Author Ben Verwer

# BD FACSDiVa Option

## White Paper

**BD Biosciences**
Clontech
Discovery Labware
Immunocytometry Systems
Pharmingen

BD

## 1. Introduction

The BD FACSDiVa option for the BD FACSVantage SE flow cytometer digitizes the amount of light created by particles passing through one or more laser beams. However, the BD FACSDiVa option does this earlier and more directly than any other commercial flow cytometer.

The BD FACSDiVa option digitizes signals at a rate of 10 million times per second into 16,384 discrete levels. As a result, logarithmic amplifiers and the traditional analog peak-and-hold circuits are no longer required. This has two main advantages: inaccuracies introduced by the logarithmic amplifiers are eliminated (noise and deviations from a perfectly logarithmic response), and there is no longer any electronic dead time, the time during which the cytometer is processing data and cannot digitize any additional particles.

The BD FACSDiVa option was designed as an integrated hardware/software platform and introduces a number of additional benefits not directly related to the digital nature of the system: compensation between any two channels from any two lasers, ratio calculations between any two channels from any two lasers, four-way sorting, and a completely new software platform and graphical user interface.

This white paper describes the hardware architecture, how the electronics digitize the data (Section 2), the compensation methodology (Section 3), and how the electronics process data during sorting (Section 4). Illustrative data is presented in Sections 3 and 4.

## 2. Digitization

### 2.1 Sampling

Figure 1 shows analog signals from two spatially separated lasers: three channels (FSC/SSC/FITC) from a blue laser and a fourth channel (APC) from a red laser at a later point in time. The voltage corresponding to each signal is digitized into one of 16,384 possible levels 10 million times per second by analog-to-digital (A/D) converters. Signals are continuously digitized during normal operation, whether a pulse is present or not, and all digitized signals are represented as numbers in memory.

BD FACSDiVa electronics create an enormous amount of data, about 1,000 MB of data every minute. Therefore, new data continuously displaces old data and is kept in memory only for a short amount of time (approximately 0.5 msec).

To process signals from a single event passing sequentially through two or more lasers, data from the earlier lasers is delayed with respect to the last laser. When a single event is processed, the data in different channels is aligned even when acquired at different times.

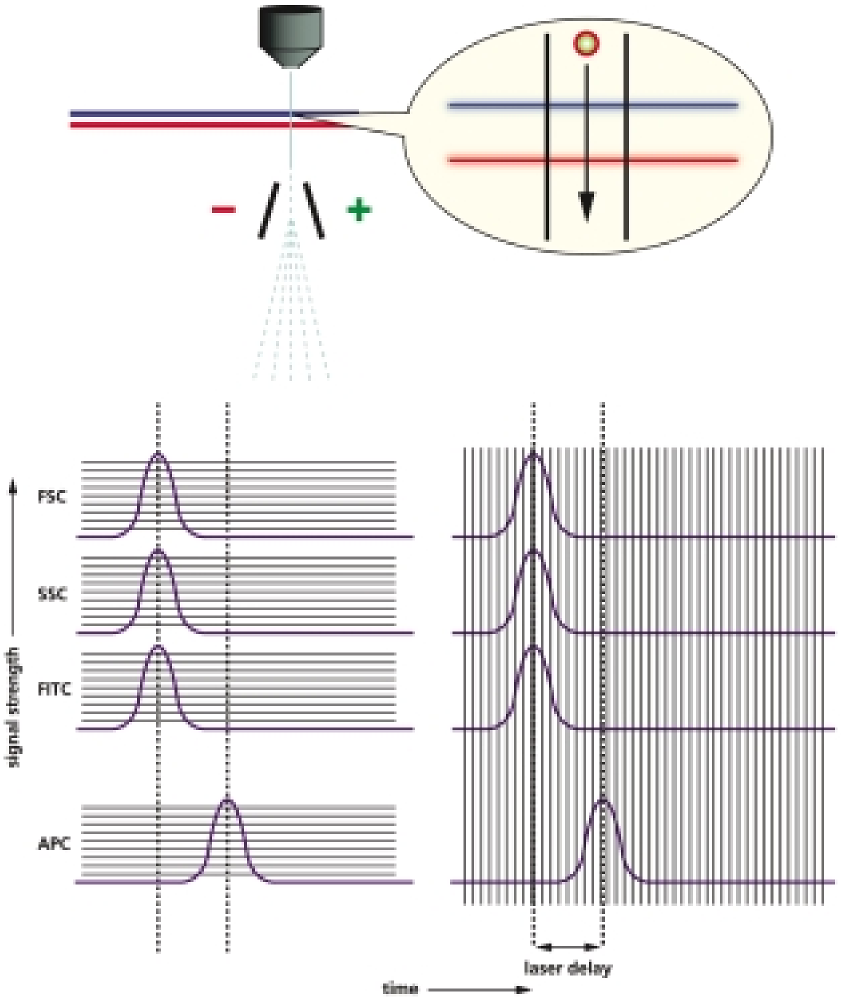
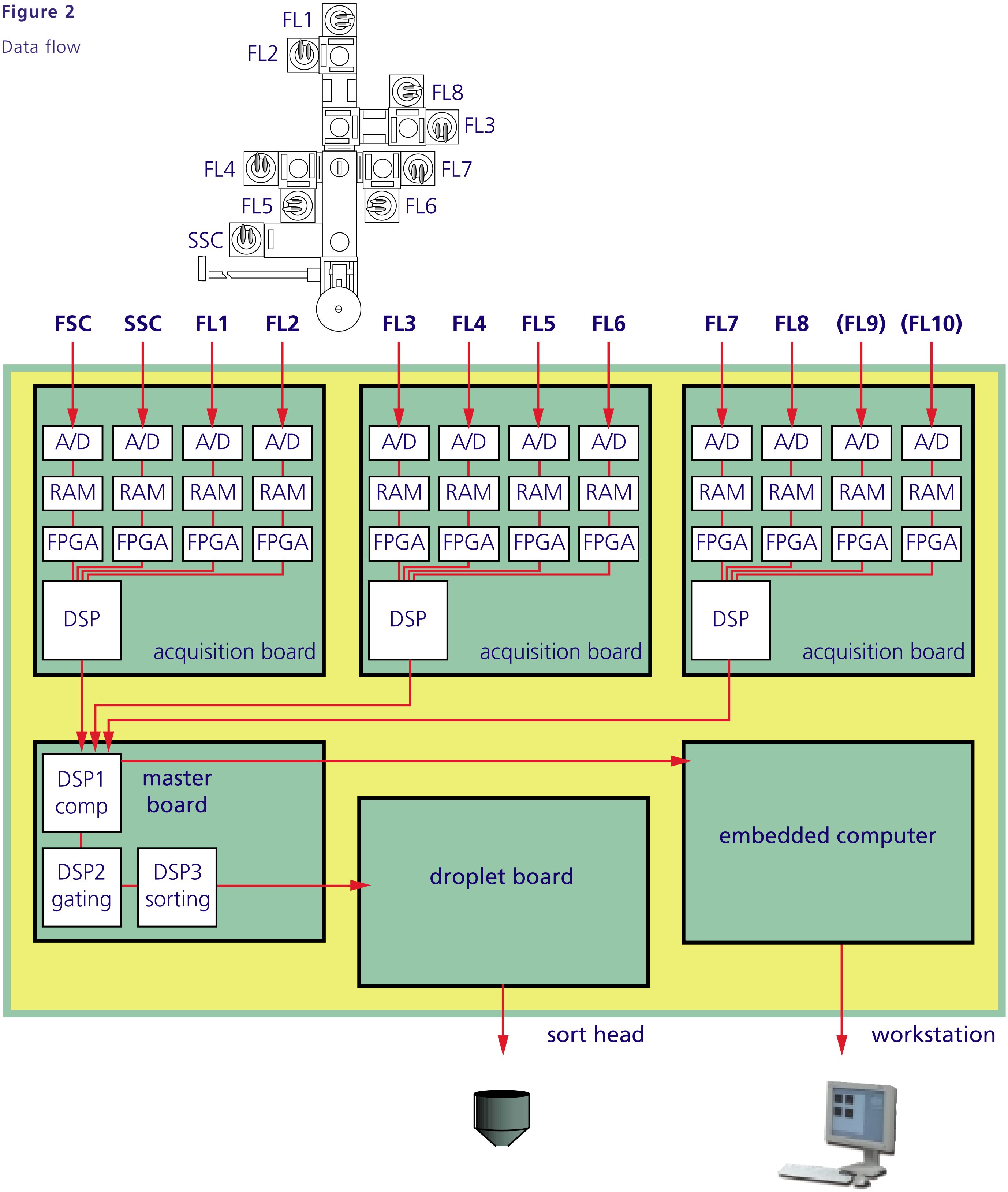


**Figure 1**

Signal generation and processing: digitization, lower left, sampling, lower right. Number of lines for illustration only (16,384 levels and 1 sample every 0.1 µsec)

**Figure 2**

Data flow



## 2.2 Data Flow

Figure 2 shows the hardware architecture that implements digital processing. Signals from the photomultiplier tubes (PMTs) travel to acquisition boards. Each acquisition board can handle up to four input channels. For every input channel there is an A/D converter running continuously at 10 MHz that converts the input into digital data and stores the data in memory (dual-port RAM).

Digital data is first processed by field programmable gate arrays (FPGA) that compute area and height for each pulse based on a global thresholding circuit. Area and height measurements are relayed to a single digital signal processor (DSP) on each acquisition board. This DSP is in direct contact with the first of three additional DSPs that reside on the master board.

The first DSP on the master board collects the data from all the input channels, performs compensation, calculates ratios, and sends the data to a second DSP for sorting and to an embedded computer for transfer to the host computer. The embedded computer communicates with the host computer and controls the system hardware (PMT voltages, droplet generation).

Sorting is performed by the second and third DSPs. The second performs log lookup and gating; the third classifies drops. Once a drop is classified, the system will wait an appropriate amount of time (the drop delay) before charging the drop packet containing the event of interest (see Section 4).

## 2.3 Threshold and Parameter Extraction

The threshold defines the level at which the system starts looking for pulses in order to extract parameters of interest from all the data continuously streaming into the system. The BD FACSDiVa option extracts area and height parameters for signals that exceed the user-defined threshold (Figure 3). The system continuously monitors the data and simultaneously calculates area and height for all channels each time a signal exceeds the threshold.

All incoming data is stored in memory, thus a threshold signal can originate from the first, second, or third laser. Any logical combination of thresholds can be used. For example, the system can be triggered when the signal exceeds user-defined values in the FITC channel and the APC channel simultaneously.

The finite period of time during which the system is measuring height and area for an event is known as the window gate. At first glance, the window gate might seem to be comparable to the dead time in an analog system, but the BD FACSDiVa option is not dead (or more applicably *blind*) while it is extracting parameters. The process of digitizing and sampling is occurring continuously.

Height is the maximum value of all data points in a given channel within the window gate. The raw pulse height value is a number between 0 and 16,384 (14 bits). For logarithmic transformation and display (see next section), the raw height value is first multiplied by 16 in order to be scaled to the range of 0 to 262,144 (18 bits).

Area is the sum of all the data points within the window gate. (For information about extending the window gate, see Section 2.6). The area value for a typical pulse of 3 µsec is in the range of 0 to 262,144.

When pulses are longer than 3 µsec, such as when running at a lower pressure and sheath velocity, an area scaling factor (less than 1) can be used to bring the signal back on scale. At higher pressures and therefore faster sheath velocities, pulses are shorter and an area scaling factor greater than one can be used.

The best use of the area scaling factor is to make area and height measurements the same magnitude. One can then measure only area, while monitoring that the A/D converters do not reach saturation. Saturation can be directly measured and detected only by the height signal.

Width is measured as the number of samples in the window gate in 0.1-µsec (10 MHz) increments. Only one value is given per event since the window gate width is determined by the threshold. Width is also scaled to 262,144, but it has limited resolution because there are only 500 possible values (maximum pulse width and maximum window gate extension add up to a maximum width of 50 µsec).

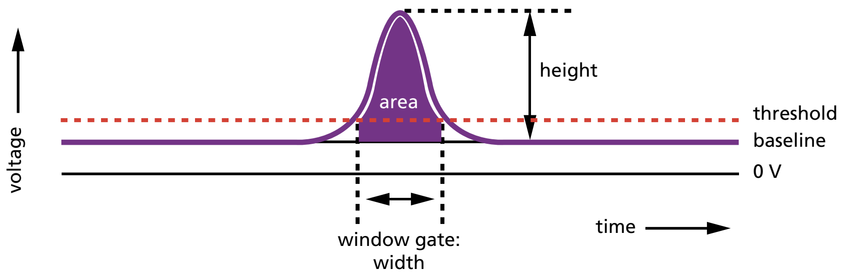


**Figure 3**

Window gate: period of time in which the signal is over threshold

## 2.4 Log Lookup

The BD FACSDiVa option handles log transformation and display just as it handles compensation (see Section 3). Both are mathematical processes that can be performed or repeated at any time. The system uses 18-bit precalculated log lookup tables, which explains the discrete nature of the histogram at the lower end (see Figure 4). In the lowest decade of the log plot, only ten possible values are available for display (Figure 5). On the analog system, the available values are distributed evenly over all decades because log amplification has occurred before digitization.

The histogram peaks are higher at the low end because these events can only be digitized into certain discrete bins (visible for the lowest peak in the PE-H log histogram). Notice that in the top four decades for the area signal, comparable to the dynamic range of current analog systems, the discrete nature of the data is not apparent.*

The main advantage of using log lookup tables is accuracy. Analog systems use logarithmic amplifiers that are manufactured to deviate no more than 5% from a perfect logarithmic response in its optimal range. At the beginning and end of the response curve, even greater deviations can be observed.

During sorting, log is calculated by the cytometer in order to classify events according to regions drawn by the user. The incoming data is sent to the workstation and stored in linear form. Log is recalculated on the host computer for display purposes, although at any time during or after acquisition, the user can switch from linear to log and the display will update.

The software calculates statistics only on linear data. Thus, changing from linear to log display will not affect the statistics, except as a result of slight gating differences. A polygon in a lin-lin plot transforms into something other than a polygon in a log-log plot, but the software only recomputes the vertices to go from one domain to the other.

## 2.5 Baseline Restoration

PMT signals can contain a high level of background from a variety of sources: light from unbound fluorophores, PMT dark current, and ambient light. Background signal is eliminated in two stages. The first, gross adjustment is made during the initial conversion of the signal from current to pulse. (Photomultiplier tubes are a source of current.) After pulse conversion in the preamp, the output signal falls in the range of 0 to 5 V. This initial gross adjustment preserves the dynamic range of the A/D converters for signals of interest, and maintains a safety margin of 100 mV.

The second, final adjustment is made just before area and height are measured. The FPGAs (Figure 2) calculate a running average of the data outside any window gate to remove the last 100 mV (Figure 3).

---

* Future versions of the software might include jittering to improve the visualization of logarithmic data.

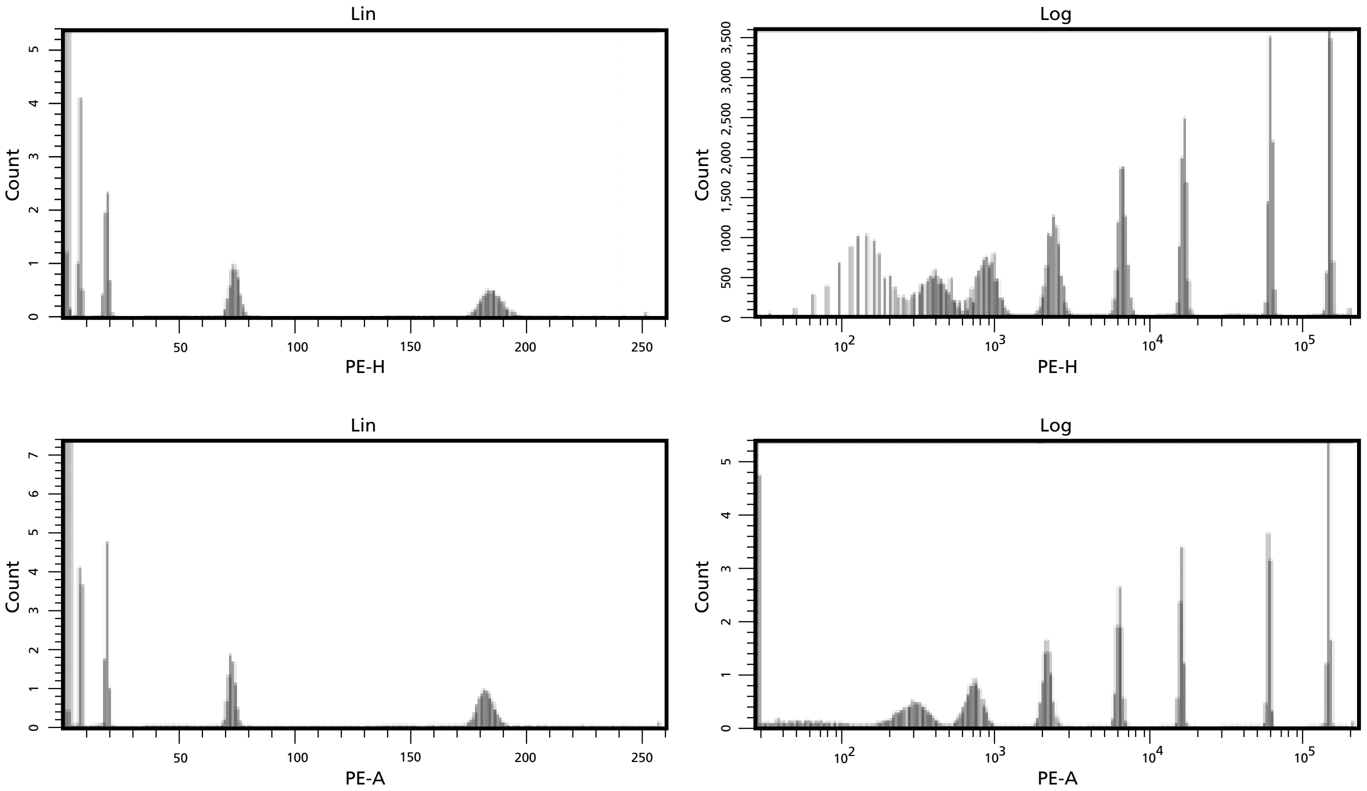


**Figure 4**

Linear and log display of height and area signals

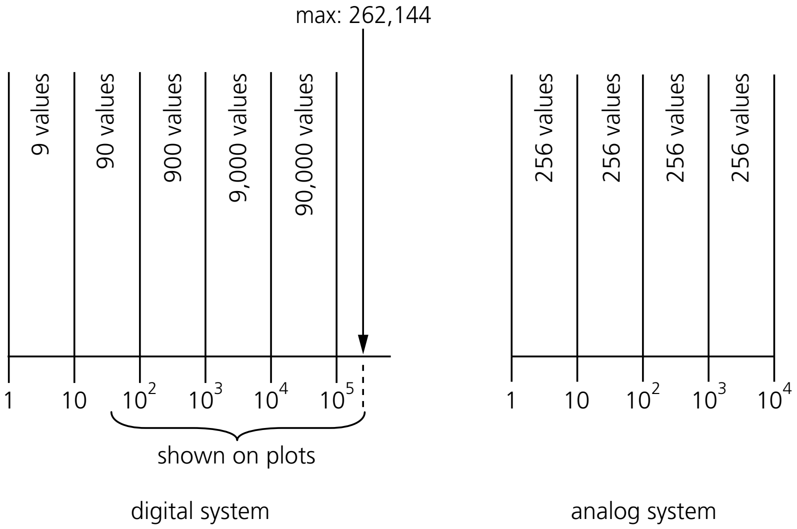


**Figure 5**

On the digital system, data is digitized before log transformation and display.

At the low end, fewer possible values can be measured.

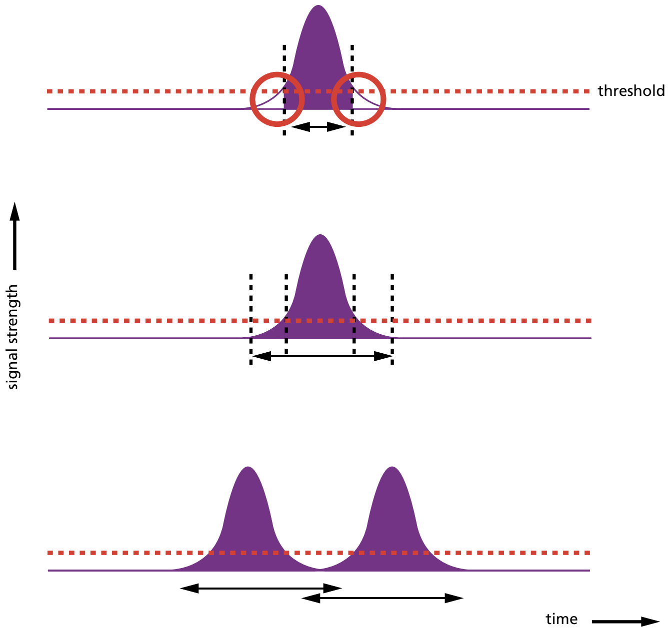


**Figure 6**

a. Initial window gate on the basis of threshold; missing area circled

b. Window gate extended; area measured correctly

c. Rare case: extended window gates overlap; pulses aborted

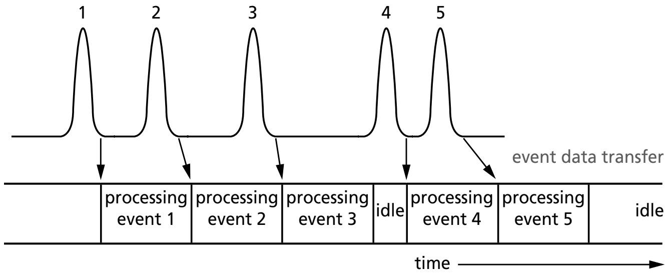


**Figure 7**

Event 2 arrives before processing of event 1 is finished; event 3 arrives before processing of event 2 is finished. The system is then idle until the data from event 4 is in. Processing of event 5 has to wait until event 4 is finished. All events are processed and none are aborted.

## 2.6 Window Gate Extension

The window gate can be too narrow to capture the entire pulse (Figure 6a), especially if the threshold is high. To correct this, a user-defined window gate extension can be used to increase the window gate by a fixed amount of time at both ends of the pulse (Figure 6b). The increased window gate allows the entire pulse to be processed.

A window gate extension is one of the two possible reasons the system can abort an event. When two events arrive so close together that their window gate extensions overlap, both events will be aborted (Figure 6c). Setting the window gate extension at zero ensures that this type of abort does not occur. The second reason for an abort is when the event rate becomes too high. This is described in more detail in the next section.

## 2.7 Event rate

The BD FACSDiVa option does not exhibit electronic dead time.* Because all raw data streams through memory whether or not a pulse is present, all pulses can be discriminated, even when a pulse arrives while the system is still processing an earlier pulse. With traditional flow cytometers, the system holds the peak signal electronically for a fixed amount of time. If another particle arrives during this time, both events are aborted.

BD FACSDiVa electronics process events sequentially. Even if events arrive in rapid succession (see Figure 7), the system will process them sequentially. Note that all parts of the system work in parallel. While one DSP is classifying an event, another DSP is working on compensating the next event, and the acquisition boards are already acquiring the event after that.

There is a limit to how much the system can buffer. If processing falls more than four events behind, the system will start aborting events as required in order to keep up (see Figure 8). (Aborted events require less processing time.) The limit of four events prevents the system from falling so far behind that the drop to sort will have already broken off. When events are aborted, drops will not be sorted during a purity sort.

---

* There is a minimum pulse width that can be set in the firmware. It is currently 1.2 μsec. The absolute minimum is 0.1 μsec, the sampling rate.

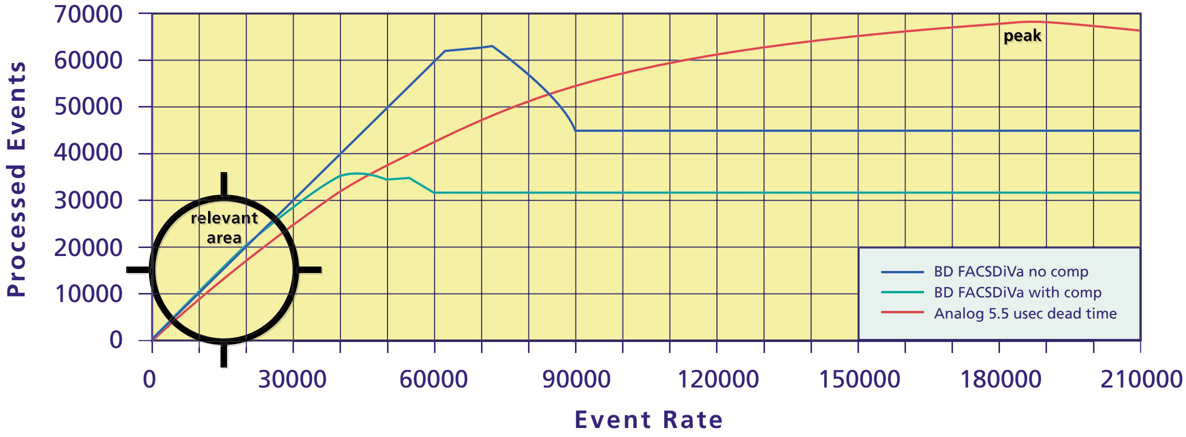


**Figure 8**

Number of processed events as a function of event rate

The rate at which the system falls behind is dependent upon how many parameters are being acquired and how many calculations need to be performed. Figure 8 shows the performance of the system for all event rates and compares it with a traditional analog system. When acquiring 10 parameters without compensation, the BD FACSDiVa option processes *all* events up to 60,000 events/sec. With compensation, aborts start to occur around 30,000 events/sec. Compare this to an analog system with a dead time of 5.5 µsec: at 30,000 events/sec, about 5,000 events/sec will be aborted.

BD FACSDiVa event processing is optimized for sorting. During sorting, the fundamental parameter limiting yield is the drop frequency. To get reasonable yields, the event rate should be less than four times the drop frequency. Considering the frequency range within which the BD FACSDiVa operates, 30,000 events/sec is close to the maximum event rate the system needs to run. At that event rate, the BD FACSDiVa option will have a greater yield than a traditional flow cytometer because it will be able to characterize all events. (This is explored in more detail in Section 4.)

Note that traditional systems with a dead time of 5.5 µsec have a theoretical maximum at about 180,000 events/sec, with a maximum number of processed events of 70,000 events/sec. However, even at a drop frequency of 150,000 drops/sec using a 1.5 drop envelope, the sorted yield of a 10% population will be less than 10%, that is, over 90% of the sample will not be sorted.

## 3. Compensation

### 3.1 Introduction

BD FACSDiVa software provides an improved compensation method using matrix inversion.

Compensation is the process by which the effect of spectral overlap among fluorochromes can be estimated and corrected. Traditionally, compensation has been performed by entering compensation coefficients, which are the percentages of one channel to subtract from another channel in order to get the other channel to measure only the fluorochrome of interest. Mathematically, compensation can be described as the solution to a set of equations, where the compensation percentages are the inverse matrix of the so-called spillover coefficients. A spillover coefficient is the amount of signal that is read by channels other than the channel in which it is intended to be measured. For two-color flow cytometry, compensation coefficients are the negatives of the spillover coefficients. But for three or more colors, this is not always the case, and it is often easier, or even necessary, to work in the spillover domain. There are other, more complete publications available on this topic. [1-3*]

For example, when running PE beads, some of the PE emission spectrum is visible in the FITC and PerCP channels. The percentage of signal in the FITC channel with respect to the signal in the PE channel is the FITC–%PE spillover coefficient. When this value is entered in the appropriate place in the software, along with the spillover coefficient for PerCP–%PE, the system correctly compensates for the spectral overlap of PE, regardless of the other colors used. This is not the case with compensation coefficients, where correctly compensating one color can negatively affect other colors.

### 3.2 Compensation Algebra

The algebra used for compensation will be illustrated here with a three-color example. Three is the minimum number of colors where there is a benefit from matrix inversion. All algebraic expressions can be scaled up for more colors.

---

[*] There are slight differences in the notation and assumptions between these various publications and this white paper, but the basic ideas are the same.

### 3.2.1 Spillover Matrix

Let *P* represent each of the stains on a particle. *P* is a collection of numbers, since there can be multiple stains on a particle. A vector is an often-used mathematical abstraction for such a collection. Thus, $P = (P_1,P_2,P_3)$, where the subscripts 1, 2 and 3 represent FITC, PE and PerCP.*

Let *F* be the fluorescence measured. *F* is a vector as well, since fluorescence is measured in multiple channels: $F = (F_1,F_2,F_3)$.

*F* is linked to *P* by the following formulas, where $s_{ij}$ are the spillover coefficients:

$$\begin{aligned} F_1 &= S_{11} * P_1 + S_{12} * P_2 + S_{13} * P_3 \\ F_2 &= S_{21} * P_1 + S_{22} * P_2 + S_{23} * P_3 \qquad (1) \\ F_3 &= S_{31} * P_1 + S_{32} * P_2 + S_{33} * P_3 \end{aligned}$$

In other words, the fluorescence measured in PE ($F_2$) is determined by the amount of all three fluorochromes on the particle passing through the laser beam. The biggest contributor to $F_2$ is the PE stain $P_2$. However, other fluorochromes, when present on a particle, can also emit light in the PE channel. Thus, $P_1$ and $P_3$ will contribute to $F_2$ via $s_{21}$ and $s_{23}$.

Matrix algebra was created as a convenient notation to express the formulas in [1]:

$$F = M * P \qquad (2)$$

Where *M* is the matrix:

$$M = \begin{pmatrix} S_{11} & S_{12} & S_{13} \\ S_{21} & S_{22} & S_{23} \\ S_{31} & S_{32} & S_{33} \end{pmatrix} \qquad (3)$$

### 3.2.2 Compensation Matrix

To estimate the spillover coefficients $s_{ij}$, singly-stained cells or beads are measured. Assume a particle contains 100,000 units of PE and no FITC or PerCP. Thus, P is (0; 100,000; 0). If the system measures F as (15,000; 100,000; 5,000) (15% of the PE light spills over into the FITC channel, and 5% of the PE light spills over into the PerCP channel), equation 2 becomes:

$$\begin{pmatrix} 15{,}000 \\ 100{,}000 \\ 5{,}000 \end{pmatrix} = \begin{pmatrix} S_{11} & S_{12} & S_{13} \\ S_{21} & S_{22} & S_{23} \\ S_{31} & S_{32} & S_{33} \end{pmatrix} \begin{pmatrix} 0 \\ 100{,}000 \\ 0 \end{pmatrix}$$

Because $P_1$ and $P_3$ are zero (singly-stained cells or beads), the spillover coefficients $s_{21}$, $s_{22}$, and $s_{23}$ can be computed (the exact units of F and P are not important; the factors cancel out later):

$$\begin{aligned} s_{12} &= 15{,}000/100{,}000 \\ s_{22} &= 100{,}000/100{,}000 \\ s_{32} &= 5{,}000/100{,}000 \end{aligned}$$

Next, when an unknown particle is measured with a fluorescence of F, the vector P can be determined (assuming that PE is the only channel that has spillover; in the next section another channel will be added):

$$\begin{aligned} P_1 &= c_{11}\,F_1 + c_{12}\,F_2 + c_{13}\,F_3 \\ P_2 &= c_{21}\,F_1 + c_{22}\,F_2 + c_{23}\,F_3 \\ P_3 &= c_{31}\,F_1 + c_{32}\,F_2 + c_{33}\,F_3 \end{aligned}$$

In these formulas, $c_{ij}$ are the compensation coefficients.

Written in linear algebra, we can solve equation [2] for P, with $M^{-1}$ being the inverse of M:

$$P = M^{-1} * F$$

Each of the compensation coefficients $c_{ij}$ in the compensation matrix $M^{-1}$ is dependent on all of the spillover coefficients. To give the expression for just one coefficient[4]:

$$C_{11} = \frac{S_{22}S_{33} - S_{23}S_{32}}{\text{Det } M}$$

where *Det M* is the determinant of the matrix M:

$$\text{Det } M = s_{11}(s_{22}s_{33}-s_{23}s_{32}) - s_{22}(s_{11}s_{33}-s_{13}s_{31}) + s_{33}(s_{11}s_{22}-s_{12}s_{21})$$

As one can see, the expressions become very large and are better left to the software.

The spillover matrix M was:

$$M = \begin{pmatrix} 1.00 & 0.15 & 0.00 \\ 0.00 & 1.00 & 0.00 \\ 0.00 & 0.05 & 1.00 \end{pmatrix}$$

And the compensation matrix $M^{-1}$ becomes:

$$M^{-1} = \begin{pmatrix} 1.00 & -0.15 & 0.00 \\ 0.00 & 1.00 & 0.00 \\ 0.00 & -0.05 & 1.00 \end{pmatrix}$$

---

* To be mathematically correct, P should be written as $P^T$, since P is a column vector.

In this case, the compensation matrix looks very similar to the spillover matrix. This will change in the next section when we add a second color.

To calculate the original stains *P*, given the measured fluorescence *F*:

$$P_1 = F_1 - 0.15\ F_2$$

$$P_2 = F_2$$

$$P_3 = F_3 - 0.05\ F_2$$

These formulas express the traditional compensation principle, where the original stains *P* are recovered by subtracting percentages of the channel that spilled over.

### 3.2.3 A Second Color Spills Over

Now, let's take into account PerCP spillover. When fluorescence is measured for PerCP beads, 2% of the PerCP signal spills over into the FITC channel and 18% of the PerCP signal spills over into the PE channel.

The new matrix M becomes:

$$M = \begin{pmatrix} 1.00 & 0.15 & 0.02 \\ 0.00 & 1.00 & 0.18 \\ 0.00 & 0.05 & 1.00 \end{pmatrix}$$

The inverse of M is now:

$$M^{-1} = \begin{pmatrix} 1.000 & -0.1504 & 0.0071 \\ 0.000 & 1.0091 & -0.1816 \\ 0.000 & -0.0505 & 1.0091 \end{pmatrix}$$

This leads to a revised estimate of the original stains P, given measured fluorescence F:

$$P_1 = 1.0000\ F_1 - 0.1504\ F_2 + 0.0071\ F_3$$

$$P_2 = 1.0091\ F_2 - 0.1816\ F_3$$

$$P_3 = 1.0091\ F_3 - 0.0505\ F_2$$

Notice the differences between the old and the new $M^{-1}$. First, the PE coefficients have slightly changed and are no longer simply the negatives of the spillover coefficients. Furthermore, the compensation coefficient $c_{13}$ is *positive* 0.7%, although PerCP spills over 2% into the FITC channel. Thus, we have to add part of the PerCP channel to the FITC channel to calculate the correct amount of FITC fluorescence.

Although the differences are minor in this case (and therefore most often neglected in three- or four-color cytometry), they can become quite large when more colors are used.

BD FACSDiVa software requires only that the spillover coefficients be entered; the application computes the inverse matrix before applying compensation.* One can then correct for spillover one color at a time, without having to worry about interactions between the different fluorochromes.

### 3.3 Compensation in Practice

Figure 9 shows the mean values for a set of beads acquired using the BD FACSDiVa option. Notice that the FITC-labeled beads show a fluorescence of 915 in the FITC channel whereas the negative beads only emit 107 units of fluorescence. As a percentage of the amount of fluorescence in the FITC channel (4485 – 148), the spillover coefficient to enter is therefore 808/4337 = 18.6%.

Alternatively, one can interactively change the spillover coefficient until the means are equal.† While viewing the means of the FITC and unstained beads in the PE channel, one can increase the PE–%FITC spillover coefficient until the mean of the FITC beads is equal to the mean of the unstained beads. One can sequentially go through each of the beads and change the spillover coefficients until each bead shows up only in its own channel. The different beads can be run simultaneously or separately. You can also run singly-stained cells separately (in this example, CD8 FITC, CD8 PE, CD8 PerCP, CD8 APC) to determine the correct spillover coefficients.

The means of the beads can be used because internally, numbers are not clipped to zero after compensation. However, if there are outliers, either because of doublets or carryover, medians can be used as a less sensitive estimator.

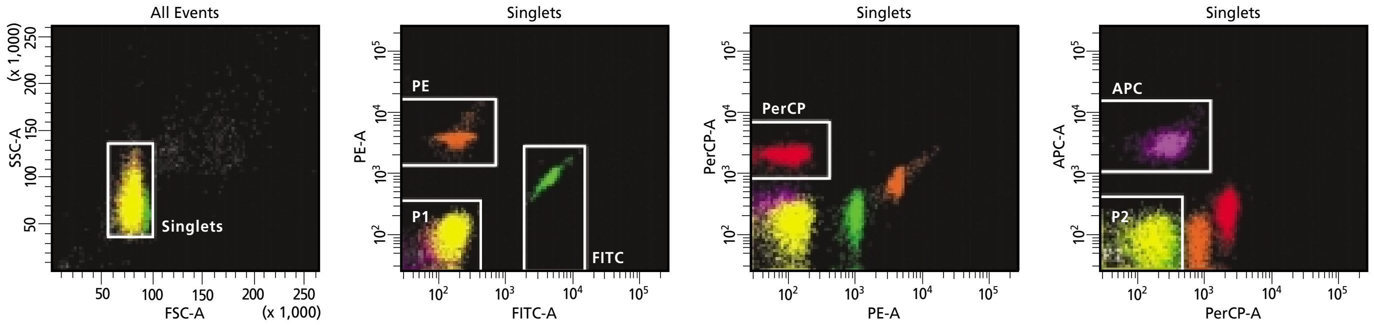
BD FACSDiVa software accepts the spillover coefficients entered in the user interface, puts them in a matrix, calculates the inverse matrix, and uses the resulting compensation coefficients to perform the compensation. During acquisition, these coefficients are sent to the instrument, which calculates compensation real time (required for sorting), and sends both raw data and compensated data to the host computer.

The host computer displays the compensated data, but stores only the raw data. It also stores the calculated compensation coefficients. When a data file is read back for analysis, the raw data is recompensated before display. However, the user can at any time enter new spillover values. In this case, the system retains the original raw data to recalculate the compensation without accumulating rounding errors.

---

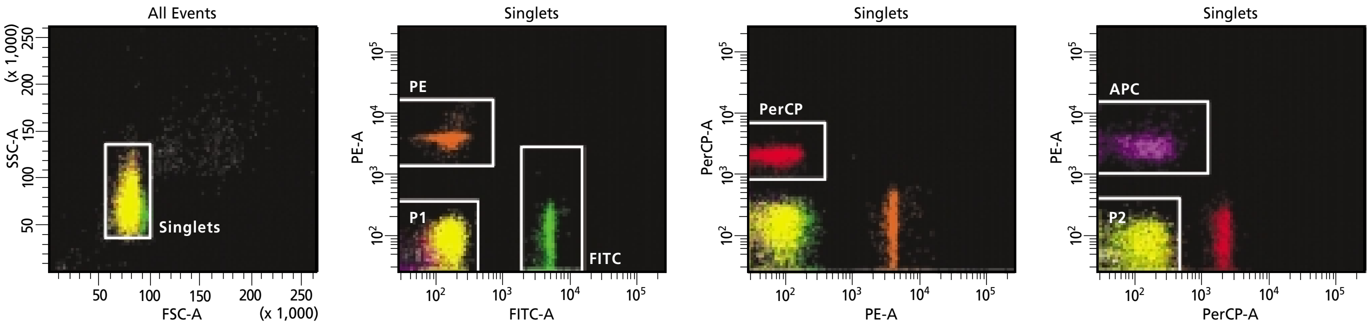
* BD FACSDiVa software makes one modification to this procedure. In order not to have to multiply with the diagonal coefficient for each color, it normalizes each row by the diagonal coefficient.

† It is not useful to adjust the compensation more precisely than the variation in the acquisition of a new data set. Because of statistical variation, the mean of the negatives is sometimes higher than the means of the positives even before applying spillover. This can also be due to a difference in autofluorescence, which is not addressed in this paper.



| Populaton | #Events | FITC-A<br>Mean | PE-A<br>Mean | PerCP-A<br>Mean | APC-A<br>Mean |
|---|---|---|---|---|---|
| Negatives | 16,648 | 148 | 107 | 144 | 63 |
| FITC | 8,591 | 4,485 | 915 | 215 | 50 |
| PE | 10,595 | 181 | 3,982 | 746 | 80 |
| PerCP | 9672 | 127 | 94 | 2,015 | 285 |
| APC | 6,003 | 93 | 56 | 285 | 3,065 |

(before compensation)



| Populaton | #Events | FITC-A<br>Mean | PE-A<br>Mean | PerCP-A<br>Mean | APC-A<br>Mean |
|---|---|---|---|---|---|
| Negatives | 16,657 | 147 | 79 | 127 | 50 |
| FITC | 8,591 | 4,478 | 79 | 127 | 39 |
| PE | 10,591 | 147 | 3,949 | 127 | 50 |
| PerCP | 9,637 | 126 | 70 | 1,993 | 50 |
| APC | 6,005 | 93 | 39 | 127 | 3,041 |

(after compensation)

**Figure 9**

BD CaliBRITE™ beads before and after compensation.

Negatives defined as P1 AND P2

## 4. Sorting

### 4.1 Conflict Resolution

The BD FACSDiVa option adds new sorting capabilities to the BD FACSVantage SE flow cytometer. It enables four-way sorting and expands flexibility in making sort decisions. The option also allows more particles to be sorted, because there is no dead time.

During sorting, particles arrive at random, whereas the drop rate is constant. The probability of a drop containing one or more events is a function of the drop rate and the average event rate. Table 1 shows the estimated distribution for a drop frequency of 50,000 per second. Most of the time, there are zero or one events in a drop, but at higher event rates, the chance of having more than one event in a drop increases. If these events need to be sorted in different directions and one is interested in having a pure population of particles, such a drop will not be deflected.

A compounding factor is that the system can charge discrete drops only at specific times (as a drop forms and breaks off). The system generates drops at a fixed frequency, and particles can reside anywhere within a drop (Figure 10). By setting the drop delay, the user empirically estimates in which drop a particle will most likely appear. For particles arriving near a drop boundary or between drops, this estimate is not 100% accurate. There is always some degree of uncertainty as to in which drop these events will ultimately fall. The system must take neighboring drops into account when making sort decisions.

If one is interested in purity, the system can be programmed to ensure that the deflected drop and parts of the adjacent drops are free of non-target particles. If one is interested in yield,[*] the system can be programmed to sort an adjacent drop if a target particle is close to a drop boundary. If one is interested in counting accuracy or maximum drop stability, the system can sort only particles that arrive in the middle of a drop. With the BD FACSDiVa option, the user has complete flexibility in setting the sort priorities. Sort decisions are determined by sort masks.

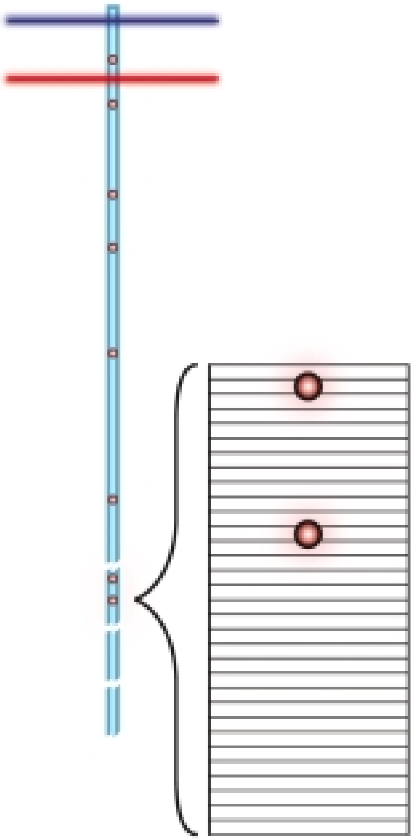
**Table 1**

Probability of having *n* particles in a drop for four different event rates at a drop frequency of 50 kHz

| No. Particles | Event Rate (events/sec)<br>1,000 | 10,000 | 20,000 | 30,000 |
|---|---|---|---|---|
| 0 | 98% | 82% | 67% | 55% |
| 1 | 2% | 16% | 27% | 33% |
| 2 | 0% | 2% | 5% | 10% |
| 3 | 0% | 0% | 1% | 2% |
| 4 | 0% | 0% | 0% | 0% |
| 5 | 0% | 0% | 0% | 0% |

**Figure 10**

Target particles in a drop with 1/32-drop resolution



---

[*] Yield is defined as the number of target cells sorted as a percentage of the number of target cells passing through the flow cytometer. Recovery is defined as the number of target cells sorted as a percentage of the counters on the system. This paper uses the term counting accuracy to indicate that a recovery of 100% is important.

## 4.2 Sort Masks

BD FACSDiVa software provides three mask settings to determine sort results: a yield mask, a purity mask, and a phase mask.

### 4.2.1 Yield Mask

The yield mask determines how many drops the system will deflect when a target particle is detected. When a particle is expected to be close to the edge of a drop, it is beneficial to sort the adjacent drop. The user can set the size of the edge segment in 1/32-drop increments. Half of each yield mask setting defines an equal area at each end of the drop. With a yield mask of zero, only one drop is deflected for each target particle; with a yield mask of 32, two drops are sorted. For yield masks in between, sometimes one drop and sometimes two drops will be deflected, depending on the position of the target particle.

Figure 11 shows three examples of target event positions. In (a), the target event is near the center of the middle drop, and only the middle drop is deflected. In (b), the target event is in the yield mask, close to the trailing drop. The trailing drop is deflected along with the middle drop. In (c), the target event is in the yield mask, close to the leading drop, so the leading drop is deflected along with the middle drop. In the given example, the yield mask is 8/32: on the average 1.25 drops are deflected per target event.

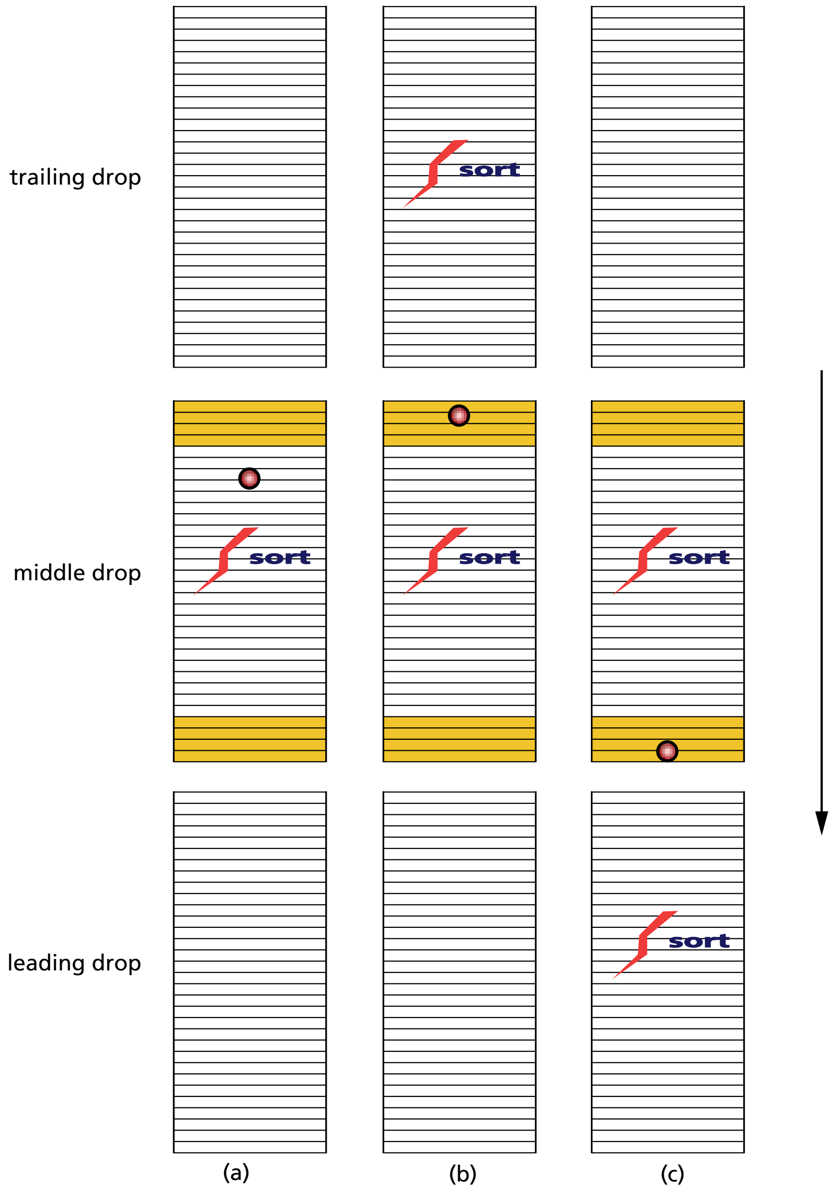


**Figure 11**

Yield mask of 8/32 indicated in yellow; target particle shown in red

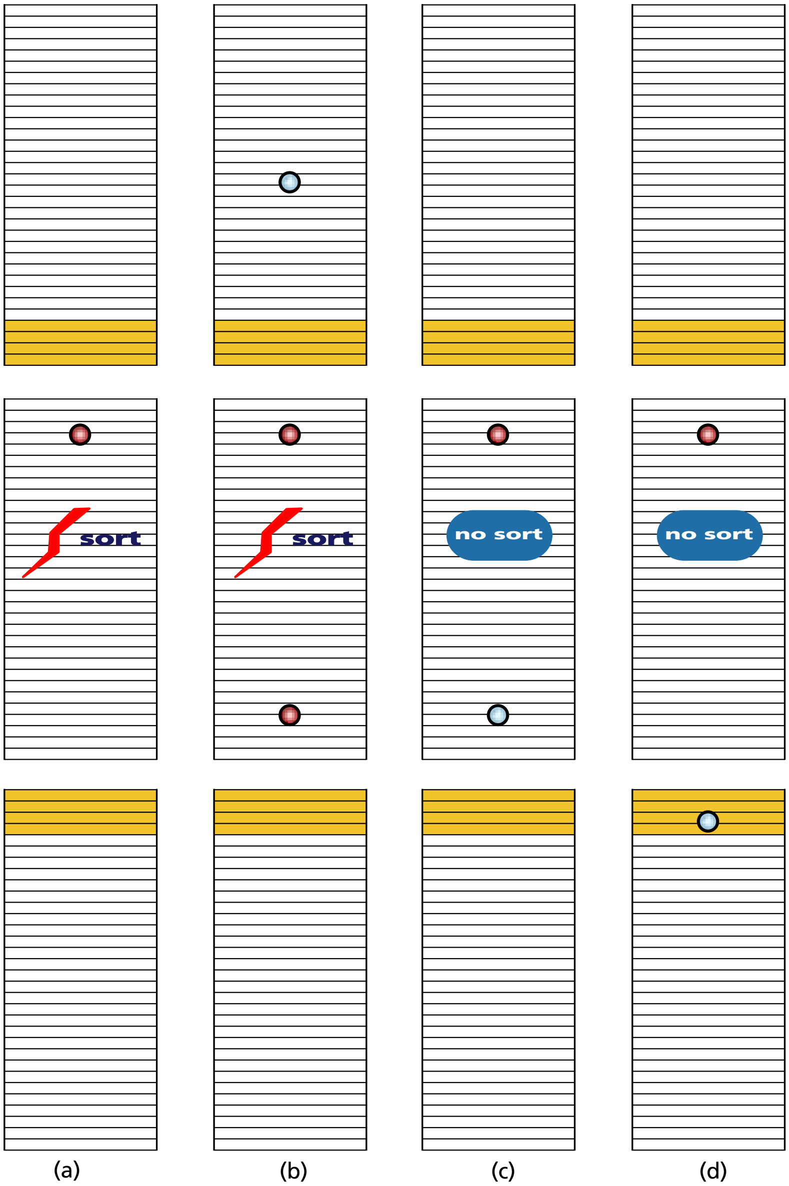


**Figure 12**

Purity mask of 8/32 In yellow, 4/32 in each adjacent drop; target particles in red, non-target particals in blue

### 4.2.2 Purity Mask

The purity mask prohibits the system from deflecting drops if non-target particles are present. The user can specify the size of the segment that has to be clear of non-target particles in the adjacent drops. When sorting for purity, the minimum size is 2/32 of a drop. The maximum size is 32/32, in which case the mask extends midway into the leading and trailing drops. Obviously, the drop to be sorted has to be clear of non-target particles as well.

Figure 12 shows a purity mask of 8/32. In (a), only a target particle is present, so the middle drop can be sorted. In (b), the middle drop contains two target particles and the purity mask is clear, so the drop can be sorted. In (c), the middle drop cannot be sorted because a non-target particle is present. In (d), the middle drop cannot be sorted because a non-target particle is present in its purity mask.

Drops that cannot be sorted can be recovered in a separate tube using the Abort Save mode.

If both a yield and a purity mask are set, the yield mask is first applied to determine which drops are candidates for sorting. The purity mask is then applied to each of the candidate drops.

Figure 13 shows two cases. In both cases, the green target particle is in the yield mask, so two drops are candidates for deflection. In (a), the middle drop can be sorted, but the trailing drop cannot because of a conflicting red particle. In (b), a conflicting particle is in the middle drop's purity mask, so the drop cannot be sorted, but the trailing drop still can. Note that for the trailing drop, the purity mask extends into the drop after the trailing drop.

Using a yield and purity mask together maximizes the number of sorted cells but does not optimize counting accuracy. In both Figures 13a and 13b, only one drop is deflected. In (a), the sort counter is incremented as the deflected drop most likely contains the target particle. In (b), the counter is not incremented, but the trailing drop is deflected nonetheless because the green target particle might slip into the trailing drop.

If counting accuracy is to be emphasized, one has to prohibit sorting cells near the edge of a drop by applying a phase mask (next section), at the cost of decreased yield.

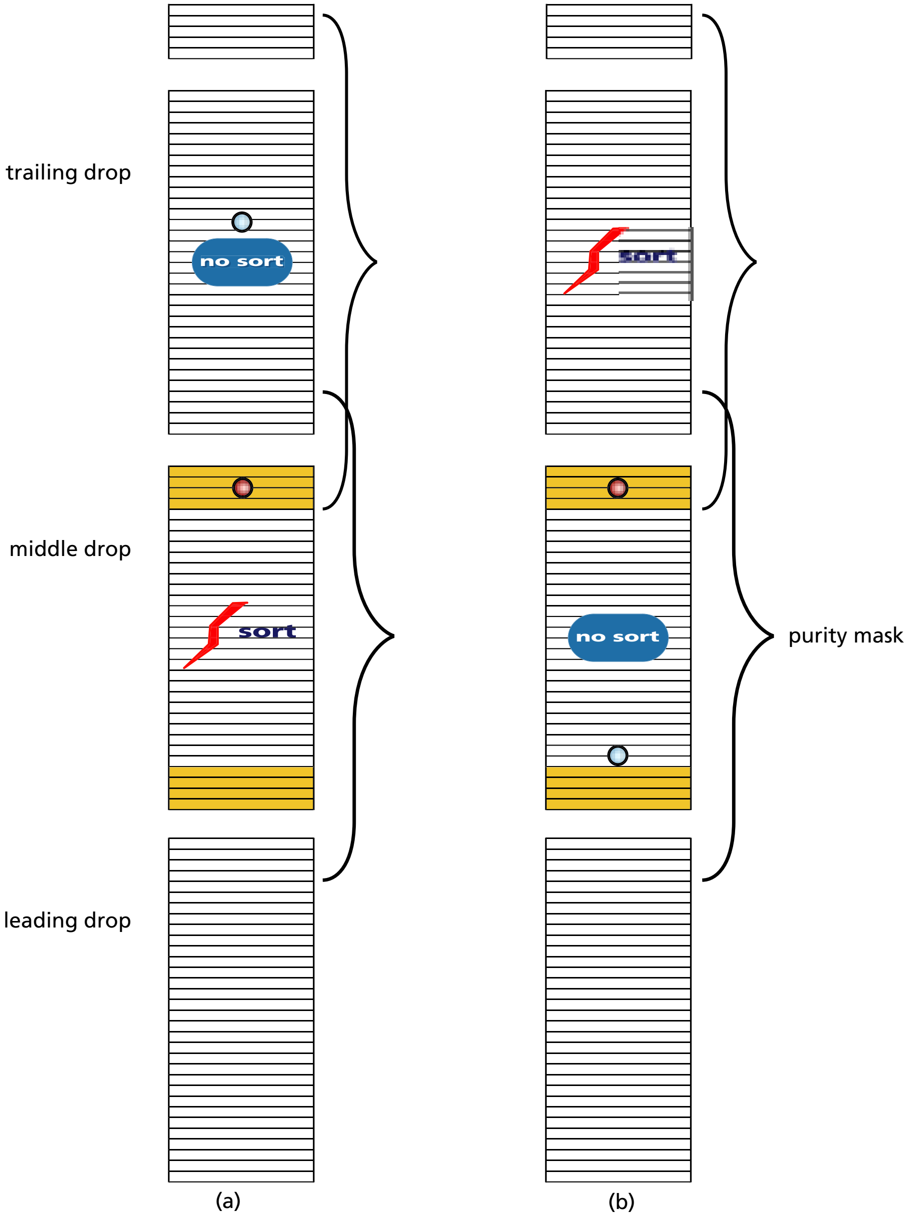


**Figure 13**

Yield and purity masks combined (both 8/32). The yield mask is indicated in yellow; the purity masks with curly brackets.

### 4.2.3 Phase Mask

A phase mask ensures that only drops that have no particles near their edges are deflected. The edges of the adjacent drops must be empty as well (Figure 14). This ensures accurate sort counters, but sacrifices yield. An additional benefit is that side streams are stabilized, since particles near the edges of drops can modify the drop breakoff. A phase mask is often applied to improve the side stream quality during single cell sorting into plates or when sorting larger particles.

A phase mask cannot be used with a yield mask, but it can be used with a purity mask. When used with a purity mask, the purity mask ensures that there are no conflicting particles that can contaminate the sort.

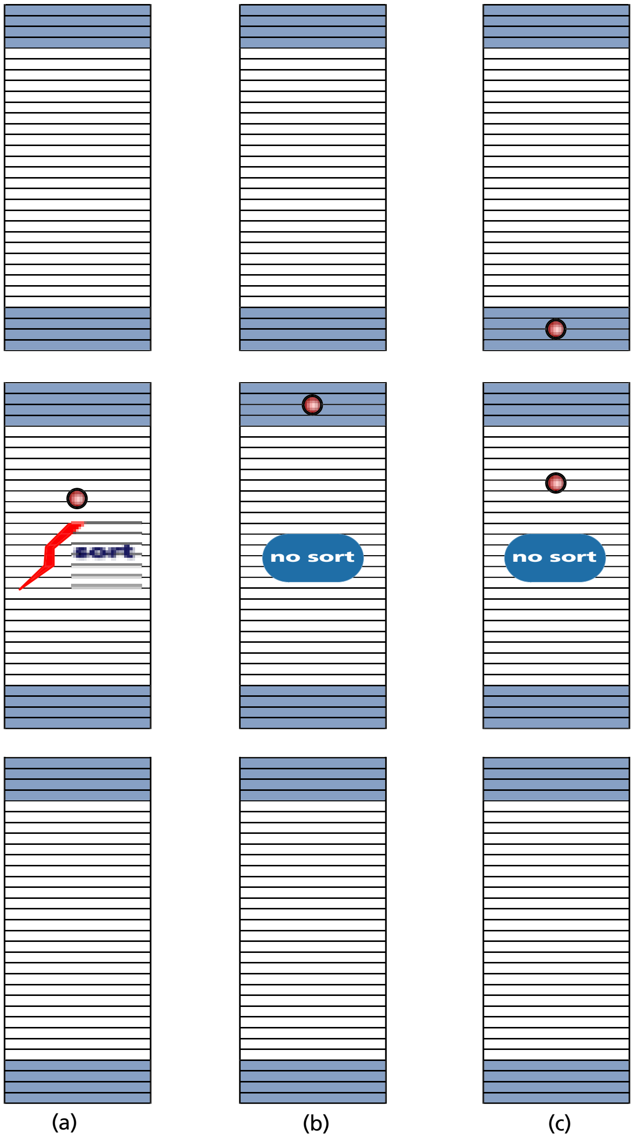


**Figure 14**

Phase mask of 8/32 shown in blue. In (a), the middle drop is deflected; in (b) and (c), the middle drop is not deflected.

## 4.3. Experiments

### 4.3.1. Zero Dead Time, Better Yield

BD FACSDiVa electronics have no dead time. To investigate the effect on yield, FITC-positive and unstained beads were acquired simultaneously on the analog and digital sides. Both sides made sort decisions, and the acquisition and sort counters were noted for each side.* Tables 2 and 3 show the results for both a small and a large subpopulation.

Theoretical yield *Y* is estimated by the following Poisson formula[5]:

$$Y = \exp^{-(1-s)rT}$$

where *s* is the fraction to sort, *r* the event rate and *T* the drop envelope time. With a drop frequency of 60,000 per second and purity mask of 16/32 (similar to normal R mode with a 1.5 drop envelope), *T* was 25° µsec.

It is clear from the tables that the BD FACSDiVa sort counter follows the predicted theoretical yield more closely than the analog sort counter and that this effect is more pronounced at higher event rates. One can modify the theoretical yield formula to a priori accept the losses due to dead time (by adding an extra term -*rt* to the exponent in the formula above, where *t* is the dead time), but for this exercise no modification was made.

At the higher event rates, the BD FACSDiVa sort counter begins to deviate from the theoretical yield because it starts to abort events (as explained in Section 2.8). Nonetheless, recovery remains significantly higher than the analog system. As shown in Table 2, the theoretical yield was only 50% at the highest event rate, so it is not advisable to run at these high event rates to achieve reasonable yield.

In summary, the BD FACSDiVa option can sort significantly more cells than the analog system, because it has no dead time. Figure 15 shows the yield as a function of event rate for Table 3.

### Table 2

Sort counters at five different event rates for a small subpopulation

| | | | | | |
|---|---|---|---|---|---|
| **Sorted fraction** | 6.3% | 6.1% | 6.1% | 6.0% | 5.8% |
| **Event rate** | 4400 | 14700 | 19800 | 23500 | 30200 |
| **System threshold** | 262936 | 879860 | 1186430 | 1409888 | 1871886 |
| **BD FACSDiVa sort counter** | 14640 | 39411 | 46416 | 47862 | 45711 |
| **Analog sort counter** | 13633 | 32811 | 37604 | 39057 | 39670 |
| **More cells with BD FACSDiVa** | 7% | 20% | 23% | 23% | 15% |
| **Theoretical yield** | 90% | 71% | 63% | 58% | 49% |
| **BD FACSDiVa yield** | 88% | 74% | 65% | 57% | 42% |
| **Analog yield** | 82% | 61% | 52% | 46% | 37% |

### Table 3

Sort counters at five different event rates for a large subpopulation

| | | | | | |
|---|---|---|---|---|---|
| **Sorted fraction** | 43.0% | 45.3% | 43.9% | 43.9% | 39.5% |
| **Event rate** | 8800 | 15000 | 19700 | 24000 | 30800 |
| **Total events** | 527768 | 902873 | 1184739 | 1440858 | 1849682 |
| **BD FACSDiVa sort counter** | 197755 | 330663 | 381931 | 411483 | 368453 |
| **Analog sort counter** | 170876 | 274150 | 307815 | 332678 | 307464 |
| **More cells with BD FACSDiVa** | 16% | 21% | 24% | 24% | 20% |
| **Theoretical yield** | 88% | 81% | 76% | 71% | 63% |
| **BD FACSDiVa yield** | 87% | 81% | 73% | 65% | 50% |
| **Analog yield** | 75% | 67% | 59% | 53% | 42% |

NOTE: Sorted fractions are not exactly the same across all columns because of sample changes. System threshold and sorted fraction counters were read in BD FACSDiVa software. Both systems sorted in Normal-R mode with a 1.5 drop envelope at 60 kHz. System threshold was set such that both acquisition systems detected the same particles.

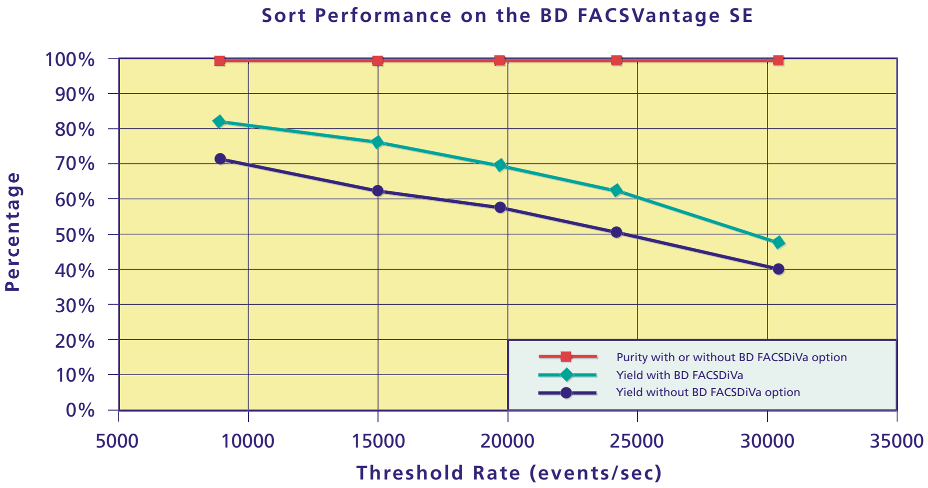


**Figure 15**

Purity and yield on the FACSVantage SE. Yield data obtained by multiplying data from tables 3 (counters) and 4 (recovery versus counter).

---

* Simultaneous acquisition is possible since the BD FACSDiVa preamp splits the signal and feeds it to both the analog BD FACSVantage SE and the digital BD FACSDiVa electronics. Obviously, only one system can control drop charging, so they cannot physically sort simultaneously. For now, we are assuming that the recovery (versus counter) is good on both systems. This will be investigated in more detail for the BD FACSDiVa option in the next section.

### 4.3.2 Recovery

In the previous section, we showed that the yield as determined by the counters is better on the BD FACSDiVa option than on the standard system. To demonstrate counter fidelity, we sorted beads and verified the count under a microscope. We sorted with a purity mask of 16 and a yield mask of 16, which are recommended values for a bulk sort. These settings minimize the number of cells that go to waste, yet ensure a pure result (more on purity in the next section). Note for depositing an exact number of particles on a slide, a phase mask would also be used (as explained in Section 4.2.3 and as will be shown in the next section), but the purpose of this experiment was to simulate the conditions during a typical bulk sort where purity is most important, followed by yield, and then counting accuracy.

At 35 psi and 50 kHz, 100 YellowBrite beads (12% target population) were sorted into five spots on a slide. Beads were sorted in Normal-R mode at a rate of 8,000 events/sec with a purity mask of 16, a yield mask of 16, and no phase mask. The sorted beads were then counted under a fluorescence microscope. Results are shown in Table 4.

The actual recovery as a function of the system counter was in all cases >95%. A small percentage of beads are lost because of variations in the time of flight between the laser intercept and the point at which a drop is charged. The system will try to sort two drops in order to catch a target particle if the particle is near the edge (yield mask), but it is not always able to because the adjacent drop can contain a non-target particle.

**Table 4**

Bulk sort recovery for five sorts

| |
|---|
| 96 |
| 98 |
| 99 |
| 97 |
| 98 |

### 4.3.3 Counting Accuracy

To obtain exactly the number of particles requested, a phase mask must be used. Counting accuracy is achieved at the cost of yield because the system will not sort any particles close to a drop boundary.

Table 5 shows the results of a single-cell sort onto a slide in a grid of 3 by 9. YellowBrite beads were sorted onto a slide and counted under a fluorescence microscope. Three regions were used to ensure that only singlets were sorted (based on scatter and fluoresence). The drop frequency was 33 kHz at 18 psi, the event rate was 5,000 events/sec. A phase mask of 16 was used, so only events in the middle half of a drop were sorted. The yield mask was 0 (by definition when a phase mask is on) and the purity mask was 16.

As shown in Table 5, the first column contained exactly two particles per spot, the second column exactly five particles per spot, and the last column exactly ten particles per spot.

### 4.3.4 Purity

Lastly, we ran an experiment to demonstrate good purity during a bulk four-way sort (Figure 17). The cytometer ran at 20,000 events/sec with a drop frequency of 64 kHz at 45 psi. A purity mask of 32 and a yield mask of 0 was used. These settings ensure a high purity, a reasonable yield, and optimal side streams.

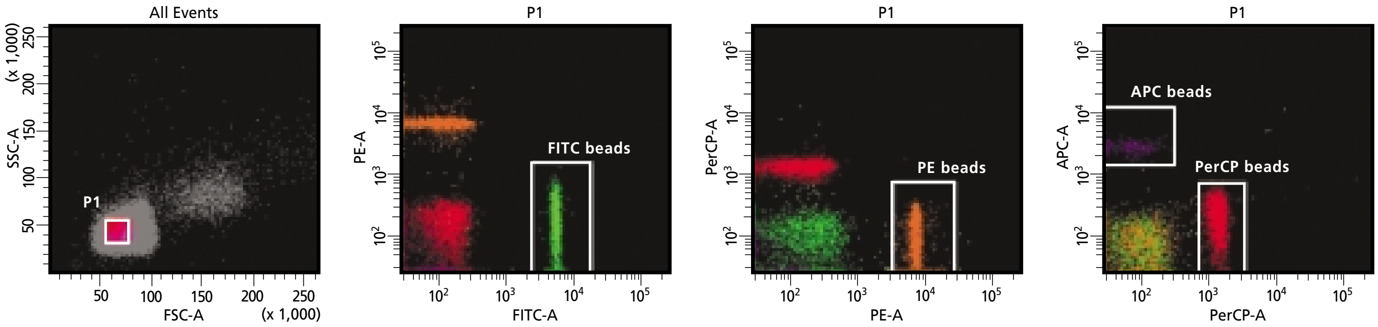
## 4.4 Summary

The BD FACSDiVa option allows the user to define exactly how sort decisions should be made. Yield masks, Purity masks, and Phase masks can be used to make an optimal tradeoff between yield, purity, counting accuracy, and side stream quality.

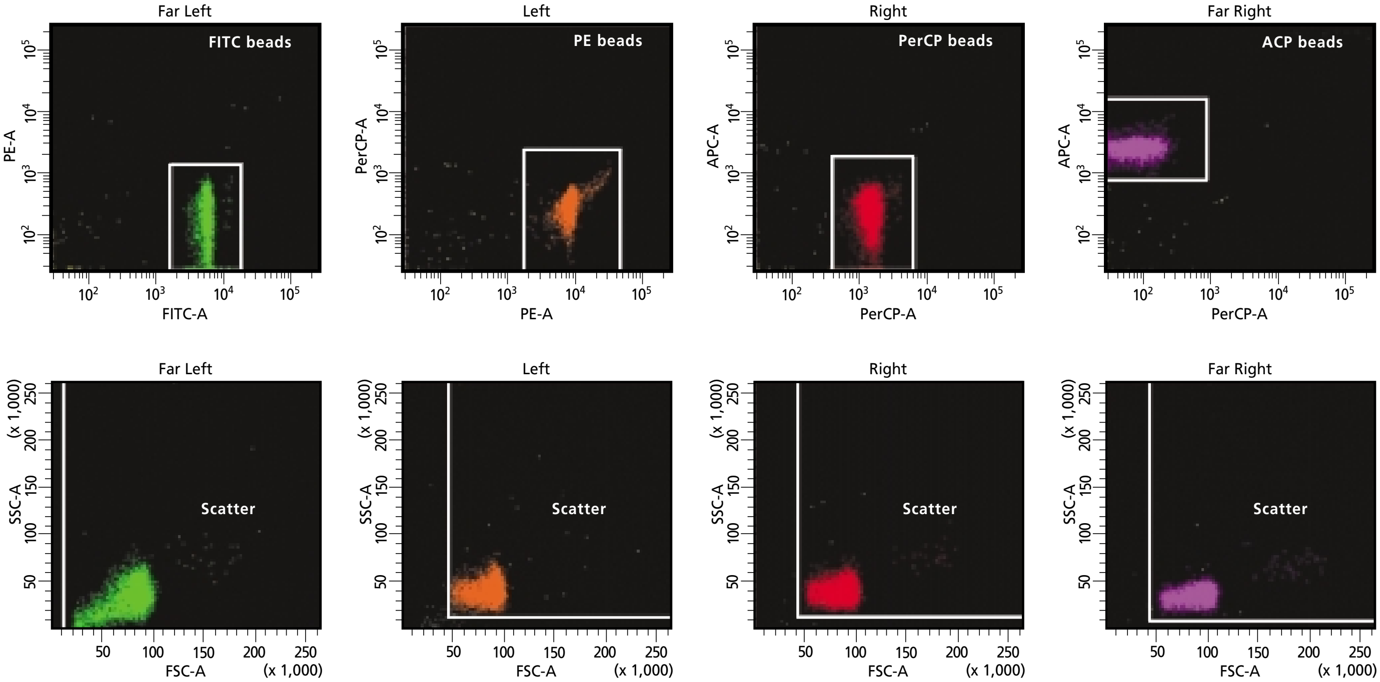
**Table 5**

Single-cell sorting recovery

| Column 1 | Column 2 | Column 3 |
|---|---|---|
| 2 | 5 | 10 |
| 2 | 5 | 10 |
| 2 | 5 | 10 |
| 2 | 5 | 10 |
| 2 | 5 | 10 |
| 2 | 5 | 10 |
| 2 | 5 | 10 |
| 2 | 5 | 10 |
| 2 | 5 | 10 |



(a) Pre-sort with sort gates



(b) Post-sort analysis on each of the four output tubes

| | FITC beads | PE beads | PerCP beads | APC beads |
|---|---|---|---|---|
| **Pre-sort fraction** | 8.2% | 9.5% | 8.0% | 0.6% |
| **Post-sort fraction** | 99.1% | 99.2% | 99.5% | 99.4% |

**Figure 17**

Sort results from a four-way purity sort at 45 psi and 64 kHz

The creation of the BD FACSDiVa option was the work of a large group of people from all parts of the BD Biosciences organization and from a large number of beta customers. Thanks to all of them. The author would also like to thank all the reviewers of this white paper, which has benefitted greatly from the time they invested.

## References

[1] Bagwell CB, Adams EG. Fluorescence spectral overlap compensation for any number of flow cytometry parameters. *Ann NY Acad Sci*. 1993;677:167-184.

[2] Roederer M, Derosa S, Gerstein R, Anderson MT, Bigos M, Stovel RT, Nozaki T, Parks DR, Herzenberg LA, Herzenberg La. 8 color, 10-parameter flow cytometry to elucidate complex leukocyte heterogeneity. *Cytometry.* 1997;29;328-339.

[3] Bigos M, Baumgarth N, Jager GC, Herman OC, Nozaki T, Stovel RT, Parks DR, Herzenberg LA. Nine color eleven parameter immunophenotyping using three laser flow cytometry. *Cytometry.* 1999;36;36-45.

[4] Lang S. *Linear Algebra*. 2nd ed. Reading, Massachusetts: Addison-Wesley. 1980.

[5] Hoffman RA, Houck DW. High speed sorting efficiency and recovery: theory and experiment. *Cytometry.* 1998;9;142.

# BD Biosciences

Clontech
Discovery Labware
Immunocytometry Systems
Pharmingen

### Asia Pacific
**BD Singapore**
Tel 65.6861.0633
Fax 65.6860.1590

### Japan
**Nippon Becton Dickinson**
Tel 81.24.593.5405
Fax 81.24.593.5761

### Canada
**BD Biosciences**
Toll free 888.259.0187
Tel 905.542.8028
Fax 905.542.9391
**www.canada@bd.com**

### Europe
**Becton Dickinson European HQ**
Tel (32) 53-720211
Fax (32) 53-720450

### United States
**BD Biosciences**
**Clontech**
Fax 650.354.0775
**Discovery Labware**
Fax 978.901.7493
**Immunocytometry Systems**
Fax 408.954.2347
**Pharmingen**
Fax 858.812.8888
**Customer/Technical Service**
Toll free 877.232.8995
**www.bdbiosciences.com**

---

### Africa
Becton Dickinson
Worldwide Inc
Kenya
Tel (254) 2 449 608
Fax (254) 2 449 619

### Australia
Becton Dickinson Pty Ltd
Tel (612) 9978-6800
Fax (612) 9978-6850

### Austria
Becton Dickinson Austria
Tel (43) 1 706 36 60-0
Fax (43) 1 706 36 60-11

### Benelux
N.V. Becton Dickinson S.A.
Belgium:
Tel (32) 53 720211
Fax (32) 53 720200
The Netherlands:
Tel (31) 76 5037720
Fax (31) 76 5014133

### China
Becton Dickinson Asia Ltd.
Tel 8610-6593 3072-77
Fax 8610 6593 3070

### Denmark
Becton Dickinson AS
Tel (45) 43 434566
Fax (45) 43 434166

### Eastern Europe
Becton Dickinson International
Germany
Tel (49) 6221 3050
Fax (49) 6221 305 388

### France
Becton Dickinson SA
Division Immunocytométrie
Tel (33) 476 683730
Fax (33) 476 683544

### Germany
Becton Dickinson GmbH
Tel (49) 6221 3050
Fax (49) 6221 303798

### Greece
Becton Dickinson Hellas SA
Tel (30) 1 940 77 41
Fax (30) 1 940 77 40

### Hong Kong
Becton Dickinson Asia Ltd
Tel (852) 2575-8668
Fax (852) 2803-5320

### Hungary
Becton Dickinson
Tel (36) 1 216 48 93
Fax (36) 1 216 48 93

### Iceland
Icelantic American Trad Co
Tel (354) 168 27 00

### India
Becton Dickinson India Pvt Ltd
Tel (91-11) 6913092
Fax (91-11) 6831783

### Indonesia
Becton Dickinson Asia Ltd
Tel (6221) 577 1920
Fax (6221) 577 1925

### Ireland
Becton Dickinson
Diagnostic Systems
Tel (353) 1 285 48 00
Fax (353) 1 285 43 32

### Israel
Bactlab Diagnostics Ltd.
Tel (972) 6-6309600
Fax (972) 6-6230777

### Italy
Becton Dickinson
Italia SpA
Tel (39) 02 482401
Fax (39) 02 48200323

### Korea
Becton Dickinson
Korea Inc
Tel (822) 5694030
Fax (822) 5694048/9

### Latin America
Becton Dickinson
Immunocytometry
Systems USA
Tel (408) 954-2157
Fax (408) 526-1804

### Malaysia
Becton Dickinson Sdn
Bhd
Tel (0203) 7571323
Fax (0203) 7571153

### Middle East
Becton Dickinson
Tel (971) 4 379525
Fax (971) 4 379551

### Norway
Laborel A/S
Tel (47) 23 05 19 30
Fax (47) 22 63 07 51

### Philippines
Becton Dickinson
Philippines, Inc
Tel (632) 818 9727
Fax (632) 810 5687

### Poland
Becton Dickinson Polska
Tel (48) 22 6517921
Fax (48) 22 6517924

### Portugal
ENZIfarma, Diagnostica
e Farmaceutica
Tel (351) 1 4420100
Fax (351) 1 4420110

### South Africa
BD Immunocytometry
Systems
Tel (27) 11 807 1531
Fax (27) 11 807 1953

### Spain
Becton Dickinson España SA
Tel (34) 91 8488100
Fax (34) 91 8488104

### Sweden
Becton Dickinson AB
Tel (46) 8 775 5100
Fax (46) 8 645 08 08

### Switzerland
Becton Dickinson AG
Tel (41) 61 385 44 22
Fax (41) 61 385 44 00

### Taiwan
Becton Dickinson
Worldwide Inc
Tel (8862) 722-5660
Fax (8862) 725-1772

### Thailand
Becton Dickinson Thailand Ltd
Tel (662) 643 1371-80
Fax (662) 643 1381

### Turkey
BD Turkey
Tel (90) 212 222 87 77
Fax (90) 212222 87 76

### United Kingdom
Becton Dickinson UK Ltd
Tel (44) 1865 748844
Fax (44) 1865 781635

---

PE: US Patent Nos. 4, 520,110; 4,859,582, and 5,055,556; European Patent No. 76,695; and Canadian Patent No. 1,179,942
PerCP: US Patent No. 4,876,190
PerCP-Cy5.5: US Patent Nos. 5,268,486; 5,486,616; 5,569,587; 5,569,766; and 5,627,027
APC-Cy7: Cy7 is licensed under US Patent Nos. 5,268,486; 5,486,616; 5,569,587; 5,569,766; and 5,627,027. APC-Cy7 is licensed under US Patent No. 5,714,386.

The BD FACSVantage SE flow cytometer and the BD FACSDiVa option are For Research Use Only. Not for use in diagnostic or therapeutic procedures.

03/02 23-6579-00