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Time Interval Gating for Analysis of Cell Function Using Flow Cytometry[1]

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We propose a method which significantly shortens the time required for both the collection and analysis of data derived from multiple sample, flow cytometric kinetic assays. We have defined the term Time Interval Gating (TIG) to describe this method. TIG effectively allows one flow cytometer to concurrently monitor several samples over the course of a kinetic assay. Data for all samples are stored in a single FCS 2.0 compatible listmode data file which we refer to as the TIG data file. TIG is adaptable to most commerical flow cytometers. Standard listmode analysis software can be used to analyze the TIG data files and correlate any combination of tubes and/or time intervals from the assay. Results for the entire assay can be displayed on a single two parameter plot. This paper describes how TIG is applied to neutrophil oxidative burst measurement using a standard EPICS Elite flow cytometer. In this assay, 11 samples were each monitored for 30 min to identify the extent to which volatile organic chemicals (VOCs) inhibited the oxidation of DCFH in stimulated neutrophils. TIG makes the oxidative burst assay practical for high volume screening by reducing the overall flow cytometer and analysis time required by a factor of ten. In addition, TIG provides an organized approach to managing data acquisition on instruments equipped with automated sampling systems.

Key terms: Kinetics, oxidative burst, time, neutrophil function

INTRODUCTION

Physiological response mechanisms in neutrophils have been examined through the use of kinetic flow cytometric analyses (1–4,6,7,9,12–14). It has been shown that both the baseline and final activation state of neutrophils can be characterized by assaying the ability of the cells to carry out oxidative burst (1,5). By measuring the activation of neutrophils over a time period, information related to the rate of response, as well as maximum responsiveness, can be obtained. Thus, several effects of cell activation can be determined simultaneously.

We have developed a flow cytometric assay to investigate the degree to which certain volatile organic chemicals (VOCs) or other environmental agents alter the responsiveness of human neutrophils. This was achieved by evaluating the oxidative burst and therefore the ability of the neutrophils to respond to invading pathogens. To accomplish this, various concentrations of VOCs were added to suspensions of neutrophils loaded with dichlorofluorescin diacetate (DCFH-DA) according to the method of Bass et al (1). The neutrophils in each sample were then stimulated using phorbol myristate acetate (PMA) and the rate that nonfluorescent dichlorofluorescin (DCFH) was converted to fluorescent dichlorofluorescein (DCF) was monitored by flow cytometry for 30 min.

Our initial application of the above assay procedure, while technically successful, was cumbersome. Unfortunately, testing each inhibiting agent required so much flow cytometry and data analysis time, that the method was impractical for screening a large number

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of agents. For example, a typical 11 sample oxidative burst assay consists of 11 samples each with incremental concentrations of the test reagent or control. Since the reaction is time dependent, every sample must be monitored individually for 30 min. Sequential measurement of all samples would require 330 min of flow cytometer time, and the subsequent analysis of 11 separate data files. Even if samples were only monitored for 15 min, the time taken for the assays would be unacceptable in most laboratories. Because of the length of the assay, it is also very difficult to ensure that control conditions such as instrument calibration and sample temperature are held constant. Further, a limited "viability window" is available for cells such as neutrophils outside which normal function is altered.

To address these problems, and to make the oxidative burst assay more suitable for high volume screening, we have developed the Time Interval Gating (TIG) method for flow cytometric kinetic assays. The TIG method is an extension of the Tube Identifier Parameter (TIP) system which we have previously described (10). It effectively allows concurrent measurement on the flow cytometer of the 11 samples described in the above assay. Thus, the complete assay can be run in 30 min rather than 330 min. In addition, all data from the sample set are stored in a single FCS 2.0 compatible TIG data file. Standard listmode data analysis software can then be used to make direct comparisons between any tubes in the set through simple gating on time and/or the TIP.

To demonstrate the application of TIG an 11 tube oxidative burst assay was prepared as described in the following section.

MATERIALS AND METHODS

Flow cytometric data were collected on an EPICS Elite flow cytometer (Coulter Cytometry, Hialeah, FL) using instrument standard computer, optics and electronics. All studies were carried out using an air cooled 15 mW argon laser (Cyonics Model 2201, San Jose, CA) operating at a wavelength of 488 nm. Optical filters were placed in the fluorescence collection path in the following order starting from the flowcell and ending at the photomultiplier tube: 488 dichroic; 488 laser blocking; 550 dichroic; 525 bandpass. Correlated fluorescence, 90° light scatter, forward light scatter and elapsed time measurements were made. A standard EPICS Elite workstation computer (Intel 80386 PCAT BUS) was used for all list data analysis. The TIG data files were analyzed using the standard EPICS Elite workstation software. All statistical analysis was done using RS-1 (BBN Software Products; Cambridge, MA).

Tube Identifier Hardware

The only modification of the instrument was the addition of a TIP parameter device which has been described in more detail previously (10). Briefly, this device produced a series of programmable DC voltage levels. The output of the device was connected to the input of one of the ADC channels on the instrument. The DC voltage level was successively incremented for each tube in the sample set, thus providing a "unique ADC channel ID" for each tube. This channel ID was saved as an additional parameter with the data collected for each cell event. The parameter created by this reference voltage was referred to as the tube identifier parameter (TIP).

Reagents and Buffers

Phosphate buffered saline (PBS) contained 0.147 M NaCl, 4.1 nM $Na_2HPO_4.7H_2O$, 2.3 mM NaH_2PO_4, 15 mM $NaNO_3$, pH 7.4. PBS-gel is PBS which also contains 2 mM EDTA, 10 mM glucose and 1% gelatin (Difco Laboratories, Detroit, MI) and was used for all cell incubations. Lysing solution consisted of 0.15 M NH_4Cl, 10 mM $NaHCO_3$, 10 mM ethylenediamine tetraacetic acid (EDTA) at pH 7.4.

Reagents

2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes, OR) was dissolved in absolute ethanol at a concentration of 20 mM. Phorbol myristate acetate (PMA) (Consolidated Midland, Brewster, NJ) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 1 mM (2mg/ml) and stored in 20 μl aliquots at −20°C. Toluene was made up in absolute ethanol at a concentration of 1 mM immediately before each assay.

Assays

In this assay H_2O_2 was measured using the fluorescence of dichlorofluorescein as described below. The assay consisted of 11 tubes as shown in Table 1. After activation of neutrophils by PMA at zero time, measurements were made on each of the 11 tubes every 3.5 min. The assay was designed to provide information relating the effects of chemicals such as toluene on neutrophil activation over 30 min.

Functional Assay—Oxidative Burst

Human peripheral blood was used for all assays. Preparation of cells for flow cytometry was achieved by lysing blood by adding 3 ml blood to 47 mls of NH_4Cl lysing solution, rocking on a benchtop rocker for 10 min followed by centrifuging at 200 × g for 7 min and resuspending in PBS. Cells were washed once more in PBS before being resuspended at 2×10^6 cells per ml.

Fluorescence Assay—Flow Cytometry

Cell populations (1×10^6/ml) were incubated with DCFH-DA (20 μM final concentration) for 15 min at 37°C according to the original method of Bass (1) with modifications previously described (9). These loaded cells were then placed into 12 × 75 mm polypropylene tubes. Twenty minutes prior to activation of the cells with PMA, a range of volatile organic chemicals were added to certain tubes to assess the effects on the oxidative burst of the neutrophils. Tubes remained in a

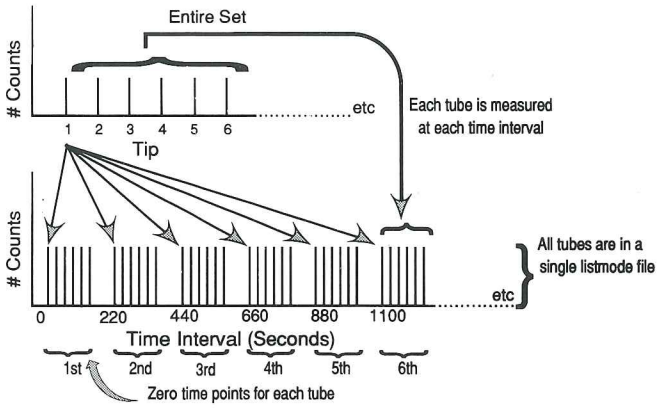

FIG. 1. Example histograms of both the TIP and TIME parameters, similar to those displayed on the instrument, are shown above for only the first 6 tubes in the set. All of the events recorded by the instrument for a particular tube in the set will have exactly the same TIP parameter value. Therefore, each spike in the TIP histogram is correlated with a particular tube in the set. Since this assay includes 11 separate tubes there are 11 spikes in a complete TIP histogram from this assay instead of the 6 spikes shown in the example above. The TIME histogram shows that data acquisition continued for 15 sec each time a tube was placed on the instrument. The example TIME histogram contains data for the first 6 tubes in the set. The six arrows from tube 1 in the TIP histogram show where tube 1 events appear in the TIME histogram. Tube 1, the first tube in the set, was run six separate times during the assay or every 220 sec. Since there are 11 tubes in this assay and each tube is run six times, a complete TIME histogram contains 66 separate 15 sec spikes. By gating on the TIP and TIME parameters, standard list data analysis software can be used to create fluorescence histograms for any tube, set of tubes and/or time period in the assay.

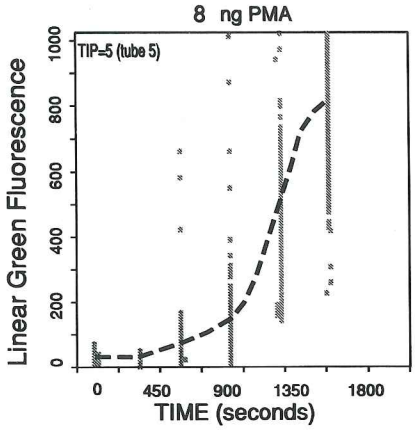

FIG. 2. The plot in Figure 2 shows the correlation of fluorescence intensity and time for neutrophils in the fifth tube from the set. The plot was generated with the standard EPICS Elite list data analysis software by placing a single gate on the fifth spike in the TIP histogram, and another gate around neutrophils on the scatter plot. The dotted line in the figure indicates the mean of the fluorescence distribution for each of the six time points. Plots such as this can be generated for up to six separate tubes simultaneously, presenting a quick summary of the experiment. One unique feature of this approach is that these plots can be viewed in real time as samples are run on the instrument. This gives the investigator the opportunity to interactively modify the experiment at the cytometer.

37°C water bath when not on the cytometer. Samples were gently vortexed prior to each window of flow cytometry measurement. Samples were activated immediately before being run on the cytometer for the first measurement; this insured that each sample's zero time measurement was a true zero for that tube.

Ungated list mode data were collected for the following parameters: forward angle light scatter (FALS); 90° light scatter (90LS); green fluorescence (GFL), TIP; and Elapsed Time (Time) at 1.8 sec per channel resolution.

RESULTS

Using TIG, flow cytometry was performed on the 11 tube assay described in Table 1. The manner in which the 11 samples were repeatedly measured on the flow cytometer is shown in Figure 1. A 15 sec "window" of data was collected from each sample and five seconds of "dead time" was left before the next sample was started. After data had been collected from each sample in turn, the first sample was started again. In this manner the entire sample set was repeatedly measured every 220 sec over the 30 min period. The instrument time parameter clock ran continuously throughout the experiment providing a real time precision of ± 0.9 sec for each cell measurement. With this configuration, each histogram channel represented an increment of 1.8 sec in real time. The lower histogram in Figure 1 is an example of the TIME histogram for only the first six tubes in the set. Each tube placed on the instrument generates a 15 sec spike of events. Since 11 tubes are used in this assay, and each tube is run 6 times, the actual TIME histogram contains 66 individual spikes. The TIP parameter was used to uniquely identify each tube in the set. The upper histogram in Figure 1 shows the distribution of the TIP parameter for tubes 1 through 6 in the set. The TIP parameter value for any events recorded by the instrument from the first tube appear as the first spike in the TIP histogram. The second spike contains all events from the second tube and so on. The TIP parameter device was set for the corresponding tube during the 5 sec dead time between samples.

Approximately 2,000 total events were measured during each acquisition window. Each sample was measured 6 times over the course of the assay accumulating a total of approximately 12,000 events per sample. When the assay was completed, the TIG data file contained a total of 132,000 events for all 11 samples.

DATA ANALYSIS

Listmode gates on TIME and TIP were used to discriminate either an individual sample or a set containing any combination of samples. For example, Figure 2 shows how a plot of fluorescence vs. time gated on TIP for sample 5 can be used to display all of the time points for only the 5th sample. Figure 3 shows how a

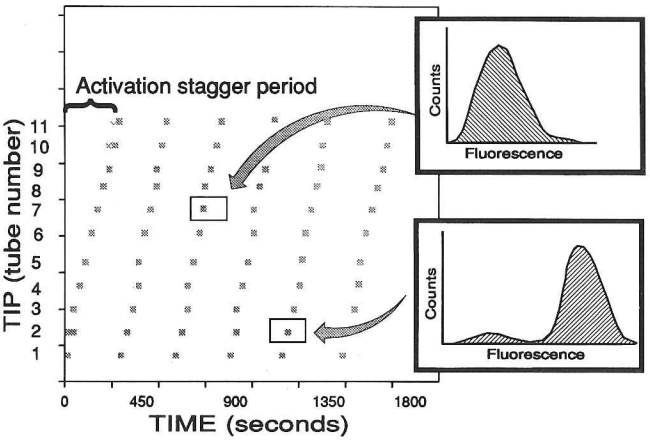

FIG. 3. Figure 3 shows a Time-View display which provides a unique view of all of the time points for each of the tubes in the assay. Each row has a specific TIP parameter value and represents a particular sample in the set. The 20 sec separation between placing each tube on the cytometer causes the stagger in the columns. The "activation stagger period" represents the time difference between the first time point for tube one and the first time points for the last tube (tube 11). Since samples are activated immediately before they are run the first time, this time does represent a true zero time point for each tube in the assay. The actual time that each of the 66 measurement periods occurred during the assay can easily be seen from this display. A fluorescence histogram for any single measurement period can be generated by placing a gating region on the Time-View display. The two single parameter fluorescence histograms shown can be generated simultaneously by gating on the regions shown. The upper histogram is for tube 7 at 440 sec after activation. The lower histogram is for tube 2 at 1,100 sec after activation.

matrix of TIPs vs Time can be created to allow easy selection of any discrete sample-time point(s). We have designated this plot as a Time-View display since all tubes run for the entire assay are shown in a time-ordered manner.

Data reduction was achieved by generating a series of dual parameter histograms of time vs fluorescence for each sample as in Figure 4. A program was written which computed the mean fluorescence value for each measurement window and stored the mean values in an RS-1 compatible data file. From this file the mean fluorescence for each sample was automatically plotted as a function of time. Once the light scatter gates were set correctly on the original TIG data file, generation of the output shown was accomplished through a series of automated batch files.

DISCUSSION

The primary benefits of TIG are reduction in sample analysis time, improved assay control, simplification and automation of data analysis. TIG reduces by a factor of 10 the time required to perform data acquisition and analysis for an 11 tube, oxidative burst, flow cytometry assay. Control of the assay is improved because all samples in the assay are exposed to similar environmental and sample handling conditions during a common 30 min period. Analysis of data is reduced to simple list mode analysis of a single FCS 2.0 compatible list data file. Mean fluorescence/time values for the entire set are transferred to RS-1. An RS-1 batch file produces both a graphic and tabular summary of the assay. It is also possible to place gating regions around each fluorescence histogram to rapidly generate mean channel numbers for fluorescence populations using traditional methods. User interaction is required only for light scatter gating during listmode analysis. The remaining oxidative burst data reduction process can be completely automated.

By choice, each sample was measured at 220 sec intervals; however if a greater degree of chronological precision per sample was required, fewer samples could be run, or sampling could be done more frequently. There are 6 discrete time points for each sample in the assay described. A fast, automated sample introduction system would be a useful addition to TIG, and could significantly increase sample throughput, by eliminating the need to manually load each sample on the instrument.

The study used to demonstrate this method was a typical neutrophil evaluation assay. In this case, several concentrations of the test chemicals were used to evaluate their effects on cell function. The advantages of using TIG are in the tightly controlled conditions of the procedure. We were interested in the rate of responses, and therefore time was required. We were also interested in determining maximum response of cells. The effect of toluene on neutrophil function, as shown in Figure 2, provided us with both the rate and the maximum responses of the neutrophils. Comparing this to the tube with no toluene provided an evaluation of the difference. Because of the way the assay was designed, we were able to compare the responses of any tube with any other by using the time-view display for gating as shown in Figure 3. Measurements were all real time, which required a well planned and organized method, which TIG provided.

The use of a real time parameter for kinetic monitoring has become more common in the past few years by investigators using flow cytometry. Examples of such use are calcium flux measurements (8), membrane potential (11), oxidative respiratory burst (9) and enzyme kinetics (15). The cytometer must be able to collect TIME as a real embedded parameter for this concept to be of use. In addition, coupled with an automated sample handling system, TIG provides a workable approach for using flow cytometry in high volume screening applications.

The ability to compare results across different tubes may provide additional information difficult to derive presently. Further, any particular set of results can be generated in a single report sheet without having to combine multiple histograms from different files. We have demonstrated this in immunophenotyping of leukocytes where application of the principles discussed in this paper can reduce the time for phenotypic analysis to minutes (10).

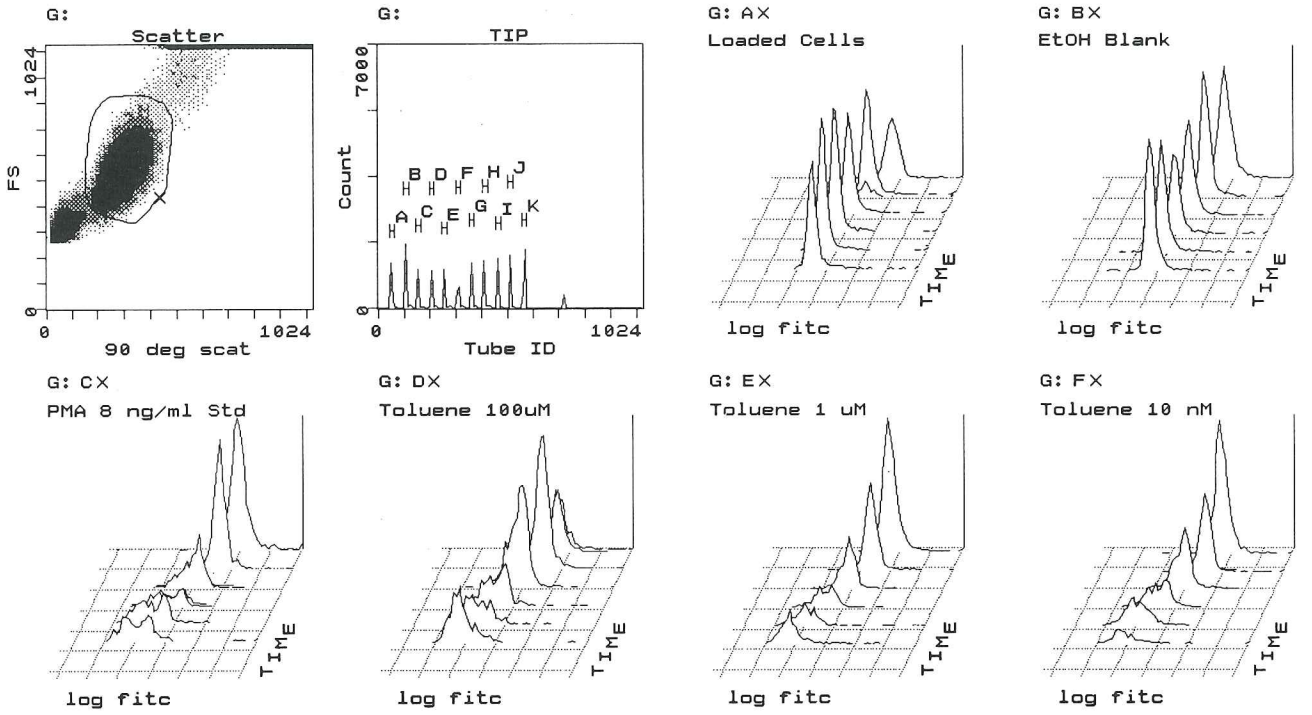

FIG. 4. Figure 4 shows an example of the 8 plot display from the EPICS Elite. The first plot shows the light scatter gating used for all tubes. The second plot is a histogram of the TIP parameter for all cells in the assay. The next series of six fluorescence versus time plots are for the first six tubes in the set. These six plots summarize the complete 30 min assay for the first six tubes in the set. They can be generated in real time during data acquisition, or with a single replay of the list data. The Loaded Cells plot is produced by gating on the region labelled X in the scatter plot and the region labelled A in the TIP histogram. In the same manner the ETOH Blank plot is produced by gating on the region X in the scatter plot and the region B in the TIP histogram. Several combinations of displays and histograms can be created for any sample(s) in the set.

An additional reason for development of the single file assay system was the very nature of many flow cytometric assays. With TIG, samples which arrive at the cytometer in logical batches can remain a batched set throughout data acquisition, analysis and report generation. Data from this experiment, which included 66 tubes, can remain grouped in a single listmode file instead of being split into 66 separate files by the cytometer. A major bonus during analysis is the ability to compare multiple tubes within the set contingent upon the capabilities of the software. Since the Elite software is capable of handling 24 gates and 8 single or dual parameter histograms simultaneously, analysis protocols can be designed to provide much of the necessary analysis. By batching these protocols, several sets of analyses can be performed rapidly.

The TIG methodology described, provides a solution to many of the sample handling difficulties encountered in fast moving kinetic assays. It is adaptable to most commercial flow cytometers, and provides a workable approach for organizing analyses of kinetic assays.

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