

Protocol for FACS analysis of HeLa cell transfectants

You can refer to: Marks *et al.*, 1995, J. Cell Biol. 131: 351-369; Voorhees *et al.*, 1995, EMBO J. 14: 4961-4975; or Marks *et al.*, 1996, J. Cell Biol. 135: 341-354 for the protocol.

Reagents

Release buffer: Phosphate buffered saline pH 7.4
10mM EDTA

FACS buffer: Phosphate Buffered Saline pH 7.4
5% fetal bovine serum (or 0.5% BSA)
0.02% sodium azide
Ice cold

Antibodies - either directly conjugated or with secondary antibodies (mostly use fluorescein [FITC]- and/or phycoerythrin [PE]- conjugated antibodies. Can use Cychrome as a third colour, and there are others that can be used as 4th and 5th colours).

For intracellular staining:

Medium
1% FBS
0.5% saponin
Ice cold

Fixative: Phosphate buffered saline pH 7.4
2% (w/v) formaldehyde (note - stock is 37%)
(for intracellular staining, use 1% formaldehyde)

Procedure

Note 1: This procedure is for HeLa cells and some other adherent cell lines. For staining of non-adherent cells, simply spin out the desired number of cells and proceed from step # .

Note 2: You can get away with as few as 20,000 cells in total. It is best if you can start with 5×10^5 - 1×10^6 , and if you use less, you may have to titrate down your antibody. This protocol assumes you have prepared for unlimited supply of cells.

1. Release cells from plate. I try to get cells at about 50% confluence - this gives around $2-5 \times 10^6$ cells, which is great for doing staining and controls. Obviously, you do not want to trypsinize, since this may remove your cell surface molecules. HeLa and many other adherent cells will release from dishes by treatment with EDTA. It generally does not harm the cells, and they can be washed out of EDTA and replaced back in culture, if necessary.

- suck off medium from 10cm dish
- add back 3ml of PBS/10mM EDTA to each plate. Incubate for about 5-10 min at RT.
To speed things up, place plates at 37°C.
- when cells are rounded and loose (look under microscope to be sure that they are rounded and are beginning to detach from the plate), add back at least 4ml of medium with FBS to each plate
- pipette up and down to get all cells off of plate and to create single cell suspension

- count an aliquot of cells and spin out the rest.
- resuspend in a convenient amount of medium with FBS and leave on ice

1a. IF YOU ARE DOING INTRACELLULAR STAINING: You must fix the cells 1st, and then do all subsequent incubations with 0.5% saponin present.

- resuspend cell samples (see below for #s) in PBS/ 1% formaldehyde. Incubate 15 min, RT
- pellet cells and wash 2X with medium/1%FBS/0.5% saponin
- follow procedure below, but include 0.5% saponin in all incubation and washing steps

2. Staining with primary antibody. It is critical to think of controls here. If you are doing simple single staining with directly conjugated antibodies, then it is kosher to use unstained cells as a negative, as long as you have some negative cell population (*e.g.* untransfected cells) from which you can determine a baseline of staining with your antibody. If you do not, then you must use an irrelevant antibody as a control. If you are doing single staining with a secondary antibody, then you must include (i) secondary antibody alone and (ii) an irrelevant antibody and secondary antibody. If you are doing double staining (*e.g.* one Ab conjugated with PE, another with FITC), then in addition to these controls, you must include at least one sample stained with each antibody alone in order to set your compensation on the FACScan - preferably a sample that you know (or think) will be very positive. It is always a good idea to have an unstained sample of each cell type to set scatter and gates without wasting precious stained samples.

Depending on the number of samples, I will do the staining in either FACS tubes (12x75 Falcon 2052 tubes), microcentrifuge tubes (easiest, I think, for small number of samples - spins are fast), or 96 well plates (easiest, I think, for >18 samples); eventually, all samples must be transferred to FACS tubes, so many people do the staining in these

Finally: this is my protocol. Other people will do things like only adding 10 μ l of antibody, but at 5X the concentration. Obviously, this works too. To me, this is easiest and standardizes everything the best, but then again, what do I know.

- spin out equal cell numbers for control and experimental staining (like I said, I try to shoot for 5x10⁵ or 1x10⁶, if possible; if not possible, you can go as low as 2-5x10⁴)
- resuspend in 50 μ l of appropriately diluted primary antibody (you must pre-determine the titer of the antibody)
- incubate on ice, 30-60 min
- dilute with 500 μ l of FACS buffer; spin out cells
- wash 2X more with FACS buffer

3. Staining with secondary antibody. If using a secondary antibody, it's pretty much the same as the primary. Again, be sure to have at least one sample stained with only one each of >1 antibody to set compensations.

- resuspend cells in 50 μ l of appropriately diluted secondary antibody
- incubate on ice, 30 min (you can cut to 15 if you are in a hurry)
- dilute with 500 μ l of FACS buffer; spin out cells
- wash 1x more with FACS buffer

4. Optional: Fix cells. If you are scheduled to go on the FACScan now, all you need to do is resuspend your cells in 0.5-1ml FACS buffer (as little as 200 μ l if you have very few cells) and make sure your samples are in FACS tubes. However, you can stain the cells

one day and examine them one or two days later if you fix the cells after staining.

- resuspend cells in PBS/ 2% formaldehyde (~100 μ l) - incubate on ice, 15-20'
- you can leave the cells in the fridge, or spin out and resuspend in 0.5-1ml FACS buffer -
I prefer the latter, but it probably doesn't matter
- just be sure to wash out fix before you apply to FACScan

5. Optional: stain with PI. You need to exclude dead cells from you analysis. You can do this either by gating on cells of an appropriate size and granularity by using the side scatter and forward scatter measurements, or by excluding dead cells that take up the vital DNA stain, propidium iodide (PI). If you have fixed your cells, you cannot use PI - it will stain all of your cells. If you have not fixed your cells, add PI to _____. You must then use two- or three- colour fluorescence on the FACScan. Be sure to have one sample unstained completely, and one sample stained with PI alone for setting compensation.

Running the FACScan using CellQuest 1.2 software

1. If machine is off, turn it on. Make sure dial is set to "Standby", that there is a tube of deionized water in the chamber.
2. Check that the reservoir is full of FACS buffer (PBS) and the waste reservoir is empty.
3. Turn on computer. Select "Chooser" from under apple. Select "Appleshare", and open up either of our lab's computers. You will transfer data to here when done, and you would be well off to transfer the settings files and any file templates into your CellQuest document.
4. When FACScan reads "Standby" (instead of "Not ready"), fill and drain the cuvette three times to clear out any possible air bubbles. Now set dial to "Run", and allow water to go through for 2-5 min. Then turn back to standby.
5. Open a CellQuest setup file appropriate for your analysis. Otherwise, open up appropriate graphs in a new file.
6. Under **Acquire** menu, click on "Connect to cytometer". If someone has used the FACScan before you, it may already be done. If it reads "cytometer connected" and yet there is no "Acquisition window", open the "Acquisition window" from under the **Windows** menu.
7. Under **Settings** menu, click on " " and on "compensation". These should pull down new menus on the screen. Drag them to convenient sites on the screen. Under **Acquire** menu, click on "counters". Drag the counter menu to a convenient site on the screen.
8. Under **Acquire** menu, click on "Parameter description". This will pull down a large menu. Drag this to a convenient site on the screen.
 - set the folder that you want to save your files under (save it to: BD Applications: CellQuest: Principle investigators: Marks: name&date folder - you may have to start a new Marks folder, and give your set of files a recognition name with your initials and the date in format of "MM970301" for Mickey Marks, 1997, March 1st)
 - set the filename that you want to save your files under (usually the same as the file set name) and set the file number to "01". This will automatically save your files as a series with the same name, numbered from 01 to whatever in series.

- set the description for the first sample (I give it the cell type, transfection, and what it's stained with in abbreviated format)
 - set the axes for the first sample
9. If you have a pre-set file of settings, then under **Settings** menu, open "settings file". Click on "open", then choose the file from the appropriate drive and folder (usually on our computer via AppleTalk); then click on "set" - this will convert the settings to your previous settings
- NOTE: THIS ONLY GIVES AN APPROXIMATE SETTING!!! YOU MUST RESET EACH TIME TO YOUR PARTICULAR SAMPLE AND FACScan INSTRUMENT!!!! NEVER ASSUME THESE SETTINGS ARE IT!!!!
10. Begin the process of setting. Make sure the box marked "Setup" on the Acquisition window is clicked - otherwise, you will start saving your stuff to files.
- Place your tube with the sample in the chamber. Turn the dial on the FACScan to "Run". I usually run the setup with the switch set to "Low"; if you have lots of sample, you can go "High".
 - adjust both the forward and side scatter until the bulk of the cells are in the center of a FSCxSSC dot plot. The voltage on the SSC is a fine adjustment; the amplitude is a course adjustment.
 - if using FSC and SSC for gating out dead cells, set your gates now. Draw a gate using either the circle, square, or non-descript options. Set your FL1 and FL2 graphs to these gates by using the "format" command under the **Plots** menu.
 - make sure that the level of FITC (FL1 channel) and/or PE (FL2 channel) and/or PI or Cychrome (FL3 channel) is at a nice negative looking area, but off of the axes (all below around 10 units)
 - now place in a FITC stained sample only. Adjust the voltage (FL1) until you have a decent reading. Now adjust the compensation for FL2-FL1 until FL2 is negative and off of the axis, but FL1 is still positive. You may want to fiddle both with the voltage of FL1 and the compensation.
 - now place in a PE stained sample only. Adjust the voltage (FL2) and compensation (FL1-FL2) so that you have a positive reading in FL2 but negative (and off axis) with FL1.
 - recheck both FITC and PE-only stained samples to make sure everything looks OK. Make minor adjustments as necessary.
 - if doing a third colour, need to impose this on top - it's a pain in the butt.
11. Once voltage and compensation are set, and gates are drawn, you are ready to acquire your data. First, open the **Acquisition** menu, and open the "Acquisition and storage" menu. Set how you want to save your data - usually I collect all events, but count based only on gated events. You may change these parameters later in your run.
12. Click off of the "Setup" switch under the acquisition window - the first file should appear in the window. Place in your first sample, and start to read.
13. For each subsequent sample, you can change the name of the sample and the names of the axes under the "Parameter Description" window. I find this very helpful. The program should automatically prepare you to save each subsequent sample in a new file (001, 002,

etc.).

- it is not necessary to switch to “Standby” between samples, but don’t leave the chamber empty for more than a few seconds
- you can definitely switch the speed to “high” while collecting your data.

14. WHEN FINISHED:

- rinse 5% bleach through the machine for 5 min at high rate
- follow with dI water for 5 min
- drain/fill 3X
- turn to standby. Empty the waste container and re-fill the reservoir.
- if you are the last person signed up, turn off the FACScan
- Copy the data files to our computer using Appletalk. If nobody is waiting, you can print out your files using one of the pre-set printout files or a new one that you set up.
- make sure you dump our computer icon when you are done, and exit CellQuest