APPLICATION NOTE

Bacterial Detection and Live/Dead Discrimination by Flow Cytometry

MICROBIAL CYTOMETRY BD FACSCalibur, BD FACSort Flow Cytometers

Introduction

Accurate determination of live, dead, and total bacteria is important in many microbiology applications. Traditionally, viability in bacteria is synonymous with the ability to form colonies on solid growth medium and to proliferate in liquid nutrient broths. These traditional, culture-based tests are time-consuming and can work poorly with slow-growing or viable, but non-culturable organisms. They do not provide real-time results or timely information that is needed in applications such as industrial manufacturing.

Flow cytometry, a technique first applied to eukaryotic cells, has been adapted to the analysis of viability, metabolic state, and antigenic markers of bacteria.[1-4] In particular, flow cytometry can be readily applied to the enumeration of viable bacteria in a sample.

Live cells have intact membranes and are impermeable to dyes such as propidium iodide (PI), which only leaks into cells with compromised membranes. Thiazole orange* (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. Thus a combination of these two dyes provides a rapid and reliable method for discriminating live and dead bacteria. If enumeration of the bacteria is important, BD Liquid Counting Beads (BD Biosciences, San Jose, CA), a flow-cytometry bead standard, can be used to accurately quantify the number of live, dead, and total bacteria in a sample.

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

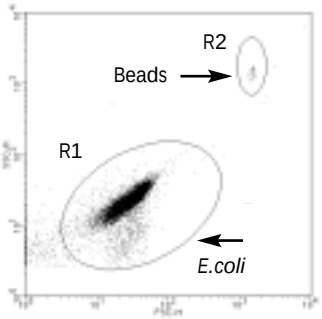

Figure 1A

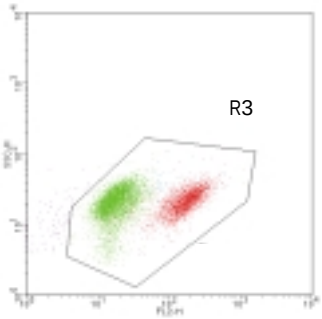

Figure 1B

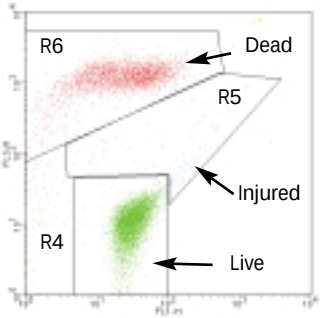

Figure 1C

Figure 1 Gating strategy for bacterial populations, using a sample of *E. coli* stained with TO and PI in a staining 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. The 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

Bacteria

For cultured bacteria, dilute to an approximate concentration range of 5×10^5 to 9×10^6 bacteria/mL in staining buffer.

For other sample types, such as pharmaceutical, food or environmental samples, at least 100 organisms per mL are required to be detected using flow cytometry. If necessary, samples can be brought into this range by an initial concentration step. We recommend that you optimize your sample preparation using samples spiked with known concentrations of your target organism(s) before attempting to analyze unknowns.

To prepare killed bacteria, mix 0.5 mL of culture before dilution with 0.5 ml of SPOR-KLENZ™ (Steris Corporation, St. Louis, MO, Catalog No. 6525-01) disinfectant for 5 minutes.

Reagents

1. Thiazole orange solution, 17 µM (BD Biosciences Catalog No. 349483 or 349480), or equivalent at 8.1 µg/mL (FW 476.6) in DMSO
2. Propidium iodide solution, 1.9 mM (BD Biosciences Catalog No. 349483 or 349480), or equivalent, at 1.3 mg/mL (FW 668.4) in water
3. BD Liquid Counting Beads (BD Biosciences Catalog No. 349480), for counting
4. Staining buffer: Phosphate-buffered saline, 1 mM EDTA, 0.2% Pluronic™ F-68 (BASF Corporation, Mount Olive, NJ, Catalog No. 51554728), 0.1% sodium azide, pH 7.4.
   Tween-20 at 0.01% can be substituted for Pluronic F-68. The staining buffer should be passed through a 0.22-µm filter and used within 2 weeks.

Equipment

1. Disposable 12 x 75-mm capped BD Falcon™ polystyrene test tubes (BD Labware Catalog No. 352052), or equivalent
2. Vortex mixer
3. Micropipettor with tips (Pipetman™, Rainin Instrument Company, Emeryville, CA, or equivalent)
4. BD FACS™ brand flow cytometer (BD FACSCalibur™ flow cytometer or equivalent) equipped with 488-nm laser excitation

Procedure

Staining

1. Label 12 x 75-mm polystyrene tubes.
2. Vortex bacterial suspension or sample and dilute at least 1:10 in staining buffer.
3. Add 200 µL of bacterial suspension, diluted as above in staining buffer.
4. Add 5.0 µL of each dye solution to the tubes. The final staining concentrations are 420 nM for TO and 48 µM for PI.
5. Vortex and incubate for 5 minutes at room temperature.

6. Reverse pipet 50 µL of BD Liquid Counting Beads into the staining tube if you want to determine the concentration of live, dead and total bacteria.
7. Analyze on a BD FACS brand flow cytometer (BD FACSCalibur flow cytometer or equivalent).
8. Dispose of stained samples and extra dye solution according to local regulations.

Flow Cytometer Setup

1. Use BD CaliBRITE™ 3 beads (BD Biosciences Catalog No. 349502) and the appropriate software, such as BD FACSComp™ or BD AutoCOMP™ software, for setting the photomultiplier tube (PMT) voltages and the fluorescent compensations, and for checking instrument sensitivity prior to use.

2. Initial instrument settings should be 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

3. Actual settings can vary with the application and should be optimized as follows: Set threshold on side scatter (SSC), and adjust PMT voltages and threshold levels using an unstained sample of diluted bacteria. The bacterial population should be positioned so that it is entirely on scale on an FSC vs SSC plot (Figure 1A). Individual FSC and SSC histograms should be checked to be sure that the bell-shaped populations are not cut off on the display (Figures 2 and 3). If the entire population is not present, adjust PMT values to position the peak on the histograms and decrease the threshold until the entire population is visible. As the voltage is further increased, the background noise should become evident on the lower end of the histogram. The balance of PMT voltage and threshold should allow the entire peak to be observed with at least a portion of the valley between the bacteria and the noise. Actual peak shapes and resolution from noise will vary with bacterial morphology and sample matrix.

4. Set FL1 and FL3 PMT voltages to place the unstained population in the lower left quadrant of an FL1 vs FL3 plot (Figure 4).

Data Acquisition and Analysis

1. Acquire prepared samples on a BD FACS brand flow cytometer using an SSC threshold. Acquire data with BD CellQuest™ Pro or BD LYSYS™ II software, in Acquisition-to-Analysis mode. Set up an FSC vs SSC plot with a live gate around the bacterial population R1 (Figure 1A). If BD Liquid Counting Beads are used, set a region R2 around the beads on the FSC vs SSC plot. Set another region R3 around the stained bacterial population in the FL2 vs SSC dot plot (Figure 1B) and an FL1 vs FL3 plot, gated on combined parameters FSC, SSC, and FL2 (FL1 vs FL3

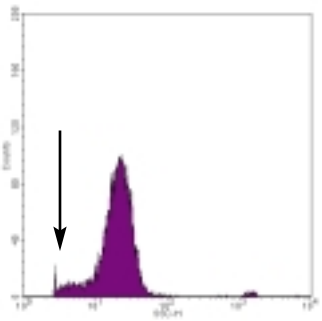

Figure 2 SSC histogram of a sample containing *E. coli*, with the threshold set at the point where the noise level is just starting to increase (arrow)

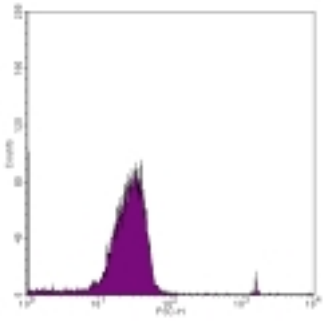

Figure 3 FSC histogram of a sample containing *E. coli*

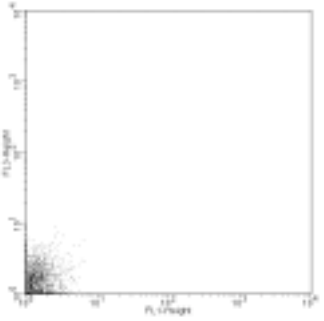

Figure 4 FL1 vs FL3 dot plot of unstained *E. coli* gated on the bacterial region in FSC vs SSC, with the unstained bacteria placed in the first decade on FL1 and FL3

* US Patent No. 5,232,828

gated on [R1 OR R2] AND R3) to display the stain results (Figure 1C).

2. Acquire a total of 10,000 events.
3. In Analysis mode, draw rectilinear regions around the live, dead, and injured populations (Figure 1C).
4. If you used BD Liquid Counting Beads and want to determine the absolute count, use the following equation:

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

Controls

Use an unstained bacterial sample to confirm that PMT voltages are set appropriately (Figure 4). Dilute, stain, and acquire an aliquot of culture media or sample matrix, diluted the same as a bacterial sample, to confirm that assay background is low. Use a mixture of live and killed bacteria to confirm that stained live, injured, and dead bacterial populations are sufficiently resolved.

Results and Discussion

Live/Dead Discrimination of Bacteria

The TO and PI dye combination provides significant resolution between live and dead cell populations (Figure 1C). An intermediate or *injured* population can often be observed between the live and dead populations. These designations were confirmed by index sorting. Only live and injured populations produced colonies on plates (data not shown).

Figure 5 shows staining patterns for five different bacteria. Dead cells were produced using SPOR-KLENZ treatment. Approximately equal amounts of live and dead cells were mixed in the samples. Stain results are shown for *Escherichia coli* (ATCC Catalog No. 25922), *Bacillus globigii* (ATCC Catalog No. 51189), *Salmonella choleraesuis* (ATCC Catalog No. 10708), *Pseudomonas aeruginosa* (ATCC Catalog No. 15442), and *Staphylococcus epidermidis* (ATCC Catalog No. 12228). Resulting populations are similar, but there are organism-specific differences. In particular, two of the gram negative bacteria, *E. coli and S. cholerasuis*, demonstrated less efficient uptake of TO than the other bacteria.

The live and dead populations do not line up in a rectilinear fashion and analysis is best accomplished by drawing regions in the appropriate areas.

Comparison of Flow vs Plate count

Figure 6 shows the relationship between the concentration of viable *B. globigii* determined by flow cytometry and plate counts of the same bacterium. A serial dilution was made on a *B. globigii* culture grown in BD Trypticase™ Soy Broth (BD Diagnostic Systems, Sparks, MD, Catalog No. 221092) and the suspensions were analyzed either by flow cytometry using TO, PI, and BD Liquid Counting Beads or by plating with appropriate dilutions on Trypticase Soy Agar (BD Diagnostic Systems Catalog No. 221185). When concentrations were corrected for dilution and plotted, flow cytometric results were linear with plate count results down to the range of 0.1 to 1 bacterium per microliter. Sensitivity of detection by flow cytometry will vary with the noise present in a sample matrix and should be evaluated for each experimental situation.

* This value is found on the BD Liquid Counting Beads label and might vary from lot to lot.

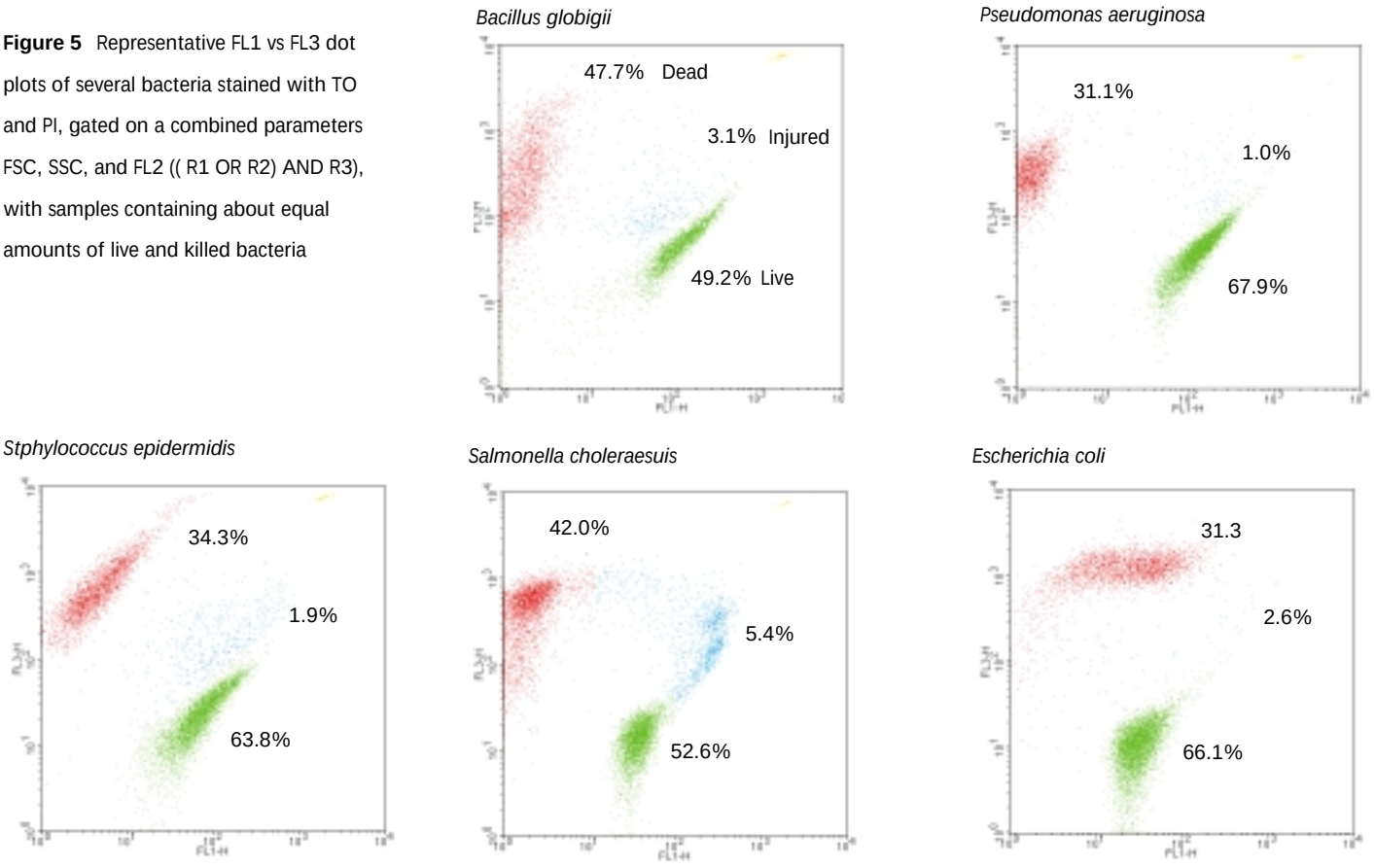

Figure 5 Representative FL1 vs FL3 dot plots of several bacteria stained with TO and PI, gated on a combined parameters FSC, SSC, and FL2 ((R1 OR R2) AND R3), with samples containing about equal amounts of live and killed bacteria

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.

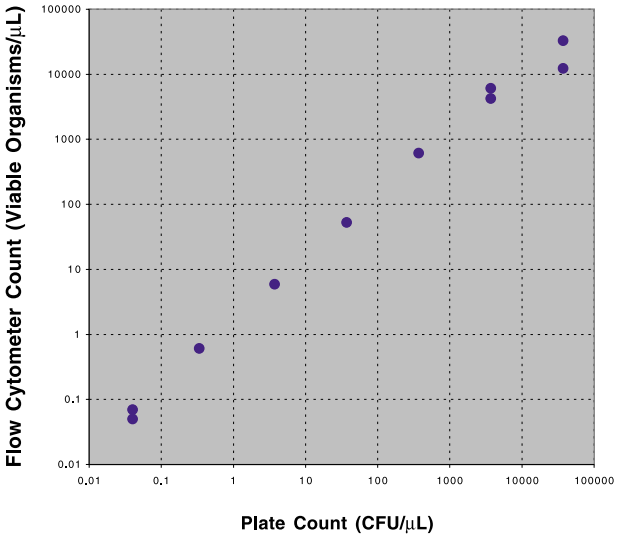

Figure 6 Correlation between the concentration of *B. globigii* determined by flow cytometry and by plating on BD Trypticase Soy Agar

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.

- 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.
- As the concentration of bacteria decreases, background noise will become progressively more prominent.
- 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 fluorescence microscopy to confirm that target organisms are stained.

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 cytometry and fluorescent probes. *Proceedings of the Royal Microbiological Society.* 1999;34:321-327.

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23-6287-01 4/2002