Simplifying Complex Flow Cytometric Analyses

Technical Bulletin

Contents

Abstract

Due to the wide variations in fluorescence and light scatter signals produced by different types of cells and particles, researchers using flow cytometry often devote significant time and sample optimizing amplifier gains and PMT voltage settings. It would simplify operation if these settings could be locked down at the factory, but how would the cytometer then capture this wide range of variation?

We demonstrate here that a flow cytometer such as the BD Accuri™ C6, equipped with high-resolution digital signal processing and over seven decades of dynamic range, can be used to analyze a wide variety of samples, ranging from bacteria to platelets to large, highly fluorescent cell lines. With this "wide-angle lens" on data, detector settings can be pre-optimized and calibrated to operate within a linear range, eliminating a significant source of experimental variability and making spillover predictable between experiments.

Researchers can employ software and hardware tools to manage this wide data spectrum and tease out detail. With a Zoom tool, they can focus in on very small areas of data display and set gates precisely. In rare cases where the fluorescence is off scale, such as GFP-transfected cell lines, easily inserted attenuation filters bring signals back on scale, further increasing the usable range of the instrument. Finally, with the VirtualGain™ function, researchers can re-align the data distribution of any parameter to a user-defined channel.

This technical bulletin reports on experiments from three common applications—blood immunophenotyping, fluorescent protein analysis, and DNA/cell cycle analysis—that illustrate how these tools can enhance and simplify flow cytometric analyses. All samples were collected and analyzed on a BD Accuri C6 flow cytometer using BD Accuri™ C6 software.

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Introduction: A wide-angle lens on your data

Flow cytometry can process and analyze a wide variety of samples, ranging from submicron-sized bacteria with varying levels of autofluorescence, to micron-sized, fluorescence-labeled platelets, to large, >30 micron, highly fluorescent cell lines. These cells and particles can produce fluorescence and light scatter signals that vary over a vast range. Because many cytometers cannot analyze such a broad range of signals, researchers often expend significant time and sample optimizing amplifier gains and photomultiplier tube (PMT) voltage settings to capture their data of interest.

On the BD Accuri C6 personal flow cytometer, in contrast, amplifier gains and voltage settings are locked down at the factory. Eliminating the need to adjust settings significantly simplifies operation, but how can the cytometer capture this wide range of variation?

The answer is the wide dynamic range of the BD Accuri C6. A high resolution (24-bit), digital signal processor (DSP) provides the ability to collect 16.7 million channels of signals spread across more than seven decades. Even after omitting the first decade of data (channels 0–9), which is often uninformative, this represents an extraordinary dynamic range on all detectors. In effect, the BD Accuri C6 serves as a "wide-angle lens" for gathering and viewing a panorama of fluorescence data.

Figure 1 shows the benefits of this arrangement. Dim, 1-micron beads and highly fluorescent Chinese hamster ovary (CHO) cells, labeled with FITC or PE, were mixed together and analyzed on flow cytometers with three different dynamic ranges. (Beads stood in for unstained or control cells in this experiment.) The BD Accuri C6, with 6.2 log decades of effective dynamic range, appears at the bottom. On the two instruments with narrower dynamic ranges, gains were adjusted to capture the unstained beads at the low end of the x-axis.

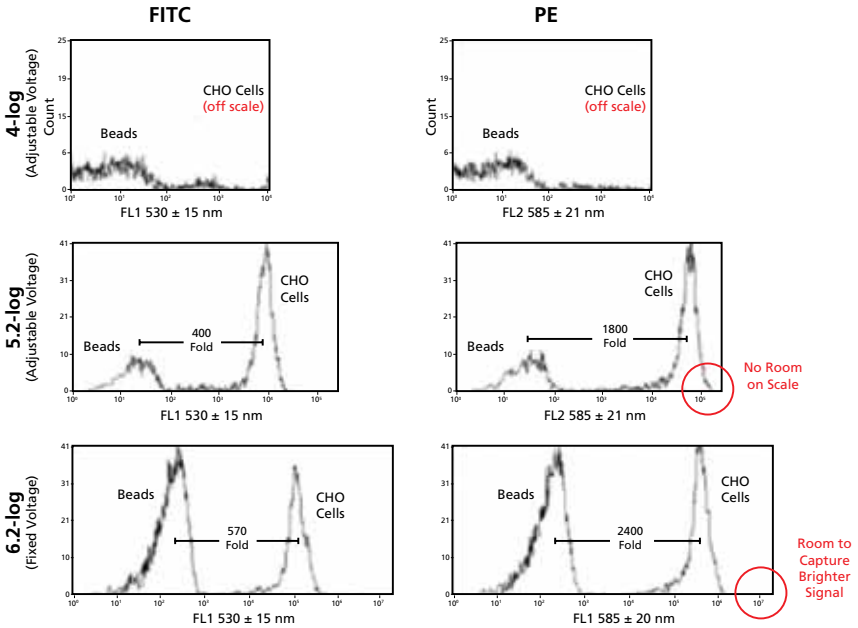

Figure 1. Analyzing bright and dim signals on three flow cytometers with different dynamic ranges.

Unstained 1-micron beads and CHO cells labeled with FITC (left) or PE (right) were mixed, acquired, and analyzed on three flow cytometers. ***(Top)*** *On an analog, 4-log cytometer, when gains were adjusted to capture the beads, the CHO cells were off scale.* ***(Middle)*** *On a digital, 5.2-log cytometer with gains similarly adjusted, the CHO cells were on scale, but with PE staining there was no room for brighter signals.* ***(Bottom)*** *On the digital, 6.2-log BD Accuri C6, both beads and cells were on scale with room to spare.*

An analog cytometer with a 4-log dynamic range (top) cannot simultaneously capture the fluorescence of both the beads and the CHO cells stained with either fluorophore. The separation between the distributions is simply too great. Gains can be adjusted before data collection to show the beads (as in the plots) or the cells, but not both, and whichever population is excluded is irretrievably lost.

A 5.2-log digital cytometer (middle) can capture both distributions, but with PE, there is no room at the top of the scale for any events with brighter signals. Gains can be adjusted to focus higher but, again, any excluded events are lost.

In contrast, the BD Accuri C6 (bottom) can capture both distributions with room to spare. There is no need to adjust gains, so voltage and amplifier settings are locked down at the factory. This not only simplifies the process and makes instrument operation easier. It also eliminates gain setting as a source of variability between experiments, and prevents data loss due to improper settings. Finally, it makes fluorescence spillover constant and predictable between experiments.

Of course, it would be unusual to mix beads and cells in a single sample. The objective is to have enough range to capture both stained and unstained (or control) cells, which might vary similarly depending on the cells.

Once the entire 6.2-log data range is available, researchers can employ software and hardware tools to manage it and tease out detail. The remainder of this technical bulletin reports on experiments from three common applications—blood immunophenotyping, fluorescent protein analysis, and DNA/cell cycle analysis—that illustrate how these tools can enhance and simplify flow cytometric analyses. In all three experiments, samples were collected and analyzed on a BD Accuri C6 flow cytometer using BD Accuri C6 software.

Human peripheral blood immunophenotyping: The Zoom tool

One common task in human blood analysis is to identify and enumerate populations of platelets, lymphocytes, monocytes, granulocytes, and eosinophils. Flow cytometry is a quick and reliable methodology for identifying these populations based on their immunophenotypes (Table 1).

Table 1. Defining immunophenotypes for five blood cell populations.

Population	Immunophenotype
Platelets	$CD41^{+}CD45^{-}$
T lymphocytes	$CD3^{+}CD45^{+}$
Monocytes	$CD11b^{+}CD14^{+}$
Granulocytes	$CD11b^{+}CD14^{-}$
Eosinophils	$FL1^{++}SSC^{high}$

Several features of the BD Accuri C6 flow cytometer system make it a good fit for no-wash, differential analysis of human peripheral blood samples. First, the fixed voltage detectors of the BD Accuri C6 simplify data collection and reduce the potential for data loss due to signal over- or under-amplification. Second, the instrument's broad dynamic range makes it easy to analyze populations as varied in size as platelets and eosinophils in the same data file.

Finally, the BD Accuri C6 software Zoom tool can help focus in on a particular subset of channels or events within the cytometer's broad dynamic range. Zoom is indispensable for locating cell populations and drawing accurate gates. Thus, Zoom complements the wide-angle data lens provided by the BD Accuri C6.

In Figure 2, an FSC vs SSC plot of a whole blood sample, you might find it difficult to locate the lymphocyte population. To zoom in, click the Zoom tool and draw a wide region that includes the lymphocyte population. Repeat as needed, drawing progressively smaller regions until you can easily identify the lymphocytes and draw an accurate gate. Then zoom back out again.

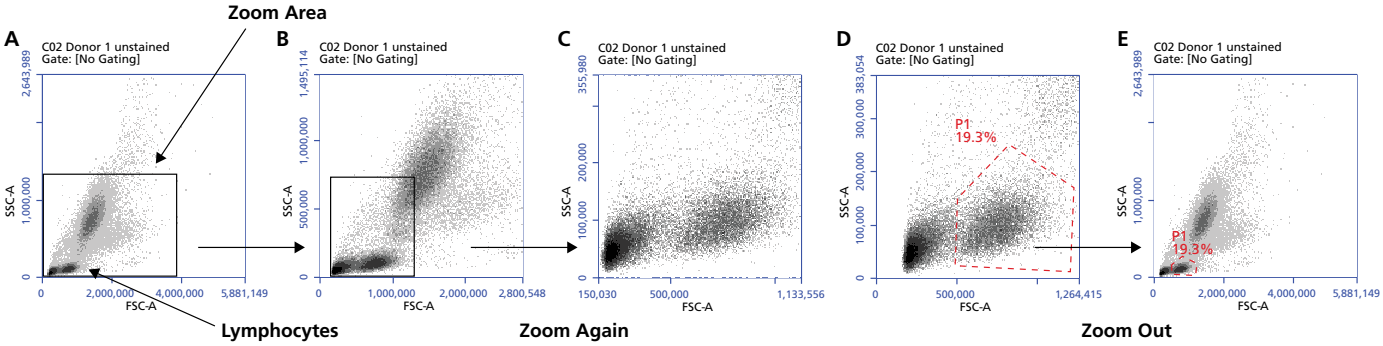

Figure 2. Use the Zoom tool to locate populations.

Human blood cells were acquired and analyzed for light scatter on the BD Accuri C6. ***A.*** *FSC vs SSC plot at original scale, with wide zoom area drawn around several cell populations including lymphocytes.* ***B.*** *The zoomed plot, with a new, narrower zoom area drawn around the lymphocyte and red blood cell populations.* ***C.*** *After zooming twice, the lymphocytes are easily recognized.* ***D.*** *A gate is drawn around the lymphocytes.* ***E.*** *When the plot is zoomed out again, the gate remains in place.*

In the following experiment,[1] whole human blood was immunophenotyped with fluorescent stains to identify five cell populations. Backgating was used to place all five populations on a CD45 vs SSC plot, but the gates were not precise and the polygons overlapped. By zooming in on the plot, the polygons can be adjusted to avoid overlap. The Zoom tool allows precise control when setting gates.

Materials and methods

Whole human peripheral blood (HPB) was drawn into heparinized tubes. 100-µL aliquots were stained in 12 x 75-mm polypropylene tubes with properly titrated, pre-diluted volumes of appropriately combined, directly conjugated antibodies as shown in Table 2.

Table 2. BD Biosciences reagents for differential analysis of HPB.

Marker	Fluorochrome	Clone	Cat. No.
CD3	FITC	UCHT1	555332
CD4	PE	RPA-T4	561843
CD8	APC	RPA-T8	561952
CD11b	PE	ICRF44	561001
CD14	FITC	M5E2	555397
CD41	FITC	HIP8	555466
CD45	PE-Cy™7	HI30	557748

The blood was incubated with antibodies at room temperature (RT) in the dark for 20 minutes. Red cells were lysed and cells fixed by adding 2 mL of BD FACS™ lysing solution (Cat. No. 349202) directly to the whole blood and allowing lysis to proceed, after gentle mixing, at RT in the dark for 15 minutes.

Results

In Figure 3A–E, platelets, T lymphocytes, monocytes, granulocytes, and eosinophils were identified using surface markers. Then, in Figure 3F–J, backgating was used to locate and gate each population on a CD45 vs SSC-A plot. Finally, in Figure 4, the Zoom tool was used to adjust overlapping polygonal gates.

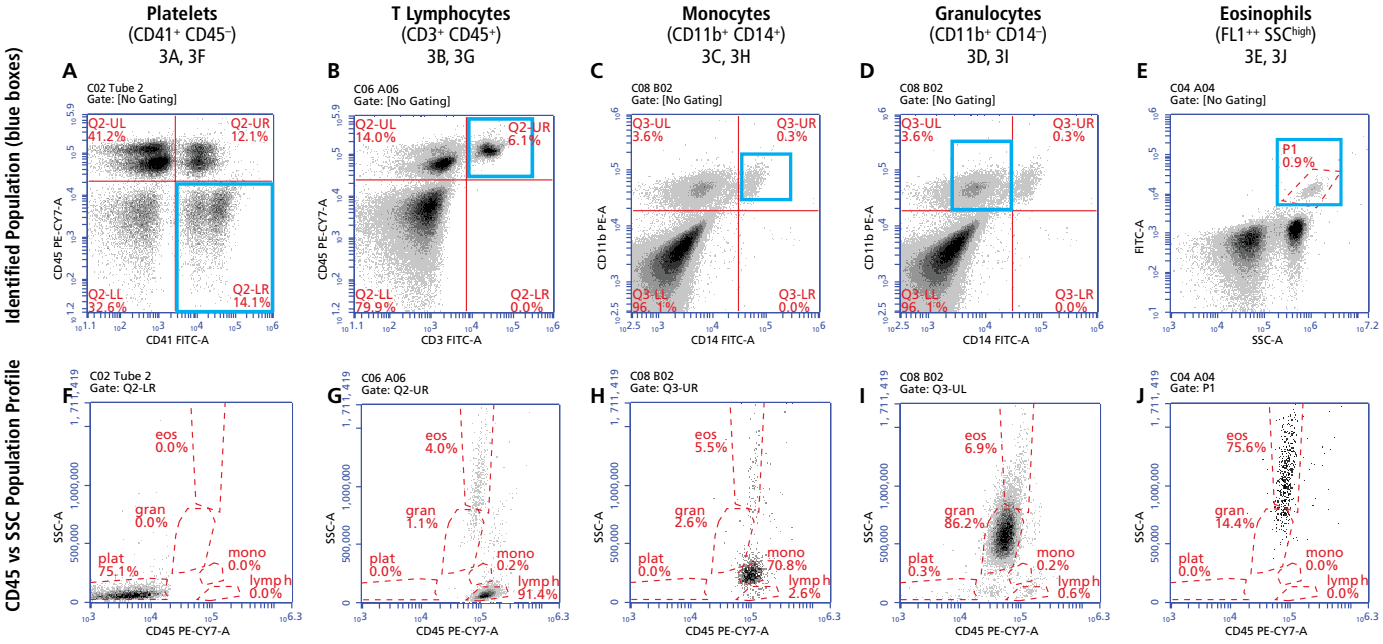

Figure 3. Identification and gating of human peripheral blood populations on the BD Accuri C6.

Whole human peripheral blood was stained, red cells were lysed in a no-wash protocol, and events were collected on the BD Accuri C6. ***A–E (top).*** *Platelets, lymphocytes, monocytes, and granulocytes were identified by their surface marker fluorescence, while eosinophils were identified by a combination of autofluorescence and side scatter properties.* ***F–J (bottom).*** *Backgating was used to locate platelets (plat), T lymphocytes (lymph), monocytes (mono), granulocytes (gran), and eosinophils (eos) on a CD45 vs SSC plot. Gates were adjusted using the Zoom tool (Figure 4).*

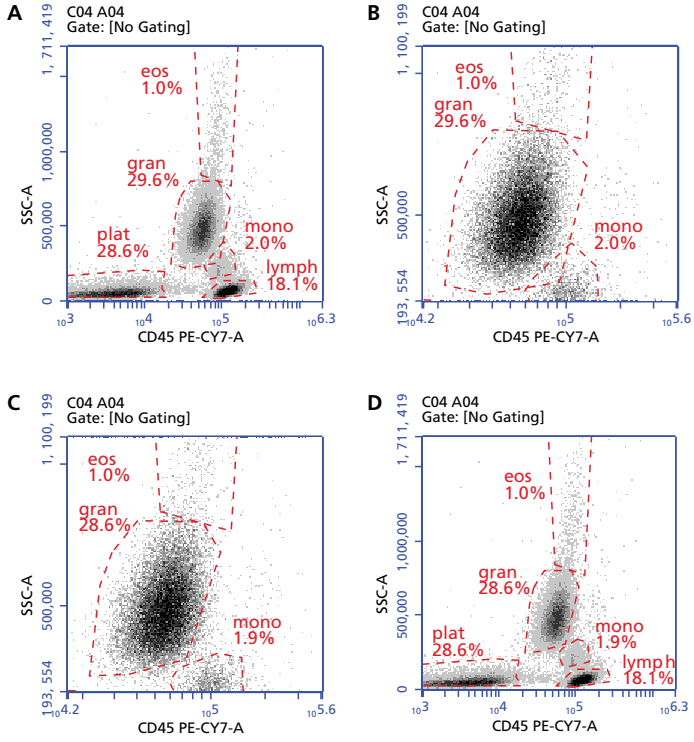

Figure 4. Using the Zoom tool to adjust gates on five populations of human peripheral blood.

A. *Original polygon placement.* ***B.*** *Zoomed plot shows overlap of polygons.* ***C.*** *Adjusted polygons.* ***D.*** *Plot zoomed back to original scale.*

Fluorescent protein analysis: Attenuation filters

Transfection of foreign DNA, RNA, or proteins into cells has become an important tool in studying gene and protein expression and function. Because of variability in transfection efficiency, a reporter molecule such as green, yellow, or red fluorescent protein (GFP, YFP, or RFP) is often co-transfected into the cells. Mutagenesis has produced enhanced variants of these reporter proteins (for example, eGFP, eCFP, eYFP, mCherry) with varying emission peaks across the blue, cyan, green, yellow, and red wavelengths.

The BD Accuri C6 can detect GFP and other fluorescent proteins using the standard filter configuration. However, signals from fluorescent proteins—which depend on transfection efficiency, the level of expression within the cells, and the excitation wavelength used—can be extremely bright.

In rare cases in which the fluorescence is off scale, easily inserted attenuation filters can bring the signals back on scale, further increasing the usable range of the instrument. This is advantageous because the detectors used to read these bright signals will still be operating within their linear range. Use of an attenuation filter (as opposed to reducing voltage) also means that the fluorescence

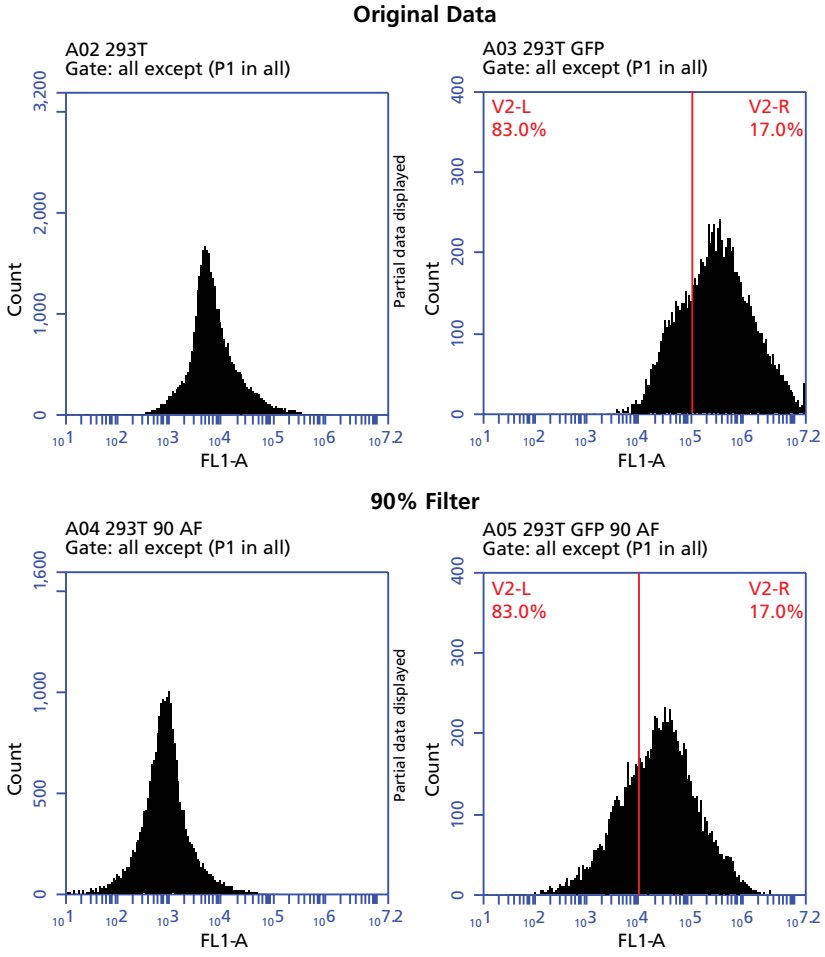

Figure 5. Use attenuation filters to bring a bright signal on scale.

GFP was transfected into an EC cell line. Untransfected (left) and transfected (right) samples were acquired and analyzed on a BD Accuri C6. ***(Top)*** *Original data with a standard FL1 filter = 533/30 BP. Some brightly fluorescent cells are off scale, positive.* ***(Bottom)*** *A 90% attenuated (OD1) 533 BP filter was placed in front of FL1 and the data re-collected. All data is now on scale.*

reduction is predictable and reproducible, and is not affected by subjective operator decisions about how much signal reduction is "enough."

Attenuation filters for the BD Accuri C6 are available at 90% (OD1) and 99% (OD2) efficiency for each fluorescence detector, reducing brightness by one or two magnitudes.

Materials and methods

GFP was transfected into a highly autofluorescent embryonal carcinoma (EC) cell line. Samples were acquired and analyzed on a BD Accuri C6 using the standard optical filter, with and without a 90% attenuation filter.

Results

Figure 5 shows the value of attenuation filters when analyzing brightly fluorescent cells on a pre-optimized detector system such as the BD Accuri C6. When analyzed only with the standard optical filter (top), some cells are off scale and therefore off the plot. Inserting a 90% attenuation filter brings all cells back on scale and onto the plot.

DNA/cell cycle analysis: VirtualGain

In certain instances, a particular fluorescence peak should have the same position across different samples or be located at a specific channel number, regardless of staining. For example, to compare DNA and cell cycle distributions of different samples stained with propidium iodide (PI), their peaks should be aligned. With most flow cytometers, you must adjust voltage and amplifier gain controls to alter peak position from sample to sample.

Since BD Accuri C6 voltages are locked down at the factory, there are no gain controls to adjust. Instead, the VirtualGain tool in BD Accuri C6 software can be used to align the peaks. VirtualGain mimics voltage and amplifier gain adjustments to grossly reposition histogram data on the x-axis after data collection.

VirtualGain is strictly an analysis tool and is not used while collecting data. It can be toggled on and off by clicking the asterisk under the parameter name. VirtualGain is applied only on histogram plots, and only on one parameter at a time. However, once VirtualGain is applied, you can view the transformed data in any type of plot. The tool affects only the displayed data and does not alter the raw data that is collected and saved in the FCS files.

Materials and methods

Jurkat cells in logarithmic growth phase were washed and fixed with 70% ice-cold ethanol, added by drops while vortexing gently, for 60 minutes on ice. Cells were pelleted by centrifugation for 5 minutes at 300*g*, washed once with PBS, and re-suspended at 1 x 10^6 cells per mL in PBS containing 5 µg/mL of PI for at least 60 minutes at RT, covered, before analysis.

Chicken erythrocyte nuclei (CEN) and calf thymocyte nuclei (CTN) were prepared according to package instructions for BD™ DNA QC Particles (Cat. No. 349523).

Results

In the left column of Figure 6, three types of samples were stained with PI, but their peaks do not align sufficiently to compare their DNA distributions. In the right column, VirtualGain was used to align the first peak of the CEN and CTN distributions with the first peak of the Jurkat distribution. The DNA distributions can now be compared.

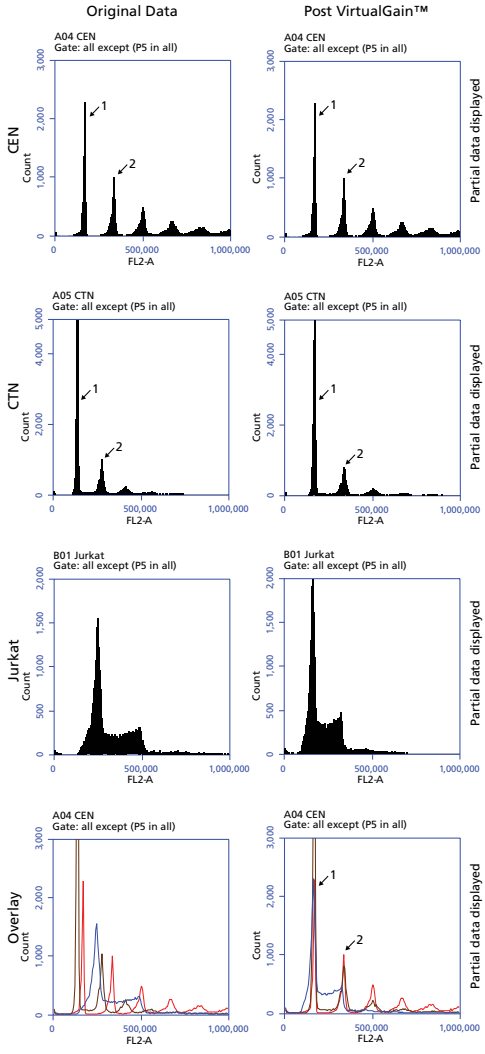

Figure 6. Use VirtualGain to align peaks of different samples.

CEN and CTN from BD DNA QC Particles (Cat. No. 349523) and Jurkat cells were prepared and stained with PI by standard methods. Data was collected on a BD Accuri C6 on different days. ***(Left column)*** *In the original data, DNA peaks from PI staining do not align.* ***(Right column)*** *VirtualGain was applied after data collection to align Peak 1 of the CEN and CTN distributions to Peak 1 of the Jurkat distribution. In row 4, overlays of DNA distributions show that after VirtualGain was applied, the peaks are aligned properly.*

Conclusions

The BD Accuri C6, a fully digital flow cytometer with pre-optimized detectors, is capable of analyzing a wide range of cell types, from small, dim platelets to large, highly fluorescent, GFP-transfected embryonal carcinoma cell lines. Fixed amplifier gains and voltage settings simplify operation, while a "wide-angle lens" spanning more than seven decades of dynamic range ensures that both bright and dim signals can be collected in the same run.

Several tools make it easy for researchers to navigate and manage the broad range of BD Accuri C6 data. The ability of the BD Accuri C6 software to "zoom" in and out on populations allows researchers to effectively magnify the view of a population for more precise gating, while retaining all data within the data set.

In samples where fluorescence signals are so bright that they are off scale, attenuation filters are an effective way to bring the signals back on scale while remaining within the linear operational range of the detectors.

Finally, the software's VirtualGain function allows researchers to shift the entire data set up or down the axis for any parameter, post acquisition. This allows the re-alignment of data to an internal standard as required in DNA/cell cycle analyses.

References

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

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