An Introduction to Window Extension on Digital Flow Cytometers

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Technical Bulletin

Contents

Abstract

The optical detectors of a flow cytometer convert all detected light into electronic signals that are subsequently converted into digital data. A sample pulse is the electronic representation of a signal with measurable height, width, and area values. In a BD digital system, the detector output is continuously digitized, and threshold defines the signal level at which the system begins to measure a pulse within the continuous data stream. The initial window gate (WG) is the time interval during which the input signal is greater than the threshold and during which the acquisition system looks to sample a pulse. To ensure that a pulse is completely integrated, additional time, called the window extension (WE), is added before and after the initial WG to extend the total integration time. Incorrect WE settings can affect proper data acquisition and lead to incorrect data measurements.

BD™ Cytometer Setup and Tracking (CS&T) is a fully automated system unique to BD digital flow cytometers that provides instrument characterization, tracking, and quality control. CS&T is a module within BD FACSDiva™ software (v6.0 and later). CS&T characterization features automatically define the instrument baseline, set correct laser delays, and fully characterize the instrument. When setting up a new cytometer configuration in CS&T, the software automatically defaults to an instrument-specific WE. For example, on the BD FACSCanto™ II cytometer, the WE defaults to a value of 7 (3.5 µs before and after the initial WG). The operator can manually set the WE to an alternate value prior to baseline determination. The appropriate WE value for a given application is specific to that application (for example, particle size and dim populations).

BD FACSCanto™ clinical software, used in conjunction with BD FACS™ 7-color setup beads, provides automated cytometer setup and quality control for in vitro diagnostic (IVD) use. This system automatically sets the WE to a value of 7, which the operator cannot change. Certain BD Multitest™ reagents are designed for use with this software system. Use of certain BD products on an instrument with different software that might have different settings, including but not limited to WE, might alter the results.

In summary, the manipulation of WE in conjunction with the use of BD FACSDiva software's CS&T performance checks allows an operator to optimize cytometer settings for a given application. However, certain BD products must be used with a WE of 7. If an operator chooses to perform these tests using a different WE (or other setting), the application must be fully validated prior to use.

Digital Flow Cytometry

Digital data collection

The optical detectors of a flow cytometer convert light signals into electronic signals that travel to the acquisition boards, where an analog-to-digital converter converts the input signal into digital data. A voltage pulse is the electronic representation of a signal with height, width, and area values, as shown in Figure 1.
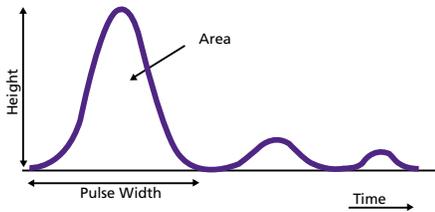
The pulse characteristics can be defined as follows:

Figure 1. Visual representation of a pulse.

- Width. The period of time that it takes for a particle to pass through the laser interrogation point.

- Height. The intensity of the pulse as it passes through the laser interrogation point.

- Area. Calculated by integration and represents the whole pulse (change in intensity over time). The use of area measurements allows for better representation of signals from dim populations or doublets compared to using height (intensity) on its own.

To correctly calculate pulse area, WG, WE, threshold, and laser delay need to be considered.

Window gate

In a BD digital system, the detector output is continuously digitized, whether a pulse is present or not. The threshold defines the signal level at which the system begins to measure a pulse within the continuous data stream. The window gate (WG) is the time interval during which any given signal is greater than the threshold (Figure 2), and the system samples the input signal to calculate the area of a pulse (shaded in blue). Any signals that do not cross the threshold are ignored and are not processed (shaded in orange). Once a signal exceeds the threshold, data is recorded until the signal falls to 75% of the initial threshold level.
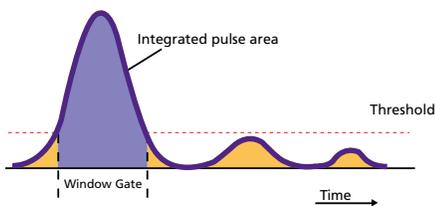

Figure 2. Window gate and pulse integration.

Window extension

As shown in Figure 2, the generated WG will not span the entire pulse, since the pulse actually begins before the WG opens (threshold) and ends after it closes. To ensure that the pulse is completely integrated, additional time is added before and after the WG (Figure 3).
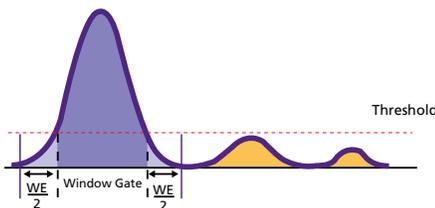
The time added to each end of the WG is called the window extension (WE). Half of the WE time (WE/2) is added before the WG and half afterward (shaded in light blue). For example, on the BD FACSCanto II system, the WE defaults to a value of 7, with 3.5 µs added before the WG and 3.5 µs afterward. The total time span for integrating pulse area is WG + WE (Figure 3) (shaded in blue and light blue). When the WE is set to 0, the pulse width of the processed signal is equivalent to the WG. If the WE is too short, pulses might not be fully integrated.
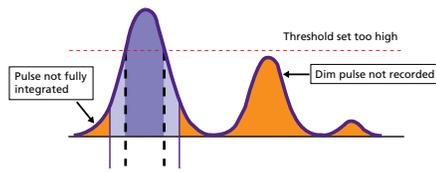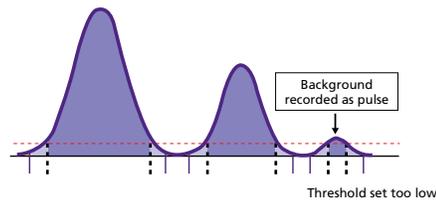

Figure 3. Visual representation of window extension.

Figure 4. Threshold set too high.

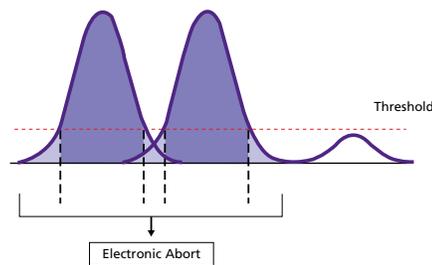**Figure 5.** Threshold set too low.

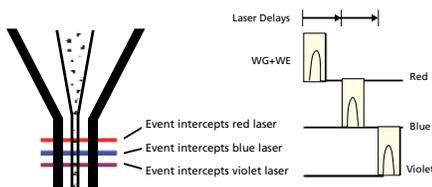**Figure 6.** Pulse overlap.

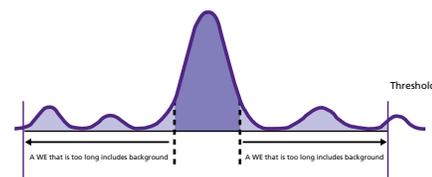

Figure 7. Laser delay.

Figure 8. A long WE increases background.

Threshold

There is a very clear interdependent relationship between threshold and WE. Once the WE for a given application has been fixed, the operator should take care not to adjust threshold values to a level that might result in the WE being inappropriate. Generally, an operator adjusts the software-defined threshold value up to exclude "debris" or unwanted data, or down to include smaller particles of interest in a scatter detector immediately above the "noise." Operators often use a FSC vs SSC plot to do this. However, the threshold can be adjusted using any detector or combination of detectors simultaneously.

If the threshold is set too high, any relevant particles generating dim signals might be inadvertently excluded and/or the preset WE might not encompass the pulse width, resulting in incomplete integration of the pulse (shaded in orange) (Figure 4).

If the threshold is set too low, the system is burdened by an excessive event rate from background (unwanted particles or noise) (Figure 5), which increases the possibility of electronic aborts (see the following section).

Electronic aborts

Under recommended operating conditions, all pulses are characterized regardless of how closely they follow a previous pulse. The rate of electronic aborts should be close to zero. If an operator increases the WE or decreases the threshold, the possibility of pulse overlap from different events also increases. If pulses overlap, the signals are not processed and are aborted (electronic abort). A visual representation of this is shown in Figure 6. Processed events are those that cross the threshold and do not overlap with another pulse.

Laser delay

To process signals from a single event (particle) passing through two or more lasers, data from the earlier laser is delayed with respect to the last laser once both (or all) events are in the data queue. Laser delays are established in CS&T based on the average arrival times of test particles at the interrogation point and are measured relative to the laser delay reference point. This is visually represented in Figure 7.

WE can also be set manually, and this is commonly undertaken on the BD™ LSR and BD FACSAria™ families of instruments. Operators should refer to their instrument user's guides for detailed instructions on how to set up laser delay manually on their particular instrument(s). Care should be taken to ensure that the WE is set correctly. If the WE is set to be too long, it can hide the effects of inaccurate laser delays. If the WE is set too short, an increase in signal coefficient of variation (CV) might occur due to variance in particle arrival times (at the interrogation point) that might cause incomplete pulse integration.

Background

Optical background contributions to signals can originate from a variety of sources such as unbound fluorochromes, Raman scatter, and ambient light. A consequence of an excessively long WE is an increase in signal CV, because the system integrates background noise before and after the pulse, as shown in Figure 8. An increase in background noise might result in decreased resolution of dim signals.

Summary

In summary, an inappropriate WE might result in unwanted effects on signal processing and data generation. A summary of these potential consequences is shown in Table 1.

Table 1. Potential consequences of inappropriate WE values.

Potential consequences if WE is set too short	Potential consequences if WE is set too long
• Incomplete integration of any given pulse, especially if the threshold is set too high (Figure 4) • Variance in particle arrival times might cause incomplete pulse integration	• Decreased resolution of dim signals due to unnecessary integration of optical background and electronic noise (Figure 8) • High background resulting in inaccurate (lower) signal-to-noise ratio • Increase in the incidence of electronic aborts due to overlapping WEs (Figure 6) • Hidden effects from inaccurate laser delays

BD FACSCanto Clinical Software

BD FACSCanto clinical software provides limited flexibility in cytometer setup and quality control. BD FACS 7-color setup beads are for in vitro diagnostic (IVD) use on the BD FACSCanto or BD FACSCanto II systems and are run with BD FACSCanto clinical software to provide application-specific setup, including automated compensation and cytometer QC. This system automatically sets the WE to a value of 7, and cannot be changed by the operator.

For certain BD Multitest reagents are designed for use on the BD FACSCanto and BD FACSCanto II flow cytometers. BD FACS 7-color setup beads and BD FACSCanto clinical software should be run to set PMT voltages, perform compensation, and check instrument sensitivity before use.

Implications for user-defined assays

For certain BD products, use of a WE other then 7 on a BD FACSCanto or BD FACSCanto II cytometer with BD FACSDiva software might alter the results. If operators choose to perform these tests using a different WE (or other setting), the assay must be fully validated by the operator prior to use.

BD FACSDiva Software and CS&T

Cytometer setup and tracking (CS&T)

CS&T is a fully automated software and beads system unique to BD digital flow cytometers that provides instrument characterization (optical and electronic), tracking, and quality control. CS&T is a module within BD FACSDiva software (v6.0 and later). After baseline determination has been performed, daily performance checks, stored and plotted on Levey-Jennings charts, allow the user to track cytometer performance and spot any trends in instrument performance.

Baseline determination

CS&T software automatically defines the instrument baseline by executing steps to set correct laser delays, and fully characterizes the instrument with respect to detector linearity, detector efficiency for specific fluorochromes (Qr), optical backgrounds (Br), and electronic noise (SD_{EN}). The software automatically sets PMT voltages to optimally run CS&T beads for subsequent performance checks. Baseline determination is specific to the optical configuration of the cytometer.

Window extension

Upon performing baseline determination, CS&T software automatically sets the WE to an instrument-specific default value. For example, on the BD FACSCanto II system, the WE defaults to a value of 7 (3.5 µs). On the BD™ LSR II and BD LSRFortessa™ systems, the default WE is 10. If an alternative WE is required, the operator must set it prior to baseline determination. Details of how to change WE are given in the workflow section of this document.

The appropriate WE value for a given application is specific to that application (for example, particle size and dim populations). Operators should optimize and validate any change in WE to ensure that it is appropriate for a given application. The Application Settings feature in BD FACSDiva software is useful in facilitating this process.

Workflows

Accessing WE settings

In BD FACSDiva software, the administrator controls access to WE settings. WE is displayed in the **Cytometer** window under the **Laser** tab for a particular tube within an experiment. See the *BD FACSDiva Software Reference Manual* for additional information.

Cytometer status report

The cytometer status report provides a list of all cytometer settings (including WE) at the time the report was created. The software must be connected to the cytometer to create the report. In an open experiment, set the current tube pointer, then select **Cytometer > Cytometer Status Report**. The report is displayed in a separate window with a menu bar above the header. The **Cytometer Info** section lists values for laser delay, area scaling, WE, and FSC scaling. See the *BD FACSDiva Software Reference Manual* for additional information.

Setting WE values in CS&T

Upon performing baseline determination using a newly created configuration, CS&T software automatically sets the WE to an instrument-specific default value. For example, on the BD FACSCanto II cytometer, the WE defaults to a value of 7 (3.5 µs). If an alternative WE is required, the operator must set it *prior* to baseline determination as follows:

1. In CS&T, set up a new configuration that matches the physical configuration of your cytometer. See the *BD Cytometer Setup and Tracking Application Guide* for additional details.

2. Change the window extension value in the display at the bottom left of the window as required.

Tip: Including the WE value in the configuration name might prevent confusion when choosing configurations.
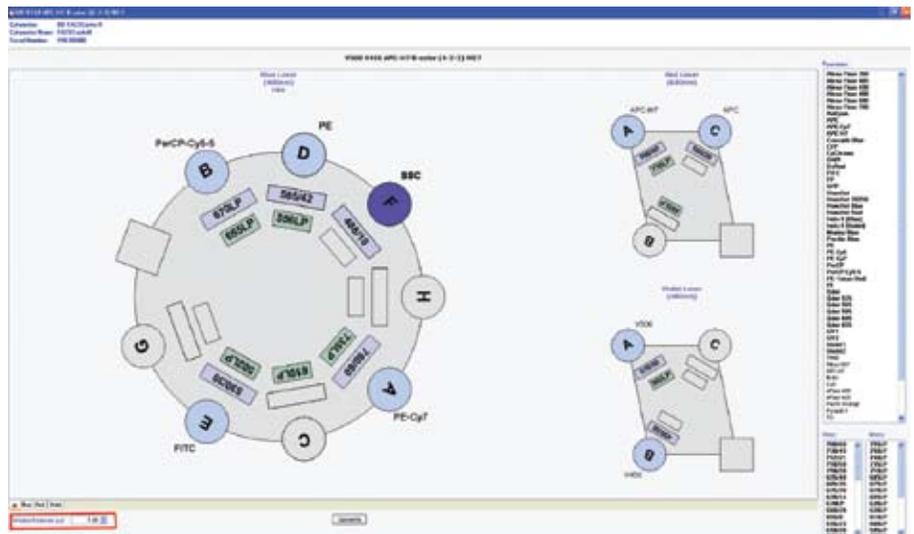
3. Close the configuration window.

4. Click **Set Configuration**.

   The Current Configuration name changes at the top of the **Cytometer Configuration** window.

   You must click **Set Configuration** for the new configuration to apply. For accurate data results, always verify that the cytometer optics match the current cytometer configuration.

5. Click **OK** to finish.

6. Define the baseline as outlined in the *BD Cytometer Setup and Tracking Application Guide.*

CS&T performs instrument setup using the configuration-specific WE setting. This WE setting is applied whenever the operator selects that configuration.

Application Settings

The Application Settings feature in BD FACSDiva software provides an easy, reproducible way to deliver consistent, performance-based cytometer setup for commonly used applications. Application settings are associated with a cytometer configuration and include all parameters, including WE, needed for the application. Note that application settings do not include compensation values. Each time you run a performance check for a configuration, application settings associated with that configuration are updated. Before you can create application settings, you must first run a performance check using the cytometer configuration that will be used for the application. The performance check optimizes the cytometer for the detection of the CS&T beads, and the additional steps for optimization in Application Settings fine tune the setup so that the settings are appropriate for a particular application.

Note: Each time you define a new baseline, verify that the application settings are still appropriate. Adjustment might be necessary.

See the *BD Cytometer Setup and Tracking Application Guide* for details on how to create application settings.

Technical Support and Customer Training

- Our experts are here to help you with questions you may have regarding any aspect of multicolor flow cytometry. Please contact your BD representative in your geographic location. In the US, reach experienced support staff at 877.232.8995 or by email at **answers@bd.com**.

- BD training courses offer the highest quality education in cell analysis using state-of-the-art flow cytometry equipment and methodologies. Find out more about our training courses, eLearning courses, and training tools at **bdbiosciences.com/support/training**.

References

1. *An Introduction to Compensation for Multicolor Assays on Digital Flow Cytometers.* BD Biosciences Technical Bulletin. Part number 23-10905-00

2. *BD Cytometer Setup and Tracking Application Guide.* Part number 642342 Rev. A

3. *BD FACSDiva Software Reference Manual.* Part number 642218 Rev. A

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