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CHAPTER 53

Flow Cytometric Analysis of Microorganisms

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I. Introduction

Conventional techniques (i.e., growth on laboratory media) employed for the detection and enumeration of microbes in clinical and environmental samples require time (24 to 48 hr), and they have a strong bias in that these methods detect only organisms that grow under a selected set of conditions. Problems with the current technology for microbial cell analysis led to development of alternative techniques that include flow cytometry. Flow cytometry allows rapid, multiparameter data acquisition and analysis of individual cells.

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Although flow cytometry was rapidly accepted into hospital pathology and immunology laboratories, microbiology laboratories have remained essentially oblivious to the use of this technology. With few exceptions (Dubelaar *et al.*, 1999; Steen, 1980, 1983; Steen and Boye, 1980), flow cytometers were not designed to measure microorganisms, but rather mammalian cells in the range of 5 to 15 μm, the general size of blood cells. In practice the measurement of smaller particles, while possible, often required modifications to the instrument or a greater understanding of and interest in the technological aspects of cytometry than generally possessed by those with expertise in clinical microbiology. In addition, clinical microbiologists generally found the technology expensive and inappropriate for their cells of interest.

Improvements in the sensitivity and specificity of flow cytometric instrumentation have made possible a wide range of techniques to rapidly characterize microbial populations. More importantly, microbiologists have started to recognize the potential of flow cytometry to study the responses of individual cells in environmental and clinical samples and to report their findings. An excellent review describing applications of flow cytometry in the field of microbiology has been published (Davey and Kell, 1996).

This chapter discusses experimental approaches that have been or could be used to study individual microbial cells using flow cytometry and key factors that may impact these studies, including instrument setup, instrument operation, and sample preparation. A brief discussion of flow cytometric applications to the field of medical and food microbiology is also included.

A. Instrument Setup for Microbes

Flow cytometers designed specifically for small particles (i.e., Bio-Rad Bryte HS, Hercules, CA; Skatron, Oslo, Norway) are no longer commercially available, and technical support for existing instruments is limited. An ordinary flow cytometer optimized for mammalian cells can be adapted for microbial cell analysis with a few simple changes in instrument setup and operation. For example, sheath fluid, sample buffer, media used to grow bacteria, and other reagents (i.e., dyes, antibodies) must be filtered (0.2-μm filter or smaller) to remove any particles that could interfere with bacterial measurements. The laboratory water system used to prepare sample buffer and sheath fluid should also be rigorously cleaned and maintained to prevent bacterial contamination.

Daily quality control procedures should include the instrument alignment beads recommended by the manufacturer and latex beads of size similar to that of the microbe of interest (1.0, 1.5, 2.0, 4.0, 6.0 μm). Because small latex beads can give a scatter signal quite different from that of bacteria of similar size, ethanol- or heat-fixed vegetative cells (i.e., *Escherichia coli*) or unfixed spores in water (i.e., *Bacillus subtilis*) should also be used as an internal laboratory standard to check light scatter parameters. Fixed cells or spores can be stored at 4°C for up to 6 months.

Initial light scatter parameters should be established using target microorganisms spiked with latex beads. For example, in Fig. 1 *Bacillus subtilis* cells were spiked with a small number of 1.0-μm beads. Bacteria and beads in the spiked sample were separated using a dual-parameter histogram of log forward scatter (FS) and log side scatter (SS). A region was established for the bacterial population and used as a gating parameter to exclude cell aggregates and debris from further analysis. Sterile, filtered sample buffer was used to set the discriminator or threshold on forward light scatter to eliminate background particles.

In order to reduce the risk associated with analyzing potentially hazardous microorganisms, certain protective measures should be followed and strictly enforced. In particular, the protective doors that shield the instrument sample probe should be kept closed to reduce aerosolization of bacterial particles, bleach should be added to the waste container to kill any harmful organisms, and personal protective gear (i.e., gloves, mask, laboratory coat) should be worn at all times. Laboratory personnel should also avoid contaminating the computer keyboard and mouse with bacteria. Instrument maintenance should include frequent flushing of the system between samples to reduce instrument carryover of bacteria and dyes and rigorous shutdown/cleaning procedures.

B. Sample Preparation

Biological characteristics of bacteria such as size, shape, DNA, RNA, and protein content can change depending on growth conditions and cell source. For example, exponentially growing cells are larger than dormant or starved cells and contain considerably higher levels of nucleic acids. Growing cells have a wide light scatter distribution with a cometlike tail in the direction of increasing

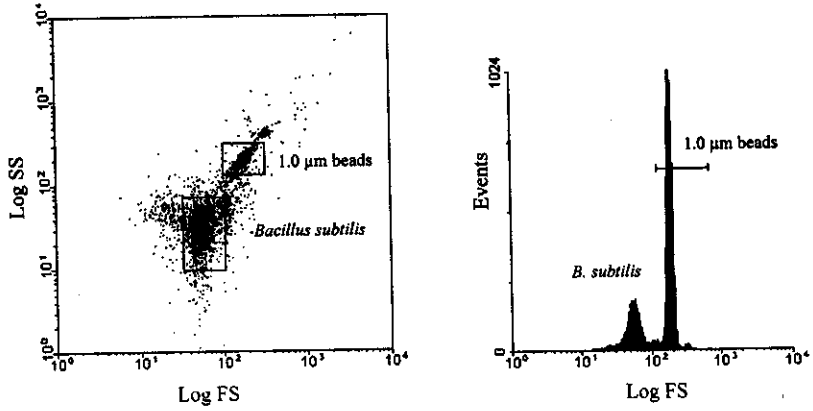

Fig. 1 Light scatter measurements of a mixture of *Bacillus subtilis* cells and latex beads. Fluorescent microspheres (1.0 μm) were added as an internal standard.

scatter (Thomas *et al.*, 1997). Prior to flow cytometric analysis, growing cells should be washed with sterile, filtered buffer to remove debris and reduce cell clumping (Fig. 2). A washing step will also remove medium that may interfere with staining. Bacteria can grow as single cells or in pairs, chains, or clusters. Gentle pipetting or vortexing may be necessary to disrupt the chains or clusters and form a single cell suspension.

Some bacteria have considerable permeability barriers (i.e., cell walls, endospores, capsules, efflux pumps) to fluorescent dyes or DNA probes and may require use of fixatives or EDTA. However, sample preparation methods necessary for efficient penetration of a fluorochrome into target cells may significantly affect light scatter profiles. For example, alcohol fixation can cause considerable cell shrinkage and a reduction in cell size.

II. Experimental Approaches

The basic problem in developing flow cytometric protocols for microbial cell analysis is the assumption that procedures developed and optimized for mammalian cells will work for bacteria. In some cases, ignorance of traditional flow methods is an advantage; however, the fundamentals of microbiology must always be understood. In this chapter, we have outlined a few experimental approaches for using flow cytometry to study microbial cells. These approaches included generic detection of microorganisms, specific identification of target organisms, cell viability determinations, and Gram staining.

A. Detection of Microbes

Nucleic acid dyes can be combined with light scatter measurements to detect bacteria using flow cytometry. A detailed discussion of bacterial DNA appears

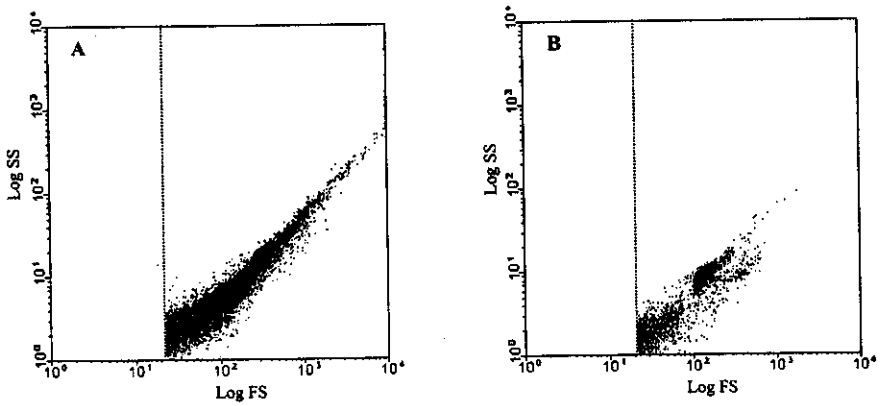

Fig. 2 Changes in light scatter profiles of bacteria due to sample preparation: (A) *Bacillus subtilis* spore slurry in water and (B) washed spores.

in Chapter 54 of this volume. The dye selected for a detection assay should have a high specificity for DNA binding, high extinction coefficient, and high quantum yield. Depending on the available excitation source, 4′,6-diamidino-2-phenylindole (DAPI), Hoechst 33258, propidium iodide, YO-PRO-1, or YOYO-1 could be used for a rapid detection assay. YOYO-1 (Molecular Probes, Inc., Eugene, OR) is a membrane impermeant cyanine dye (excitation 491 nm, emission 509 nm) that is essentially nonfluorescent unless bound to nucleic acids. Dyes that are membrane impermeant will stain only cells that are dead or have compromised membranes. Live cells must be fixed for the dye to pass through the membrane. Rapid fixation with ice-cold 70% ethanol will ensure that the selected dye will enter all cells in the sample and bind to nucleic acids. Alcohol fixation will cause some cell shrinkage and prevent further studies regarding cell viability.

Figure 3 is an example of a rapid detection assay. A "bacteria" region (region F) was created using *E. coli* cells fixed with ice-cold 70% ethanol. Cells were washed briefly with filtered 0.8% NaCl, stained with 0.1 μM solution of YOYO-1 for 5 min in the dark, and then analyzed using flow cytometry (Sincock *et al.*, 1996a). YOYO-1 stained *E. coli* cells were gated on region F; the fluorescence of the gated population was then measured and displayed as a histogram with fluorescence intensity on the *x*-axis and the number of cells on the *y*-axis. The background noise was determined using 0.2 μm-filtered 0.8% NaCl.

Samples containing dust, pollen, fungal spores, or unknown bacteria were tested with this assay. Particles in the test samples that met the light scatter requirements (bacteria region) and stained positive for nucleic acids were classified as bacteria. Although pollen, mold, and fungal spores contain nucleic acids and will stain with YOYO-1, they do not meet the light scatter gating require-

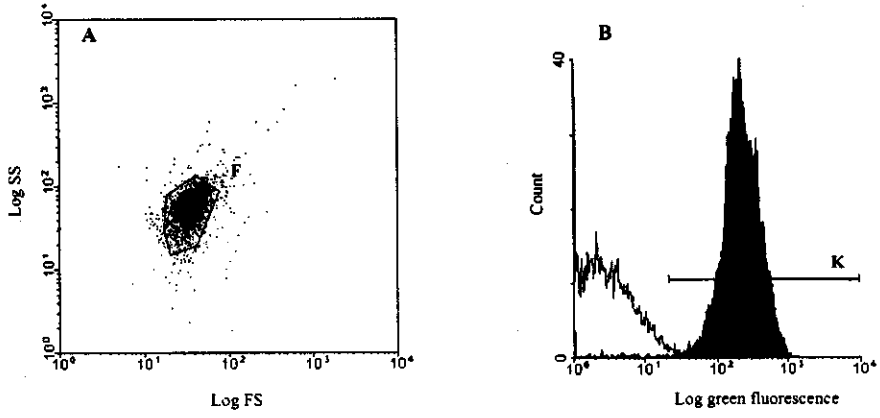

Fig. 3 Detection of *E. coli* cells in environmental samples using YOYO-1 nucleic acid stain. (A) Light scatter measurements of ethanol-fixed *E. coli* cells (region F). (B) Fluorescence histogram overlay of YOYO-1 stained (■) and unstained *E. coli* cells (□).

ments due to their large size (20–100 μm) and therefore can be excluded. YOYO-1 will not stain dust particles that fall within the bacteria region because they do not contain nucleic acids.

This assay can be used for generic detection of bacteria within a heterogeneous sample but cannot be used for specific identification. In mixed populations of bacteria (i.e., *E. coli* combined with *Staphylococcus aureus*), it was not possible to discriminate between bacteria using light scatter or by using differences in relative staining.

B. Identification of Specific Microorganisms

During the 1990s, experimental approaches to identifying microorganisms in liquid samples using flow cytometry have included light scatter profiles, DNA content, immunoassays, neural nets, and rRNA probes, with varying degrees of success. Some of these areas are discussed in detail in other chapters of this volume (Chapters 54, 55); however, a brief introduction to this material follows.

1. Light Scatter Measurements

Light scattering profiles are a function of cellular size, shape, and refractive index of a cell. Morphological features of bacteria that can influence light scatter profiles include shape (rods, cocci, vibrios, spirilla, spirochetes), flagella, pilli, and capsules. Growth conditions, cell source, and responses to stress (i.e., starvation, antimicrobial exposure) can also influence light scatter profiles.

Light scatter profiles are a useful first step in characterizing microorganisms. Identifying specific organisms within mixed populations is difficult. Allman *et al.* (1993) collected dual-parameter contour plots of forward versus side scatter for artificial mixtures of clinically relevant microorganisms using an arc lamp-based cytometer. Mixtures of vegetative cells (i.e., *Salmonella typhimurium, Legionella pneumophila, Staphylococcus aureus*) had overlapping light scatter profiles. However, light scatter profiles could be used to resolve spore-forming bacteria (i.e., *Clostridium perfringens*) from vegetative cells. Spores give a forward light scatter signal that is out of proportion to their size, which may be explained on the basis of a high value for their refractive index (Allman *et al.*, 1993). Using a cytometer specifically designed for small particles (Bio-Rad Bryte HS), light scatter profiles could also be used to resolve populations of closely related gram-positive spores (Sincock *et al.*, 1996b) (Fig. 4A). A laser-based commercial cytometer (Coulter EPICS XL, Hialeah, FL) could not resolve a mixture of *E. coli, S. aureus,* and *Bacillus subtilis* spores using light scatter measurements (Fig. 4B).

2. DNA Content

Using flow cytometry, DNA base composition of individual cells within a bacterial sample can be determined without extraction of DNA. Van Dilla *et al.*

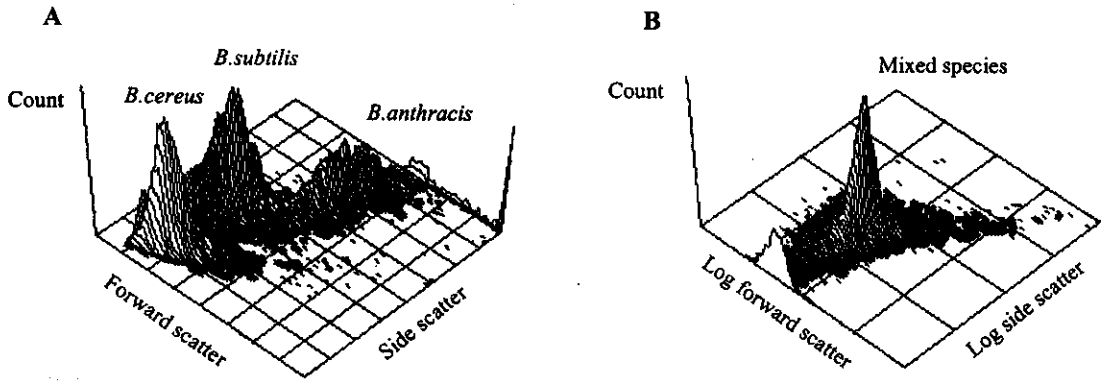

Fig. 4 Isometric plots of forward scatter versus side scatter for artificial mixtures of bacterial species: (A) mixture of three closely related *Bacillus* spores (Bio-Rad Bryte flow cytometer) and (B) mixture of *E. coli* and *S. aureus* and *Bacillus subtilis* spores (Coulter EPICS XL flow cytometer).

(1983) used a combination of DNA-specific fluorochromes to analyze six species of ethanol-fixed bacteria with differing DNA base composition. Using a combination of Hoechst 33258, a fluorochrome that binds preferentially to the regions of DNA rich in AT base pairs, and chromomycin A3, a fluorochrome that binds preferentially to regions of DNA rich in GC base pairs, this group established a direct relationship between the fluorescence dye ratio calculated by flow cytometry and the % Guanine + Cytosine (%[G + C]) content. This method was able to resolve individual species within an artificial mixture of *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* based on differences in DNA content. Each species formed a distinct cluster within the dual-parameter fluorescence histogram. However, further studies using this method suggested that flow cytometric determination of %[G+C] be limited to samples containing only one bacterial species (Sanders *et al.,* 1990).

3. Immunofluorescence Approach

Fluorescently labeled antibodies combined with light scatter measurements can be used for the specific identification of microorganisms. Microbes that have been identified using a flow cytometric immunoassay include pathogenic microorganisms found in food, water, sewage, and aerosols (Table I). An example of a direct flow cytometric immunoassay can be found in Fig. 5. *Escherichia coli* O157:H7 cells at a concentration of 10^6 cells/ml were incubated with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-*E. coli* O157:H7 polyclonal antibody for 5 min at room temperature and analyzed by flow cytometry. For this assay, the desired population of cells was selected by gating on light scatter signals. A discriminator was set on forward scatter and used to resolve bacteria from noncellular material and electronic noise. The fluorescence of the gated

Table I
Examples of Microbes Identified Using a Flow Cytometric Immunoassay

Microbe	References
Food	
Escherichia coli O157:H7	Seo *et al.* (1998a,b); Tortorello *et al.* (1998)
Listeria monocytogenes	Pinder and McClelland (1994); Donnelly and Baigent (1986)
Salmonella typhimurium	Clarke and Pinder (1998); Pinder and McClelland (1994); McClelland and Pinder (1994b)
Salmonella serotypes	McClelland and Pinder (1994a)
Oral Bacteria	
Streptococcus mutans & *Actinomyces viscosus*	Barnett *et al.* (1984)
Streptococcus pyogenes	Sahar *et al.* (1983)
Dental plaque	Obernesser *et al.* (1990)
Aerosols	
Francisella tularensis	Henningson *et al.* (1998)
Water and sewage	
Legionella pneumophila	Ingram *et al.* (1982)
Nitrosomonas serotypes	Volsch *et al.* (1990)
Salmonella spp.	Desmonts *et al.* (1990)
Fecal bacteria	Apperloo-Renkema *et al.* (1992); van der Waaij *et al.* (1994)
Cryptosporidium parvum	Vesey *et al.* (1993, 1997); Valdez *et al.* (1997); Arrowood *et al.* (1995)
Giardia spp.	Dixon *et al.* (1997); Bruderer *et al.* (1994) Heyworth and Pappo (1989)
Biowarfare Agents	
Bacillus anthracis	Sincock *et al.* (1996b); Phillips and Martin (1983, 1988)
Cell surface polysaccharides or proteins	
Bacteroides fragilis	Lutton *et al.* (1991)
E. coli lipopolysaccharide expression	Nelson *et al.* (1991)
Myxococcus virescens	Martinelli *et al.* (1995)
Pseudomonas aeruginosa outer membrane protein	Hughes *et al.* (1996)
Microsphere-based immunoassays	
Helicobacter pylori	Best *et al.* (1992)
E. coli O157:H7	Seo *et al.* (1998a,b)

population was then measured and displayed as a histogram with fluorescence intensity on the x-axis and the number of cells on the y-axis (5000 counts). Target *E. coli* O157:H7 cells were identified and enumerated within a few minutes of obtaining the sample. Culturing of the target organism was not necessary for identification in this direct immunoassay.

Low numbers of target organisms can be identified in the presence of large numbers of nontarget organisms or high levels of background particulate material

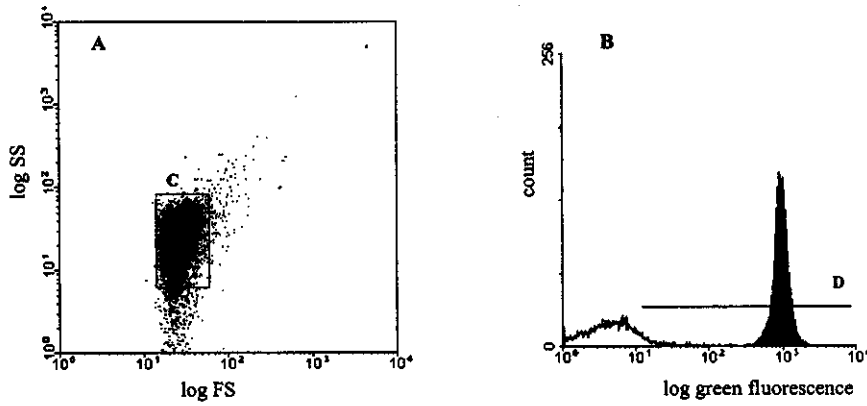

Fig. 5 (A) Dual-parameter histogram of forward versus side scatter for live *E. coli* O157:H7 cells stained with a FITC-labeled anti-*E. coli* O157:H7 polyclonal antbody. (B) Comparison of stained (■) and unstained (□) *E. coli* O157:H7 cells. Region D represents bacteria stained positive with FITC-labeled antibody. Cells were gated on region C (5000 events).

using nonselective media. Unknown samples are incubated in nonselective media for a short period of time, washed with buffer, and then stained with a specific antibody. Flow cytometry can be used to discriminate target organisms from nontarget organisms by means of specific antibody binding. Enrichment media specific to the nutritional requirements of the target organism can also be used. Ideally, only the target organism will grow. Nutritional supplements can also be used to facilitate expression of specific polysaccharides on bacterial cell surfaces that can be used to discriminate between closely related species. For example, viable *Bacillus anthracis* spores were identified after a brief incubation (20 min, 37°C) in media selected to stimulate the outgrowth and expression of specific polysaccharides on the surface of vegetative cells. After exposure to the food source, *Bacillus anthracis* spores were able to sporulate and transition to vegetative cells. Cells were stained with FITC-conjugated monoclonal antibody specific for *B. anthracis* cell wall polysaccharide and analyzed using flow cytometry (Fig. 6) (Sincock *et al.*, 1996b).

Because cell fixation is not necessary for antibody binding, the immunofluorescence approach can be combined with certain stains to identify viable target organisms. For example, the survival ratio of *Francisella tularensis,* the causative agent of tularemia, was determined before and after aerosolization using a specific anti-*F. tularensis* monoclonal antibody to identify the target organisms together with rhodamine 123 to count the number of viable or metabolically active cells (Henningson *et al.*, 1998). In a second example, Red613-conjugated anti-*Salmonella typhimurium* monoclonal antibody combined with Chemchrome, a live cell stain, was used to detect viable *Salmonella typhimurium* cells in the presence of large number of nontarget and dead organisms (Clarke and Pinder, 1998).

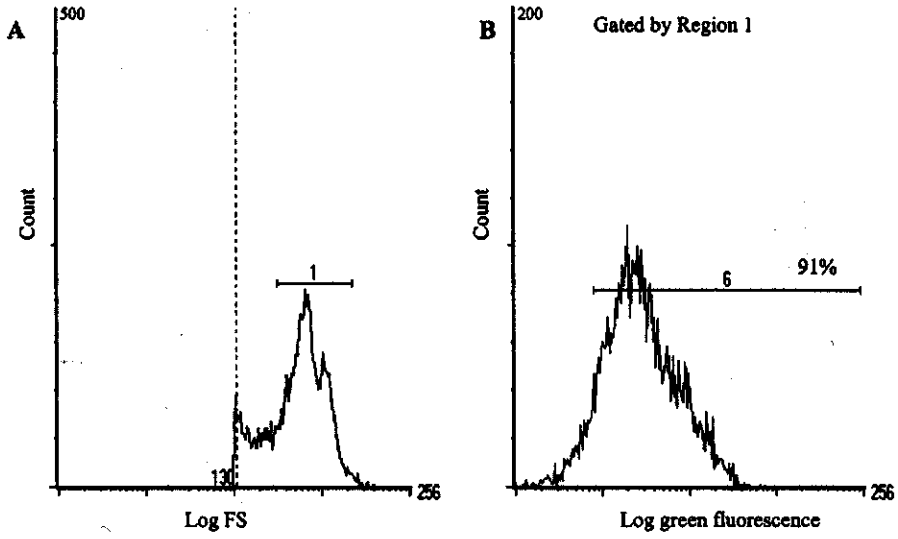

Fig. 6 Flow cytometric analysis of *B. anthracis* spores incubated in polysaccharide media for 30 min at 37°C. A specific FITC-conjugated anti-cell wall polysaccharide MAb stained emerging vegetative *B. anthracis* cells (region 6) (viable cells) but did not stain encapsulated *B. anthracis* cells, dormant *B. anthracis* spores, or other *Bacillus* species. Forward light scatter was used to identify vegetative cells population in the sample (region 1). (See color plates.)

Flow cytometric immunoassays are rapid (usually less than 10 min), sensitive (lower limit of 10^3 cells/ml), specific, require very little sample preparation, and need no cell fixation. Immunoassay-based methods rely on the specificity and sensitivity of the selected antibody to identify the target organism. Unfortunately, very few good antibodies for microbes are commercially available, and in-house antibody development can be time consuming and expensive. High levels of nonbacterial particles, bacterial debris, and antibody aggregates in the test sample can also produce sensitivity problems.

4. Automated Classification and Identification Techniques

Flow cytometry can be used to generate multiparameter data for individual cells. However, the vast quantity of information generated can make data analysis difficult. Artificial neural networks are computing technologies that can be used to discriminate between different cell types based on flow cytometry data (Boddy and Morris, 1993). A computer is "taught" how to recognize data patterns (i.e., staining profiles of different organisms) and to analyze cell populations using examples. Eventually, the neural network can identify specific cell types in real time and adapt to changing conditions (Frankel *et al.*, 1989). Artificial neural networks have been developed for chromosome classification (Errington and

Graham, 1993), leukemia subsets, (Maguire *et al.*, 1994a,b), and phytoplankton populations (Frankel *et al.* 1989). Davey *et al.* (1999) developed an artificial neural network for detection and identification of *Bacillus globigii* spores against a background of other microorganisms (*Escherichia coli, Micrococcus luteus, Saccharomyces cerevisiae*). Data sets were collected for microorganisms stained with six cocktails of fluorescent stains. These stains included Tinopal CBS-X, Nile Red, propidium iodide, FITC, $DiSC_2(5)$, Oxonol V, SYTO 17, and TO-PRO-3. Forward scatter, side scatter, and autofluorescence measurements were also included in the data sets. Careful selection of the staining cocktail and data analysis method allowed accurate identification of the target organism (*Bacillus* spores). Trained neural networks may be useful in identifying specific organisms against a high background of particulate matter or discriminating between closely related organisms in real time. Applications may include food analysis, clinical microbiology samples, and identification of biowarfare agents.

C. Cell Viability

Fluorescent dyes have been successfully used as indicators of cell viability in fluorescence microscopy and flow cytometry. Using these dyes, live and dead cells within a heterogeneous sample population can be identified and counted within a few minutes. Traditional methods employed to detect and enumerate bacteria (such as growth on laboratory media) require time (24 to 48 hr) and may underestimate the number of viable bacteria. Therefore, direct methods for the assessment of microbial viability are of increasing importance. Because each technique has its limitations, each investigator must choose the experimental approaches that are best suited for the test organisms and the specific questions being asked.

1. Membrane Integrity

Membrane integrity analysis is based on the capacity of bacterial cells to exclude certain compounds. Stains that are commonly used to determine membrane integrity include ethidium bromide, propidium iodide, and SYTOX Green dead cell stain. These dyes passively enter stressed, injured, or dead cells via damaged membranes and intercalate into DNA and RNA. The fluorescence indicates a loss of viability or membrane integrity. Flow cytometry can be used to quantify the fluorescence associated with dead or injured cells. Because the influx of the dye can be correlated with the extent of the bacterial wall permeability, the number of fluorescent cells counted using flow cytometry is inversely proportional to the number of viable cells. These dye exclusion methods have been successfully used to monitor antibiotic-induced changes in bacterial membrane permeability (Gant *et al.*, 1993). For example, the oral pathogen *Streptococcus mutans* was treated with the antibiotic clindamycin and then stained with

SYTOX Green (Fig. 7). After 2 hr of exposure to the antibiotic, a significant number of cells were dead, as indicated by strong green fluorescence.

Membrane integrity analysis is not suitable for all cell types because some bacteria can rapidly pump out dyes using an efficient efflux pump (Jernaes and Steen, 1994). In this case, damaged or injured cells would not fluoresce and would be counted as viable.

2. Membrane Potential

Membrane potential analysis is based on the selective permeability and active transport of charged molecules through intact membranes. Cells with a membrane potential actively take up lipophilic, cationic dyes or actively exclude lipophilic, anionic dyes. Using flow cytometry, any particle in the approximate size range of bacteria that is found to have a membrane potential can be identified as a viable organism. However, organisms can show considerable variation in dye uptake due to differences in membrane potential (Allman *et al.*, 1993).

Using the lipophilic cation rhodamine 123, which preferentially accumulates within viable cells, several groups have been able to discriminate between live, dead, and dormant cells in culture. Viable and nonviable cells have been enumerated using flow cytometry (Kaprelyants and Kell, 1992, 1993a,b; Kaprelyants *et al.*, 1993). Studies using this dye have determined that dye uptake is variable both between species and among cells from the same culture (Porter *et al.*, 1995). In addition, this dye can be used for gram-negative bacteria only after they have been treated with EDTA (Diaper *et al.*, 1992).

In contrast to rhodamine 123, the lipophilic oxonol dyes are anionic and preferentially accumulate within dead bacteria; they have been used to assess

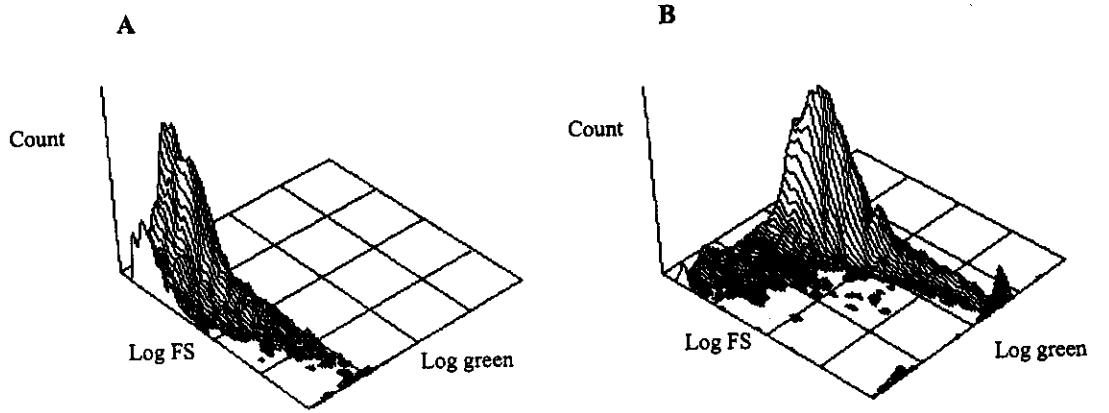

Fig. 7 Isometric plots of log green fluorescence versus log forward scatter for *Streptococcus mutans* cells stained with SYTOX Green after exposure to clindamycin (10× MIC) for (A) 0 hr and (B) 2 hr.

bacterial antibiotic susceptibility (Deere *et al.*, 1995; Mason *et al.*, 1995b) and cell viability (Jepras *et al.*, 1995; Mason *et al.*, 1995a) by flow cytometry. In these studies, either heat or bactericidal antibiotics were used to kill cells prior to oxonol staining, and comparisons were made with untreated cells. Figure 8 is a fluorescence histogram overlay of *E. coli* cells treated with gentamicin at 10 times the mimimum inhibitory concentration (10× MIC) and then stained with bis(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$]. Over time, the number of dead cells increased as indicated by an overall shift in green fluorescence.

3. Enzymatic Activity

Flow cytometric detection of intracellular enzymatic activity utilized lipophilic, uncharged, nonfluorescent derivatives such as fluorescein diacetate (FDA) that readily diffuse across cell membranes. Once inside the cell, the derivative is hydrolyzed by nonspecific esterases to release the highly fluorescent parent compound. Because the parent compound is polar and charged, it is retained inside the cells with intact membranes. Dead or dying cells with compromised membranes rapidly leak the dye.

Flow cytometry can be used to detect the number of viable bacteria and to verify the metabolic activity of these cells (Diaper and Edwards, 1994; Diaper *et al.*, 1992). However, FDA does not efficiently penetrate some types of membranes and the fluorescein product tends to leak from cells or can be actively pumped out (Edwards, 1996). Other related fluorescent compounds such as

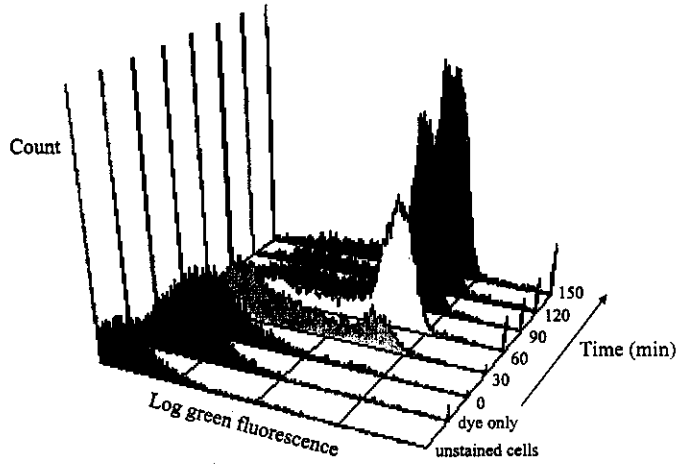

Fig. 8 Fluorescence histogram overlays of *E. coli* cells stained wiith $DiBAC_4(3)$ (oxonol) after exposure to 10× MIC gentamicin. Cells were stained with oxonol after 0, 30, 60, 90, 120, or 150 min of drug incubation.

carboxyfluorescein diacetate (CFDA) and sulfofluorescein diacetate (SFDA) exhibit similar problems (Tsuji *et al.*, 1995).

4. Bacterial Respiration

The redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was first employed for the direct microscopic enumeration of respiring bacteria in environmental samples. CTC is readily reduced via electron transport activity to insoluble, highly fluorescent, and intracellularly accumulated CTC–formazan through bacterial respiration. Actively respiring bacteria (red fluorescence) can be distinguished from nonrespiring bacteria and abiotic material. More recent studies have used flow cytometry to enumerate respiring bacteria in lakes (del Giorgio *et al.*, 1997), marine systems (López-Amorós *et al.*, 1998), and after exposure to antibiotics (Suller and Lloyd, 1999).

However, several problems associated with using CTC have been identified. The CTC assay may not be sensitive enough to detect low respiration rates of microorganisms, especially in very small bacteria. In addition, not all bacteria are able to reduce tetrazolium salts. It is also thought that CTC may have an inhibitory effect on bacterial metabolism (Ullrich *et al.*, 1996; Yu *et al.*, 1995).

D. Identification of Viable Bacteria with Fluorescent *in Situ* Hybridization

Staining with membrane-integrity or membrane-potential fluorochromes offers limited information on the numbers of viable bacterial cells within a sample and none at all about their identity. Staining with fluorochromes that preferentially bind to specific DNA base pairs offers limited information on species identification but not cell viability. Fluorescent *in situ* hybridization can be used to label specific nucleic acid sequences inside intact, viable cells and identify species of bacteria present in the sample. Probe binding to ribosomal RNA (rRNA) is perhaps the best target for bacterial cells.

rRNA can be found in all bacteria and consists of both highly conserved and variable regions. Synthetic probes have been developed that can target sections of the rRNA based on the amount of conserved and variable regions. Appropriate probes can be composed of oligonucleotide sequences that distinguish between the primary kingdoms (eukaryotes, eubacteria, archaebacteria) and between closely related organisms (DeLong *et al.*, 1989). Probes that target very conserved regions can be used as universal probes to measure total rRNA within a sample (Amann *et al.*, 1990).

The rRNA content of microorganisms is proportional to the growth rate in pure culture. Using microfluorimetry, DeLong *et al.* (1989) quantified the binding of a universal rRNA probe to *E. coli* cells grown in media that support different growth rates. The fluorescence intensity of single cells due to hybridization with the universal probe varies linearly with growth rate and can be used to estimate the growth rate of that particular organism in a natural population. Further

studies conducted by Wallner *et al.* (1993) demonstrated that 16S rRNA probe-conferred fluorescence is directly proportional to ribosome content. Because the amount of fluorescence can be correlated with cellular rRNA content, it is possible to obtain information on the physiological state (i.e., growth rate, activity, viability) of specific bacterial cells (Manz *et al.*, 1993; Wallner *et al.*, 1993). Due to the abundance of cellular ribosomes in rapidly growing cells (approximately 10^4 to 10^5 per cell), the binding of fluorescent probes to individual cells can be readily visualized (DeLong *et al.*, 1989).

After appropriate selection, rRNA-targeted oligonucleotides can be sequenced, labeled with an appropriate fluorochrome, and used as probes in hybridization experiments. After hybridization, the fluorescence conferred by rRNA-targeted oligonucleotide probes can be analyzed by flow cytometry (Rice *et al.*, 1997; Thomas *et al.*, 1997; Simon *et al.*, 1995; Lange *et al.*, 1997; Wallner *et al.*, 1993, 1995; Amann *et al.*, 1990) or confocal microscopy (Amann *et al.*, 1996).

E. Gram Stain

Gram staining is the most commonly used procedure in clinical microbiology laboratories. Specimens are smeared on glass slides, heat fixed, Gram stained, and examined microscopically. Based on the outcome of the Gram reaction, bacteria are divided into two taxonomic groups. Cells stained purple-blue are gram-positive; cells stained red are gram-negative. This technique is relatively simple, albeit messy. However, some organisms can show gram variability (i.e., *Acinetobacter* species), particularly anaerobes.

Sizemore *et al.* (1990) reported on the use of a fluorescently labeled lectin as an alternative Gram staining technique. Lectin isolated from *Triticum vulgaris*, or wheat germ agglutinin (WGA), will bind specifically to *N*-acetylglucosamine in the outer peptidoglycan layer of gram-positive bacteria. Gram-negative bacteria have an outer membrane covering the peptidoglycan layer that prevents lectin binding. Using this method, heat-fixed bacterial smears were covered with a small aliquot of FITC-conjugated WGA (100 μg/ml), washed briefly with phosphate buffer, and observed using fluorescence microscopy. Unlike the Gram staining method, culture age did not affect lectin binding, suggesting that this technique can be used directly on samples without culturing and may offer an alternative method to classify fastidious, slowly growing, or viable but nonculturable organisms. In theory, flow cytometry could be used to extend this technique.

Flow cytometry has been used to determine the Gram stain of unfixed cells using $DiIC_1(5)$ (Shapiro, 1995) or rhodamine 123 (Allman *et al.*, 1993). More recently, Mason *et al.* (1998) developed a two-color flow assay for mixed populations of bacteria in suspension. Bacterial strains isolated from clinical specimens were cultured overnight, washed, and then stained with a combination of fluorescent nucleic acid-binding dyes hexidium iodide (excitation 488 nm, emission 605 nm) and SYTO 13 (excitation 488 nm, emission 509 nm). Hexidium iodide (HI) preferentially penetrates gram-positive bacteria, whereas SYTO 13 enters both

gram-positive and gram-negative bacteria. When used in combination, these dyes allow differential labeling of unfixed gram-positive bacteria (HI and SYTO 13, red-orange fluorescence) and gram-negative bacteria (SYTO 13 only, green fluorescence) in suspension (Mason *et al.*, 1998). Using this method, artificial mixtures of *E. coli* and *S. aureus* cells analyzed using flow cytometry were clearly separated using fluorescence. Total time needed for this assay was 15 min.

III. Applications in Medical and Food Microbiology

To date, the most frequent application of flow cytometry to the study of microorganisms is the field of environmental microbiology, where rapid assessment of bacterial viability in natural samples is important. The rapid methods first described in these studies have been adapted for use in medical and food microbiology. In these areas, flow cytometry can significantly shorten the analysis time required for detection and identification of bacteria compared with conventional detection procedures and provide additional information on responses of individual cells.

A. Antimicrobial Agents

Flow cytometry permits rapid analysis of individual bacterial, fungal, or protozoan responses to antimicrobial agents. Antimicrobial agents such as antibiotics, disinfectants, and antiseptics are used to reduce the number of microorganisms to a level that is insufficient to transmit infection. Antibiotics are products of the metabolism of a microorganism that are inhibitory to other microorganisms. Disinfectants are chemical or physical agents used to kill pathogenic microorganisms on nonliving objects (i.e., sink, table); antiseptics are chemicals used to kill microbes on a living object (i.e., skin, mouth). Flow cytometry can be used to investigate physiological and morphological changes that can occur after drug exposure, even if little is known about a particular antimicrobial agent.

1. Exposure to Antibiotics

Clinical microbiology laboratories devote a great deal of resources to antibiotic susceptibility testing. Routine analysis is limited to growth inhibition assays using fast growing, nonfastidious bacteria. Flow cytometry can supply valuable additional information on the response of individual cells to antibiotic exposure within a short period of time and provide an indication of population dynamics within the heterogeneous test sample. For example, gentamicin was added to early exponential phase *E. coli* cells in broth and incubation was allowed to continue for 5 hr. Untreated *E. coli* cells were used as controls. At timed intervals, aliquots of treated and untreated cells were removed, stained, and analyzed. Membrane perturbation was assessed using the membrane potential-sensitive dye

$DiBAC_4(3)$ and the membrane integrity dye propidium iodide. Dual-parameter histograms of log forward scatter versus log fluorescence suggest that membrane potential of the treated cells collapsed after 5 hr; however, a subpopulation of treated cells maintained membrane integrity (Fig. 9).

Table II is a brief summary of work using flow cytometry to investigate the effect of antibiotic and antifungal agents on target organisms. Procedures for antibiotic susceptibility testing using flow cytometry are described in detail in Chapter 55 of this volume.

2. Exposure to Disinfectants or Antiseptics

Traditional assessment of disinfectant efficacy involves the incubation of microbes in liquid or on solid media for 24 to 48 hr. Most bacteria will not grow in the presence of low concentrations of disinfectants. To avoid this inhibitory effect, disinfectant compounds must be inactivated or neutralized before treated cells are incubated in media or plated. In addition, some cells will experience a

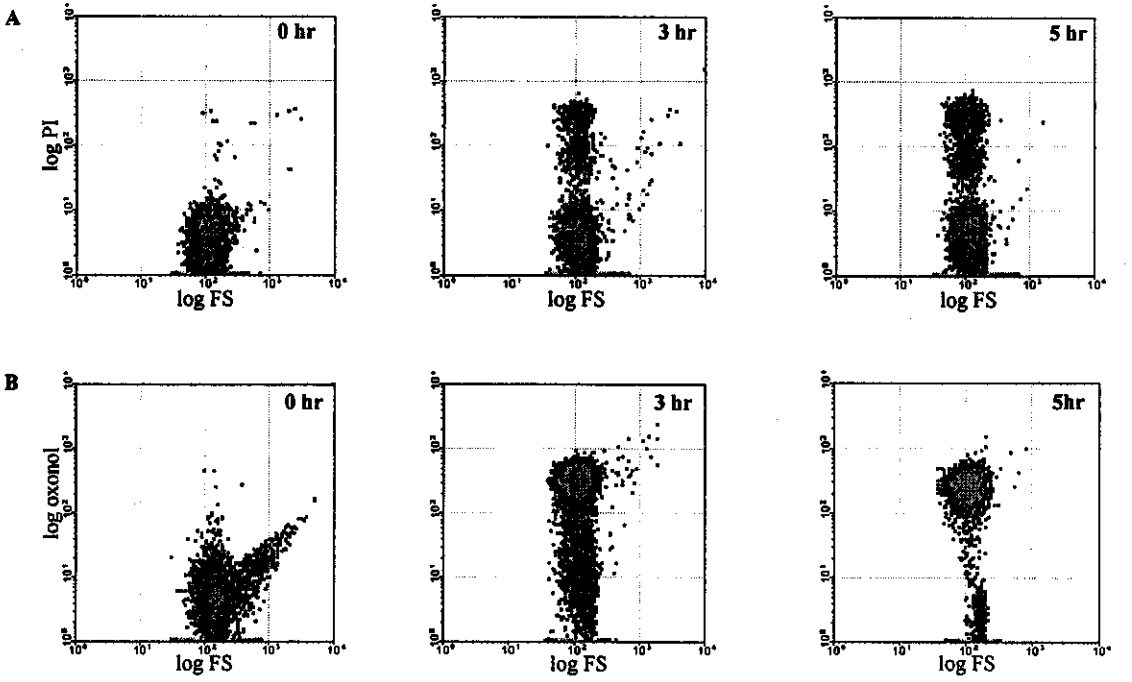

Fig. 9 Dual-parameter density dot plots of log forward scatter versus log fluorescence for *E. coli* cells stained with (A) propidium iodide (PI) or (B) $DiBAC_4(3)$ (oxonol), after exposure to gentamicin for 0, 3, and 5 hr.

Table II
Rapid Antimicrobial Susceptibility Testing Using Flow Cytometry

Bacteria: Dye	Species	Antibiotic	References
Ethidium bromide (EtBr)	*E. coli, P. aeruginosa, S. aureus, P. mirabilis, S. pyogenes*	Amikacin	Cohen and Sahar (1989)
EtBr + mithramycin	*E. coli*	Ceftazidime, ciprofloxacin, gentamicin	Walberg *et al.* (1997a)
	E. coli, K. pneumoniae	Ampicillin	Walberg *et al.* (1997b)
Acridine orange	*E. coli*	Gentamicin	Mason and Lloyd (1997)
Propidium iodide (PI)	*E. coli*	Gentamicin, mecillinam, cefotaxime, ampicillin, ciprofloxacin	Gant *et al.* (1993)
	E. coli, P. aeruginosa	Ampicillin, ceftriaxone, ciprofloxacin, rifampin, imipenem	Gottfredsson *et al.* (1998)
Live/Dead BacLight	*Propionibacterium acnes*	Lymecycline, minocycline	Arrese *et al.* (1998)
	L. monocytogenes	Bacteriocin	Swarts *et al.* (1998)
	E. coli, S. aureus, P. aeruginosa	Ceftazidime, ampicillin, vancomycin	Suller and Lloyd (1999)
SYTOX Green	*E. coli, S. aureus, B. cereus*	Ampicillin, amoxicillin, penicillin G, vancomycin	Roth *et al.* (1997)
	E. coli, S. aureus, P. aeruginosa	Ceftazidime, ampicillin, vancomycin	Suller and Lloyd (1999)
Rhodamine 123	*E. coli, P. fluorescens, E. aerogenes, A. globiformis*	Valinomycin	Porter *et al.* (1995)
$DiBAC_4(3)$ (oxonol)	*E. coli*	Azithromycin, cefuroxime, ciprofloxacin	Jepras *et al.* (1997)
	E. coli, S aureus, P. aeruginosa	Gramicidin S	Jepras *et al.* (1995)
	S. aureus	Methicillin	Suller and Lloyd (1998); Suller *et al.* (1997)
	E. coli, S. aureus	Ampicillin, gentamicin, ciprofloxacin	Mason *et al.* (1994)
	Aeromonas salmonicida	Gentamicin	Deere *et al.* (1995)
	E. coli, S. aureus, P. aeruginosa	Ceftazidime, ampicillin, vancomycin	Suller and Lloyd (1999)
Fluorescein diacetate (FDA)	*Mycobacterium tuberculosis*	Ethambutol, isoniazid, rifampin	Kirk *et al.* (1998)
CTC	*S. aureus*	Methicillin	Suller and Lloyd (1998)
	E. coli	Ciprofloxacin	Mason *et al.* (1995b)
	E. coli, S. aureus, P. aeruginosa	Ceftazidime, ampicillin, vancomycin	Suller and Lloyd (1999)
FITC	*E. coli*	Amoxycillin, mecillinam, chloramphenicol, ciprofloxacin trimethoprim	Durodie *et al.* (1995)

Yeast: Dye	Species	Antifungal Agent	References
Propidium iodide	*Candida albicans, S. cerevisiae, Cryptococcus neoformans*	Amphotericin B, fluconazole, cilofungin	Green *et al.* (1994)
	C. albicans, C. krusei, C. parapsilosis	Amphotericin B, fluconazole	Ramani *et al.* (1997)
Ethidium bromide	*Candida* spp.	Amphotericin B	O'Gorman and Hopfer (1991)
$DiOC_5(3)$ (oxonol)	*Candida* spp., *T. glabrata*	Amphotericin B	Peyron *et al.* (1997)
	C. albicans, C. tropicalis	Amphotericin B	Ordóñez and Wehman (1995)
FUN-1	*C. albicans*	Amphotericin B, flucytosine, fluconazole, ketoconazole	Wenisch *et al.* (1997)

lag of regrowth, similar to the postantibiotic effect, after exposure to disinfectants. For example, chlorhexidine delays regrowth after exposure for more than 2 hr. Flow cytometry combined with fluorescent probes allows the activity of disinfectant compounds on target organisms to be ascertained within a few minutes and provides information on the heterogeneity of the sample population.

Sheppard *et al.* (1997) used oxonol and propidium iodide to monitor chlorhexidine-induced membrane damage in stationary and log phase *E. coli* cells. Their results indicated that membrane potential (oxonol) of cells collapsed prior to loss of membrane integrity (propidium iodide). Increased light scattering properties of organisms exposed to higher chlorhexidine concentrations suggest that there are also major changes to internal cellular structure. Comas and Vives-Rego (1997) used rhodamine 123, bis-oxonol, propidium iodide, SYTO-13, and SYTO-17 to assess the effect of formaldehyde and surfactants [i.e., sodium dodecyl sulfate (SDS), benzalkonium chloride] on *E. coli.*

Paul *et al.* (1996) used oxonol to determine the effectiveness of oral antiseptics found in mouthwash and toothpaste to kill bacteria such as *Streptococcus mutans, Streptococcus sanguis,* and *Streptococcus oralis* that cause tooth decay and gum disease. Membrane potential damage after 30 sec of exposure to triclosan, chlorohexidine, or cetylpyridinium chloride at 5× MIC was assessed using flow cytometry and compared to plate-count data. Flow cytometry provided information within minutes on the immediate effect of oral antiseptics on target bacteria; plate-count data required 24 to 48 hr.

In our laboratory, we have developed a rapid flow cytometric assay to evaluate alternative disinfectant processes. Outbreaks of cryptosporidiosis have been attributed to the inability of chlorine to inactivate the oocyst form of *Cryptosporidium parvum.* Gamma (γ) irradiation may be a viable alternative to conventional chlorine-based wastewater disinfection processes.

Purified *Cryptosporidium parvum* oocysts were exposed in batch reactors to γ-irradiation from a ^{60}Co source. Exposures to γ-irradiation ranged from 50 to 800 krad. Untreated oocysts, heat-killed (70°C for 30 min) control oocysts, and irradiated oocysts were stained with SYTOX Green dead cell stain (10 μM final concentration), incubated at 37°C for 1 hr, and counted using flow cytometry (Fig. 10). Differences in light scattering properties were used to differentiate oocysts from sporozoites, ghosts (oocyst shells), and debris. After exposure to γ-irradiation, the oocysts were morphologically intact, but the process damaged the oocyst wall and allowed SYTOX Green, a membrane integrity stain, to enter and bind to nucleic acids. Nonviable oocysts with damaged but intact walls fluoresced bright green; viable oocysts and ghosts did not stain. Flow cytometry was used to count the number of damaged or inactivated oocysts after disinfectant exposure (Sincock *et al.,* 1998).

B. Food and Drink

Flow cytometry has been used to detect and identify pathogenic microorganisms in food samples and to monitor food and drink products for spoilage microor-

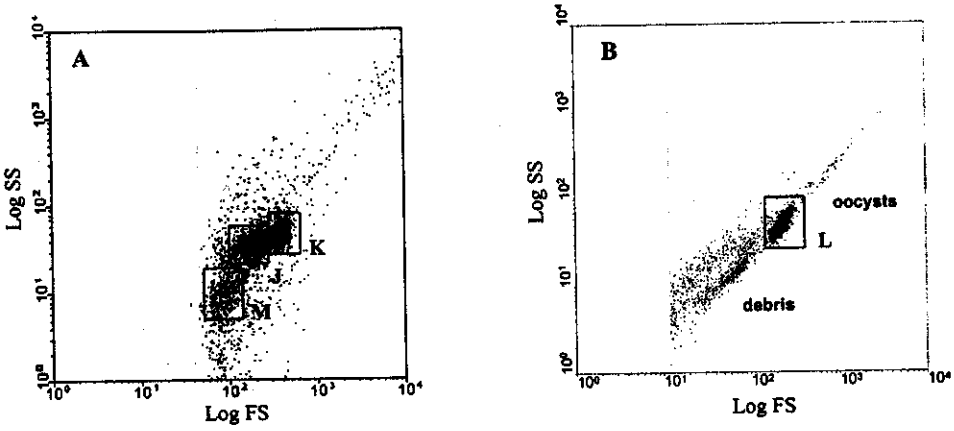

Fig. 10 Flow cytometric analysis of *Cryptosporidium parvum* oocysts exposed to γ-irradiation from a ^{60}Co source: (A) 200-krad dose: (B) 800-krad dose. Intact cysts (region L, K) can be differentiated from sporozoites (region J), ghosts (region M), and debris using light scatter measurements. High doses of γ-irradiation prevented excystation of sporozoites from oocysts.

ganisms. Food pathogens that can be detected and identified using flow cytometry include *Listeria monocytogenes* in raw milk (Donnelly and Baigent, 1986), *Salmonella typhimurium* in eggs and milk (Pinder and McClelland, 1994; McClelland and Pinder, 1994b), and *Escherichia coli* O157:H7 in ground beef, apple juice, and milk (Seo *et al.*, 1998a,b; Tortorello *et al.*, 1998). In general, flow cytometry requires specific monoclonal antibodies to detect and identify food pathogens.

Spoilage microorganisms are not necessarily harmful but can interfere with the quality of a food or drink product and can cause delays in product releases at great economic cost to the manufacturer. To guarantee that food or drink products conform to specifications, flow cytometry has been used to detect spoilage caused by yeast in soft drinks (Pettipher, 1991), yogurt, and fruit juice (Mulard, 1995), and to monitor the viability of yeast used for beer (Jespersen *et al.*, 1993; Jespersen and Jakobsen, 1994), wine (Bruetschy *et al.*, 1994), and cider (Willetts *et al.*, 1997; Lloyd *et al.*, 1996).

In order to identify sources of food contamination and spoilage, a large number of samples need to be tested. Flow cytometry allows the rapid and semiautomated analysis of heterogeneous food samples; however, extensive sample preparation is needed to isolate target organisms from high background levels of nonpathogenic microflora and particulate matter found in food samples. Sample preparation may include homogenization of solid food using a stomacher, filtering of large food particles, serial dilutions, or special reagent addition (i.e., clearing solution to remove micelles in milk and egg samples). After the cells have been isolated from the food sample, enrichment media can be used to increase the number of

target organisms and to allow recovery of stressed or injured cells. Increasing the number of target organisms in food samples is extremely important because the infective dose for some foodborne illnesses can be as low as 10 cells.

IV. Conclusion

It is clear that there are a tremendous number of excellent uses of flow cytometry in the field of microbiology; however, there are some valid problems in implementing this technology. Listed below are what are considered to be the main advantages and disadvantages in the application of flow cytometry to microbial systems.

1. Advantages of using flow cytometry to analyze microbes

a. Technology is clinically proven in areas such as leukemia/lymphomas, HIV monitoring, platelet studies, and functional studies. Most hospitals and research centers have already purchased the instrument.

b. Assays can be performed rapidly, usually taking less than 1 min with little to no sample preparation.

c. Sensitivity is high, as low as 10^3 cells/ml reported.

d. Direct detection and identification can be achieved without elaborate or time-consuming culturing of microbes.

e. Cost per test, after initial investment in instrument, is low.

f. Automation/walk-away capability is available in most instruments as well as report generation for clinicians.

g. Flow cytometry is user friendly once the initial protocol is developed.

h. Instrument maintenance (daily/monthly quality control) is minimal.

2. Disadvantages to using flow cytometry to analyze microbes

a. Most microbiologists are not comfortable with using nontraditional, high technology procedures to run routine tests.

b. Initial cost of instrument is high.

c. Few microbial reagents or kits are commercially available for use with flow cytometry. Reagents (i.e., antibodies, DNA probes, control cells) must be developed in-house.

d. Protocols for microbes need to be developed in-house. Because most instruments were designed for mammalian cells, instrument setup and operation must also be modified.

e. Few, if any, instruments are designed specifically for small particles.

f. Little support is available from instrument manufacturers. Service technicians and technical support personnel are not familiar with procedures or methods utilized in microbial flow cytometry.

Clearly, the application of flow cytometry to the field of microbiology involves many unresolved problems; however, the continuing development of detailed protocols, appropriately designed instruments, and fluorescent probes will enable flow cytometry to solidify its position as the technology of preference.

References

Allman, R., Manchee, R., and Lloyd, D. (1993). Flow cytometric analysis of heterogeneous bacterial populations. *In* "Flow Cytometry in Microbiology" (D. Lloyd, ed.), pp. 27–48. Springer-Verlag, New York.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., and Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56,** 1919–1925.

Amann, R., Snaidr, J., Wagner, M., Ludwig, W., and Schleifer, K.-H. (1996). In situ visualization of high genetic diversity in a natural microbial community. *J. Bacteriol.* **178,** 3496–3500.

Apperloo-Renkema, H. Z., Wilkinson, M. H. F., and van der Waaij, D. (1992). Circulating antibodies against faecal bacteria assessed by immunomorphometry: Combining quantitative immunofluorescence and image analysis. *Epidemiol. Infect.* **109,** 497–506.

Arrese, J. E., Goffin, V., Avila-Camacho, M., Greimers, R., and Piérard, G. E. (1998). A pilot study on bacterial viability in acne. Assessment using dual flow cytometry on microbials present in follicular casts and comedones. *Int. J. Dermatol.* **37,** 461–464.

Arrowood, M. J., Hurd, M. R., and Mead, J. R. (1995). A new method for evaluating experimental cryptosporidial parasite loads using immunofluorescent flow cytometry. *J. Parasitol.* **81,** 404–409.

Barnett, J. M., Cuchens, M. A., and Buchanan, W. (1984). Automated immunofluorescent speciation of oral bacteria using flow cytometry. *J. Dent. Res.* **63,** 1040–1042.

Best, L. M., Veldhuyzen van Zanten, S. J. O., Bezanson, G. S., Haldane, D. J. M., and Malatjalian, D. A. (1992). Serological detection of *Helicobacter pylori* by a flow microsphere immunofluorescence assay. *J. Clin. Microbiol.* **30,** 2311–2317.

Boddy, L., and Morris, C. W. (1993). Neural network analysis of flow cytometry data. *In* "Flow Cytometry in Microbiology" (D. Lloyd, ed.), pp. 159–170. Springer-Verlag, New York.

Bruderer, T., Niederer, E., and Köhler, P. (1994). Separation of a cysteine-rich surface antigen-expressing variant from a cloned *Giardia* isolate by fluorescence-activated cell sorting. *Parasitol. Res.* **80,** 303–306.

Bruetschy, A., Laurent, M., and Jacquet, R. (1994). Use of flow cytometry in oenology to analyse yeasts. *Lett. Appl. Microbiol.* **18,** 343–345.

Clarke, R. G., and Pinder, A. C. (1998). Improved detection of bacteria by flow cytometry using a combination of antibody and viability markers. *J. Appl. Microbiol.* **84,** 577–584.

Cohen, C. Y., and Sahar, E. (1989). Rapid flow cytometric bacterial detection and determination of susceptibility to amikacin in body fluids and exudates. *J. Clin. Microbiol.* **27,** 1250–1256.

Comas, J., and Vives-Rego, J. (1997). Assessment of the effects of gramicidin, formaldehyde, and surfactants on *Escherichia coli* by flow cytometry using nucleic acid and membrane potential dyes. *Cytometry* **29,** 58–64.

Davey, H. M., and Kell, D. B. (1996). Flow cytometry and cell sorting of heterogeneous microbial populations: The importance of single-cell analyses. *Microbiol. Rev.* **60,** 641–696.

Davey, H. M., Jones, A., Shaw, A. D., and Kell, D. B. (1999). Variable selection and multivariate methods for the identification of microorganisms by flow cytometry. *Cytometry* **35,** 162–168.

Deere, D., Porter, J., Edwards, C., and Pickup, R. (1995). Evaluation of the suitability of *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol, ($diBAC_4(3)(^-)$), for the flow cytometric assessment of bacterial viability. *FEMS Microbiol. Lett.* **130,** 165–169.

del Giorgio, P. A., Prairie, Y. T., and Bird, D. F. (1997). Coupling between rates of bacterial production and the abundance of metabolically active bacteria in lakes, enumerated using CTC reduction and flow cytometry. *Microb. Ecol.* **34,** 144–154.

DeLong, E. F., Wickham, G. S., and Pace, N. R. (1989). Phylogenetic stains: Ribosomal RNA-based probes for identification of single cells. *Science* **243,** 1360–1362.

Desmonts, C., Minet, J., Colwell, R., and Cormier, M. (1990). Fluorescent-antibody method useful for detecting viable but nonculturable *Salmonella* spp. in chlorinated wastewater. *Appl. Environ. Microbiol.* **56,** 1448–1452.

Diaper, J. P., and Edwards, C. (1994). The use of fluorogenic esters to detect viable bacteria by flow cytometry. *J. Appl. Bacteriol.* **77,** 221–228.

Diaper, J. P., Tither, K., and Edwards, C. (1992). Rapid assessment of bacterial viability by flow cytometry. *Appl. Microbiol. Biotechnol.* **38,** 268–272.

Dixon, B. R., Parenteau, M., Martineau, C., and Fournier, J. (1997). A comparison of conventional microscopy, immunofluorescence microscopy and flow cytometry in the detection of *Giardia lamblia* cysts in beaver fecal samples. *J. Immunol. Methods* **202,** 27–33.

Donnelly, C. W., and Baigent, G. J. (1986). Method for flow cytometric detection of *Listeria monocytogenes* in milk. *Appl. Environ. Microbiol.* **52,** 689–695.

Dubelaar, G. B. J., Gerritzen, P. I., Beeker, A. E. R., Jonker, R. R., and Tangen, K. (1999). Design and first results of CytoBuoy: A wireless flow cytometer for *in situ* analysis of marine and fresh waters. *Cytometry* **37,** 247–254.

Durodie, J., Coleman, K., Simpson, I. N., Loughborough, S. H., and Winstanley, D. W. (1995). Rapid detection of antimicrobial activity using flow cytometry. *Cytometry* **21,** 374–377.

Edwards, C. (1996). Assessment of viability of bacteria by flow cytometry. *In* "Flow Cytometry Applications in Cell Culture" (M. Al-Rubeai and A. N. Emery, eds.), pp. 291–310. Dekker, New York.

Errington, P. A., and Graham, J. (1993). Application of artificial neural networks to chromosome classification. *Cytometry* **14,** 627–639.

Frankel, D. S., Olson, R. J., Frankel, S. L., and Chisholm, S. W. (1989). Use of a neural net computer system for analysis of flow cytometric data of phytoplankton populations. *Cytometry* **10,** 540–550.

Gant, V. A., Warnes, G., Phillips, I., and Savidge, G. F. (1993). The application of flow cytometry to the study of bacterial responses to antibiotics. *J. Med. Microbiol.* **39,** 147–154.

Gottfredsson, M., Erlendsdottir, H., Sigfusson, A., and Gudmunsson, S. (1998). Characteristics and dynamics of bacterial populations during postantibiotic effect determined by flow cytometry. *Antimicrob. Agents Chemother.* **42,** 1005–1011.

Green, L., Petersen, B., Steimel, L., Haeber, P., and Current, W. (1994). Rapid determination of antifungal activity by flow cytometry. *J. Clin. Microbiol.* **32,** 1088–1091.

Henningson, E. W., Krocova, Z., Sandström, G., and Forsman, M. (1998). Flow cytometric assessment of the survival ratio of *Francisella tularensis* in aerobiological samples. *FEMS Microbiol. Ecol.* **25,** 241–249.

Heyworth, M. F., and Pappo, J. (1989). Use of two-colour flow cytometry to assess killing of *Giardia muris* trophozoites by antibody and complement. *Parasitology* **99,** 199–203.

Hughes, E. E., Matthews-Greer, J. M., and Gilleland, H. E., Jr. (1996). Analysis by flow cytometry of surface-exposed epitopes of outer membrane protein F of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* **42,** 859–862.

Ingram, M., Cleary, T. J., Price, B. J., Price, R. L., and Castro, A. (1982). Rapid detection of *Legionella pneumophila* by flow cytometry. *Cytometry* **3,** 134–137.

Jepras, R. I., Carter, J., Pearson, S. C., Paul, F. E., and Wilkinson, M. J. (1995). Development of a robust flow cytometric assay for determining numbers of viable bacteria. *Appl. Environ. Microbiol.* **61,** 2696–2701.

Jepras, R. I., Paul, F. E., Pearson, S. C., and Wilkinson, M. J. (1997). Rapid assessment of antibiotic effects on *Escherichia coli* by *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol and flow cytometry. *Antimicrob. Agents Chemother.* **41,** 2001–2005.

Jernaes, M. W., and Steen, H. B. (1994). Staining of *Escherichia coli* for flow cytometry: Influx and efflux of ethidium bromide. *Cytometry* **17,** 302–309.

Jespersen, L., and Jakobsen, M. (1994). Use of flow cytometry for rapid estimation of intracellular events in brewing yeasts. *J. Inst. Brew.* **100,** 399–403.

Jespersen, L., Lassen, S., and Jakobsen, M. (1993). Flow cytometric detection of wild yeast in lager breweries. *Int. J. Food Microbiol.* **17,** 321–328.

Kaprelyants, A. S., and Kell, D. B. (1992). Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. *J. Appl. Bacteriol.* **72,** 410–422.

Kaprelyants, A. S., and Kell, D. B. (1993a). The use of 5-cyano-2,3-ditolyl tetrazolium chloride and flow cytometry for the visualisation of respiratory activity in individual cells of *Micrococcus luteus*. *J. Microbiol. Methods* **17,** 115–122.

Kaprelyants, A. S., and Kell, D. B. (1993b). Dormancy in stationary-phase cultures of *Micrococcus luteus:* Flow cytometric analysis of starvation and resuscitation. *Appl. Environ. Microbiol.* **59,** 3187–3196.

Kaprelyants, A. S., Gottschal, J. C., and Kell, D. B. (1993). Dormancy in non-sporulating bacteria. *FEMS Microbiol. Rev.* **104,** 271–286.

Kirk, S. M., Schell, R. F., Moore, A. V., Callister, S. M., and Mazurek, G. H. (1998). Flow cytometric testing of susceptibilities of *Mycobacterium tuberculosis* isolates to ethambutol, isoniazid, and rifampin in 24 hours. *J. Clin. Microbiol.* **36,** 1568–1573.

Lange, J. L., Thorne, P. S., and Lynch, N. (1997). Application of flow cytometry and fluorescent in situ hybridization for assessment of exposures to airborne bacteria. *Appl. Environ. Microbiol.* **63,** 1557–1563.

Lloyd, D., Moran, C. A., Suller, M. T. E., and Dinsdale, M. G. (1996). Flow cytometric monitoring of rhodamine 123 and a cyanine dye uptake by yeast during cider fermentation. *J. Inst. Brew.* **102,** 251–259.

López-Amorós, R., Comas, J., García, M. T., and Vives-Rego, J. (1998). Use of the 5-cyano-2,3-ditolyl tetrazolium chloride reduction test to assess respiring marine bacteria and grazing effects by flow cytometry during linear alkylbenzene sulfonate degradation. *FEMS Microbiol. Ecol.* **27,** 33–42.

Lutton, D. A., Patrick, S., Crockard, A. D., Stewart, L. D., Larkin, M. J., Dermott, E., and McNeill, T. A. (1991). Flow cytometric analysis of within-strain variation in polysaccharide expression by *Bacteroides fragilis* by use of murine monoclonal antibodies, *J. Med. Microbiol.* **35,** 229–237.

McClelland, R. G., and Pinder, A. C. (1994a). Detection of low levels of specific *Salmonella* species by fluorescent antibodies and flow cytometry. *J. Appl. Bacteriol.* **77,** 440–447.

McClelland, R. G., and Pinder, A. C. (1994b). Detection of *Salmonella typhimurium* in dairy products with flow cytometry and monoclonal antibodies. *Appl. Environ. Microbiol.* **60,** 4255–4262.

Maguire, D., King, G. B., Kelley, S., and Robinson, J. P. (1994a). Neural network classification of acute leukemias using flow cytometry analysis, data. *Proc. 13th Southern Biomed. Eng. Conf.,* 645–648.

Maguire, D. J., King, G. B., and Robinson, J. P. (1994b). A comparison of hard and soft boundaries of intensity regions for the statistical classification of acute leukemias. *Cytometry* 7(Suppl.), 48 (Abstract).

Manz, W., Szewzyk, U., Ericsson, P., Amann, R., Schleifer, K.-H., and Stenström, T.-A. (1993). In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. *Appl. Environ. Microbiol.* **59,** 2293–2298.

Martinelli, F., Pizzi, R., Cabibbo, E., Licenziati, S., Dima, F., Canaris, A. D., Crea, G., Ravizzola, G., Caruso, A., and Turano, A. (1995). Monoclonal antibodies against antigens exposed on the surface of vegetative forms and spores of *Myxococcus virescens. Microbiologica* **18,** 399–407.

Mason, D. J., and Lloyd, D. (1997). Acridine orange as an indicator of bacterial susceptibility to gentamicin. *FEMS Microbiol. Lett.* **153,** 199–204.

Mason, D. J., Allman, R., Stark, J. M., and Lloyd, D. (1994). Rapid estimation of bacterial antibiotic susceptibility with flow cytometry. *J. Microsc.* **176,** 8–16.

Mason, D. J., López-Amorós, R., Allman, R., Stark, J. M., and Lloyd, D. (1995a). The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. *J. Appl. Bacteriol.* **78,** 309–315.

Mason, D. J., Power, G. M., Talsania, H., Phillips, I., and Gant, V. A. (1995b). Antibacterial action of ciprofloxacin. *Antimicrob. Agents Chemother.* **39,** 2752–2758.

Mason, D. J., Shanmuganathan, S., Mortimer, F. C., and Gant, V. A. (1998). A fluorescent gram stain for flow cytometry and epifluorescence microscopy. *Appl. Environ. Microbiol.* **64,** 2681–2685.

Mulard, Y. (1995). Flow cytometry: Real time microbiology testing. *Food Technol. Eur.* **2,** 72–76.
Nelson, D., Bathgate, A. J., and Poxton, I. R. (1991). Monoclonal antibodies as probes for detecting lipopolysaccharide expression on *Escherichia coli* from different growth conditions. *J. Gen. Microbiol.* **137,** 2741–2751.
Obernesser, M. S., Socransky, S. S., and Stashenko, P. (1990). Limit of resolution of flow cytometry for the detection of selected bacterial species. *J. Dent. Res.* **69,** 1592–1598.
O'Gorman, M. R. G., and Hopfer, R. L. (1991). Amphotericin B susceptibility testing of *Candida* species by flow cytometry. *Cytometry* **12,** 743–747.
Ordóñez, J. V., and Wehman, N. M. (1995). Amphotericin B susceptibility of *Candida* species assessed by rapid flow cytometric membrane potential assay. *Cytometry* **22,** 154–157.
Paul, F., Jepras, R., Hynes, D., Smith, A., and Marken, B. (1996). Activity of common oral antiseptics against bacteria assessed using the oxonol $DiBAC_4(3)$. *Cytometry* 8(Suppl.), 117 (Abstract).
Pettipher, G. L. (1991). Preliminary evaluation of flow cytometry for the detection of yeasts in soft drinks. *Lett. Appl. Microbiol.* **12,** 109–112.
Peyron, F., Favel, A., Guiraud-Dauriac, H., el Mzibri, M., Chastin, C., Duménil, G., and Regli. P. (1997). Evaluation of a flow cytofluorometric method for rapid determination of amphotericin B susceptibility of yeast isolates. *Antimicrob. Agents Chemother.* **41,** 1537–1540.
Phillips, A. P., and Martin, K. L. (1983). Immunofluorescence analysis of Bacillus spores and vegetative cells by flow cytometry. *Cytometry* **4,** 123–131.
Phillips, A. P., and Martin, K. L. (1988). Limitations of flow cytometry for the specific detection of bacteria in mixed populations. *J. Immunol. Methods* **106,** 109–117.
Pinder, A. C., and McClelland, R. G. (1994). Rapid assay for pathogenic salmonella organisms by immunofluorescence flow cytometry. *J. Microsc.* **176,** 17–22.
Porter, J., Pickup, R., and Edwards, C. (1995). Membrane hyperpolarisation by valinomycin and its limitations for bacterial viability assessment using rhodamine 123 and flow cytometry. *FEMS Microbiol. Lett.* **132,** 259–262.
Ramani, R., Ramani, A., and Wong, S. J. (1997). Rapid flow cytometric susceptibility testing of *Candida albicans. J. Clin. Microbiol.* **35,** 2320–2324.
Rice, J., Sleigh, M. A., Burkill, P. H., Tarran, G. A., O'Connor, C. D., and Zubkov, M. V. (1997). Flow cytometric analysis of characteristics of hybridization of species-specific fluorescent oligonucleotide probes to rRNA of marine nanoflagellates. *Appl. Environ. Microbiol.* **63,** 938–944.
Roth, B. L., Poot, M., Yue, S. T., and Millard, P. J. (1997). Bacterial viability and antibiotic susceptibility testing with SYTOX Green nucleic acid stain. *Appl. Environ. Microbiol.* **63,** 2421–2431.
Sahar, E., Lamed, R., and Ofek, I. (1983). Rapid identification of *Streptococcus pyogenes* by flow cytometry. *Eur. J. Clin. Microbiol.* **2,** 192–195.
Sanders, C. A., Yajko, D. M., Hyun, W., Langlois, R. G., Nassos, P. S., Fulwyler, M. J., and Hadley, W. K. (1990). Determination of guanine-plus-cytosine content of bacterial DNA by dual-laser flow cytometry. *J. Gen. Microbiol.* **136,** 359–365.
Seo, K. H., Brackett, R. E., and Frank, J. F. (1998a). Rapid detection of *Escherichia coli* O157:H7 using immunomagnetic flow cytometry in ground beef, apple juice, and milk. *Int. J. Food Microbiol.* **44,** 115–123.
Seo, K. H., Brackett, R. E., Frank, J. F., and Hilliard, S. (1998b). Immunomagnetic separation and flow cytometry for rapid detection of *Escherichia coli* O157:H7. *J. Food Protect.* **61,** 812–816.
Shapiro, H. M. (1995). "Practical Flow Cytometry," 3rd ed. Wiley-Liss, New York.
Sheppard, F. C., Mason, D. J., Bloomfield, S. F., and Gant, V. A. (1997). Flow cytometric analysis of chlorhexidine action. *FEMS Microbiol. Lett.* **154,** 283–288.
Simon, N., LeBot, N., Marie, D., Partensky, F., and Vaulot, D. (1995). Fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. *Appl. Environ. Microbiol.* **61,** 2506–2513.
Sincock, S. A., Anderson, P. E, and Stopa, P. J. (1996a). New fluorescent stains for flow cytometric detection of bacteria. *Cytometry* 8(Suppl.), 128 (Abstract).
Sincock, S. A., Anderson, P. E., Stopa, P. J., and Ezzell, J. (1996b). Rapid detection of pathogenic bacteria using flow cytometry. *Cytometry* 8(Suppl.), 117 (Abstract).

Sincock, S. A., Thompson, J. E., Blatchley III., E. R., Ragheb, K. E., and Robinson, J. P. (1998). Flow cytometry can detect inactivation of *Cryptosporidium parvum* oocysts by γ irradiation. *Cytometry* 9(Suppl.), 46–47 (Abstract).

Sizemore, R. K., Caldwell, J. J., and Kendrick, A. S. (1990). Alternate gram staining technique using a fluorescent lectin. *Appl. Environ. Microbiol.* **56,** 2245–2247.

Steen, H. B. (1980). Further developments of a microscope-based flow cytometer: Light scatter detection and excitation intensity compensation. *Cytometry* **1,** 26–31.

Steen, H. B. (1983). A microscope-based flow cytophotometer. *Histochem. J.* **15,** 147–160.

Steen, H. B., and Boye, E. (1980). Bacterial growth studied by flow cytometry. *Cytometry* **1,** 32–36.

Suller, M. T. E., and Lloyd, D. (1998). Flow cytometric assessmant of the postantibiotic effect of methicillin on *Staphylococcus aureus. Antimicrob. Agents Chemother.* **42,** 1195–1199.

Suller, M. T. E., and Lloyd, D. (1999). Fluorescence monitoring of antibiotic-induced bacterial damage using flow cytometry. *Cytometry* **35,** 235–241.

Suller, M. T. E., Stark, J. M., and Lloyd, D. (1997). A flow cytometric study of antibiotic-induced damage and evaluation as a rapid antibiotic susceptibility test for methicillin-resistant *Staphylococcus aureus. J. Antimicrob. Chemother.* **40,** 77–83.

Swarts, A. J., Hastings, J. W., Roberts, R. F., and von Holy, A. (1998). Flow cytometry demonstrates bacteriocin-induced injury to *Listeria monocytogenes. Curr. Microbiol.* **36,** 266–270.

Thomas, J.-C., Desrosiers, M., St.-Pierre, Y., Lirette, P., Bisaillon, J.-G., Beaudet, R., and Villemur, R. (1997). Quantitative flow cytometric detection of specific microorganisms in soil samples using rRNA targeted fluorescent probes and ethidium bromide. *Cytometry* **27,** 224–232.

Tortorello, M. L., Stewart, D. S., and Raybourne, R. B. (1998). Quantitative analysis and isolation of *Escherichia coli* O157:H7 in a food matrix using flow cytometry and cell sorting. *FEMS Immunol. Med. Microbiol.* **19,** 267–274.

Tsuji, T., Kawasaki, Y., Takeshima, S., Sekiya, T., and Tanaka, S. (1995). A new fluorescence staining assay for visualizing living microorganisms in soil. *Appl. Environ. Microbiol.* **61,** 3415–3421.

Ullrich, S., Karrasch, B., Hoppe, H.-G., Jeskulke, K., and Mehrens, M. (1996). Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride. *Appl. Environ. Microbiol.* **62,** 4587–4593.

Valdez, L. M., Dang, H., Okhuysen, P. C., and Chappell, C. L. (1997). Flow cytometric detection of *Cryptosporidium* oocysts in human stool samples. *J. Clin. Microbiol.* **35,** 2013–2017.

van der Waaij, L. A., Mesander, G., Limburg, P. C., and van der Waaij, D. (1994). Direct flow cytometry of anaerobic bacteria in human feces. *Cytometry* **16,** 270–279.

Van Dilla, M. A., Langlois, R. G., Pinkel, D., Yajko, D., and Hadley, W. K. (1983). Bacterial characterization by flow cytometry. *Science* **220,** 620–622.

Vesey, G., Slade, J. S., Byrne, M., Shepherd, K., Dennis, P. J., and Fricker, C. R. (1993). Routine monitoring of *Cryptosporidium* oocysts in water using flow cytometry. *J. Appl. Bacteriol.* **75,** 87–90.

Vesey, G., Deere, D., Weir, C. J., Ashbolt, N., Williams, K. L., and Veal, D. A. (1997). A simple method for evaluating *Cryptosporidium*-specific antibodies used in monitoring environmental water samples. *Lett. Appl. Microbiol.* **25,** 316–320.

Volsch, A., Nader, W. F., Geiss, H. K., Nebe, G., and Birr, C. (1990). Detection and analysis of two serotypes of ammonia-oxidizing bacteria in sewage plants by flow cytometry. *Appl. Environ. Microbiol.* **56,** 2430–2435.

Walberg, M., Gaustad P., and Steen, H. B. (1997a). Rapid assessment of ceftazidime, ciprofloxacin, and gentamycin susceptibility in exponentially-growing *E. coli* cells by means of flow cytometry. *Cytometry* **27,** 169–178.

Walberg, M., Gaustad, P., and Steen, H. B. (1997b). Rapid discrimination of bacterial species with different ampicillin susceptibility levels by means of flow cytometry. *Cytometry* **29,** 267–272.

Wallner, G., Amann, R., and Beisker, W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14,** 136–143.

Wallner, G., Erhart, R., and Amann, R. (1995). Flow cytometric analysis of activated sludge with rRNA-targeted probes. *Appl. Environ. Microbiol.* **61,** 1859–1866.

Wenisch, C., Linnau, K. F., Parschalk, B., Zedtwitz-Liebenstein, K., and Georgopoulos, A. (1997). Rapid susceptibility testing of fungi by flow cytometry using vital staining. *J. Clin. Microbiol.* **35,** 5–10.

Willetts, J. C., Seward, R., Dinsdale, M. G., Suller, M. T. E., Hill, B., and Lloyd, D. (1997). Vitality of cider yeast grown micro-aerobically with added ethanol, butan-1-ol or iso-butanol. *J. Inst. Brew.* **103,** 79–84.

Yu, W., Dodds, W. K., Banks, M. K., Skalsky, J., and Strauss, E. A. (1995). Optimal staining and sample storage time for direct microscopic enumeration of total and active bacteria in soil with two fluorescent dyes. *Appl. Environ. Microbiol.* **61,** 3367–3372.