Flow Cytometry SOP

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Revised: DJE 2007

General Information

Flow cytometry is a powerful technique for characterizing cells and quantifying aspects about their components (e.g. size, internal complexity, and surface markers). Samples of cells, usually stained with antibodies to cell-surface molecules or proteins, are injected into the cytometer. Once there, the cells are focused into a one-cell-wide beam by a fluidics setup consisting of a fast-moving sheath fluid (usually PBS) flowing around the slower moving solution of cells. The beam of cells is directed to an observation area where two general methods are used to analyze each individual cell. First, light scattering techniques (1. forward scatter (FSC), which is proportional to the size of each cell, and 2. side scatter (SSC), which is proportional to cell-complexity, or number of internal compartments) give information on the size and composition of each cell. Leukocyte sub-populations can be determined using scattering techniques (especially for determining between lymphocytes, monocytes, and neutrophils). The second detection technique relies on fluorescence. Excitation sources within the cytometer (usually lasers) excite fluorophores in probes attached to the cells, and several detectors measure the resulting emitted photons.

General notes:

- Does not need to be sterile
- Coulter Epic XL can process > 1000 cells/sec
- Beckman FACSCalibur is generally better than the XL (more detectors, two lasers, etc.)
- Note: when wash cells and dump out supernatant, ~150 µL remains with the sample
- When dumping out supernatant, turn over tube and shake – but don't re-suspend cells by letting excess supernatant contact the pellet. Also, DON'T DUMP TWICE!! You will lose your pellet, which is bad…
- Normally, ~10,000 cells is a good number to process in a flow cytometry experiment
- Limitation is antibody amount – too many cells can be bad
- When it comes to probes, Pharmingen, Invitrogen (Molecular Probes) make good markers. Ancell, R&D Systems (which is GREAT for ELISA) are not as good
- Steve Lundy uses PBS w/ 2% NCS/(very small amount of azide – prevents capping of antibodies) as his wash, FACS solution – according to the flow core, it is actually better to NOT use serum. We use dPBS (no Ca, and no Mg) with 2% FBS.
- Fixing cells, which can be accomplished with 4% paraformaldehyde or 70% EtOH (added drop-wise), is useful for looking at cells a long time after they are drawn from blood
- Usually, for FACS, we want a concentration of 1 millions cells/mL, and probably only 0.5 mL total volume
- Don't have less than 100,000 cells!!

- The order of brightness of fluorescence: unlabeled < biotin < FITC < PE < APC / cychrome

Staining Procedure:

Work fast and work cold (4 degrees) – keep buffer, cells, and antibodies all at 4 degrees C

1. We have cells that are either PBMCs or separated cells from magnetic bead separations
2. Divide up cells into cytometry tubes (5 mL Falcon Tubes) – put ~100,000 - 1,000,000 cells in each tube, or as many cells as possible (but less than 1,000,000)
3. Fill each cytometry tube (containing cells) to around 3 mL level, using the cytometry wash buffer (98% dPBS (no Ca, no Mg), 2% FBS)
3. Centrifuge at 250g for 6 min – dump out supernatant and resuspend cells by vortexing each tube at level 6 for about 8 seconds
4. Cells should be in ~150 µL buffer in each cytometry tube
6. Label cells – all of our antibodies are BD/Pharmingen, we want to add 20 µL of each antibody of interest to each cytometry tube (with a pellet of cells)
    - Add 20 µL of each antibody to the correct tube – repeat for every antibody being used
    - Mix gently (vortex at about 4 for a few seconds)
    - Incubate for 30-45 minutes at 4 degrees C in the dark (ie the refrigerator)
7. Take out of the refrigerator and fill each tube to 3 mL using cytometry wash buffer
8. Centrifuge at 250 g for 6 minutes
9. Dump out supernatant (exactly as before) and resuspend by vortexing each tube at level 6 for 8 seconds
10. Repeat steps 7-9 twice
11. You should have 150 µL in each cytometry tube, and the cells should be resuspended
12. For the final suspension, add 200 - 350 µL wash buffer (depends on the initial number of cells) to each tube, cap each tube, and let sit in the refrigerator until taken to the Michigan flow cytometry core

Using the FACSCalibur

General

- Detectors:
    - FL1 => FITC
    - FL2 => PE
    - FL3 => PI/Cychrome
    - FL4 => APC
- The Sphero beads are in the fridge – they have a blue cap, and usually we should use 1/3 mL to calibrate the machine
- If the machine is idle for more than a moment, put the H2O tube on the machine and press “STANDBY”

- For every color we use, we need a negative control (NOT PE, FITC, APC, or CYCHROME) and a single positive control for each color we are using

Procedure

1. Turning on the machine
   - Before turning on the machine, check the saline levels and the waste levels
   - Open the top shelf of the machine – saline is on the left (looking at the machine), waste is on the right (saline = pressurized, waste = not pressurized)
   - If saline is low, first, depressurize the machine, then remove the black plate, and then fill the saline container (while placing the probe on a glove to not contaminate it) with the saline in the blue jars by the sink
   - If the waste is high, remove the container and pour down the sink (making sure to put the opening of the container near the drain so as to not splash waste outside the sink)
   - With the waste container, pour in a layer of bleach into the container, and replace in the machine
   - To reduce air bubbles, pull back the clip on the clear tube near the saline line

2. Make sure that machine is on when the computer starts up
   - Username: FlowCoreUsers
   - Password: flowlab

3. Open the CellQuestPro program to run the system-check program (sphero)
   - a. Open the sphero setup file: File ->Open->Data1->Setup-> sphero
   - b. Connect to the Cytometer: Acquire -> Connect
   - c. Open the instrument settings for sphero: Cytometer -> Instrument Settings -> Open -> Data1 -> Sphero Inst -> Set -> Done

4. Make sure the machine is primed
   - If we replaced the saline container, let's prime the machine with saline
   - Take the H_2O tube off of the machine, and press "PRIME"
   - Let the machine prime for a few moments
   - To get rid of air bubbles, switch between "PRIME" and "RUN" 3x [CONFLICTING REPORTS – ONE PERSON SAID THIS WORKS, ONE SAID NO]

5. Run the beads sample
   - Put machine in the "Hi" position and hit "RUN"
   - On the software, hit "Acquire"
   - If using APC, then we need to check the second laser: go Cytometer => Time Delay
   - Look at the peaks of the beads – should be tightly focused peak – otherwise, might need to get new beads

6. Now we're ready to get ready to run our samples – let's check the voltages for the cells in our samples. We want to have the negative control be in the first decade for all detectors

- Cytometer => Instrument Settings => Open => Sample Files C1 => Mayer => Set => Done
- If not open, let's open some windows for our setting of voltages: 1) Voltage window: Cytometer => Detector/Voltages; 2) Compensation Window: Cytometer => Compensation; 3) Parameter Descriptions: Acquire => Parameter Description
- Note: in the parameters box, if "Setup" is checked, then there will not be a save
- Change the Parameter Descriptions in the Parameters window to reflect the current sample being examined
- In the parameters box, we should set up file names to save data. We should click **"Save Data" and create a folder with the name template "C1MMOCT3105"**, with the last part signifying the date. Under **"file name", we should create the template header: "C1MM103105"**. Under file count, we should start at 1.
- Note: to edit labels on the quadrant stat sheet, just click on the label box and go "STATS"=> Edit Quadrant Stats.
- To see different gating regions, just go "Gates" => Gate List
- To change gating for a graph, just go "Windows" => Show Inspector
- To draw new gates, just go Toolbox (left side) => draw regions/gates
- To change the number of cells to count, just find "Acquisition and Storage"

7. Put the sample containing the negative control into the reading slot
    - Adjust the voltages in the voltages window, such that each graph displays the negative control in the first decade (ideally, centered in this decade)
    - Also note: we want the SSC and FSC graph to be consistent – to adjust voltages on these two graphs, we adjust the voltage for SSC and the gain on FSC (E00 => lymphocytes)

8. Once voltages are setup, we need to adjust compensation
    - Use positive control for each color (run one at a time)
    - Note: in the compensation box, the color to the right (eg FL1) is the main color being detected, and the color to the left (FL2) is the color that is erroneously being detected
    - Try and make mean of negative-negative the same as the mean of the negative-positive (the dots should be roughly along the same vertical or horizontal azis)
    - Be sure to save graphs of each compensation run

9. Now, we're ready to run our samples
    - It should be as easy as placing the sample under the machine and pressing run and acquire!!

10. After finishing (for the day I think), we should clean the machine by placing first H2O under the machine and pressing "RUN" => "HI" for 3 minutes. Then place bleach in the machine and do the same thing. Finally, wash with H2O once again…