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Identification of B-Cell Subsets

An Exposition of 11-Color (Hi-D) FACS Methods

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Summary

In the last few years, the effectiveness of developmental and functional studies of individual subsets of cells has increased dramatically owing to the identification of additional subset markers and the extension of fluorescence-activated cell sorter (FACS) capabilities to simultaneously measure the expression of more markers on individual cells. For example, introduction of a 6–8 multiparameter FACS instrument resulted in significant advances in understanding B-cell development. In this chapter, we describe 11-color high-dimensional (Hi-D) FACS staining and data analysis methods that provide greater clarity in identifying the B-cell subsets in bone marrow, spleen, and peritoneal cavity. Further, we show how a single Hi-D FACS antibody reagent combination is sufficient to unambiguously identify most of the currently defined B-cell developmental subsets in the bone marrow (Hardy fractions A–F) and the functional B-cell subsets (B-1a, B-1b, B-2, and marginal zone [MZ] B cells) in the periphery.

Although we focus on murine B-cell subsets, the methods we discuss are relevant to FACS studies conducted with all types of cells and other FACS instruments. We introduce a new method for scaling axes for histograms or contour plots of FACS data. This method, which we refer to as *Logicle visualization*, is particularly useful in promoting correct interpretations of fluorescence-compensated FACS data and visual confirmation of correct compensation values. In addition, it facilitates discrimination of valid subsets. Application of Logicle visualization tools in the Hi-D FACS studies discussed here creates a strong new base for in-depth analysis of B-cell development and function.

Key Words

FACS; flow cytometry; Hi-D; B cells; B-1a; B-1b; marginal zone B cells; follicular B cells; fluorescence compensation; Fluorescence-Minus-One (FMO); Logicle visualization; FlowJo; contour plot; quantile contours.

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1. Introduction

The fluorescence-activated cell sorter (FACS) can be seen as one of the first molecular biology instruments because it was designed and developed to measure the expression of sets of genes by individual cells. Since the introduction of FACS instruments (*1*), they have been widely used to identify and sort lymphocyte subsets defined by the selective expression of genes encoding surface proteins and other markers. Studies discussed here have made use of the latest advances in FACS technology to identify B-cell developmental and functional subsets.

The B-cell subset analyses discussed here are broadly useful in that they serve as an atlas of B-cell subsets in bone marrow and the periphery. In addition, they provide detailed examples of how we use 11-color (Hi-D FACS) methods with a new FACS data-visualization method to resolve subsets that may represent considerably less than 1% of the cells in a given sample. Thus, this chapter provides a guide both to modern Hi-D FACS methodology and to the isolation and study of the development and functions of B-cell subsets.

1.1. Using FACS to Identify B-Cell Subsets

FACS measures expression levels of proteins and other molecules in and on cells by their intrinsic fluorescence (e.g., green fluorescent protein [GFP]) or, more usually, by their ability to bind fluorochrome-conjugated antibodies or other fluorescent probes. When cells stained with fluorescent reagents/probes are passed through the FACS, the fluorochromes are excited by lasers at appropriate wavelengths and the amount of fluorescence emitted is detected by detectors filtered to accept the appropriate emitted wavelength. Over a period of roughly 30 years, FACS has evolved from a one-laser machine capable of detecting two fluorochrome colors and a size marker to a three-laser machine capable of detecting 12 (or more) fluorochromes plus indicators of cell size and granularity.

The identification of B-cell subsets has been intimately tied to the development of the FACS. Antibody-secreting (plasma) cells were the first cells to be identified and sorted with the single-laser FACS (1969) (*1*). Later (1982), the basic B-cell subsets in spleen and lymph nodes were defined in the first immunology studies conducted with a dual-laser FACS instrument (*2–4*). Since these early studies, a series of B-cell development subsets, including pro-B, pre-B, immature, and recirculating B-cell populations, have been identified and characterized in the bone marrow (*5–7*). Similarly, several functionally distinct peripheral B-cell populations, including B-1a, B-1b, B-2, and marginal zone (MZ) B cells, have been recognized (*4,9–12*).

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B-cell development in bone marrow has been classified into six to seven sequential subsets designated Fractions A, B, C, C', D, E, F by Hardy, Hayakawa, and colleagues (5). Fractions A–C', all of which express B220 and CD43, are subsets of pro-B cells actively undergoing heavy chain rearrangement. CD43 surface expression is downregulated after successful Ig heavy chain rearrangement as cells progress to fraction D (pre-B) cells.

Cells in fraction D rearrange Ig light chains and differentiate to fraction E (immature B) cells, which express surface IgM, exit the bone marrow, and continue maturation in the spleen. Bone marrow fraction F contains mature recirculating B cells that display the same phenotype as splenic and lymph node follicular (FO) B cells (relatively low levels of surface IgM and high levels of surface IgD) and probably represents a recirculating pool of these cells.

The B-cell subsets (in some cases lineages) in the periphery include immature B cells recently migrated from bone marrow to spleen, FO B cells, MZ B cells, and small numbers of B-1a and B-1b cells (11–13). Collectively, these subsets/lineages (referred to hereafter as subsets) are distinguished by differential surface expression of CD21–CD24, CD43, and CD5. The origins, anatomical locations, and functions of these subsets have been widely discussed (8,13–18). Here, we focus on their phenotypes and demonstrate the Hi-D FACS methods for resolving them from one another when they are resident in the same organ.

In the examples presented in **Subheading 3.**, we show how the simultaneous staining and Hi-D FACS detection of 11 surface markers enables resolution of fractions A–F and the mature B-cell subsets in bone marrow, spleen, and in the peritoneal cavity (PerC). In addition, we show how Hi-D FACS methods enable detailed exploration of heterogeneity within the known B-cell subsets.

1.2. Fluorescence Compensation in FACS Studies

In discussing methods for resolving the B-cell subsets, we pay particular attention to problems introduced by fluorescence compensation and to related factors that influence the accuracy of conclusions drawn from FACS analyses. These considerations apply to data collection and analysis with all FACS instruments, not just Hi-D FACS, and to all FACS studies in which fluorescence compensation is necessary.

For many years, standard FACS data collection was based on analog (hardware) “compensation” to correct for overlap in the fluorescence emission spectra of fluorochromes excited by the same laser. However, these methods introduce nonlinear systematic errors into the evaluations and cannot resolve between-laser (excitation) spectral overlaps. These problems can be avoided with computed compensation based on uncompensated primary measurements.

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This can be done in real time on some newer instruments and is supported for off-line analysis by most current cytometry software. In measurements using analog logarithmic amplifiers the accuracy of computed compensation is dependent on the accuracy of the logarithmic amplifier (logAMP) calibration. Therefore, the most reliable method is to use high-dynamic-range linear digital electronics with computed compensation. The Logicle data-visualization tool (developed in our laboratory, offered commercially by FlowJo, and incorporated into other software packages; Parks, Moore, et al., in preparation), provides crucial help in evaluating compensated data and is useful in assuring that correct compensation values are being used.

Compensation effects and background (autofluorescence) levels also limit our ability to accurately delineate (gate) subsets deemed “positive” or “negative” for a given marker. Therefore, we discuss methods for choosing staining combinations that optimize gating accuracy, minimize compensation problems, and facilitate determination of background staining levels for individual subsets within an overall population. Together, the methods we describe offer a series of readily applicable techniques that can improve all multiparameter and Hi-D FACS studies, regardless of the number of fluorochromes detected and whether the studies are conducted with traditional or digital flow cytometry instruments.

2. Materials

1. Newborn calf serum (NCS), keep at -20°C until use.
2. Staining medium: deficient hRFMI, 3% NCS; optional: 1 mM ethylenediaminetetraacetic acid (EDTA) (for aggregation-prone cells). Store at 4°C .
3. 100X propidium iodide (PI): 100 $\mu\text{g}/\text{mL}$ PI in 1X phosphate-buffered saline (PBS). Store at -20°C .
4. For staining: FACS tubes (5-mL polystyrene round-bottom tubes), 96-well plates, fluorochrome-conjugated antibodies (these are light sensitive so keep in dark at 4°C).
5. For data collection: We use a modified flow cytometer (Hi-D FACS) capable of detecting 11 colors with 3 lasers (488, krypton, and dye laser). The 488-nm laser excites fluorescein isothiocyanate (FITC), phycoerythrin (PE), Cy5PE/Cy-Chrome, Cy5.5PE (or Cy5.5 peridinin chlorophyll protein [PerCP]), and Cy7PE reagents. The dye laser, emitting at 598 nm, excites Texas Red/Alexa594, allophycocyanin (APC), Cy5.5APC, and Cy7APC reagents. The krypton laser (407 nm) excites Cascade blue and Cascade yellow/Alexa430 reagents. Calibration beads (Spherotech, Libertyville, IL); store at -20°C .
6. For data analysis: FlowJo software (TreeStar, San Carlos, CA). Version 4.3 (includes Logicle visualization) or above is preferable.

3. Methods

The following methods include (1) staining procedure, (2) collection of 11-color data on flow cytometer, and (3) data analysis using FlowJo software.

Table 1
11-Color Stain Combination Used to Identify B-Cell Subsets

Specificity	Fluorochrome	Second step
CD21	FITC	–
B220	PE	–
CD5	Cy5PE	–
CD24	Biotin	SA-Cy5.5PerCP
IgD	Cy7PE	–
BP-1	Alexa594	–
CD43	APC	–
CD23	Cy5.5APC	–
IgM	Cy7APC	–
CD3 ϵ , CD8 α , Gr-1	CasBlu	–
Mac-1	CasYel	–

3.1. Basic Staining Protocol (see Note 1)

1. Prepare cell suspension from Balb/c spleen, peritoneal cavity, or bone marrow. Resuspend 0.5–1 million cells in 50 μ L staining media for each stain combination. Add Fc block (CD16/CD32) prior to staining to prevent Fc receptor binding to antibodies.
2. Make up the stain combination: Use the recommended titer for each reagent and add an appropriate amount of the fluorochrome-conjugated antibodies to 50 μ L of staining media. See **Table 1** for 11-color B-cell staining combination.
3. Add cells to the stain and incubate for 15 min on ice.

If FACS tubes are used for staining, add 4 mL staining medium to wash away unbound antibodies. Centrifuge at approx 800g for 5 min. Aspirate and resuspend cells in 200 μ L staining medium, and add PI to a final concentration of 1 μ g/mL.

If 96-well plates are used for staining, stain cells in alternate columns and rows to avoid potential contamination from adjacent wells.

Add 150 μ L staining medium, and spin down cells after the first incubation. Aspirate, add 200 μ L staining medium for washing, and centrifuge. Repeat the washing step twice. Aspirate and resuspend cells in 200 μ L staining medium and add PI to a final concentration of 1 μ g/mL. Transfer the stained cells to FACS tubes.
4. If biotin-conjugated antibody is used in the staining cocktail, then a second step is required. Add an appropriate amount of streptavidin-fluorochrome (SA-fluorochrome) to the stained cells (stained with fluorochrome-coupled and biotin-coupled antibodies), and incubate on ice for 15 min. Wash away the unbound second-step reagent with 4 mL staining media (in FACS tubes). Centrifuge at approx 800g for 5 min. Aspirate and resuspend cells in 200 μ L staining medium and add PI to a final concentration of 1 μ g/mL.

5. The stained cells can be covered and kept at 4°C for 3–4 h until analysis.
6. A compensation sample (cells or antibody-capturing beads) must be made fresh for each fluorochrome used in the staining combination. A compensation sample consists of cells or capturing (antirat Ig or antimouse Ig) beads and only one staining reagent. Generally, a single-stain sample should be included for each reagent in an experiment. In particular, tandem dye lots differ in spectrum so a separate single stain sample is almost always necessary for each tandem reagent.
7. Fluorescence-minus-one (FMO) samples are made by staining cells with a reagent cocktail from which a selected reagent is omitted. FMO-stained cells are necessary for determining the effective background signal distribution in the fluorescence channel of the omitted reagent. FMO methodology is discussed in **Note 2**.

3.2. Data Collection on 11-Color Flow Cytometer (Hi-D FACS)

3.2.1. Instrumentation Standardization

Before collecting data, the three lasers in the Hi-D FACS are carefully aligned. If an automated standardization system is available, the instrument is standardized with stable fluorescent microspheres (“beads”). If automated standardization is not available (most flow instruments), the beads are used to manually standardize the instrument by tuning each photomultiplier tube (PMT) voltage in turn until the beads in each channel are at the standard location assigned for the channel.

Except under very special circumstances, no changes are made to the PMT settings after standardization is completed. If the instrument develops a nozzle clog or other mechanical problem, it must be restandardized before data collection is resumed. After the standardization is completed, it is wise to collect data for a calibration bead sample (10,000 events will do) and to store this standardization data along with the data collected for the samples in the experiment.

In our experience, the multiple dye-containing rainbow beads obtained from Spherotech (*see Subheading 2., step 5*) provide a very convenient, uniform, and stable reference for standardization.

3.2.2. Fluorescence Compensation

The emission spectrum of each fluorochrome is not restricted to the wavelength band collected for that fluorochrome. In most cases, some fluorescence is detectable at wavelengths collected for other fluorochromes. Because the amount of the spectral overlap fluorescence is proportional to the emission in the primary channel, the signal owing to the overlap fluorescence can be estimated and subtracted from the overall fluorescence collected in a given channel. This process, through which signals owing to overlap fluorescence are removed, is called *fluorescence compensation*. Fluorescence compensation is necessary whenever a dye generates signal on a detector other than its primary detector.

Our method of setting fluorescence compensation parameters has changed recently, owing to introduction of software that provides good user support in specifying correct compensation values and owing to increased appreciation of the pitfalls and irreversibility of data manipulation by analog compensation. Previously, instrument hardware was used to subtract the amount of overlap fluorescence and adjust the net signal to match background/autofluorescence. However, this subtraction introduces significant artifacts for two reasons:

Hardware subtraction with peak detection becomes biased as the net corrected signal approaches zero.

Logarithmic scales do not contain a zero point and cannot display negative numbers. However the single stain data used to set compensation values yield populations of events distributed around zero and a mean value close to zero. Logarithmic displays of such populations give views that are not suited to selection of correct compensation values. In addition, measurements of compensated signals using logarithmic amplification distort results by reporting all very low and negative values at the minimum of the logarithmic scale.

Therefore, good practice dictates that the hardware compensation utility on FACS instruments be turned off and that uncompensated data be collected. In this case, data must also be collected from compensation samples and stored so that it can be used later to do the fluorescence compensation off-line. The method for doing this is discussed in the next section.

Analog hardware compensation must still be used for most multicolor sorting on instruments that are without real-time software-computed compensation. In all cases data on control samples should be recorded with the same settings as the test samples.

3.2.3. Data Collection on Multicolor Experimental Samples

After standardizing the FACS instrument and collecting the data on calibration beads, adjust the FACS instrument forward scatter gain to bring cell samples to the proper scale. Select number of cells to be analyzed. Collect data on individual samples. Adjust the sample flow rate to a proper rate as the accuracy of measurements and fluorescence compensations are degraded if the sample flow rate ($\mu\text{L/s}$ more than cells/s) is too high. Minimize changes in sample flow rate between samples.

3.3. Data Analysis Using FlowJo Software

1. Create a new FlowJo workspace with the data that is collected.
2. Live cell gate: double-click on a sample to view the collected data in a new window. Dead cells (PI positive) will show up as very bright cells on the Cy5PE (488 nm excitation, approx 665 nm emission) channel. Set a gate that includes all cells except the dead cells. Click on down arrow to bring up a new window showing all cells within the gate. It is preferable to view the analysis on contour plots

or color density plots instead of the dot plots because the structure of the data in dense regions is obscured in dot plots owing to contiguous placement and overwriting of dots (**Fig. 1**). Both contour plots (with outliers, **Fig. 1**) and color density plots with outliers (not shown) in FlowJo maintain resolution regardless of cell numbers and are thus better suited for viewing populations.

3. Compensation gates: each fluorescent reagent must have a compensation sample associated with it. Gate on fluorochrome-positive cells (or antibody-capturing beads) for each of the 11 colors. However, very bright cells (at the upper boundary of the plot) must be excluded, even when they are part of a continuous population.
 - a. To define compensation matrix, go to *Platform* → *Compensate sample* → *Define matrix*. Using the populations defined in the previous step, place the gate name for each of the fluorochrome-positive cell samples in the appropriate boxes on the menu. Use unstained cells for fluorochrome-negative cells. Use gated unstained beads if capturing beads are used for compensation. Click “compute” to generate a compensation matrix for the staining combination.

One special caution: Different lots of tandem fluorochromes (i.e., Cy5PE, Cy5.5PE, Cy7PE, Cy5.5APC, and Cy7APC) will have different emission spectra. Similarly, different conjugates with the same or different APC lots may have different emission spectra. Therefore, it is necessary to generate separate compensation matrices if different lots of tandem fluorochromes are used in different stain combinations (**Fig. 2**).
 - b. Apply the compensation matrix to the cells stained with 11 colors (*Platform* → *Compensate Samples* → *Comp Matrix*). Gate on live cells before analyzing each sample.

3.4. Logicle Visualization

The Logicle data display addresses problems introduced by the absence of a zero point and negative values on the logarithmic axes traditionally used to display FACS data (David Parks, Wayne Moore, et al., manuscript in preparation) (see **Fig. 3**). Logarithmic plots “pile up” at baseline all events with values at or below that level and often show a peak above the true center of a population with a low-to-negative mean (**Fig. 3A**, upper right panel). Logicle axes, in contrast, enable display of the data on mathematically defined axes that are essentially linear in the region around zero and essentially logarithmic at high data values.

Note that the Logicle transformation does not change the data. It only provides a method for visualizing data that cannot be displayed well on a logarithmic scale.

Figure 3A illustrates the usefulness of Logicle display of single stain control data in assessing the accuracy of fluorescence compensation. In the logarithmic view the data is correctly compensated (as confirmed by statistical evaluation), but there is no way to determine that from the visual display. The

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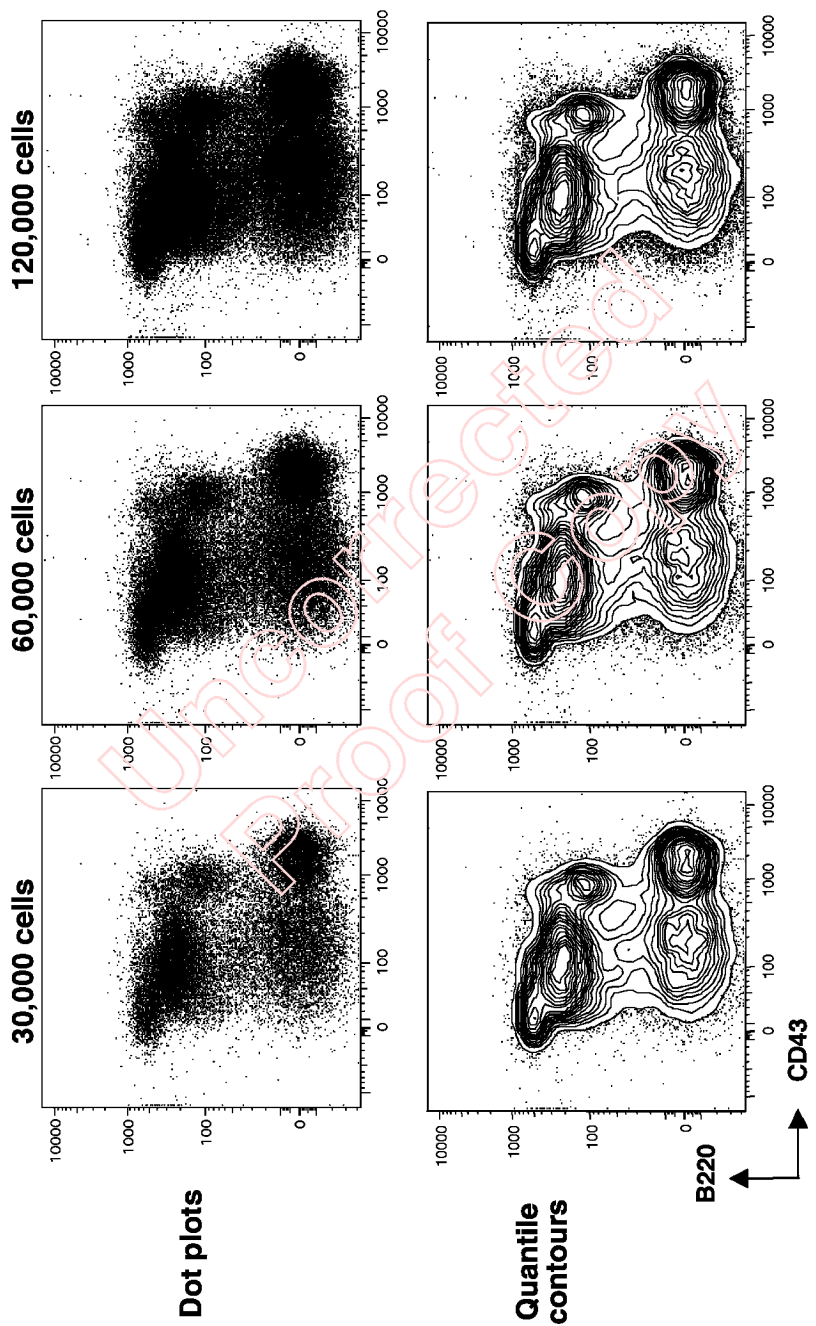


Fig. 1. Dot plots are sensitive to cell numbers. Dot plots tend to lose resolution when cell numbers are too high or too low. Contour plots (with outliers) maintain the resolution of subsets regardless of cell numbers.

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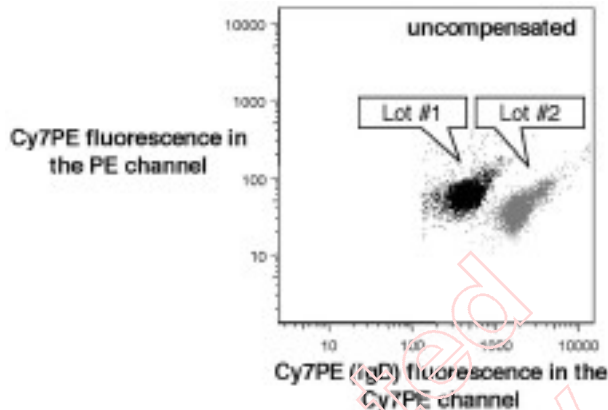


Fig. 2. Different lots of tandem dyes have different fluorescence properties. IgD conjugated to two different lots of Cy7PE (shown in *gray* and *black*) gives different Cy7PE to PE fluorescence ratios.

Logic plot, however, provides clear visual confirmation that the FITC high and FITC low/negative populations are matched in terms of their average PE channel signal.

Deviation from this pattern will indicate errors in compensation (**Fig. 3B**).

Logic visualization tool has recently been established as the default in our laboratory. It has only been added to FlowJo in version 4.3, March 2003. Therefore, in future versions the method for accessing it may be different than that described here.

In essence, a Logic transformation is defined to match a user-selected, compensated, and gated data set and then applied to related compensated data sets. Live, size-gated populations work well as the selected data set. Using cells from the same source as those to which the transformation will be applied is advisable. Logic is currently accessed in FlowJo by turning on the *Allow custom visualization* option in the FlowJo Preferences. Once Logic is turned on and compensation has been done, select the gated data set (fully stained) intended as the Logic base (i.e., live, size-gated lymphocytes), then select *Platform* → *Compensate Sample* → *Define Transformation*. FlowJo will then apply the transformation to all samples compensated with the compensation matrix used for the initial transformation sample.

Note that to use different transformation base populations for samples compensated with the same matrix, it is necessary to copy and rename the matrix and use the copy to compensate the target subgroup. Using different base populations may be useful because the Logic transformation is determined by the selected base population.

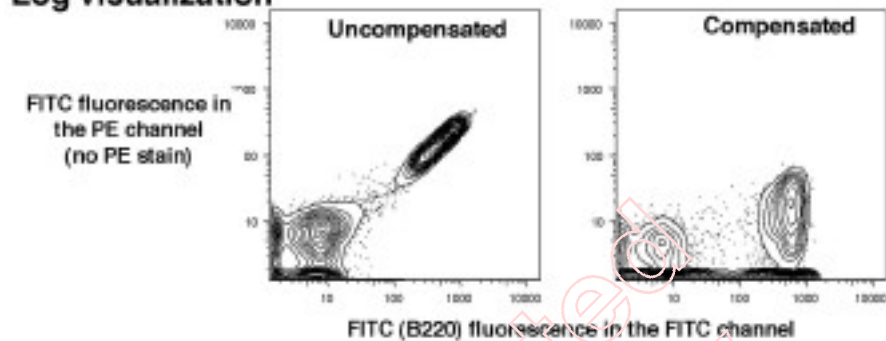
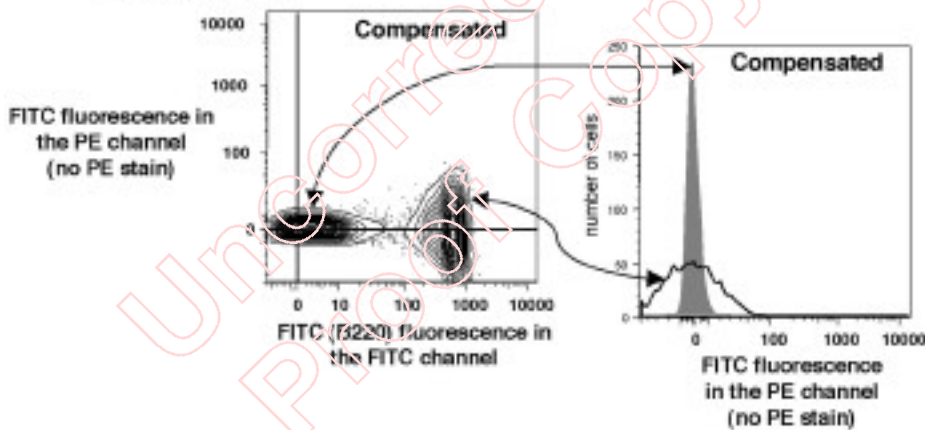
A**Log visualization****visualization**

Fig. 3A. Visualization methods. Spleen cells are singly stained with B220-FITC and shown in two-dimensional plots (FITC vs PE) on top panels. *Top left*: uncompensated data. *Top right*: compensated data with standard logarithmic scale. Note that while the computed median in the PE dimension of the FITC-positive population is near the baseline of this plot, the apparent center of the population is well above this. *Bottom left*: compensated data with custom visualization. *Bottom right*: Histograms of compensated PE fluorescence detected in FITC⁺ and FITC⁻ cells. Note that both FITC⁺ and FITC⁻ cells are distributed symmetrically around a common center near zero.

3.5. Gating and Visualizing B-Cell Subsets

Figs. 4–6 show data for bone marrow, spleen, and peritoneal cavity samples stained with an 11-color staining combination designed to distinguish subsets of developing and mature B cells. We point out the advantages of using this single

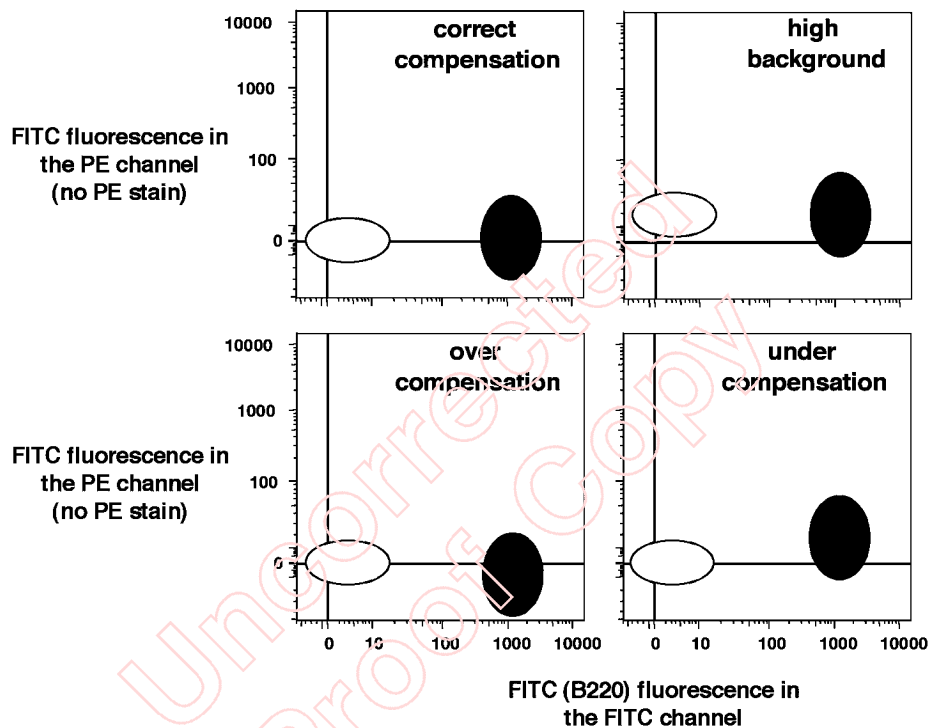
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Fig. 3B. Visualization methods (*continued*). Deviation from proper compensation can be visualized on Logicie axes. *Upper left panel*: cells that are properly compensated. *Upper right panel*: high background or autofluorescence level on the PE channel. *Lower left panel*: FITC⁺ cells that are overcompensated (too much fluorescence subtraction). *Lower right panel*: FITC⁺ cells that are undercompensated (too little fluorescence subtraction).

Hi-D FACS stain, as opposed to using several combinations with fewer colors in each, as we move through the gating methods.

1. In adult Balb/cN bone marrow (**Fig. 4A**), live cells are gated from total cells (in FSC and Cy5PE channels). Within the live cells, a lymphocyte gate is applied to gate out the large, nonlymphocytic cells. Within the live, lymphocyte gate, a B220⁺ dump (CD3ε⁻, CD8α⁻, GR-1⁻) gate is applied to gate on B cells. This live, lymphocyte, B220⁺ gating scheme is also used for bone marrow, splenic, and peritoneal B cells.

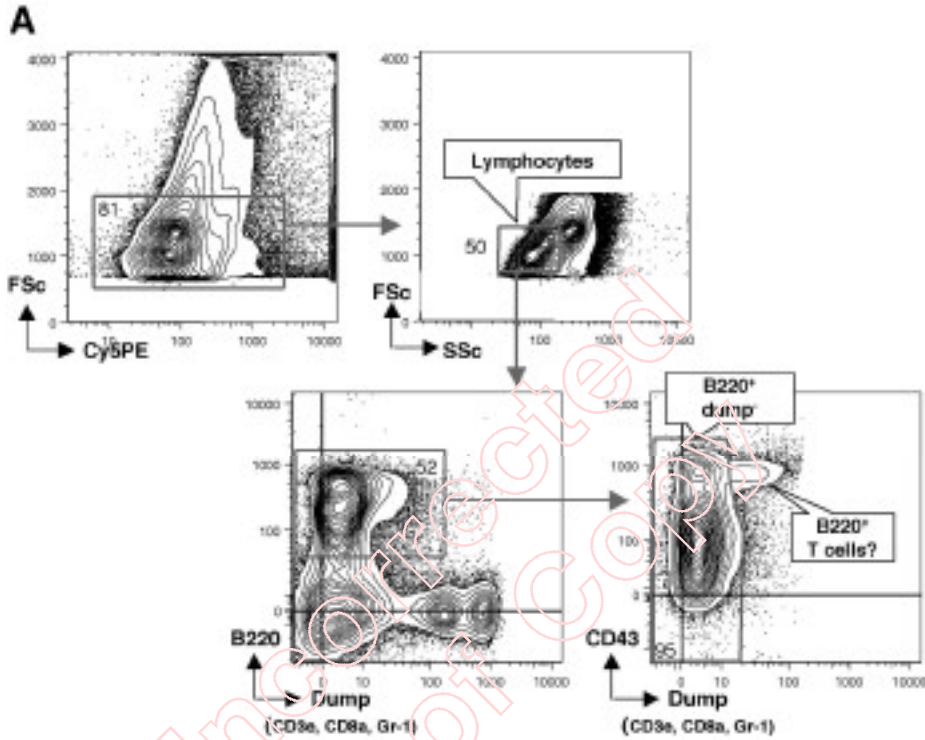


Fig. 4A Identification of bone marrow B-cell populations. Bone marrow cells are first gated on live cells (*top left panel*), on lymphocyte size (*top right panel*), followed by eliminating contaminating non-B cells by gating on B220 and the dump channel ($CD3e^-$, $CD8\alpha^-$, and $Gr-1^-$) (*bottom panels*).

The classic gating scheme for identifying the B-cell developmental subsets is shown in **Fig. 4B**. In this figure, CD43 and B220 (*upper panel*) jointly identify two major B-cell developmental subsets within the live, lymphocyte, B220⁺ population: Hardy fractions A–C', which contain the earliest developing B cells; and Hardy fractions D–F, which contain pre-B, immature and recirculating B cells.

Fraction A–C is then further separated into fractions A, B, C, and C' by CD24 and BP-1 (**Fig. 4B**, *lower right panel*). Fractions D–F (**Fig. 4B**, *lower left panel*) is separated into fractions D, E, and F by staining for IgM and IgD. Further gating (data not shown) demonstrates that fractions D (pre-B) and E (immature B) continue to express CD24 and do not express CD5, CD21, and CD23. Fraction F (recirculating B) cells express CD23 and low levels of CD21.

Fig. 4C presents an alternate gating scheme that better resolves fractions A–C' and reveals a "new" subset akin to fraction D but expressing higher levels of

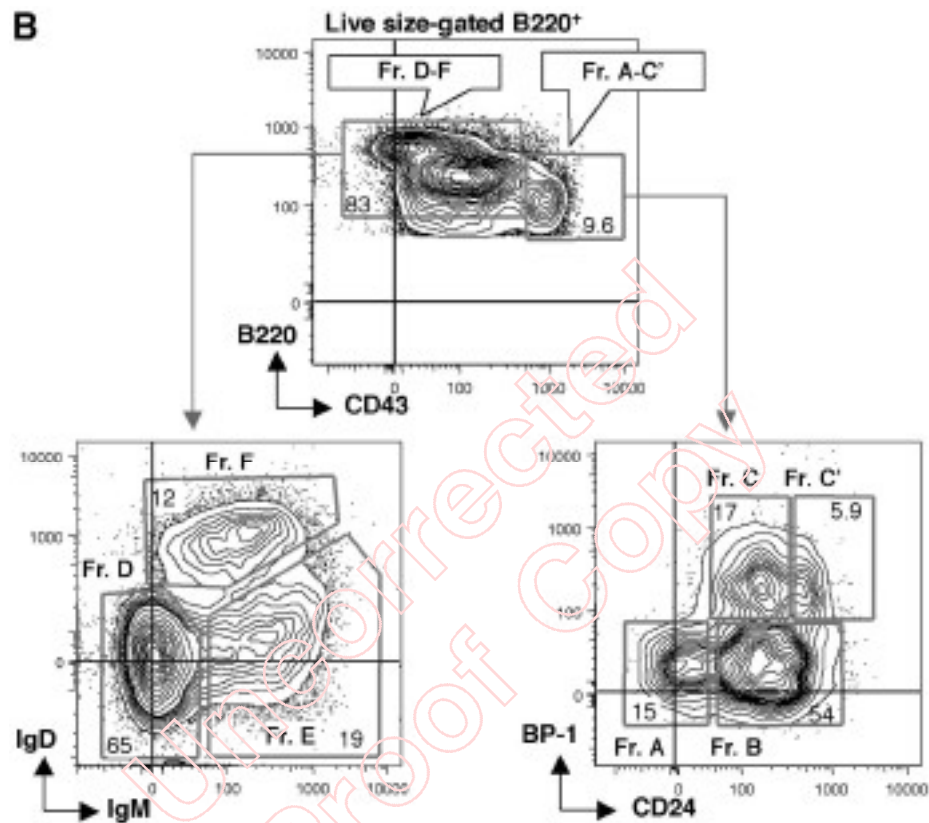
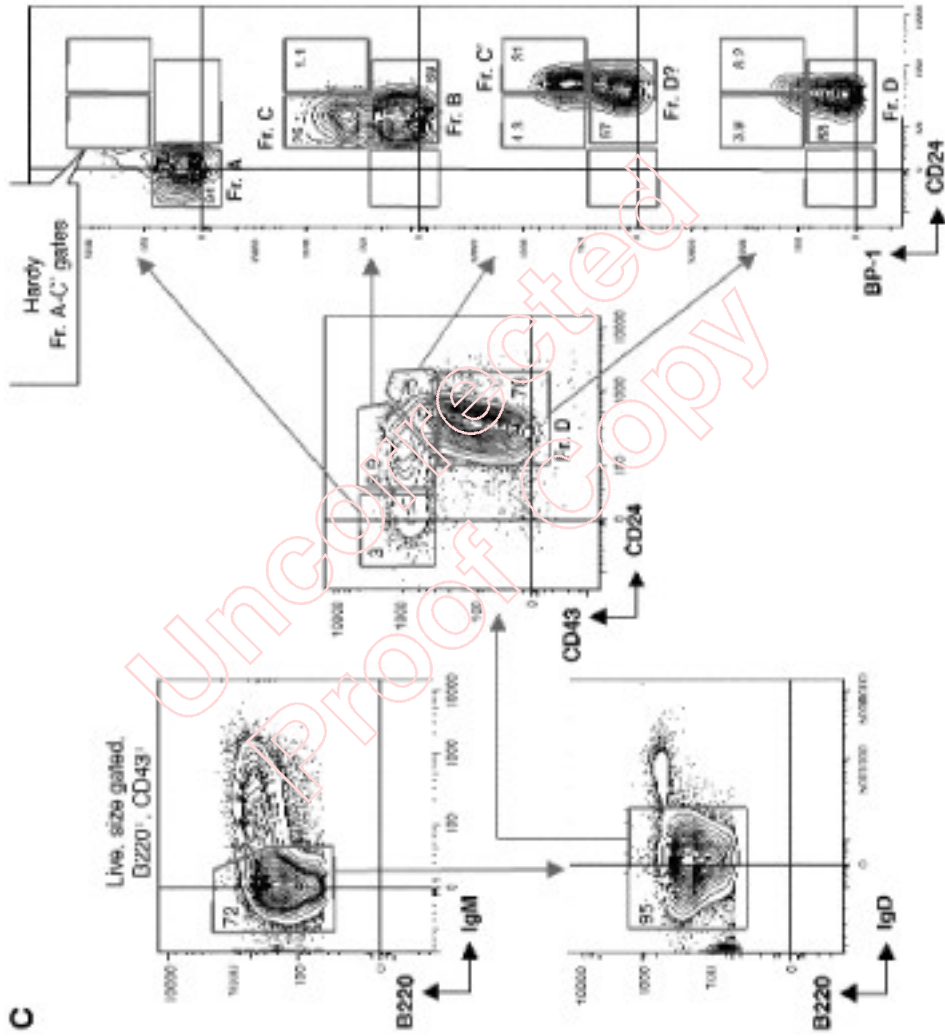


Fig. 4B. Identification of bone marrow B-cell populations (*continued*). In live, size-gated, B220⁺ dump⁻ bone marrow cells, fractions A–C are identified as CD43⁺ B220⁺, whereas fractions D–F are CD43⁻ B220⁺. Fractions A–C can be separated into individual subsets by CD24 and BP-1. Fractions D–F can be separated based on the IgM and IgD surface expressions.

CD43 and CD24. In this scheme, fractions E and F (B220⁺ cells expressing IgM and IgD) are sequentially gated out. The remaining cells are gated on CD24 and CD43 and are resolved into four subsets that can then be further gated to resolve fractions A–D and reveal a previously unresolved subset labeled “D” in the *third panel* down on the right. In addition to revealing this “new subset,” this gating scheme has the advantage of cleanly resolving fraction C from fraction C’.

Fig. 4C. (*see facing page*) Identification of bone marrow B-cell populations (*continued*). An alternative way of separating fractions A–C’ based on CD24 and CD43.

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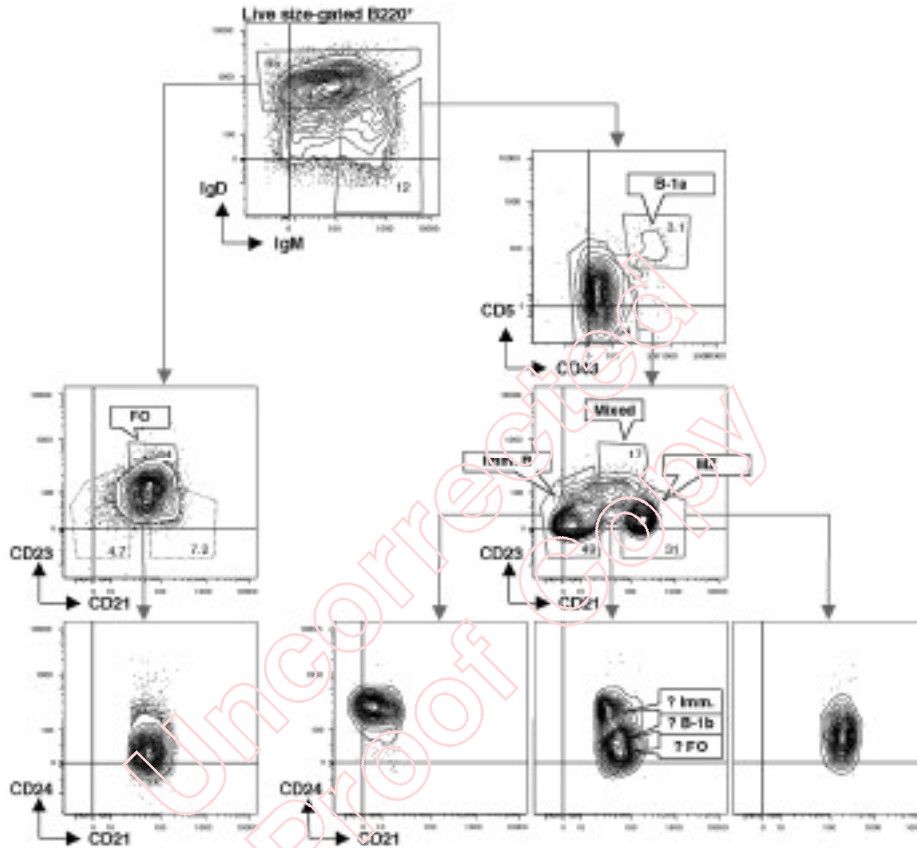


Fig. 5. Identification of splenic B-cell populations. Live, size-gated, B220⁺ dump⁻ splenic B cells are separated based on IgM and IgD expression. The IgM^{lo} IgD^{hi} cells are mainly FO B (B-2) cells. The IgM^{hi} IgD^{lo} cells can be further separated into MZ, B-1a, and immature B populations.

2. **Figure 5** presents the gating scheme for mature B-cell subsets in adult Balb/cN spleen. Live, B220⁺ cells are selected using the gating scheme described in the previous section and then resolved according to surface Ig expression into two major subsets.
 - a. FO B cells (*left panels*) are a fairly homogeneous IgM^{lo} IgD^{hi} subset. They express intermediate levels of CD21, little or no CD24 (*lower left panel*), high levels of CD23, and they do not express detectable levels of CD5, BP-1, or Mac-1 (data not shown).
 - b. The IgM^{hi} IgD^{lo} subset (*right panels*) contains two subsets:
 - (i) B-1a cells, which express CD5 and CD43, are readily resolved by visualizing CD5 and CD43 expression.

- (ii) The remaining cells, which express neither of these markers (*center right panel*). CD21 and CD23 expression resolves this latter subset into three smaller subsets (*lower panels on right*).
- Immature B cells, which do not express either CD21 or CD23 but express high levels of CD24.
 - A small subset that expresses intermediate levels of CD21 and CD23 and appears to be a mixture of immature B, B-1b, and perhaps some follicular B cells.
 - Marginal zone (MZ) B cells, which express high levels of CD21 but little or no CD23 and CD24.
3. **Figure 6** presents a gating scheme for resolving B-cell subsets in the Balb/cN peritoneal cavity. The Ig⁺ population that constitutes the vast majority of the B220⁺, size-gated live cells is resolved into three subsets by B220 and CD5 expression levels (*top right panel*).
- B-1a cells, which express low levels of CD5, high levels of CD43, low to intermediate levels of CD24, high levels of IgM, low levels of IgD, and little or no CD21 or CD23 (although a few cells may express intermediate CD23 levels).
 - B-1b cells, which do not express detectable levels of CD5 but are otherwise similar in phenotype to B-1a for the markers tested.
 - A subset with the same phenotype as FO B cells in the spleen.

4. Notes

- Considerations in designing reagent combinations: FACS data collection consists basically of using a laser to excite a particular fluorochrome and collection of the emitted fluorescence via a fluorescence detector. However, as the number of lasers and detectors increase, the complexity of the data collection and the analysis also increases because of the spectral overlap that occurs between fluorochromes. Designing staining combinations for multiparameter and Hi-D FACS experiments therefore requires careful choice of reagent–fluorochrome combinations to minimize data uncertainty owing to high background and fluorescence compensation limitations.

When the staining cocktail is being designed to detect two or more determinants of the same cells, reagent–fluorochrome pairs should be chosen to minimize the need to subtract large fluorescence compensation values. Ideally, the fluorochromes associated with reagents that detect determinants on the same cells should be excited by separate lasers (and hence the fluorescence signals are collected into different detectors at different times). Initial studies can be done with pairwise stains to determine the spectral overlap between the two reagents when excited by the separate lasers. For example, in **Fig. 3B**, because PE and APC have minimal spectral overlap, B220-PE and CD43-APC are chosen to identify fractions A–C and D–F in the bone marrow so that overlap from the bright B220 (488 nm laser) signal will not have to be subtracted from the CD43 (dye laser) signal, which may be dull or negative on some subsets (fractions D–F).

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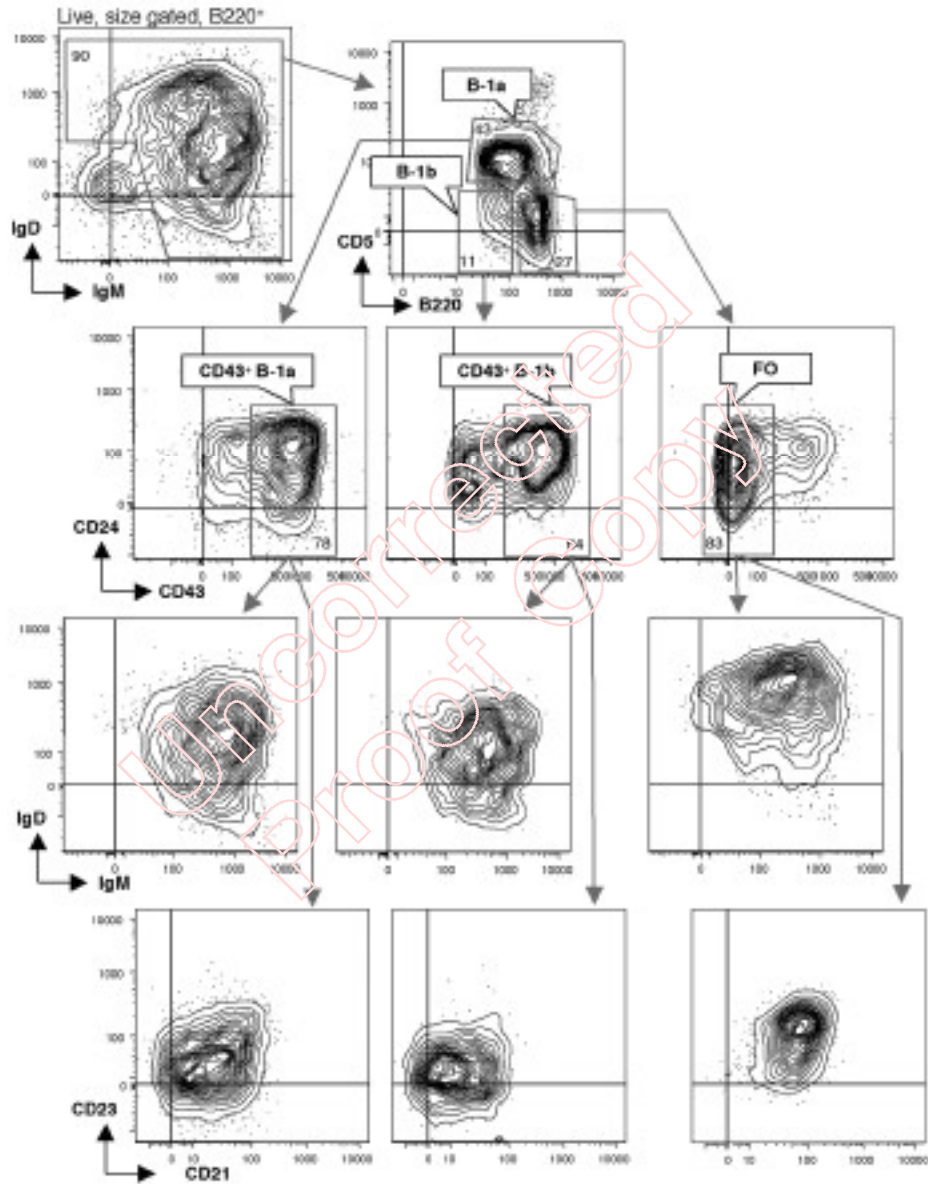


Fig. 6. Identification of peritoneal B-cell populations. Live, size-gated, B220⁺ dump⁻ peritoneal cells are separated based on B220 and CD5 expression. These B220⁺ cells can be separated into FO B, B-1a, and B-1b cells.

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If the only reagents available to stain a pair of markers on a given cell type must be excited by the same laser, choice of which fluorochrome is used to mark the reagent detecting the duller of the two markers is quite important. In general, efforts should be made to assign markers giving high levels of staining to dyes that do not have strong spectral overlap onto several other detectors. Conversely, markers expected to yield low or negative staining on cells of interest can be preferentially assigned to dyes that show strong spectral overlaps.

In addition, it is important to titrate the antibody reagents before staining cells with a reagent cocktail. Generally, staining concentrations are selected to stain the cell type of interest at saturation for the target determinant. Minimization of background staining is also taken into account. Titrations also enable estimation of the amount of target determinant expressed on subsets of interest and thus provide key information for designing efficient reagent cocktails.

2. Background signal estimation and fluorescence-minus-one (FMO) controls: it is always difficult to distinguish cells that do not express a given determinant from cells that express low levels of the determinant because cells may have different levels of intrinsic autofluorescence or nonspecifically bound fluorochrome-conjugated reagents. For estimating background owing to nonspecific binding of fluorochrome-coupled reagents, staining samples with a nonreactive antibody that otherwise mimics the staining reagent (i.e., an “isotype control”) can be useful (although interpretation can be confounded by differences in “stickiness” of the two putatively similar antibodies). However, background staining tends to be minimal when appropriate Fc and other blocking antibodies are used before staining, and if staining reagents are prepared and stored properly and centrifuged when necessary to remove aggregates.

Autofluorescence contributions to background can be estimated by collecting data for an unstained cell sample. However, in multiparameter and Hi-D FACS studies, overall background estimation is more complicated because spectral overlap and the statistics of fluorescence compensation differentially affect subsets of cells. For example, consider a sample that contains two subsets of cells, one of which expresses a determinant detected by a FITC-coupled reagent and the other does not. If no PE-conjugated reagent is included in the cocktail, both subsets would *a priori* be expected to show the same level of background fluorescence in the PE channel. However, because FITC reagents give a substantial signal in the PE channel, the FITC-positive population will have a higher fluorescence in this channel.

On average, fluorescence compensation reduces the corrected fluorescence in the PE channel to the background level for both subsets in this example. Thus, both subsets will be distributed symmetrically around the center of the negative population, but because of the statistics of the compensation process, the FITC-negative population will have a substantially smaller spread than the FITC-positive subset (see Fig. 3A).

The compensation effects shown in Fig. 3A can be readily visualized because the cells in this example were only stained with B220-FITC. Therefore, the amount of FITC fluorescence represents the total fluorescence on each subset

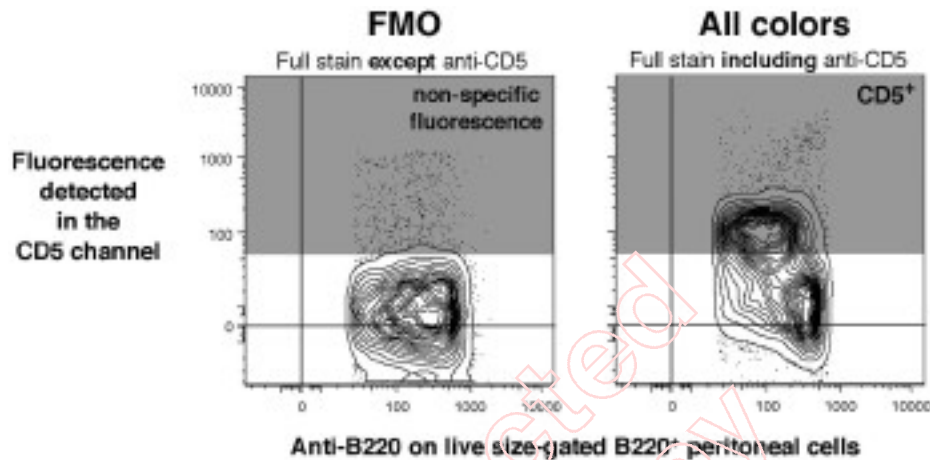


Fig. 7. Fluorescence-minus-one (FMO). An FMO stain is performed with peritoneal cells to determine background level in the CD5 (Cy5PE) channel. The standard live, size-gated, B220⁺ gating is applied to both 11-color and FMO-stained cells. The B220⁺ subsets in each stain are then compared to determine background levels.

above the unstained autofluorescence. The FITC primary signal can be shown on the *x*-axis, and the compensated values can be shown on the *y*-axis (PE channel). In multiparameter and Hi-D FACS experiments, however, the expression of multiple markers in individual subsets often means that several stains may contribute to signals obtained for a particular subset in a given channel, necessitating compensation for several fluorescence signals in that channel. For this reason, the boundary between no detectable expression and low expression of a particular determinant must be determined directly.

FMO staining, that is, staining the cells with all reagents in the cocktail except the one that registers in the channel of particular interest, is critical for this threshold determination (19,20). In the example shown in **Fig. 7**, FMO staining is used to determine CD5 expression in peritoneal B cells (live, size-gated B220⁺) stained with the 11-color staining cocktail shown in **Table 1**. The upper bound of the B220⁺ cells in the FMO stain (no CD5 reagent) is 55 fluorescence units. Therefore, when the CD5 reagent is present (the full cocktail), B cells that show more than 55 fluorescence units can be taken as CD5⁺. The CD5 threshold for B220⁻ cells (not shown in this example) could be considerably lower if they do not express any other determinant that contributes signals for which compensation is necessary. In all studies shown in the figures in this publication, FMO stains were done to evaluate thresholds for positivity whenever they were in question.

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