CytoFLEX Research Cytometer Event Rate Settings

TECHNICAL INFORMATION BULLETIN

Digital flow cytometers use optical sensors to collect light signals and convert them into electronic signals to represent the light events occurring in the flow cell of the instrument. The conversion of the light signal into an electronic signal allows for the digital quantification and measurement of the signal event.

The Voltage Pulse

The digital information recorded by a flow cytometer indicates the total light signal collected across the event as a particle moves into and across a laser's path.

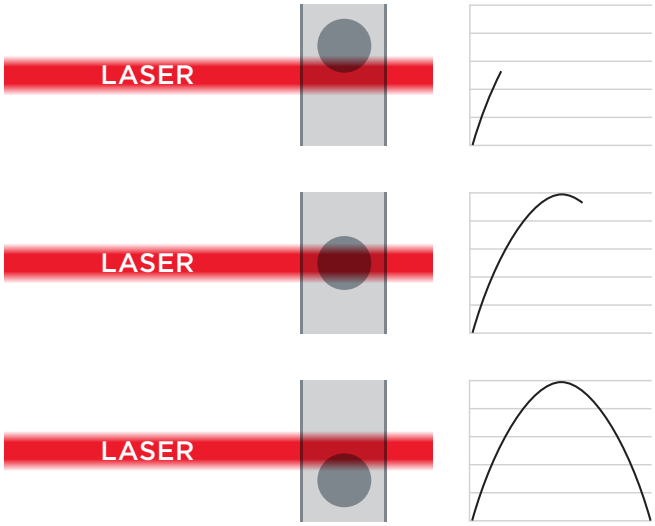

Figure 1

The entire pulse has three characteristics:

Pulse Intensity: The strength of the signal as it passes through the data collection point

Pulse Width: The period of time that it takes for a particle to pass through a data collection/interrogation point.

Pulse Area: The integration of the area under the curve formed from the pulse width and intensity as the particle moves through the collection point.

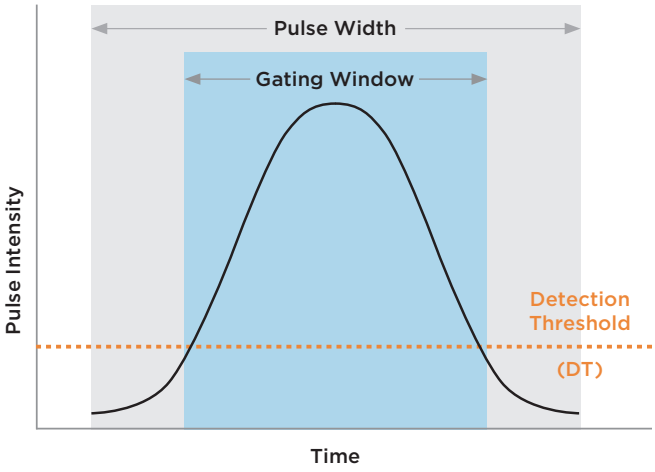

Figure 2

The Pulse Parameters: The Gating Window

In most digital flow cytometers electronic signals are continuously being collected even if no particle is being passed through the collection point. To distinguish background from real signals, an electronic cutoff called the detection threshold is established for each experiment and this determines the signal intensity where the electronic system will begin to record and measure a voltage pulse (Figure 2, orange dotted line, labeled Detection Threshold (DT). The length of time that the machine records the signals above threshold is referred to as the gating window (GW). (Figure 2, and shaded light blue). The electronics within the machine are thus programmed to process any signals that are greater than the DT and occur

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within the timing of the GW, producing an integrated area or signal (Figure 3, shaded blue). Any signals that do not cross the detection threshold or occur within the window gate are not recorded.

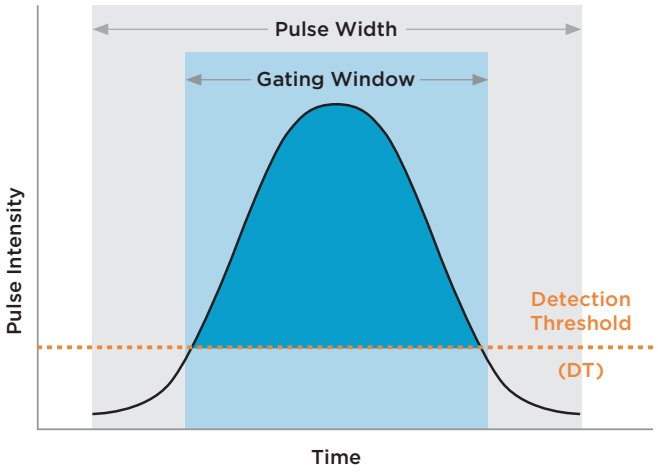


Figure 3

Event Rate Setting

As a consequence of the detection threshold, the entire electronic pulse created by a particle moving across an interrogation point is not always captured. This is because the electronic pulse actually starts before and finishes after the gating window closes. To ensure that the entire signal of an event is captured and integrated, additional time is added to both sides of the gating window. This additional time for the CytoFLEX instrument is termed the **Event Rate** setting (**ER**). (Figure 4, ER, shaded red regions). Thus the signal for an entire pulse is the sum of the GW and ER areas (Figure 4, red + blue shaded regions) and together the values constitute what is often termed the detection window or pulse width of a flow cytometer event (Figure 4).

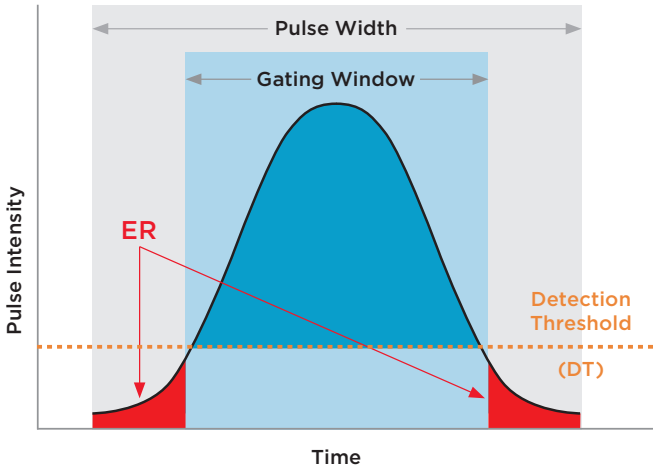


Figure 4

Importance of Event Rate Settings

By adding additional time and capturing the entire pulse, the additional data is often useful for obtaining the greatest fluorescence measurement and scatter measurements between samples, thereby facilitating the highest level of signal discrimination between samples. The proper event rate setting is important for resolution of dim versus negative cells and for resolving DNA peaks in cell cycle studies. Ultimately the highest sample fluorescence measurement and scatter measurement is desired with the lowest noise for all pulses, however, many factors affect the ER value settings and ultimately the resolution of the signal data generated.

Factors Influencing Event Rate Setting Selection

Timing

The event rate setting is an adjustable factor that influences the amount of time during which a pulse from a sample is collected in addition to the gating window (GW) time adjustment. There is a risk that a loss of useful sample information will occur when the event rate setting is too small. In contrast, increasing the event rate setting extends the detection time to allow for a more complete sampling of the signal pulse. However, if the event rate setting is too large, the area of integration stretches and the instrument collects information that really should be considered "noise", leading to a high coefficient of variation (CV) between samples. The overly large event rate setting can lead to a condition called "event overlap" where information from the next event is collected with the current event (Figure 5b). In those cases the cytometer's software automatically determines what is termed an "abort" and eliminates the offending data from the sample population. If the abort rate becomes too high, the value of the data obtained from the population will be adversely affected.

CytoFLEX signal processing uses a proprietary algorithm. Dr. Yong Chen, founder of Xitogen and Chief Technology Officer of Beckman Coulter Life Sciences, describes the Event Rate setting feature found in the CytoFLEX this way, "Flow cytometers are multi-parameter instruments. The operator is required to select one of the parameters, typically the main threshold parameter (DT, figure 2). Based on that, the CytoFLEX digital electronics searches in all parameter space for "peak intensities" or maximum voltages within the specified or system suggested event rate settings and delays. The narrower the event rate setting, the faster the search, therefore the higher system throughput. But narrower ER settings may risk mischaracterization of "peak intensity". For example, due to the inherent statistical fluctuation of particle velocity in a flow system, the true maximum may fall outside of the event rate setting. A wider event rate setting clearly reduces the risk. However, it would increase "abort rate", due to the fact that the digital electronics will be confused if two pulses happen

to fall into the event rate setting of a given parameter". Thus timing of the ER settings is critical for proper and complete pulse width processing.

Threshold Values & Background

Because the threshold value (DT) determines the signal strength at which the software will begin to integrate the sample information there is considerable interplay between the two factors. If the threshold value is set too low, the data collected will be burdened with excessive events and noise which will increase the overall sample CV and lead to excessive electronic aborts because signals cannot be distinguished from one another (Figure 5b). If the threshold is set too high however, then relevant samples expressing weak signals will be ignored and more importantly strong signals will not be completely integrated leading to incorrect data (Figure 5a). Finally, because ER and threshold values essentially govern the extent of sample data collected surrounding the window gate, they have an undeniable interrelationship with one another for a particular experiment, and thus once set they should not be changed during an experiment or data integrity could be compromised.

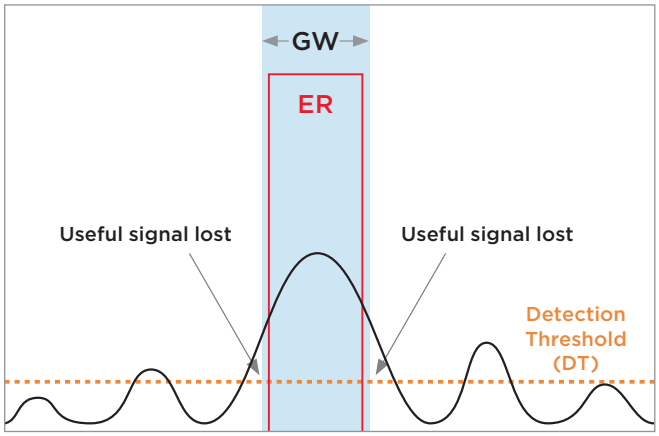


Figure 5a Event Rate (ER) set too short

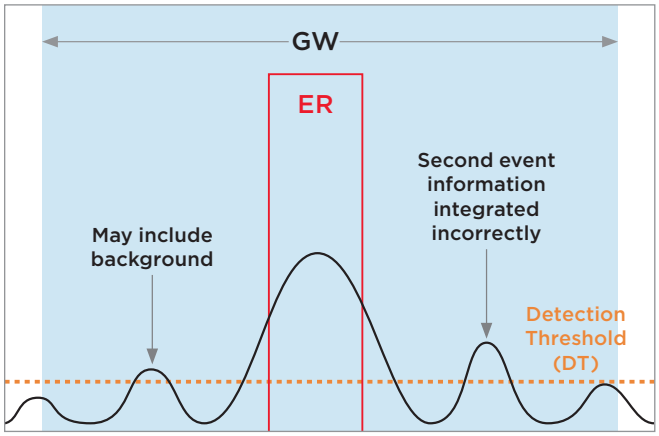


Figure 5b Event Rate (ER) set too long

Laser Delay

In flow cytometers with multiple lasers, by necessity, data from preceding lasers (the first lasers contacting the stream) will be delayed with respect to the last laser once all the data is collected. The actual laser delays settings are typically instrument specific and do not need to be changed once optimized. However, because the event rate setting influences the amount of time data is collected surrounding an event, incorrectly set laser delay values can affect the data obtained surrounding these values.

Incorrectly configured laser delay settings can affect the detection threshold determination such that small events may not appear above the threshold and therefore not recorded. In addition, an ER value that is too short will have the effect of appearing to increase sample CV because it will be "cutting off" too early before a particle has completely passed a specific laser leading to incomplete integration of the pulse. Conversely, an ER value that is too long may assemble data from one or more lasers and thus again produce inaccurate data, and "hiding" incorrect laser delay timings.

Sample Concentration

Because the event rate setting influences the length of time that sample data is collected, it is easy to understand how the concentration of a sample in particular experiment can have an impact upon the event rate setting used to obtain good, reliable, and consistent data with tight CV's and reproducibility. Samples run at a fixed flow rate that are low in concentration will have less of an opportunity to have overlapping signals than samples run at high concentration simply because there is less chance in the detectable events arriving at the detector at the same time (Figure 6, Low Concentration). As such, each detection event will occur separately even with a relatively high (long) ER setting value. If the sample concentration is too high, then even at a low fixed flow rate there is an increased chance of detectable events arriving at the same time and having overlapping pulses. Such overlapping pulses can lead to electronic aborts which results in data loss and incomplete data on the population as a whole, (Figure 6, High Concentration).

Detection Rate (EPS)

The event rate setting also impacts, or is impacted by the detection rate or events per second parameter in a similar fashion to sample concentration primarily because the two are closely related. Typically, the detection rate, or the number of cells detected per second (EPS), is determined by the concentration of cells in a sample, the flow rate, and the capabilities of the machine's electronics to process and manipulate the data stream. Because the ER setting is part of the parameters that set the length of time that a signal is collected and integrated, changes in the number of events per

second can have a great impact upon what ER setting is used, and conversely what ER setting time used, will impact the amount and fidelity of the data collected. In general, the higher the detection rate the shorter the ER settings times should be to maximize the data integrity. This is because too long of an ER setting value will lead to an increase in the frequency of electronic overlaps between pulses (Figure 6), which will lead to aborts. A high abort rate impacts not only amount of data but the quality of the data obtained as often times rare events in particular could be removed as well as otherwise good more common events discarded. Shortening the ER setting value as the detection rate is increased helps to compensate for the necessary "bunching" of the signals and helps ease the occurrence of electronic overlaps that will naturally occur as a consequence of the time between discrete events shortens.

Event Rate Settings Value Summary

As discussed in the proceeding paragraphs, the event rate setting (ER) values, because they impact the amount of time that data is collected and integrated from flow events and can be impacted or make impacts on a wide range of parameters used in everyday flow cytometry, are important parameters to set correctly to maximize not only data signal but data quality from a flow experiment. A summary of the principal consequences surround ER settings values is given in the Table 1.

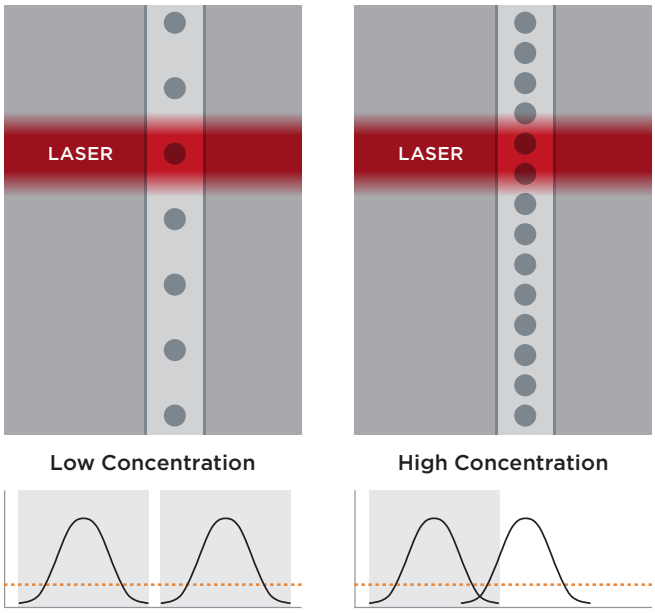
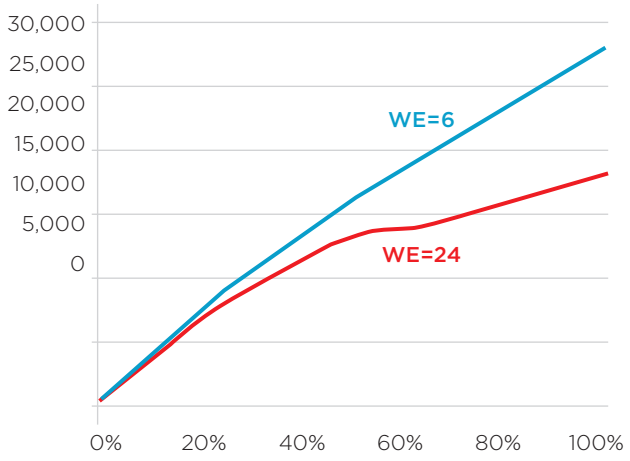


Figure 6 High Concentration

Table 1

ER SETTING TOO SHORT	ER SETTING JUST RIGHT	ER SETTING TOO LONG
Incomplete integration of a pulse	**Maximal data integration**	Decreased resolution: dim signals are masked by high noise/background events
Lower than necessary signals	**Tight sample CVs**	Backgrounds grow higher, making lower (Poorer) signal to noise ratios
Compounds high threshold problems	**Low background noise**	Potentially false MFI readings because longer than necessary data is integrated
Increased sample CV caused by early cut because of conflict with built in laser delays	**High MFI readings**	Increased incidence of electronic aborts
Lower MFI readings	**Stable readings**	Poor data quality and integrity because of increased abort rate
Lower quality data	**High Quality data**	Hides other incorrect settings such as laser delays because ER masks the data

		Bead Concentration							
ER setting value		**1.56%**	**3.13%**	**6.25%**	**12.50%**	**25.00%**	**50.00%**	**66.67%**	**100.00%**
>10,000 events/sec	Detection Rate	642	1058	2233	4436	8982	16006	20091	28022
	Abort Rate (%)	**0.06**	**0.11**	**0.22**	**0.43**	**0.94**	**1.89**	**2.72**	**5.68**
DEFAULT	Detection Rate	636	1082	2074	4141	7990	13216	14298	18228
	Abort Rate (%)	**0.39**	**0.91**	**1.51**	**3.17**	**6.66**	**12.83**	**16.71**	**24.01**



The CytoFLEX Flow Cytometry System

The CytoFLEX flow cytometer system is targeted for research users wanting a high quality instrument that has multiple laser capability combined with a level of robustness and ease of use that is unprecedented in the industry at large. Moreover the CytoFLEX is designed to provide researchers with the unmatched usability in the industry at its price point. CytoFLEX is designed to handle both high and low sample volumes and has an extremely broad detection rate (EPS) scale. CytoFLEX can operate from as low as 100 events per second (EPS) to up to 30,000 EPS with simple software and user controls.

With such a large dynamic range one might expect that optimizing the event rate setting would play a critical role in proper data measurement with the CytoFLEX, and this is indeed the case; however the process of setting ER values is made exquisitely simple because of the CytoFLEX's exclusive CytExpert software. The CytExpert software uses specialized integration algorithms for setting ER values thus allowing the CytoFLEX to properly measurement events over a large range of sample concentration and flow rate speeds.

By default, the CytoFLEX ER setting value is optimized for low event rates in the range of 100 events per second to 5000 events per second, and is a reasonably broad time amount that is added simultaneously to both ends of the gating window allowing for complete data integration. However the CytoFLEX also has an advanced special user feature that allows users to optimize event rate settings for rates greater than 10,000 events per second and still maintain robust and quality data set.

The table and graph above demonstrate how event rate (ER) influences the abort rate as the detection rate (EPS) increases. The table shows how, when properly set, the CytExpert's software's ability to properly maintain an acceptable level of low abort rates even when events exceed > 20,000 EPS. The default setting has been optimized for event rates between 100 to 5000 EPS. (Red line in graph, red boxes in table). From

Table 3

	HIGH ER SETTING	LOW ER SETTING
Advantge	1. Integrated pulse area 2. Stable; more room is given for laser delay change 3. Threshold setting change does not affect the area signals too much, unless the setting is too high	1. Low abort rate to achieve higher data acquisition rate 2. Less noises/background are integrated
Disadvantage	1. Increased abort rate 2. More noises/background are integrated	1. Incomplete pulse area integration, especially when the threshold is set high 2. Laser delay change and threshold change effect can be more obvious
Suggested Conditions	1. Normal conditions 2. Data acquisition rate < 10,000 events/second	1. High concentration samples 2. Small particles 3. Very dim signal detection

the analysis one can see that as the detection rate increases with the default event rate setting, the abort rate increases and data integrity compromises. However, by adjusting the event rate value to a shorter value, the new event rate setting allows drastically reduced sample abort rate to an acceptable level of < 3% even at >20,000 EPS. Thus this result demonstrates that with the CytoFLEX users can run at high EPS without compromising event measurement and abort rate.

From similar experiments and extensive testing a table of suggested recommendations for ER value use on the CytoFLEX machine has been compiled in Table 3.

Moreover the CytoFLEX cytometer is not as greatly affected by ER parameters because the CytExpert software integrates signals based on the position of the primary peak and then dynamically searches all the data in the ER around the peak position (after appropriate laser delay adjustment) for peaks in other channels. Integration width in the CytoFLEX, unlike other cytometers, is thus entirely determined by the trigger pulse shape, and therefore independent of "event rate value," thus less sensitive to it. This is why the CytoFLEX can operate over a variety of sample parameters without a great need to continuously adjust event rate setting parameters.

Because CytoFLEX uses a dynamic auto-search algorithm for peak finding and area integration, the system performance is less sensitive to ER settings; therefore there is no need to continuously adjust the ER parameter. The majority of applications will use either of the two primary default settings. It is hoped that this brief discussion surrounding the parameters that influence the event rate settings and how the CytoFLEX innovative solutions simply their impact will help both novice and experienced users appreciate the advantages of the CytoFLEX flow cytometry system and its benefits for their flow cytometry research needs.

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