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An Example of Staining Cells for Multicolor Flow Cytometry

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Let's say I want to look at T cell, B cell, Granulocyte and Macrophage populations in the spleens of 4 different mice, and I have the following 5 antibodies:

Population	Antigen	Clone	Fluorochrome	Dilution *(from previous titration)*
Helper T cells	CD4	GK1.5	APC	1:100
Cytotoxic T cells	CD8	53-6.7	PerCP	1:200
B Cells	CD45R (B220)	RA3-6B2	FITC	1:200
Granulocytes	Gr1 (Ly6b)	RA6-8C5	PE-Cy7	1:100
Macrophages	CD11b	M1/70	Biotin	1:50

Notice that one of my antibodies (CD11b) is a biotin conjugate, so I will be using the PE conjugate of StreptAvidin to visualize it on the cytometer.

First I make my 4 spleen cell preparations and adjust each of them to $2x10^7$ cells/ml in PBS4. Then I begin the staining process…

I. Preliminary Steps – Diluting the Antibodies

A) I have a 5-color panel that I want to stain my samples with, and I know my dilutions.

1) Determine the total number of samples to be stained with each cocktail **(4 spleens)**
2) Determine the total volume of each cocktail (V_c) required for the experiment as:

*Total Cocktail volume needed (V_c) = (**4** x 50µl) + 50µl =* **250µl**

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)

I will be making 250µl Total Volume of cocktail, so I will need the following amounts of antibody.

Antigen	Fluorochrome	Dilution *(from previous titration)*	Total Volume	**Amount Needed**
CD4	APC	1:100	250 µl	**2.5 µl**
CD8	PerCP	1:200	250 µl	**1.25 µl**
CD45R (B220)	FITC	1:200	250 µl	**1.25 µl**
Gr1 (Ly6b)	PE-Cy7	1:100	250 µl	**2.5 µl**
CD11b	Biotin	1:50	250 µl	**5 µl**

Note: You can determine the final amount by dividing the Final Volume by the dilution ratio. For example 250/100=2.5; 250/200=1.25, 250/50=5, etc.

4) Add up the volumes from each antibody to get the total volume of antibody (V_a)

Total Antibody volume (V_a) = ***2.5*** *+* ***1.25*** *+* ***1.25*** *+* ***2.5*** *+* ***5*** *=* **12.5µl**

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

$$V_{PBS4} = \mathbf{250} - \mathbf{12.5} = \mathbf{237.5\mu l}$$

6) Make the cocktail by …
   a) adding 237.5μl of PBS4 to a small tube
   b) adding to the tube …
      i. 2.5μl of $CD4^{APC}$
      ii. 1.25μl of $CD8^{PerCP}$
      iii. 1.25μl of $B220^{FITC}$
      iv. 2.5μl of $Gr1^{PE\text{-}Cy7}$
      v. 5μl of $CD11b^{Biotin}$

B) I am using a biotin-conjugated primary antibody in this experiment. Six (6) tubes will receive the StreptAvidin-PE solution (4 samples + 2 controls) in step 5 of the staining protocol below. A previous titration has shown that the StreptAvidin-PE should be used at a dilution of 1:50. I need 600μl (6 tubes x 100μl each), so I will make a little bit more (650μl) in case my pipetter is not quite accurate. So I add 13μl of StreptAvidin-PE to 637μl of PBS4 in a small tube.

II. Controls

For this experiment I will need the following controls…

1. Cells Only
2. Avidin Only
3. FITC Only
4. PE Only
5. PerCP Only
6. PE-Cy7 Only
7. APC Only

The antibodies I will be using are to common antigens, and each of them should be found on normal mouse spleen cells. Therefore, I can use cells for my controls (not CompBeads).

I will need to make up antibody dilutions for most of these controls (#'s 3-7). I will need to use 50μl of each single color antibody dilution in my staining protocol (step 2 below), so I will make a minimum of 100μl for each.

FITC Only Ab ($B220^{FITC}$)

Dilution is 1:200. My pipette only accurately measures down to 1μl, so I will take out 1μl and add it to 199μl of PBS4. (This results in a total volume of 200μl. I only need to use 50μl, but 200μl is the least amount I can make accurately.)

PE Only Ab ($CD11b^{Biotin}$)

Dilution is 1:50. I take out 2μl and add it to 98μl of PBS4.

PerCP Only Ab ($CD8^{PerCP}$)

Dilution is 1:200. As with the $B220^{FITC}$ antibody (FITC Only Ab) above, I take out 1μl and add it to 199μl of PBS4.

PE-Cy7 Only Ab ($Gr1^{PE\text{-}Cy7}$)

Dilution is 1:100. I take out 1μl and add it to 99μl of PBS4.

APC Only Ab ($CD4^{APC}$)

Dilution is 1:100. I take out 1μl and add it to 99μl of PBS4.

III. Staining Protocol

In this experiment I will have 4 mouse spleens each labeled with a 5-color cocktail, and 7 controls. Thus 11 total tubes (4+7=11). They will be labeled as follows…

1. Cells Only
2. Avidin Only
3. FITC Only
4. PE Only
5. PerCP Only
6. PE-Cy7 Only
7. APC Only
8. Spleen 1
9. Spleen 2
10. Spleen 3
11. Spleen 4

I have made a table of what goes into each tube and placed it at the end of the protocol to summarize each tubes contents.

For convenience I will use spleen cells from Mouse 1 (a "normal" mouse) for all 7 control samples.

1) Place 50μl of the cells from …
    a. Mouse 1 into "Cells only", "Avidin only", "FITC only", "PE only", "PerCP only", "PE-Cy7 only", "APC only" and "Mouse 1" tubes.
    b. Mouse 2 into "Mouse 2" tube.
    c. Mouse 3 into "Mouse 3" tube.
    d. Mouse 4 into "Mouse 4" tube.
2) Add …
    a. 50μl of the antibody cocktail to "Mouse 1", "Mouse 2", "Mouse 3" and "Mouse 4" tubes.
    b. 50μl of FITC Only Ab antibody to "FITC Only" tube
    c. 50μl of PE Only Ab antibody to "PE Only" tube
    d. 50μl of PerCP Only Ab antibody to "PerCP Only" tube
    e. 50μl of PE-Cy7 Only Ab antibody to "PE-Cy7 Only" tube
    f. 50μl of APC Only Ab antibody to "APC Only" tube
    g. 50μl of PBS4 to "Cells Only" tube
    h. 50μl of PBS4 to "Avidin Only" tube
3) Incubate 30 minutes in the dark on ice.
4) Wash
    a. Centrifuge for 3 minutes at 300g and 4^{o}C.
    b. Remove the supernatant from each tube with vacuum aspiration and a pulled Pasteur pipette.
    c. Wash each tube by resuspending in 200μl of fresh cold PBS4.
    d. Centrifuge for 3 minutes at 300g and 4^{o}C.
    e. Remove the supernatant from each tube with vacuum aspiration and a pulled Pasteur pipette.
    f. Wash each tube by resuspending in 200μl of fresh cold PBS4.
    g. Centrifuge for 3 minutes at 300g and 4^{o}C.
    h. Remove the supernatant from each tube with vacuum aspiration and a pulled Pasteur pipette.
5) Add…
    a. 100μl of the diluted StreptAvidin-PE to the "Avidin Only", "PE Only", "Mouse 1", "Mouse 2", "Mouse 3" and "Mouse 4" tubes.
    b. 100μl of PBS4 to the "Cells Only", "FITC Only", "PerCP Only", "PE-Cy7 Only" and "APC Only" tubes.
6) Incubate 5-15 minutes in the dark on ice.
7) Wash
    a. Centrifuge for 3 minutes at 300g and 4^{o}C.
    b. Remove the supernatant from each tube with vacuum aspiration and a pulled Pasteur pipette.

c. Wash each tube by resuspending in 200μl of fresh cold PBS4.
d. Centrifuge for 3 minutes at 300g and 4°C.
e. Remove the supernatant from each tube with vacuum aspiration and a pulled Pasteur pipette.
f. Wash each tube by resuspending in 200μl of fresh cold PBS4.
g. Centrifuge for 3 minutes at 300g and 4°C.
h. Remove the supernatant from each tube with vacuum aspiration and a pulled Pasteur pipette.

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

What Goes Into Each Tube

Tube	Step 1: 50μl cells from …	Step 2: 50μl	Step 5: 100μl
Cells Only	Mouse 1	PBS4	PBS4
Avidin Only	Mouse 1	PBS4	PE-StreptAvidin
FITC Only	Mouse 1	FITC Only Ab	PBS4
PE Only	Mouse 1	PE Only Ab	PE-StreptAvidin
PerCP Only	Mouse 1	PerCP Only Ab	PBS4
PE-Cy7 Only	Mouse 1	PE-Cy7 Only Ab	PBS4
APC Only	Mouse 1	APC Only Ab	PBS4
Mouse 1	Mouse 1	Cocktail	PE-StreptAvidin
Mouse 2	Mouse 2	Cocktail	PE-StreptAvidin
Mouse 3	Mouse 3	Cocktail	PE-StreptAvidin
Mouse 4	Mouse 4	Cocktail	PE-StreptAvidin