Basic Multicolor Flow Cytometry Wet Lab

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SURFACE STAINING PROTOCOLS for cells and beads

CELLS (peripheral blood cells)

3 ml EDTA anti-coagulated freshly collected human peripheral blood.

Antibodies and Buffers:

Reagent	Other details
Anti CD3 antibodies conjugated to different fluorochromes	In this wet lab we will use: CD3 FITC, CD3 PE, CD3 ECD, CD3 PeCY5, PerCP-CY5.5, CD3 Pe-CY7, CD3 APC, CD3 APC-AL750, CD3 Pacific Blue
Lysing solution (1x)	Manufacturer's protocol
PBS or stain buffer (PBS with 1% BSA)	

STAIN:

- Add 100 ul of whole blood (about 1 million cells) to 12x15 round bottom tube
- Add antibody to the appropriate concentration as determined by titration or as recommended by manufacturer.
- Incubate in dark at room temperature (RT) for 10-15 minutes

LYSE:

- Add 2 ml lysing (1x) solution.
- Vortex
- Incubate in dark at RT for 10-12 minutes (no less and no more!).
- Centrifuge cells at 300 g for 5 minutes.
- Discard supernatant and break the pellet by gently flicking the bottom of tube.

WASH:

- Add 2ml of PBS
- Vortex.

- Centrifuge at 300g for 5 minutes
- Discard the supernatant and break the pellet.
- Resuspend cells in 0.5 ml of Stain Buffer or PBS.

BEADS

Use Compensation beads appropriate to your antibody;

- some compensation beads are generic and will bind any species of antibody
- others are species-specific and will bind mouse, rat, or hamster antibodies
- many kinds available from different vendors

STAIN:

- Add 1 drop of positive and 1 drop of negative compensation beads to a 12 × 15–mm round-bottom tube.
- Add 100 µl of PBS or staining buffer
- Add antibody to the appropriate concentration as determined by titration or as recommended by manufacturer.
- Incubate in dark at room temperature (RT) for 10-15 minutes

WASH:

- Add 2ml of PBS
- Vortex.
- Centrifuge at 300g for 5 minutes
- Discard the supernatant and break the pellet.
- Resuspend in 0.5 ml of Stain Buffer or PBS.

Exercise 1: STAIN INDEX

In this exercise we will calculate stain index (Bigos, 2007), which is used to quantify the effective brightness of a fluorochrome/antibody on a cytometer.

$$\text{Stain Index} = \frac{\text{median positive} - \text{median negative}}{2 \times \text{rSD negative}}$$

The stain index uses the separation of medians of the positive and the negative populations, normalized to the width (robust Standard Deviation: rSD), of the negative population.

Stain Index is fluorochrome and cytometer specific: it is affected by the intrinsic fluorochrome brightness, antigen density, antibody affinity, and importantly here, by cytometer characteristics and detector voltage or gain.

SAMPLES:

Prepare cells or compensation beads stained with CD3 FITC antibody as in Staining Protocols

DATA ACQUISITION AND ANALYSIS

1. Ensure cytometer is functioning correctly by running daily quality-control procedure.

2. Create a new experiment

3. Create dot plots and histograms as follows:

    a. Create Forward scatter vs side scatter dot plot

    b. Set fsc ssc gate on lymphocytes or beads

    c. Create histogram in log scale for FITC parameter, gated on fsc ssc

    d. Set appropriate detector gain (PMT Voltage) (see Exercise 2) and threshold

e. Record data on 5000 beads/cells.

f. Create gates on negative and positive populations.

g. Create statistics view to show median fluorescent intensity and rSD (robust standard deviation) of positives and negatives

4. Calculate stain index according to the formula:

$$\text{Stain Index} = \frac{\text{Median positive} - \text{median negative}}{2 \times \text{rSD negative}}$$

Exercise 2: VOLTRATION

DETERMINATION OF BEST GAIN OR PMT VOLTAGE SETTINGS

In this exercise we will determine the optimum gain setting or photomultiplier tube (PMT) voltage for one parameter (FITC in this case), in order to maximize the sensitivity of the FITC detector. Beads or cells stained with FITC coupled antibody will be run at a range of gain or voltage settings, and the Stain Index calculated for each setting. This would generally be done for all parameters on a cytometer to determine the best settings for general use.

Hint: If doing this on all parameters, it saves time to create a generic template with a tube for each gain setting, with the same gain on all the parameters. For example, tube 'gain 400' would have a setting of 400 V on all parameters. This also gives a good idea of how much spillover you are seeing in the other detectors at equivalent gain settings.

SAMPLE:

Prepare cells or compensation beads stained with CD3 FITC antibody as in "Staining Protocols"

DATA ACQUISITION AND ANALYSIS (as in Exercise 1)

1. Ensure cytometer is functioning correctly by running daily quality-control procedure.
2. Create a new experiment
3. Create dot plots and histograms as follows:
    a. Create Forward scatter vs side scatter dot plot
    b. Set fsc ssc gate on lymphocytes or beads
    c. Create histogram in log scale for FITC parameter, gated on fsc ssc
4. Set FITC detector gain at low setting, eg 350 volts
    a. Record data on 5000 beads/cells.
5. Increase FITC detector gain by 50 volts, eg to 400 volts

a. Record data on 5000 beads/cells

6. Continue creating tubes in this way and record data at 50 volt increments for the entire voltage range

7. Analysis: (as in Exercise 1)

    a. Create gates on negative and positive populations.

    b. Create statistics view to show median fluorescent intensity and rSD (robust standard deviation).

8. Calculate stain index according to the formula:

$$\text{Stain Index} = \frac{\text{Median positive} - \text{median negative}}{2 \times \text{rSD negative}}$$

9. Plot stain index versus gain or PMT voltage setting. The optimal gain is the lowest gain that gives a maximal stain index, where it reaches a plateau.

If the positive population goes off the top of the scale before a maximum stain index plateau, for example at a medium gain setting, then your sample is too bright to test the full range of gain settings. Restain the beads/cells with either less antibody or diminish staining by adding unlabeled antibody.

Voltage/gain	Positive median	Negative median	rSD negative	2 x rSD	Stain Index
25					
50					
100					
200					
300					
400					
500					
800					
1000					
1500					
2000					

Exercise 3: Fluorochrome Brightness Comparison

This exercise uses calculation of the Stain Index to compare different fluorochromes coupled to the same antibody. The differences seen depend on the intrinsic fluorochrome brightness and the individual cytometer characteristics. Spillover can also be visually evaluated. This information will aid in choosing fluorochromes to detect antigens, bright fluorochromes are usually used for low density antigens and vice versa.

SAMPLES:

Prepare cells or compensation beads stained with CD3 antibody coupled to different fluorochromes (see table below), as in Staining Protocols

DATA ACQUISITION AND ANALYSIS

1. Ensure cytometer is functioning correctly by running daily quality-control procedure.

2. Create a new experiment

3. Create dot plots and histograms as follows:

    a. Create Forward scatter vs side scatter dot plot

    b. Set fsc ssc gate on lymphocytes or beads

    c. Create histograms in log scale for all parameters, gated on fsc ssc. Set these up in order, laser by laser, so that all parameters for each laser are lined up in rows. This makes it easy to see where the fluorochromes spillover into other detectors.

    d. Set appropriate detector gains (PMT Voltage) (see Exercise 2) and threshold

    e. Record data on 5000 beads/cells for each sample. `

    f. Create gates on negative and positive populations for each parameter.

    g. Create statistics view to show median fluorescent intensity and rSD (robust standard deviation) of positives and negatives

4. Calculate stain index according to the formula:

$$\text{Stain Index} = \frac{\text{Median positive} - \text{median negative}}{2 \times \text{rSD negative}}$$

CD3 Fluorochrome	Positive median	Negative median	rSD negative	2 x rSD	Stain Index
FITC					
PE					
ECD					
PE-Cy5					
PerCP-Cy5.5					
PE-Cy7					
APC					
APC-Al750					
Pacific Blue					

Which fluorochromes are the brightest?

Which show the most spillover?

Which would be most appropriate for a high density antigen?

Which for a low density antigen?

Exercise 4 : Manual Compensation

This exercise will show how to perform manual compensation for a simple 2 color experiment. Compensation is the procedure by which the level of fluorochrome spillover into other channels is calculated using single color controls and then corrected for, so that only the fluorochrome of interest is measured in each detector.

Keep in mind that this is an exercise to understand the compensation process, and it is preferable to use the automatic compensation calculation available in almost all software. Automated software calculation is more accurate, as all colors are compensated simultaneously, not sequentially as in the manual procedures.

In part B we will look at the effect that changing the gain or voltage has on the calculated compensation.

SAMPLES:

Prepare cells or compensation beads stained with CD3 FITC, or CD19 PE, or double stained with both, as per Staining Protocols.

DATA ACQUISITION AND ANALYSIS

1. Ensure cytometer is functioning correctly by running daily quality-control procedure.

2. Create a new experiment

3. Create dot plots and histograms as follows:

    a. Create Forward scatter vs side scatter dot plot

    b. Set fsc ssc gate on lymphocytes or beads

    c. Create dot plot in log scale for FITC vs PE, gated on fsc ssc.

    d. Set appropriate detector gain (PMT Voltage) (see Exercise 2) and threshold

    e. Record data on 5000 beads/cells.

    f. Create gates on negative and positive populations.

    g. Create statistics view to show median fluorescent intensity of positive and negative populations.

A Compensation calculation:

h. Using the FITC single color: adjust the compensation PE- x% FITC so that on the PE axis (i.e. in the PE channel), the median of the FITC positive is the same as the FITC negative. This correctly compensated sample now shows the single color FITC shows a positive population only in the FITC channel: all cells are negative in the PE channel.

i. Vice versa, on the PE single color, adjust the compensation FITC-x% PE so that on the FITC axis (i.e. in the FITC channel), the median of the PE positive population is the same as the PE negative.

j. These are the compensation values (PE- x% FITC and FITC-x% PE) that should be applied to the double stained cells.

B The effect of voltage change on compensation

1. Rerun the same FITC single color using the compensation value calculated above, but this time, increase the FITC PMT voltage by 50 volts. What happens to the medians? Is the compensation still valid?
2. Rerun the same FITC single color using the compensation value calculated above, but this time, decrease the PMT voltage by 50 volts. What happens to the medians? Is the compensation still correct?
3. Repeat this test with the PE single color using the compensation value calculated above, but increase or decrease the PMT voltage. What happens to the medians? Is the compensation still valid?

The lesson here is that compensation is dependent on the voltage or gain setting. If you change your voltage or gain setting for your samples, you **must** recalculate the compensation values using your single colors.

Supplementary Protocol

ANTIBODY TITRATION

Each antibody should be titrated before use to determine optimal antibody concentration to maximize discrimination of positive and negative populations. A serial dilution of the antibody is prepared, the cells are added, incubated and washed and the stain index at each dilution is calculated. This protocol is an 8-point doubling dilution series starting at 2× the manufacturer's recommendation; more points can be added or the dilution factors changed if needed.

Staining Buffer: PBS/1% BSA

SAMPLES:

Cells are prepared and treated exactly the same as the experimental sample (fixation, permeabilization, FC block etc.) at 1–5 × 10^6 cell/ml, and should contain 2 populations: 1 positive for the antigen and 1 negative for the antigen.

ANTIBODY SERIAL DILUTIONS: Dilute the antibody as per the Table below

1. Add 50 µl of staining buffer to each tube
2. Add 50 µl of antibody at 4× the manufacturer's recommendation to the first tube
3. Mix well and transfer 50 µl to the next tube according to the Table
4. Discard the excess 50 µl from tube 7
5. All tubes should contain 50 µl of antibody except tube 8 which is an unstained control.
6. **Add cells:** Add 100 µl of the cell suspension to all tubes and mix well.
7. Incubate 30 min at room temperature in the dark, or the recommended conditions for your antibody.
8. Wash: Add 2 ml staining buffer, centrifuge 5 min at 300 × *g*, 4°C, remove the supernatant
9. Vortex the pellet, and resuspend in 200 µl staining buffer

DATA ACQUISITION AND ANALYSIS

1. Create a new experiment

2. Create dot plots and histograms as follows:

    a. Create Forward scatter vs side scatter dot plot

b. Create gate on cells

c. Create histogram in log scale for antibody/ fluorochrome used, gated on fsc ssc.

d. Set appropriate detector gain (PMT Voltage) (see Exercise 2) and threshold

e. Create gates on negative and positive populations.

f. Create statistics view to show median fluorescent intensity and rSD (robust standard deviation).

g. Record data on 5000 cells for each antibody dilution

3. Calculate stain index according to the formula:

$$\text{Stain Index} = \frac{\text{Median positive} - \text{median negative}}{2 \times \text{rSD negative}}$$

4. Plot stain index against antibody dilution/concentration. Choose the lowest antibody concentration that gives the highest stain index.

Tube	Stain Buffer	Transfer from previous tube	Temporary volume	Transfer to next tube	Final Ab volume	Antibody concentration	Stain Index
1	50 ul	50 ul Antibody	100 ul	50 ul	50 ul		
2	50 ul	50 ul	100 ul	50 ul	50 ul		
3	50 ul	50 ul	100 ul	50 ul	50 ul		
4	50 ul	50 ul	100 ul	50 ul	50 ul		
5	50 ul	50 ul	100 ul	50 ul	50 ul		
6	50 ul	50 ul	100 ul	50 ul	50 ul		
7	50 ul	50 ul	100 ul	discard 50 ul	50 ul		
8	50 ul	0 ul (no antibody)	50 ul	NA	50 ul		