

C.C. Stewart, S.J. Stewart, Immunophenotyping, In: Current Protocols in Cytometry, (J.P. Robinson, Z. Darzynkiewicz, P. Dean. L. Dressler, P.Rabinovitch, C. Stewart, H. Tanke, L. Wheelless, eds.) J.Wiley & Sons, Inc., New York, 6.2.1 - 6.2.15, 1997.

IMMUNOPHENOTYPING

INTRODUCTION

There are four basic methods for staining cells with antibodies for immunophenotyping by flow cytometry. The first method is an indirect method that employs a neat antibody followed by a second fluorochrome conjugated polyclonal antibody. Because it is not conjugated with a fluorochrome, a second fluorochrome conjugated polyclonal antibody derived from a second animal species and directed to the IgG from the first antibody species is used. The fluorochrome conjugated F(ab)₂ fragment should always be used, never use an intact polyclonal antibody preparation for immunophenotyping.

The second method is also an indirect method that employs a hapten conjugated first antibody followed by a fluorochrome conjugated polyclonal antibody to the hapten. Examples of haptens are digoxigenin (1), di or trinitrophenol (2,3) and biotin (3,4). The third method is also an indirect method that employs a biotinylated antibody followed by a fluorochrome conjugated avidin. The method of choice is the fourth method that employs a directly conjugated monoclonal antibody to the desired antigen (see Unit 4.3).

Materials

normal mouse IgG at 3 mg/ml

normal goat IgG at 3 mg/ml

Monoclonal Antibodies:

a) unconjugated

b) hapten conjugated

c) biotinylated

d) fluorochrome conjugated

polyclonal fluorochrome conjugated goat anti-mouse IgG F(ab)₂

polyclonal fluorochrome conjugated goat anti-hapten F(ab)₂

fluorochrome conjugated avidin

directly conjugated Mabs

phosphate buffered saline

lysing reagent

2% ultrapure Formaldehyde

Basic Staining Procedure

A. Neat antibody with fluorochrome conjugated second antibody

1. To a tube containing the target cell suspension at a concentration of $5 - 10 \times 10^6$ /ml, add 67 μ l of goat IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.

2. To empty tubes add the appropriate amount of properly titered neat antibody (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. then add 3 ml of lysing reagent.
3. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend the cells in residual solution.
4. Add the appropriate amount of fluorochrome conjugated goat anti-mouse IgG F(ab)'₂, incubate cells 15 min. and then add 3.0 ml PBS.
5. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend the cells in residual solution
6. Add 200 μ l 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.

B. Biotinylated or hapten conjugated antibodies

1. To a tube containing the target cell suspension at a concentration of 5 - 10 x 10⁶/ml, add 67 μ l of mouse IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.
2. To empty tubes add the appropriate amounts of properly titered antibodies (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. then add 3 ml of lysing reagent.
3. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
4. Add 10 μ l of appropriately titered avidin or anti-hapten antibody with associated conjugated fluorochrome. Incubate 15 minutes and then add 3 ml PBS.
5. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
6. Add 200 μ l 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.

C. Directly conjugated antibodies

1. To a tube containing the target cell suspension at a concentration of 5 - 10 x 10⁶/ml, add 67 μ l of mouse IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.
2. To empty tubes add the appropriate amounts of properly titered antibodies (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. then add 3 ml of lysing reagent.
3. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
4. Add 200 μ l 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.

Multicolor immunophenotyping

The four basic procedures for staining cells with antibody can be combined to produce simultaneous evaluation of multiple antigens. There are two general rules that must be adhered to for obtaining valid results.

1. Always perform the neat antibody second antibody step separately and first.
2. When the first reaction is complete but before addition of additional antibodies always block with IgG from the species the neat antibody was derived. e.g. mouse IgG for mouse antibodies.

There are several strategies that can be performed for combining antibodies and the following list contains a few examples.

Two color:

A. Neat antibody combined with biotinylated antibody

1. To a tube containing the target cell suspension at a concentration of $5 - 10 \times 10^6$ /ml, add 67 μ l of goat IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.
2. To an empty tube add the appropriate amount of properly titered antibody (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. then add 3 ml of lysing reagent.
3. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend the cells in residual solution.
4. Add the appropriate amount of fluorochrome conjugated goat anti-mouse IgG F(ab)'₂, incubate cells 15 min. and then add 3.0 ml PBS.
5. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend the cells in residual solution.
6. Now add 10 μ l mouse IgG (3 mg/ml) to each tube, incubate 10 minutes, then add the appropriate amount of properly titered antibody (unit 4.2). Incubate 15 min. and then add 3 ml of lysing reagent.
7. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
8. Add 10 μ l of appropriately titered avidin with associated conjugated fluorochrome. Incubate 15 minutes and then add 3 ml PBS.
9. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
10. Add 200 μ l 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.

B. Biotinylated or hapten conjugated antibodies

1. To a tube containing the target cell suspension at a concentration of $5 - 10 \times 10^6$ /ml, add 67 μ l of mouse IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.
2. To empty tubes add the appropriate amounts of properly titered biotinylated and hapten conjugated antibody (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. and then add 3 ml of lysing reagent.
3. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
4. Add 10 μ l of appropriately titered avidin and anti-hapten antibody with associated conjugated fluorochrome. Incubate 15 minutes and then add 3 ml PBS.

5. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
6. Add 200 μ l 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.

C. Biotin antibody combined with directly conjugated antibody

1. To a tube containing the target cell suspension at a concentration of 5 - 10 x 10⁶/ml, add 67 μ l of mouse IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.
2. To empty tubes add the appropriate amounts of properly titered biotinylated and directly conjugated antibodies (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. and then add 3 ml of lysing reagent.
3. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
4. Add 10 μ l of appropriately titered avidin with associated conjugated fluorochrome. Incubate 15 minutes and then add 3 ml PBS.
5. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
6. Add 200 μ l 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.

Three or Four Color

A. Neat, biotin and directly conjugated antibodies

1. To a tube containing the target cell suspension at a concentration of 5 - 10 x 10⁶/ml, add 67 μ l of goat IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.
2. To empty tubes add the appropriate amounts of properly titered antibodies (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. and then add 3 ml of lysing reagent.
3. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend the cells in residual solution.
4. Add the appropriate amount of fluorochrome conjugated goat anti-mouse IgG F(ab)'₂, incubate cells 15 min. and then add 3.0 ml PBS.
5. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend the cells in residual solution.
6. Add 10 μ l of mouse IgG (3 mg/ml) per ml to each suspension. Incubate 10 minutes, then add the appropriate amounts of properly titered biotinylated and fluorochrome (1 or 2) conjugated antibodies (unit 4.2).
7. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
8. Add 10 μ l of appropriately titered avidin with associated conjugated fluorochrome. Incubate 15 minutes and then add 3 ml PBS.
9. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.

10. Add 200 μ l 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.
- B. Biotinylated, hapten conjugated and directly conjugated antibodies
1. To a tube containing the target cell suspension at a concentration of $5 - 10 \times 10^6$ /ml, add 67 μ l of mouse IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.
 2. To empty tubes add the appropriate amount of properly titered biotinylated, hapten conjugated and one or two directly conjugated antibodies (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. and then add 3 ml of lysing reagent.
 3. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
 4. Add 10 μ l of appropriately titered avidin and anti-hapten antibody with associated conjugated fluorochrome. Incubate 15 minutes and then add 3 ml PBS.
 5. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
 6. Add 200 μ l 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.
- C. Biotin antibody combined with two or three directly conjugated antibodies
1. To a tube containing the target cell suspension at a concentration of $5 - 10 \times 10^6$ /ml, add 67 μ l of mouse IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.
 2. To empty tubes add the appropriate amounts of properly titered biotinylated and two or three directly conjugated antibodies (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. and then add 3 ml of lysing reagent.
 3. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
 4. Add 10 μ l of appropriately titered avidin or anti-hapten antibody with associated conjugated fluorochrome. Incubate 15 minutes and then add 3 ml PBS.
 5. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
 6. Add 200 μ l 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.
- D. Neat antibody with a biotinylated second antibody and two or three directly conjugated antibodies.
1. To a tube containing the target cell suspension at a concentration of $5 - 10 \times 10^6$ /ml, add 67 μ l of goat IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 μ g/ml. Incubate 10 minutes.
 2. To empty tubes add the appropriate amounts of properly titered neat antibody (unit 4.2). Then add 50 μ l of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. and then add 3 ml of lysing reagent.
 3. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend the cells in residual solution.

4. Add the appropriate amount of biotin conjugated goat anti-mouse IgG F(ab)'₂, incubate cells 15 min. and then add 3.0 ml PBS.
5. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend the cells in residual solution
6. Add 10 µl of mouse IgG (3 mg/ml) to each and incubate 10 minutes.
7. Then add the appropriate amounts of properly titered directly conjugated antibodies (unit 4.2) and 10 µl of appropriately titered avidin with associated conjugated fluorochrome. Incubate 15 min. and then add 3 ml of lysing reagent.
8. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant, blot tube lip on absorbent towel and resuspend cells in residual solution.
9. Add 200 µl 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.
- 10.

E. All Antibodies Directly Conjugated

1. To a tube containing the target cell suspension at a concentration of 5 - 10 x 10⁶/ml, add 67 µl of mouse IgG (3 mg/ml) per ml of suspension to produce a final concentration of 200 µg/ml. Incubate 10 minutes.
2. To empty tubes add the appropriate amounts of properly titered antibodies (unit 4.2). Then add 50 µl of blocked cell suspension. Prepare an isotype control tube and a tube with no added reagents. Incubate 15 min. and then add 3 ml of lysing reagent.
3. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and resuspend cells in residual solution.
4. Add 200 µl 2% ultrapure formaldehyde and incubate at least one hour before acquiring data.

EMA Procedure For Detecting Nonviable Cells In A Cell Population To Be Fixed

Dead cells bind antibodies non-specifically. Each antibody, however, binds to a different extent that could lead to misinterpretation. It is important, therefore, to minimize dead cells that are found in the analysis window.

Materials

Ethidium Monoazide (Molecular Probes, Eugene, OR)

Phosphate Buffered Saline

12 x 75 mm tubes

1. Add 50 µl of cells to a 12 x 75 mm test tube and lyse erythrocytes, if necessary using 3.5 ml of lysing reagent.
2. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend cells in residual solution.
3. Add 5 µl of EMA (5 µg/ml) to the EMA tube. (Titering of the working solutions is required.)
4. Put tube(s) 20 cm. beneath a fluorescent light for 10 minutes. (Lay flat in white pan under a desk lamp). Then add 3.5 ml PBS.
5. Centrifuge at 1500 x g for 3 min. Decant the supernatant and resuspend cells in residual solution.
6. Fix with 0.5 ml of 2% formaldehyde.

EMA tubes are analyzed on the flow cytometer along with the other panels on immunophenotyping panels as described in the Data Analysis Section. EMA stains the dead cells making them much brighter than the viable cells.

Data Analysis

Two basic ways to analyze flow cytometry data are the marker approach, wherein a marker is placed on a histogram to designate positive and negative cells and the template approach, where a region is drawn circumscribing the geometric pattern created by the cell population. The marker approach is well suited for the measurement of 1 or 2 colors of fluorescence, but for 3 and 4 colors, the template approach may be more meaningful.

Materials

List Mode Files

For PCs

Coulter Elite or XL Software

Ortho Cyteron Software

Winlist by Verity Software House

MSA WinLAS

For Macintosh

BDIS Cell Quest

BDIS FACSCConvert

MSA MacLAS

Marker Approach

We will illustrate this approach using five parameter data as this is the most complex of the three. There are two approaches to analyzing with the marker approach. The first is to establish a “population” gate using FSC vs SSC. The second is to establish a “Cell” gate using SSC vs cell specific antibody. We will illustrate the procedure using human leukocytes.

1. Display bivariate plots of all parameters:

3 parameters = FSC vs SSC and FL1 univariate histogram

4 parameters = FSC vs SSC and FL1 vs FL2 as shown in fig. 1

5 parameters = FSC vs SSC, FL1 vs FL2, FL3 vs FL2 and FL3 vs FL1
as shown in figures 2 and 3.

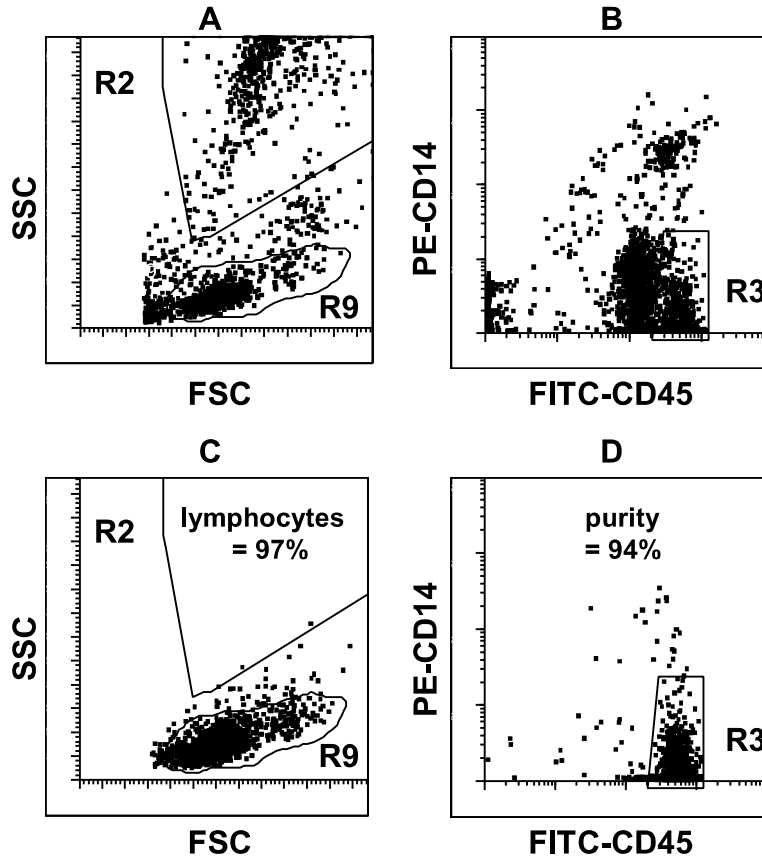


Figure 1. Bivariate Plots of All Parameters.

2. To establish the desired population, e.g. lymphocytes that express CD45 brightly, load the list mode file of cells stained with CD45 and CD14.
3. Refer to figure 1A and draw a region R9 to circumscribe the lymphocyte cluster and a region R2 to circumscribe the granulocytes completely in the FSC vs SSC plot. This latter region is to exclude some granulocytes that overlap B-cells that are less CD45 bright than T-cells and NK cells. Draw a region R3 (figure 1B) to circumscribe the CD45 bright CD14 negative cells in that bivariate plot. Create the boolean logical gate “R3 and not R2” and gate the FSC vs SSC display as shown in figure 1C.
4. Gate the CD45 vs CD14 display using R9, figure 1D. Create results and determine the percentage of lymphocytes (CD45bright) inside the region R9 and their purity from the cells inside R3.
5. Load the file containing the unstained cells (or an isotype control file, figure 2), gated on region R9 and adjust quadrant markers in the FL1 vs FL2 and FL3 vs FL1 display so that <1% of the events are beyond the marker.
6. Create the boolean logical gates for all the combination as shown in Table 1 and assign a color to each.
7. Load each file containing stained cells and record the results as shown in figure 3 and Table 1.

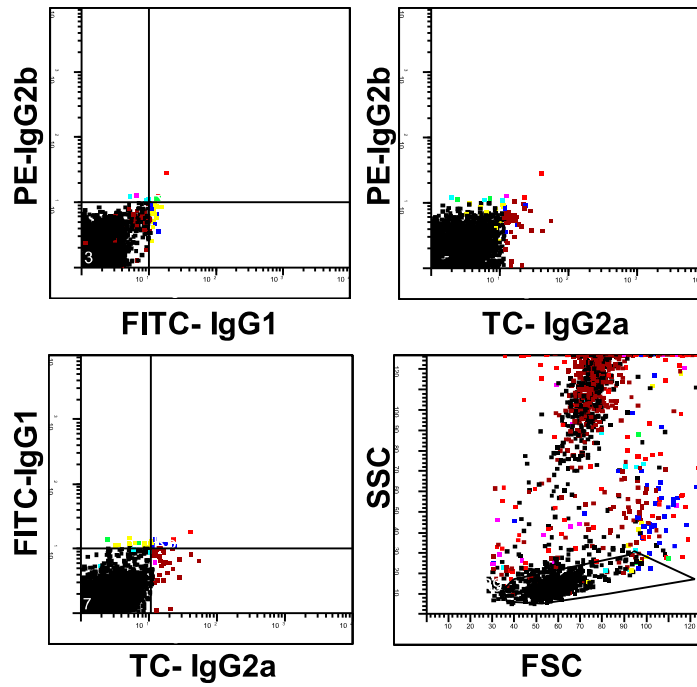


Figure 2. Isotype Control Bivariate Displays

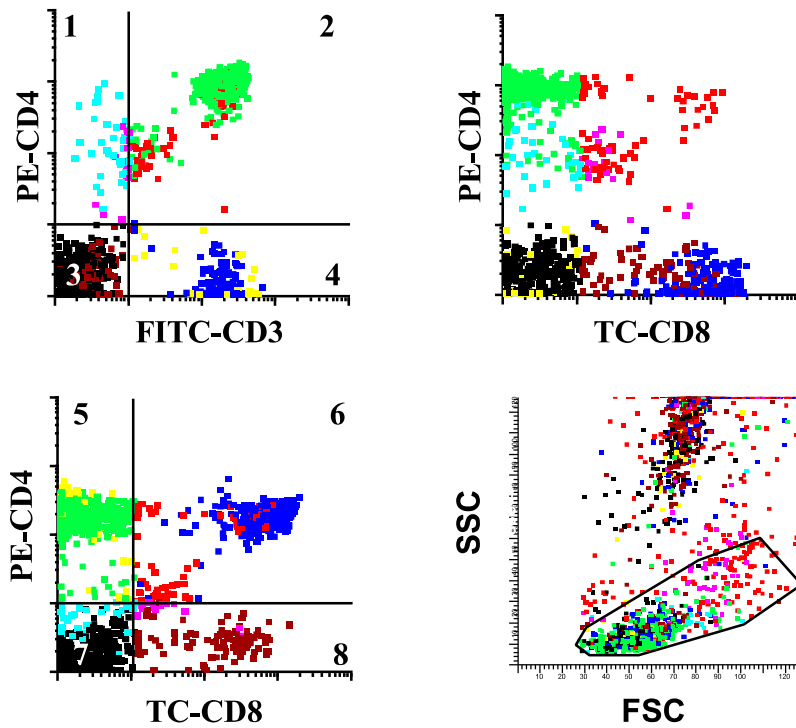


Figure 3. Bivariate Displays of Stained Cells

Cell Gating

Instead of using a population gate in FSC vs SSC, cells stained with a specific antibody can be gated. This requires that the gating antibody is present in every tube.

Display bivariate plots of SSC vs the gating antibody, FSC vs SSC and Ab1 vs Ab2.. For four color data, the SSC vs gating antibody, Ab1 vs Ab2, Ab3 vs Ab2 and Ab1 vs Ab3 are displayed as shown in figure 4.

Table 1 Analysis of Data Using Quadrant Markers

		boolean expression	cluster color	% positive from fig.3
ONE COLOR	- - -	R3 AND NOT R8	BLACK	1
	+ - -	R4 AND R5	YELLOW	3
TWO COLORS	- + -	R1 AND NOT R6	CYAN	2
	+ + -	R2 AND R5	GREEN	49
THREE COLORS	- - +	R8 AND R3	BROWN	6
	+ - +	R6 AND R4	BLUE	17
	- + +	R1 AND R8	VIOLET	1
	+ + +	R2 AND R6	RED	4

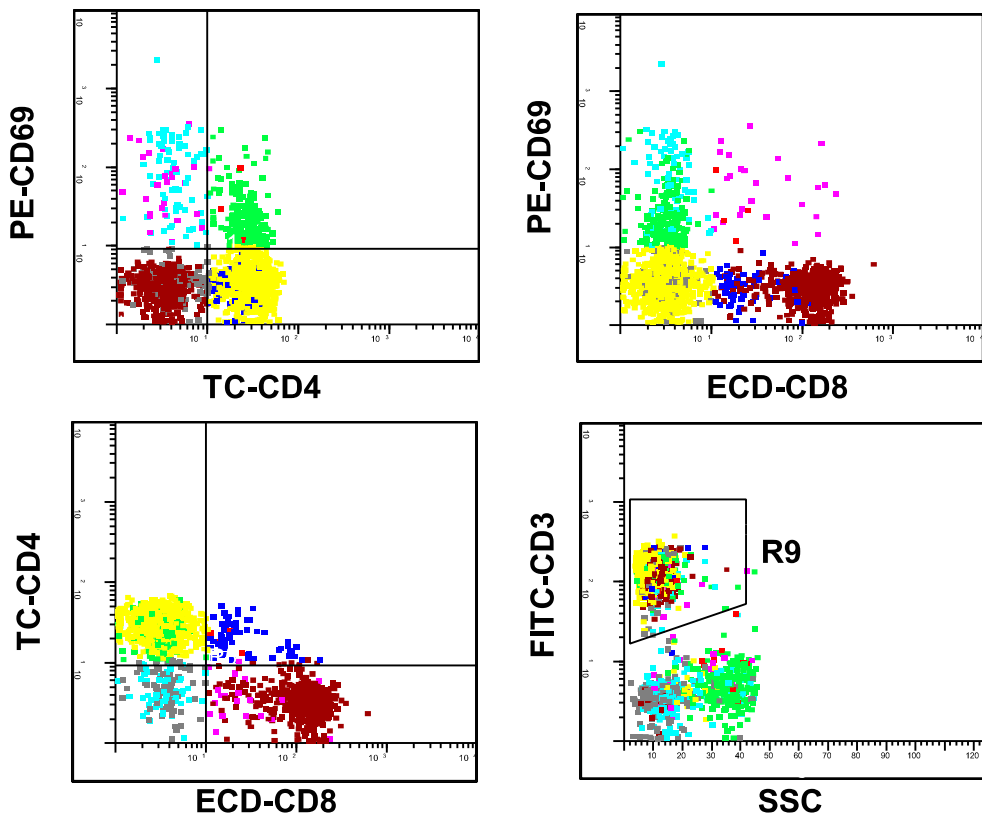


Figure 4. Four Color Bivariate Displays

1. In the SSC vs gating antibody view, draw region R9 and gate all other views on R9.
2. Set quadrant markers using file from cells stained with only the gating antibody with or without an isotope control so that <1% of events are beyond the marker.
3. Create the boolean logical gates for all the combinations and assign a color to each, as shown in Table 1
4. Load each file containing stained cells and record the results.

The gating antibody could be a lineage specific one like CD3 for T-cells, CD19 for B-cells, CD34 for hematopoietic progenitor cells. It might also be a combination like CD56 and NOT CD3 for NK cells, or CD45 for all leukocytes.

Template Approach

As more parameters are acquired, the boolean combinations increase exponentially. Indeed, some analysis programs only provide eight regions and could not be used for any more than three parameters (excluding FSC vs SSC) because four parameter data requires at least 16 regions to obtain all binary combinations provided by the quadrant markers. The goal of any analysis, however, is to determine the frequency of each distinct cell population resolved by the antibody combination, so it actually makes better sense to produce a template by drawing regions around the geometric shape they produce in multidimensional space.

Materials

Six parameter list mode files

For PCs: Winlist by Verity Software House, WinLAS by Management Science Associates (MSA)

For Macintosh: Attractors by BDIS or MacLAS by MSA

1. Display FSC vs FL1, FSC vs FL2, FSC vs FL3, SSC vs FL1, SSC vs FL2, SSC vs FL3, FSC vs SSC, FL1 vs FL2, FL1 vs FL3 and FL2 vs FL3, as shown in figure 5
2. Load list mode file of stained cells. There is no requirement for controls.
3. Using any view, select a well resolved cluster of cells and apply an “attractor” (or region) to it. A cluster is any geometric shape with clear boundaries separating it from others. Classify the data. The attractor will automatically find the Euclidean center of the cluster.

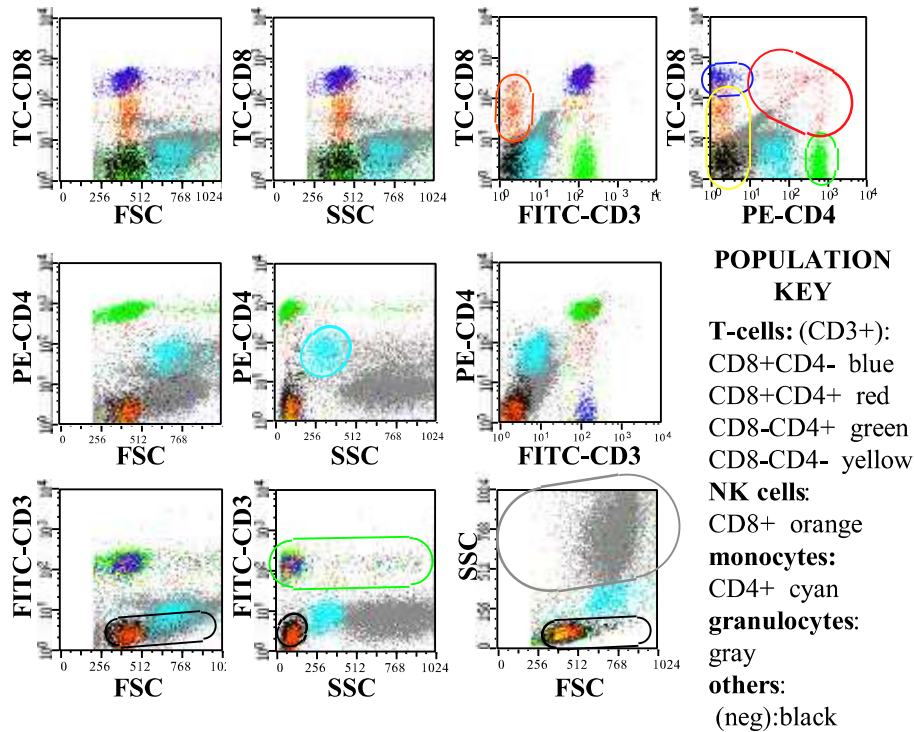


Figure 5. Display of All Parameters.

4. The cluster is now colored, determine if the cluster forms additional ones in any of the other ten views. If it does, there are two strategies to use.
 - a) The first strategy is to make each cluster unique. Using the attractor (or region) editor, assign the new parameters to the attractor (or region) and adjust it to surround one of the divided clusters. Create a new attractor for each additional cluster, applying the assigned attractor to them, until each one has a unique attractor (or region). Classify the data. Make sure all regions are linked by a boolean “AND”.
 - b) The second strategy is to make each additional cluster a subset of the initial attractor (or region). Select “subset attractor” (or connect by boolean “AND” subsequent regions) and adjust it to surround one of the divided clusters. Repeat for each subset.
5. Go to “Display” and choose “selected population only” (or gate on the boolean combination of regions). Click on any view so the unclassified events (i.e. “NOT”) will be shown. Select another well resolved cluster of cells and apply an attractor (or region combination) to it. Classify the data and repeat step 4 above.
6. Repeat step 5 until no more homogeneous clusters are found in the entire display. Any unclassified events are predominantly noise in the file from debris or cells about the margins of the clusters.
7. Save the template and use it to analyze cells stained with that specific combination of antibodies.

Determining Viable Cells

Because EMA is a separate test (so the color parameter is not lost), we determine where dead cells are in the FSC vs SSC plot so they can be gated out of the analysis. Refer to figure 6.

1. Display FSC vs SSC and FSC vs FL3. Draw a region R2 around the dead cells.

2. Gate FSC vs SSC on R2. Draw a gating region R9 so that >95% are viable. By gating the FSC vs FL3 display on R9, the percent of dead cells in R9 can be determined.

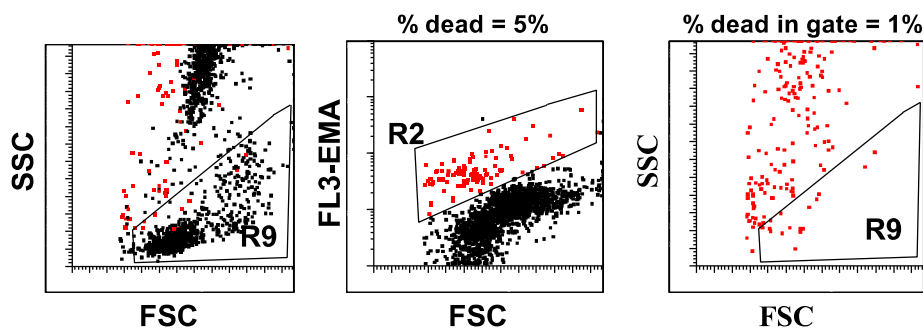


Figure 6. Determination of Dead Cells Using EMA

BACKGROUND INFORMATION

Antibodies used as second antibodies or antibodies to haptens are almost always polyclonal antibodies derived from the serum of mammals that have been immunized with the immunoglobulin fraction of serum from the animal species of the first antibody (5) or a hapten that has been conjugated to a large protein such as keyhole limpet haemocyanin (2). Serum derived polyclonal antibodies consist of IgM, all subclasses of IgG and IgA with specificities for the immunogen. Because pentameric IgM and quadrameric IgA are present, the F(ab)₂ fragments should always be used as all antibodies will then be dimeric. This produces a uniform preparation with the lowest amount of undesired cell binding. Polyclonal antibodies are usually purified by passing the F(ab)₂ preparations over a column composed of protein A or protein G (6) or anti IgG directed against the animal's purified IgG light chains or heavy chains. The latter method is best because only those Ig molecules specific for the immunogen are recovered.

Propidium iodide stains DNA and is commonly used to detect dead cells in a fixed preparation because it is excluded by viable cells with intact membranes. Unfortunately, if cells are fixed all cells become stained because it reversibly binds to DNA and stains the previously viable but now fixed cells. Other dyes like 7-AAD behave the same way. Acquiring viable cells presents both a health hazard and may not be convenient because immediate evaluation is required.

Ethidium monoazide (EMA) provides a solution to this problem (7). Like PI and 7AAD, it stains the DNA of dead cells, but does not stain viable cells. By exposing the dye to light, its photoactivated azide group covalently links the dye to the DNA and histones. After exposure, non-linked dye can be washed away and the dead cells are permanently stained.

Software for the analysis of immunophenotyping data has evolved over the years as the number of antibodies combined together has increased. The fundamental approach is to set a marker to resolve antibody positive cells from negative ones. The most primitive analysis strategy is to create a gate in the FSC vs SSC view and apply it to all files. Next, the antibody fluorescence histogram is displayed. For a single antibody a univariate histogram is used. For antibodies in combination, bivariate histograms are displayed and quadrant markers are inserted. The position of markers is set using unstained cells or an isotype control. There is no rule for the percentage of events allowed above the marker, but usually it is less than 1 - 2%.

The number of bivariate histograms (BH) increases with the number of antibodies (n) according to the formula: $BH = (n-1) + (n-2) + \dots + (n-n)$ where n is the number of antibodies. The number of bivariate histograms that require quadrant markers is (n-1). The number of binary populations that can be resolved by the quadrant markers expressed in terms of boolean algebra is 2^n . Thus, three antibodies provide eight populations, four provides 16, five provides 32, etc. Clearly our ability to visualize all of these populations becomes increasingly difficult.

As interest in measuring more of parameters increases, new approaches to data analysis will be required. One new approach is cell gating where one of the antibodies is used to resolve the cells of interest such as CD45 for lymphocytes, CD3 for T-cells, CD19 for B-cells, CD14 for monocytes and CD34 for hematopoietic progenitor cells. Other antibodies are combined such as CD4 and CD8 for T-cells or anti-kappa and anti-lambda for B-cells to resolve the population's subsets.

Another approach is to use a template composed of regions that define all the populations resolved by the antibody combination. When using this approach it is assumed that distinct clusters occur as well resolved geometric shapes in the various bivariate views into multidimensional space. Thus, in one bivariate view the cluster may appear homogeneous but in a second view, two or more clusters are found. Regions linked by boolean algebra are used to define all the distinct clusters until all of them are homogeneous. Homogeneity does not imply any particular geometric shape only that no further separation occurs and all kinds can be found.

The template approach can be automated using clustering algorithms (8). While several attempts have been made to apply these mathematical approaches, the results have not been promising, mainly because each file represents an entirely new experience for the algorithm. Applying neural networks (9.10) or classification and regression trees (11) provides the added dimension of experience so the necessary sophistication required to rapidly analyze high dimensional data automatically occurs.

CRITICAL PARAMETERS

Recently, several suppliers have introduced procedures that only require lysing erythrocytes, but not washing the specimen when fluorochrome conjugated antibodies are used. There are several reasons why this may seem to be a good idea (even though it is really a bad idea). First, the supposition is that cells are not lost because they are not washed. Sounds good, but in reality positive cells are lost to detection because the non specific binding increases markedly for most antibodies. While the stained cells do not change their brightness, the increased nonspecific binding and fluorescence in the cell stream causes a decreased signal to noise discrimination and this results in lost cells to detection. In contrast, washed cells may be discarded if they are in the supernatant fraction. This problem can be markedly reduced by centrifuging them faster (1500 x g, rather than 300 x g) as recommended in this protocol. This increased speed does not increase cellular aggregation and no intact cells can be found in the discarded wash supernatant.

Proponents of the lyse/no wash system also believe it saves time, after all there is no wash. This is also a false belief. A minimum of one ml of lysing reagent is required and this amount is almost

always marginal, increasing intact erythrocyte frequency and producing an invalid denominator when computing the percentage of positive cells. In tests performed with the recommended procedure in this protocol, 23 minutes (Average time for three different technicians) is required to lyse and wash 100 tubes. For lyse-no wash, this time equals zero. We resuspend our cells in 100 μ l of 2% formaldehyde for a final volume of approximately 300 μ l. To acquire the same number of cell it will take 3.3 times longer for the lyse-no wash specimens. If it takes 20 seconds per sample (or 33 min.) to acquire data using the lyse-wash procedure described here, it will take 110 min. for the lyse-no-wash procedure and that is 77 min. longer, so where did we save the time? We recommend washing all samples and discourage the use of any procedure that does not include a wash step to remove unreacted antibody.

Intact antibodies, especially monoclonal antibodies, bind to any cell that has unoccupied Fc receptors. Even when derived from blood that contains all immunoglobulin isotypes, these receptors are not fully saturated because the immunoglobulin concentration in blood is non saturating. Therefore, it is necessary to add a higher concentration of IgG to block all receptors (refer to Chapter 4.1) so the antibody binds only to its epitope and not to Fc receptors as well. All hematopoietic cells have Fc receptors except T-cells and erythrocytes.

When a second conjugated polyclonal antibody is used, that staining reaction must always be performed first and the IgG block must be from the same species as the polyclonal antibody. When this reaction is followed by additional staining steps, mouse IgG must be added to block any available binding sites on the polyclonal antibody before addition of any of the other conjugated antibodies. Failure to correctly perform this procedure in the correct order will result in flawed data.

The temperature of staining has not been indicated. Many protocols recommend room temperature, which is about 22^o C. We recommend placing the rack of tubes in an ice bath (4^o C). Many cell types, like myeloid cells, still function at room temperature, although slower, and can internalize their bound antibodies.

The FSC vs SSC distribution of aldehyde fixed cells changes markedly during the first eight hours. Do not expect their distributions to look the same over this time period because the continuous crosslinking of proteins occurs causing the cells to change shape and granularity. We recommend a 12 hour fixation time for complete stabilization. Fixed suspensions should be evaluated in less than five days because increasing autofluorescence can occur with longer storage times.

Dead cells bind antibodies non-specifically and their presence can lead to misinterpretation. By using EMA, it is possible to establish a gate that excludes nearly all dead cells based on a FSC vs SSC gate most instances. We recommend there be no more than 5% dead cells (95% viable cells) in the gated region. A stock solution of EMA is prepared in PBS and stored at -20^oC in total darkness by wrapping aluminum foil around the container. The working dilution of 5 μ g/ml is prepared biannually and 50 μ l is aliquoted into 0.5 ml eppendorf vials and stored at 4 ^oC. One vial is thawed at a time, as necessary. While EMA can be added as a third color after staining cells with FITC and PE conjugated antibodies, we stain cells in a separate tube so that three or four antibodies can be combined for immunophenotyping.

For single color analysis, a simple univariate histogram can be displayed because cells are either negative or positive for the antibody. When two antibodies are combined, four populations, shown in Table 1 can be resolved. When three antibodies are combined, eight populations, shown in Table 1, can be resolved, but because two bivariate views are required to resolve their combination, boolean expressions are required and a color assigned to them so each cell population can be visualized in all the bivariate views.

The reason two regions R7 and R2 are drawn in the FSC vs SSC plot for determining lymphocyte purity is the region R3 must be extended somewhat into the space occupied by granulocytes (CD45dim) if all B-cells are to be included. Some CD45 Mabs do not resolve B-cells, that are dimmer than T-cells and NK cells, from the brighter CD45 dim granulocytes. Since these granulocytes are excluded when forward scatter gating on R9, they also need to be excluded when backgating on R3. This is accomplished using the boolean combination “R3 and not R2”. In this way the percentage of lymphocytes in the gate R9 can be determined. The cells in R3, when gated on R9, contain the purity of the lymphogate. In the example shown in figure 1, 97% of lymphocytes, the cells in R3 that are inside R9, are counted with a purity of 94% for cells in R9 that are also in R3. These percentages should be above 90% for lymphocytes (12). Large lymphocytes, that are most important in disease are frequently excluded from the analysis. Note that large cells with low SSC have been included in R9 to accomplish this. These cells are very important and should always be included even though they often reside under the monocyte cluster.

R9, rather than R1, is used as the gate region because most analysis software assign colors in a hierarchical fashion. If R1 were the gate, it would not be possible to assign colors to the various cell populations resolved by the boolean logical gates shown in Table 1.

One problem with multiparameter data analysis is the restriction on the number of regions available. While most data analysis software now provides more than eight regions, some do not. Any software limited to eight regions is not useful for evaluating more than five parameters. Region 8 is used as the gating region for this limited software and the triple negatives are determined by adding up all the other seven subsets and subtracting them from 100%.

For software providing more than eight regions, as shown in figure 1 and 2 and Table 1, regions 1 - 8 are used for the quadrant markers and regions 9 and higher are used for gating.

Most software attaches a color, based on a Venn Spectrum to the gate logic, in a hierarchical order rather than to allow user selected colors for each population. When viewing multiparameter data, this software restriction is often not desirable because the color mixtures can produce the background color causing the population to completely disappear from the view and the hierarchical nature means the gate must always be last. Thus, when using quadrant markers combined by boolean equations, color assignments must be assigned by the user and not the software.

REAGENTS AND SOLUTIONS

Lysing Solution:

4.13 g Ammonium Chloride (NH₄Cl) -- Sigma A-5666
0.5 g Potassium Bicarbonate (KHCO₃) -- Sigma P-4913
0.0185 g Tetra Sodium EDTA -- Sigma ED4SS
500 ml double distilled water

2% UltraPure Formaldehyde:

200 ml Ultrapure, E. M. Grade 10% solution (Polysciences, cat. # 04018)
800 ml Dulbecco's PBS GIBCO (cat#450-1300EC)

REFERENCES

1. Hartig, W. Bruckner, G., Holzer, M., Brauer, K., Bigl, V. Digoxigenylated primary antibodies for sensitive dual-peroxidase labeling of neural markers. *Histochem-Cell Biol.* 104:467-472, 1995
2. Makela, O, and Seppala, I.J.T. (1986) Haptens and Carriers. In *Immunochemistry*. Blackwell Scientific Publications. Chapter 3.
3. Jassni, B, Thomas, N.D., Navabi, H., Millar, D.M., Newman, G.R., Gee, J. and Williams, E.D. Dinitrophenyl (DNP) hapten sandwich staining (DHSS) procedure. A 10 year review of its principle reagents and applications. *Journal of Immunological Methods.* 150:193-198, 1992
4. Van der Loos, C.M. Das, P.K., Van den Oord, J.J., Houthoff, H.J. Multiple immunoenzyme staining techniques. Use of fluoresceinated, biotinylated and unlabelled monoclonal antibodies. *Journal of Immunological Methods.* 117:45-52, 1989
5. Paterson, Y. (1992) Production of antibodies. *Current Protocols in Immunology*, Chapter 2.4
6. Andrew, SM and Titus, JA (1992) Purification and Fragmentation of Antibodies. *Current Protocols in Immunology*, Chapter 2.7
7. Riedy, M. C., Muirhead, K. A., Jensen, C. P., and Stewart, C. C. The Use Of A Photolabeling Technique To Identify Nonviable Cells In Fixed Homogenous Or Heterologous Cell Populations. *Cytometry* 12:133-139, 1991
8. Salzman, R. J. Beckman, C. C. Stewart and S. J. Stewart, Expert Assistance for Flow Immunophenotyping. In: *NATO ASI Series, Flow Cytometry*, Villejuif, France, (A. Jacquemen- Sablon, ed.), Springer-Verlag, 67:413-420, 1993.
9. Frankel, D.S., Olson, R.J., Frankel, S.L., Chisholm, S.W., Use of a neural net computer system for analysis of flow cytometric data of phytoplankton populations. *Cytometry* 10:540-550, 1989

10. Boddy, L., Morris, C.W., Wilkins, M.F., Tarran, G.A., Burkill, P.H. Neural network analysis of flow cytometric data for 40 marine phytoplankton species. *Cytometry*, 15:283-293, 1994
11. R.J.Beckman, G.C. Salzman, C.C. Stewart, Classification and Regression Trees for Bone Marrow Immunophenotyping, *Cytometry*, 20:210-217, 1995.
12. Centers for Disease Control and Prevention. 1997 Revised guidelines for performing CD4+ T-cell determinations in persons infected with human immunodeficiency virus (HIV). *MMWR* 1997: 46(No. RR-2) pp. 18-19

KEY REFERENCES

C.C. Stewart, S.J. Stewart, Cell Preparation for the Identification of Leukocytes. In: *Methods In Cell Biology*, (Z. Darzynkiewicz, J. Robinson, H. Crissman, eds.) Academic Press, Inc. New York, 41:39-60, 1994.

C. C. Stewart, S.J. Stewart, Multiparameter Analysis of Leukocytes by Flow Cytometry. In: *Methods in Cell Biology*, (Z. Darzynkiewicz, J. Robinson, H. Crissman, eds.) Academic Press, Inc. New York, 41: 61-79, 1994.