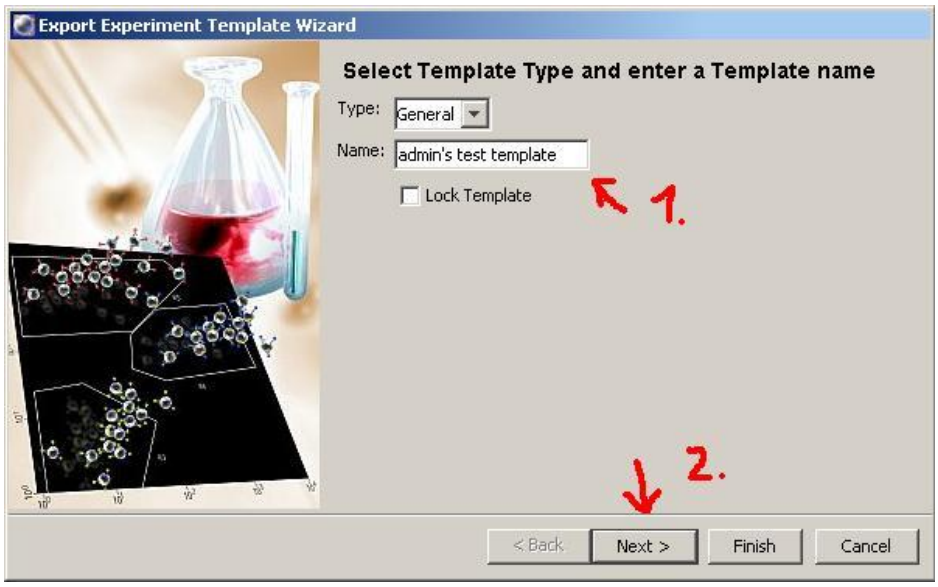
Exporting experiments as “Experiment template” in FACS DIVA

Creating an experiment template

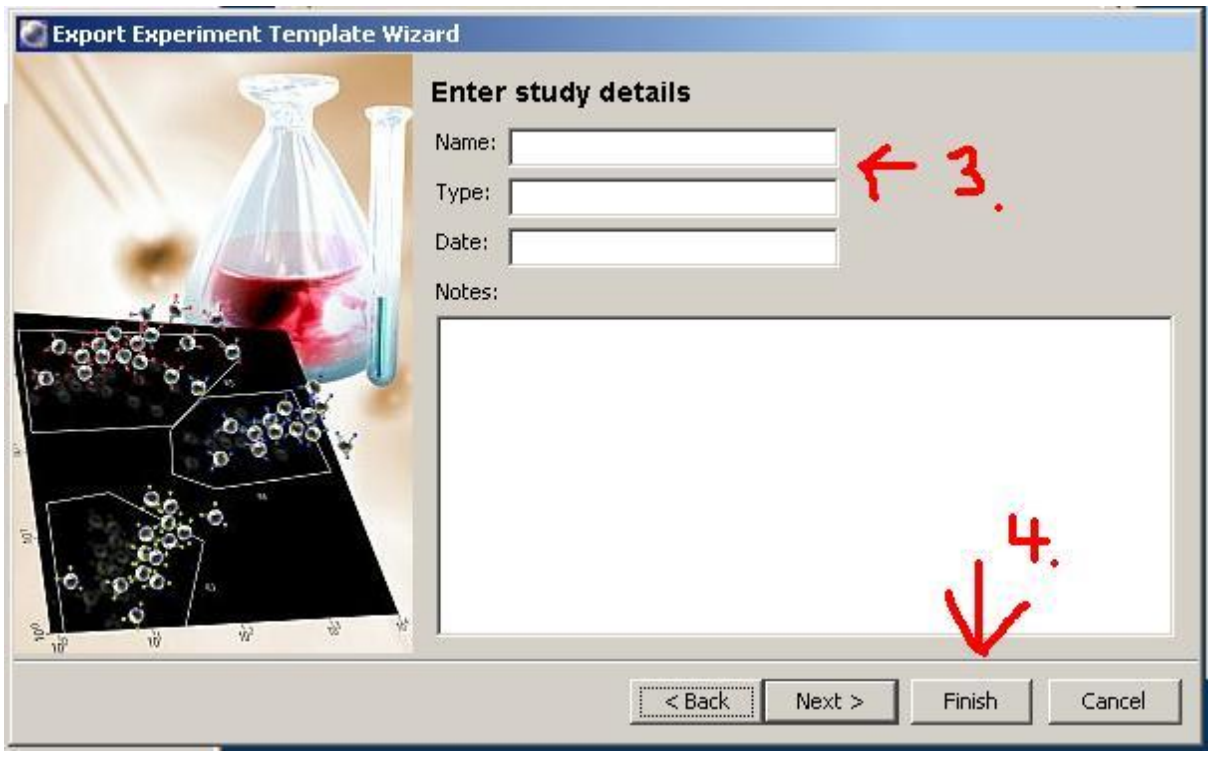
Step 1: select & right click on the experiment you wish to re-use in the future as a template, select ‘*Export’* and then ‘*Experiment Template*’



Step 2: give it a name that you'll be able to identify in the future (template name should include your name as well!) & press '*Next*'

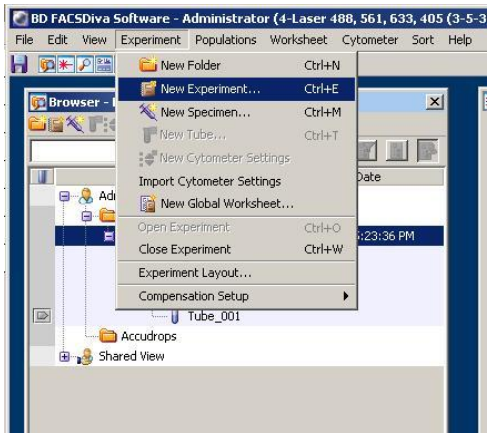


Step 3: Enter further details for your future reference (optional but highly recommended) & click '*Finish'*. Now your experiment template is saved and is safe from database maintenances!

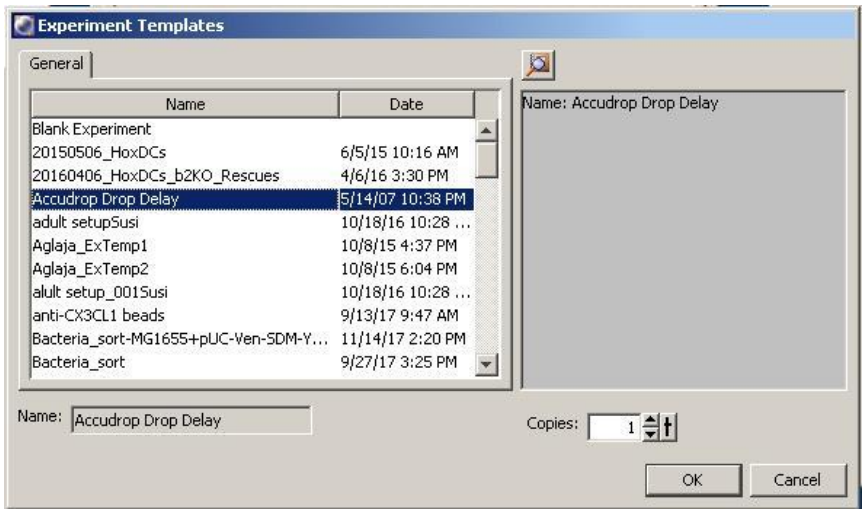


Loading an existing experiment template

Step 1: Select '*Experiment*' in the menu bar & click on '*New Experiment*'



Step 2: Select the template you need (this is why you should describe it as accurately as possible…) and press 'OK'.



References

https://www.bdbiosciences.com/documents/BD_FACSAria_III_User_Guide.pdf

https://med.stanford.edu/content/dam/sm/stemcell/documents/facs/sop/Stanford%20AriaII%20User%20Guide%2020170901.pdf

https://med.nyu.edu/research/scientific-cores-shared-resources/sites/default/files/pdf/ariaII-self-serve-quick-guide.pdf

Laser Safety Instructions

During operation of class 3B and class 4 lasers red warning lights have to be switched on manually

Red warning light at the door of the room, containing laser-based equipment, prohibits the entrance

Optical path of the laser beam at all setups has to stay intact and should never be disassembled by a user. Users are never permitted to disconnect optical connections (pipes, fibers etc), remove protective coverings or disassemble any parts of the setups, especially those parts that are labeled with laser-warning signs.

User has to make sure, that objectives mounts are blocked by objectives or light- blocking plugs, before switching the system on or starting the work

Any cleaning activities (objectives, stage cleanings) as well as changing of objectives or filters have to be performed only after blocking of the laser light is ensured. This can be ensured by closing the scanhead shutter or switching off the laser.

Laser class-specific warnings at each setup have to be observed and considered

Eye contact with direct beam of Class 3B laser, or eye contact with mirror reflection from class 3B laser, should be avoided at all times

Eye or skin contact with direct or diffuse light of Class 4 laser, should be avoided at all times

Laser safety goggles are situated at all workspaces and should be used in any situation where potential contact of eyes with Laser light of the classes 3B or 4 is possible, according to the previous two points

Laser safety goggles have to be worn at all times of operation of Laser Class 4

Laser safety goggles have to be worn at all times of operation laser Class 3 at the optogenetic setups

Laser safety goggles are assigned to each setup and matched to the corresponding laser wavelengths. Matching laser safety goggles should be used at all times, and should not be carried over between the setups

Only one person is allowed to be in the corresponding compartment during Laser Class 4 operation and optogenetic setup operation

Users are not allowed to wear any reflective objects (rings, watches etc) during laser operation

Using of the equipment is only allowed after the introduction from a laser safety officer of IST Austria. Introduction has to be done individually for each setup.

Changing experimental conditions, that involves changes in the laser application, have to be reported to the laser safety officer prior to the start of experiment

Users have to understand that any violations against the instructed rules and also withholding information leading to safety hazards will ultimately result in denial of admission to all laser equipped instruments at the IST Austria.