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Bacterial analysis using the Attune® Acoustic Focusing Cytometer

Flow cytometry has been widely used in cell biology for analysis of eukaryotic cell cycle, expression of surface proteins, and expression of intracellular proteins in isolated or cultured cells. In recent years the application of flow cytometry to the study of microbiological phenomena has increased, and has been used in studies including detection and quantification of viable organisms and those that cannot be cultured [1], analysis of host-microbe interactions [2], analysis of microbial cell cycle [3], and detailed spatial and temporal analysis of microbial metabolism in different environments [4], as well as many others. Flow cytometry is particularly suitable for the study of environmental microbiology and microbial physiology because of its ability to analyze large numbers of cells in a short period of time. The speed of sample acquisition coupled with the ability to measure multiple parameters simultaneously makes flow cytometry an invaluable technique for researchers interested in studying time-sensitive processes [4].

Table 1. Flow cytometry products for microbiology.

Application	Product
Bacterial staining	*Bac*Light™ Green Bacterial Stain (B35000) *Bac*Light™ Red Bacterial Stain (B35001) SYBR® Green I Nucleic Acid Stain (S7563) SYTO® BC Nucleic Acid Stain (S34855) SYTO® 9 Nucleic Acid Stain (S34854)
Gram character	LIVE *Bac*Light™ Bacterial Gram Stain Kit (L7005)
Cell viability	LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7007, L7012, L13152)
Cell count	LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit (L34856) Bacteria Counting Kit (B7277)
Cell vitality • measured by membrane potential	*Bac*Light™ Bacterial Membrane Potential Kit (B34950)
Cell vitality • measured by metabolism	*Bac*Light™ RedoxSensor™ Green Vitality Kit (B34954) *Bac*Light™ RedoxSensor™ CTC Vitality Kit (B34956), for fixed samples

Life Technologies offers a wide range of Molecular Probes® products for microbiology studies that emply flow cytometry (Table 1). From simple to complex, coupled with the innovative Attune® Acoustic Focusing Cytometer, these products represent a complete solution for cytometric analysis of microbial physiology. Here, we describe how to analyze bacteria using the Attune® Acoustic Focusing Cytometer, and highlight one important and widely used measure in microbiology—cell viability using the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit.

Using flow cytometry to analyze small particles

Eukaryotic cells range in size from 2–40 µm, and cells from multicellular organisms average 10 µm in diameter [5]. In comparison, prokaryotic cells are much smaller; on average only 1 µm in diameter [5]. The small size of bacteria necessitates careful instrument setup in order to differentiate the bacterial population of interest from electronic "noise" events and debris. In all flow cytometers, electronic noise is due to stray light collected by the system optics and/or low-level electronic signals. Noise may be eliminated or reduced through correct setup of instrument threshold and photomultiplier tube (PMT) voltage. The easiest way to differentiate a bacterial population from other small particles and electronic noise is to label the bacteria with a fluorescent stain and use the fluorescence emission to identify the population of interest.

To demonstrate the effect of instrument threshold on acquisition of small particles such as bacteria, a sample of *Escherichia coli* (*E. coli*) expressing green fluorescent protein (GFP) was run on the Attune® Acoustic Focusing Cytometer equipped with a 488 nm laser and a 530/30 bandpass filter (BL1) to detect GFP fluorescence emission. In this experiment, PMT voltage was kept constant and threshold was varied. Sample plots are shown in Figure 1. In the first plot (A), GFP-expressing *E.coli* are easily identified using a BL1-fluorescence threshold. Only fluorescence events above the threshold are detected, and no electronic noise is observed. The alternative method to using a fluorescence threshold is shown in (B), in which a combination of forward scatter (FSC) and side scatter (SSC) thresholds are used to identify the population of interest. In this example, noise events are observed outside the cell gate, and can be removed from analysis by increasing the FSC threshold.

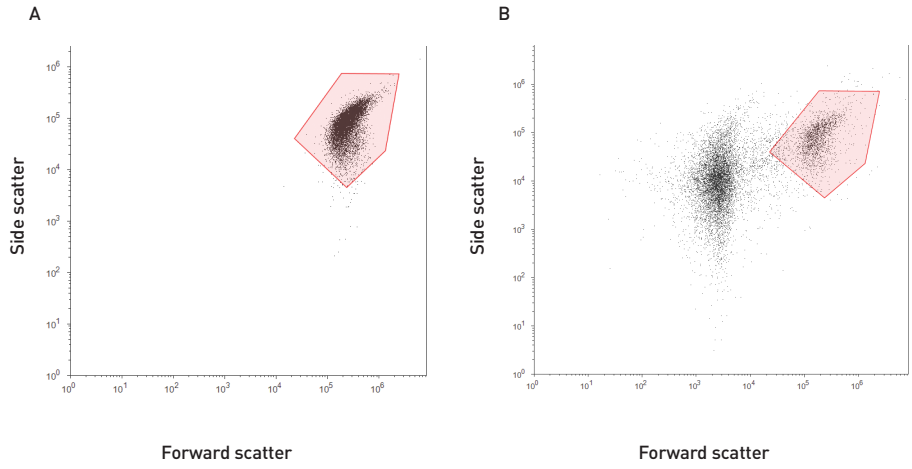

Figure 1. The effect of instrument threshold on forward and side scatter of *E.coli* using the Attune® Acoustic Focusing Cytometer. A culture of *E. coli* cells expressing GFP was diluted in 0.2 µm filtered PBS to 5 x 10^5 colony forming units (CFU)/mL and analyzed at a standard collection rate of 25 µL/min using the Attune® Acoustic Focusing Cytometer. (**A**) BL1 threshold set to 10 (equal to 10,000). (**B**) A noise population is observed when scatter thresholds (FSC and SSC) are set to 2 (2,000).

Experiment focus—LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit

In the following example, we provide in-depth instructions for analyzing bacteria stained using Molecular Probes® LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. This kit provides a novel two-color fluorescence assay for bacterial viability that allows researchers to easily, reliably, and quantitatively distinguish live and dead bacteria in minutes, even in a mixed population containing a range of bacterial types. Conventional direct-count assays of bacterial viability are based on metabolic characteristics or membrane integrity; these methods often only work for a limited subset of bacterial groups [6], have high levels of background fluorescence [7], and are sensitive to growth and staining conditions [8]. The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit has been used in numerous applications, including characterization of bacteria from different aquatic environments [9], detection of bacterial contamination [10], analysis of antimicrobial susceptibility testing [11] and to study vital bacterial cellular functions during solar disinfection [12].

Sample preparation

1. It is important to reduce the amount of particulates in the sample prior to analysis using flow cytometry. All liquids used in sample preparation (buffers, growth media, etc.) should be filtered through a sterile 0.2 µm filter before use.
2. Prepare bacterial samples and stain using SYTO® 9 dye and propidium iodide following kit instructions. For best results, dilute samples to approximately 1 x 10^4–5 x 10^6 CFU/mL. A minimum of four samples are required: one unstained control, two single-color compensation controls and one test sample.
3. SYTO 9 fluorescence emission is collected in the BL1 channel using the 530/30 bandpass filter, and propidium iodide fluorescence emission is collected in the BL3 channel using the 640 LP filter. Follow the steps outlined below to optimize Attune® instrument settings.

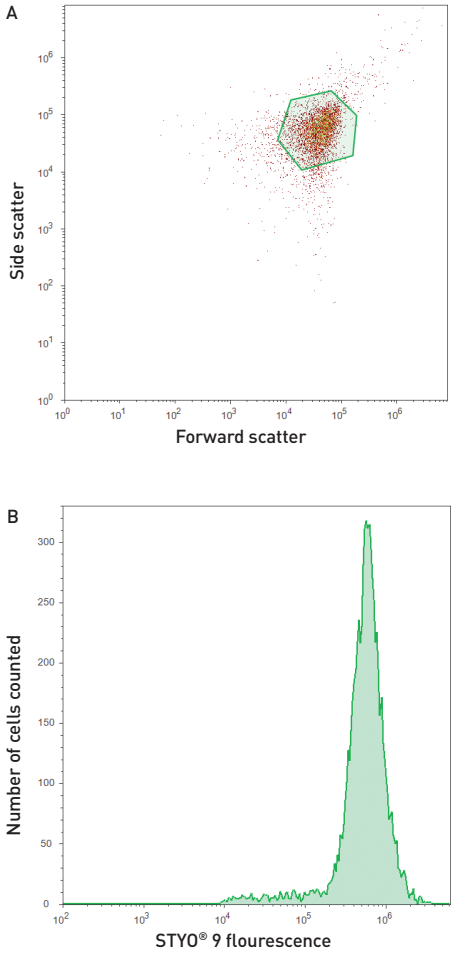

Figure 2. *E. coli* cells stained with SYTO® 9 dye and propidium iodide are run on the Attune® Acoustic Focusing Cytometer at the Standard 25 µL/min collection rate using a BL1 threshold set to 10,000. (A) The region outlined identifies the bacterial population of interest. (**B**) Histogram indicating SYTO® 9 fluorescence of *E. coli* population gated in.

Attune® instrument setup and data acquisition:

1. Open instrument settings from the "View" tab and change the following parameters from the default instrument settings:
   a. Threshold: change the default FSC threshold setting to "Ignore" and select a BL1 threshold by changing the Boolean operator to "And" and setting the numeric value to "5".
   b. PMT voltages: set forward and side scatter to 3,000 (FSC) and 3,500 (SSC).

Further adjustments will be made to threshold and PMT voltage settings during initial sample acquisition.

2. On the collection panel, set collection rate to Standard 25 µL/min and set draw volume to 50 µL.
3. Create plots in the Attune® software workspace for sample analysis:
   a. Density plot: SSC (y-axis) vs. FSC (x-axis); both axes in logarithmic scale. Include a polygon gate on this plot to identify the population of interest (Figure 2A).
   b. Histogram plot: Count (y-axis) vs. BL1 (SYTO® 9 dye fluorescence; x-axis).
   c. Histogram plot: Count (y-axis) vs. BL3 (propidium iodide fluorescence; x-axis).
   d. Dot plot: BL1 (SYTO® 9 dye fluorescence) vs. BL3 (propidium iodide fluorescence). Include a quadrant gate on this plot for discrimination of live and dead cells. Statistics generated from this plot will be used to determine culture concentration and culture viability.
   e. Statistics: insert a global statistics box by selecting "Statistics" under the "Insert" tab. Ensure that "Concentration (ev/µL)" is selected as statistical output.
4. Use the SYTO® 9 dye–stained sample to adjust settings. Place the sample in the tube lifter, raise the tube lifter to the SIP, and press "Run".
5. Note the appearance of events on the density plot of SSC vs. FSC. Adjust forward and side scatter PMT voltage as necessary to place the bacterial population in the upper right quadrant of the density plot to differentiate this population from noise events.
6. Observe SYTO® 9 dye florescence as indicated in the BL1 histogram (Figure 2B) and adjust the PMT voltage of BL1 so that the events are on scale in the BL1 histogram. In this example, the BL1 voltage is set to 2,200.
7. Dependent on the sample, it may be necessary to further increase the BL1 threshold setting to reduce or eliminate noise events. If the sample contains a great deal of debris, incorporate FSC and SSC thresholds to reduce noise events.

When instrument settings are correctly adjusted, the event rate should be between 500 and 1,000 events per second for stained cells at a concentration of ~1 x 10^6 CFU/mL.

8. Ensure that PMT voltage settings for BL3 are correct. Stop acquisition of the SYTO® 9 dye–stained sample and replace it with the sample stained with propidium iodide alone. Using this sample, adjust the BL3 PMT voltage so that the events are on scale in the BL3 histogram.
9. Open the compensation module in the Attune® software and perform compensation following the steps outlined in the compensation module for the BL1 and BL3 channels using single-color labeled cells and using the instrument settings determined in the steps above.

The Attune® instrument settings are now correctly adjusted for sample acquisition.

10. Analyze dual-stained test samples using the Attune® cytometer, collecting

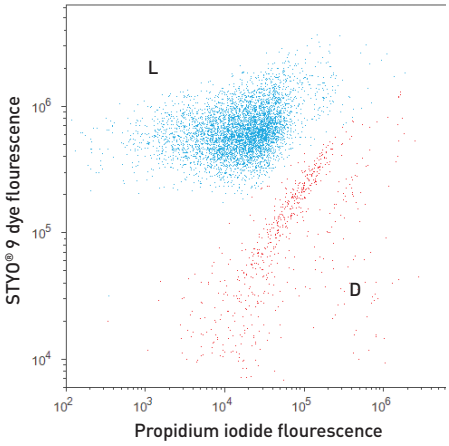

Figure 3. *E. coli* cells were stained with SYTO® 9 dye and propidium iodide and analyzed on the Attune® cytometer as described. Live cells (L) are shown in blue; dead cells (D) are shown in red. Using the Attune® software, viable cell concentration was determined to be 6.37 ± 0.289 x 10^4 CFU/mL. This value is not significantly different than that determined using a traditional plating method, 6.28 ± 0.665 x 10^4 CFU/mL.

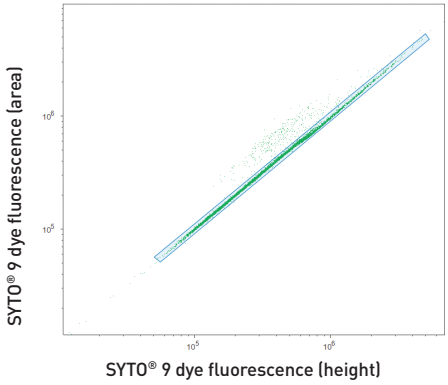

Figure 4. *E. coli* cells at ~1 x 10^6 CFU/mL stained with SYTO® 9 dye are analyzed for aggregation. Singlet cells fall on the diagonal of a SYTO® 9 dye fluorescence (area) versus SYTO® 9 dye fluorescence (height) dot plot. Aggregates have a greater area measurement and will fall above the diagonal. At a concentration of 1 x 10^6 CFU/mL, very little cell aggregation should be observed.

an appropriate number of events (≥10,000).

11. Calculate percent viability from the statistics generated from the SYTO®9 dye fluorescence vs. propidium iodide fluorescence dot plot (see Figure 3) using the calculation: % viability = [Number of events in live gate/(number events in live gate + number events in dead gate)] x 100%. Viable cell concentration can be determined from the Attune® software under the "concentration" heading. Note that concentration is reported as events per µL and does not account for any culture dilution prior to sample acquisition.

Hints for a successful experiment

- To thoroughly optimize staining, we recommend experimenting with a range of concentrations of SYTO® 9 dye, each in combination with a range of propidium iodide concentrations.
- The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit has been used successfully to stain a variety of bacteria including *Bacillus cereus, Bacillus subtilis, Clostridium perfringens, E. coli, Klebsiella pneumoniae, Micrococcus luteus, Mycobacterium phlei, Pseudomonas aeruginosa, Psudomonas syringae, Salmonella oranienburg, Serratia marcescens, Shigella sonnei, Staphylococcus aureus,* and *Streptococcus pyogenes*.
- To reduce the possibility of coincidence, we recommend using a concentration ≤1 x 10^6 CFU/mL at a standard collection rate of 25 µL/min.
- If sample aggregation is suspected, look for aggregates in an area-versus-height dual parameter plot (see Figure 4).
- If sample dilution is required, dilute with 0.2 µm filtered buffer and include SYTO® 9 dye at a final concentration of 5 µM and propidium iodide at a concentration of 20 µM.
- As sample concentration decreases, background noise will become progressively more prominent. The bacterial sample can be centrifuged at >10,000 x *g* to concentrate prior to staining and analysis.

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