FACSARIA FUSION INSTRUCTIONS FOR USE **23.2.2018 Laura Kummola**

A. BEFORE TURNING ON THE INSTRUMENT

1. Check the last logbook entry for anything that could affect your run.
2. Inspect the instrument: Check that the sample line is in its holder. If the loading chamber or deflection plates are dirty, clean them.
3. Fill the FACS Flow (sheath solution) and ethanol tanks to the tank seam, if needed.
4. Detach the gas inlet (transparent tube) from the ethanol tank. Detach the fluid outlet (blue tube) from the ethanol tank filter. Attach the fluid outlet to the sheath filter. Attach the gas inlet to the sheath tank.
5. Open the air pressure lock on the wall.

B. BASIC STARTUP PROCEDURE

1. Turn on the computer. Select Admin account. The password is BDIS#1. Wait until the computer has finished loading.
2. Turn on the FACSAria Fusion.
3. Open up the FACSDiva software and wait until the cytometer has finished connecting.
4. Whenever a CST mismatch window appears, click *use CST settings*.
5. From the toolbar, select *Cytometer -> Fluidics Startup*. Follow the instructions carefully one by one. Optional: clean the nozzle with water before putting it in place. Check that it is dry before inserting! Do not wipe or touch the red o-ring as it is very sensitive!
6. Check that the configuration matches the current filters and lasers. If not, select *Cytometer -> View configuration* and select the one you want, click *Set configuration*.
7. Turn on the stream and see that it enters the waste directly and there is no funkiness. If something is wrong, clean the nozzle and reinsert it.

CST check (once in 24 hours)

8. Before proceeding to the CST check, wait until lasers have finished warming up (takes 20-30 min from turning on the power).
9. From the toolbar, select *Cytometer -> CST*. Cytometer disconnects, opens up the CST view and connects again.
10. Prepare the CST-beads: add one drop of beads (shake the bottle well, do not vortex) into 350 µl of PBS. You can (and should) now vortex the beads in the tube.
11. Check that the bead bottle number matches the lot number on the screen. Check that from the drop-down menu the option *Check performance* is chosen. Click run and insert the tube when prompted. Close the (upper) grey cytometer door!
12. Verify that the CST report is adequate. If failed or passed with warnings, cleaning the flow cell is likely to help.
13. For sorting experiments: check if the stream is now stable. If not, adjust the amplitude and frequency. Wait for 5-10 minutes. Write the values to the white boxes and put on *Sweet spot* if stable. For 100 µm nozzle the optimal gap is 11-12. Frequency should usually be around 30. Drop 1 should remain stable.
14. Proceed to creating your experiment (**section C or D**). You can begin it while the stream is stabilizing.

C. SETTING UP A NEW EXPERIMENT, SAVING TEMPLATES AND APPLICATION SETTINGS

Go to section D if you are opening up a template (and possibly using already saved application settings).

1. In the *Browser* menu, open your folder and create a new experiment (*brown folder icon* on the *Browser* icon bar).
2. Create specimens *(syringe icon)* and tubes under specimens *(tube icon).* Name your experiment, specimens and tubes.
3. Activate any tube (the arrow next to it turns green) in the *Browser* menu and select *Cytometer window* from the *Browser* icon bar (*red laser icon*) if not yet open. Choose Parameters tab and delete any detectors you are not using. Tip! Check FSC and SSC W and H boxes for doublet discrimination option.
4. Open your plots, histograms and statistical views the way you want them in the *Global worksheet*. Statistical view can be added by accessing the mouse right button menu.
5. From the toolbar, select *Experiment -> Experiment layout*. Add labels to your fluorochromes. Select the amount of cells you want to collect (Acquisition tab). You can now give correct labels for your plots in the *Global Worksheet* by clicking the axis labels.
6. Set the detector voltages: activate any tube in the *Browser* menu and run an unstained sample on acquire (use *Acquisition dashboard)*. Flow rate can be low as long as you see enough events. Run an unstained sample to set the negative population, and a stained sample to check that the positive population is on scale. Adjust the voltages in the Parameters tab in the *Cytometer window*.
7. In a simple experiment, you can now draw your gates of interest. Stop acquisition and unload the tube when you are happy with your gates and voltages.
8. If you want to set a stopping or storage gate, selects from the toolbar *Experiment -> Experiment layout.* Select the Global Worksheet column and choose “Global sheet 1” from the drop-down menu. Now you can choose the stopping gate. Do this when you have decided on final gating. You can always come back after compensation to finish it.
9. You can save application settings and experiment template at this point (see **section D** for explanation). To save experiment template, right-click your experiment name in the *Browser* menu. *Select Export -> Experiment Template* and do as prompted in the appearing window. To save application settings, right-click *Cytometer settings* under your experiment name in the *Browser.* Select *Application Settings -> Save*. Name the settings.
10. Proceed to running compensation samples if needed (see **section E**), sort setup (**section F**) or just recording your sample data (**section G**), if no more adjustments are needed.

    - *What do you want to have in your template? Certain tubes with names? A compensation matrix that cannot be updated? Saving the template before adding these saves you the time and effort of editing and deleting them the next time.*

D. USING TEMPLATES AND APPLICATION SETTINGS

If you already have set up your experiment previously and want to reuse its settings, these are the best options.

- *If you want your experiment to be updated by the latest CST to ensure data comparability, you must use application settings – not just copies and templates!*
- *Application settings include parameters, area scaling and PMTV. Templates and copies include all these, and also gates and compensation. Saved compensation matrices can never be updated so compensation must be run anew!*

1. Either copy an old experiment (right-click and *Duplicate without data*), or open up a previously saved template: in the toolbar menu, choose *Experiment -> New experiment*. Choose your template from the appearing list.
2. In the *Browser* menu, right-click *Cytometer settings* below your experiment name. Make sure to have run CST at this point (if not valid from yesterday). Select *Unlink from (the name of your previously saved compensation data)*, if applicable.
3. Right-click again *Cytometer settings* below your experiment name. Choose *Application settings -> Apply*, and choose previously saved settings from the list that appears. If asked about compensation, *Set to zero*. If eg. Area scaling has changed, choose *Overwrite*. The text *Cytometer settings* has now changed to *Application settings*.
4. If compensation is not needed, proceed to either sort setup (**section F**) or just recording your sample data (**section G**).
5. Otherwise check that compensation in your samples is now zero: activate a sample, open the *Cytometer window (red laser icon)* and see Compensation tab. Diva is sometimes tricky here!
6. Run new compensation controls (see **section E**). Check that the new compensation is now applied to your samples.

- *Application settings can also be applied to a completely new experiment (see **section C**).*

E. COMPENSATION

Perform compensation whenever you have two or more fluorochromes with overlapping spectra in the same sample.

1. From the toolbar, select *Experiment -> Compensation setup -> Create compensation controls*. If you have only one marker per fluorochrome, delete everything that does not have the label "general". If you use beads, check the box that includes a separate unstained tube. If compensating with cells, leave it unchecked. If you need an empty channel, delete it here. Click OK.
2. Switch to *Normal Worksheet* (icon on the worksheet corner). Activate the first compensation sample in the *Browser* menu.
3. Load the correct single-stained sample. Set the gate to singlets and press *Record*. Collect preferably at least 10 000 events (can be set in the *Experiment layout* or one by one in the *Acquisition dashboard*). Unload sample.
4. Right-click the P1 gate and select *Apply to all*. Click the next sample either in the *Browser* or in the *Acquisition dashboard*. Load new sample and record. Repeat until you have run all the compensation tubes.
5. Verify correct positioning of the P2 gates. If you did not include a universal negative, create P3 gate on the negative peak by using the *Snap-to-gate icon*.
6. In the toolbar menu, select *Experiment -> Compensation setup -> Calculate compensation*. If you forget this, software will say nothing and you will not have any compensation! In the pop-up window, choose *link and save*. You can change the name if you wish.
7. Change back to *Global Worksheet*.
8. Now you can verify and fine tune correct gating and set gating hierarchy (for sorting) by running (on acquire) an appropriate sample, if necessary. Gating hierarchy can be added by accessing the mouse right button menu. You must not change any voltage other than FSC or SSC from now on – it would make the compensation invalid.
9. Proceed to setting up sorting (**section F**) or recording your data (**section G**).

F1. SORTING TO TUBES

- *Use polypropylene tubes as recipient tubes to avoid cells adhering to tube walls.*
- *Tip! Record 10 000-20 000 events (while sorting) to have visual data of what you actually sorted.*

Setup of side streams

1. Remove the splash shield under the sort block. Stream should now be stable and the *Sweet spot* on!
2. Insert the tube holder with four tubes in it. *Side stream window* should be in Four tube mode. This can be changed from the icon in the lower taskbar of the computer.
3. Activate any sample and open a *Sort layout* (*Blue plate icon* in the *Browser* toolbar). Choose four tubes or two tubes from the Device drop-down menu in the sort layout.
4. Perform test sort: in the *Side stream window*, turn on *Voltage*, close *Waste drawer* (icon now shows stream going into tubes), press *Test sort*.
5. Open the sort block door and check that the streams enter the tubes. If needed, adjust the streams with sliders in the *Side stream window.* The streams should be placed in between the guidance lines.
6. Turn off *Voltage* and open *Waste drawer* (icon now shows blocked stream and the stream enters the waste drawer).

Setting drop delay

7. Open the Accudrop template and add a *Sort layout*.
8. Prepare Accudrop beads: shake the Accudrop bottle and add one drop into 500 µl of PBS. Vortex and acquire on flow rate 1. If event number is over 1500/s on 100 µm nozzle, dilute the sample a bit.
9. Add P1 population to the left tube in the *Sort layout* (Right-click -> *Add*).
10. Turn on *Voltage. Waste drawer* can be left open – no need to collect the beads. Turn on *Optical filter* (two boxes appear in the *Side stream window*). Press *Sort* in the *Sort layout.* Click *Cancel* when a dialogue box appears.
11. All or nearly all beads should go to the left box. Press *Auto delay* and *Start run.* A few rounds might be needed to get the beads to the left box. If several attempts fail, disable the *Optical filter* and adjust the camera screw until center stream is sharp and side streams visible.
12. When drop delay is set, stop sorting, close *Optic filter*, turn off *Voltage* and unload tube. Amplitude and frequency should not be adjusted after the drop delay is set – otherwise you must set it again.

Sorting

13. Return to your experiment.
14. Activate the sample you want to sort first and open *Sort layout*. Verify correct amount of tubes (two or four) and correct precision. Set target events to the number you wish or continuous.
15. Add your target populations (gates) to the wells in the Sort layout. Double check gating and sorting logic!
16. Run some FACSClean before sorting (at least 10 min), check for debris in gates. You can also run 70% ethanol to sterilize fluidic lines. Run PBS 5 min.
17. Place the polypropylene tubes to the tube holder. There should be some medium on the bottom. Wet also the tube walls!
18. Vortex your (filtered!) sample and load it. Press *Sort*.
19. Record some data if necessary by pressing *Record*.
20. Save only the sort reports you need.

F2. SORTING TO PLATE

- *Note that Falcon well plates fit the ACDU best!*
- *Tip! Record 10 000-20 000 events (while sorting) to have visual data of what you actually sorted.*

Initial setup of side streams (for Accudrop)

1. Remove the splash shield under the sort block. Stream should now be stable and the *Sweet spot* on!
2. Insert the tube holder with four tubes in it. *Side stream window* should be in Four tube mode. This can be changed from the icon in the lower taskbar of the computer.
3. Activate any sample and open a *Sort layout* (*Blue plate icon* in the *Browser* toolbar). Choose four tubes from the Device drop-down menu in the sort layout.
4. Perform test sort: in the *Side stream window*, turn on *Voltage*, close *Waste drawer* (icon now shows stream going into tubes), press *Test sort*.
5. Open the sort block door and check that the streams enter the tubes. If needed, adjust the streams with sliders in the *Side stream window.* The streams should be placed in between the guidance lines.
6. Turn off *Voltage* and open *Waste drawer* (icon now shows blocked stream and the stream enters the waste drawer).
7. Begin setting drop delay: open the Accudrop template and add a *Sort layout*.
8. Prepare Accudrop beads: shake the Accudrop bottle and add one drop into 500 µl of PBS. Vortex and acquire on flow rate 1. If event number is over 1500/s on 100 µm nozzle, dilute the sample a bit.
9. Add P1 population to the left tube in the *Sort layout* (Right-click -> *Add*).
10. Turn on *Voltage. Waste drawer* can be left open – no need to collect the beads. Turn on *Optical filter* (two boxes appear in the *Side stream window*). Press *Sort* in the *Sort layout.* Click *Cancel* when a dialogue box appears.
11. All or nearly all beads should go to the left box. Press *Auto delay* and *Start run.* A few rounds might be needed to get the beads to the left box. If several attempts fail, disable the *Optical filter* and adjust the camera screw until center stream is sharp and side streams visible.
12. When drop delay is set, stop sorting, close *Optic filter*, turn off *Voltage* and unload tube. Amplitude and frequency should not be adjusted after the drop delay is set – otherwise you must set it again.
13. Remove the tube holder under the sort block, put back in the splash shield.

Side stream adjustment in ACDU mode for plate

14. Return to your experiment.
15. Switch the *Side stream window* to ACDU mode. Use the icon in the lower taskbar of the computer to do this.

16. Adjust the far left side stream to the same value as the proximal left side stream.
17. From the toolbar, select *Sort -> Home Device*. A window with arrows appears (the *Device setup window*). Select you well plate from the list on the left.
18. Activate any sample, open your *Sort layout*. In the *Sort layout* choose your well plate from the drop-down menu. Press *Access stage* ("eject" button). Plate holder comes to the front. Place your test well plate (lid on) into the holder, A1 at the front left corner.
19. In the arrow window, press *Go to home*. The ACDU moves to the starting position for sorting into A1.
20. Turn on *Voltage*, close *Waste drawer*. Press the button in the middle of the arrows (*Test sort*) two times quickly, to sort a small droplet onto the lid. The droplet should be directly on A1. Side stream should fall between the lines – you can check this by keeping the test sort on longer (remove plate lid!).
21. Continue fine tuning of the ACDU position. When the ACDU is in home position, move it with the arrows and repeat test sorting until droplet is in the middle of the A1 well. Double arrow moves the ACDU five steps and single arrow moves it one step. Press Set home in the arrow window to save the new A1 coordinates. Use the "eject" button to bring it back to the front for inspection between adjustments, if needed. Press set home only when the plate is in A1 position – not in the front unless you want extra work!

Final adjustment of sorting in rows and columns (with beads)

22. Put plate into home position and click *Apply* to link the position coordinates to *Sort layout*.
23. Load and run some beads (Accudrop or CST, or use cells if you have spare).
24. Create a gate for the beads and add it to horizontal and vertical well rows. Choose 10-50 events for sorting. Press *Sort*.
25. If droplets are not in a satisfactory row on the lid, click *Go to home* and do the needed adjustments with arrows. Click *Apply* and *Set home*. Acquire sample, sort. Repeat this whole step until position is good.
26. Unload the bead tube, check that *Voltage* is off and *Waste drawer* open. Delete the bead gate, if necessary.

Sorting to plate

27. Replace test plate with your sorting plate. There should be some medium on the well bottom.
28. In the *Sort layout*, select the desired precision. Set target events to the number you wish.
29. Add your target populations (gates) to the wells in the Sort layout. Double check gating and sorting logic!
30. Run some FACSClean before sorting (at least 10 min), check for debris in gates. You can also run 70% ethanol to sterilize fluidic lines. Run PBS 5 min.
31. Vortex your (filtered!) sample and load it. Press *Sort*. Record some data if necessary.
32. Save only the sort reports you need!

G. RECORDING YOUR SAMPLE DATA AND EXPORTING FSC-FILES

- *Filtering your samples might be a good idea – it is always less annoying to filter them than to open a clogged system***.**

1. Activate your first sample in the *Browser* and place the tube into the loading chamber. Click *Load*.
2. Set the flow rate so that you get enough events per second. Generally, lower flow rate is better, but with dilute samples it might take too long.
3. Press *Record*. You can change the event number to be recorded in the *Experiment layout* or in the *Acquisition dashboard,* if needed*.*
4. Move to the next sample by activating it in the *Browser* or by clicking *Next tube* in the *Acquisition dashboard*. Load and record, repeat for the rest of the samples.
5. After the last sample, insert a USB storage device and right-click your experiment in the *Browser*. Select *Export -> FSC Files*.
6. A window opens, click OK after checking that file format is FSC 3.0.
7. Choose where to save your files and click OK.

H. BASIC SHUTDOWN PROCEDURE

1. After sorting, remove the tube holder and replace it with the splash shield. Clean the holder if dirty.
2. Load a tube of FACS Clean and run on acquire for 10 minutes on flow rate 11.
3. Repeat with FACSRinse.
4. Repeat with mqH_2O.
5. *Optional but highly recommended: clean flow cell as instructed below.*
6. *Stop the stream.*
7. *Remove the nozzle and clean it carefully as during startup (see section B). Insert the broken 100 µm nozzle (in the drawer below the desk).*
8. *Place a tube filled with FACSClean into the loading chamber. From the toolbar, select* Cytometer -> Cleaning modes -> Clean flow cell*, click OK.*
9. *Repeat Clean flow cell two more times. If you let the flow cell soak for 5-20 minutes, the cleaning will be more effective.*
10. *Clean flow cell three times with mqH_2O. No soaking is needed.*
11. From the toolbar, select *Cytometer -> Fluidics shutdown*. Follow the instructions one by one. It is safest to first detach the gas inlet, then the fluid outlet. Then attach them to the EtOH tank in reverse order: fluid outlet first!
12. Clean the nozzle now, if you did not do it already. Dry it!
13. When prompted during fluidics shutdown, use mqH_2O as the final cleaning solution.
14. Turn off the FACSAria Fusion.
15. Clean all spatter from the loading chamber, sorting chamber, deflection plates and fluidics cart. Ethanol and lint-free lens paper can be used on the camera lenses, if necessary.
16. Close the air pressure lock on the wall.
17. Empty the waste tank. Rinse away all foam. Pour some FACSClean on the bottom (around 100 ml is enough). Attach the tank back to the fluidics cart.
18. Check that you do not have too many old experiments in the *Browser*. 1-3 is fine as templates. Delete the ones that are already exported.
19. Close FACSDiva and turn off the computer.
20. Fill in the logbook (both of them).