

1. Flow Cytometry

1.1. Introduction

Flow cytometry is a fluorescence technique, using antigens with a fluorescence tag, expressing a particular colour, to attach to genes on the surface of the cell under analysis (as in figure 1). These molecules are specific for a certain gene or transcription factor, usually characteristic of the type of cell being analyzed, while they will absorb the light emitted by a laser beam on a certain extent, proportional to the amount of expressed genes in the sample. This quite advanced technique is capable of performing several different tests (one for each colour) on each cell, making the analysis of about 300 cells/sec (new machines can actually go until an incredible rate of 8000 cells.s⁻¹).

Therefore, by using the proper staining procedure for a cell culture, it is possible to count the amount (or percentage) of cells that express a particular gene, thus evaluating the differentiation extent of the cells, as previously described, by determining and distinguishing between sub-populations. Though not yet fully described,² the expression of particular surface markers can give an idea of the type of cells under observation. Hence, according to previous studies, which have been dedicated to confirm the expression of certain markers on particular cells, one can infer on the expected sub-populations expressing particular markers. There are several stains available commercially, while the flow cytometer available in the lab (a Coulter® Epics® Altra™) has 2 lasers. These are capable of emitting 3 different wavelengths that in turn are used for measuring the fluorescence of 4 different wave lengths, of which the following three will be used: fluorescein isothiocyanate or **FITC** (351-364 nm UV, 50 mW Power); phycoerythrin or **PE** (488 nm UV, 180 mW Power); phycoerythrin-Cy5 or **PE-Cy5** (633 nm UV, 10 mW Power).

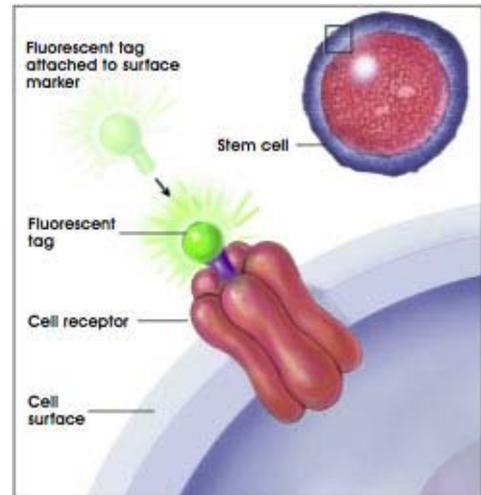
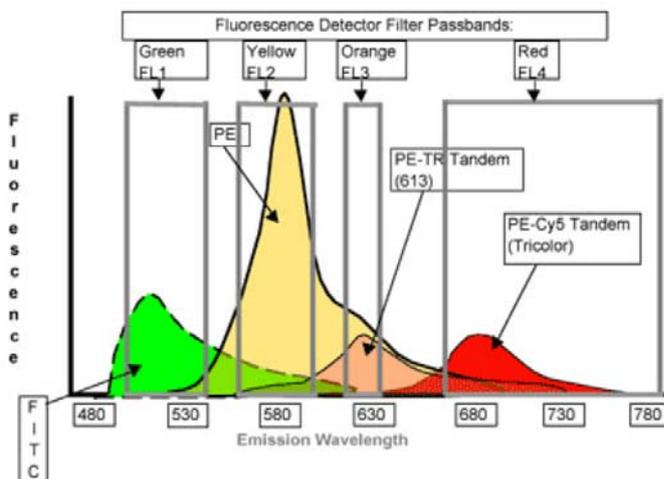


Figure 1: Identifying Cell Surface Markers Using Fluorescent Tags. (From The National Institutes of Health, 2006)¹

Each of these stains can then be used to trace the anti-genes, by tailoring the experiment so to have each anti-gene with a different fluorescence tag. However, as it can be seen from figure 2, the colours usually overlap, thus requiring the use of positive controls for measuring the



effective fluorescence of each stain, a procedure that is taken into account on the results section.

Figure 2: Emission spectra obtained from flow cytometry, showing the overlap of the different stains used, which in turn requires the use of positive controls, in order to trace the real amount of expression of each of these, separately. (From University of Berkeley, 2007)³

Antigen Markers

The markers chosen for running a 3-colour protocol in flow cytometry have been screened, by marking the cell lines used with 5 different surface markers, available in the laboratory, in order to assess which of these markers the cell lines would express: anti-human CD235a (marked with PE-Cy5), anti-human CD33 (marked with FITC), anti-human CD34 (marked with PE), anti-CD71 (marked with PE) and anti-human CD45 (marked with FITC). This experiment has allowed establishing the phenotype of the cell lines TERA-2 and K562 before inducing their differentiation. It was assessed that TERA-2 cells only express CD71, and none of the other tried markers, and that K562 cells express CD71, CD235a and CD45, but don't express CD33 or CD34 (data not shown).

Therefore, the three markers that are going to be used in this experiment are the following:

- ✓ Glycophorin-A (CD235a) – PE-Cy5 (isotype control IgG_{2b})
 - This antigen is expressed exclusively on human erythroid cells and their progenitors, being very useful to identify red blood cells due to its absence on the lymphoid or granulocytic progenitor cells; it is thought that its expression is not shown on erythroid colony forming cells in bone marrow, but starts to be up-regulated during maturation from pro-erythroid blast cells to mature erythrocytes.⁴ Being a well known red blood cell marker, CD235a is a valuable tool to assess the presence of red blood cells' lineage.⁵
- ✓ CD71 – PE (isotype control IgG₁)
 - This human Transferrin Receptor is expressed on proliferating lymphoblasts, majority of human tumour cell lines and normal peripheral blood monocytes. Cell surface expression of transferrin receptors is correlated with relative cell growth rate of malignant and non-malignant cells.⁴;
 - This surface marker is also expressed on early or immature erythroid cells, but the differentiation of reticulocytes into mature red blood cells is followed by a down regulation of this transferrin receptor. Hence, the down-regulation of CD71, combined with the up-regulation of CD235a, can indicate the presence of red blood cells (erythrocytes = CD71⁻/CD235a⁺).^{5, 6}
- ✓ CD45 – FITC (isotype control IgG₁)
 - CD45, also known as the leukocyte common antigen, is a transmembrane protein-tyrosine phosphatase (PTPase) that is expressed in haematopoietic cells except erythrocytes and platelets.⁴ Thus, the non-expression of this marker would allow to confirm the data obtained from the CD71⁻/CD235a⁺ phenotype (erythrocytes = CD45⁻/CD71⁻/CD235a⁺).

1.2. Sample Preparation

Materials

- Test antibody: mouse monoclonal conjugated to FITC, PE, PerCP or PE-CY5 Tandem (tradenames: Cy-Chrome or Tri-Color). If you're planning to label cells with 2 or more antibodies simultaneously, you need a negative control for each fluorochrome conjugate;
- Negative control sera: usually, purified mouse IgM or IgG of the same subclass as the test antibodies and conjugated to the same fluorochrome. One usually purchases them from the same manufacturer to get a similar fluorochrome-to-protein ratio as the test antibodies. *[In flow cytometry, fluorescence is relative. We need a negative control to determine where "positivity" begins]*
- Cells in suspension, counted and viability-checked. Keep them in culture medium supplemented with antibiotics and 2-5% fetal bovine serum, on ice. If viability is less than 90%, consider adding another fluorochrome to identify dead cells during analysis;
- FC Buffer (PBS + 2% FBS + 0.1% sodium azide). The sodium azide assists in preventing capping and shedding or internalization of the antibody-antigen complex after the antibodies bind to the receptors;
- FcR Blocking Reagent (Fetal bovine serum, FBS)
- 2X Paraformaldehyde Stock Solution:
 - Add 2 g. paraformaldehyde to 100 ml. PBS+azide;
 - Heat to 70°C in a fume hood, or in a 56°C water bath, just until the paraformaldehyde goes into solution;
 - Allow to cool to room temperature, and then adjust to pH 7.4 using 0.1 M. NaOH or 0.1 M. HCl, as needed;
 - Store at 4°C.

Procedure for Direct Immunofluorescence Labelling:

1. Prepare cell suspension: 5×10^5 - 1×10^6 cells.mL⁻¹ in each tube;
2. Centrifuge for 5 mins at 2x1000 rpm;
3. Use a pipette to remove the liquid. Be careful not to disturb the pellet. A slight amount of liquid can remain;
4. Add to each tube:
 - i. 20uL FBS
 - ii. 80uL Buffer

5. Staining:

a. 3-colour protocol:

Tube 1: [<i>Negative control</i>]	Unstained cells (don't add any stain)
Tube 2: [<i>FITC isotype control</i>]	100uL cells + 10uL FITC isotype
Tube 3: [<i>PE isotype control</i>]	100uL cells + 10uL PE isotype
Tube 4: [<i>PC5 isotype control</i>]	100uL cells + 10uL PC5 isotype
Tube 5: [<i>FITC positive control</i>]	100uL cells + 10uL FITC stain
Tube 6: [<i>PE positive control</i>]	100uL cells + 10uL PE stain
Tube 7: [<i>PC5 positive control</i>]	100uL cells + 10uL PC5 stain
Tube 8: [<i>Sample</i>]	100uL cells + 10uL CD34-PE + 10uL CD45-FITC + 10uL CD38-PC5

b. 2-colour protocol:

Tube 1: [<i>Negative control</i>]	Unstained cells (don't add any stain)
Tube 2: [<i>FITC isotype control</i>]	100uL cells + 10uL FITC isotype
Tube 3: [<i>PE isotype control</i>]	100uL cells + 10uL PE isotype
Tube 4: [<i>FITC positive control</i>]	100uL cells + 10uL FITC stain
Tube 5: [<i>PE positive control</i>]	100uL cells + 10uL PE stain
Tube 6: [<i>Sample</i>]	100uL cells + 10uL CD34-PE + 10uL CD45-FITC

c. Notes:

i. Isotype controls

- Use isotype controls of the same conjugate as the color stain. E.g. CD45-FITC IgG1 will use a IgG1 isotype control for FITC.
- If conjugates on isotype are dissimilar, all isotypes can be combined in one tube (i.e. just stain one tube of cells).
- If conjugates on isotypes are the same, use separate tubes of cells for each isotype control.

ii. Positive controls

- Positive controls do not have to be the same as the marker of interest. If an alternate positive stain can be used that gives good signal, it can be used as a positive control
- The purpose of the positive control is mainly for color compensation in this case therefore a good signal (nice broad band over the ranges) will be ideal

6. Vortex. Incubate for 30mins on the fridge at 4°C;

7. Centrifuge and wash cells with 1mL PBS (wash 2x);

8. Centrifuge again and then re-suspend cells in FC Buffer (1mL) for direct analysis or cells may be fixed in 1mL of 1% p-formaldehyde (50% buffer – 50% 2% p-formaldehyde).

1.3. Flow Cytometer Settings

In the flow cytometer being used, EPICS Altra from Beckman Coulter, the configurations of the PMT's (photo-multiplier tubes), which detect the signal variations received from the laser light, converting these into electrical signals, readable by the flow cytometer, are as follows:

- PMT1 – side scatter;
- PMT2 (Green) – 525nm (FL1) for FITC (fluorescein isothiocyanate);
- PMT3 (Orange) – 575nm (FL2) for PE (phycoerythrin);
- PMT4 – 610nm (FL3) for PI (Propidium iodide), used for DNA analysis and viability;
- PMT5 (Red) – 675nm (FL4) for PEcy5 (phycoerythrin-Cy5);

1.4. Flow Cytometer operation

1. Turn Flow Cytometer ON
 - a. First, turn ON flow cytometer, on top;
 - b. Laser key turn to ON;
 - c. Lift button on the left of laser key (to turn ON laser cooling);
 - d. Turn air conditioning ON to 18°C;
 - e. Turn ON laser on the control device of Coherent;
 - f. Wait for 30 minutes until laser warms-up.
2. Check flow cytometer fluids:
 - a. Tanks underneath flow cytometer: the one further to you should be emptied (waste tank); the one closer should be filled with Sheath fluid;
 - b. The reservoir, located on the left side of the tanks and in the bottom, should contain distilled water (rinse tank);
3. Turn valves ON:
 - a. After having distilled water and sheath fluid enough to run the samples, go to the right touch-screen of the flow cytometer;
 - b. Press “Control” → “Valves Screen” → “Power up valves”;
4. De-bubble system:
 - a. Press the black button, near the distilled water tank, for 10 seconds, in order to purge any air out from the tubing;
 - b. Press the “de-bubble” button on the main panel of the flow cytometer;
 - c. Repeat step 4c two more times, so to make sure there are no bubbles on the hydro-dynamic focusing chamber;

5. Start FC software on computer:
 - a. Click “Expo 32 for Altra”;
 - b. Always turn on cytometer before starting up the software!
6. Cytometer control screen (where FC setup is made):
 - Acquire Setup – differentiators;
 - Detectors – gain voltage;
 - Compensation – colour compensation, when two colours overlap;
 - Gated Amp. – gated amplifier;
 - Sorting – sort settings.
7. Run Flow-Check, to align the fluorescence signals:
 - a. Double-click “Flow-check” Protocol on the software;
 - b. Fill one sample tube with a small amount of “flow-check beads”;
 - c. Wash the sample tubing by pressing the “RUN” button on the cytometer and leave it running for about 30 seconds. Always perform this step before running new samples;
 - d. Place beads in the sample holder, located on the right side of the cytometer;
 - e. Reduce “sample flow” to 15;
 - f. Press the “RUN (▶)” button on the cytometer to start collection;
 - g. Choose “Settings” on the software to open the dialog box;
 - h. Check “setup mode” to allow vision of live data without recording (this will be helpful to configure the acquisition settings);
 - i. Click “START (▶)” in the software to see live data from the cytometer;
 - j. Make sure to adjust the sample flow on the touch-screen to a data rate of around 150;
 - k. Configure the parameters of every channel, by adapting the standard deviations of the program to the fluorescence peaks being read;
 - l. Uncheck “setup mode” to start collecting 10.000 events;
 - m. When done, stop sample flow on the cytometer and remove the vial from the sample holder;
 - n. Note the file name generated by the software (this will be localized in your directory, under “LMD” folder, and contains the data acquired).

8. Run first sample (negative control):
 - a. Wash the sample tubing by pressing the “RUN” button on the cytometer and leave it running for about 30 seconds Always perform this step before running new samples;
 - b. Place the first negative control on the sample holder;
 - c. Press the “RUN (▶)” button on the cytometer to start collection;
 - d. Check “setup mode” to allow vision of live data without recording;
 - e. Click “START (▶)” in the software to see live data from the cytometer;
 - f. Make sure to adjust the sample flow on the touch-screen to a data rate of around 300;
 - g. Configure the parameters of every channel, by selecting “detectors” in the settings dialog box, first by changing both forward scatter (FS) and side scatter (PMT1) in order to have your cell population within the plot FS vs. PMT1. Afterwards, change settings of PMT2, PMT3, PMT4 and PMT5 in order to have a signal just below 10^0 , in order to have a “zero” response of fluorescence;
 - h. Uncheck “setup mode” to start collecting 30.000 events;
 - i. When done, stop sample flow on the cytometer and remove the vial from the sample holder;
 - j. Note the file name generated by the software (this will be localized in your directory, under “LMD” folder, and contains the data acquired).

9. Run samples:
 - a. After fixing settings for the first sample (negative control), the parameters of the detectors shouldn't be changed any more;
 - b. Wash the sample tubing by pressing the “RUN” button on the cytometer and leave it running for about 30 seconds Always perform this step before running new samples;
 - c. Press the “RUN (▶)” button on the cytometer to start collection;
 - d. When a data rate of 300 is reached, click “START (▶)” in the software to start collecting 30.000 events from the cytometer;
 - e. When done, stop sample flow on the cytometer and remove the vial from the sample holder;
 - f. Note the file name generated by the software (this will be localized in your directory, under “LMD” folder, and contains the data acquired).
 - g. Repeat steps 9b to 9e until all samples are measured. For every new cell type, step 8 should be performed again.

10. Cleaning the Flow Cytometer:

- a. When finishing acquiring data, remove the last sample from the holder;
- b. Change “sample flow” to 100;
- c. Add a vial filled with **Bleach** to the sample holder and press “RUN (▶)”;
- d. Wait for 10 minutes;
- e. Add a vial filled with **Blue Clenz** to the sample holder and press “RUN (▶)”;
- f. Wait for 10 minutes;
- g. Add a vial filled with **Distilled Water** to the sample holder and press “RUN (▶)”;
- h. Wait for 10 minutes;

11. Turn OFF Flow Cytometer:

- a. Turn OFF laser on the control device of Coherent;
- b. Turn valves OFF: Press “Control” → “Valves Screen” → “Shutdown valves”;
- c. Put down button on the left of laser key (to turn OFF laser cooling);
- d. Laser key turn to OFF;
- e. Last, turn OFF flow cytometer, on top;

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1.6. Reference List

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