Taking Advantage of Fluorescence Spillover to Analyze More Markers on Fewer Detectors: Thinking Outside the Orthogonal Box

Technical Bulletin

Contents

Abstract

The conventional approach to multicolor flow cytometry is to assume that each fluorochrome will be defined by signal in only one detector: for example, FITC will be detected only in FL1, PE only in FL2, and so on. From this perspective, fluorescence spillover (FITC detected in FL2 and PE in FL1) is an artifact to be removed using a fluorescence compensation algorithm.

This paper demonstrates that spillover—far from being a nuisance—can be exploited to increase the number of fluorochromes that can be analyzed simultaneously with a given number of detectors. By selecting two fluorochromes that do not spill over into the other's channel, and one that emits into both channels, all three fluorochromes can be resolved using just two detectors. This strategy is especially suited to an instrument such as the BD Accuri™ C6 flow cytometer, in which detector voltage, optical filters, and laser power are fixed and spillover is predictable.

Using staining protocols based on this strategy, three human lymphocyte populations (B cells, NK cells, and T cells) were successfully identified using two detectors. The approach was then extended using strategic gating techniques to characterize subpopulations and assess cell viability within these three populations. The results show that up to seven markers can be analyzed simultaneously using only the four fluorescence detectors of the BD Accuri C6.

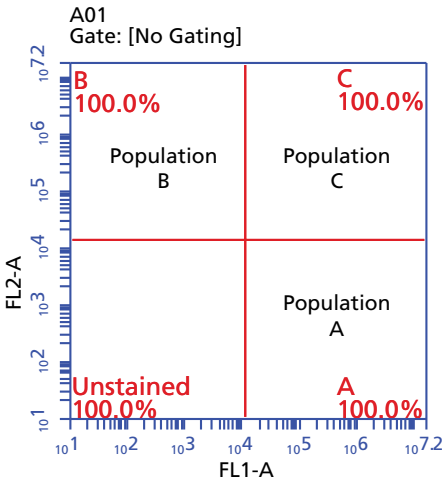

Figure 1. Detecting three populations using two detectors.

Introduction and strategy

In conventional multicolor flow cytometry, each fluorochrome is defined by signal in a single detector. On an instrument with four fluorescence detectors, you can design up to a four-color experiment. If you need more than four markers to distinguish cell populations, you might be able to double up markers on a single fluorochrome and use backgating to identify all the populations on a single plot.[1]

Another innovative way to analyze more markers (and maximize your instrument's capabilities) is to break the paradigm of assigning one marker/fluorochrome to each detector. Suppose three different populations can be identified using three markers, each unique to one population. If population A's marker can be identified by a fluorochrome that is detected only in FL1, it will appear in the lower right quadrant of a bi-fluorescence plot (Figure 1). Similarly, if population B's marker is detected only in FL2, it will appear in the upper left quadrant. Finally, if population C can be identified using a fluorochrome that is detected in both detectors—that is, one that has substantial spillover between the two detectors—it will appear distinctly in the upper right quadrant.

Conventionally, fluorescence spillover from a fluorochrome's primary detector into a neighboring detector is considered an artifact that is usually removed using a fluorescence compensation algorithm. This paper shows how spillover can be exploited to increase the number of fluorochromes that can be analyzed simultaneously with a given number of detectors.

Because the strategy depends on spillover between channels being predictable and reproducible, it requires that voltage and gain settings be held constant. A fixed-voltage flow cytometer such as the BD Accuri C6 is particularly suited for this strategy. Accordingly, the paper begins by demonstrating such predictability for six common fluorochromes across multiple sample types, fluorochrome sources, and instruments. In fact, spillover on the BD Accuri C6 can be mathematically modeled by a best-fit line with a slope indicating the magnitude of spillover from the primary into the secondary detector.

The models show that, on the BD Accuri C6, certain fluorochromes such as PE-Cy™7 (FL3) and APC (FL4) exhibit very little spillover into the neighboring detector channels. PE-Cy™5, in contrast, has substantial spillover from FL3 into FL4, resulting in a "diagonal population." Thus, these three fluorochromes can readily be distinguished using only FL3 and FL4. Similarly, propidium iodide (PI) shows diagonal spillover between FL2 and FL3.

These findings are used to develop staining protocols for two immunophenotyping applications with human peripheral blood (HPB). Both begin by identifying B, NK, and T cells using just two detectors. Following this, additional markers are added to characterize subpopulations and/or assess cell viability. In all, seven markers are analyzed simultaneously using the four fluorescence detectors of the BD Accuri C6.

Overview of the BD Accuri C6

The BD Accuri C6 personal flow cytometer is powerful, versatile, compact, and affordable. A state-of-the-art digital signal processing (DSP) system allows it to simultaneously collect 16 million channels of digital data, displayed as six full decades. This means that it can finely resolve both faint and bright signals at once and analyze the entire scope of biological variations in a single run, from dim, micron-sized platelets through large, >30-micron, highly fluorescent cell lines.

Figure 2. The BD Accuri C6 flow cytometer system.

The key to analyzing multiple fluorochromes on each detector is standardization of the BD Accuri C6 system. The broad dynamic range allows voltages and gains to be optimized and locked when the instrument is manufactured, so that data display and fluorescence spillover are consistent and predictable. The intuitive BD Accuri™ C6 software includes a Zoom tool and other features that aid researchers in analyzing this broad range of data.

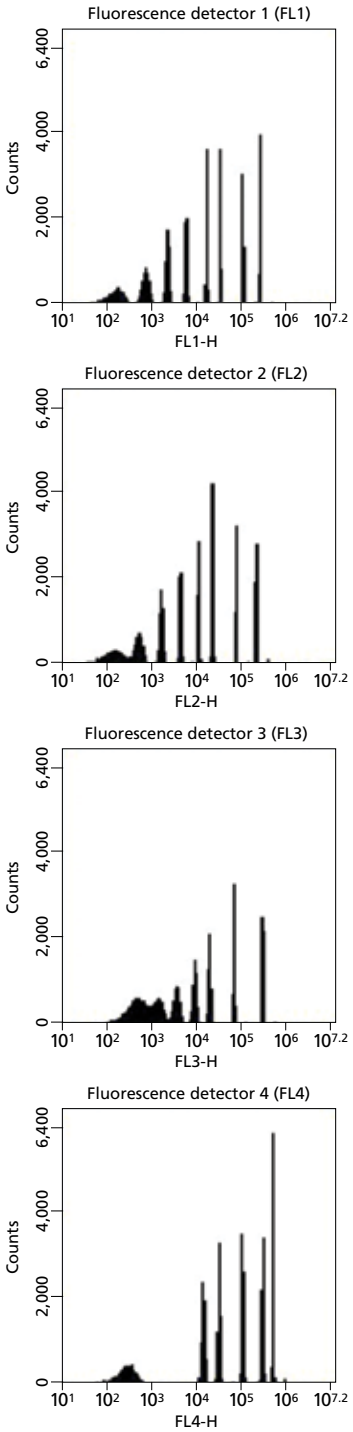

Figure 3. Bead distributions for four detectors on the BD Accuri C6.

Representative plots of SPHERO 8-Peak (for FL1, FL2, and FL3) and 6-Peak (for FL4) Rainbow Bead distributions for the four fluorescence detectors of a BD Accuri C6 after each detector has been optimized for sensitivity and dynamic range. Standard optical filters were used in front of each detector. Actual mean channel value for individual instruments should not vary by more than 5% from day to day.

Detector optimization and fluorescence consistency

Each BD Accuri C6 instrument is calibrated for optimal performance to the same bead standard, and detector voltages are locked down at the factory. As long as instrument performance is validated regularly, fluorescence measurements will be directly comparable across instruments and over time.

Figure 3 shows representative plots of distributions for 6- and 8-Peak SPHERO™ Rainbow calibration particles (beads) for four BD Accuri C6 detectors. Figure 4 shows mean fluorescence bright peak values for the Rainbow Beads, measured on three fluorescence detectors of 32 different BD Accuri C6 instruments, taken at installation and then again 6–24 months later. The data shows that the instruments maintain their optical alignment and detector performance over time when properly installed and maintained.

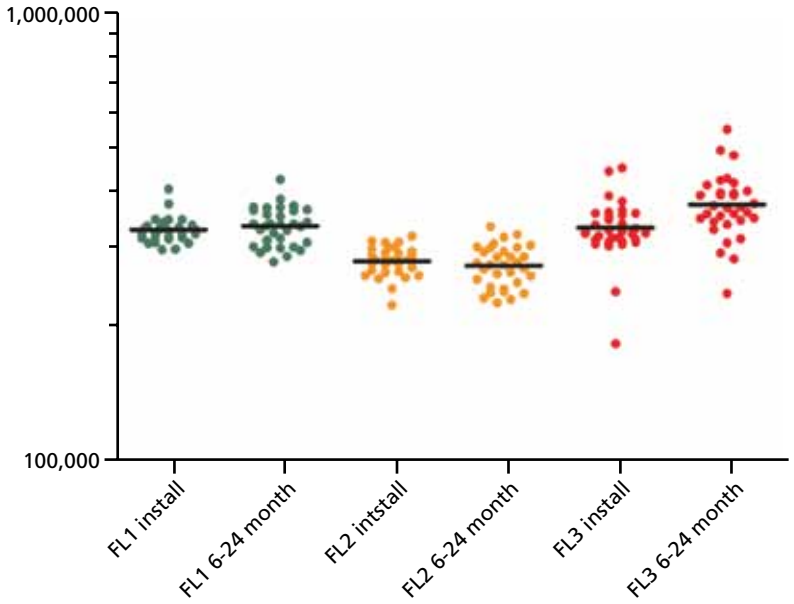

Figure 4. Fluorescence bright peak position over time.

Mean fluorescent bright peak values (SPHERO 8-Peak Rainbow Beads) from 32 BD Accuri C6 instruments were compared at the time of customer installation and then at 6–24 months post-installation. A 1-paired Student's t-test showed no statistically significant difference in top peak mean values over time.

Mathematical modeling and spillover equations

On a fixed-voltage flow cytometer such as the BD Accuri C6, fluorescence spillover can be mathematically modeled. Figure 5 shows FL1 vs FL2 plots of samples stained with a range of FITC or PE fluorescence intensities, while Figure 6 shows FL3 vs FL4 plots for samples stained similarly with APC, PE-Cy5, or PE-Cy7. Unstained samples are included for comparison.

Table 1 shows best-fit linear regression equations for the fluorochrome spillover data in Figures 5 and 6. The slope of each equation indicates the magnitude of the fluorochrome spillover into the indicated channel. A high r^2 value indicates that the equation can be used to predict the channel value of a single-stained event in the spillover detector (y-value) based on its channel value in the primary detector (x-value). The negligible slope (0.0013) for the PE-Cy7 data indicates that there is very little spillover into the FL4 detector, and therefore its FL3 value does not predict its fluorescence in FL4 (low r^2).

Table 1. Best-fit linear equations for fluorochrome spillover on the BD Accuri C6.

Fluorochrome	Equation	Correlation
FITC into FL2	y = 0.0734x + 311	r^2 = 0.98
PE into FL1	y = 0.037x + 983	r^2 = 0.97
APC into FL3	y = 0.007x + 643	r^2 = 0.96
PE-Cy5 into FL4	y = 0.4613x + 6016	r^2 = 0.98
PE-Cy7 into FL4	y = 0.0013x + 480	r^2 = 0.19

Based on the data in Figures 5 and 6.

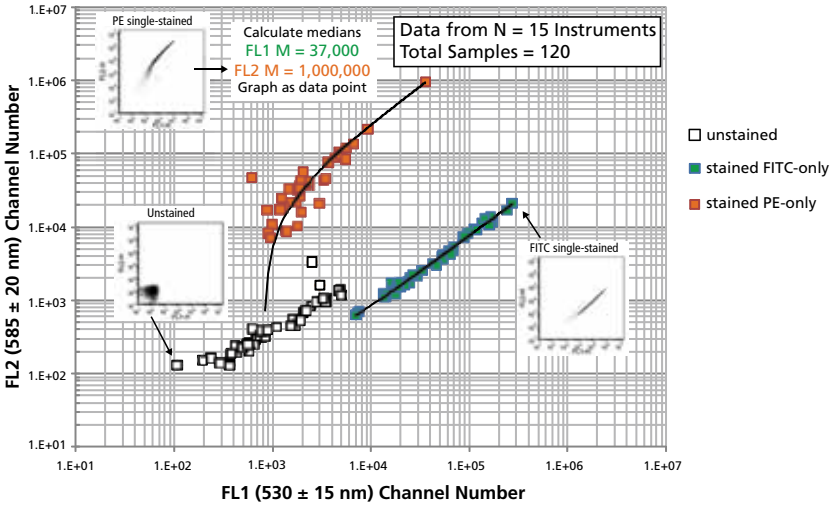

Figure 5. Predictability of FITC and PE spillover into FL2 and FL1.

Forty data sets consisting of 3 samples each (unstained, FITC single-stained, and PE single-stained) were acquired on 15 different BD Accuri C6 flow cytometers actively in use in the field. Sample types included human peripheral white blood cells, isolated platelets, mouse splenocytes and bone marrow, bacteria, antibody-labeled beads, and human and mouse cell lines. Plotted points represent the median channel values (MCVs) on FL1 and FL2 for unstained (□ autofluorescence), FITC-only (■), or PE-only (■) stained samples. The plots show that FITC spillover into FL2, and PE spillover into FL1, were highly consistent across a range of fluorescence intensities and instruments.

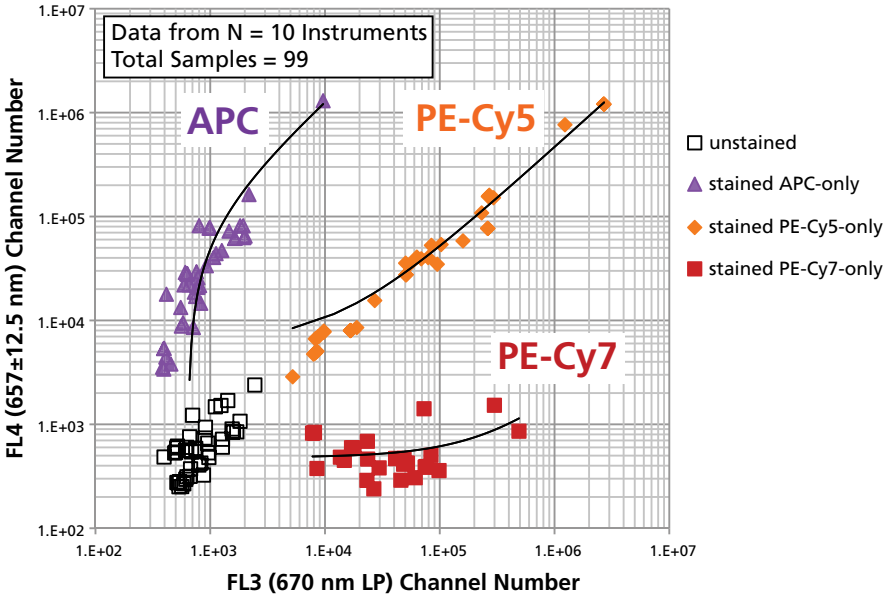

Figure 6. Predictability of APC, PE-Cy5, and PE-Cy7 spillover into FL3 and FL4.

Ninety-nine stained samples plus unstained controls from a variety of cell types were acquired on 10 different BD Accuri C6 flow cytometers. Sample types included lysed human peripheral red blood cells, mouse splenocytes, antibody capture beads, and cell lines. Plotted points represent MCVs on FL3 and FL4 for either unstained (□), APC-only (▲ n = 40), PE-Cy5-only (◆ n = 32), or PE-Cy7-only (■ n = 27) stained samples. The data show that APC spillover into FL3, and PE-Cy5 spillover into FL4, were highly predictable across a range of fluorescence intensities and instruments. PE-Cy7 has little spillover into FL4 and therefore its FL3 fluorescence does not predict its FL4 fluorescence.

The equations in Table 1 can be used to assess events having unknown fluorochrome labels. Comparing the actual channel numbers for any two modeled detectors to their expected channel values can determine whether they are likely to belong to single- or double-stained populations.

Live cells: PI^-
Lymphocytes: $CD45^+$ FITC
B cells: CD19 APC
T cells: CD3 PE-Cy5
Helper: CD4 APC
Cytotoxic: CD8 PE
NK cells: CD16+56 PE-Cy7

Table 2. Recommended BD Biosciences reagents for HPB immunophenotyping application 1.

Marker	Fluorochrome	Clone	Cat. No.
CD45	FITC	H130	555482
CD3	PE-Cy5	UCHT1	555334
CD19	APC	HIB19	555415
CD8	PE	HIT8a	555635
CD56	PE-Cy7	B159	557747
CD16	PE-Cy7	3G8	557744
CD4	APC	RPA-T4	555349
PI	—	—	556463

Immunophenotyping application 1: TBNK, T-cell subsets, and viability

Notice from the equations in Table 1 that PE-Cy7 (FL3) and APC (FL4) have minimal spillover into the other's channel (slopes = 0.0013 and 0.007 respectively). In contrast, PE-Cy5 has a large amount of spillover (FL3/FL4 slope = 0.4613) resulting in a "diagonal population" in Figure 6. If this diagonal space between FL3 and FL4 is not needed to detect double-positive ($FL3^+FL4^+$) events—that is, if the cells stained with PE-Cy7 and the cells stained with APC are mutually exclusive—then the space is available to detect PE-Cy5. Thus, these three fluorochromes can be distinguished using only FL3 and FL4.

Similarly, the viability dye propidium iodide (PI) shows a diagonal spillover between FL2 and FL3. If the diagonal space between FL2 and FL3 is not needed to detect double-positive ($FL2^+FL3^+$) events, it is available to detect PI.

This example shows how researchers can exploit these unique spectral characteristics. It begins by using PI and CD45 to gate on live cells and lymphocytes, respectively. We then use PE-Cy5, APC, and PE-Cy7 to distinguish T cells, B cells, and NK cells—three mutually exclusive lymphocyte subpopulations—on two detectors. Finally, we apply the same principles to ultimately analyze six markers and a viability dye using just four detectors.

A sample of 50 µL of human peripheral blood (HPB), freshly drawn from a single donor, was added to 14 tubes already prepared with reagents as shown in Tables 2 and 3 using recommended dilutions. The red blood cells were lysed with BD FACS™ lysing solution (Cat. No. 349202), and samples were washed and collected on a BD Accuri C6.

Table 3. Staining panel for HPB immunophenotyping application 1.

Tube	FL1	FL2	FL3	FL4
Laser & Filter	530/30	585/40	670 LP	675/25
1	—	PI	PI	—
2	CD45 FITC	PI	PI	—
3	—	PI	CD3 PE-Cy5 PI	—
4	—	PI	PI	CD19 APC
5	—	PI	CD56+16 PE-Cy7 PI	—
6	—	PI	PI	CD4 APC
7	—	CD8 PE PI	PI	—
8	CD45 FITC	PI	CD3 PE-Cy5 CD56+16 PE-Cy7 PI	CD19 APC
9	CD45 FITC	CD8 PE PI	PI	—
10	CD45 FITC	CD8 PE PI	CD3 PE-Cy5 CD56+16 PE-Cy7 PI	CD19 APC
11	CD45 FITC	PI	CD3 PE-Cy5 CD56+16 PE-Cy7 PI	CD4 APC
12	CD45 FITC	PI	CD3 PE-Cy5 CD56+16 PE-Cy7 PI	CD4 APC CD19 APC
13	CD45 FITC	CD8 PE PI	CD3 PE-Cy5 CD56+16 PE-Cy7 PI	CD4 APC CD19 APC
14	CD45 FITC	PI	CD56+16 PE-Cy7 PI	—

Sample was collected from all 14 tubes both before and after adding propidium iodide (PI).

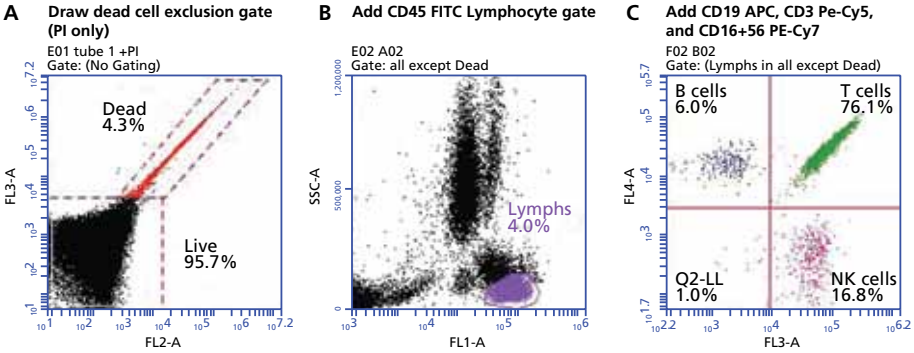

Figure 7, Step 1. Set initial gates for live cells and lymphocytes, and use spillover to distinguish three cell populations on two detectors.

*Single-stained controls are used to set up gates for (**A**) dead cell exclusion (PI⁺) and (**B**) lymphocytes (CD45⁺). **C**. After adding antibodies to CD19, CD3, and CD16+56, an FL3 vs FL4 plot clearly distinguishes B cells (purple, UL quadrant), NK cells (magenta, LR quadrant), and T cells (green, UR quadrant). PE-Cy5, which has high fluorescence spillover from FL3 into FL4, is used along with APC (detected primarily in FL4) and PE-Cy7 (detected primarily in FL3) to distinguish three mutually exclusive cell populations using two detectors.*

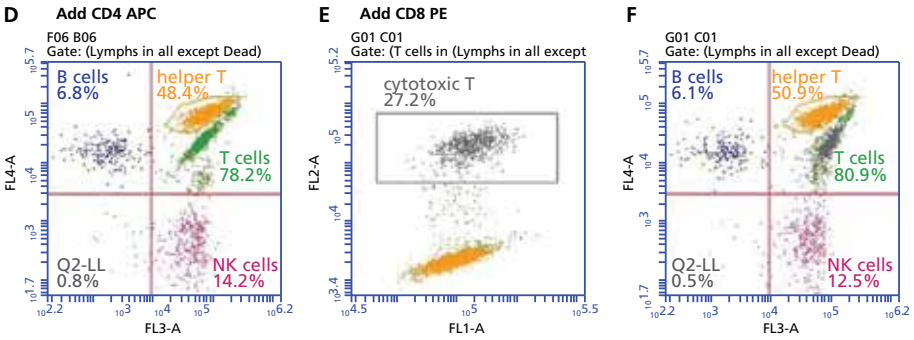

Figure 7, Step 2. Add additional markers to distinguish T-cell subpopulations.

*D. Adding CD4 APC allows identification of an exclusive CD4⁺ subset of the CD3 cell population. These helper T cells (orange, CD3⁺CD4⁺) can be distinguished from other T cells (green, CD3⁺CD4⁻) as well as B cells (purple, CD3⁻CD19⁺) on an FL3 vs FL4 plot. In this sample, helper T cells make up 48.4% of the total live lymphocyte population. **E**. Similarly, adding CD8 PE allows identification of an exclusive CD8⁺ subset of the CD3 population. These cytotoxic T cells (gray, CD3⁺CD8⁺) can be distinguished from helper T cells (orange, CD3⁺CD4⁺) on an FL1 vs FL2 plot, gated on T cells. **F**. Five different kinds of lymphocytes—B cells (purple), NK cells (magenta), and helper (orange), cytotoxic (gray) and other (green) T cells—are now visible on the FL3 vs FL4 plot.*

Lymphocytes: FSC/SSC
- B cells: CD19 APC
  - IgD PE
- NK cells: CD16+56 PE-Cy7
- T cells: CD3 PE-Cy5
  - Helper: CD4 APC
  - Cytotoxic: CD8 PE
  - CD127 FITC

Immunophenotyping application 2: TBNK, T-cell subsets, and B-cell subsets

This example shows how, using the same strategy, unique spectral characteristics and mutually exclusive subpopulations can be exploited to analyze seven markers on four detectors. Again, a sample of 50 µL of HPB, freshly drawn from a single donor, was added to 17 tubes prepared with reagents as shown in Tables 4 and 5. The red blood cells were lysed with BD FACS lysing solution (Cat. No. 349202) and collected on a BD Accuri C6.

Table 4. Recommended BD Biosciences reagents for HPB immunophenotyping application 2.

Marker	Fluorochrome	Clone	Cat. No.
CD127	FITC	HIL-7R-M2	560549
IgD	PE	IA6-2	555779
CD8	PE	HIT8a	555635
CD16	PE-Cy7	3G8	557744
CD56	PE-Cy7	B159	557747
CD3	PE-Cy5	UCHT1	555334
CD19	APC	HIB19	555415
CD4	APC	RPA-T4	555349

Table 5. Staining panel for HPB immunophenotyping application 2.

Tube	FL1	FL2	FL3	FL4
Laser & Filter	530/30	585/40	670 LP	675/25
1	—	—	—	—
2	CD127 FITC	—	—	—
3	—	IgD PE	—	—
4	—	CD8 PE	—	—
5	—	—	CD56+16 PE-Cy7	—
6	—	—	CD3 PE-Cy5	—
7	—	—	—	CD19 APC
8	—	—	—	CD4 APC
9	CD127 FITC	CD8 PE	—	CD19 APC
10		IgD PE	CD3 PE-Cy5	—
11	—	—	CD56+16 PE-Cy7 CD3 PE-Cy5	CD19 APC
12	—	IgD PE	CD56+16 PE-Cy7 CD3 PE-Cy5	CD19 APC
13	—	—	CD56+16 PE-Cy7 CD3 PE-Cy5	CD19 APC CD4 APC
14	CD127 FITC	—	CD56+16 PE-Cy7 CD3 PE-Cy5	CD19 APC CD4 APC
15	—	CD8 PE	CD56+16 PE-Cy7 CD3 PE-Cy5	CD19 APC CD4 APC
16	CD127 FITC	CD8 PE	CD56+16 PE-Cy7 CD3 PE-Cy5	CD19 APC CD4 APC
17	CD127 FITC	IgD PE CD8 PE	CD56+16 PE-Cy7 CD3 PE-Cy5	CD19 APC CD4 APC

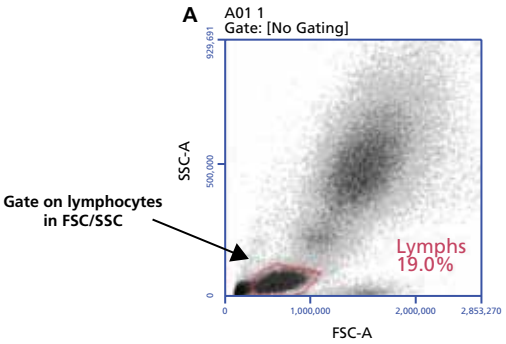

Figure 8, Step 1. Set initial lymphocyte gate.

A. In this example, lymphocytes are distinguished using an FSC vs SSC scatter plot rather than with a CD45 antibody.

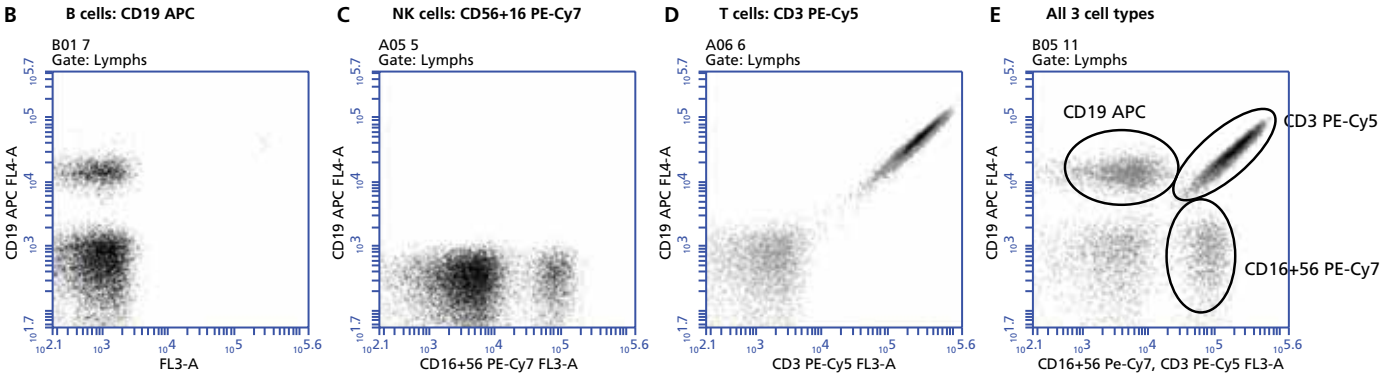

Figure 8, Step 2. Use spillover to distinguish three lymphocyte populations on two detectors.

*As in Figure 7C (immunophenotyping application 1), fluorescence spillover is used to distinguish three mutually exclusive populations of lymphocytes—(**B**) B cells, (**C**) NK cells, and (**D**) T cells. **E.** All three cell subpopulations are resolved in two detectors.*

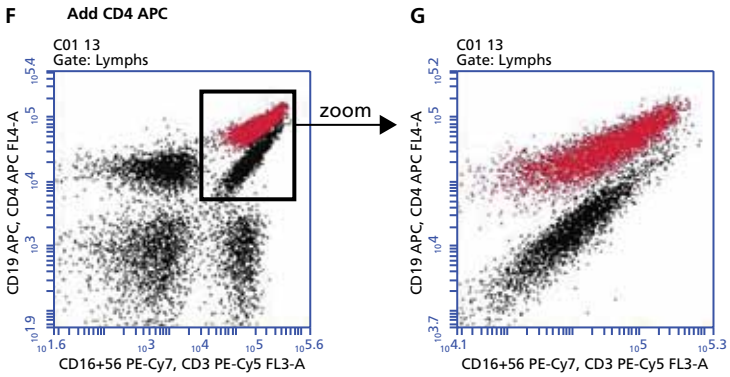

Figure 8, Step 3. Double up markers using population subsets.

*F. After adding CD4 APC, the CD3⁺CD4⁺ (double-positive) helper T cells (red) can be distinguished from both single-positive CD3⁺ non-helper T cells (black upper right) and CD19⁺ B cells (black upper left). They appear as a "stacked" population. The two markers conjugated to APC (in this case, CD19 and CD4) must be mutually exclusive. This step essentially duplicates Figure 7D in immunophenotyping application 1. **G.** The software Zoom tool shows the clear distinction between the two T-cell subpopulations.*

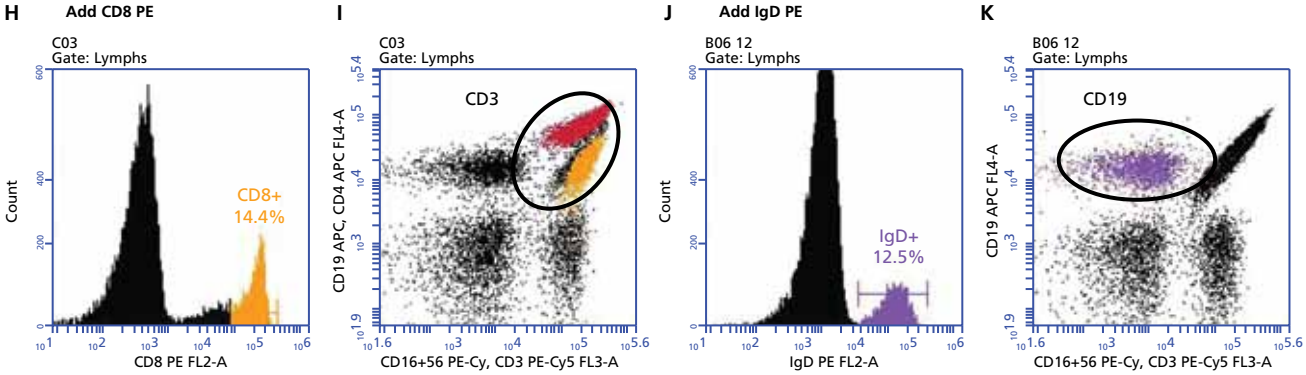

Figure 8, Step 4. Verify that subset populations on the same fluorophore are exclusive by using fluorescence-minus-one (FMO) controls.

H, I. *Adding CD8 PE (without IgD PE) allows identification of an exclusive CD8⁺ subset of the CD3 cell population. These cytotoxic T cells (orange) can be distinguished from other T cells (red, black) within the CD3⁺ area.* ***J, K.*** *Similarly, adding IgD PE (without CD8 PE) verifies exclusivity of IgD to B cells. The IgD⁺ cells (purple) appear only within the CD19⁺ B-cell area. In samples with PE antibodies, FL3 signal was corrected by subtracting 25% of FL2 signal. Histograms represent total lymphocytes. Event coloring illustrates subset exclusivity.*

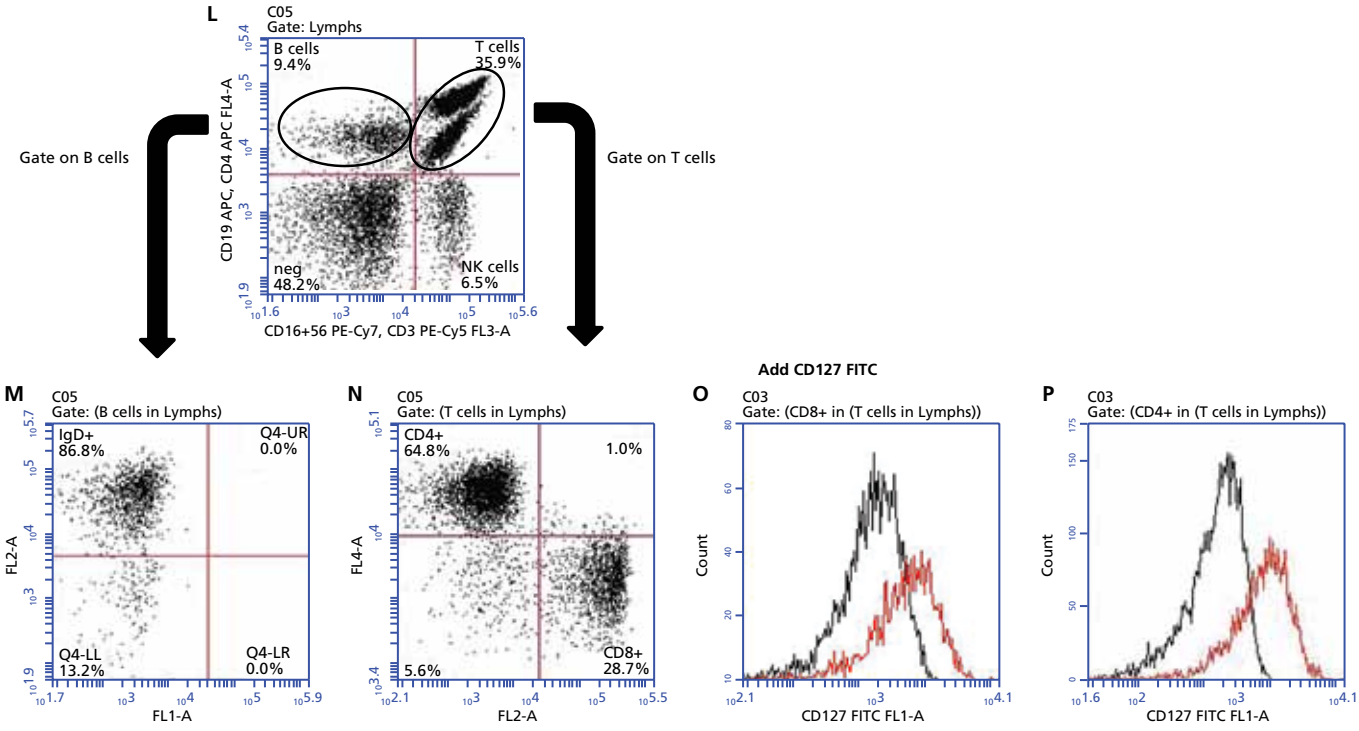

Figure 8, Step 5. Create gated plots for each population to further analyze subpopulations.

L. *Gating on B cells or T cells allows further breakdown and analysis.* ***M.*** *An FL1 vs FL2 plot of B cells characterizes them based on IgD expression.* ***N.*** *An FL2 vs FL4 plot characterizes T cells as cytotoxic (CD8⁺) or helper (CD4⁺).* ***O, P.*** *Adding CD127 FITC and gating further on CD8⁺ (**O**) or CD4⁺ (**P**) enables histogram plots of CD127 expression in stained T cells (red) vs unstained (black) FMO controls. In this sample, helper T cells (**P**) express somewhat more CD127 than cytotoxic T cells (**O**). In samples with PE antibodies, FL3 and FL1 signals were corrected by subtracting 25% and 4% of FL2 signal, respectively.*

The characterization of CD127 in panels 8O and 8P shows how the FITC detector (FL1)—unused until this step—can be used for further analysis of any marker of interest. In fact, FITC could also have been used to analyze another B-cell marker in panel 8M, as long as that marker and CD127 are mutually exclusive in B cells and T cells.

Discussion

Although fluorescence spillover is often considered to be a nuisance in flow cytometric analysis, it can be highly useful on a fixed-voltage system such as the BD Accuri C6 flow cytometer. For stable fluorochromes, fluorescence spillover is predictable and reproducible between multiple instruments, regardless of sample type, provided that detector voltage, optical filters, and laser power are fixed. The key is to pair two fluorochromes that have little spillover into the other's detector with a third that emits into the same two detectors.

This technical bulletin has shown how such spillover patterns can be exploited to identify three different subpopulations (B cells, NK cells, and T cells) on just two detectors using human peripheral blood stained with CD19 APC, CD3 PE-Cy5, and CD16+56 PE-Cy7. In fact, when analyzing mutually exclusive subpopulations, this approach can be extended by applying strategic gating techniques and staining panels. Two immunophenotyping examples illustrate how up to seven markers can be analyzed on the four fluorescence detectors of the BD Accuri C6.

References

1. For an example, see *Identification of human peripheral blood cell populations with the BD Accuri™ C6 flow cytometer.* BD Biosciences technical bulletin, November 2011. Available at bdbiosciences.com under Support > Resources > BD Accuri™ C6 System.

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