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Rapid Enumeration of Live and Dead Bacteria by Flow Cytometry Using Thiazole Orange, Propidium Iodide and Counting Beads

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Abstract

Growth-based methods to determine bacterial viability require significant culture periods. Permeant and impermeant DNA-binding dyes, such as thiazole orange and propidium iodide, can be used in combination with counting beads to rapidly enumerate live and dead bacterial populations by flow cytometry. This reagent combination can label cells in 5 minutes and quantify populations down to 1 bacterium per microliter. Surfactant in the staining medium improves dye uptake and EDTA-mediated release of lipopolysaccharide is critical for the staining of gram-negative bacteria. Although the staining patterns of live and dead cells are characteristic, bacterial species demonstrate subtle differences. This dye combination can be used to demonstrate the effectiveness of bacterial killing by an antimicrobial agent like SPOR-KLENZ. Flow cytometry provides a rapid and powerful means to assess the concentration and viability of bacterial populations.

Introduction

Accurate determination of live, dead, and total bacteria is important in many microbiology applications. Traditionally, bacterial viability is synonymous with culture-based tests, which are time-consuming and can work poorly with slow-growing organisms.

Flow cytometry has been adapted to the analysis of viability, metabolic state, and antigenic markers of bacteria.[1-4] Thiazole orange (TO) and propidium iodide (PI) provide a rapid flow cytometric viability screen. PI is am impermeant dye that only leaks into cells with compromised membranes; TO is a permeant dye and enters all cells, live and dead, to varying degrees. With gram-negative organisms, depletion of the lipopolysaccharide layer with EDTA greatly facilitates TO uptake. BD Liquid Counting Beads (BD Biosciences Immunocytometry Systems), a flow-cytometry bead standard, can be used to quantify the concentration of bacterial populations in the sample.

As an example application, this reagent combination has been used to measure the efficacy of the antimicrobial compound SPOR-KLENZ™ (Steris Corporation, St. Louis, MO), which contains the active ingredients hydrogen peroxide and peroxyacetic acid. Viability results from a 50-minute assay involving exposure to SPOR-KLENZ showed comparable sensitivity with a standard 48-hour plate count assay.

Methods

Bacteria. *Bacillus globigii* (ATCC Cat. # 51189), *Escherichia coli* (ATCC Cat. # 25922), *Pseudomonas aeruginosa* (ATCC Cat. # 15442), *Salmonella choleraesuis* (ATCC Cat. # 10708), *Staphylococcus aureus* (ATCC Cat. # 6538), and *Staphylococcus epidermidis* (ATCC Cat. # 12228) were cultured using Trypticase™ Soy Agar (TSA) and Trypticase Soy Broth (TSB, BD Diagnostic Systems, Sparks, MD). Prior to use, cultures were inoculated from a single colony.

Killed bacteria were prepared by mixing bacteria 1:1 with SPOR-KLENZ for 5 minutes.

Reagents. *BD Cell Viability Kit* (BD Biosciences Immunocytometry Systems), containing 17 µM thiazole orange solution in DMSO and 1.9 mM propidium iodide solution in water. *BD Liquid Counting Beads* (part of the BD Cell Viability Kit), for counting. *Staining Buffer:* Phosphate-buffered saline, 1 mM EDTA, 0.2% Pluronic™ F-68 (BASF Corporation, Mount Olive, NJ), pH 7.4. Tween-20 at 0.01% can be substituted for Pluronic F-68.

Staining. Bacterial suspensions were diluted at least 1:10 in staining buffer to bring them into an appropriate concentration range for cytometry (5 x 10^5 to 9 x 10^6 bacteria/mL). A 200-µL sample was reacted with 420 nM TO and 48 µM PI (5 µL of each) for at least 5 minutes at room temperature. A known quantity of fluorescent counting beads was added to the tube before analysis.

Preservative Efficacy . Bacteria were rotated for 10 min with SPOR-KLENZ, diluted 1:2 to 1:512. Treated bacteria were split into two groups. Dilutions of one group were plated on RODAC™ D/E Neutralizing Agar (BD Diagnostic Systems) to neutralize residual SPOR-KLENZ and checked for growth after 48 hours. The second group was prepared for flow cytometric analysis as above.

Flow Cytometer Setup. Samples were analyzed on a BD FACSort™ System equipped with 488-nm laser excitation. 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 1A). Individual FSC and SSC histograms were checked to be sure that the bell-shaped populations were not cut off on the display (Figures 1B). FL1, FL2, and FL3 PMT voltages were adjusted to place the unstained population well within the first decade, as seen in an FL1 vs FL3 plot (Figure 1C). Data files of 10,000 events were acquired and analyzed using BD CellQuest™ software. The bacterial population was gated using a combination of FSC, SSC, and FL2 (Figure 1A and 1D). Dead, live and injured populations were discriminated using an FL1 vs FL3 plot (Figure 1E). A separate analysis region was drawn around the counting beads and the absolute count of the target population was determined using the following equation:

$$\frac{\#\text{ events in region containing cell population}}{\#\text{ of events in bead population}} \times \frac{\#\text{ beads per test*}}{\text{test volume}} \times \text{dilution factor} = \text{Concentration of bacterial population}$$

* Value is found on the BD Liquid Counting Beads label.

Discussion

Flow cytometry with viability dyes and counting controls allows the rapid enumeration of live and dead bacteria in a sample. All bacteria tested showed similar staining patterns , although species differences were exhibited. One application is the measurement of disinfectant or preservative efficacy in under an hour and allows the quantitation of live and dead cells. Results are comparable with standard microbiological plate counts, and significantly faster than traditonal methods such as the hard-surface carrier method (AOAC 991.47-49) and use-dilution method (AOAC 955.14-15, 964.02),[5] which rely on exposure of the test organism to a disinfectant with growth generally observed after 48 hours. Flow cytometry 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.

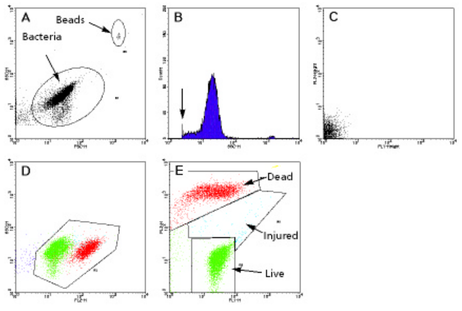
Things to Consider

- There will be differences between bacteria in their abilities to take up dyes such as TO and PI. Interference by the LPS on gram negative bacteria can be largely overcome by 1 mM EDTA in the staining buffer.
- TO staining is adequate for analysis at 2 to 5 minutes but requires at least 15 minutes to achieve maximum intensity.
- 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.
- As the concentration of bacteria decreases, background noise will become progressively more prominent.

References

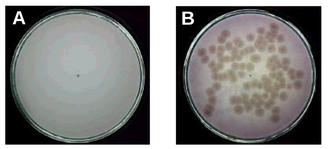
1. Davey HM, Kell DB. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single cell analyses. *Microbiological Reviews.* 1996;60:641-696.
2. Nebe-von-Caron G, Stephens PJ, Hewitt CJ, Powell JR, Badley RA. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. *Journal of Microbiological Methods.* 2000;42:97-114.
3. Shapiro HN. Microbial analysis at the single-cell level: tasks and techniques. *Journal of Microbiological Methods.* 2000;42:3-16.
4. Nebe-von-Caron G, Stephens PJ, Badley RA. Bacterial detection and differentiation by cytometryand fluorescent probes. *Proceedings of the Royal Microbiological Society.* 1999;34:321-327.
5. *Horwitz W. Official Methods of Analysis of AOAC International.* 17th Edition. Association of Official Agricultural Chemists. 2000.

1 Setup and Gating Strategies



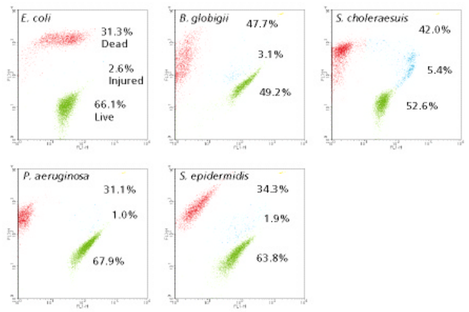
Sample of *E. coli* stained with TO and PI, counting beads added (except as noted). The TO and PI dye combination provides significant resolution between live and dead cell populations (Figure 1E). An intermediate or injured population can often be observed between the live and dead populations. A) FSC vs SSC dot plot with the bacterial population on-scale and with regions set around the target population and the beads. B) SSC histogram of bacteria with the threshold set at the point where the noise level is just starting to increase (arrow). C) FL1 vs FL3 dot plot of unstained bacteria gated on the population in FSC vs. SSC and with the unstained bacteria placed in the first decade on FL1 and FL3. D) The FL2 vs SSC dot plot with region set around the stained bacteria. E) FL1 vs FL3 dot plot gated on FSC, SSC and FL2 with regions set around the live, injured, and dead bacterial populations.

2 Cultures of Sorted Bacteria



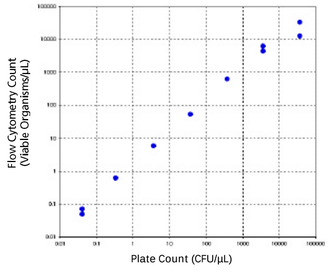
Live and dead designations were confirmed by index sorting. Only live and injured populations produced colonies on plates. *B. globigii* were plated on TSA after being sorted on a BD FACS Vantage™ System for live (A) and dead (B) organisms, gated as in Figure 1E.

3 Staining of Various Bacteria



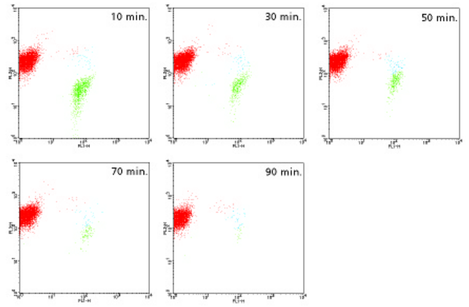
Representative FL1 vs FL3 dot plots of several bacteria stained with TO and PI, gated on FSC, SSC, and FL2, with samples containing about equal amounts of live and killed bacteria. The resulting populations are similar, but with organism-specific differences. The gram-negative bacteria, *E. coli* and *S. choleraesuis*, demonstrated less efficient uptake of TO than the other bacteria.

4 Flow Cytometry vs Plate Count



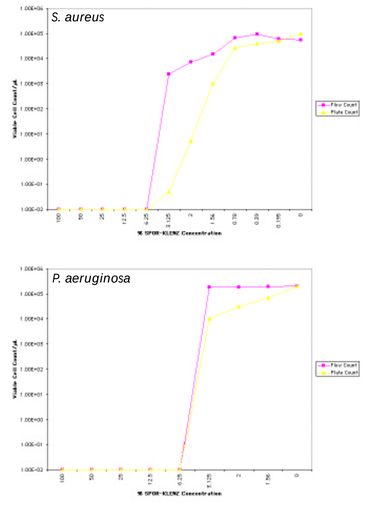
Relationship between the concentration of *B. globigii* determined by flow cytometry and by plating on Trypticase Soy Agar. Replicate serial dilutions were either prepared with TO, PI and counting beads for flow cytometry or plated, with appropriate dilutions, on TSA. The calculated concentrations from both methods were linear to the range of 0.1 to 1 bacterium per microliter.

5 Exposure of P. aeruginosa to SPOR-KLENZ



Preservative Efficacy. Progressive FL1 versus FL3 plots show the change in viability of *P. aeruginosa* over 90 minutes of exposure to 3.1% SPOR-KLENZ.

6 SPOR-KLENZ: Flow Cytometry vs. Plate Count



The effect of various concentrations of SPOR-KLENZ on *S. aureus* and *P. aeruginosa* by flow cytometry and plate counting after a 30 minute exposure. 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.