

Flow Cytometric Analysis of Microorganisms

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The application of flow cytometry to microorganisms is as old as the technique itself, but it has historically been under-exploited for microbial applications. This is now being reversed and microbiologists are ideally placed to benefit from recent technological advances. While earlier papers demonstrated the use of flow cytometry for studies of viability and taxonomy, recent developments in bioinformatics and reporter gene technologies are leading to novel applications in microbiology. Variants of green fluorescent protein have been used for the study of conditional microbial gene regulation in medically important host–pathogen interactions and fluorescence-activated cell sorting is being applied to the isolation of novel mutants in directed evolution studies. This paper reviews the reasons for the delay in the application of flow cytometry to microbial problems, the range of applications, and their limitations and considers the progress made in developing new strategies for use in microbiological investigations. © 2000

Academic Press

In 1947 Gucker and colleagues (1) developed an instrument for the analysis of dust particles and airborne bacterial spores. This instrument is often quoted as being the first flow cytometer (see, e.g., (2)). Gucker stated in the summary to his paper that “The principle (of flow cytometry) should have wide application in (. . .) bacteriology.” Despite this, flow cytometry (FCM) is still regarded primarily as a technique for the analysis of mammalian cells.

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The small size of microbial cells and the low concentrations of constituents within these cells have historically limited applications of FCM in the field of microbiology. The DNA contents of *Saccharomyces cerevisiae* and *Escherichia coli*, being some 200 and 1000 times less than a normal diploid human cell, were below the detection limits of the early flow cytometers. Twenty years ago the published literature on the subject comprised little more than a series of demonstrations of proofs that the technique could be used with microbial cells (3, 4).

While these papers were useful in their own right, it was not until instruments were designed with the analysis of microorganisms in mind that applications of FCM to clinical, industrial, or even research microbiology really began. These specialized instruments are discussed below.

Specialized Instruments for Microbial Flow Cytometry

The development by Steen and co-workers of a sensitive arc-lamp-based instrument designed specifically for work with bacteria heralded a new age for microbial FCM. The instrument, later to be commercialized as the Skatron Argus flow cytometer, was first described by Steen and Lindmo in 1979 (5). An updated version of this machine was available as the Bio-Rad Bryte HS during the mid-1990s but this too has now unfortunately disappeared from the marketplace. Nevertheless, a number of important developments have been made using these instruments.

Early work by Steen and co-workers (6) demonstrated the role that FCM had to play in monitoring

the cell cycle of bacteria. Prior to this, the cell cycle model had been developed using synchronized cultures. This has the disadvantage that, however carefully synchronization is achieved, some perturbations to the cells and thus the cell cycle may be expected. By studying *individual* cells flow cytometrically, the cell cycle can be studied in a meaningful way using unsynchronized and therefore unperturbed cultures. A comprehensive review of this work can be found in Skarstad *et al.* (7). Steen and colleagues, and indeed most other users of arc-lamp-based flow cytometers, have found the combination of mithramycin and ethidium bromide to be optimal for measurements of DNA contents of microbial cells (see also Fig. 1).

Extending the use of DNA staining beyond that of cell cycle analysis, the arc-lamp-based flow cytometers have also been used to assess the effects of antibiotics on bacterial cells (8, 9). This approach has potential clinical benefits as a rapid pretreatment screen to ensure that the most appropriate drug is used. In a lateral approach, it may also be used as a means of distinguishing between different organisms based on their differing responses to the antibiotics (10). While the majority of work with antibiotics has studied their effects on DNA replication, Suller and Lloyd (11) have used a Skatron Argus to investigate the effects of antibiotics on bacterial viability using a variety of stains. Two groups independently found rhodamine 123 to be an effective stain for bacterial viability measurements (12, 13). Providing that the concentration of rhodamine 123 is kept below $0.5 \mu\text{M}$ (14) and appropriate controls are used (15) effective measurements of cell viability can be obtained.

While FCM is generally a laboratory-based technique, there are some situations in microbiological applications (e.g., environmental sampling) where a portable instrument would be advantageous. One approach has been to use conventional flow cytometers in vehicles or on-board ship (16). An alternative and more flexible approach was adopted by Gjelsnes and Tangen (17) who developed the Microcyte flow cytometer. The Microcyte is a portable flow cytometer, designed primarily for the analysis of microorganisms (18, 19). The instrument is small, with dimensions of $33 \times 43 \times 16$ cm and a weight of ~ 15 kg. The light source is a low-power 635-nm laser diode and the instrument can be powered from internal batteries. The portable nature of this in-

strument should enable the technique of FCM to be used for a broad range of environmental applications without the necessity for transporting samples to the laboratory.

The Partec PAS III particle analyzing system (Partec GmbH, Münster, Germany) has recently been launched and offers a modular FCM system for a variety of applications including the analysis of bacteria and yeast (for more information see <http://www.partec.de/indexre.html>).

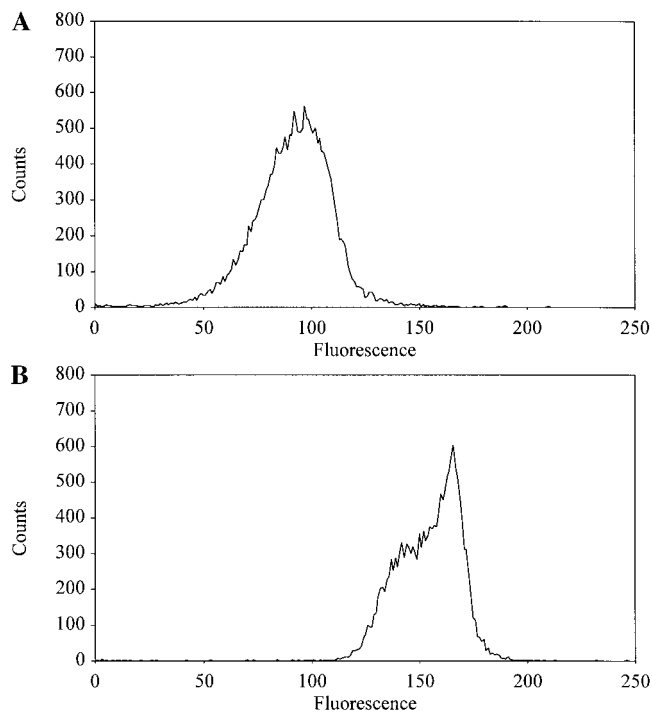


FIG. 1. Fluorescence distribution of ethanol-permeabilized yeast cells stained with (A) $100 \mu\text{g ml}^{-1}$ mithramycin or (B) $100 \mu\text{g ml}^{-1}$ mithramycin plus $10 \mu\text{g ml}^{-1}$ ethidium bromide. Flow cytometry was performed using a Skatron Argus 100 flow cytometer. The B1 filter block (excitation 395–440 nm, band stop 460 nm, emission >470 nm) was used for all measurements. Mithramycin (excitation maximum 445 nm) was thus excited at close to its optimum wavelength but, importantly, ethidium bromide (excitation maximum 493 nm) was not well excited. Under these conditions, DNA-bound ethidium bromide is excited by fluorescence resonance energy transfer from mithramycin. Mithramycin has a high specificity for DNA, but weak fluorescence. In contrast ethidium bromide is strongly fluorescent but binds to other polyanions in the cell. The dye mixture thus gives a valuable combination of high specificity and cells with a detectable fluorescence (99% of unstained cells analyzed under the same conditions appeared below fluorescence channel 100).

In addition to these specialized instruments, improved technology has resulted in mainstream instruments that are useful for a range of measurements on bacteria (20, 21) and viruses (22, 23). More recently FCM has been extended to genome fingerprinting for bacterial discrimination (24).

FLOW CYTOMETRY FROM THE MICROBIOLOGIST'S VIEWPOINT

Single-Cell Analysis

One of the most important characteristics of the flow cytometric method from the point of view of the microbiologist is that data are collected for each individual cell. This enables the investigator to measure the distribution of a property or properties within the population, in marked contrast to the majority of analyses, which only allow a mean value to be recorded. Heterogeneity in microbial populations results from several distinct sources. First, genetic differences may arise as the result of a mutation or the loss of a plasmid. The earlier in the growth of the culture that this happens, the greater will be the effect on the overall population. Second, heterogeneity results in part from cell-cycle differences. In bacteria, new rounds of DNA replication may be initiated before a previous round has been completed and thus heterogeneity of DNA content will be much larger in prokaryotes than in eukaryotes. The variable gene copy number that results from this adds to culture heterogeneity (7). Flow cytometric measurement of DNA content and, for example, the effect of an antibiotic or of other environmental stimuli may be valuable in understanding and quantifying the heterogeneity of response. Withers and Nordström (25) recently used FCM to show that the initiation of chromosome replication is regulated by an extracellular factor in *E. coli*. A third source of heterogeneity is at the physiological level; cells at the center and the edge of a colony growing on an agar plate are exposed to different local environments and their genetic regulation will be adjusted to reflect this.

Microbial cultures therefore consist of a series of subpopulations, with overlapping distributions in a variety of characteristics. Multiparametric FCM, involving the use of a cocktail of stains with different cellular targets, can be used to define the subpopu-

lations of interest. This approach has been used to detect mutants or contaminants which, under the right measurement conditions, will appear as a separate subpopulation of cells. Van Dilla *et al.* (26) reported an elegant method for distinguishing between bacterial species based on differences in DNA GC content. By using chromomycin A3 (which has a guanine-cytosine binding preference) and Hoechst 33258 (which has a preference for adenine-thymine-rich DNA) discriminatory bivariate contour plots were produced that distinguished clearly between *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Where more than two or three parameters are measured, data visualization and interpretation become problematic, although multivariate data analysis methods are applicable in this case (27). The measurement of population heterogeneity can be coupled with cell sorting to isolate cells of interest (see below).

Cell Sorting

Flow cytometric analysis permits the investigator to perform a rapid and quantitative version of the experiments that could otherwise be performed by fluorescent microscopy. Fluorescence-activated cell sorting (FACS) allows the process to be taken one very important step further. Cells with a specific characteristic or indeed a combination of characteristics can be separated from the sample for further analysis or growth.

While sorting of cells into tubes or into the wells of microtiter plates is the norm, the ability of some flow cytometers to sort directly onto agar plates is advantageous to microbiologists. This approach was first demonstrated for assessing methods of flow cytometric bacterial viability determination (28). The value of single-cell analyses is made evident to the microbiologist on production of the familiar plate of discrete colonies; however, the experimenter must be cautious of the fact that microbial cells may grow in clumps or form aggregates during sample preparation. If this is the case any colony produced on the plate may not be the result of growth of a single sorted cell and a mixed phenotype may result or the undesired cell may outcompete the selected cell of interest. In viability tests, for example, if one cell in a clump has a leaky membrane it will take up an exclusion dye such as propidium iodide and thus the clump will be fluorescent and score as "dead" in the assay. However, if this aggregate also contains one

or more live cells, a colony will result when it is sorted onto an agar plate. One method of overcoming this problem is to use forward scatter as an indication of the size of the scattering particle. However, size variation in microbial cells (even within a single species) can be large. As a consequence of this variability, log amplification is usually required for the forward scatter signal; the compression that this exerts on the higher channel numbers can make the discrimination between single cells and aggregates difficult. Consequently more robust methods (e.g., using two or more complementary viability stains) should be sought wherever possible (15).

Similar considerations must be made when analyzing or sorting environmental samples. These often require preprocessing to ensure that there are no large particles present that would block the flow cell (which is typically 100–250 μm in cross-section). This limitation of course also precludes the analysis of filamentous bacteria and fungi in their natural state. Other microorganisms grow as biofilms and these must be disrupted prior to analysis. In instances such as these, interpretation of results will not be straightforward, as the sample preparation will necessarily perturb the cells.

While FACS can be used to isolate mutants it is generally not practical, at least on mainstream instruments, if the mutation frequency is less than 1 in 10^5 – 10^7 . In these circumstances alternative methods such as the use of an antibiotic resistance marker may be more appropriate.

Autofluorescence

Many naturally occurring cellular substances fluoresce when excited with light of a suitable wavelength. For example, NAD(P)H has a (near-)UV-excited blue fluorescence while flavins impart a blue-excited green fluorescence to cells. In studies of phytoplankton or photosynthetic bacteria the autofluorescence profile can be used to discriminate between species. However, when the excitation and/or emission of the cellular autofluorescence overlap with that of extrinsic fluorescent molecules, autofluorescence can prevent useful measurements from being performed. In a few cases it is possible to monitor a cellular product simply by using its light scattering or autofluorescence characteristics. FACS can be used to sort rare, natural higher producers or, in combination with forced or natural molecular techniques, to select evolved mutants with a hyper-

producing phenotype. Clearly, success with this technique is dependent on the particular autofluorescent properties of the product being analyzed and it is therefore not generally applicable. Using the relationship between natural fluorescence of carotenoids and the forward scatter in FCM, An *et al.* (29) isolated carotenoid-producing mutants of the yeast *Phaffia rhodozyma* after chemical mutagenesis. Through the use of FACS, they achieved a 10,000-fold improved efficiency in isolation of astaxanthin hyperproducers compared to random isolation on agar plates. Generally though, as discussed below, analysis of cellular products by FCM depends on an indirect method of analysis, such as staining with a fluorescent dye or identification of (native or reporter) enzyme activity using a fluorogenic substrate.

Fluorescent Stains

DNA is perhaps the most commonly stained cellular constituent in flow cytometric analyses. However, as mentioned above, the amount of DNA in a bacterial cell is much lower than that found in mammalian cells. While nucleic acid stains such as ethidium bromide and propidium iodide are adequate for the analysis of eukaryotic cells, they are less favorable for use with microorganisms. In addition to the lower DNA content, there is the complication that bacteria have a relatively much higher, and more variable, RNA content (RNA:DNA ratio) than typical mammalian cells. Thus stains such as ethidium bromide and propidium iodide, although highly fluorescent, are not easily used for studies of bacteria since they bind not only to DNA, but also to RNA. Some successful studies of bacteria using propidium iodide have been achieved by incubating the cells with an RNase solution prior to staining (4) although other workers (e.g., 30) report that the results obtained from such samples are inconsistent. While mithramycin, discussed above, has been used successfully with arc-lamp-based flow cytometers it is poorly excited by the argon-ion laser used in the majority of commercial systems.

The DNA-specific Hoechst dyes (26) and 4',6-diamino-2-phenylindole (DAPI) (31) have been used for the analysis of microorganisms, but since they require UV excitation they are not appropriate for use with all flow cytometers. The range of fluorescent stains available is continuously expanding and Molecular Probes (32) has produced a range of dyes with different spectral characteristics and high spec-

ificities for nucleic acids. These are appropriate for the flow cytometric analysis of microorganisms (33, 34).

A common application of FCM in microbiology is determination of viability. While viability is one of the most fundamental properties of a cell, it is difficult both to define and measure. Exclusion of propidium iodide or ethidium bromide by the intact membrane of viable cells is a common approach with mammalian cells but it is less useful with bacteria due to leakage of these molecules into viable cells and the presence of efflux pumps (35). An alternative approach is to use lipophilic cations that are accumulated by microbial cells or nonfluorescent substrates that are converted to fluorescent products as a result of metabolic (enzymatic) activity. These approaches have been reviewed elsewhere (15) but we discuss the enzymatic approach below from the viewpoint of reporter gene technology.

One further development that has made flow cytometric measurements more readily available to the microbiologist is the recent development of a variety of "kits." These kits enable the nonspecialist to develop protocols rapidly, and since they correspond to routine methods (enumeration, viability determination, Gram status determination, etc.) they are particularly attractive. The measurement of viability can be accomplished using the LIVE/DEAD BacLight bacterial viability kit from Molecular Probes. This kit is becoming widely used in microbial FCM, although appropriate controls are essential to obtain useful results (15, 32).

Flow Cytoenzymology

Many of the qualities that make fluorescently labeled substrates suitable for use in enzyme assays in solution render them inappropriate for use in FCM. Conventional solution assays for measuring enzymatic activity in cell extracts utilize substrates yielding a highly fluorescent water-soluble product with substantially different fluorescent properties from the substrate. However, for FCM analysis it is necessary for the probe to enter the cell and for the product to be retained.

Ideally after enzymatic processing inside the cell the fluorogenic precursor should become immediately fluorescent and either be insoluble or form a complex with a cellular component which effectively renders it insoluble. The label should preferably be nontoxic, stable, persist within the cell for sufficient duration to

allow the level of fluorescence to be monitored, and be correlated easily with the enzyme activity.

Although enzyme-generated intracellular fluorescence or "flow cytoenzymology" has found wide application in mammalian cells, microbiological investigations have been more limited. However, techniques for discriminating between bacteria and yeasts (36), monitoring cellular viability, and increasingly, using reporter enzymes in transcriptional fusions have been developed. Nir *et al.* (36) used native β -galactosidase activity to show that *E. coli* and *Candida pseudotropicalis* encapsulated in agar beads could be distinguished by FCM. In this example, the solubility of the fluorescent product was not an issue as any fluorescein leakage was retained in the encapsulating agar bead and thus for FCM detection was still associated with the producer cell.

Dehydrogenase activity has been used as an indicator of cellular viability with the substrate 5-cyano-2,3-ditolyltetrazolium chloride (CTC) (37) to demonstrate cell heterogeneity in a culture of *Micrococcus luteus*, and viability in a wide range of bacterial species has been demonstrated using fluorogenic esters to monitor esterase activity (38).

Recent advances in molecular biological methods now permit the detailed study of genes that were previously difficult to analyze because of the nature of the cognate protein product. The rapid progress in bioinformatics and whole genome sequencing now enables either rational design of transcriptional fusions or the random insertion of a reporter downstream of the promoter of interest on a plasmid or in the chromosome, in order to monitor expression of the target gene. Translational fusions producing chimeric proteins can also be generated. The reporter gene typically encodes an enzyme that is stable, that has an activity which is easy to assay, and for which there is little endogenous activity in the host. As FCM analysis monitors enzyme activity through fluorescence of single cells there are a number of additional criteria that need to be satisfied for the successful measurement of reporter enzyme activity by this technique, especially in microbes. In particular, a fluorogenic rather than a chromogenic substrate is required; the substrate must be capable of being taken up by the cell and the fluorescent product retained, and there must be a low level of cellular fluorescence (autofluorescence) at the excitation and emission wavelengths used.

The *lacZ* gene encoding β -galactosidase has been used extensively as a reporter for the study of gene expression. In spectrophotometric assays the chromogenic product *ortho*-nitrophenol derived from 2-nitrophenyl β -D-galactopyranoside is typically used to assay activity of this enzyme. However, a more sensitive substrate recently applied to the measurement of β -galactosidase in single cells by FCM is fluorescein di- β -D-galactopyranoside (FDG). The nonfluorescent FDG substrate is hydrolyzed initially to fluorescein monogalactoside (FMG) and finally to the highly fluorescent product fluorescein.

Although FDG has been used extensively for FCM in mammalian cells (39) only a few examples of its use have been reported for microbial cell analysis (40). Despite the advantage of increased sensitivity in using substrates such as FDG, methods that are based on the release of fluorescein are limited by problems with substrate uptake and retention of the fluorescent product in reporter-active cells. The relationship between observed fluorescence and the actual number of β -galactosidase molecules per cell may thus be complex as variations in cell envelope permeability to FDG can influence the intracellular substrate concentration (41). In addition many workers have noted that fluorescein can leak from β -galactosidase-active bacteria into inactive cells in heterogeneous populations. Alvarez *et al.* (42) found that leakage of fluorescein (released after enzymatic hydrolysis from *E. coli* stained with FDG) could be observed as a decrease in fluorescence after 45 min of incubation at 37°C. Lipophilic derivatives of FDG which yield products that are retained by cells are available (32), and their utility has been demonstrated in the analysis of plasmid segregation in *E. coli* (43) and during investigation of differentiating cultures of *Bacillus subtilis*. In the latter case problems with background fluorescence were overcome by using C8-FDG (5-octanoylamino fluorescein) in combination with the lipophilic tracking dye PKH26 (44). Cn-FDG substrates are derived from FDG but contain a saturated hydrocarbon tail that allows the released fluorescent product to remain in association with the cell. C8-FDG was found to be the optimal substrate from $n = 2, 4, 6, 8, 12,$ and 16 for detecting β -galactosidase activity in sporulating cultures of *B. subtilis* using FCM.

An alternative label to FDG, resorufin β -D-galactopyranoside, can be used for continuous monitoring of enzymatic activity at physiological pH, it is

hydrolyzed to a fluorescent product in a single step, and its fluorescence emission (585 nm) is easier to separate from cellular autofluorescence than that of fluorescein (525 nm).

Although the *lacZ* reporter system is widely used in *E. coli* and many other bacterial species, it is less well suited to FCM studies on yeast such as *S. cerevisiae* and *Candida albicans* because the FDG is unable to enter cells unless they are permeabilized (and their viability thus reduced). Cid *et al.* (45) overcame this problem by exploiting the *S. cerevisiae* EXG1 gene (and a homologous gene in *C. albicans*) encoding an exo-1,3- β -glucanase. Yeast cell walls are permeable to the fluorescein di(β -D-glucopyranoside) (FDGP) substrate and the activity of the exoglucanase reporter enzyme results in the release of fluorescein. Gene expression in yeast can be regulated differentially at various stages of the mitotic or meiotic phases, and this system was used successfully to sort cells with different levels of gene promoter activity from a heterogeneous population. However, one drawback of the exoglucanase reporter system is its reduced activity above 30°C.

A major development in substrate design has recently been reported by Zlokarnik and co-workers (46) who utilized β -lactamase as a reporter enzyme. Although the β -lactamase system has similar sensitivity to β -galactosidase, the major advantage is in the design of a membrane-permeant substrate precursor, enabling its product to be retained in the cell. The nonfluorescent, fully esterified substrate CCF2/AM is sufficiently nonpolar to allow it to cross the cell membrane. The four ester groups are then hydrolyzed by cytosolic, nonspecific esterases to release and trap within the cell the actual substrate for β -lactamase, CCF2. CCF2 is a novel resonance energy tandem (RET) fluorogenic substrate consisting of covalently linked donor and acceptors. In this form the complex has the donor excitation properties and the acceptor emission spectrum. When the covalent linkage is broken by hydrolytic activity of β -lactamase the fluorophores are separated and exhibit their own independent fluorescence excitation/emission profiles. Using the ratios of intensities at two or more wavelengths is a significantly more robust approach than single-wavelength measurement, reducing possible interference from other variables such as cell size variation. Although this method was designed to measure gene expression in mammalian cells the principles of using RET and

trapping of the substrate would also be applicable in analysis of microbial gene expression and heterogeneity. The method could be used without modification for the rapid analysis of cells expressing native β -lactamase activity and for screening for inhibitors.

Green Fluorescent Protein

The gene encoding green fluorescent protein (GFP) from *Aequorea victoria* has become an important reporter in the study of the dynamics of gene expression and localization of proteins *in vivo* (47). The main advantage of GFP over other commonly utilized reporters that depend on additional cofactors or substrates is that it requires only the presence of oxygen for its characteristic green fluorescence (emission max. 509 nm). The light absorbing chromophore, within a hexapeptide located at positions 64–69, is an intrinsic part of the protein. Modification of the amino acid sequence of GFP, particularly of the Ser65-dehydroTyr66-Gly67 cyclic tripeptide fluorophore, has enabled the isolation of a number of proteins with increased fluorescence or altered excitation/emission properties. These include red-shifted (excitation maximum shifted from 395 to 490 nm), blue-shifted (emission max. ~448 nm), intermediate (cyan FP), and other variants (sapphire and yellow FP). The different categories of GFP variants have been comprehensively reviewed by Tsien (48).

Through appropriate choices of filter sets, combinations of GFP variants can be utilized for applications requiring dual reporters, increasing the permutations of useful parameters for detection by FCM. This approach can be applied to monitoring expression of multiple genes in the same cell, the same gene in different genetic backgrounds, and in cell mixtures. The use of spectrally distinct fluorescent protein reporters (49) thus allows for the simultaneous measurement of a number of bacterial genes under different environmental conditions and is particularly useful for the study of intracellular pathogenicity.

Analysis of Intracellular Pathogens

Many bacterial pathogens are known to survive in phagocytes by coordinately regulating expression of a wide spectrum of genes. Valdivia and Falkow (50) used GFP as a selectable marker in conjunction with FACS. Macrophages infected with *Salmonella typhimurium* cells containing a transcriptionally active

GFP fusion were separated by FACS and lysed. The bacteria were grown and isolated by FACS and then the fluorescence intensity of individual bacteria grown in tissue culture medium was compared with the same clone after release from infected cells. It was found that 14 *S. typhimurium* genes under the control of at least four independent regulatory circuits were induced selectively in host macrophages.

A major advantage of using GFP is that it can be used to label living cells and monitor them in real time. GFP has been used in FCM studies to monitor promoter strength by *in vivo* analysis of mycobacteria in macrophages containing GFP transcriptional fusions. Dhandayuthapani *et al.* (51) examined the possibility of using the GFP-based fluorescence to sort bacterial cells from macrophages according to their GFP expression level. They found that enrichment of those bacteria containing plasmids with active transcriptional fusions was possible when the ratio of GFP-expressing to nonexpressing bacterial cells was 1:1000. In these investigations it was necessary to homogenize mycobacterial clumps and the fluorescence data were gated by forward-angle light scatter, pulse height, and pulse width; only the smallest particles were analyzed for fluorescence intensity. FCM analysis revealed distinct distributions of activities in mycobacterial cell populations, with the ranges depending on the type of promoter. For example, little cell-to-cell variation was found with a GFP transcriptional fusion to the *hsp60* promoter, whereas the *mtrA* promoter showed a wide range of fluorescence intensities.

Barker *et al.* (52) overcame problems associated with isolating a mixed population of organisms from intact macrophages by using FACS to isolate macrophage vesicles rather than intact macrophages. They identified 12 clones from GFP fusions containing mycobacterial promoter constructs differentially expressed in the macrophage.

Many GFP variants have been improved for use in eukaryotic cells but are less satisfactory for prokaryotes, especially where weak promoters are being studied. A GFP cloning cassette (green TIR) specifically designed for prokaryotic transcriptional fusions and containing the S65T “red-shift” and F64L “protein solubility” mutations has been developed to overcome this limitation (53). The modified *gfp* gene, flanked by convenient restriction sites and appropriate prokaryotic expression sequences, is reported to fluoresce 40- to 80-fold more intensely than

the wild-type GFP. The need to develop specific plasmid constructs for diverse groups of bacteria has also limited the widespread application of GFP in microbiological investigations. Moreover, plasmid-based reporters generally require antibiotic selection and can cause problems with multicopy effects. Single-copy chromosomally integrated reporters of gene expression are more appropriate as they maintain the native promoter environment. Such reporters can be constructed rationally by recombination using available sequence information or randomly, using transposons. *lacZ* and GFP-based mini-Tn5 reporters (54, 55) are available for creating mutant libraries in a wide range of gram-negative bacteria and these can be utilized in FACS to isolate conditionally expressed genes for further investigation.

Enzymatic transcriptional reporters are extremely sensitive because the catalysis of a large number of nonfluorescent precursors to fluorescent products amplifies the original expression signal. In contrast each GFP molecule produces at most one fluorophore and the limit of detection is therefore lower. However, because of its intrinsic fluorescence, stability, small size, and the lack of requirement for substrates, GFP is ideal for making in-frame fusions both to the amino and to the carboxy termini resulting in chimeric proteins and this is where it has found widest application (48). The persistence of GFP (with a half-life usually in excess of 24 h) may be an advantage in some cases, particularly in protein localization studies. However, in microbiological FCM applications this is of less significance and where transient gene expression is being monitored it may actually be a hindrance. Andersen *et al.* (56) have developed unstable variants of GFP for real-time analysis of temporal gene expression by the addition of short peptide sequences to the C-terminal end of intact GFP. This renders the modified protein susceptible to the action of endogenous housekeeping proteases and reduces the protein half-life to as little as 40 min.

The success of directed molecular evolution of GFP to produce variants with novel properties that can be isolated by FACS provides an excellent model for evolving other proteins. DNA shuffling is technically simple in bacteria and the combination of this technique with high-throughput screening and FACS-based enrichment has great potential for optimizing commercially important enzymes for which there are no selection methods currently available

(57). Advances in FRET (fluorescence resonance energy transfer) technology enables GFP variants to be used as FRET partners in investigating protein-protein interactions, opening up a range of screening possibilities for molecules capable of inhibiting the activities of important proteins. The development of new types of fluorescence proteins as secondary fluorophores in FRET, LRET (luminescence RET) (58), and BRET (bioluminescence RET) (59) pairs will be of particular significance in extending the range of combinations available for FCM multiparametric analysis in microorganisms.

In considering the use of FACS for microbial analysis it is important to note that it is essentially a serial technique. As such, although it is ideal for selecting, sorting, and enriching cells and has the advantages of being rapid (100–10,000+ cells may be analyzed in 1 s) and multiparametric, at present the technique is not well adapted to the demands of high-throughput screening. For screening of a large number of isolates a parallel sample processing technique that could be performed in a 96-well microtiter plate would generally be more rapid and less labor intensive.

CONCLUDING REMARKS

Traditionally, analysis of microorganisms has been outside of the mainstream of flow cytometric studies and is often regarded as a niche area with fairly esoteric applications. However, the microbial world is extremely diverse, incorporating single-cell and multicellular prokaryotes and eukaryotes. Different strategies for staining and cell sorting must be adopted for different species of bacteria, yeast, and fungi and their various states (e.g., spores). While FCM has many advantages for the microbiologist the main barriers to its widespread usage have been the expense and complexity of the equipment and the fact that many of the methodologies and staining techniques have been designed principally for studies on mammalian cells. However, the development of new fluorescent stains appropriate for use with microorganisms and technological advances leading to reasonably priced flow cytometers capable of multiparametric detection and cell sorting will soon make FCM and FACS suitable for widespread use by microbiologists.

FCM and FACS have considerable potential for application in the evolution of microbial strains and recombinant proteins by classical and modern mutagenic methods. The development of altered green fluorescent proteins (in some cases evolved by FACS), in addition to being an excellent model system for development of novel enzymes, is ideal for multiparametric analysis of the complex regulation of gene expression in microbes. The potential of the combination of FACS and fluorescent proteins in investigating host-pathogen interactions and the importance of cell heterogeneity is only just being realized.

ACKNOWLEDGMENT

H.D. thanks the European Research Office of the United States Army for financial support.

REFERENCES

- Gucker, F. T., O'Konski, C., Pickard, H. B., and Pitts, J. N. (1947) *Am. J. Chem.* **69**, 2422-2431.
- Shapiro, H. M. (1995) *Practical Flow Cytometry*, 3rd ed., A. R. Liss, New York.
- Pauu, A. S., Cowles, J. R., and Oro, J. (1977) *Can. J. Microbiol.* **23**, 1165-1169.
- Hutter, K.-J., and Eipel, H. E. (1978) *A. van Leeuwenhoek* **44**, 269-282.
- Steen, H. B., and Lindmo, T. (1979) *Science* **204**, 403-404.
- Steen, H. B., and Boye, E. (1980) *Cytometry* **1**, 32-36.
- Skarstad, K., Bernander, R., Wold, S., Steen, H. B., and Boye, E. (1996) in *Flow Cytometry Applications in Cell Culture* (Al-Rubeai, M., and Emery, A. N., Eds.), pp. 241-255, Dekker, New York.
- Steen, H. B., Boye, E., Skarstad, K., Bloom, B., Godal, T., and Mustafa, S. (1982) *Cytometry* **2**, 249-257.
- Walberg, M., Gaustad, P., and Steen, H. B. (1996) *J. Antimicrobial Chemother.* **37**, 1063-1075.
- Walberg, M., Gaustad, P., and Steen, H. B. (1997) *Cytometry* **29**, 267-272.
- Suller, M. T. E., and Lloyd, D. (1999) *Cytometry* **35**, 235-241.
- Diaper, J. P., Tither, K., and Edwards, C. (1992) *Appl. Microbiol. Biotechnol.* **38**, 268-272.
- Kaprelyants, A. S., and Kell, D. B. (1992) *J. Appl. Bacteriol.* **72**, 410-422.
- Davey, H. M., Kaprelyants, A. S., and Kell, D. B. (1993) in *Flow Cytometry in Microbiology* (Lloyd, D., Ed.), pp. 83-93, Springer-Verlag, London.
- Davey, H. M., Kaprelyants, A. S., Weichart, D. H., and Kell, D. B. (1999) in *Current Protocols in Cytometry: Microbial Cytometry*, Vol. 11, pp. 3.1-3.20, Wiley, New York.
- Olson, R. J., Vault, D., and Chisholm, S. W. (1985) *Deep-Sea Res. Part A - Oceanogr. Res. Pap.* **32**, 1273-1280.
- Gjelsnes, O., and Tangen, R. (1994) Patent WO 94/29695 Norway.
- Davey, H. M., and Kell, D. B. (1996) *Microbiol. Rev.* **60**, 641-696.
- Rønning, Ø. (1999) *Genetic Eng. News* **19**, 18.
- Bernander, R., Stokke, T., and Boye, E. (1998) *Cytometry* **31**, 29-36.
- Roth, B. L., Poot, M., Yue, S. T., and Millard, P. J. (1997) *Appl. Environ. Microbiol.* **63**, 2421-2431.
- McSharry, J. J. (1994) *Clin. Microbiol. Rev.* **7**, 576.
- Marie, D., Brussaard, C. P. D., Thyraug, R., Bratbak, G., and Vault, D. (1999) *Appl. Environ. Microbiol.* **65**, 45-52.
- Huang, Z. P., Jett, J. H., and Keller, R. A. (1999) *Cytometry* **35**, 169-175.
- Withers, H. L., and Nordström, K. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 15694-15699.
- Van Dilla, M. A., Langlois, R. G., Pinkel, D., Yajko, D., and Hadley, W. K. (1983) *Science* **220**, 620-622.
- Davey, H. M., Jones, A., Shaw, A. D., and Kell, D. B. (1999) *Cytometry* **35**, 162-168.
- Nebe-von-Caron, G., Stephens, P., and Badley, R. A. (1998) *J. Appl. Microbiol.* **84**, 988-998.
- An, G., Bielich, J., Auerbach, R., and Johnson, E. A. (1991) *Biotechnology* **9**, 69-73.
- Steen, H. B., Skarstad, K., and Boye, E. (1990) in *Flow Cytometry* (Darzynkiewicz, Z., and Crissman, H. A., Eds.), pp. 519-526, Academic Press, London.
- Boucher, N., Vault, D., and Partensky, F. (1991) *Marine Ecol.-Prog. Ser.* **71**, 75-84.
- Haugland, R. P. (1996) *Molecular Probes Handbook of Fluorescent Probes and Research Chemicals*, 6th ed., Molecular Probes Inc., Eugene, OR.
- Marie, D., Vault, D., and Partensky, F. (1996) *Appl. Environ. Microbiol.* **62**, 1649-1655.
- Guindulain, T., Comas, J., and VivesRego, J. (1997) *Appl. Environ. Microbiol.* **63**, 4608-4611.
- Jernaes, M. W., and Steen, H. B. (1994) *Cytometry* **17**, 302-309.
- Nir, R., Yisraeli, Y., Lamed, R., and Sahar, E. (1990) *Appl. Environ. Microbiol.* **56**, 3861-3866.
- Kaprelyants, A. S., and Kell, D. B. (1993) *J. Microbiol. Methods* **17**, 115-122.
- Diaper, J. P., and Edwards, C. (1994) *J. Appl. Bacteriol.* **77**, 221-228.
- Nolan, G. P., Fiering, S., Nicolas, J. F., and Herzenberg, L. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2603-2607.
- Plovins, A., Alvarez, A. M., Ibanez, M., Molina, M., and Nombela, C. (1994) *Appl. Environ. Microbiol.* **60**, 4638-4641.
- Nwoguh, C. E., Harwood, C. R., and Barer, M. R. (1995) *Mol. Microbiol.* **17**, 545-554.

42. Alvarez, A. M., Ibanez, M., and Rotger, R. (1993) *Biotechniques* **15**, 974.
43. Miao, F., Todd, P., and Kompala, D. S. (1993) *Biotechnol. Bioeng.* **42**, 708–715.
44. Chung, J. D., Conner, S., and Stephanopoulos, G. (1995) *Cytometry* **20**, 324–333.
45. Cid, V. J., Alvarez, A. M., Santos, A. I., Nombela, C., and Sanchez, M. (1994) *Yeast* **10**, 747–756.
46. Zlokarnik, G., Negulescu, P. A., Knapp, T. E., Mere, L., Burres, N., Feng, L. X., Whitney, M., Roemer, K., and Tsien, R. Y. (1998) *Science* **279**, 84–88.
47. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) *Science* **263**, 802–805.
48. Tsien, R. Y. (1998) *Annu. Rev. Biochem.* **67**, 509–544.
49. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. (1995) *TIBS* **20**, 448–455.
50. Valdivia, R. H., and Falkow, S. (1997) *Science* **277**, 2007–2011.
51. Dhandayuthapani, S., Via, L. E., Thomas, C. A., Horowitz, P. M., Deretic, D., and Deretic, V. (1995) *Mol. Microbiol.* **17**, 901–912.
52. Barker, L. P., Brooks, D. M., and Small, P. L. (1998) *Mol. Microbiol.* **29**, 1167–1177.
53. Miller, W. G., and Lindow, S. E. (1997) *Gene* **191**, 149–153.
54. Matthyse, A. G., Stretton, S., Dandie, C., McClure, N. C., and Goodman, A. E. (1996) *FEMS Microbiol. Lett.* **145**, 87–94.
55. Tombolini, R., Unge, A., Davey, M. E., de Bruijn, F. J., and Jansson, J. K. (1997) *FEMS Microbiol. Ecol.* **22**, 17–28.
56. Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjørn, S. P., Givskov, M., and Molin, S. (1998) *Appl. Environ. Microbiol.* **64**, 2240–2246.
57. Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. C. (1996) *Nature Biotechnol.* **14**, 315–319.
58. Xiao, M., Li, H., Snyder, G. E., Cooke, R., Young, R. G., and Selvin, P. R. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 15309–15314.
59. Xu, Y., Piston, D. W., and Johnson, C. H. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 151–156.