A) Starting the instrument

1) Turn on the computer, log into the operation system. Password: Welcome#1

2) Turn on the SpectroFlo program, it’s either on the desktop or the bottom menu bar.

3) Log into the SpectroFlo program, use the username and password provided by the flow core.

4) Select the “Acquisition”.
5) At the bottom right corner, the status is “Disconnected”.

6) Turn on the Aurora on the left hand side of the instrument. There is only one button. The status will change to “Connecting” and then “Connected”.

7) Sheath and Waste: the core staffs will take care of the sheath and waste in the morning and evening. In case you need to run the instrument in the weekend or late night, the core staff will show you how to change the sheath and empty the waste.
B) Cleaning the instrument

1) Fill the bleach labeled tube with 2ml. DON’T fill more than 2ml. The tube has a 2ml labeled mark. Plug into the sample injection tube (SIT).

2) Go to the left hand side of the software, click “Cytometer”, then “Clean Flow Cell”.

3) Wait until it’s done.
4) Then click “SIT Flush”, wait until it’s done. Repeat “SIT Flush”

5) Fill the water labeled tube with 3ml of water. The tube has a 3ml labeled mark. Plug into the sample injection tube and repeat step 2) to step 4).

6) Repeat the water cleaning cycle one more time, i.e. step 5.

7) The machine is ready.
C) Setting up the template

1) Go to the left hand side of the software, click “Experiment”, then “New”.

2) A new window, “Create New Experiment” pops out, first rename the “Name”
3) Click open the arrow in front of the laser, you will see a list of the color you may use.

![Image of a list of colors]

4) Click the color in your panel individually (a) from the list in the blue, violet and red laser, and hit "Add" (b). The color will appear in the “Selection” box. Double check the list and hit “Next” (d). In this instruction, only FITC, PE, and PerCP are used.

![Image of a selection box]
5) In the new page, Click “Reference Group”.

![Create New Experiment]

6) Regardless what you use in the single control, beads or cells, leave the setting and click “Save”

![Create Reference Group]

7) The single color control will be created under the Experiment.
8) Click “Group” (a), a new group with a tube is generated (b). Enter the name for individual colors (c) and click “Next” (d).

9) The experiment hierarchy will open. There are three levels: experiment level, folder level and tube level. If you apply anything at experiment level, it applies to all samples in the experiment; if you apply anything at folder level, it just applies to all samples in that folder; if you apply anything at the tube level, it just applies to a particular sample tube. You can set the stopping criteria based on gate, events, time, etc.

10) You can leave all the settings under the reference group. At the Group_001 level, a) change the worksheet to “Default Unmixed Worksheet (Unmixed)” and b) enter the cell number you want
for each tube at “Events To Record”. The “Default Unmixed Worksheet (Unmixed)” will be modified later for specific experiment.

<table>
<thead>
<tr>
<th>Name</th>
<th>Worksheet</th>
<th>Stopping Gate</th>
<th>Storage Gate</th>
<th>Events To Record</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstained (Cells)</td>
<td>Default Raw Worksheet (Raw)</td>
<td>P1</td>
<td>P1</td>
<td>10,000,000</td>
</tr>
<tr>
<td>FTC (Cells)</td>
<td>Default Raw Worksheet (Raw)</td>
<td>P1</td>
<td>P1</td>
<td>10,000,000</td>
</tr>
<tr>
<td>P1 (Cells)</td>
<td>Default Raw Worksheet (Raw)</td>
<td>P1</td>
<td>P1</td>
<td>10,000,000</td>
</tr>
<tr>
<td>P2 (Cells)</td>
<td>Default Raw Worksheet (Raw)</td>
<td>P1</td>
<td>P1</td>
<td>10,000,000</td>
</tr>
<tr>
<td>P2CF (Cells)</td>
<td>Default Raw Worksheet (Raw)</td>
<td>P1</td>
<td>P1</td>
<td>10,000,000</td>
</tr>
</tbody>
</table>

**Group 111**
- Default Unmixed Worksheet (Unmixed)
- All Events
- All Events
- 10,000,000

11) Then click “Save and Open”, the template will be opened. Four windows are required for the data acquisition, A) Experiment; B) Acquisition Control; C) Instrument Control; and D) Worksheet. You may double click the header of the “Experiment” window and change the name of the experiment.

![Diagram](image.png)
12) Sometimes the “Acquisition Control” and “Instrument Control” are hiding at the bottom left corner. Click the “Acquisition Control” title a), then the pull down manual b), and finally “Float”, the window will come out, repeat the same for the “Instrument Control”.

13) The control for two windows are displayed now and you can move them freely to anywhere in the template.
14) Under the Instrument Control window, click the pull down menu and select “Cytek Assay Settings for Users”.

15) Immediately hit the “Save As” a). A window pops out b), give the setting a new name that is meaningful to you c) and hit “Save” d). Now the user setting has changed to the new name. This is going to be the settings for your SAME experiment moving forward and it stays in the pull down menu under “User Settings”.
16) For the “Acquisition Control”
   a) “Start”: if there is a sample tube in the SIT, a beige tube will come down from the machine to
      the bottom of the sample tube for sample uptake. It’s the time to adjust the FSC and SSC in the
      Instrument Control”. DON’T take out the sample tube while the “Start” button is active.
   b) “Stop”: The beige tube will retract into the machine. It’s the time to do take out the sample tube.
   c) “Record”: after the FSC and SSC are adjusted, you can start to record the data.
   d) “Flow Rate”: click the flow rate pull down menu and you will be able to adjust the flow rate.
      ATTENTION: you need to change the flow rate before you hit “Start”. You are allowed to change
      the flow rate while you are running the sample, however, the change of flow rate won’t kicks-in.
   e) “Flow rate”:
      Low: around 10ul/min
      Medium: around 28ul/min
      High: around 60ul/min
      If it’s far from the value above, please contact the flow core.
   f) “Events to Display”: the number of event to display real time.
17) Changing from sample to sample. If there is no tube in the SIT, the orange light is on, which means you cannot acquire the sample and in the “Acquisition Control”, the “Start” button is inactivated. Once you put in the tube, the orange light is off and the “Start” button is activated. If you put in the tube and the orange light is still on, it means the machine is doing SIT Flush and you will hear noise from the machine. Just wait until the noise disappear and the “Start” button will be activated again.
D) Data acquisition

1) Put in the unlabeled cells into the sample injection tube (SIT), make sure you click the space in front of the “Unstained (Cells)”. The green arrow indicates the active tube. Please see Appendix A for the single color control preparation guideline.
2) Hit “Start” in the “Acquisition Control” window. Adjust the FSC and SSC a) so that your population appears in the desired position in the FSC/SSC plot in the “Default raw worksheet”. You will notice that an asterisk will appear in the settings name b). Hit save c) and the asterisk will disappear d). The asterisk is the reminder for any changes. It applies to the “User Settings”, “Worksheet” and “Experiment”. Once the FCS and SSC is set, hit “Record” in the “Acquisition Control”.

3) Once the FCS and SSC is set, hit “Record” in the “Acquisition Control” window. The software will record the data until it reaches the assigned number. Please pay attention to the sample tube, don’t let the sample run dry. If the sample volume is getting low, hit the “Stop” in the “Acquisition Control” window.
4) Once the sample tube has saved data, the tube change to “filled” tube a), which is different from the sample tube without data b). If the cell number reaches the designed number, the “green arrow” will automatically switch to the next sample. Otherwise, you will need to activate the next sample tube manually by clicking the space in front of the tube.

5) Continue with all the single color control under the “Reference Group”. While you are running each single color control, make sure you see the distinctive signature for each color. Please refer to Appendix B for the signatures.

6) After you saved the complete set of unstained and single control a), hit “Unmix” b).
7) A new window pops out, hit “Next”.

8) The number of row in the new window depends on the number of color of your experiment. In this example, there are four row, FITC, PE, PerCP and Unstained. Each row there are three plots: FSC/SSC a), P1 (color) b), and Positive (color) c).
9) Press the control key while adjusting the P1 gate in the FSC/SSC plot to select the right population. The P1 gate in all the colors will move together once the control key is pressed, it helps to save some effort to move individually a). Move the Positive gate in the P1 (color) plot to select the positive population b) and move the negative gate in the P1 (color) plot to select the negative population c). The distinctive signature of that color will be shown on positive (color) plot d). Normally the program automatically selects the brightest channel in the positive (color) plot d). If not, you can also move around. If you decide to move around, you may also need to move b) and c). NOTE: moving the channel in the positive (color) doesn't affect the unmixing calculation. The signature should roughly match the signatures provided by the vendor (Appendix C). Hit “Live Unmixing” e) when all the populations are selected and signatures are confirmed.

10) After unmixing, you will see the “spectral sign” in front of each sample tube.
11) Now move your green arrow to the actual sample a). The active worksheet changed to “Default Unmixed Worksheet” b), and hit the “save as” icon c). Once the window pops out, rename this worksheet to the name that you can recognize, in this case “Calibrate unmixed worksheet” and hit “saved”.

12) Then hit “Edit” a) in the experiment window. A new window pops out, hit “Acquisition” b); then in the Sample (or the name you put in) level, click the pull down manual c), the newly saved template will appear, select that newly saved template and hit “Save and Open” d).
13) Now the active worksheet will change to the newly saved worksheet a). You can delete the old worksheet b). In the template c), you may add the plots required for visualizing the data during acquisition. You can add more tubes by clicking the “Tube” d) in the experiment window. Please pay attention to the level of the new tube, it should appear the level under the Sample folder e). For unknown reason, it may appear the same level as the experiment folder f). If that’s the case, delete the tube at the wrong level, click the “Sample” folder and hit the tube d) again.

14) Continue until you finish all the samples.

15) If you see any asterisk in the experiment window, worksheet window and Instrument Control window a), you can save them individually. If you see the asterisk for “Default Raw Worksheet”, you can ignore it.
16) Now you can close the whole experiment a). If the only item that is unsaved is “Default Raw Worksheet” b), you can hit “No, do not save” c). If there is more than one item, hit “Cancel” d) and go back to step 15) above. After that repeat this step i.e. step 16).
E) Autofluorescence subtraction (Re-requisition of data is not needed)

1) You can take advantage of the spectral flow properties by subtracting the autofluorescence. After you close the experiment, hit “My Experiment” a), look for your experiment b) and right click that experiment, then “Duplicate” c). A new experiment is created with the extension “-Copy 1” d), double click on that new experiment.
2) Rename the experiment a) by adding the subtraction at the end b) (or whatever you like).

3) Go back to D) Data Acquisition and start from step 6) to 9). Only when you repeat step 7), check the “Auto Fluorescence as a Fluorescent Tag”.

![Diagram of data acquisition software interface]
4) For certain experiment, the autofluorescence subtraction helps a lot. In this case, the Calibrate beads from BD has high level of autofluorescence, so it improves the separation.

Without

With subtraction
F) Shutting down

1) After saving all the data, experiment, instrument setting, etc and close the experiment. Hit the “Cytometer” icon on the left hand side of the software a), then hit “Fluidics Shutdown” b) and follow the steps c).

2) Sign out from the program and close the program completely. Otherwise, you won’t be able to transfer the data.

3) On the Desktop, you will find a folder named “FcsFiles – Shortcut”. Click into that and look for your folder. For each experiment, there are two folders: raw and unmixed. Please transfer both in case you need to troubleshoot. Transfer the data to network drive immediately. We don’t backup anything. The Aurora follows the data policy of CCTI flow core.

G) Repeat the same experiment

Option:

1) Set a brand new template, just follow this manual from the very beginning.

2) Reuse the template (not recommended), open the old experiment and add more tubes. You can save the effort to prepare the single color control and acquire them. However, you may not have perfect data.
Appendix A

Sample preparation

The sample prep is exactly the same as conventional flow. Below are the differences:

1) For single color control, cells are always preferred over beads.

2) If you need to use beads, please use the ultracomp from Thermofisher (01-2222-41).

3) You need the unlabeled beads and unlabeled cells.

4) You need to wash the beads after the staining.

5) If you are using V500, only cells can be used. Otherwise, the unmixing will be completely wrong.

6) If you are doing intracellular staining, the single color controls need to go through the same protocol as the real sample, i.e. fix, perm, etc.

7) For surface staining, always block with Fc block (Biolegend: human: 422301; mouse: 156603).

8) For intracellular staining, if possible, block with heparin (Sigma H3393) at 100 U/ml (from 100x stock = 10 kU/ml):
   - after fixing and washing, resuspend cells in 50 μl 1x PermWash
   - add 1 μl 100x heparin stock, mix well gently, incubate on ice for 5 min
   - prepare 2x conc IC ab cocktail in 1x PermWash (50 μl per sample, 2x conc!!)
   - add 50 μl 2x IC ab cocktail to 50 μl cells and mix well gently. Incubate on ice for 20-30 min
   - wash twice with 1ml 1x PermWash
Appendix B

- Unless it is specified, this is the pattern of beads, not cells.
- You may use this as reference while acquiring the single color control.
- For unmixing, please refer to Appendix C
BB515
Alexa 532
PE-Cy5
PerCP-Cy5.5
BB700
PE-Cy7
Pacific Blue
V500 beads
NEVER USE BEADS
V500 cells
BV650
Alexa 700
NIR beads
NIR cells
Appendix C
Blue Laser Excitable Dyes Unique Signatures

- BB515- sVio515- Vio515
- AF 488, FITC, vioBright FITC
- AF 532
- PE Dazzle594, PE CF594, PE eFluor 610, PE Tx Red
- BB700
Violet Laser Excitable Dyes Unique Signatures

- BV421
- SB436
- Pacific Blue- eFluor450-vioBlue
- BV480
- eFluor506
- BV510-vioGreen
- BV510
- BV570
- SB 600-BV605
- BV605
- BV650
- SB702-BV711
- Qdot705
- BV750
- BV785
- Qdot800
- Qdot605
- Qdot655
- Qdot705
Red Laser Excitable Dyes with Unique Signatures
In house unique spectra

Live/dead

[Graph of DAPI intensity across channels]

[Graph of Zombie NIR intensity across channels]
UV excited dyes

- BUV496
- BUV563
- BUV661
- BUV737
- BUV805
Violet excited fluorescent protein

- eBFP
- eCFP
- eCerulean3
Blue excited fluorescent protein

- eGFP
- mVenus
- eYFP
- ZsGreen
Red excited fluorescent protein
Not optimally excited, test it before you use or wait until the 561 laser is installed