

Multicolor Staining Protocol for Flow Cytometry

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Equipment:

Pipettes and tips	Ice bucket with ice
12x75mm plastic tubes (Falcon #2052) or 96-well round bottom plate(s)	Vacuum source
Refrigerated centrifuge	Long glass Pasteur pipettes (pulled)

Reagents:

Cell preparation (at 2×10^7 cells/ml)	Fluorochrome conjugated streptavidin (if any antibody used is biotin-conjugated)
PBS4	FACSFix
Fluorochrome conjugated antibodies	

Methods:

Note: There are 2 basic methods for staining. One is staining in 12x75mm tubes, the other is staining in 96-well round bottom plates. Generally the method is the same for both with the only difference being how the cells are washed and decanted. The method for staining in 12x75mm tubes is described below, with a note at the end of the staining protocol describing how the technique is changed when staining in a plate. In addition an example of multicolor staining is available as a separate document entitled "An Example of Staining Cells for Multicolor Flow Cytometry".

I. Preliminary Steps – Diluting the Antibodies

A) For each "panel" of antibodies to be used, make an antibody cocktail (a tube containing a mixture of all the antibodies) for each panel by diluting the fluorochrome conjugated antibodies in PBS4 according to optimal concentrations as determined by previous titration (or according to manufacturers instructions).

- 1) Determine the total number of samples to be stained with each cocktail
- 2) Determine the total volume of each cocktail (V_c) required for the experiment as:

$$\text{Total Cocktail volume needed } (V_c) = (\# \text{ of samples for cocktail} \times 50\mu\text{l}) + 50\mu\text{l}$$

- 3) Determine the amount of each antibody needed in the total volume to make the optimal concentration of that antibody (based on previous titration experiments)
- 4) Add up the volumes from each antibody to get the total volume of antibody (V_a)

$$\text{Total Antibody volume } (V_a) = V_{ab1} + V_{ab2} + V_{ab3} + \dots + V_{abn}$$

- 5) Determine the amount of PBS4 (V_{PBS4}) needed for each cocktail

$$V_{\text{PBS4}} = V_c - V_a$$

- 6) Make each cocktail by ...
 - a) adding the appropriate amount of PBS4 (V_{PBS4}) to a small tube
 - b) adding the appropriate amount of each antibody (V_{ab1} , V_{ab2} , V_{ab3} ... V_{abn}) to the tube
- B) If you are using biotin-conjugated primary antibodies, dilute the fluorochrome conjugated StreptAvidin in PBS4 to optimal concentrations determined by previous titration experiment (or according to manufacturers instructions).

II. Controls

Controls are a very important issue for your experiment and are best determined in direct consultation with the flow cytometry lab. Without proper controls you may be unable to interpret the results of your experiment. Generally the following controls are required for each experiment:

- 1) "Cells Only" control for each cell type (*used to determine autofluorescence*).
- 2) "Avidin Only" control for each cell type (*used only when staining with biotin-conjugated 1^o antibodies, to determine background StreptAvidin binding*).
- 3) "Fluorochrome Only" control for each fluorochrome used (*used as compensation controls to determine the extent of fluorochrome spectral overlap*). This needs to be ...
 - Done for each fluorochrome used in the experiment.
 - Done on a cell type that displays the antigen.
 - Done using the brightest antigen for each fluorochrome.

Note: "Fluorochrome Only" controls can be performed using CompBeads from Becton Dickinson (for mouse Ig (BD #552843) or rat Ig (BD #552844)) instead of cells. This is strongly suggested in situations where antigen expression and/or density is questionable.

III. Staining Protocol

For each panel and for each tissue ...

- 1) Place 50 μ l of the cells into appropriately labeled 12x75mm tubes.
- 2) Add 50 μ l of the panel cocktail to the appropriate tubes and mix gently. (*Control tubes that do not receive 1^o antibodies ("Cells Only" or "Avidin Only") receive 50 μ l of PBS4. "Fluorochrome Only" control tubes receive 50 μ l of their appropriate fluorochrome-conjugated antibody alone.*)
- 3) Incubate 30 minutes in the dark on ice.
- 4) Wash
 - a. Centrifuge for 3 minutes at 300g and 4 $^{\circ}$ C.
 - b. Remove the supernatant with vacuum aspiration and a pulled Pasteur pipette.
 - c. Wash the samples by resuspending in 200 μ l of fresh cold PBS4.
 - d. Centrifuge for 3 minutes at 300g and 4 $^{\circ}$ C.
 - e. Remove the supernatant with vacuum aspiration and a pulled Pasteur pipette.
 - f. Wash the samples by resuspending in 200 μ l of fresh cold PBS4.
 - g. Centrifuge for 3 minutes at 300g and 4 $^{\circ}$ C.
 - h. Remove the supernatant with vacuum aspiration and a pulled Pasteur pipette.

Note: *If you are not using any biotin-conjugated antibodies in your cocktails, skip to step 8, otherwise continue with step 5.*

- 5) Add 100 μ l of the diluted StreptAvidin to each tube that received a biotin-conjugated 1^o antibody. (*Tubes that do not receive StreptAvidin must receive 100 μ l of PBS4.*)
- 6) Incubate 5-15 minutes in the dark on ice.
- 7) Wash
 - a. Centrifuge for 3 minutes at 300g and 4 $^{\circ}$ C.
 - b. Remove the supernatant with vacuum aspiration and a pulled Pasteur pipette.
 - c. Wash the samples by resuspending in 200 μ l of fresh cold PBS4.
 - d. Centrifuge for 3 minutes at 300g and 4 $^{\circ}$ C.
 - e. Remove the supernatant with vacuum aspiration and a pulled Pasteur pipette.
 - f. Wash the samples by resuspending in 200 μ l of fresh cold PBS4.
 - g. Centrifuge for 3 minutes at 300g and 4 $^{\circ}$ C.
 - h. Remove the supernatant with vacuum aspiration and a pulled Pasteur pipette.

- 8) Resuspend the cells in 500 μ l of FACSfix.
- 9) Store samples covered and refrigerated prior to analysis. Samples are best analyzed within 48 hours of completion of staining protocol.

IV. Notes on using 96-well Plates

96-well plates can be a real time saver if you are staining a lot of samples. The decision to stain in tubes or plates is usually based on the number of samples to be stained. As a general rule I suggest staining in tubes if you are staining 15-20 samples or fewer and in plates if you are staining more than that.

Staining in plates saves time during the wash steps. You can “aspirate” 96 samples in about 2 seconds.

It helps to have a “plate washer” available to add PBS4 to the plate. A multichannel pipette can do the job, but be sure to shoot the PBS4 directly into the center of each well to make sure to resuspend the cell pellet. You can check to see how well you’ve resuspended by holding the plate above your head and looking at the bottom of the plate against a ceiling light fixture.

Specifically steps 1, 2, 3, 5 and 6 remain unchanged. When you wash (steps 4 and 7) use the following method...

- a. Centrifuge for 3 minutes at 300g and 4°C.
- b. Remove the supernatant by “flicking” the plate into the sink.
- c. Wash the samples by resuspending in 200 μ l of fresh cold PBS4.
- d. Centrifuge for 3 minutes at 300g and 4°C.
- e. Remove the supernatant by “flicking” the plate into the sink.
- f. Wash the samples by resuspending in 200 μ l of fresh cold PBS4.
- g. Centrifuge for 3 minutes at 300g and 4°C.
- h. Remove the supernatant by “flicking” the plate into the sink.

The technique to “flick” the plate is as follows...

1. Firmly hold the plate upright in the palm of your hand.
2. In a single motion, move the plate upward, turn it over, and bring it straight down, stopping abruptly above the sink.

Hints for the “flick” technique:

- I usually move the plate about 12” upward before I turn it over, and bring it down about the same amount before I stop.
- From the time I start my upward motion to the time I stop my downward motion you can count “one-thousand one”.
- Don’t be bashful. Your cells will stay in the plate.
- Be careful to “stop abruptly” and don’t let the plate “bounce” at the bottom.
- Believe it or not, this technique routinely results in better cell yields that vacuum aspiration in tubes.

Finally, as the samples are stained in a 96-well plate but the cytometer reads them in 12x75mm tubes, an additional step to transfer the samples into tubes is required. Thus steps 8-9 in the protocol above become steps 8-10 as follows...

- 8) Resuspend the cells in 100 μ l of FACSfix.
- 9) Transfer samples to labeled 12x75mm tubes containing 400 μ l of FACSFix.
- 10) Store samples covered and refrigerated prior to analysis. Samples are best analyzed within 48 hours of completion of staining protocol.