Protocol for sorting budding yeast using the SONY SH800 Sorter

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Most of these steps are taught to you by the training course. However, you can find them here too. Book the machine on https://sv-ppms.epfl.ch/

FACS machine setup

1. Write your name, date, start time, and lab's name on the notebook prepared for this. The last column of the notebook is for you to write down any problems that were encountered during use. Put on a lab coat and gloves.
2. Open the left side door of the FACS and take out the water tank (Figure 1). Empty the bottle in the sink and refill it with fresh Milli-Q water (Figure 2 and 3). The Milli-Q will most likely be in "standby" mode. To use, simply press the right arrow next to the screen to switch to "ready" mode. After using, switch back to "standby" mode by pressing the same arrow again.

Figure 1

Figure 2

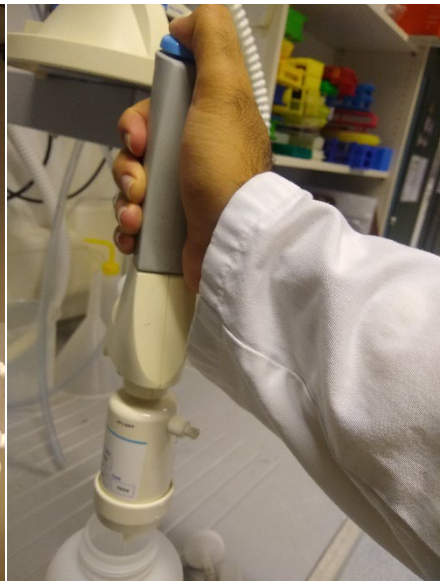

Figure 3

3. Check the three tanks under the table of the FACS (Figure 4). The left tank is the waste tank, the middle is ethanol (used by staff for cleaning), and the right is the sheath tank. Check that the sheath tank is full (it should be full, but check to be sure by lifting it up to weigh it) and well connected. If the tank is not full, switch to a full tank (there should be a full tank nearby) by disconnecting the two

lines and reconnecting them to the new tank. Check that the waste tank is empty. If it is not empty, empty it in the dedicated waste collection bin.

4. Turn on the FACS with the power button (Figure 5) as well as the computer screen (if it is not already on).
5. Double-click the software icon (Figure 6).

Figure 4

Figure 5

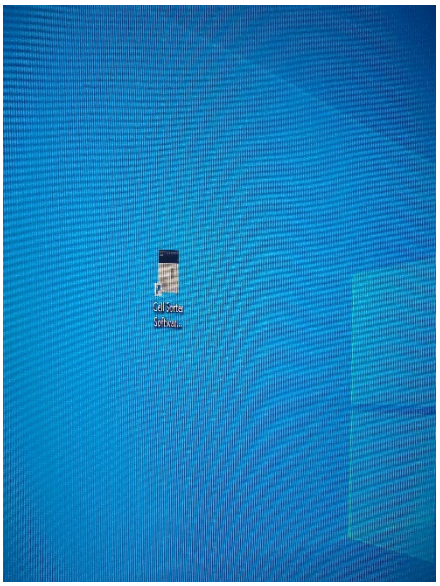

Figure 6

6. Log in with your account information. User: LPBS Password: usual lab password with an extra # at the end.
7. For yeast cells, use a 100 um chip. If there is already a chip in the machine and the time written on the wrapper of the chip (should be in front of the computer) is less than 24h ago, you can still use that one. But, if the chip is older than 24h, change the chip (the box of chips is stored in the closet, Figure 7). It is recommended to use a new chip if the lab before you sorted out something other than yeast cells. In any case, put the QR of the chip wrapper (Figure 8) in front of the camera (Figure 9).

Figure 7

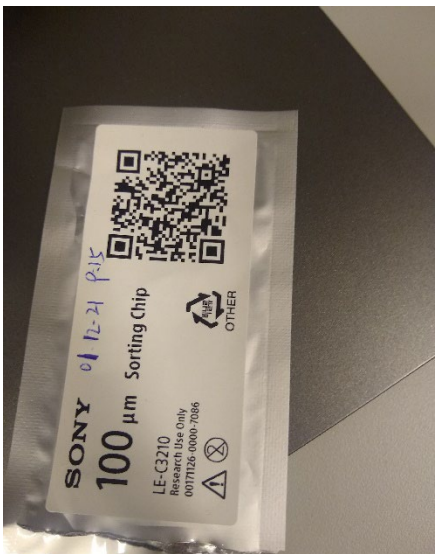

Figure 8

8. Check if the details shown in the screen match the information written on the pack and press Next (Figure 10).

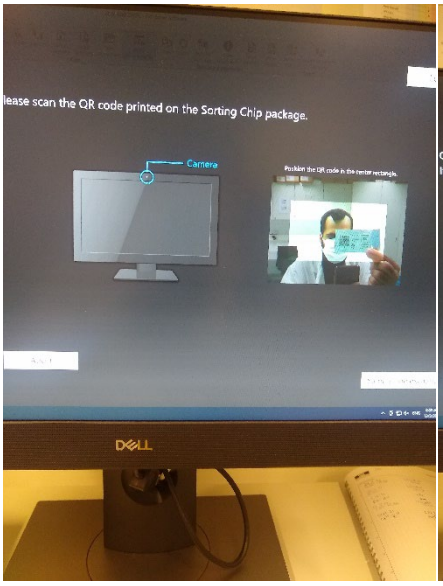

Figure 9

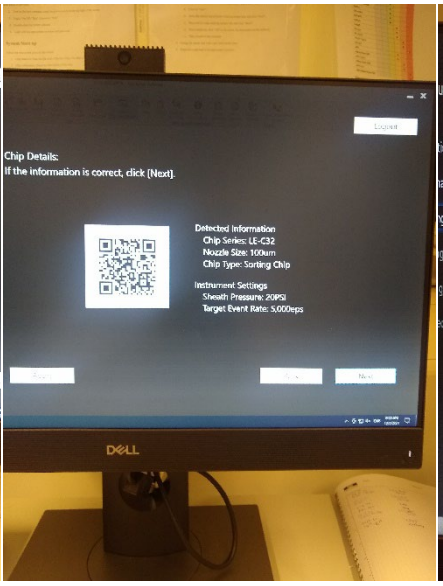

Figure 10

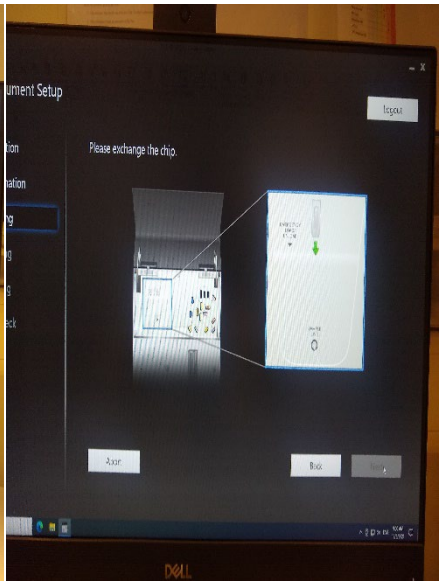

Figure 11

9. As shown in the Figure 11, you are asked to put the chip into the machine. For that, open the top door of the machine (Figure 12, “PUSH OPEN”). Either push the used chip back into its dedicated place or replace with a fresh one. Simply push once (DO NOT force), the machine will insert the chip by itself. Be sure that the chip is placed in the proper orientation - the ‘XX um’ label of the chip must be readable if standing in front of the machine (Figure 13). If you used a new chip, write down the time and date on the wrapper and place it in front of the computer for the next user. Throw away the used chip and its wrapper.

Figure 12

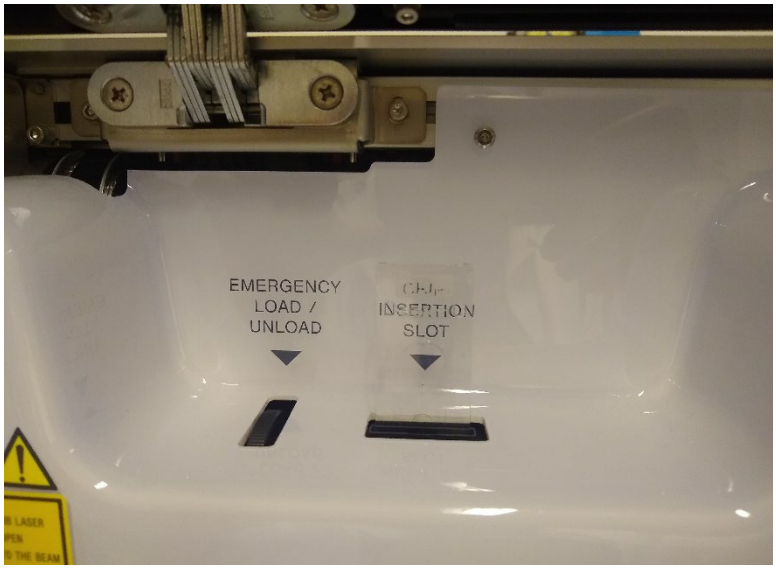

Figure 13

10. Press Next (Figure 11).
11. Press Next (Figure 14).

12. Select the middle option (‘With 405nm Laser’) (Figure 15). This is the configuration of the machine. You can check it by opening the top door of the machine, but for us it will always be the middle option. Press Next.

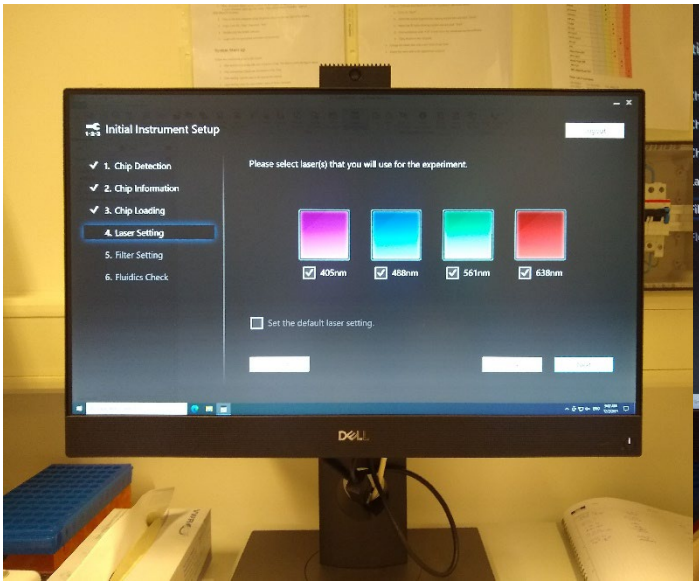

Figure 14

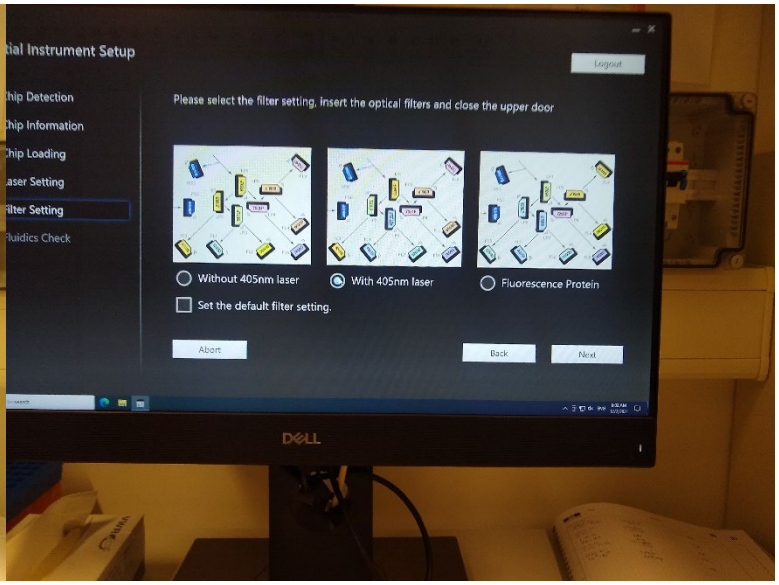

Figure 15

13. Before the process of the “Fluidic check is in progress” finishes, press “sheath filter de-bubble” (Figure 16).
14. Press Start (Figure 17).

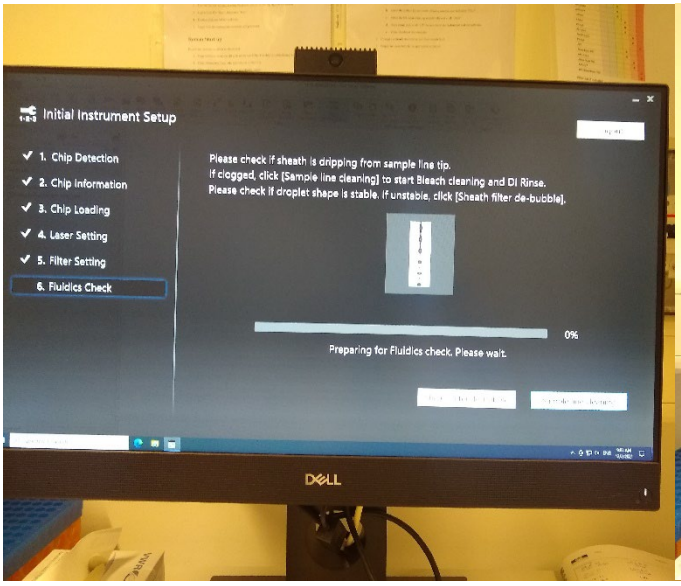

Figure 16

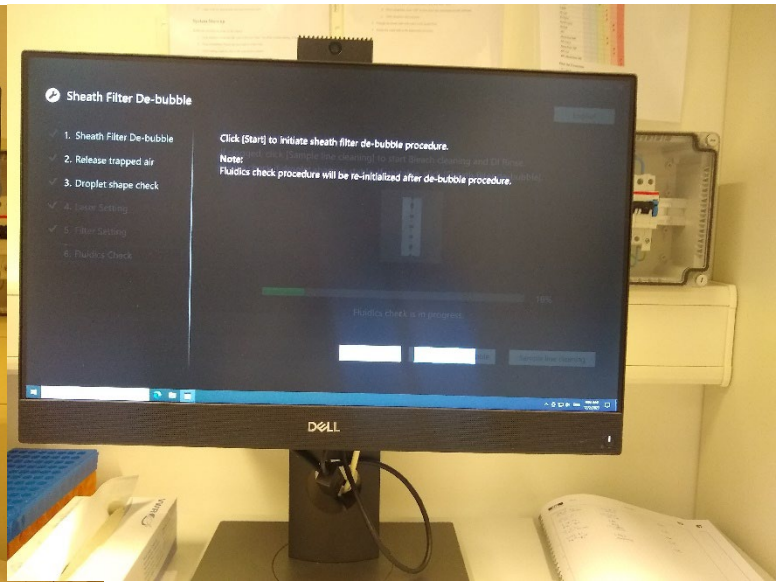

Figure 17

15. Press Start (Figure 18).
16. Next.

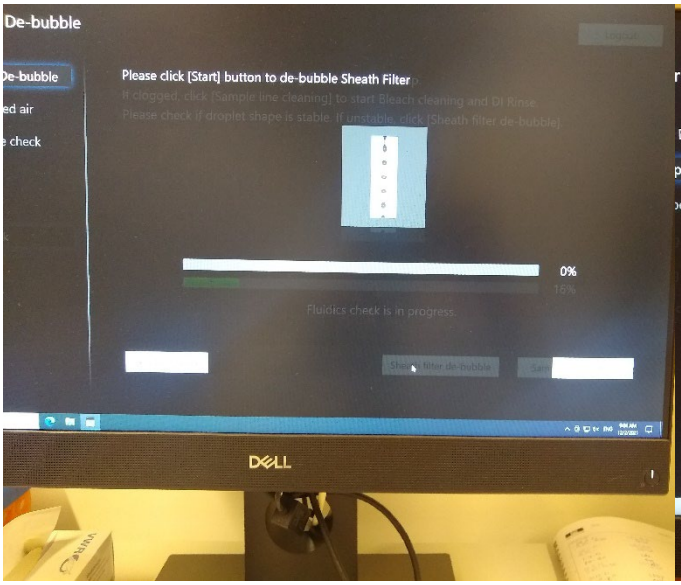

Figure 18

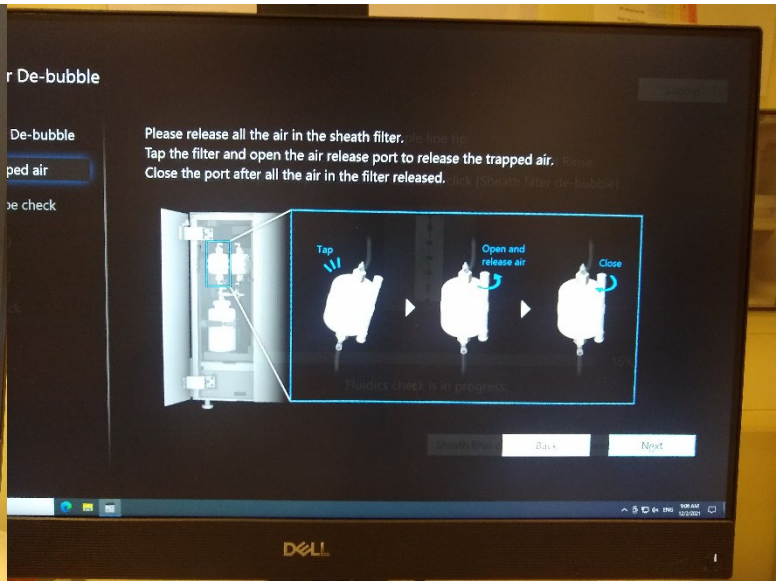

Figure 19

17. Release trapped air in the sheath filter. There is a bug in the software so the instructions (Figure 19) appear only after you finish the program. When the computer runs the debubbling program, it increases the pressure to 40psi (usually the pressure is at 20psi). When the pressure is around 40psi, pull out the sheath filter (small bottle connected to the blue line on the left side of the machine; Figure 20), tap the top of the filter with your hand, and open the white valve a little bit so that the trapped air flows out. In doing so, there will also be a bit of liquid that will flow out. Close the valve, dry off the filter, and lock it back in its place.

Figure 20

18. Next.
19. Debubble the sheath filter again. You should always do this step at least twice.
20. Look at the stream of droplets on the computer. If the stream is stable (i.e., it is not moving up and down), continue to the next step. If it is not stable, debubble again. If after 5 times debubbling the stream is still not stable, ask for help.
21. Next (if the droplets are stable).

22. Ok.
23. Press “Sample line cleaning”. This part takes around 10 min.
24. Take the bleach tube which should be on the table next to the computer (Figure 21) and fill it with bleach up to 10ml (Figure 22).

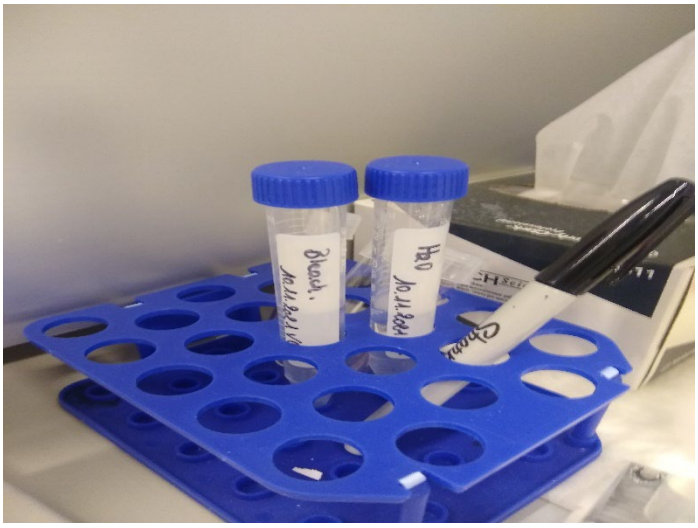

Figure 21

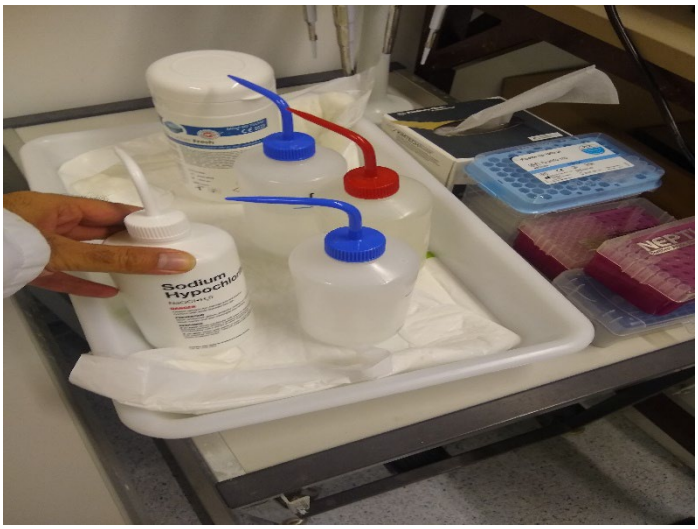

Figure 22

25. Find the proper holder that fits the bleach tube (Figure 23) and install the whole thing in the sample area (Figure 24). (At the bottom of each holder there is a number that corresponds to the tube size that holder is used for)

Figure 23

Figure 24

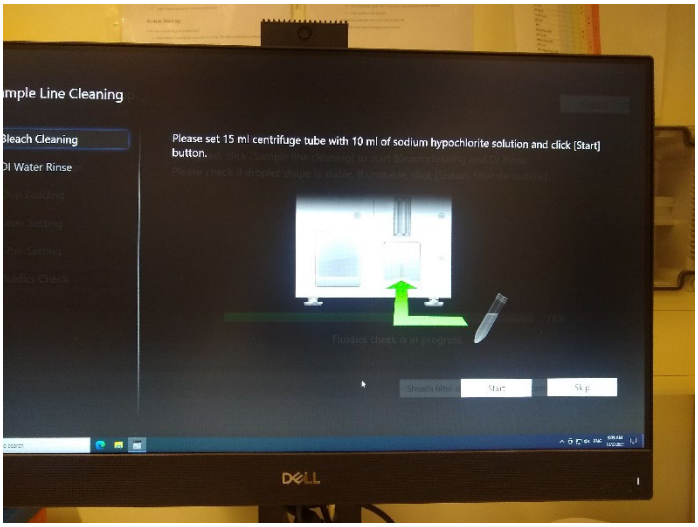

Figure 25

26. Press Start (Figure 25).
27. Next.
28. Fill the DI water tube with 12 ml of DI water. Replace the bleach tube in the sample area with the DI water tube.
29. Start.
30. Take out the DI water tube when the cleaning is done.
31. Next.
32. Ok.

Auto Calibration

1. In the box of calibration beads found in the fridge (Figure 26), there should be 3 black bottles of beads (Figure 27) and potentially a 5 mL tube with some leftover drops of beads (Figure 28).

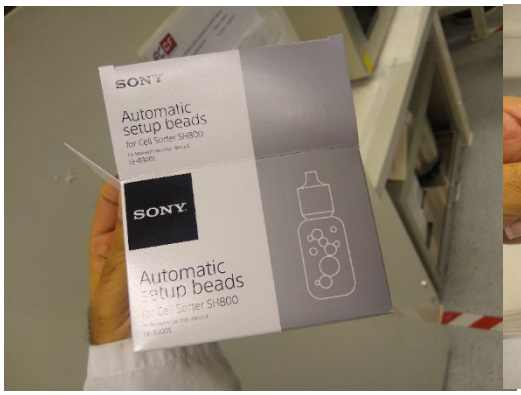

Figure 26

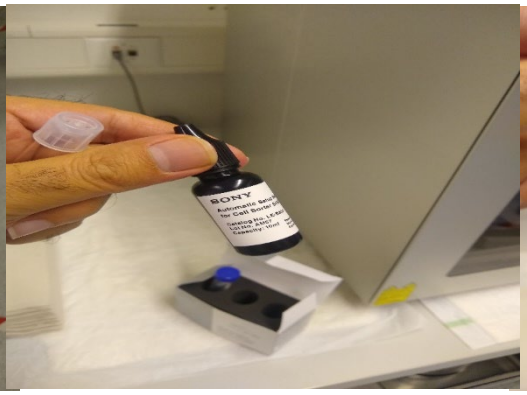

Figure 27

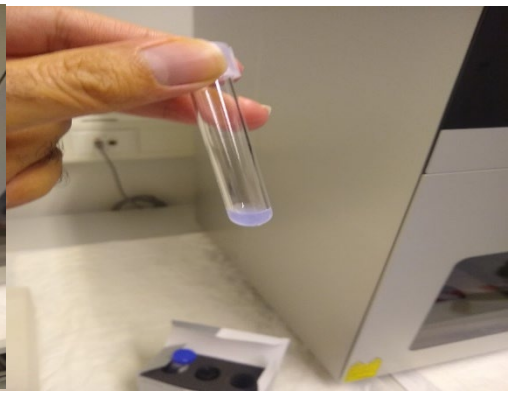

Figure 28

2. Make a new tube with 10 drops of beads or use the tube in the fridge (Figure 28) if it is < 5 days old and add 5-10 drops depending on how much is left in the tube. Be sure to shake/vortex the bottle of beads before adding drops into the tube.
3. Take the proper holder that fits the tube (Figure 29).

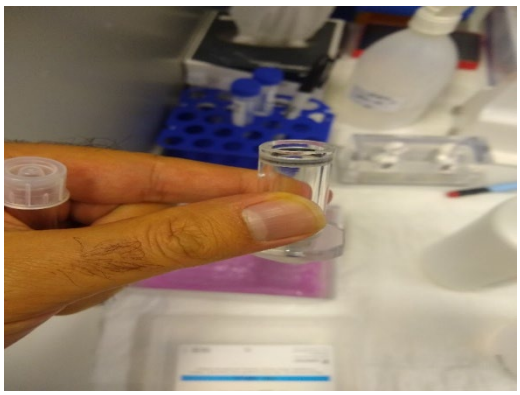

Figure 29

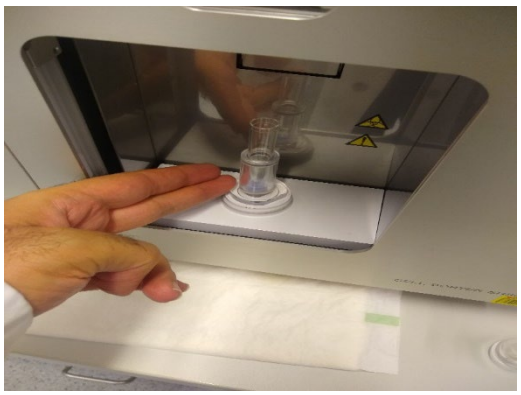

Figure 30

4. Put the whole thing in the sample area (Figure 30).
5. Start (Figure 31).
6. Standard (Figure 32).

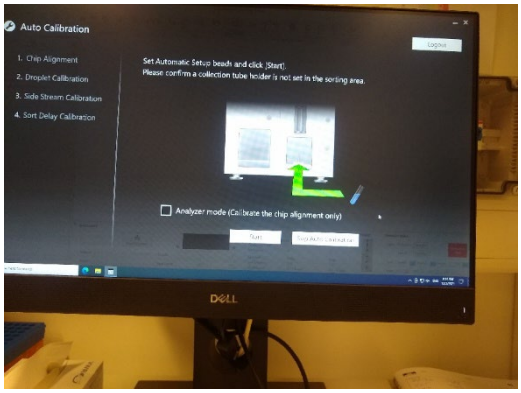

Figure 31

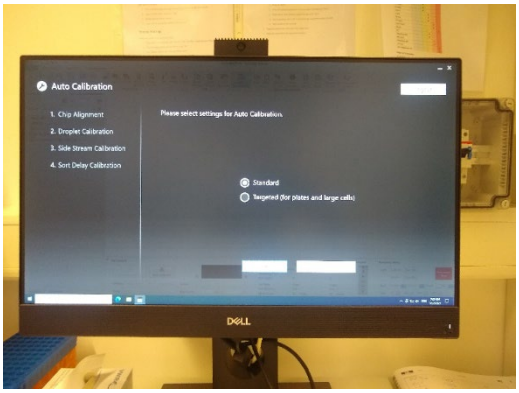

Figure 32

7. Wait for the calibration process to finish (takes around 15 min).
8. Take out the tube, write the date on it, and put it back in fridge.
9. You can now either use a template or create a new experiment. These templates can be found in the left panels of Figure 33. If creating a new experiment, change the name of the experiment (Figure 34a) and measurement settings (Figure 34b-c). You can fill out the rest of the fields as well but it is not necessary. In the measurement settings, select area, height, and width for both BSC and FSC, and select area for each fluorescence you will use. Unselect everything you will not use. For the fluorescence, there is a table on the wall which tells you which fluorescence uses which channel. Press “Create Experiment” in the bottom-right corner of the page (Figure 34e).

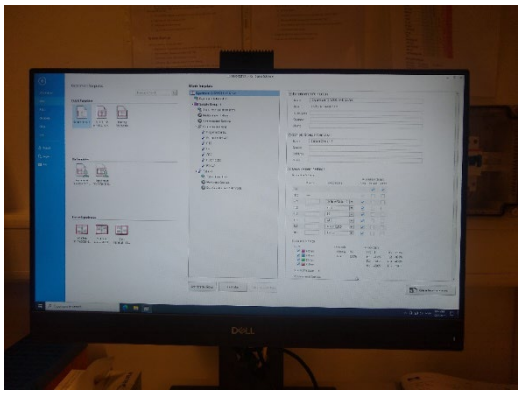

Figure 33

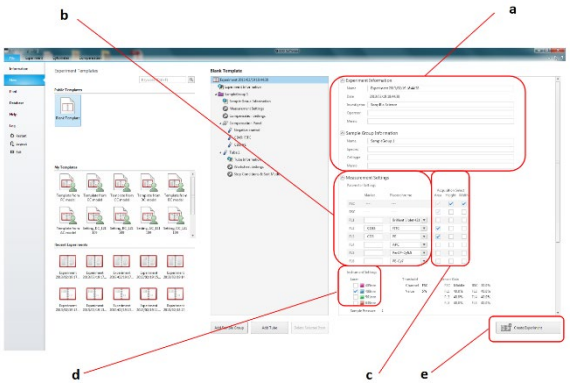

Figure 34

Before starting the experiment

1. Optional but recommended: Before beginning the experiment, go to cytometer (Figure 35a) >settings > maintenance > debubble DI water and follow the instructions. This process is similar to the sheath filter debubble.
2. For yeast cells, the 100 um chip is used (the lower the um, the more pressure is applied to the cells). When inserting the chip, the computer tells you the maximum event rate. For the 100 um chip, it is 5000 events per seconds. When first starting your experiment, be sure the event rate is not higher than this value, or else the sorting will be less precise. If it is, either decrease the sample pressure (button on left

panel of the software) or dilute your cell suspension. The event rate appears on the left panel of the software whenever it is running (Figure 35b).

3. If you have more than one fluorescent protein/dye, follow the compensation instructions below. If not then:
    a. Click “start” (Figure 36e).
    b. Click on the “detection threshold” button (below the “start” button).
    c. Change the values of BSC and FSC to get your cell population at the bottom left corner of the graph.
    d. After changing these values, always click on “restart” so that you can visualize how it affects the graph.
    e. Draw a gate around the majority of the cells (=gate A). This separates the cells from debris.
    f. Follow the “gating singlets” instructions (skip if your cells are expected to be budded).

Compensation (skip if the cells only have 1 fluorescent protein or dye)

1. When sorting/analyzing cells that have more than one fluorescent protein or dye, it is important to follow the “compensation wizard” (button on the top left side of the toolbar in the software). This is to remove any contribution one fluorescent color may have to the other (if the spectra have a bit of an overlap). If you use fluorescent colors which have spectra where the peaks are indistinguishable, you will not be able to compensate and therefore will not be able to know which light comes from which protein or dye.
2. In order to follow the compensation wizard, you will need to have one negative control sample (cells with no fluorescence) and one sample with only one fluorescence for each of the fluorescent proteins or dyes in the cells.
3. Click the “compensation wizard” button.
4. Follow the instructions. It will first ask you to put your negative control tube in the sample area, then click start.
5. Open the “detection threshold” on the left of the software.
6. Change the values of BSC and FSC so that your cell population appears on the bottom left of the left graph. After changing any value, click “restart” to see how it modifies the graphs. If necessary, move gate A to encompass your cell population.
7. For the right graph DO NOT move the gates. Simply move the peaks by changing the threshold values.
8. Change the record stop condition to around 5000.
9. Then you will be asked to put in your first single fluorescent sample.
10. Now, you only change the gates of the left graph so that it is centered on the two peaks.
11. Same idea for your additional single fluorescent samples.
12. When you are done with the compensation wizard, click on “calculate matrix values”.
13. From now on, do not touch the threshold values for the fluorescent channels (this will undo the compensation). If necessary, you may still change the BSC and FSC values.

Gating singlets (skip if sorting cells that have buds)

1. It is important that before taking any measurement, you gate the singlet cells only. If not, you will end up measuring/sorting doublets (=a drop with 2 cells in it instead of one) which will lead to erroneous results. To gate singlets, simply make a dot plot with either FSC-A vs FSC-H/FSC-W or BSC-A vs BSC-H/BSC-W on the axis. To do this either double click on gate A of the main plot, or click on "new dot plot" and change the title so that it only takes cells from gate A.
2. Then click on the axes to change them.
3. Singlets will have a height/width proportional to the area whereas doublets will not. Therefore, draw a gate (polygon) around the diagonal of the graph.

General measurements

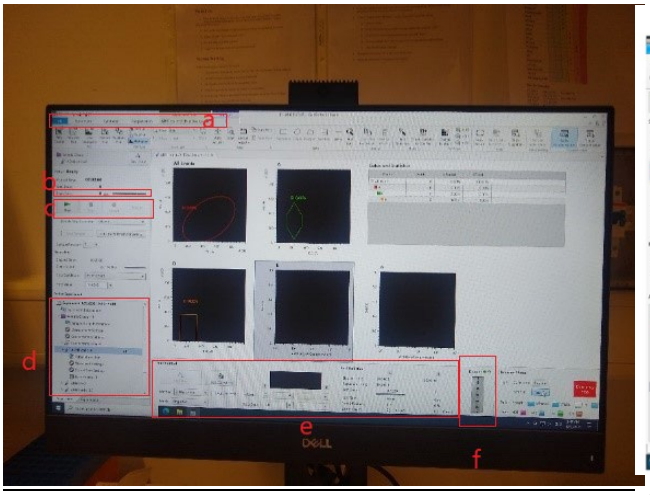

Figure 35

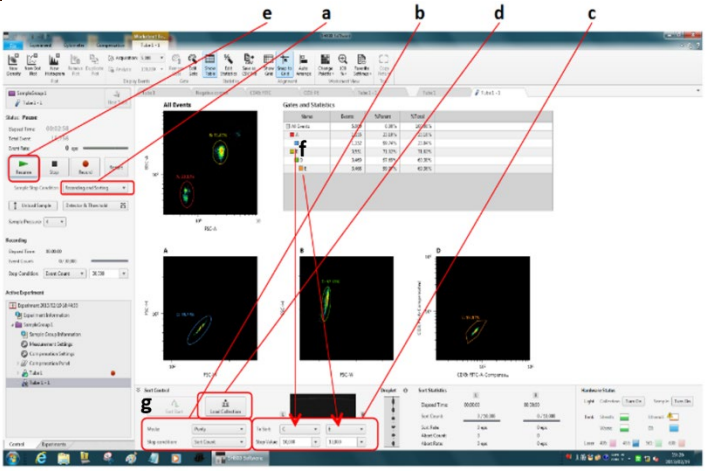

Figure 36

1. Set the stop condition (on the left of the software) and click "start" (Figure 35c and 36e) to visualize some cells.
2. Click "pause" (Figure 35c).
3. Draw your desired gates (they should all be subgates of the singlet gate if separating singlets).
4. If you only want to record: set the recording stop conditions, then click "start", then click "record" (Figure 35c).
5. If you want to sort (Figure 35e and 36g):
    a. Set the recording conditions, the sorting conditions (Figure 36f), and the sorting mode (Figure 36b; there is a PDF of the manual on the desktop where each sorting mode is explained in detail).
    b. Enter the two-tube holder in the sample collection area and click "load collection" (Figure 36d). Be sure that the tubes have a bit of liquid in them to collect the falling cells (prevents cells from being damaged).
    c. Click on "start" then "record" (Figure 35c) then "sort" (Figure 35e). You will be notified when each stop condition is satisfied.
    d. If you want to double check the sorting, you can do a purity check:
        i. Take one of the two sorted tubes (usually if one side is well sorted so is the other).
        ii. Place it in the sample area and click "new tube" then "start".
        iii. 90-100% of the cells should appear in the gate that was sorted.

6. To go to the next tube, either press “next tube” button or right click on the tube name (Figure 35d) and select “duplicate tube” and then right click on the new tube that was created and click “assign”. If you are using a template where the tubes are already defined, simply right click the next tube name and click “assign”. It is also useful to right click the first tube and click “create tube template”. You can then apply this template to every other tube by right clicking the next tube, selecting “apply tube template” and then “assign”. This ensures that the exact same gates are used for each tube.

Saving and shutting down

1. To save:
    a. The experiment: right click on the tubes you want to save or the experiment name if you want to save everything, click “save FCS file”, and save in your lab’s folder (Figure 35d).
    b. The template: on the top toolbar, click on “save as template” and save in “my templates”.
    c. A PDF per tube: on the top toolbar, click on “print” then “print as PDF” or ctrl+p. This only prints the open workbook (i.e. 1 tube). You need to open the workbook of each tube individually in order to print each tube (right click tube name > open workbook).
2. To shut down:
    a. Click the Cytometer tab (Figure 35a) and find the icon in red box shown in Figure 37 and click and follow the instruction steps. Be sure to do the bleach and DI water rinse (again, this takes around 10 min) and keep DI water in the probe.

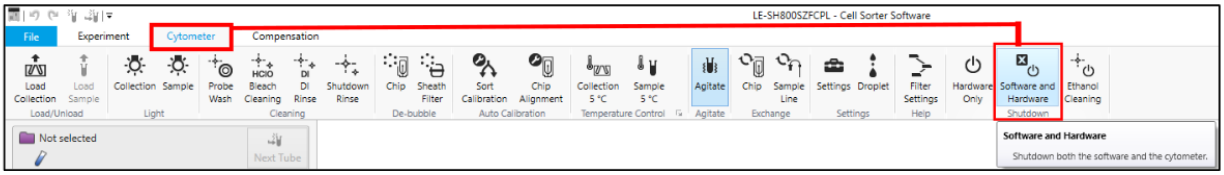

Figure 37

    b. After shutting down the machine, discard the waste of the waste tank into the dedicated bin (this will be shown to you in training class) and change the sheath tank to a new one. Optionally, fill up the used sheath tank: pull the pin (Figure 38b) to release the pressure, pull up the handle (Figure 38a) and remove the lid (Figure 39), fill with sheath (found in the cardboard box on the table across from the FACS), close the lid by putting the lid back in the hole and pushing down the handle to secure.

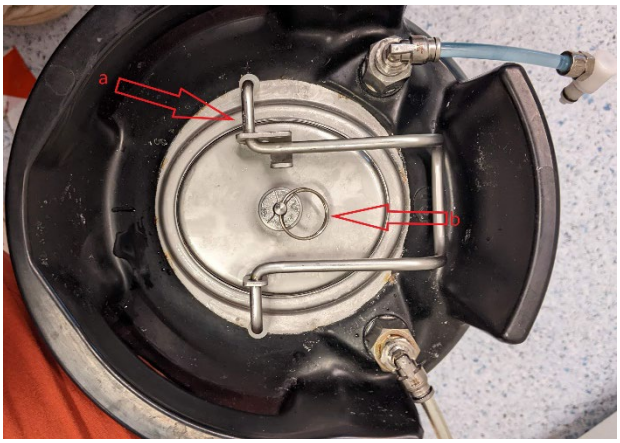

Figure 38

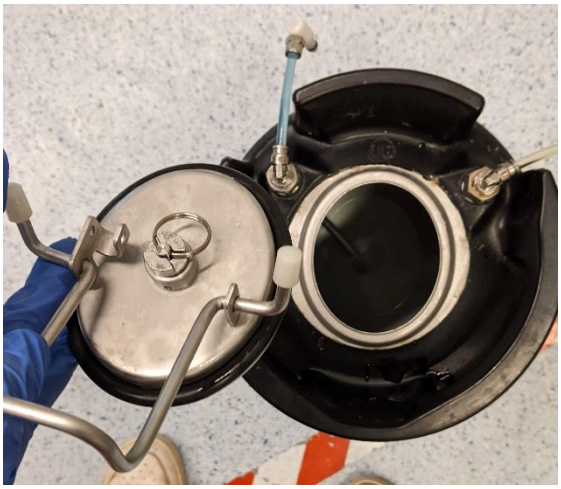

Figure 39

c. Write the end time in the notebook.

Checkpoint experiments - timepoints

In the following protocol, keep in mind that for light-sensitive (LIP) strains, you need to keep the tubes wrapped in aluminum foil, starting at the latest after the addition of gal. Tape aluminum foil on the windows of the Sony machine (Figure 42) as well to block outside light during sorting. Transport the tubes to and from the FACS room in a closed cardboard box.

Additionally, to increase efficiency, leave to go get your samples once the auto calibration is started. This way the 15 min of auto calibration will be done while you are preparing your samples and when you get back to the Sony machine it should be immediately ready for use.

1. Culture cells for two days (or one day, depends on the freshness of the cells) in liquid R-M medium.
2. The night before the experiment, dilute them so that the next morning they are in early log phase. This depends on how fast your strains grow and may need a few attempts to know how much and when to dilute.
   Example: Add 30uL stationary phase cells to 30mL R-M around noon to start next day at 7am.
3. In the morning of the experiment, check the OD. Ideally it is around 0.1-0.2. Anything below 0.5 and above 0.05 should be ok.
4. Add 5x Met to the solution.
   Example: Per 30mL cell culture, add 150uL of 1000x Met.
5. Incubate for 2h.
6. After 2h in R+M, add 1x Galactose.
   Example: Per 30mL cell culture, add 3.33mL of 10x Gal.
7. Incubate for another 1h.
8. Wash with 750g centrifugation: spin down, decant/aspirate as much liquid as possible, mix with several mL of D-M, spin down, decant/aspirate as much liquid as possible.
9. Add D-M.
   Example: Add 40mL D-M to the cells spun down from 30mL cell culture.

10. Incubate for 1.5h
11. Add 5x Met again to the whole solution.
    Example: Per 40mL cell culture, add 200uL of 1000x Met.
12. Incubate until sort
13. 15 min before desired sorting time, sonicate each tube and spin down. Remove most of the liquid, leaving around 1 mL (you need a very high density of cells to have efficient sorting). Go to FACS (or do this step in the FACS room if there is an incubator & sonicator there).
    Example: For 1 timepoint, 10-12 mL of cell culture is enough. E.g. a 40 mL tube can be split up into 3-4 timepoints. To do this, pour 10-12 mL of the 40 mL into a 15 mL tube and keep the big tube in the incubator. Sonicate + spin down the small tube. This ensures that the same conditions were applied to all timepoints.
14. Sort out the desired number of cells:

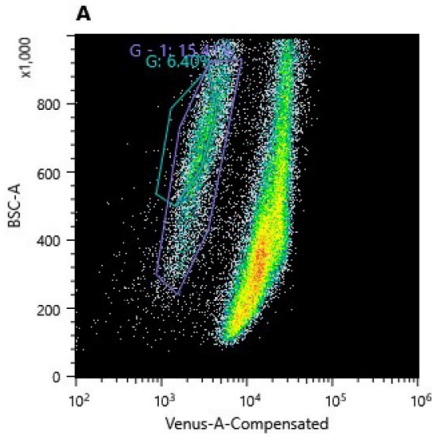

Figure 40

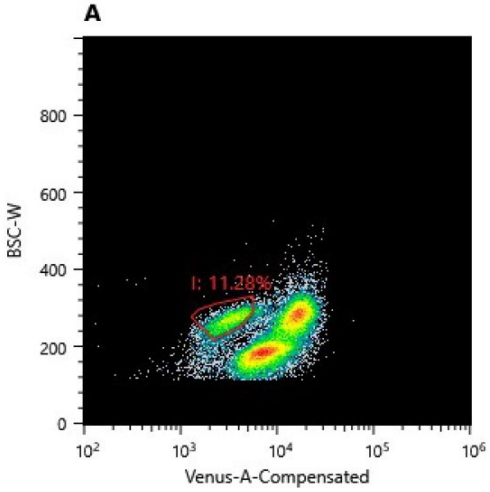

Figure 41

a. Plot FSC-A/BSC-A vs Venus-A and gate around the left population (Figure 40) to sort only via YFP level OR FSC-W/BSC-W vs Venus-A and gate around the top left population (Figure 41) to sort with YFP levels and size (this is what I did, as I wanted cells arrested at checkpoint = budded and YFP-).
b. Put D-M in sorting tubes (100-200 uL) to buffer the cells' impact as they are sorted. Put the tube in the sorting area. Load.
c. Sort the desired number of cells, always checking that the event rate does not exceed 5000 eps. I typically sorted 2500 cells per plate.

15. Back in the lab, quickly spin down the tubes to collect the droplets and spread on the corresponding plates.

Figure 42

Checkpoint experiments – knockdown

Follow same steps as timepoint experiments with the following differences:

- 2 h after wash and switch to D-M, split the sample in half and add 1x doxy and the desired concentration of NAA to one half
  Example: Per 20mL cell culture, add 20uL of 1000x doxy and 40uL of 10x NAA.
- Add the same concentrations of doxy and NAA to the sorting tubes and plates which are used for the "+aux" samples.

Troubleshooting

- It is very important that there are NO air bubbles in the machine. This is why we go through the process of debubbling all the filters. Air bubbles cause the droplets to destabilize. If you suspect any air bubbles, redo DI water filter debubble and/or sheath filter debubble.
- When turning off the machine, check that the "keep DI water in sample probe" is on. This is very important, because if there is no water in the sample probe and the machine is not in use for a long time, the excess sheath in the sample probe can crystallize and clog the probe.
- If someone is using the machine after you, email them to ask if they want you to keep the machine on. If that is the case, then before leaving do the following:
  - "bleach rinse"
  - "shutdown rinse"
  - Settings > adv. Settings > pressure options > standby
  - Do not log out, or else the calibration will no longer be valid
  - The next person can then use the same calibration by: Settings > adv. Settings > pressure options > ready + wait until the blinking green dot at the bottom of the screen next to the image of the stream (Figure 35f) stops blinking. Log out + log in with their own account.

- Similarly, if you leave for >20 min, or there is some problem with the machine and you want to fiddle around with it, put the stream in standby
- If the sample probe is clogged:
    - Turn off machine/put the stream in standby
    - Open the top
    - Disconnect the probe and unscrew it
    - Carefully pull it out
    - Fill a small syringe with DI water and attach it to the probe
    - Squeeze the syringe until the probe unclogs
    - Screw the probe back in (CAREFUL not to press down on the probe when you screw it in as this will change the position of the probe) and attach it
    - Close the top
- If the probe bends in the sample tube or is too high up and doesn't reach the sample: open the top and very carefully push/pull the probe up or down a bit
- If there is a problem with the side stream calibration: clean the 3 sensors in the sorting area with the special paper that is on the table and ethanol
- If the sorting efficiency is too low, diluting the sample can help.
- For a long experiment, you might encounter the following errors:
    - A warning that asks you to calibrate the sorting lasers again. You should not ignore this warning. Press sort calibration and follow the instruction (it is pretty much the same as auto calibration).
    - A warning that warns about the sheath tank running low. If you plan on doing a long experiment, be sure that the spare sheath tank is full in case you need it. Change the tank when this warning appears. This shouldn't be an issue if you start with a full tank and always place the machine in standby between timepoints.
- If you are unable to insert/remove a chip from the machine, use the "emergency load/unload" dial (Figure 13).