APPLICATION NOTE

Bacterial Disinfectant Efficacy Using Flow Cytometry

MICROBIAL CYTOMETRY BD FACSCalibur, BD FACSort

Introduction

Flow cytometry is a rapid method to quantitate live and dead bacteria in a sample.[1-4] It can be used to measure the efficacy of antimicrobial compounds by exposing organisms to the disinfectant and subsequently staining with permeant and impermeant DNA dyes. Test organism viability, measured using a 30-minute flow cytometry assay, showed comparable sensitivity with a standard 48-hour plate count assay. Flow cytometry provides a rapid and quantitative method to determine the efficacy of disinfectants.

Current methods, such as the hard-surface carrier method (AOAC 991.47-49) and use-dilution method (AOAC 955.14-15, 964.02),[5] rely on exposure of the test organism to a disinfectant, with growth generally observed after 48 hours. These methods require substantial labor and time and can be susceptible to inaccurate results. In contrast, a flow cytometry assay can be completed in less than one hour and can provide rapid quantitation of live, dead, and injured organisms. The assay can be readily adapted to a variety of analytes and test organisms, and the process can be automated.

Flow cytometry discriminates live cells from dead cells by simultaneously measuring light-scatter and fluorescence characteristics at rates of thousands of events per second. In this assay, all cells containing DNA are stained with the permeant dye, thiazole orange* (TO), and fluoresce yellow to orange. Cells with damaged membranes are also stained with the impermeant dye, propidium iodide (PI), and fluoresce orange to red.

The disinfectant SPOR-KLENZ™ (Steris Corporation, St. Louis, MO) was used as a model system in this study. The active ingredients in SPOR-KLENZ are hydrogen peroxide and peroxyacetic acid.

* US Patent Nos. 4,883,867 and 4,957,870

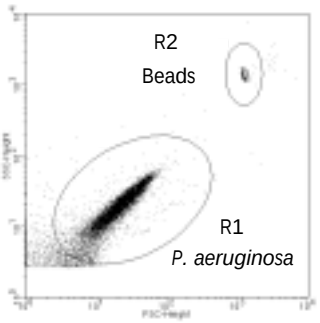


Figure 1A

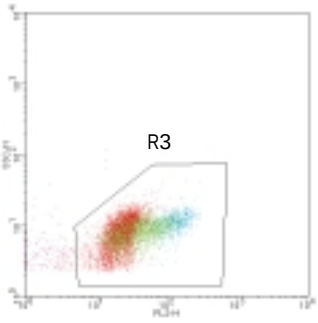


Figure 1B

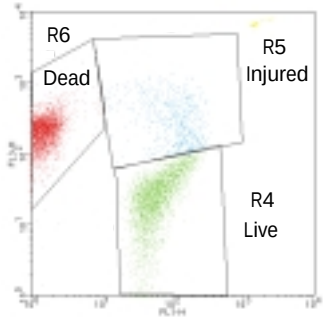


Figure 1C

Figure 1 Gating and analysis strategy for bacterial populations, using a sample of *P. aeruginosa* stained with TO and PI in a TruCOUNT Tube

A. FSC vs SSC dot plot with the bacterial population on-scale, with region R1 set liberally around the target population, region R2 set around the beads

B. FL2 vs SSC dot plot with region R3 set around the stained bacteria

C. FL1 vs FL3 dot plot gated on (R1 OR R2) AND R3, with regions set around the live, injured, and dead bacterial populations

Materials and Methods

Bacteria

Pseudomonas aeruginosa (ATCC Catalog No. 15442), *Salmonella choleraesuis* (ATCC Catalog No. 10708), *Staphylococcus aureus* (ATCC Catalog No. 6538) were obtained from ATCC and cultured using Trypticase™ Soy Agar (BD Diagnostic Systems, Sparks, MD, Catalog No. 221185) and Trypticase Soy Broth (BD Diagnostic Systems Catalog No. 221092). Prior to use, cultures were inoculated from a single colony.

Treatment and Staining

Bacteria were exposed for 10 minutes with rotation to SPOR-KLENZ (Steris Catalog No. 6525-01) that was diluted serially between 1:2 and 1:512 in sterile water. Treated bacteria were split into two groups. One group was plated on RODAC™ D/E Neutralizing Agar (BD Diagnostic Systems, Catalog No. 221232) to neutralize residual SPOR-KLENZ and checked for growth after 48 hours. Several dilutions were plated to assure that at least one culture had an acceptable number of colonies. The second group was prepared for flow cytometric analysis. An aliquot was diluted 10- to 20-fold in phosphate buffered saline containing 0.2% Pluronic™ F-68 (BASF Corporation, Mount Olive, NJ, Catalog No. 51554728) and 1 mM EDTA and incubated at 37°C for an additional 10 minutes. This additional incubation proved to be necessary for the efficient uptake of propidium iodide (data not shown), presumably because the membrane integrity of damaged cells had to degrade to readily admit the dye.

A 200-µL sample was added to a TruCOUNT™ Tube (BD Biosciences, San Jose, CA, Catalog No. 340567), containing a known quantity of fluorescent beads. The beads were required to determine the actual volume of sample analyzed. Thiazole orange and propidium iodide were added to final concentrations of 420 nM and 48 µM, respectively. Samples were vortexed and analyzed after a 5- to 10-minute incubation.

Dye solutions used: Thiazole orange solution, 17 µM (BD Biosciences Catalog No. 349483), or equivalent at 8.1 µg/mL (FW 476.6) in dimethyl sulfoxide (DMSO). Propidium iodide solution, 1.9 mM (BD Biosciences Catalog No. 349483), or equivalent at 1.3 mg/mL (FW 668.4) in water.

Flow Cytometer Setup

A BD FACSCalibur™ flow cytometer was optimized using CaliBRITE™ 3 beads (BD Biosciences Catalog No. 349502), to verify instrument performance.

PMT voltages and threshold levels were adjusted using an unstained sample of diluted bacteria. The bacterial population was positioned so that it was entirely on scale on an FSC vs SSC plot (Figure 1). For fluorescence measurements, FL1, FL2, and FL3 PMT voltages were adjusted to place the unstained population in the lower left quadrant of two parameter plots (data not shown).

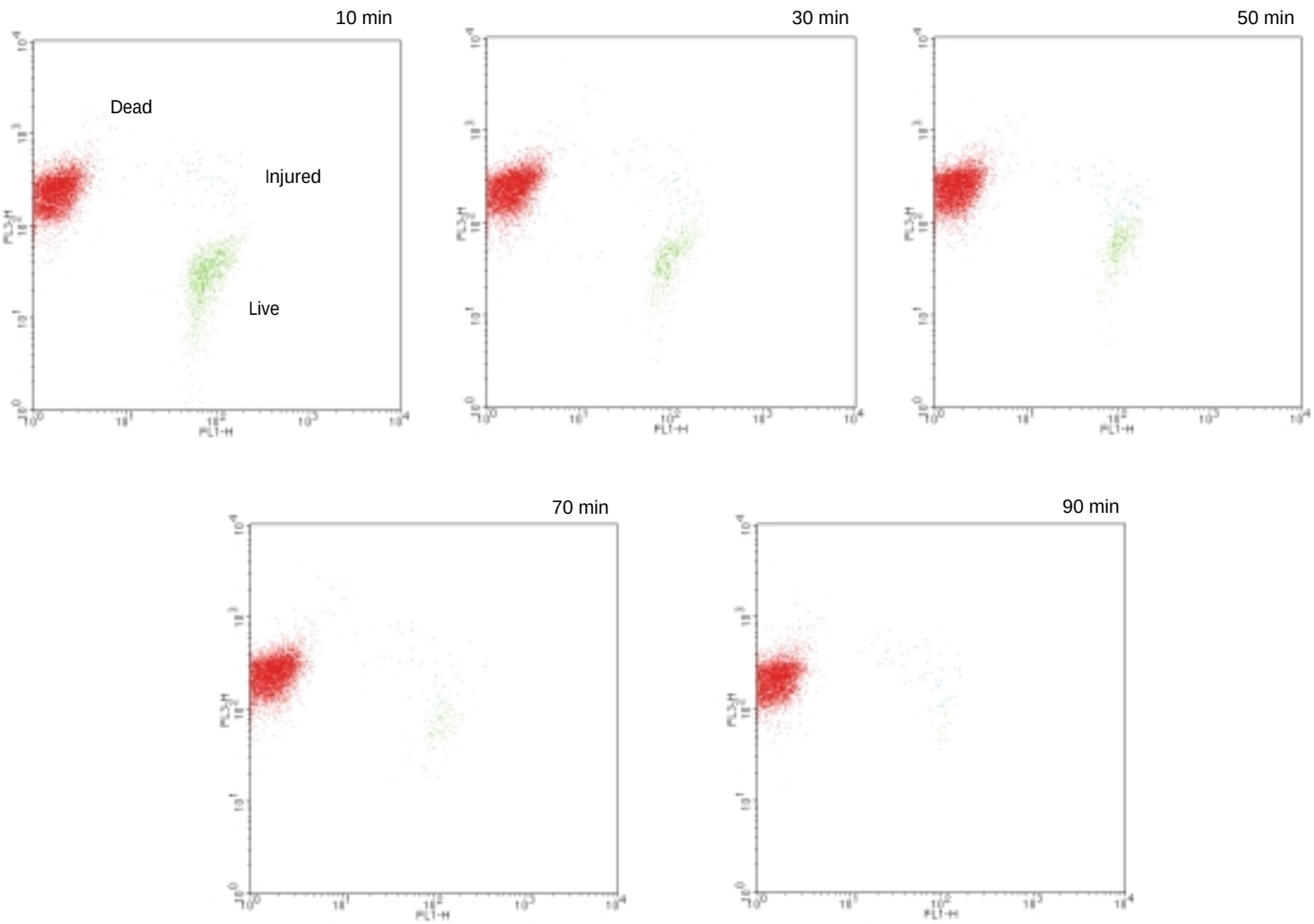
Instrument settings for the results below were as follows:

- Threshold—SSC
- FSC—E01, logarithmic amplification
- SSC—375 V, logarithmic amplification
- FL1—600 V, logarithmic amplification
- FL3— 800 V, logarithmic amplification
- Compensation—none used

Data Acquisition and Analysis

Data files were acquired and analyzed using BD CellQuest™ software. Ten thousand events were acquired per sample in Acquisition-to-Analysis mode. The bacterial population was gated using a combination of FSC, SSC, and FL2 (Figure 1, R1 and R3). Dead, live and injured populations were discriminated using FL1 vs FL3.

Figure 2 Change in live and dead populations of *P. aeruginosa* with 10- to 90- minute exposure to 3.1% SPOR-KLENZ



Results and Discussion

Figure 2 shows the change in viability of *P. aeruginosa* populations over 90 minutes of exposure to SPOR-KLENZ. Figures 3 and 4, respectively, compare the effect of SPOR-KLENZ on *S. aureus* and *P. aeruginosa* by flow cytometry and plate counting. The observed change in viability with *S. aureus* was equivalent in both methods. *P. aeruginosa* showed a greater difference in response between the methods than *S. aureus*, perhaps due to differences in the cell envelope. Similar results were observed for *Salmonella* (data not shown). Maximal killing was observed with both methods at the same disinfectant concentration.

Flow cytometry allows evaluation of disinfectant or preservative efficacy in as short as 30 minutes and allows the quantitation of live and dead cells. Results are comparable with standard microbiological plate counts. The approach could be applied to a variety of applications, including prediction of disinfectant stability and potency, and microbial studies where greater than 100 organisms per mL need to be detected, such as antimicrobial effectiveness, nutritional studies, and evaluation of non-sterile products.

Figure 3 Comparison of flow cytometry and plate counting on *S. aureus*

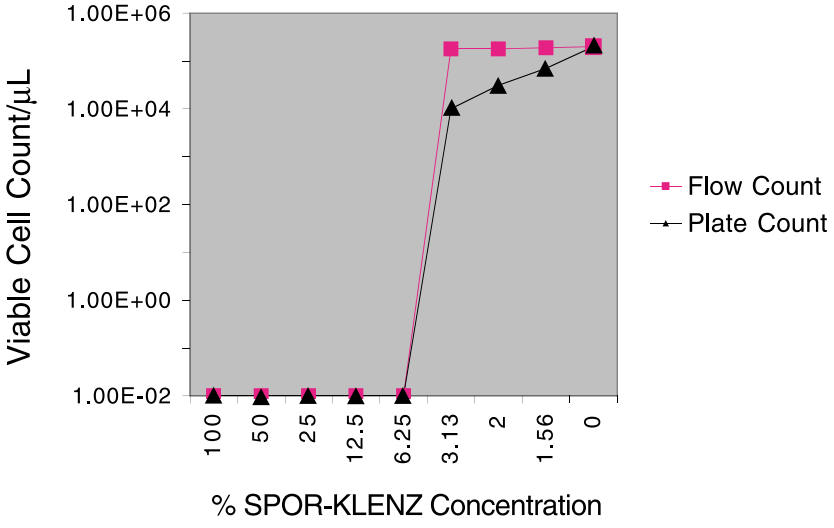
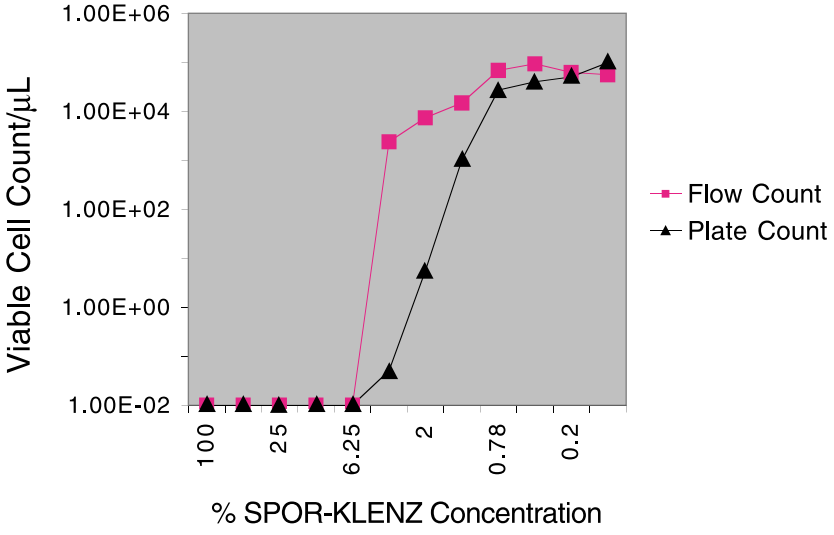


Figure 4 Comparison of flow cytometry and plate counting on *P. aeruginosa*



Hints

- TO fluoresces primarily in FL1 and FL2; PI fluoresces primarily in FL3. Therefore, the best discrimination of live and dead populations is on an FL1 vs FL3 plot. This method can be applied with a variety of buffer systems, but optimal resolution requires some surfactant to be present in the staining buffer.
- There will be differences between bacteria in their abilities to take up TO and PI. The LPS on gram-negative bacteria can interfere with the uptake of TO and other permeant dyes. Interference can be largely overcome by inclusion of 1 mM EDTA in the staining buffer, which has been reported to remove the LPS from the bacteria.
- In practice, staining protocols must be adjusted to the bacteria being analyzed.
- Thiazole orange is hydrophobic. Stock solutions should be maintained in DMSO or alcohol. TO concentration will decrease over time in aqueous solution due to adsorption to surfaces.
- TO staining is adequate for analysis at 2 to 5 minutes but requires at least 15 minutes to achieve maximum intensity. PI stains very quickly, while TO enters the cells more slowly.
- Setting FSC and SSC on logarithmic amplification assures that a wide range of bacterial sizes can appear on-scale and helps present recognizable populations for gating.
- An event rate of ≤1000 events per second minimizes the chance of coincidence and improves population resolution. High event rates can be corrected either by dilution or by decreasing the instrument flow rate.
- At least 1000 bead events should be collected to provide reliable concentration data.
- If the population of interest cannot be adequately resolved using an SSC threshold alone, a secondary threshold on FL1 can be used. An FL1 threshold alone might not be adequate due to the large amount of small fluorescent debris that can be present in a stained bacterial sample.
- If high background counts are observed on an instrument, the staining buffer and sheath fluid should be checked for particles. An instrument cleaning cycle and a drain/fill cycle can also reduce noise.
- Samples can be checked by flourescence microscopy to confirm that target organisims are stained.
- Stained samples and extra dye solution should be disposed according to local regulations.

BD Biosciences publishes this method as a service to investigators. Detailed support for non–flow cytometric aspects of this procedure might not be available from BD Biosciences.

References

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