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Flow Cytometry and Cell Sorting of Heterogeneous Microbial Populations: the Importance of Single-Cell Analyses

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INTRODUCTION—MICROBIAL HETEROGENEITY

> "The principle (of flow cytometry) should have wide application in . . . bacteriology."
>
> Gucker et al., 1947 (381)

> "Flow cytometry of bacteria is still in its infancy."
>
> Jernaes and Steen, 1994 (462)

Preamble: the Fundamental Problem of Analyzing Heterogeneous Microbial Cultures, and the Requirement for Single-Cell Analyses

Since the time of J. Willard Gibbs, the treatment of macroscopic systems as ensembles of microscopic particles that, averaged over time, are identical has underpinned most of even modern thermodynamics (see, e.g., references 1020 and 1024). Implicitly, similar assumptions have been taken as axioms for the analyses performed by most microbial physiologists: we usually describe our cultures as having a certain growth yield or respiratory rate or rate of glucose catabolism, with the implication that this represents a full description of these variables. However, this would be true only if our cells were not only identical but in fact at equilibrium, constituting what thermodynamicists call an ergodic system. Since we know that growing cells are nonequilibrium in character, it is usual, even within

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the framework of nonequilibrium thermodynamics, to ascribe a "local" equilibrium to the macroscopic parameters and variables (forces and fluxes) in which we are interested, thus permitting us to refer to them as possessing a "sharp" value (i.e., one that does not change significantly as a result of fluctuations). This approach is generally thought acceptable (but compare references 478 and 1020), since the large numbers of molecules participating in say the ATP "pool," even in a single cell, means that spontaneous thermodynamic fluctuations in their "instantaneous" value will normally be negligible in the steady state. It turns out, however, that the problem of analyzing heterogeneous microbial cultures correctly is far worse than this.

Consider a study of bacterial starvation in which one might wish to establish the relationship between one or more metabolic properties of interest and the loss of viability (as judged by the ability to form a colony) that must necessarily occur (with appropriate kinetics) in the absence of any free energy input. We might, for instance, monitor the ATP content of the culture. However, if the total or average ATP content of a population of starving cells declines by one-half, this could be because all of the cells have lost half of their ATP or because half of the cells have lost all of their ATP, or any combination in between (147, 495, 501). From what we know of the behavior of the adenylate energy charge (518), the first is unlikely to be accompanied by any necessary loss in viability, the second is almost certainly accompanied by a complete loss in viability, and the third is of course indeterminate. Thus, the relationship between a macroscopic property, such as ATP concentration, and a microscopic property (i.e., a property of an individual cell), such as viability on a plate, is variable, and even if the ATP level of an individual cell did in fact correlate well with its culturability, we should not expect to be able to observe this relationship when the ATP measurements are made macroscopically. However, if measurements of the ATP concentration of each cell in the population could be made independently, one would be able to determine the distribution of ATP loss within the population and hence the extent to which a decrease below a "threshold" value correlated with viability. Thus, a technique which enables measurements to be made on individual cells in heterogeneous populations provides incomparably more useful information than do traditional biochemical assays, which study only large populations of cells. A major theme of this review is thus that the circumstances in which such measurements are required for the correct, quantitative analysis of microbial systems are widespread to the point of ubiquity and that technical advances, especially in the area of flow cytometry, now allow substantial progress to be made toward their realization (although we recognize that most of what we have to say applies equally to other forms of individual cell analysis [376, 557, 691, 692, 1027]).

The essence of the problem is that one is trying, typically, to correlate a rate of change (v) of a certain variable with respect to the value of a certain property (p) and that a correlation may be expected between the mean values $\bar{v}$ and $\bar{p}$ only if v is kinetically of first order with respect to p (501), which is rarely the case in biology. Indeed, when one studies the extent to which the activity of an enzyme determines the growth rate of a cell, for instance, one has to consider the possibility that the activity of that enzyme is distributed heterogeneously among individual cells (503, 506). Macroscopic analyses will always tend to give erroneous answers under these circumstances. Similarly, the heterogeneity (in viability) of chemostat populations at low dilution rates can lead to substantial errors in the estimation of Monod coefficients (874). Because of the typical existence of thresholds (146) and other nonlinearities in biological systems, other cases in which unsuspected heterogeneity may be expected to have significant effects upon the kinetic analyses of microbial processes include fluctuating systems (219, 1023) and stochastic systems in which small numbers of repressor molecules or other elements may control gene expression.

We are aware that it could be considered that much of the scientific progress which we review in what follows is technology led or hinges upon technical and instrumental advances, rather than being driven purely by more conceptual and scientific or intellectual advances. While this is partly true, in the same sense it differs little from the types of advance in molecular biological methods that have been responsible for the revolution in our recent understanding of the behavior of microbes at the molecular level. Before discussing flow cytometry, however, we briefly outline the main sources of heterogeneity in microbial (and other) cell cultures.

Sources of Heterogeneity

Leaving aside mixed natural populations and considering simply an axenic laboratory culture, microbial heterogeneity arises from three principal sources: (i) genotypic through mutations, (ii) phenotypic via progression through the cell cycle, and (iii) phenotypic due to changes in the exact local environment (and its history). We briefly consider each of these.

Basic mutational variability of cells. The basic mutational variability of cells is reasonably well understood, at least for *Escherichia coli* (327) (and it is likely that mutation rates are to a first approximation reasonably constant amongst different DNA-based microbes [271, 693]), and (per cell division) is some 10^{-7} per bp in the absence and some 10^{-10} per bp in the presence of postreplicative repair systems. Even with the latter value (and by no means all cells may be expected to have non-error-prone repair mechanisms), and assuming that *E. coli* contains some 2,000 genes of 1,000 bp each, we find that in each generation a mutation will have arisen on average in 5×10^{-3} of the population; i.e., after 1 generation, 0.995 of the population is "wild type". (Lethal mutations constitute ca 1% of the total and for present purposes may be ignored.) After n generations, the proportion of "wild-type" organisms will, statistically, be 0.995^n. This becomes <0.5 after 140 generations, or five sequential cultures in which cells are grown from single colonies to 10^9 cells (ca. 1 mg). Obviously, these numbers may be modified, but the conclusion remains that mutational variability will always contribute to physiological performance via selection in a way that is not easy to analyze by present approaches, and one might add that the ongoing debate (see, e.g., references 143, 315, 318, 386, 553, 816, 817, and 1035) about "directed mutation" (now called adaptive mutation) serves amply to reinforce this view. In particular, adaptive mutation rates, as may occur in nongrowing cells, may be enormously greater than those estimated on the basis of fluctuation tests (385).

Prokaryotic cell cycle. For reasons of space and scope, we do not discuss the eukaryotic cell cycle. To date, the literature on the prokaryotic cell cycle (71, 188, 259, 261, 449, 510, 538, 571, 628, 636, 668) has largely been curiously divorced from the mainstream of work in microbial physiology, in which we typically study (and exploit) nonsynchronised batch and continuous cultures. Indeed, even at the most fundamental level, there has been recent debate among experts concerning whether biomass accretion through the cell division cycle of *E. coli* is more nearly linear or exponential (187, 188, 537), models which in fact differ only by some 6% (132, 217) but are accompanied by a vastly different biology. However, the studies cited

above do illustrate that the expression of particular proteins is far from independent of the phase of the cell cycle, and although our knowledge of this is still very rudimentary, the distribution of cells throughout the cell cycle must necessarily affect microbial performance to a substantial degree (and indeed vice versa [548]).

Physiological level. On top of the genotypic properties alluded to above, the expression of microbial activities is of course modulated by the local environment and its history. Indeed, it has been shown that even in laboratory-scale fermentors, which are usually considered to approximate "perfectly mixed" reactors to a fairly high degree, the dropwise addition of nutrient to glucose-limited chemostats is accompanied by observable "bursts" in respiration which indicate that not every cell sees every drop (673). Similarly, a cell "seeing" 10 μM O_2 all the time will be very different from one seeing on/off pulses of 20 μM O_2 once per s, a timescale that will never be picked up by a conventional O_2 electrode but will easily be transduced by the respiratory chain (495), and it is very likely that considerations such as these contribute significantly to the problem of scale-up in cultures of industrial interest (494). Even for relatively high-performance laboratory-scale fermentors, which are usually considered to approximate perfectly mixed reactors to a high degree (but see reference 873), it would seem that the relevant microscale (50 to 300 μm), below which turbulence is not manifest and thus the access of substrates to the cell surface remains diffusion limited, is far greater than that of the dimensions of typical microorganisms (278, 317), necessarily providing a substantial contribution to heterogeneity (298). In other words, the addition of a volume element of nutrient to a culture will not in fact lead to perfect mixing on any timescale that is short relative to many intracellular reactions, so that not all cells will have the same nutritional history. Lloyd (569) refers to fluctuations on such "physiological" timescales as "ultradian" rhythms and has reviewed their likely importance in single-celled organisms more generally (572).

Regardless of the above, more mechanistic considerations, another simple and quite general statistical argument (based on that in reference 1032) serves strikingly to point up the heterogeneity necessarily observable in (micro)biological populations. If we assume a population that is normally distributed, only 95.45% or 0.9545 of the population will, by definition, possess a quantitative attribute that is within ± 2 standard deviations of the mean (116). Indeed (however unsatisfactorily, since this obviously hides systematic biases that a population may possess), the central 95% of a population is often taken in clinical practice, chemical pathology, and so on, as the normal or reference range of values for that attribute (95). For an individual to have two quantitative attributes each within ± 2 standard deviations of the mean, i.e., to be "normal" by these biochemical criteria, the probability is 0.9545^2, and for n quantitative attributes all to be normal, the probability is 0.9545^n. This decreases below 50% for $n = 15$; i.e., if we consider only 15 attributes, the chance of an individual possessing normal values for each of them is less than 50%, and the chances drop below 10 and 1% for $n = 50$ and $n = 99$, respectively. Given that bacteria contain thousands of genes, it is clear (i) that we can no longer expect to understand the physiology of cultures by treating them as ensembles of identical cells (172, 501, 523, 524) and (ii) that the physiological state of a culture (592) must be defined as a "physiological pattern" in terms of the distribution of physiological states throughout the population. Evidently, this means screening, as far as is possible, large numbers of cells in a culture of defined overall physiological state for their distributions in size and macromolecular content as a first step toward obtaining a fuller and more accurate description of what is going on in our cultures, and flow cytometric procedures nowadays permit this to be done with some grace. As will become abundantly clear, the heterogeneity of microbial populations, even in axenic laboratory cultures, is far greater than is normally assumed, and this has substantial implications for the quantitative analysis of microbial performance. The literature review is taken to be to the end of July 1996.

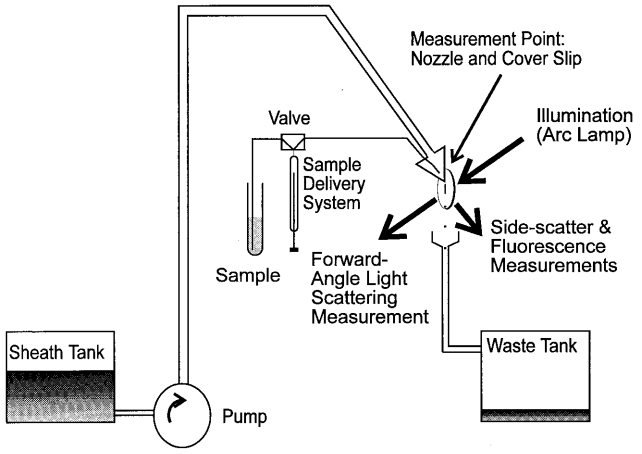

FIG. 1. The principle of flow cytometry. The pump passes fluid through a narrow tube, into which a more slowly moving sample is injected. Hydrodynamic focusing causes the sample to be constrained to the middle of the sheath fluid. In the system illustrated (a Skatron Argus 100 flow cytometer), the sample impinges on a circular coverslip and the illumination source is a mercury arc lamp. Forward and right-angle light-scattering events are detected by photomultipliers, as (via suitable filters) is fluorescence, and are stored on a computer.

FLOW CYTOMETRY

What Is Flow Cytometry?

In a typical microbial flow cytometer (Fig. 1) (627, 865), individual particles pass through an illumination zone, typically at a rate of some 1,000 cells $\cdot$ s^{-1} (although much higher rates are possible in specialized instruments [58, 865, 867]), and appropriate detectors, gated electronically, measure the magnitude of a pulse representing the extent of light scattered. The magnitudes of these pulses are sorted electronically into "bins" or "channels," permitting the display of histograms of the number of cells possessing a certain quantitative property versus the channel number (Fig. 2). Although many of the purposes for which one might use flow cytometry, such as microbial discrimination, require only a qualitative output, the essential character of the flow cytometric approach is strictly quantitative (see also reference 587).

The angular dependence of scattered light provides further information on the nature of the scattering particles, but, more importantly, appropriate fluorophores may be added to the cell suspension. These may be stains which bind to (or react with) particular molecules such as DNA, RNA, or protein; fluorogenic substrates which reveal distributions in enzymatic activity; indicators which change their property as a function of pH_{in} or which are taken up in response to membrane energization; or, increasingly, antibodies or oligonucleotides tagged with a fluorescent probe. Two- or three-variable histograms or contour plots of, for example, light scattering versus fluorescence from a DNA stain (versus fluorescence from a protein stain) may also be generated, and thus an impression may be

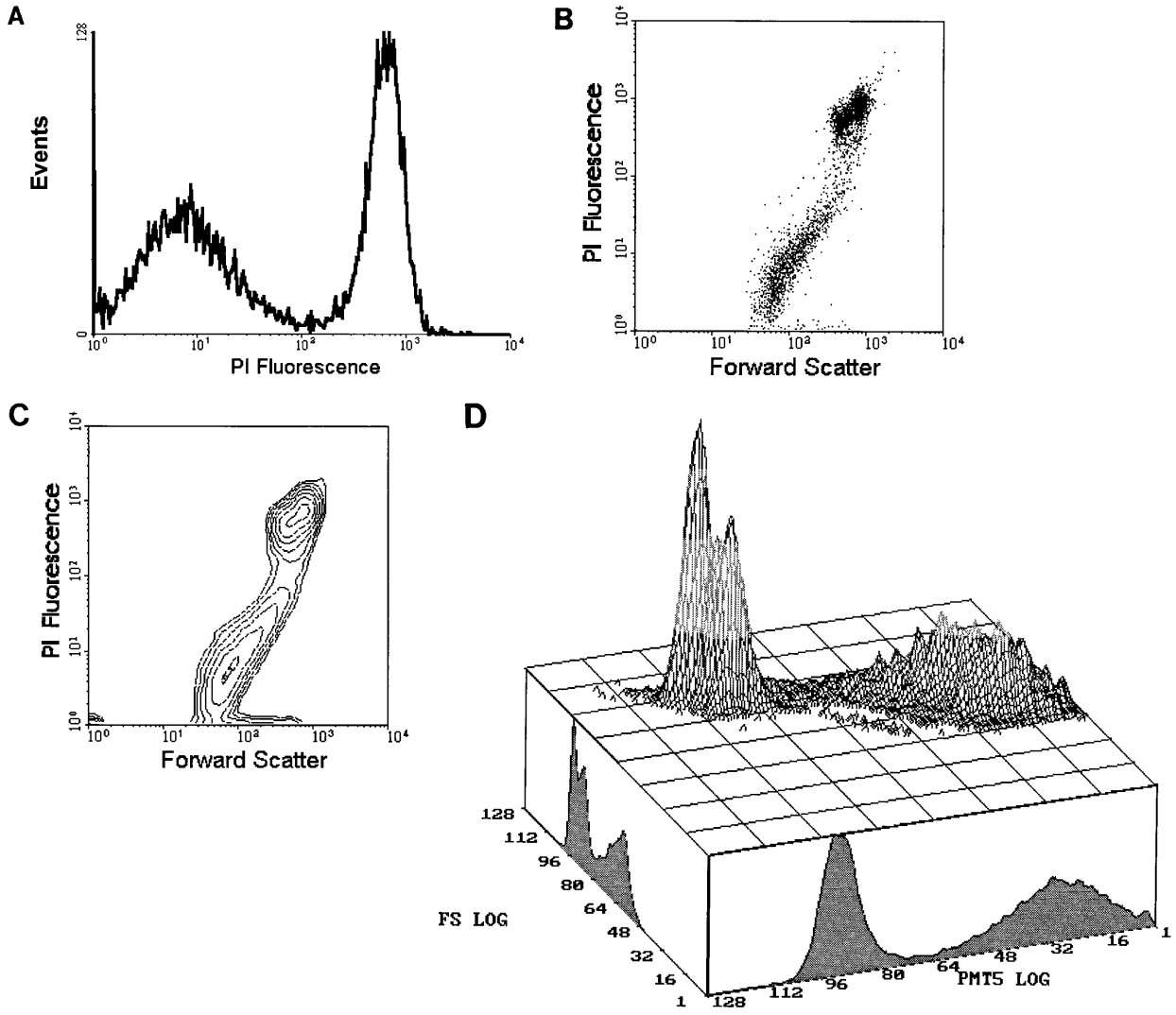

FIG. 2. Representations of flow cytometric data. The data are from a mixture of *Bacillus globigii* spores and vegetative cells of *S. cerevisiae*. (A) Single-parameter histogram of propidium iodide (PI) fluorescence (the yeast cells show the greater staining). (B) Dot plot of propidium iodide fluorescence versus forward light scattering. Each dot represents the position of a single particle in this two-dimensional space. (C) Contour plot of the data in panel B, where contours represent regions of increasing frequency of events. (D) Three-dimensional isometric plot of the data in panel B. FS LOG, logarithmic forward light scattering; PMT5 LOG, log PI fluorescence. Note how each representation brings out different features of the data.

gained of the distribution of a variety of properties of interest among the cells in the population as a whole. A number of displays of such multiparameter flow cytometric data are in common use, and because these will be unfamiliar to a general audience, we consider it worthwhile to illustrate them in Fig. 2.

Some Historical Aspects

The automated flow analysis of single cells had its modern beginnings some 60 years ago, when Moldovan reported a photoelectric method for counting individual cells flowing through a capillary tube mounted on a microscope stage (638). The system described suffered from some serious technical problems in that the narrow tubes required tended to block easily and also caused interference problems as a consequence of the difference in refractive index between the glass walls of the tube and the fluid they contained. A more successful advance was the development by Gucker et al. (381) of an instrument for the analysis of dust particles, primarily for determining the efficiency of gas mask filters, and it is this instrument that is often quoted as being the first flow cytometer (862, 924). Although designed for the analysis of dust, Gucker's particle counter was also applied to the study of microbiological specimens when, during World War II, it was used in collaboration with the U.S. Army for the detection of airborne bacterial spores. Gucker stated in the summary to his paper that the apparatus should have wide application in bacteriology, yet it was to be many years before the flow cytometer became a practical instrument for microbiological research.

In the mid-1950s, Wallace Coulter developed a flow instrument for the electrical measurement of cell volume. The model A Coulter Counter was used to determine the number of cells in a suspension by measuring the difference in electrical conductivity between the cells and the medium in which they were suspended. From this, the model B Coulter Counter, which related the amplitude of the signal produced as the cell passed through the measuring point to the size of the cell, was developed. The modern Coulter Counter developed from these

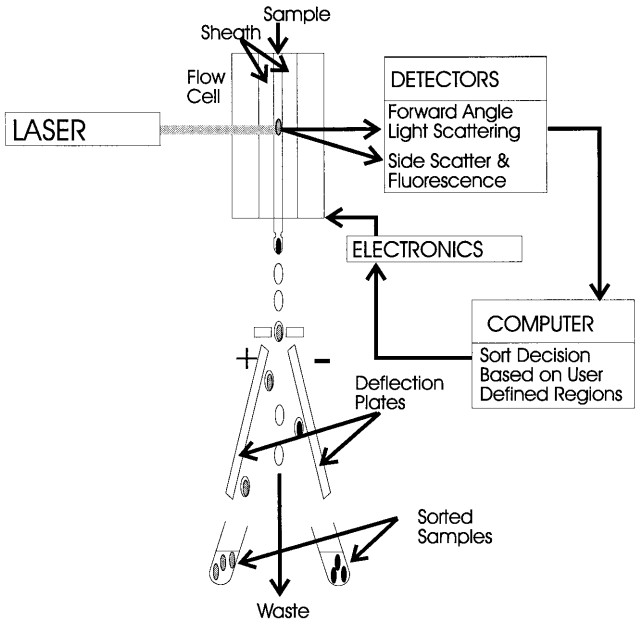

FIG. 3. Flow cytometric cell sorting. A cell passes the interrogation zone, where its flow cytometric light scattering and fluorescence behavior are assessed. Typically, the flow cell is vibrated at some 18 kHz to ensure that a uniform stream of droplets emerges from the end of the flow cell. The cell concentration is made sufficiently low that almost all droplets contain zero or one cell. If a cell or droplet is of interest, it is charged electrostatically, causing the droplet to be sorted. Since the fluid is moving at some 1 to 10 ms^{-1} and the distance from the flow cell to the deflector is, say, 5 mm, the sort decision needs to be made in less than 0.5 to 5 ms, typically allowing the sorting of some hundreds or thousands of cells per second.

early models still enjoys wide use today, but it is with optical rather than electrical techniques that flow cytometry is used to best advantage (although see, e.g., reference 916). Advantages of optical flow cytometry over Coulter counting include the following. The Coulter Counter approach can only easily handle objects with a diameter between, say, 1 to 2 and 20% of the orifice diameter; for smaller objects, there is no signal (or it is buried in the noise), and for larger objects, the danger of blockage can become extremely acute (through clumps and other material which it is hard to have filtered out as a result of their closeness in size to the sample of interest). This means that the dynamic range in terms of size is rather limited. In addition, the apparent size of a nonspherical cell depends on its orientation as it goes through the orifice, no discrimination of viable and nonviable cells is possible (even chalk dust gives a blip), and no easy method for species determination exists. By contrast, optical flow cytometry gives a scattering signal that is largely independent of orientation, whereas because of the substantial amount of sheath fluid around the cells, clumping and blocking is much less of a problem and the dynamic range can be much larger, while viable and nonviable cells are easily discriminated by using appropriate stains and species determination is possible with the use of fluorescent antibodies and oligonucleotides.

In 1965, Kamentsky and colleagues used spectrophotometric techniques to measure the nucleic acid content and light scattering of unstained mammalian cells in a flow stream (477). Later the same year, the first flow sorter was described by Fulwyler (330). The instrument separated biological cells according to their volume, which was determined as they passed through a Coulter aperture. The development of the cell sorter (Fig. 3) was made possible by advances in a very different field; in fact, the technology required to sort the droplets had first been developed for ink jet printers but was adapted by Fulwyler for the very useful function of cell separation. Using this technology, Fulwyler successfully separated a mixture of mouse and human erythrocytes whose mean volumes were 50 and 100 μm, respectively, and also demonstrated the separation of mouse lymphoma cells from debris present in their growth medium. It was also shown that the viability of the separated cells was high (at least 96% in all samples tested) and that following sorting, they grew with a generation time identical to that of an untreated control.

Throughout the 1970s, applications of flow cytometry to the study of mammalian cells were reported (see, e.g., references 66 and 195), and these almost exclusively used laser illumination for measurement. Meanwhile, applications of flow cytometry in the field of microbiology were somewhat limited by the small size and consequently the low concentrations of cellular constituents of microbial cells. The DNA contents of *Saccharomyces cerevisiae* and *E. coli*, being some 200 and 1,000 times less than that of a normal diploid human cell (326, 904), were below the detection limits of the early flow cytometers.

In the late 1970s, improvements in optics technology and the development of better fluorescent stains resulted in the technique of flow cytometry being applied successfully to microorganisms. In 1977, for example, Pauu et al. (723) reported the use of flow cytometry for the study of the cell cycle of three bacterial species with different growth rates (*E. coli*, *Rhizobium meliloti*, and *R. japonicum*) by using a combination of light scattering and ethidium bromide (DNA) fluorescence signals. The results of studying the cell cycle by the analysis of single cells by flow cytometry largely confirmed the results that had previously been obtained from (synchronized) population studies by earlier workers (260, 409).

Hutter and Eipel demonstrated the use of flow cytometry for the study of several types of microorganisms including an alga (*Chlorella* sp.), a bacterium (*E. coli*), several yeasts (*Saccharomyces cerevisiae*, *S. pastorianus*, and *Schizosaccharomyces pombe*) and a (sieved) filamentous fungus (*Nectria coccinea*) (439). The DNA content of these organisms was determined by staining with propidium iodide, and the total cell protein was stained with fluorescein isothiocyanate (FITC). Dual staining for DNA and protein was also performed. These investigators demonstrated the measurement of autofluorescence from algal chlorophyll and in a later study used similar techniques to extend the study to a wider range of bacteria and yeasts (440).

Although flow cytometers designed for the study of mammalian cells produced some useful results with microbes, the development by Steen and coworkers of a sensitive arc lamp-based instrument designed specifically for work with bacteria was an important step in increasing the use of the technique in microbiology. The instrument, later to be commercialized as the Skatron Argus flow cytometer (and now available in an updated design as the Bio-Rad Bryte HS instrument), was first described by Steen and Lindmo in 1979 (909). One of the factors important for the success of the new flow cytometer was the redesigned flow chamber (Fig. 4). The "open" flow chamber used in the Skatron Argus flow cytometer results in a flat, laminar flow of sheath fluid (and sample) across the surface of a glass coverslip. This flow has only two interfaces—the glass/water interface and the water/air interface—and thus results in less background light scatter than that in the fully enclosed flow chambers found in other flow cytometers. Of the two interfaces, only the glass/water interface can become contaminated by deposited particulate matter which would cause background light scatter, and the orientation of the surfaces perpendicular to the optical axis means that the surfaces them-

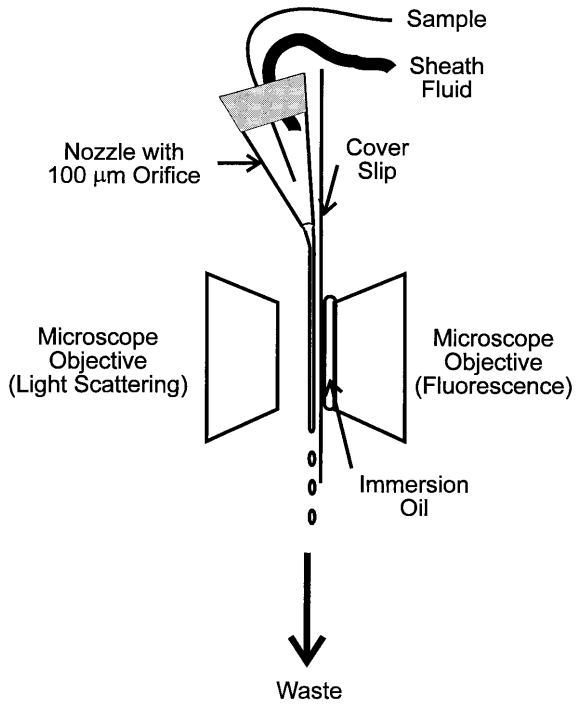

FIG. 4. The flow chamber of a microbial flow cytometer. At the measuring point of the Skatron Argus 100 flow cytometer, a hydrodynamically focused stream of cells hits a glass coverslip and forms a laminar flow across the surface. In the early instrument designed by Steen et al. (909), the coverslip was mounted on the stage of a fluorescence microscope. In the commercial version of the instrument, the coverslip and nozzle assembly have been rotated through 90°. The "open" arrangement of this flow chamber leads to a high signal-to-noise ratio, which is very important for measurements of microbial cells.

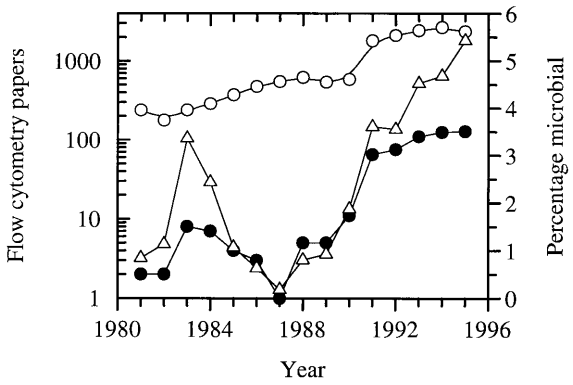

FIG. 5. Bibliometric analysis of flow cytometric studies. A survey was made of the database of the Institute for Scientific Information, counting all papers whose title, keyword, or abstract contained the words flow and cytometr* (the * indicates any characters following in the word) (open circles). Papers relating to the flow cytometry of microorganisms (solid circles) required that one or more of the following also be present: bacteri*, microorganism*, prokaryot*, procaryot* or yeast (where * again represents any characters). The percentage of all flow cytometry papers which, on this basis, deal with microorganisms is also shown (open triangles). Data before 1991 do not include a search of abstracts. Since 1987, microbial flow cytometry shows a steady growth, and in 1995, more than 5% of flow cytometry articles included studies of microbes.

selves scatter only the minimum of light. Although the first application of their newly designed flow cytometer was for the measurement of animal cells (909), it has subsequently been shown both by Steen and by other groups using instruments based on Steen's design, that the high signal-to-noise ratio of the new flow chamber results in an instrument that is ideal for making measurements on microorganisms (see, e.g., references 15, 114, 218, 220, 248, 249, 289, 462, 720, 910–912, and 914).

Throughout the last decade, a growing number of papers describing various applications of flow cytometry in the field of microbiology have been published (87, 113, 289, 316, 326, 863) (Fig. 5), and a book devoted to the subject has recently been published (568). While some current workers in the field make use of the arc-lamp/open-flow chamber design (15–17, 196, 218, 220, 249, 774, 987), others use laser-based systems with closed flow chambers (836, 837, 844). The two types of illumination have been compared (728), and the selection of one rather than the other depends mainly upon the range of wavelengths required for excitation of the selected fluorescent stains, although the arc lamp has the additional advantage that it is a less expensive light source than most lasers (Table 1). Although the early laser-based instruments were not sufficiently sensitive for many microbiological applications, continually improving technology has resulted in instruments that may be successfully applied to a range of measurements on bacteria (316, 326, 347, 568, 836), viruses (783, 784, 837), and even fragments of DNA (368, 372).

Although not normally devoting much of their space specifically to microorganisms (the recent third edition of Shapiro's wonderful book is a most honorable exception [865], which we particularly recommend, and not only for this reason), a number of useful flow cytometry textbooks (20, 212, 213, 355, 378, 627, 713, 808, 1004, 1005) and review articles (19, 206, 337, 588, 653, 861, 915) may also be pointed up. Reviews concentrating on the applications of flow cytometry to microbial systems include references 11, 288, 316, 326, 670, 863, and 948). Finally, in the Spirit of the Age, we would mention that increasing volumes of information about flow cytometry are available on the Internet via the World Wide Web. Since many of the relevant sites purposely include links to each other, it is appropriate just to mention the Home Page of the Society for Analytical Cytology, whose Uniform Resource Locator (URL) is http://nucleus.immunol.washington.edu/ISAC.html (with a U.K. mirror site at http://www.cf.ac.uk/uwcm/hg/hoy/ISAC__Web__mirror/ISAC.html), and the Purdue Flow Cytometry Labs (http://flowcyt.cyto.purdue.edu/). Our own Flow Cytometry Page, which contains links to many sites around the world, is at http://pcfcij.dbs.aber.ac.uk/home.htm/ or via the group's home page at http://gepasi.dbs.aber.ac.uk/home.htm/.

ADVANTAGES OF FLOW CYTOMETRY

As well as the specific and invaluable benefits of microscopic analysis, which we stressed above as a crucial feature of flow cytometric procedures and that follow from the ability to analyze individual cells, flow cytometric methods enjoy the benefit of at least three major factors which we believe also contribute substantially to the power of these approaches. Although we shall illustrate these in more detail below, especially in the sections covering applications, it is appropriate here just to collate and outline these advantages, which include multiparameter data acquisition and multivariate data analysis, high-speed analysis, and the ability to effect cell sorting.

Multiparameter Data Acquisition and Multivariate Data Analysis

Many conventional wet chemical analyses (although not those based on chromatography) tend to measure only one determinand in a sample at a time, and of course they rarely

TABLE 1. Comparison of conventional laser-based and arc lamp-based flow cytometers

Parameter	Light source	
	Laser	Arc lamp
Excitation wavelength	Selectable by laser choice (e.g., HeCd, 325 nm; Ar^+, 488 nm; HeNe, 633 nm)	Selectable by filter block (e.g., in Skatron Argus, A1, 366 nm; B1, 395–440 nm; FITC, 470–495 nm; G1, 530–550 nm)
Parameters measured	Forward light scattering, side scattering, frequently four or more fluorescence parameters	Forward light scattering, side scattering, one or two fluorescence parameters
Fluorescence emission collection	Wavelengths selected by use of one or more optical filters	Wavelengths for collection fixed by choice of filter block
Relative cost of light source	Typically expensive; modern laser diodes can be cheap	Usually cheap
Sorting	Available in higher-specification machines	Not available in commercial instruments of this type

possess the sensitivity necessary to assay single cells. By contrast, appropriate combinations of stains and exciting wavelengths permit the estimation of multiple determinands on each cell, permitting one, for instance, conveniently to distinguish between cell types in mixed cell populations or to determine the detailed relationships between different cellular variables. Progress in realizing these advantages is such that Kachel et al. (475), for instance, described an eight-parameter system based on a simple personal computer, Robinson et al. (809) acquired a set of multicolor immunofluorescence data from a given sample incubated with 11 tubes containing multiple phenotypic markers, and the so-called Optical Plankton Analyzer (58, 274, 275, 426, 427, 724, 990; also see below) can permit the acquisition of even more variables from each cell. As we shall discuss in more detail below, coupled with the ability to acquire multivariate data from each cell is the much greater discriminating power that follows from the ability to exploit chemometric data reduction techniques than simple univariate analyses alone (see, e.g., references 121, 429, 465, 536, 605, and 616).

High-Speed Analysis

Given the need to avoid both coincidence counting and mechanical damage to the cells of interest, the combination of the cell concentrations and linear flow rates usually used means that most flow cytometers analyze some 100 to 1,000 cells $\cdot$ s^{-1}, with some trade-off (inevitably) between speed and precision. This is frankly more than adequate for most purposes, in which one is merely interested in obtaining an adequate level of precision in the assessment of the distributions of different populations of cells, when the acquisition of 10,000 events may be effected within 10 to 100s. When one is particularly interested in detecting very rare cells, the ability to do so is not in fact greatly limited by the rate at which individual cells may be analyzed but by other factors. Thus, Gross et al. (379) seeded the pre-B-cell line REH into 250×10^6 peripheral blood mononuclear cells at frequencies of 10^{-4}, 10^{-5}, and 10^{-6} and could detect the REH cells with a background of about 1 event per 10^8 peripheral blood mononuclear cells. The four main sources of false-positive events which had to be removed to achieve this sensitivity were nonspecific immunofluorescence, autofluorescence, background particles from previous experiments, and bursts of nonspecific photon "events" during acquisition. The use of fewer parameters is less effective, but even with two parameters, 2 events per 10^7 cells can be determined (795). We are not aware of comparable studies with microorganisms, but it is not obvious that any such findings would be radically different.

Sorting

As indicated above (and see also Fig. 3), it was early recognized that the data accruing from flow cytometric measurements could be analyzed (electronically) sufficiently rapidly that electronic cell-sorting procedures could be used to sort cells with desired properties into separate "buckets," a procedure usually known as fluorescence-activated cell sorting. As we shall see later, this has profoundly beneficial consequences for a number of areas of microbiology and biotechnology.

ARE THERE ANY DISADVANTAGES OF FLOW CYTOMETRY?

While the focus of this review concerns the intellectual, scientific, and practical benefits that accrue from the ability to analyze large numbers of microbial cells individually, it would be wrong to gloss over the two main features of this approach which could be construed as disadvantages. The first is the substantial cost of many of the various flow cytometers presently available: a typical laser-based flow cytometer capable of analyzing but not sorting might work out at $100,000, while the addition of a sorting facility might double that. The arc-lamp-based cytometers are somewhat cheaper than this (but as implemented commercially cannot sort) and might work out at $75,000. Such sums are very much at the high end of the current costs of typical advanced laboratory instrumentation such as mass spectrometers. However, the continuing rise in computer power and in the installed base of flow cytometers is leading to a fall in costs in real terms, and we believe that much cheaper instruments, more directly tailored to the microbiologist and using laser diodes as sources and photodiodes as detectors of photons, will shortly become commercially available. Indeed, preliminary data from such a device are given later. The second potential disadvantage is that because these are sophisticated instruments, especially the laser-based cell sorters, skilled operators are usually required to obtain the optimum (and sometimes even any kind of acceptable) performance. Nevertheless, the increasing number of articles on mi-

crobial flow cytometry does serve to indicate that reliable and useful data are being obtained in an increasing number of laboratories, and the most modern instruments are carefully designed to maximize their user friendliness.

BASICS

Light Scattering and Its Angular Dependence

Light scattering is of course widely used in almost all microbiology laboratories, via optical density measurements, as a means of estimating microbial biomass (593). Under these circumstances, of course, it is simply assumed (correctly) that the scattering of light means that fewer photons arrive at a photodetector collinear with the source than would have done in the absence of the scatterers. Such measurements do not therefore attempt to discriminate photons scattered at different angles. The elastic scattering of light by microbial and similarly sized particles depends (nonlinearly) on a number of factors, including, in particular, the illuminating wavelength, the relative size of the scatterer, and the difference in refractive index between the scatterer and the medium (see, e.g., references 104, 152, 389, 390, 511, 520–522, 547, 834, 867, 960, 1042, and 1044). In brief, Rayleigh scattering occurs when the particle sizes are significantly less than the wavelength of the light, while a modified form of Rayleigh scattering, usually referred to as Rayleigh-Debye-Gans scattering, occurs when, as in the case of many bacteria, the particle size and wavelength of light are of the same order (519, 520, 1042). One result of these considerations is that at lower cell concentrations, direct measurement of the scattered light (nephelometry), typically at right angles to the illuminating beam, provides a much more accurate measure of particle density than does the optical density (389, 394, 492, 593, 1044). However, given the dependence on the above factors of the scattering at different angles, it is clear that much more accurate and informative analyses might be obtained if one were to measure light scattering at many angles simultaneously and to use these data in combination with modern methods of multivariate analysis to obtain information not only on overall biomass but also on its size, contents, metabolic status, and morphology.

The generalized approach of multiangle light scattering was largely developed within microbiology under the term "differential light scattering" by Wyatt (1044; also see reference 1047), and a commercial instrument, the DAWN, is available (1045, 1046). This instrument has the particular merit that the data acquisition is carried out simultaneously by 18 separate detectors. However, the general approach has still been little exploited (1045, 1046), and we believe that this is mainly because of the inability, contingent on the complexity of the biological systems involved, to account adequately for the differential light scattering in terms of the physical theories alluded to above (1043). Similarly, the angular dependence of polarized light scattering provides a rich source of information (122, 123). Another excellent example is provided by the study of individual spores of *Bacillus sphaericus* by Ulanowski et al. (955), using a scanning laser diffractometer in which the scattering was logged serially at every 1° between 4 and 176° (this is actually rather too many [632] and would probably cause problems of collinearity in multivariate calibration!). Despite the morphological simplicity of the target biological system and the lack of complications from studying heterogeneous suspensions containing many different cells, the light-scattering data obtained (see Fig. 1 of reference 955) were not at all well fitted by theoretical (Lorenz-Mie) scattering curves containing four free parameters (two radii and two refractive indices). Indeed,

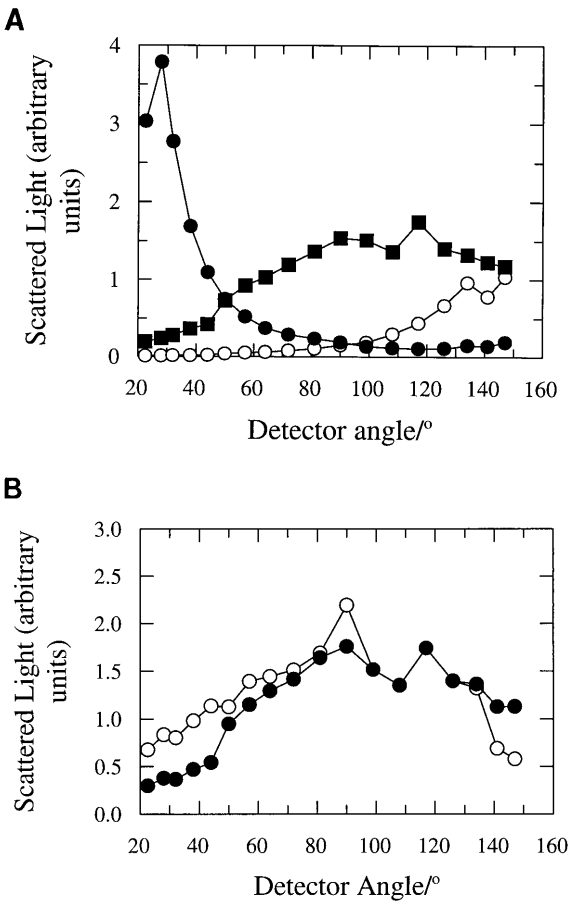

FIG. 6. Angular dependence of light scattering by microbial cells. Cells of baker's yeast were suspended to the concentrations indicated and the angular dependence of their light scattering was measured in a DAWN instrument (1045, 1046). (A) Dependence of differential light scattering on the concentration of viable cells. Symbols: ○, 50 mg · ml^{-1}; ■, 7 mg · ml^{-1}; ●, 0.1 mg · ml^{-1}. (B) Dependence of differential light scattering on viability at a concentration of 6 mg (dry weight) · ml^{-1}. Symbols: ○, viable; ●, dead. The yeast cells used had a median diameter of ca 6.5 μm. Forward scattering equates to a detector angle of 0°.

unexpectedly complex patterns can be seen even with latex beads (269, 425), although on occasion the narrow (forward) angular dependence of scattering has been exploited to obtain information on cell shape (205). The exploitation of multiangle light scattering for the analysis of pure or mixed cultures of cells was also reviewed by Salzman et al. (835); one of the systems described measured light scattered at up to 32 discrete angles between 0 and 21° (777) and (not surprisingly) found high correlations between many of the signals. Carr and colleagues have also pursued the differential light-scattering approach (459) and obtained very reproducible data from a variety of fermentations with *E. coli*, *S. cerevisiae*, and *Pseudomonas aeruginosa*, but while the same group has successfully exploited a variety of other modern optical methods for the extraction of very useful information from microbial fermentations (see, e.g., references 151, 152, 166, 178, 179, 461, and 725), they were again unable to account for the differential light-scattering data on the basis of theoretical scattering models. Finally, to illustrate the utility of measurements of this type and the potentially rich information that they contain, Fig. 6 shows the angular dependence of the light scattered from a number of cell suspensions. From this, it may be observed that

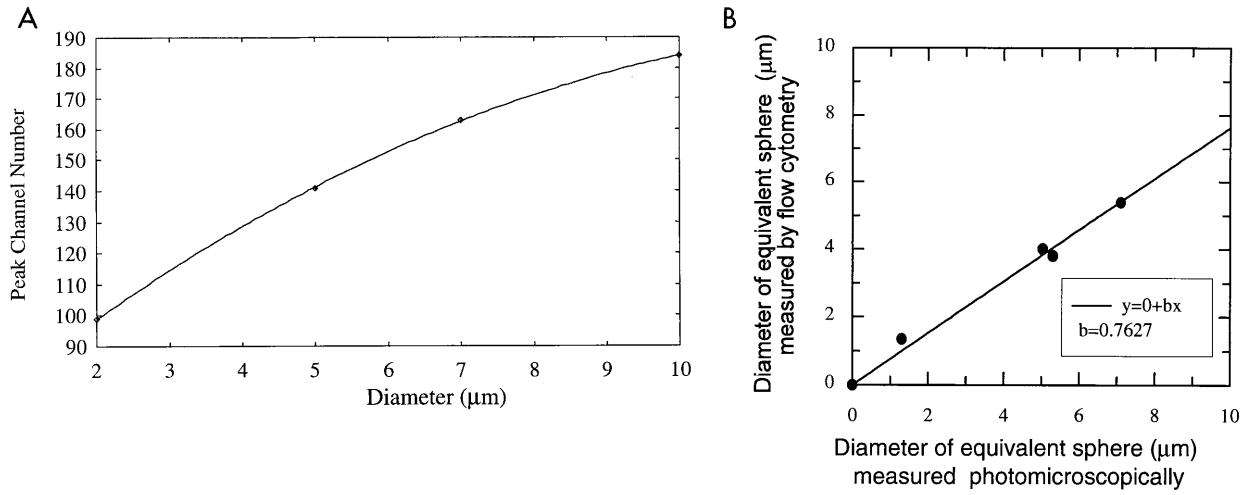

FIG. 7. (A) Relationship between particle diameter and peak channel number of forward light scatter for latex beads. The data are well fitted by the three-term polynomial ($y = 63.62 + 19.05x + 0.7x^2$), where x is the diameter in micrometers and y is the equivalent channel number. (B) A comparison of size measurement by flow cytometry and by direct measurement from photomicrographs. Data are for unfixed baker's yeast and *Micrococcus luteus* cells suspended in phosphate-buffered saline and were obtained on a Skatron Argus instrument. The graph shows that calibration of the forward scatter signal by latex beads gives an underestimation of the true cell size. However, the gradient (b) of the straight line $y = 0 + bx$ gives an additional calibration factor, which can be used to convert the apparent cell size measured by flow cytometry into a "true" cell size.

at low concentrations (more characteristic of the conditions in a flow cytometer), most light is scattered at small forward angles and that it is indeed possible to distinguish organisms in different physiological states on the basis of their differential light-scattering behaviour, even if it is not easy to interpret the differential scattering on the basis of exact biophysical theories. It should also be mentioned that there is evidence that the shape (width) of the light-scattering pulse may give more accurate estimates of cell size (202, 947), but this feature is not available on most commercial cytometers and is not discussed further.

The above types of measurement are performed under essentially steady-state conditions, and the acquisition of photons may be averaged over a relatively extended period. Partly to maximize the number of photons collected during the much shorter time that an individual particle is being interrogated, present generations of commercial flow cytometers tend to collect light scattered only at "low" and "high" scattering angles relative to the direction of the exciting beam, e.g. 2 to 15° and 15 to 90°, usually abbreviated as forward and side scattering, respectively. However, in research instruments a limited number of multiangle light-scattering devices have been developed (see, e.g., reference 777; for a review, see reference 835). In modern, sensitive flow cytometers, even single microorganisms may be detected by simple light-scattering measurements (Fig. 2). Light scattered in the forward direction is often used as a measure of cell size, although the range of angles that are considered "forward" may vary between instruments (as does the name given to such measurements). Work by Mullaney and Dean (655) showed that the light scattered at small angles (i.e., forward light scatter) could be successfully used to determine relative volume distributions for populations of cells. In their models, they used homogeneous spheres as approximations to biological cells. It has also been shown that the amount of forward-angle light scattering of dextran gel beads depends upon their diameter (867) and that although there was some change in the amount of forward scattering when beads with different refractive indices were analyzed, the effect of this on the forward-scatter signal was much smaller than it was on the scattering detected at wider angles. In general, the extent of small-angle light scattering by a cell does depend largely (though not always linearly [215, 218, 834] [Fig. 7]) on the mass or volume of the cell, while the refractive index of the cellular contents is considered to be of more importance in accounting for the extent of side scattering. In reality, the relationship between forward-scattered light and cell size is more complex, but provided that only physiologically similar cells are compared, the extent of their forward light scattering can be used to good effect for the estimation of microbial cell size (218). Different cells require different calibrations, however (168, 169), and, in growing yeast cultures, a discontinuity in the relationship between forward light scattering and cell size at the point of cell division has been noted (900). With the present instruments, the size limit for detection of individual particles is usually caused by imperfections in the optical system, leading to spurious scattering signals from the lenses and suspension fluid, and thus is not usually dominated by the brightness of the light sources or the sensitivity of the detectors (but cf. reference 281). Other important sources of extraneous noise are particulates and air bubbles in the sheath fluid (280). These limits could possibly change if more angles were used individually, more detectors with higher sensitivity and fiber-optics were exploited for collection, and the background artifacts were thereby decreased by multivariate deconvolution.

Light scattered at wider angles can be used to provide information about the internal structure of the cell. Higher-intensity wide-angle scatter signals are usually obtained from cells with the highest levels of cytoplasmic granularity, for example those that have laid down storage compounds. Microorganisms that have been genetically engineered to produce foreign proteins may deposit these as inclusion bodies within the cell cytoplasm. The deposition of these highly refractile inclusions may be expected to result in an increase in light scattering both at right angles to the incident beam and, to a lesser extent, in the forward scatter signal. Wittrup et al. have exploited this change in light-scattering behavior to design a simple yet elegant method for monitoring interferon or growth hormone production in recombinant *E. coli* by flow cytometry (1038). Since such organisms are of industrial interest, it is often desirable to select mutants or subpopulations with the

highest levels of production of the foreign protein to increase yields. The use of an inherently nondestructive probe such as light scattering, together with cell-sorting techniques, would allow such a subpopulation to be isolated from a heterogeneous population for further study. Such measurements have also proved useful for the assessment of poly-β-hydroxybutyrate formation (897) and (see below) to some extent in the discrimination of different microorganisms (15, 904, 905, 911). As well as being used for the estimation of cell size, forward-angle light scatter is frequently used as a gating parameter by which to exclude cell aggregates and debris from further analysis, although a combination of light scatter and fluorescence is sometimes better for the latter purpose.

What Stains Are Available?

While a number of useful measurements can be made on cells by using light-scattering measurements alone, it is the ability of the flow cytometer to quantify particle-associated fluorescence that makes the technique of special utility.

When a compound absorbs light, electrons are raised from the ground state to an excited state. The electrons return to the ground state via a variety of routes; some processes such as the loss of the energy by heat do not result in fluorescence, but certain molecules also lose energy by a process of radiative transfer (fluorescence). When stimulation of the fluorescent compound is stopped (by removing the exciting light source), fluorescence emissions cease very rapidly. This distinguishes fluorescence from the related phenomenon of phosphorescence, which continues for some time following removal of the excitation.

Fluorescence is always of a lower energy, and hence longer wavelength, than the exciting light, and this separation in wavelength is known as the Stokes shift. The Stokes shift enables the exciting and emitted light to be separated by optical filters, and thus the amount of fluorescence can be quantified. The magnitude of the Stokes shift varies among fluorescent molecules, and it is therefore possible to separate the fluorescence emitted by different molecules even if they are present in the same sample and excited by the same wavelength.

By adding fluorescent stains specific for cellular substances to the cell sample of interest, the full potential of flow cytometric fluorescence measurements may be realized. There are a variety of fluorescent probes, either adapted for use with flow cytometry or designed primarily for that purpose (399), and these are outlined in the relevant flow cytometry textbooks (355, 378, 568, 865). See also references 730 and 992 and, in particular, the outstanding catalog of such substances available from Molecular Probes (398) and also browsable over the Internet at the URL http://www.probes.com/.

Certain characteristics of potential fluorescent stains must be considered to determine whether they will be useful for flow cytometric assays. The extinction coefficient of a dye is a measure of the amount of light that can be absorbed at a given wavelength. The most useful fluorescent dyes have their maximal absorption wavelength (i.e., highest extinction coefficient) close to one of the spectral lines of the most common flow cytometer light sources (e.g., the argon ion laser has a powerful emission at 488 nm) but distant from the excitation maximum of autofluorescent molecules that may be present within the cells of interest. At the wavelength that is chosen for excitation, the stain should have a high extinction coefficient so that small concentrations of the stain can be detected within the cell. For example, fluorescein (a very popular dye for flow cytometric measurements) has its maximum absorption at 495 nm, which is close to the strong 488-nm line of the argon laser, and at 488 nm, fluorescein has an extinction coefficient of 8×10^4 M^{-1} cm^{-1} (714). Because fluorescence is caused by electronic transitions, which usually have relatively high energies, cellular autofluorescence tends to decrease substantially toward the red end of the optical spectrum, a fact which is driving the development both of red- and near-infrared-excited fluorophores for fluorimetry (8, 125, 154, 304, 594–596, 722, 868, 869) in which single-molecule detection is achievable (28, 29, 67, 383, 680, 839, 892–894, 934), and of Raman spectroscopy involving near-infrared excitation (157, 371, 411, 849), in which fluorescent photons can interfere drastically when excitation is in the visible part of the spectrum. (In terms of sensitivity in the absence of autofluorescence, flow cytometric single-molecule detection with a photon counter was achieved in 1987 [678].) Finally, it is worth commenting that one of the major motivations for making single-molecule fluorescence measurements is exactly that which drives the use of flow cytometry, namely, that nominally identical molecules are in fact heterogeneous, here because of interactions with their environment, a fact which bulk measurements cannot usually determine.

In addition to the extinction coefficient of a stain, its quantum yield should be considered, since fluorescence intensity is proportional to the product of these two factors. The fluorescence quantum yield of a dye is the probability that absorption of a photon leads to the emission of a photon of fluorescence. The quantum yield can be affected by environmental factors such as pH and may increase on binding to the target substance within the cell. For example, fluorescein has a quantum yield of 0.5 to 0.7 at pH 8 but this drops rapidly with decreasing pH (992), while the fluorescence from propidium iodide is increased over and above that found in aqueous solution following intercalation into double-stranded nucleic acid (714).

Another factor that may be considered is the photostability of the dye, i.e., the number of times it can be excited before decomposing; for fluorescein, this number is in the region of 10^4 to 10^5 excitations (992). While this is a very important issue for the selection of dyes for use in fluorescence microscopy, provided the stains are handled correctly prior to use, photostability is not usually considered to be a problem with flow cytometry, since the cells are in the illumination zone of the flow cytometer for such a short time (204). However, if the excitation intensity exceeds, say, 100 mW (although modern instruments with sensitive detectors do not require such powerful light sources and a 15-mW argon ion laser is an adequate excitation source even for bacteria), each molecule may be excited on the order of 100 times as it passes through the focus; thus, if the bleaching quantum yield is 0.001 or greater, as may occur for some dyes in aerobic media, bleaching may be significant.

The length of time between excitation of and emission from the fluorescent molecule is also of some importance. Rapid emission (of the order of a few nanoseconds) may allow the fluorescent molecule to be excited more than once during its passage through the illumination zone, which typically lasts some 10 to 100 μs. Fluorescein, for example, has an excited lifetime of about 4 ns (992). Other factors that may be important in certain situations include the solubility of the stain in water, the ease with which it penetrates the cell, and the toxicity of the stain, the last being of particular importance when the technique of cell sorting is applied to obtain a subpopulation of interest for further physiological study.

There are a large number of fluorescent stains that are selective for nucleic acids. The phenanthridinium dyes ethidium bromide (2,7-diamino-9-phenyl-10-ethylphenanthridinium bromide) and propidium iodide (3,8-diamino-5-diethylmethylamino-propyl-6-phenylphenanthridium diiodide) will

bind to both DNA and RNA. Binding usually occurs by intercalation of the dye into the double-stranded helical structure, a process which results in a considerable enhancement of fluorescence over that of the free dye (1004). Since these dyes bind to both DNA and RNA, DNA quantification requires removal of the RNA by pretreatment of the cells with RNase. Both ethidium bromide (66, 723, 751) and propidium iodide (85, 300, 440, 844) have been used successfully in flow cytometric studies of microorganisms. However, for experiments in which cells are stained not only for DNA but also with a fluorescein derivative (e.g., with FITC for total protein [633]), propidium iodide is the dye of choice, since its emission spectrum, being some 10 to 15 nm further toward the red than that of ethidium bromide, is more easily separated from that of fluorescein.

There are a number of nucleic acid stains that will bind more or less specifically to DNA. These include chromomycin A3, olivomycin and mithramycin, DAPI (4′,6-diamidino-2-phenylindole), its analog DIPI [4′,6-bis(2′-imidazolinyl-4H,5H)-2-phenylindole], and the bisbenzimidazole or Hoechst dyes. The most modern DNA dyes such as the dimeric thiazole orange derivatives of the BOBO/POPO/TOTO/YOYO family based on benzothiazolium-4-pyridinium and benzoxazolium-4-pyridinium (398, 600, 828–830) bind particularly strongly and will even migrate with DNA during electrophoresis. It should be noted that these dyes can also bind to RNA. More recently, heterodimeric derivatives which have very large Stokes shifts due to resonance energy transfer have become available (75, 1068).

The fluorescent antibiotics chromomycin, olivomycin, and mithramycin all show affinity for the 2-amino group of guanine in DNA, and hence a specificity for GC-rich DNA (195, 196). All three of these dyes have been used for flow cytometric applications, but the most popular method for microbiological samples appears to be dual staining with mithramycin and ethidium bromide (17, 115, 877, 908, 913), in which the selectivity of the antibiotic for DNA is exploited by illuminating it at a wavelength at which it absorbs and allowing resonance energy transfer (which only occurs when molecules are very close to each other) to ethidium bromide, from which the emitted fluorescent photons are detected. This scheme overcomes the relatively poor quantum yields of the antibiotics and has the further advantage that the fluorescence resonance energy transfer method causes a substantial Stokes shift.

The phenylindoles (DAPI and DIPI) bind nonintercalatively to repetitive AT-rich regions of the DNA (405, 715), as do the Hoechst dyes (90, 543, 643, 757, 837, 962). The AT specificity of the Hoechst dyes, together with the GC specificity of chromomycin A3, has been exploited by Langlois and colleagues for the flow cytometric determination of the G+C content of bacteria (836), bacterial characterization (962), and chromosome classification (372), while mithramycin/Hoechst 33342 was used by others for the study of algae (550). Triple staining of DNA with mithramycin, Hoechst 33342, and propidium iodide has been reported for analyzing cell cycle-related changes in chromatin structure (194). DNA dyes suitable for animal cells and excitable by the 633-nm line of the He-Ne laser were identified by Shapiro and Stephens (866).

Recently, however, novel dimeric stains have been introduced for staining nucleic acids (single- and double-stranded DNA and RNA), which have occasionally been applied to staining bacteria in flow cytometry (981, 989) and which seem set to revolutionize the field. These stains include ethidium homodimer (357) and the BOBO/POPO/TOTO/YOYO dimer family based on benzothiazolium-4-pyridinium and benzoxazolium-4-pyridinium (398, 828–830). They have the advantages of having exceptionally stable binding constants (which may even be increased in the presence of some organic solvents), coupled with really excellent fluorescence enhancements on binding (the free dyes are practically nonfluorescent), yields, and wavelength characteristics. They are membrane impermeant, but their characteristics allow rapid, stable, and sensitive staining of permeabilized cells.

Apart from nucleic acid quantification and analysis, many other cellular constituents have been studied by flow cytometry. The total protein content of fixed cells is commonly estimated by staining with FITC, because it is conveniently excited by the 488-nm line of the argon laser (373, 633, 907). Lipids may be stained with the fluorochrome Nile red (186, 862), which fluoresces yellow when dissolved in neutral lipids and red when dissolved in the more polar lipids (e.g., phospholipids). As with fluorescein, Nile red can be excited at 488 nm, and it has recently been used for staining bacterial poly-β-hydroxyalkanoates (226, 661). In fact, many other (and better) solvatofluorochromic compounds exist (796), but they seem to have been little exploited for flow cytometric purposes (848).

An emerging group of stains for flow cytometry of microbial samples are the fluorescent brighteners. Fluorescent brighteners (362, 456, 721, 1033, 1073) are stains which are widely used as additives in domestic washing powders to improve the apparent "whiteness" of clothes, and they exert this effect by absorbing otherwise invisible UV light and giving off a blue fluorescence (as may be observed in discotheques employing UV lighting!). They have the advantages that (i) they are widely available at low cost, (ii) they are nontoxic to higher organisms (513, 721, 1033), and (iii) since they are excited by UV light, they can be used in staining cocktails in conjunction with stains such as FITC that are excited at 488 nm without significant interference (81). They have been used successfully for staining of fungal cell walls (407) and for the determination of cellular (81) and, most recently, bacterial (613) viability. The Tinopal series of dyes from Ciba-Geigy are particularly widely used, and some years ago we noted the antimicrobial action of Tinopal AN, a benzoxazole dye of this series (744–747), indicating that it must therefore have appropriate binding sites among bacteria. Figure 8 illustrates the staining of a variety of organisms with another Tinopal, Tinopal CBS-X.

As well as stains which bind to (or react with) particular molecules such as DNA, RNA, or protein, fluorogenic substrates which reveal distributions in enzymatic activity, indicators which change their property as a function of pH_{in} or which are taken up in response to membrane energization, or, increasingly, antibodies (or oligonucleotides) tagged with a fluorescent probe may also be exploited. This is of course true both for flow cytometric analyses and for cognate analyses carried out by using sensitive microscopy in situ, as are being developed by Barer and colleagues (376, 557, 691, 1027). Clearly, the possibilities are limited mainly by the ingenuity of the experimenter, and for illustrative purposes, a variety of fluorescent probes that have been used in conjunction with flow cytometry, together with representative excitation/emission wavelengths and their applications, are shown in Table 2. While details should be sought in the references cited, it is worth stressing that some, but not all, of these reagents are normally membrane impermeant and require that the cells be fixed or permeabilized with ethanol (70%), formaldehyde, or glutaraldehyde. The question whether and how to fix is a thorny one, since fixation brings both advantages and disadvantages. The chief advantages are that (i) fixed cells may be stored indefinitely before being stained (and do not seem to change their flow cytometric properties substantively even when stored for several months (280) and probably longer) and

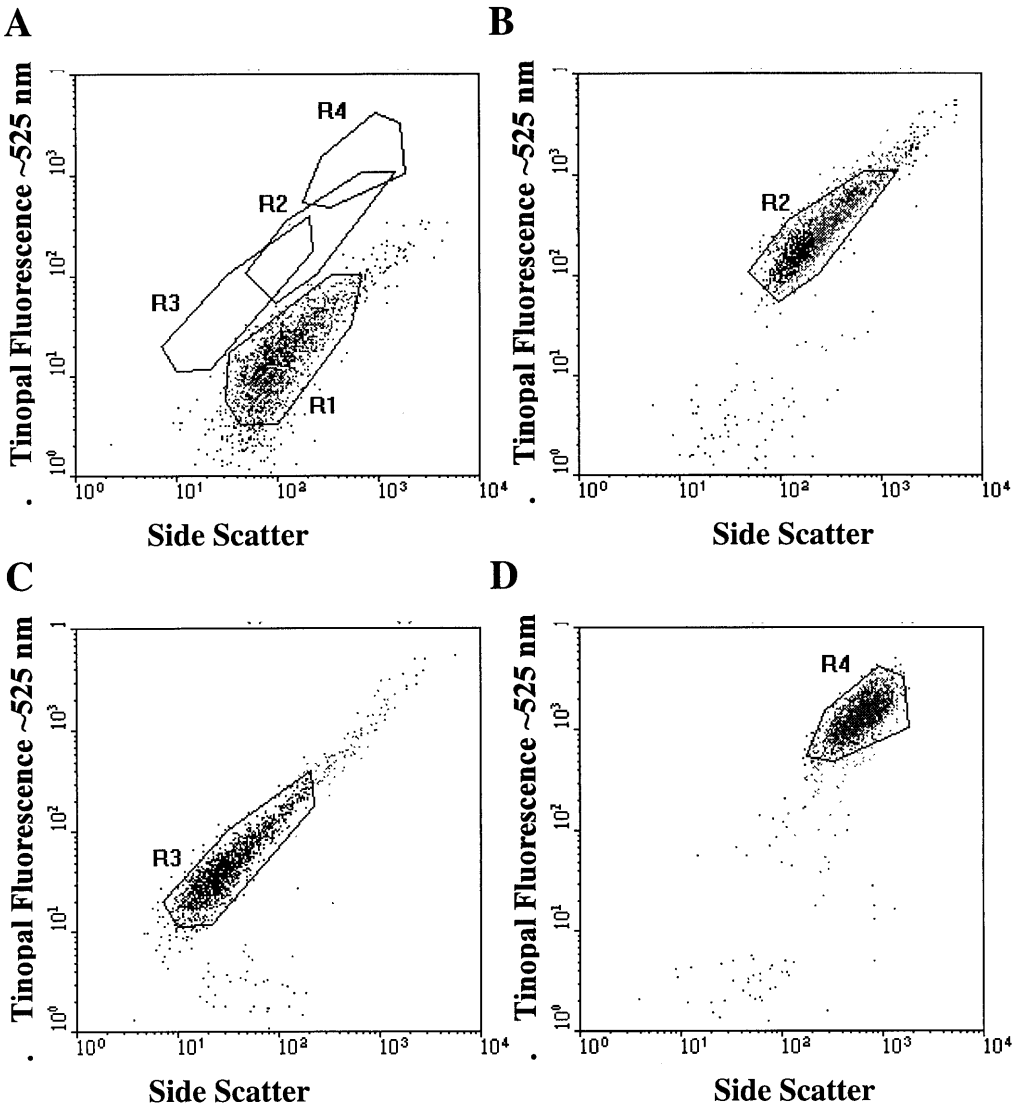

FIG. 8. Flow cytometric properties of Spores of *B. subtilis* subsp. *niger* (A), *E. coli* (B), *M. luteus* (C), and *S. cerevisiae* (D) stained with Tinopal CBS-X. Flow cytometry was performed on a Coulter Epics Elite flow cytometer, and signal was discriminated from background noise by the forward-scatter signal from the argon (488 nm) laser. Side scatter from the argon laser was collected on PMT2 by using a voltage of 400 V. The organisms were stained with Tinopal CBS-X at a concentration of 40 $\mu g \cdot ml^{-1}$ and were excited with 325-nm emission from a HeCd laser. Fluorescence at 525 nm was collected on PMT3 (570 V) via the gated amplifier. All gains were logarithmic. R1 to R4 are regions which may be used for identifying the different organisms.

(ii) fixed cells may be stained with fluorophores which are normally membrane impermeant (and thus a very much larger range of useful fluorophores is then available). Other stains will penetrate unfixed cells, but when a rapid assay is required, the kinetics of staining may be too slow. In the case of the Tinopal CBS-X stain described above, ethanol fixation allows one to obtain maximally stained cells in a few seconds rather than the several minutes required for unfixed cells (Fig. 9). Indeed, when studies are performed in vivo, one can never be sure (as indeed also applies with the question of antibiotic sensitivity [221, 682, 896]) whether a reduced extent of staining is caused by a lowered concentration or conformational change of the binding site, a lowered permeability to the stain, or (105, 462, 556, 908, 988) active efflux of the stain via a membrane efflux pump. Gram-negative cells in particular tend to exclude many cationic stains, a problem which can only partially be overcome by treatments, such as with Tris-EDTA, designed to improve their passage through the outer membrane.

The disadvantages of fixing are that (i) viable stains and other stains based on membrane energization cannot be used to assess these variables, and indeed all data on viability are lost (although parallel runs may be performed); (ii) since the cells are killed, sorting and regrowth of sorted cells is impossible; and (iii) there may be other changes in the flow cytometric behavior of the cells that are induced by the fixation process itself, in particular as a result of the conformational changes induced by the fixatives in cellular macromolecules.

When cells are stained, for example with a fluorescent DNA stain, measurements are often gated on cell size and the data are plotted as a dual-parameter histogram of DNA versus size versus counts (as in Fig. 2). It is therefore important to determine the effects of fixing on cell size and light-scattering behavior. While a major purpose of fixing cells prior to staining is to permeabilize the cell membrane to allow entry of the probes, this will also facilitate leakage of cell contents and will allow the suspension medium to enter the cell. This may be

TABLE 2. Some determinands amenable to analysis by flow cytometric fluorimetry

Determinand	Stain	Ex (max)[a] (nm)	Em (max)[a] (nm)	Application(s)	Reference(s)[b]
DNA	Ethidium bromide	510	595	Cell cycle studies, detection of biological material	*526*, *723*, *751*
	Propidium iodide	536	623	Ploidy determination, viability (membrane integrity) testing, antibiotic susceptibility testing	*306*, *440*, *544*, *633*, *758*
	Hoechst 33258/33342	340	450	Fermentation monitoring, determination of the percent G+C content, virus detection	*90*, *836*, *837*, *856*
	DAPI	350	470		*408*, *715*
	Acridine orange	480	520		210, 211
	Chromomycin	340	470		*115*, *196*, *836*, *837*, *856*, *883*
	Mithramycin (alone or combined with ethidium bromide)	425	550		
	Olivomycin	480	625		
RNA	Propidium iodide	536	623	Fermentation monitoring	*306*
	Acridine orange	440–470	650		210, 211
	Pyronine Y	497	563		992
	Thiazole orange	509	525		399
Protein	FITC	495	525	Identification	*218*, *306*, *633*
	Rhodamine 101 isothiocyanate (Texas Red)	580	620		901
Enzyme activities	Substrates linked with naphthoyl, fluorescein, umbelliferyl, coumaryl, and rhodamine groups	Depends on added label			253, 254, 535
	β-Galactosidase				*685*, *1037*
	Dehydrogenases (e.g., 5-cyano-2,3-ditolyl tetrazolium chloride [CTC])	530–550	Varies		*479*, *482*
Antigens	Fluorescently labelled antibodies	Depends on added label		Detection and identification	*252*, *739*
Nucleotide sequences	Fluorescently labelled oligonucleotides	Depends on added label		Detection and identification	*84*
pH	BCECF	460–510	520–610	Excitation and emission spectra depend strongly on pH	378, 786, 963
	SNARF-1	510–580	587–635		
Lipid	Nile Red	551	636	Poly-β-hydroxy butyrate detection	*661*
pCa	Aequorin		460	Calcium concentration	*147*
	Indo-1	330–350	390–485	Typically used in ratiometric mode	378, 730, 785
	Fluo-3	460–510	530–560	Typically used in ratiometric mode	637, 801
Membrane energization	Oxonol ($DiBAC_4(3)$)	488	525		*224*, *576*, *577*
	Rhodamine 123	510–560	580	Viability testing, respiratory activity measurements	*220*, *246*, *249*, *479–481*, *576*, *763*
Cardiolipin	10-N-nonyl-acridine orange	450–495	640	Mitochondrial assay	730
Carbohydrate polymers such as chitin	Calcofluor white	347	436	Viability testing	81, *613*

[a] Ex (max) and Em (max) are the wavelength of maximal excitation and emission respectively, although it should be noted that the excitation and emission wavelengths are dependent to a variable degree on the conditions used for measurement.
[b] References in italics pertain to analyses of microbial cells.

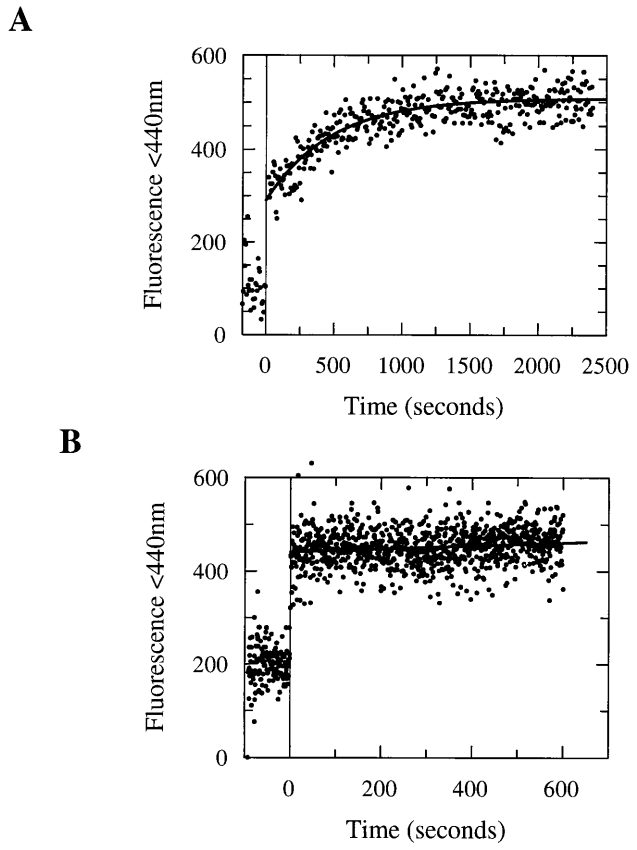

FIG. 9. (A) Following staining of unfixed *B. globigii* spores with the fluorescent brightener Tinopal CBS-X, the fluorescence continues to increase until about 1,200 s (20 min). This is far from ideal when a rapid assay is required. (B) Following treatment with ethanol, maximal fluorescence is reached almost immediately on addition of the stain. All measurements were discriminated from the background by the forward-scatter signal from the 488-nm argon laser. The spores were stained with Tinopal and excited by the HeCd (325 nm) laser, and fluorescence below 440 nm was collected on PMT1 (PMT voltage is 630 V, logarithmic gain). The data shown are means for each channel number (0 to 1023). This has the effect of removing noise form the time course plot but also, and most importantly, reduces the amount of data from, for example, 20,000 to 1,024 events per file, a number that can be more readily handled by general plotting packages. The data in panel A are fitted to an exponential function, $F_f = S\,[1 - \exp(-kt)] + F_0$, where F_f is the fitted fluorescence at a given time (t, in seconds), k is an exponential constant, S scales the exponential increase from 0 to 1 to 0 to S, and F_0 offsets the exponential curve up the fluorescence axis. The data in panel B are fitted by linear regression.

expected to affect both the "true" cell size and the light-scattering properties of the cell as compared both with an unfixed cell and with a latex bead of equivalent size (which is usually used to calibrate light-scattering signals in terms of particle size). The effect of a variety of fixatives (ethanol, formalin etc.) on samples of human tumors for flow cytometry was investigated by Alanen et al. (10), who concluded that ethanol fixation produced data for the distribution of DNA content most similar to those obtained with unfixed cells. Many workers studying microorganisms (see, e.g., references 526, 844, and 879) use 70% ethanol for fixing their cells, although the effect of this on the apparent cell size has been but little studied.

Our experience with yeast cells (218) is that although 70% ethanol is probably the best fixative for reasons such as those given above, fixation with 70% (cold) ethanol can induce a certain amount of clumping. Another effect of fixing yeast cells was to reduce their diameters; this was apparent both from flow cytometry data and from photomicrographs, although the size reduction assessed flow cytometrically was almost twofold greater. Related to the reduction in cell size caused by fixing with ethanol was a third effect on the yeast cells, namely, the effect on the axial ratios of the cells, which increased after fixing. This indicates that although the cells do shrink in both length and width, they do not do so in equal proportions. This study also showed that although unfixed cells of a given diameter showed less forward light scattering than did latex beads of the same diameter, a constant factor could be used to calibrate the light scattering in terms of cell size (as determined photomicrographically) (and see also reference 525); by contrast, no such scaling was possible in fixed cells; i.e., it was not possible to calibrate the forward light scattering in terms of any "true" cell size. Finally, this study showed that forward-angle light scattering (size) and FITC staining (for protein content) were roughly proportional to each other for both fixed and unfixed samples of *Micrococcus luteus*, as also intimated for bacteria generally by Steen (904), although not in all cases for yeast cells (900). Thus, only for unfixed cells is it possible to obtain a reliable value for their diameter by measuring the extent to which they scatter light at small angles in a flow cytometer. However, since light scattering is such a complex function of cell size, shape, structure, and refractive index, different instruments may yield significantly different histograms of the same sample consequent upon relatively minor changes in detection geometry.

DATA ANALYSIS METHODS

As mentioned above, flow cytometry enables the experimenter to analyze large numbers of cells at high speeds (15, 114, 117, 569, 627, 751, 865). As one may imagine, with typical rates of data acquisition being on the order of 100 to 1,000 cells $\cdot$ s^{-1} (thus enabling the collection of data from tens of thousands of cells per sample rather quickly), large numbers of data are produced. In addition, since flow cytometry is a multiparameter technique, i.e., data are collected reflecting two, three, or in some instruments up to eight (475, 916) or even more (426, 427, 724, 809) different parameters, sophisticated data-processing techniques are often required to extract the most useful information from the data and in any event to optimize the utility of the information so obtained. Data analysis is thus an integral part of flow cytometry and, as such, deserves special treatment in any discussion of the approach. Classical data-handling methods for flow cytometry are well treated in the textbooks (see, e.g., references 51, 627, 865, and 1005), but there have been a number of important and pertinent recent developments in the areas of machine learning and the deconvolution of complex, multivariate data to which we shall also draw attention.

The initial data-processing step may be carried out at the acquisition stage, as many flow cytometers (or, rather, the computer software packages that control them) allow the experimenter to set electronic thresholds. A threshold (sometimes referred to as a discriminator) may be defined as a level which a signal must exceed in order to be recorded, and thus it is a means of minimizing the recording of background noise. In addition, electronic gates may be set around an area defining a subpopulation of interest in one histogram, thus resulting in the data histograms for the other measured parameters (which are said to be gated on the first parameter) displaying data reflecting only the subpopulation of interest.

While data may be collected on many different parameters of each cell, for visualization purposes it is usually desirable to display the data in two or sometimes three dimensions. The

usual method used to display flow cytometric data is as frequency distributions in which the abscissa represents the magnitude of the parameter measured and the ordinate displays the number of cells with a given value for that parameter. As indicated in Fig. 2, a two-parameter (three-dimensional) histogram, in which the x and y axes represent two different measured parameters and the z axis represents the number or proportion of cells that may be described by a given combination of the two parameters, may be used.

A growing number of specifically written data visualization programs are available on the Internet, and a catalog of free flow cytometry software is available at http://www.bio.umass.edu/mcbfacs/flowhome.html.

As the number of measured parameters (n) increases, the number of dual-parameter plots that need to be inspected if one is to explore the data fully (950) increases as $n(n - 1)/2$. Thus, when one measures even a modest six parameters, one should inspect 15 plots for each sample. Consequently, methods have been sought to reduce the manual data analysis, and a range of statistical methods and models for flow cytometric analysis has been reviewed by Bagwell (51). Often, the approach is to reduce the dimensionality of a multiparameter data set to a more easily interpretable number of dimensions (238, 529, 928). For instance, the method of principal-components analysis (see, e.g., references 158, 313, and 470) has been utilized to effect data compression from four-dimensional to two-dimensional space (530), thus allowing cluster analysis to be performed to identify subpopulations. Methods such as principal-components analysis are referred to as unsupervised learning methods, since they group individuals merely on the basis of the overall variance in the properties under study (303), without any attempt to incorporate prior knowledge. By contrast, supervised learning methods (often referred to in the statistical literature as discrimination) exploit the prior knowledge of class membership possessed by a Supervisor in the formation of a set of learning rules, which can then be applied to the classification of new data. Overall, these methods may be construed to fall within the area of pattern recognition, excellent introductions to which may be found in the books by Duda and Hart (276), Fukunaga (329), McLachlan (626), and Everitt (303). According to the jargon, the purpose of these methods is to classify individuals (or groups) in a population into particular classes (known as the y data) on the basis of a multivariate set of measurements known as the x data; in general, then, when one has a set of standards with which to establish the classification rules, supervised methods must always perform better than the unsupervised methods do, in that they take the variance of both the x and y data into account when setting up the classification boundaries (605). The types of supervised learning methods in common use are usually themselves classified into statistical, symbolic (rule based/machine learning), and neural, and comparative introductions to these methods may be found in the books by Anzai (34), Weiss and Kulikowski (1019), and Michie et al. (631). Statistical and neural methods bear many similarities and are well compared in the review papers by Ripley (803), Næs (667), Sarle (838), and Cheng and Titherington (161) and the books by Bishop (92) and Ripley (804). Useful introductions to the related approaches of genetic algorithms (363, 428), genetic programming (532, 533), and fuzzy systems (see, e.g., references 393 and 1064) are also available. Other important aspects of all such methods, which should be considered when implementing them and which are covered in the relevant statistical literature, include the speed of deriving and implementing the learning rules, the accuracy with which they perform, the "prior" frequency distributions themselves, their robustness to small changes in the measurands, and the "cost" of making incorrect analyses.

In all of these cases, what we are essentially trying to do with the multivariate data obtained flow cytometrically from each individual in the population, from subpopulations, and from the overall population itself is to classify. In the flow cytometric analysis of microbial systems, it makes sense to classify the types of problem themselves, in particular into studies designed (i) to distinguish microbes from each other (and from nonmicrobial particles), i.e., classification as usually used in the taxonomic sense; (ii) to assess something about the physiological state of the cells, most fundamentally whether they are alive or not, and more generally about the distributions of their genes, gene products, and other metabolic properties; and (iii) to assess the interactions between microbial cells and other entities, such as drugs or macrophages.

An example of the set of such supervised methods is the use of artificial neural networks (Fig. 10) for the processing of flow cytometric data sets. In this approach, multivariate inputs of known samples together with the identity of the sample (as an output) are presented in order to "train" the network. The trained network may then be presented with the data collected by flow cytometry of a sample of unknown identity and, using its "knowledge" of previous samples, will deduce the identity of the unknown. This approach was first applied to flow cytometric analyses for phytoplankton (323) and was later used to good effect by Balfoort et al. to develop a system for the automatic identification of groups of algae (e.g., cyanobacteria) and to some extent to identify to the species level (59). Neural networks have also been applied to the analysis of animal cell cytograms (791, 865) and of mixtures of fungal spores or bacterial species (100, 101, 651), and the approach has recently been reviewed for flow cytometry by Morris and Boddy (650) and Frankel et al. (322), for other areas of biotechnology by Montague and Morris (644) and ourselves (367), and more generally in a substantial number of books of which we would mention (92, 343, 402, 404, 414, 804, 824, 872, 1002, 1021). The particular power of these methods derives from the fact that typical artificial neural networks of an arbitrary size containing at least one hidden layer and using a nonlinear activation function can effect any nonlinear mapping from an input vector to an output vector, i.e., to learn any nonlinear relationship (1025, 1026). It should be stressed, however, that the optimal performance of a neural network requires that many possible parameters of the model be considered and tuned to their optimal values (Table 3) (367, 502). In addition, and in contrast to the stated goals of machine learning methods (see, e.g., references 631 and 799), it is difficult to extract from such trained neural networks an explanation of exactly how they are deriving their classifications, i.e., a set of meaningful rules relating the inputs to the outputs. This is known as the assignment problem. In these circumstances, linear multivariate methods, whose models may be interrogated more easily to provide a meaningful output, are to be preferred (605), provided, of course, that they actually form an adequate model.

The use of a two-level, hierarchical neural network was investigated by Boddy et al. (102) for its use in the identification of 40 marine phytoplankton species. The first level of the network was trained to distinguish between the major taxonomic groups, and then a network for that taxonomic group was used to effect a species-level identification. This system was shown to compare favorably with a single, larger network trained to reach a one-step identification to the species level. Boddy and colleagues have also demonstrated the applicability of neural networks to the study of other microorganisms (100, 101) and have tested a number of the most popular neural net

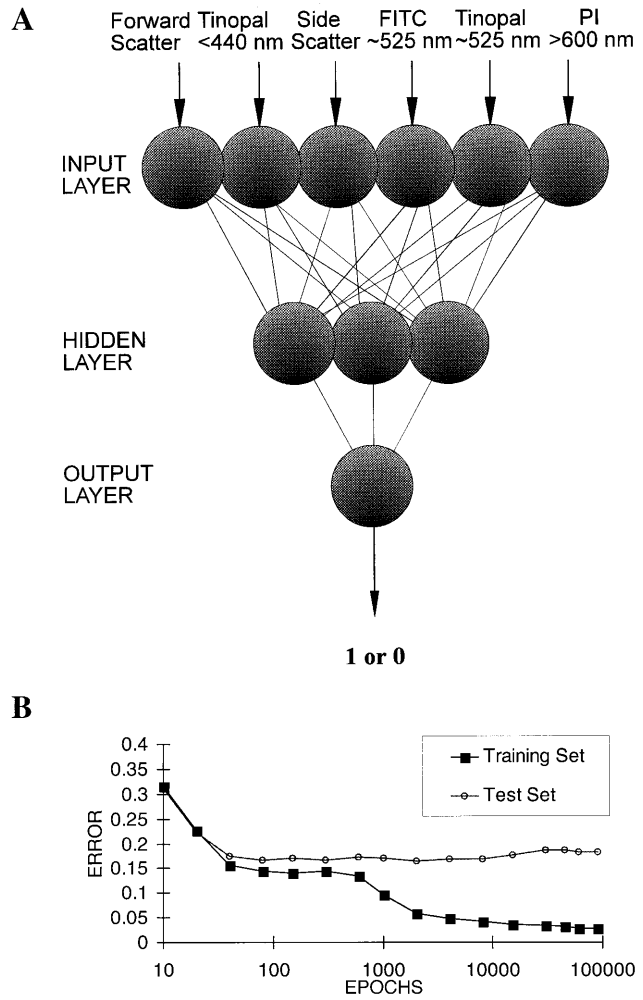

FIG. 10. Application of neural networks to flow cytometric data. (A) Typical architecture of a neural net, exemplified by a fully interconnected feedforward perceptron, together with some appropriate inputs and an output in which the identify of a target bacterial species is coded 1 and all other particles are coded 0. (B) Training curve for such a neural net, showing that with an increasing number of epochs (presentations of the training set), the error of both the training set and the test set falls rapidly. On repeated presentation (beyond about 2,000 epochs in this case), however, the net begins to (over)learn the training set more and more perfectly, at the expense of its ability to generalize and accurately identify the members of the test set. The flow cytometric data were generated as described in the legend to Fig. 8. Illumination was with a HeCd laser at 325 nm and an argon ion laser at 488 nm; forward and side scatter and fluorescence from FITC and propidium iodide (PI) were from the Ar^+ laser, and the two Tinopal signals were from the HeCd laser. The neural network software used was a Windows-based package called NeuDesk (367).

TABLE 3. Some hints in choosing parameters during the production of a feedforward back-propagation neural network calibration model to improve learning/convergence and generalization

1. No. of hidden layers
   One is thought sufficient for most problems. More can give a big increase in computational load.

2. No. of nodes in hidden layer
   Partial least squares (PLS) models (605) can give a reasonable upper bound; i.e., the number of hidden nodes is less than or equal to the optimum number of PLS factors. If this is not possible or desirable, a suitable (but often pessimistic) rule of thumb says the number of hidden nodes = ln (number of inputs).

3. Architecture
   Fully interconnected feedforward net is most common. Many others exist such as adaptive resonance theory, Boltzmann machine, direct linear feedthrough, Hopfield networks, Kohonen networks.

4. No. of exemplars in training set
   Need enough to fill parameter space evenly and to allow generalization. When fewer are used, the network can "store" all the knowledge, predicting the training set superbly but unseen data badly.

5. No. of input variables
   Those that are weakly related or unrelated to the output data may impair generalization and are best removed from the input data set before training commences.

6. Scaling of input and output variables
   Individual scaling on inputs improves learning speed dramatically and often improves the accuracy and precision of the predictions also. There is a need to leave headroom, especially on the output layer.

7. Updating algorithm
   There are many variants on the original backpropagation, some of which give small but worthwhile improvements. Others include radial basis functions and quick-prop. Standard backpropagation (824) is still the most popular. For the nonspecialist, there is little point in using the others unless satisfactory results cannot be obtained with this.

8. Learning rate and momentum
   Need to be carefully chosen so that the net does not get stuck in local minima or "shoot" off in the wrong direction when encountering small bumps on the error surface. For standard backpropagation, a learning rate of 0.1 and a momentum of 0.9 are a good starting point. These parameters may need adjusting before or during training if the net gets stuck in a suspected local minimum.

9. Stability
   Best to remove a representative sample of the training data for validation. If there are insufficient samples to allow this, an approximation to the correct place to stop training in order to prevent overfitting can be made at the point where the training error bottoms out after the first major drop.

algorithms (1029, 1030). Although the populations studied did not lend themselves optimally to demonstrate this, the investigators also make the important point (1030) that physiological differences, e.g., between summer and winter, might cause two populations of the same organism to be disjoint and not easily modelled by a standard multilayer perceptron; however, radial basis function networks (124, 402, 645) can provide an effective model in this case.

Although the neural net approaches have perhaps been the most popular of the artificial intelligence methods exploited to date, decision tree methods may also be used to discriminate between individuals on the basis of a multivariate set of properties. Thus, Dybowski et al. (283) used the classification and regression tree method of Breiman et al. (120) to discriminate antibiotic-sensitive from antibiotic-resistant bacteria on the basis of the cytograms produced by using forward- and side scatter and oxonol fluorescence.

The foregoing discussions have served to give an overview of the most common measurements that are made with flow cy-

tometers. Our task now is to review the various types of study which have been undertaken by the technique. The strategy we have chosen is to review studies, as far as is possible, in approximately the order of increasing complexity, either of the methods used or of the problems addressed. The first area concerns the simple detection and identification of microbial cells.

APPLICATIONS

Microbial Discrimination and Identification by Using Light Scattering and Cocktails of Fluorophores

It is often necessary to detect microorganisms present within a given sample, not least when the presence of a pathogen is suspected, and, as outlined above, modern, flow cytometers are sufficiently sensitive to detect the light scattered by microorganisms. It was probably the development by Steen (909) and coworkers of the Skatron Argus flow cytometer that first enabled microbiologists to make reliable light scatter measurements on bacterial cells. In their early work (906), Steen's group demonstrated the applicability of dual-parameter flow cytometric measurements of light scattering and fluorescence to a range of microbiological problems. Using the total protein stain FITC, they demonstrated a linear relationship between protein content and light scattering, permitting light scattering alone to be used as a measure of protein content in some of their later work and thus freeing the single fluorescence channel of the early instruments for other measurements. In this way, they showed that there was an essentially linear relationship between DNA and protein content for normal *E. coli* cells, and a deviation from this pattern was observed for cells grown in the presence of chloramphenicol (a protein synthesis inhibitor). Later work on the flow cytometric analysis of the interaction between microbial cells and antibiotics is described in detail below. Steen et al. also showed that dual-parameter measurements of light scattering at two angles could be used to distinguish between *P. aeruginosa* and *Staphylococcus aureus* in an artificial mixture (911). The ratio between the small- and large-angle scattering was also shown to be a measure of the viability of the cells. Similarly, Allman et al. (15) could discriminate a number of organisms, while Héchard et al. (403) discriminated various cocultures of *Listeria monocytogenes* and an antagonistic *Leuconostoc* strain by dual-angle light scattering.

As well as light scattering, of course, the next level of sophistication is to consider fluorescence signals, initially in the absence of any added fluorophore. Many naturally occurring cellular substances fluoresce when excited with light of a suitable wavelength. These substances include reduced pyridine and flavin nucleotides, which impart (near-)UV-excited blue fluorescence and blue-excited green fluorescence, respectively, to the cells. The autofluorescence from NAD(P)H has been measured flow cytometrically in yeast cells for determination of the redox state (938). Flow cytometric monitoring of the autofluorescence of *Micrococcus luteus* following excitation at 395 to 440 nm has also been demonstrated (501), and a bimodal distribution of autofluorescence magnitude, which was essentially independent of cell size, was observed; it was suggested that this might reflect oscillations in pyridine nucleotide fluorescence that can be observed macroscopically in appropriately synchronized cultures (35, 601, 800). In favorable circumstances, a combination of light scattering and autofluorescence measurements may be sufficient to effect a discrimination between microorganisms (Fig. 11). As is well known, autofluorescence of pyridine nucleotides has been proposed as a means of registering the viable cellular biomass on-line in fermentations (see, e.g., reference 1063), although it is not now used for this purpose, since the method measures redox changes, which will occur independently of the biomass concentration. Such studies have, however, helped significantly to improve our understanding of the causes of autofluorescence and have led to proposals for monitoring this on-line at multiple wavelengths, some of which excite molecules such as tryptophan and pyridoxine, which are much better indicators of biomass (558). Such analyses will be extremely useful for the continuing exploitation of autofluorescence under flow cytometric conditions, and flow cytometry might serve better to inform the interpretation of macroscopic fluorescence measurements in fermentors (932).

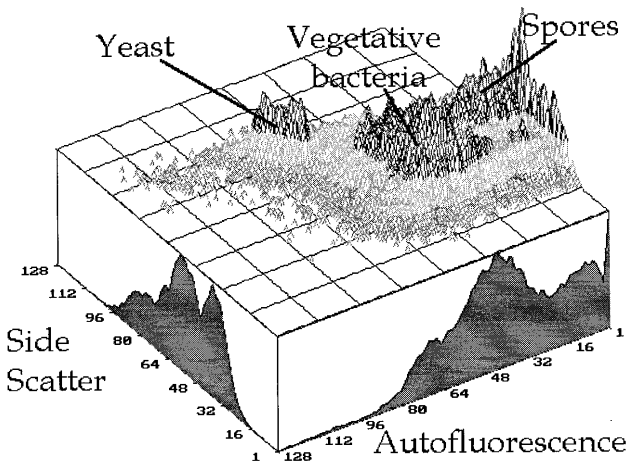

FIG. 11. Flow cytometric analysis of an artificial mixture of microorganisms. By using just side scatter (488-nm excitation from an argon ion laser) and autofluorescence measurements (<440 nm following excitation with the 325-nm line of a HeCd laser), yeasts and spores of *B. subtilis* subsp. *niger* (*B. globigii*) can be distinguished from the vegetative cells of *E. coli* and *M. luteus*. However, there is overlap for the last two microorganisms when just these two variables are considered. All samples were fixed in 70% ethanol prior to mixing, and flow cytometry was carried out on a Coulter Epics Elite flow cytometer.

Probably the most frequent application of flow cytometry to the study of autofluorescence is in the field of aquatic biology, where autofluorescence of photosynthetic pigments is used in the identification of algae (3, 4, 58, 59, 204, 274, 426, 427, 724, 931, 1054). Chlorophyll *a*, the primary photosynthetic pigment and the only pigment present in all algae (696), absorbs both at the UV-blue (<450 nm) and in the red (~680 nm). Fluorescence from chlorophyll *a* is usually collected in the far red (680 to 720 nm) (862). The other chlorophylls, *b* and *c*, together with the carotenoids, capture photons and pass them to chlorophyll *a* (204). Other pigments found in algae include phycoerythrin, phycocyanin, and allophycocyanin, which absorb blue-green, yellow-orange, and red light, respectively. Since different algal species contain different amounts and combinations of the various pigments, it is possible to use a multistation flow cytometer to collect the fluorescence from each pigment separately as an aid to the classification of phytoplankton from mixed environmental samples (204).

Despite the advantages of being able to measure cellular autofluorescence, its presence is often considered to be a problem in flow cytometric experiments. If autofluorescence is emitted at a similar wavelength to the emission of an added fluorescent stain, then it is often impossible to separate the natural autofluorescence of the cell from the fluorescence produced by the stain. In some situations, the level of autofluo-

rescence may far exceed the fluorescence from the stain and thus make it impossible to quantify the fluorescence of the stain of interest. Autofluorescent pigments situated close to the binding site(s) of the added stains may in fact cause quenching of the added stains by energy transfer. Problems of these types are most acute when using immunofluorescence techniques in which a fluorescent molecule is conjugated to an antibody that may bind to only a small number of receptors on the cell, and thus any background autofluorescence will dominate the signal of interest.

While the photosynthetic pigments of algal cells allow many of them to be distinguished readily by flow cytometry, fluorescent stains are usually required to discriminate between other microbes. The simultaneous measurement of (several) fluorescent compounds, together with light-scattering measurements (at one or more angles) on single cells, shows that the combination of flow cytometry and multiparameter data acquisition does indeed allow a certain amount of discrimination (reviewed in reference 861; also see the discussion of Allman's work, below).

The ability of flow cytometric techniques to detect selected cell types in the presence of a high biological background has frequently been exploited. For example, blood has an extremely high cellular concentration, thus making the identification of rare cells difficult. In clinical conditions such as bacteremia (the presence of bacteria in blood), concentrations of the contaminant may be of the order of 10 bacteria in 1 ml of blood, compared with 3.2×10^9 to 6.5×10^9 erythrocytes in the same volume (284), making detection by conventional microscopic methods all but impossible. Thus, although bacteremia is a potentially life-threatening condition, diagnosis usually relies on the growth of bacteria in media inoculated with samples of whole blood, and up to 7 days of incubation may be required before the results are apparent. However, by selectively lysing the eukaryotic cells in a blood sample, a sufficiently low cell concentration can be achieved to allow the rapid sample throughput capabilities of the flow cytometer to be utilized. Using this method, Mansour and colleagues (597) were able to detect *E. coli* in blood at concentrations of 10 to 100 ml^{-1}. This was a sensitivity some 100- to 1,000-fold better than that possible by microscopy methods, and the procedure took only 2 h, including sample preparation. Although the flow cytometric method offers considerable advantages over microscopy or plate growth methods, it may still not be sensitive enough to detect some cases of bacteremia where bacterial concentrations are less than 10 ml^{-1}. In these situations, a short preincubation step prior to flow cytometric analysis may be envisaged to increase the bacterial load of the sample to a level at which it may be detected.

Another instance in which flow cytometry has been used for the detection of a given cell type against a highly particulate background is in quality control applications. Yeasts are a common spoilage problem in many soft drinks, and as a consequence, their detection is desirable. The problem lies in the complex matrix of biological matter contained in drinks such as fruit juices. It was shown (738) that while contamination levels of 50 yeast cells $\cdot$ ml^{-1} could be detected in lemonade, much higher concentrations (6,000 to 14,000 cells $\cdot$ ml^{-1}) were required for detection in fruit juices. However, in some cases, it is necessary not merely to detect microorganisms but also to count them. An example of this would be in the monitoring of pasteurized milk, where the presence of (certain types of) viable bacteria is expected and acceptable below a certain threshold. Indeed, a study of the early flow cytometry papers (198, 381, 638) reveals that the driving force behind their development was the desire for a rapid method of counting particles.

The accurate enumeration of a variety of microorganisms serially diluted from axenic laboratory cultures has been demonstrated (460, 751). However, when this technique was applied to meat, paté, and milk samples (720), the results were not so convincing. With the samples of paté and milk, the background particle counts were too high to allow reliable measurements to be made, and although a good correlation between flow cytometric and plate counts ($r = 0.95$) was obtained for the meat samples, the line was skewed such that at low concentrations (as measured by plate counts) the flow cytometer overestimated the microbial load while at higher concentrations there was an underestimation.

Any method that simply compares flow cytometric counts with plate counts must assume not only that all of the cells in the sample are viable (and see below) but also that they will grow on the medium used on the plate. In an attempt partially to address this problem, Chemunex have developed a simple flow cytometer and set of reagents that could be used reliably in an industrial environment for the detection of viable microorganisms. In the so-called ChemFlow system, viable cells are detected by their ability to cleave a nonfluorescent precursor to release a fluorochrome (intracellularly). A good relationship between CFUs and viable counts in the ChemFlow system has been reported (545), although others have reported that the ChemFlow system underestimates the direct epifluorescence filter technique viable count (740), while with yeasts the ChemFlow viable count consistently overestimated the count obtained by the plate count method (130).

An alternative approach to the ChemFlow system was recently demonstrated by Jepras et al. (460). They evaluated a range of viability stains and developed a technique for distinguishing live and dead bacteria, thus enabling them to obtain both a viable count and a total count for their samples. However, while there was good qualitative agreement between the viable counts obtained flow cytometrically (using the Bio-Rad Bryte HS, the successor to the Skatron Argus instrument) and by measuring CFUs, different sources of errors involved in the two techniques coupled with the problem that different methods of "viability" assessment measure different things (discussed in more detail below) prevented absolute agreement between the two methods.

It is apparent that the accuracy with which counts can be made is very dependent upon the type of flow cytometer, and particularly the accuracy with which sample delivery can be measured (114). When the sample flow rate is unknown, it is possible to mix the microbial sample with a known concentration of fluorescent beads as an internal standard. However, Cantinieaux et al. (150) found that because of the wide distributions of properties seen in microbial cells, situations could arise in which there was overlap between the beads and bacteria. To overcome this problem, they developed a technique based upon sequential analysis of samples of known and unknown concentrations and showed that good reproducibility of counts could be obtained. Despite the successful demonstrations of determination of microbial concentrations, and while their measurement by flow cytometry remains a desirable goal (which may soon be attained in laser diode-based instruments), for the most part viable and total counts continue to be measured routinely by microscopic or Coulter Counter methods.

While in some instances the mere detection of microorganisms is sufficient, in others it is necessary to make at least a preliminary identification of the organism(s) present. This is particularly important when the microorganisms of interest may or may not be present in a sample but when the sample

will also usually contain other microorganisms and/or other biological and nonbiological particulates. This may be important if one is screening environmental samples for the presence of a pathogen, but it can also be useful for the study of nominally axenic laboratory or industrial cultures. For example, in the brewing industry, wild yeasts can produce noticeable spoilage effects even when they are present at a concentration of 1 wild yeast cell per 10^4 culture yeast cells. Jespersen et al. showed that by careful formulation of a growth medium that favored the growth of the wild yeasts, combined with flow cytometric methods, they could detect 1 wild yeast cell per 10^6 culture yeast cells within 24-72 h (463).

Multiparametric flow cytometry involving the collection of both light scattering (at one or more angles) and fluorescence has proved to be effective for discriminating between artificial mixtures of laboratory strains. It was illustrated above that some level of discrimination could be effected simply by measurements of light scattering and/or autofluorescence; however, methods involving the addition of fluorescent stains have much more resolving power. This was demonstrated for instance by Allman et al. (15), who examined the light scattering and (DNA) fluorescence profiles of eight different vegetative bacteria and two spore samples. They showed that while light scattering (at two different angles) provided a useful first step in the characterization of the bacteria, the use of a third measurement parameter greatly enhanced their ability to discriminate between the bacteria. By applying the same techniques, they were also able to effect some level of discrimination between spores of six different fungi and in some cases were also able to discriminate subpopulations within nominally homogeneous samples (14).

While staining for a single parameter (DNA) was effective for discrimination of different microorganisms in the above examples, the ability further to discriminate (sub)populations of cells will increase as the number of the measured parameters increases (501). It is becoming apparent that the use of flow cytometry, together with a cocktail of stains designed to demonstrate a reasonable level of specificity for particular organisms, requires several design decisions to be made at an early stage.

(i) Should the distinction of the extent of staining between different organisms rely on the kinetics of staining/binding (which has the advantage of greater rapidity but the disadvantage of greater variability) or the extent of binding (at or near equilibrium)? Our own view is that the latter is preferable and that efforts to speed up the kinetics by a wise choice of stains and congeners will be highly beneficial.

(ii) Should one use membrane-permeabilizing agents (see above) to speed the ingress of stains (thus allowing the use of all possible stains but killing the cells)? We again believe that the answer to this is in the affirmative, since a duplicate sample may be retained if nondestructive analyses are subsequently required, and suitable agents exist which even permit the immunological labelling of intracellular antigens (see, e.g., reference 473) (thus opening up many more possibilities than those which rely solely on access to surface antigens).

To distinguish biological from nonbiological particulates, nucleic acid stains are most suitable. Traditionally, either ethidium bromide alone (723) or propidium iodide alone (440) or the combination of mithramycin plus ethidium (15, 17, 115, 912) have been used. Ethidium and propidium do not bind particularly strongly (nor are they necessarily selective against other polyanions), while the latter, although very selective for discriminating between DNA and RNA and most widely used with the mercury arc lamp flow cytometers (14, 16, 913), is more selective than necessary (since RNA is also an adequate determinand). In some cases (988), ethidium bromide and propidium iodide are used together, the former as a supravital stain and the latter to assess membrane damage. As outlined above, mithramycin is not well excited in 488-nm argon ion laser-based instruments and has the added disadvantage of a relatively high cost.

The use of two separate DNA stains, one selective for GC-rich DNA and the other selective for AT-rich DNA, has been alluded to above. While being an elegant method of determining the percent G+C content of an axenic microbial culture, it is also a good example of a multiparameter approach for distinguishing between microorganisms. This approach has been used to distinguish between certain types of bacteria (836, 962). This technique has more potential for discriminating between bacteria than does measurement of the total DNA, since the chromosome number will vary with growth conditions. However, while the percent G+C content may be useful to distinguish between distantly related organisms, it is unlikely that such an approach could act as more than a "first step" for the discrimination of closely related organisms. In this case, however, a final identification may be achieved by more species-specific probes such as fluorescently labelled antibodies and oligonucleotides.

Microbial Discrimination and Identification with Antibodies

As with many other fields of biology the growing availability and range of monoclonal antibodies have greatly extended the utility and discriminating power of flow cytometry (see, e.g., references 113, 230, 252, 282, 287, 301, 307, 309, 619, 621, 750, 769, 907, and 993). Antibodies may be labelled with fluorescent tags, thus enabling the enumeration of surface antigens on the target cells or the detection of a given cell type in a mixed population. A particular benefit of the approach is, of course, that the cells need not be culturable (242).

Steen et al. (907) demonstrated a flow cytometric approach for the determination of the antigenicity of bacteria. As well as quantification of antibody binding, they could relate the amount of antibody binding to the cell size (light scattering) in order to determine antigenicity per unit cell weight. Barnett et al. (68) used fluorescently labelled antibodies and light scattering to detect the oral bacterium *Streptococcus mutans* and to discriminate it from another oral organism, *Actinomyces viscosus*. Determination of the antigenicity of different preparations of bacteria is an important step for the purposes of vaccine preparation. Consequently, the detection and measurement of antibody levels in the blood are used for assessing the effectiveness of vaccination procedures and also for identifying exposure to microbial pathogens. By using fluorescently labelled *Brucella abortus* cells, antibodies against these bacteria have been detected in blood samples by flow cytometry (252).

Screening for the presence of *Legionella* spp. in cooling-tower water is desirable to reduce the chances of outbreaks of Legionnaires' disease. Traditional methods which involve plating of water samples and/or animal experiments are time-consuming and costly. Flow cytometric analysis of these samples with a fluorescein-labelled antibody has been described previously (450) and has shown that flow cytometry offers several advantages over conventional techniques. This process has been further refined by using a combination of the fluorescently labelled antibody and the nucleic acid stain propidium iodide (951), and in the limited number of samples analyzed, an interesting trend was observed between the ratio of red to green fluorescence and the virulence of the strain.

The enteric protozoan *Cryptosporidium parvum* is responsible for outbreaks of waterborne disease, with an outbreak in

Milwaukee in 1993 affecting more than 100,000 people (585, 586); consequently, the detection of this pathogen is extremely desirable (974). The problem here is not only that very low levels of oocysts need to be detected (since the infective dose is <10 oocysts [981]) but also that there is the potential for confusion between immunofluorescently labelled *Cryptosporidium* oocysts and the autofluorescence signal from algae commonly found in water reservoirs (810). Consequently, *Cryptosporidium* oocysts are normally identified by laborious microscopic methods, in which nonfluorescent particulates may in unfavorable circumstances obscure the oocysts. A novel approach to the problem was developed by Campbell et al. (145) and by Vesey et al. (981, 982), who combined the fast throughput capabilities of the flow cytometer with fluorescence-activated cell sorting. By using a flow cytometer to sort all presumptive oocysts directly onto microscope slides, the microscope operator could then make a final identification. The initial flow cytometric sorting step greatly reduced the amount of material that needed to be examined, and it was found that this technique was more sensitive, faster, and far easier to perform than conventional epifluorescence microscopy. These investigators have also devised a number of useful methods, including $CaCO_3$ flocculation, for the effective concentration of such oocysts (980, 983). Although their present approach requires cell sorting, they report (981) that the exploitation of multiparameter methods will shortly allow them to effect the accurate identification of *Cryptosporidium* and *Giardia* oocysts directly. Direct measurement of excreted oocysts in fecal preparations by using antibody staining has also been demonstrated (38, 297).

Phillips and Martin have published a number of papers on the use of flow cytometric-immunofluorescence techniques for labelling *Bacillus* spp. Thus, they have demonstrated the use of fluorescent antibodies for detecting spores and vegetative cells (739, 741, 743) and the binding of FITC-labelled antibodies to *Bacillus anthracis*, *B. subtilis*, and *E. coli*. They also uncovered significant variability in the immunofluorescence of *Bacillus* samples and demonstrated a significant sensitivity of the resulting fluorescence to the staining conditions (740, 742). Antigenic overlap between *B. cereus* and *B. anthracis* is a problem in these assays, requiring careful antibody preparation if cross-reactivity is to be avoided (743). Evans et al. (301) isolated a number of monoclonal antibodies active against different parts of the gram-negative lipopolysaccharide, and many of these antibodies showed very good specificity for different classes of rough and smooth strains. The essential simplicity of the epitopes was apparent from the fact that although raised with intact cells, the monoclonal antibodies still bound to boiled bacteria. Nevertheless, the heterogeneity of binding within each strain was substantial, with the cytograms indicating a variance in binding of at least an order of magnitude between individuals.

An FITC-labelled antibody has proved to be effective for the flow cytometric detection of *Salmonella typhimurium* against a background of *E. coli* (621), even when the *E. coli* cells were in excess by a factor of 10,000:1. By introducing a second antibody raised against *S. montevideo* and labelled with phycoerythrin, the simultaneous detection of two different salmonellae was also demonstrated (622). The limit of detection was ca. 20 cells $\cdot$ ml^{-1} (750). FITC-labelled polyclonal antibodies were used by Donnelly et al. (262–264) for the flow cytometric detection in milk of *Listeria monocytogenes*. However, it has been reported that commercially available antibodies cross-react with other species, and, consistent with the problems with *Bacillus* spp. described above, Vesey et al. (981) comment (and demonstrate) that in their experience, monoclonal antibodies can exhibit far more specificity and are thus to be preferred if available. In addition, nonspecific binding may be overcome in part by blocking agents, and in contrast to enzyme-linked immunosorbent assay methods, the multiparametric nature of the flow cytometric approach permits much better discrimination between true and adventitious binding. That specificity is usually more important than detectability is well illustrated by the study of Baeza et al., who could discriminate particular arrangements or types of lipids in small liposomes (containing only lipids) by flow cytometry with antibody staining (50). However, the question of specificity and cross reactivity may sometimes mean that monoclonal antibodies can be too specific, since very small changes in surface epitopes may have little taxonomic significance (302, 581, 1055).

Immunofluorescence techniques are not limited to surface antigens but can also be readily applied to intracellular proteins. Methanol may be used to permeabilize the cell, allowing ingress of the fluorescently labelled antibody without affecting the antigenicity of the intracellular epitope (555). Immunofluorescent labelling of intracellular antigens was exploited to determine levels of gene expression in *S. cerevisiae* following the preparation of *lacZ* gene fusions to the promoters of interest (291). The use of antibodies against intracellular antigens simultaneously with DNA staining has also been demonstrated. This approach has been used with mammalian cells to identify cell-cycle-specific staining, in particular of proliferation markers (149). The use of immunofluorescence-flow cytometry techniques has also been reviewed in the context of biotechnological research (225).

By careful selection of appropriate fluorescent compounds for antibody labelling and the continuous progress toward brighter labels with a variety of Stokes shifts, it is now possible to effect three-color (230, 307, 993) or even four-color (865) immunofluorescent labelling with a single-laser flow cytometer.

Microbial Discrimination and Identification with Fluorescent Oligonucleotides

rRNA sequences exhibit a degree of variation that can be suitably exploited for phylogenetic analysis (1040), and a widespread interest in the molecular characterization of bacteria by using oligonucleotide probes has developed (9, 24–26, 232–234, 295, 296, 354, 434, 584, 727, 766, 768, 840, 845, 902, 903, 943, 1001, 1049). Substantial databases have been developed (76, 239, 590, 591, 701, 959) and are accessible electronically (http://rrna.uia.ac.be/index.html or http://rdpwww.life.uiuc.edu/ or via http://www3.ncbi.nlm.nih.gov/Taxonomy/tax.html and http://www.biophys.uni-duesseldorf.de/bionet/rt__1.html). As well as the more taxonomic issues, this has been partly brought about by the desire to release genetically engineered microorganisms into the environment and the consequential requirement that the persistence of these organisms be monitored (287). As discussed above, antibodies can be raised against bacteria, but for the purposes of identifying unknowns, these may in fact be too specific, since it is probes for the genus or even the kingdom level that are often required (415).

Fluorescently labelled rRNA probes have the advantage that they are small enough freely to penetrate fixed cells and that they hybridize specifically to intracellular rRNA. However, for the analysis of microorganisms in the natural environment, where metabolism may be slow and many exist in a nongrowing and possibly dormant state (see below), the ribosome number may be decreased such that probe binding is not detectable against the background of cellular autofluorescence (27). However, this problem will soon be overcome, since it has been

demonstrated that the use of PCR is possible prior to in situ hybridization, greatly enhancing the sensitivity of the technique and permitting clean flow cytometric estimation of single gene copies (351). Amann et al. (26) have recently given a quite excellent review of gene probe methods in this journal, and our discussion is restricted to the combination of fluorescently labelled oligonucleotide probes and flow cytometry.

Flow cytometry and fluorescent oligoribonucleotides were used to detect yeast cells by Bertin et al. (84), who found that by using a biotinylated antisense rRNA probe of about 100 nucleotides followed by streptavidin-FITC labelling, they could detect the yeast cells by flow cytometry. Fluorescently labelled oligonucleotides, together with flow cytometry, have also been exploited by Wallner et al. for the flow cytometric identification of a range of yeasts and bacteria (1000). Wallner et al. demonstrated the multiparametric use of these phylogenetic stains by simultaneously labelling mixed samples with two different oligonucleotide probes labelled with different fluorochromes. A number of studies have shown that fluorescently labelled rRNA probes can be used to study microbial consortia in situ, even in matrices as difficult as sewage sludge (599, 994–997) and for targets as difficult as the very small picoplankton (871). Thus (999), samples from a wastewater treatment plant were hybridized with fluorescein-labelled oligonucleotide probes specific for a variety of bacterial domains and counterstained with the DNA dye Hoechst 33342 for flow cytometric analysis. Forward-angle light scatter and Hoechst- and probe-conferred fluorescence were used as measures for cell size, DNA content, and rRNA content, respectively. By using a probe able to bind to all bacteria, it was shown that in the activated-sludge samples examined, 70 to 80% of the Hoechst-stained cells could be identified unambiguously by this method and that flow cytometric and microscopic counts were in general agreement. Some problems due to the formation of aggregates were encountered, but it was concluded that flow cytometry combined with rRNA-based in situ probing was a powerful tool for the rapid and automated analysis of the microbial communities in activated sludge. A eukaryote-specific 18S rRNA probe has also been used successfully by Vesey et al. (979) to assess the viability of *Cryptosporidium* oocysts; in contrast to some of the work with antibodies (in which live oocysts might shed the antigen but dead cells might retain it), there was a quite excellent correlation between staining and viability, presumably owing to the loss of rRNA in dead oocysts, holding out the hope of a very specific oocyst viability probe.

Flow Cytometric Assessment of Bacterial Viability and Vitality in Laboratory Strains and Natural Samples

> "These germs—these bacilli—are transparent bodies. Like glass. Like water. To make them visible you must stain them. Well, my dear Paddy, do what you will, some of them won't stain; they won't take cochineal, they won't take any methylene blue, they won't take gentian violet, they won't take any colouring matter. Consequently, though we know as scientific men that they exist, we cannot see them."
>
> Sir Ralph Bloomfield-Bonington
> (*The Doctor's Dilemma*, George Bernard Shaw)

Laboratory strains. Arguably the most fundamental question that a microbiologist might ask of a microorganism is whether it is alive, although this is not easily answered in all cases, even for higher organisms (1006). For microbes, it is usually considered that the ability of a microbial cell to reproduce itself on a nutrient agar plate constitutes the benchmark method for determining how many living cells may be contained in a sample of interest (773). The great problem with this method of viable counts (396, 772), however, is that incubations of 72 h or even more may be required to obtain an answer (and some cells do not grow at all under the conditions provided); it may tell us that a cell was alive, but it cannot tell us if a cell is alive (771). Especially in life-threatening situations, and for economic reasons generally, such a delay is unacceptable, and many so-called "rapid" methods have been developed to allow a speedier assessment of the "viable" microbial load in a sample (see, e.g., references 5, 331, 394, and 471). Of routine microbiological methods, only direct counting of cells (48, 328, 387, 394, 413, 690, 737, 782), usually stained with an appropriate fluorochrome and counted under an epifluorescence microscope, can give a rapid answer to the question of how many "viable" cells may exist in a sample. Such fluorochromes are usually DNA stains, and some so-called "vital" stains, such as acridine orange (211, 737), thus allow, in principle (48, 624, 625), a distinction between "living" and "dead" cells (or debris), on the basis that in the latter the nucleic acids which constituted the major binding site for acridine orange are rapidly degraded. Thus, "living" cells appear green while "dead" cells appear red or orange. In exponentially growing cultures, there is usually a good correlation between viable counts and acridine orange-based direct counts, but in natural environments these direct counts can exceed the viable counts by several orders of magnitude (26, 820; also see below). A further disadvantage of these types of stain is that they are usually administered to cells that have been fixed with formaldehyde or glutaraldehyde; it is therefore not possible to exclude an argument that the fixatives used may have affected the findings, and such fixation of course renders physiological studies impossible.

Other fluorescent stains normally excluded by living cells have also been used to assess viability on the grounds that dead cells will have leaky membranes, to which the stains will be permeable. DNA stains such as propidium iodide or ethidium bromide are indeed generally excluded by intact plasma membranes, and their uptake is often used to indicate cell death (6, 103, 374, 378, 462, 471, 544, 576, 846). Similarly, a class of viability stains widely used in mammalian cell biology, of which the prototype is fluorescein diacetate, are themselves membrane permeant but are cleaved by intracellular esterases to produce a product (in this case fluorescein) that is not. Dead cells with leaky membranes do not stain. Little is known about the extent to which microorganisms possess esterase activity. Thus, Diaper and Edwards (248) used flow cytometry to detect a variety of viable bacteria after staining with a range of fluorogenic esters including fluorescein diacetate (FDA) derivatives and ChemChrome B, a proprietary stain sold commercially for the detection of viable bacteria in suspension. No one dye was found to be universal, but the ChemChrome B dye stained the widest number of gram-positive and gram-negative species whereas the FDA derivatives preferentially stained gram-positive bacteria. Breeuwer and colleagues showed that FDA and carboxy-FDA penetrated yeast rapidly and that esterase activity was probably limiting (118); an energy-dependent efflux from viable cells of carboxyfluorescein was also observed (119, 954). Our own experience (also see references 249 and 639) is that fluorescein can be pumped out of (or at least leak rapidly from even slightly leaky membranes of) bacterial cells that are nonetheless viable. Indeed, a serious disadvantage with all these types of stain is that staining is effectively qualitative—cells are scored either as dead or alive, with no other possibilities entertained. It may also be noted here that artifactual permeabilization of cells may occur if they are stored at low temperature; conversely, the operation of efflux pumps may appear to make an intact cell impermeable to a dye to which it is in fact permeable (462, 556).

It is widely recognized that especially in nature, the distinction between life and nonlife is not absolute; many cells may exist in cryptobiotic (491), dormant (922), moribund (771), or latent (1007) forms or states, in which they will not form colonies on nutrient media (i.e., are nonculturable) but will give a "viable" direct count (64, 370, 479, 516, 589, 610, 617, 647, 697, 699, 700, 755, 773, 820, 1016). Such cells have been referred to as pseudosenescent cells (773) or somnicells (63, 820). In some cases, cells that have undergone some kind of stress are, or may be, considered injured and may be resuscitated by preincubation in nutrient media prior to plating out (see, e.g., references 31, 623, and 792). They differ from starved and dormant bacteria in being more sensitive than normally growing cells to environmental insults. Among brewers, the term "vitality" is used to refer to those properties of a culture of otherwise viable cells which reflect its capacity to metabolize rapidly after transfer from a nutrient-poor to a nutrient-rich environment (446). We have chosen to reserve the term "viable" to refer to a cell which can form a colony on an agar plate when plated out (at appropriate dilutions) directly from the sample of interest, "dormant" or "vital" to refer to one which can do so only after some kind of resuscitation protocol, and "nonviable" to refer to a cell which cannot do so under any tested condition (481), whereas Barer et al. (64) subdivide the possible physiological states into a rather greater number of categories. Von Nebe-Caron and Badley (988) distinguish reproductive viable cells (platable), vital cells (metabolically active), intact cells (with membrane integrity), and dead cells, and they nicely illustrate their flow cytometric discrimination by using a variety of cocktails of stains. The term "viable but not culturable" is often used to refer to cells which display metabolic activity but cannot be cultured (697–700, 820, 1015); however, such cells are not dormant in our definition, in that in almost no case has there been a persuasive demonstration that resuscitation could take place. In our own studies of bacterial dormancy, one interesting finding (989) was that the plasma membrane of these dormant cells was apparently permeable to normally membrane-impermeant DNA dyes and that an early part of the resuscitation process involved repair of the damaged membrane—these cells would thus have scored as dead, not even viable but not culturable, by traditional criteria.

Dormant cells, and indeed nonculturable cells of any type, can assume particular importance in the etiology of particular disease states. Thus dormancy of mycobacteria is particularly well known (33, 183, 243, 346, 1007–1009), and the difficulties in culturing *Helicobacter pylori* (708) were mainly responsible for the long lag prior to the recognition of its crucial involvement in gastritis and ulcerogenesis (96, 223, 602). The recent ability to exploit oligonucleotide probes in the assessment of the presence of nonculturable bacteria, outlined above, is well illustrated in this context by the work of Domingue et al. (258), who were interested in interstitial cystitis, an inflammatory disease of the urinary bladder with no known etiology. A microbial association with this disease had not been supported, since routine cultures of urine from patients with interstitial cystitis are usually negative. However, using a PCR method capable of amplifying 16S rRNA genes from a wide variety of bacterial genera, these authors demonstrated the presence of gram-negative bacterial 16S rRNA genes in bladder biopsy specimens from 29% of patients with interstitial cystitis but not from control patients with other urological diseases. They also discovered and cultured 0.22-μm-filterable forms from the biopsy tissue of 14 of 14 pa tients with interstitial cystitis and from 1 of 15 controls. Overall, it seems inevitable that the availability of these methods will cause the catalog of disease states recognised as having a microbial contribution to their etiology to expand enormously in the short term (257, 324), particularly as improved methods for resuscitation of small cell numbers are found (483). It seems very likely, for instance, that *H. pylori* is an important risk factor for gastric cancer (see, e.g.; references 189, 290, 314, 431, 582, 719, and 968).

As discussed above, it is at least plausible that the ability to generate ATP or other biologically relevant forms of energy declines as starving, injured, or dying cells pass through "moribund" states en route to states in which their nonviability has become permanent. Since in many cases we should like to know the proportion of dead, injured, and living bacteria in a sample, it is obvious that there is a great need for an appropriate stain or other rapid method for distinguishing these macroscopic physiological states. The traditional vital stains such as fluorescein diacetate, ethidium bromide, and acridine orange work on principles (hydrolysis by esterases or binding to nucleic acids) which may be expected not to reflect the energetic status of a cell very directly and will not therefore adequately distinguish degrees of cell viability reflecting a generalised physiological or energetic capacity.

Metabolizing bacteria, like the mitochondria of eukaryotes, have the ability to concentrate lipophilic organic cations from the external medium by passage across their cytoplasmic membranes into the cytoplasm, and it was proposed more than 15 years ago that the (uncoupler-sensitive) extent of this ability would reflect the energetic status in vivo (528). A loss of this ability would then reflect both the decline in the provision of ATP- or respiration-derived energy to the cytoplasmic membrane and/or an increase in its permeability, both of which may be expected to be characteristics of dead or dying cells. Most commentators assume that the uptake of such lipophilic cations is electrophoretic, being driven by a transmembrane potential (negative inside) generated by respiration or by ATP hydrolysis. However, since the uptake of these cations is normally accompanied by the extrusion of a similar number of protons and is thus effectively electroneutral, this assumption is probably incorrect (418, 495, 496, 933). Indeed, Chen's group has shown, in a most elegant optical study (886), that the uptake of a lipophilic cation within individual mitochondria is spatially heterogeneous and thus cannot be driven simply by a delocalized membrane potential. At all events, the method has been widely applied to bacterial cell cultures, especially when cationic derivatives of triphenylphosphine are used (see, e.g., references 47 and 620). Such a method cannot, however, usefully be applied to the problem of distinguishing differing physiological states within a given culture or sample, since, as we have stressed throughout, any reduction in uptake could be due to the complete lysis of a proportion of cells or to a reduced vitality of all of them (or indeed to any combination of these) (495).

Chen and colleagues (159, 160, 469) and subsequently many others (see, e.g.; references 294, 378, 452, 567, 730, 732, 733, 814, and 882) have shown that the polar, water-soluble, cationic, fluorescent dye rhodamine 123 (Rh123) (Fig. 12) is highly concentrated by eukaryotic mitochondria in an energy-dependent fashion (in that the accumulation is reversed by uncouplers), and Matsuyama (618) indicated that the same dye could be used to stain living bacteria in an uncoupler-sensitive manner, with the extent of uptake being assessed qualitatively by light microscopy. Resnick et al. (797) reported briefly on the use of Rh123 to stain *Mycobacterium smegmatis*, while Odinsen et al. (694) successfully used Rh123 in combination with ethidium bromide as a viability stain for *Mycobacterium leprae* and Bercovier et al. (77) exploited it for antisusceptibility testing of mycobacteria. In 1992, Diaper et al. (249) and Kaprelyants and Kell (480) recognized that it might be possible to

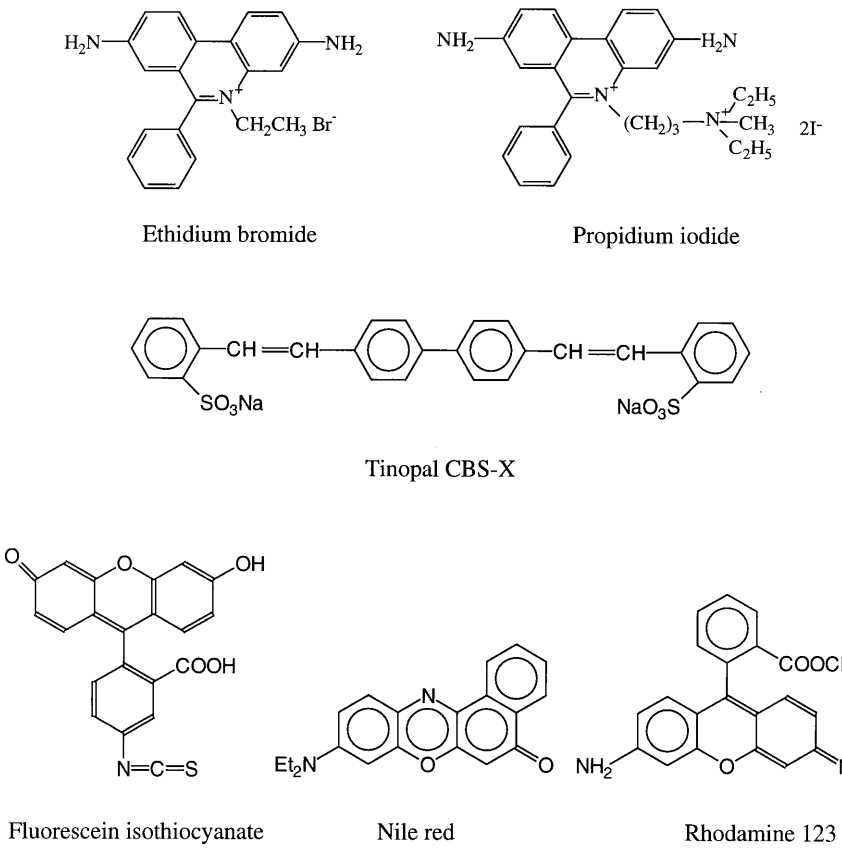

FIG. 12. Chemical structures of some of the fluorescent dyes mentioned in the text.

distinguish nonviable, viable, and dormant cells in terms of the extent to which they would take up Rh123. Of course, larger cells will accumulate more dye molecules for a given extent of concentrative uptake, so that a simple microscopic observation would be misleading. However, it seemed reasonable that a rapid assessment of cellular vitality in populations would be possible by combining Rh123 staining with flow cytometry, in which results on both the extent of light scattering, reflecting cell size, and the extent of Rh123 uptake, reflecting energetic status, could be obtained simultaneously and quantitatively. These hopes were amply fulfilled (Fig. 13), and many authors have now exploited this dye in the assessment of bacterial viability and vitality (220, 247, 460, 480, 568, 576), usually finding fairly good correlations between the ability to take up Rh123 and the three main physiological states defined above and based on colony formation. As regards resuscitation, great care must be taken to ensure that any increase in the viable-cell count at constant total cell count is not due simply to the growth of viable cells present in the sample whose numbers do not exceed the precision of the total cell count. In our own studies of dormancy and resuscitation in *Micrococcus luteus* (481), four points are worth stressing. (i) Given the constancy of the total counts, it is implausible that any lysis can be exactly balanced by growth, and anyway the percentage of dormant cells significantly exceeded the imprecision of the total cell count. (ii) During resuscitation, the percentage of small cells in the population was decreased with an exactly equivalent increase in the percentage of larger cells (as could conveniently be observed by flow cytometry), indicating the conversion of small, dormant cells into larger, active cells. (iii) The transient character of the accumulation of larger cells during resuscitation suggested that the increase in the number of viable cells in this period is not due to typical growth and division but to an unbalanced increase of cell size during this period. (iv) The rate of increase in viable cell counts was much greater than the doubling time of the cells. Dormant cells could also be separated from dead cells by flow cytometric cell-sorting procedures (484). Finally, resuscitation could be observed under conditions (in a most-probable-number measurement) in which no viable cells were present at all (485)!

While it has the virtue of giving a large range of accumulation ratios under different physiological conditions, a particular problem with Rh123 (and, indeed, most lipophilic cations) is that many bacteria, notably gram-negative organisms, may stain poorly unless their outer membrane is made more permeable to the dye by treatment with a chelating agent such as ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) or EDTA in the presence of Tris (which removes lipopolysaccharide from the outer membrane [552, 747]). It is not, of course, then possible to know whether such treatments actually changed the energetic status of the target cells. However, many other dyes have been devised for assessing the energetic status of biological cells and organelles (reviewed in references 181, 575, 733, and 991), and these, too, have been applied to the problem at hand.

Carbocyanines, like Rh123, are positively charged dyes which are slowly accumulated by cells and mitochondria in an energy-dependent manner (181, 991). Shapiro long ago proposed their use for the assay of cell viability (860) and, following improvements in the sensitivity of flow cytometers, has outlined the use of flow cytometry with the fluorescent dye hexamethylindodicarbocyanine (863, 865), which is excited at

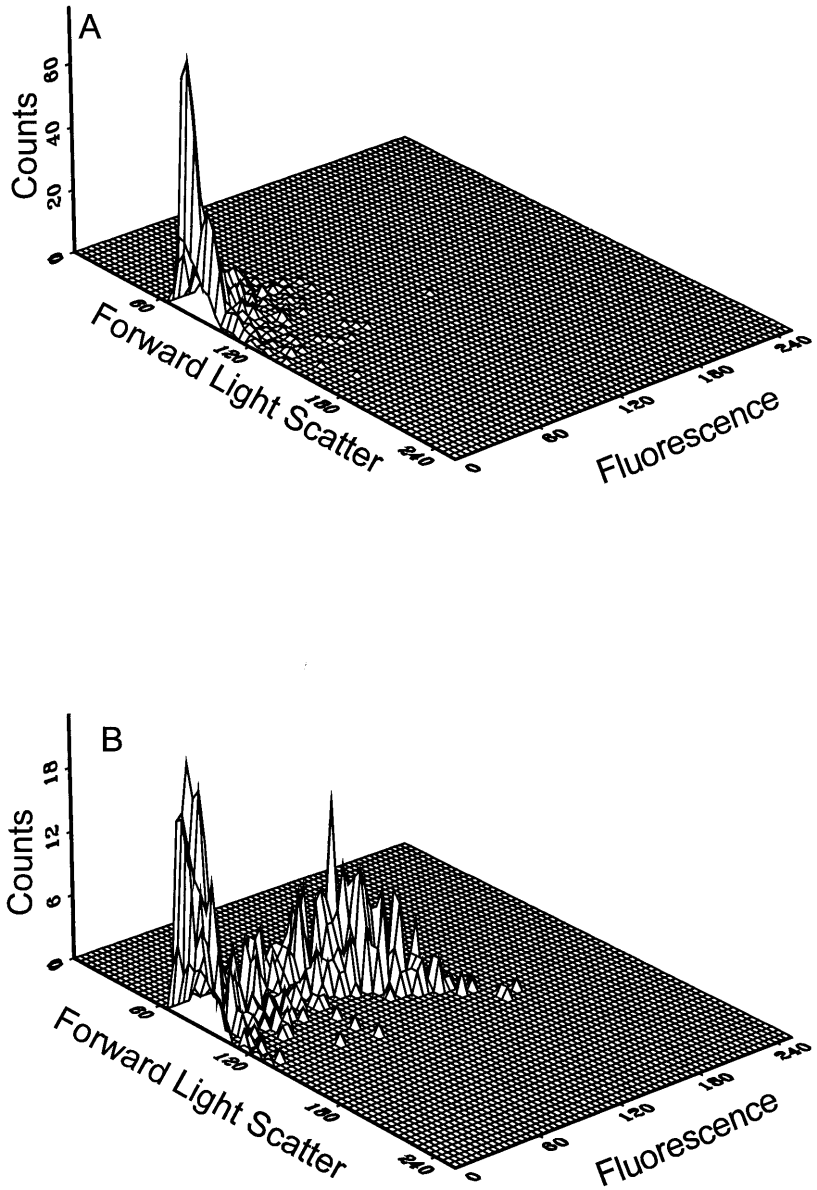

FIG. 13. Changes in light-scattering behavior and in the ability to accumulate Rh123 during resuscitation of a starved cultured of *M. luteus*. Cells were grown and starved and other measurements were carried out as described by Kaprelyants and Kell (481). Cells were starved for 2.5 months, incubated with penicillin G for 10 h, washed, and resuscitated in weak nutrient broth (481). Flow cytometry was carried out with a Skatron Argus instrument. Data represent a culture immediately after the penicillin treatment (A) and 2 days later (B).

633 nm, for assessing microbial viability in a way analogous to the Rh123 method; he also effectively used it as a Gram stain by comparing the extent of staining before and after treating the cells with chelator. Because carbocyanines are relatively hydrophobic, not all staining is likely to be uncoupler sensitive, and these and other problems are reviewed in detail by Shapiro (865).

An interesting approach to distinguishing energized from deenergised membranes by using a specific type of cyanine dye follows that taken by Chen and colleagues (796, 888), who showed that the cyanine dye JC-1 fluoresces in the green (527 nm) when monomeric but in the orange (590 nm) when aggregated as a consequence of accumulation by mitochondria with energized membranes. Shapiro (864) demonstrated similar behavior with Tris-EDTA-treated *S. aureus.*

Oxonols are negatively charged dyes whose binding (and fluorescence) is decreased upon membrane energization and increased in the presence of uncouplers. They are thus complementary to the positively charged cyanine and Rh123 dyes, are relatively nontoxic (in that they are largely excluded by live cells), and possess the very great advantage that they do not appear to need the cells to be treated with EDTA to elicit a differential energy-dependent staining response (224, 460, 577, 612, 613). On this basis and although their fluorescence is somewhat weaker than that of cyanines (864), oxonols are likely to become dyes of choice for studies of this type (570). Since they are anions, it is also unlikely that they will be substrates for drug efflux pumps, again somewhat simplifying the interpretation of their staining reaction.

There are many other fluorescent probes which were ex-

ploited as a means of measuring membrane energization during the heyday of such bioenergetic studies, including 1-anilino-8-naphthalene sulfonate (46, 688, 788, 789, 884); whether they might be of utility in the flow cytometric assessment of the activity of intact bacterial cells remains largely unexplored.

It is reasonable that "a central requirement for viability assays. . .would be that they measure a process central to cellular energy metabolism" (692, 841). For most organisms other than fermentative bacteria, the proximate cause of membrane energization is respiratory-chain activity, and since a variety of respiratory rates are accompanied by very similar steady-state extents of lipophilic cation uptake (497, 498), it is evident that a fluorescent "respiratory-chain activity" stain might be significantly more discriminating as regards energetic status than one reflecting lipophilic cation uptake (482). Such a stain is 5-cyano-2,3-ditolyl tetrazolium chloride (811), which is reduced by a variety of dehydrogenases to an insoluble fluorescent formazan derivative (542, 858, 859, 917), and a number of authors (111, 433, 481, 482, 577, 625, 841, 1060, 1061) have exploited it, together with flow cytometry, for the assessment of microbial activity, in all cases finding a substantial heterogeneity in the dehydrogenase activity of the populations studied. However, the formazan is somewhat less fluorescent than many other flow cytometric dyes, is rather cytotoxic at the concentrations which must be used (482), and may not work satisfactorily under some circumstances (937). While it has perhaps been best deployed to advantage for studies in situ, the availability of better dyes for the fluorimetric assessment of respiratory chain and dehydrogenase activity remains an urgent need.

Here, we should also mention that flow cytometry is also of use for the assessment of the interaction between pathogenic bacteria and macrophages, especially in in vitro studies of the causes of virulence. Typically, organisms (and sometimes leukocytes) are labelled with a fluorescent marker, and it is then relatively facile to monitor the phagocytosis and destruction of the microbes (or otherwise) (606, 607, 679, 753, 793, 819, 847). Thus, Raybourne and Bunning (793) labelled *Listeria monocytogenes* and *Salmonella typhimurium* with a lipophilic dye, PKH 2, and used flow cytometry to investigate phagocytosis by J774A.1 cells and short-term bacterial survival. It was shown that labelling had no effect on viability, growth kinetics, and survival within macrophages, although the recovery per macrophage was much greater for *L. monocytogenes* than for *S. typhimurium*. The survival of *L. monocytogenes* (as a prototypic facultative intracellular bacterium) during phagocytosis was estimated on the basis of fluorescence measurements of infected J774A.1 cells and the recovery of *L. monocytogenes* from sorted cells. *Listeria* enrichment recoveries, derived from individually sorted J774A.1 cells, demonstrated the substantial heterogeneity of macrophages in terms of intracellular bacterial survival, especially within heavily infected cells, and indicated that the survival of *L. monocytogenes* was dependent on the adaptations of a small fraction of bacteria within a population of macrophages which permit intracellular growth. Phagocytotic grazing of autofluorescent phytoplankton (201) and bacteria (507) and of fluorescent latex beads (44) has also been demonstrated, with the latter study proving a striking visual discrimination between *Acanthamoeba castellani* cells that had taken up different small numbers of beads.

To summarize the section on microbial viability, however, it must be concluded that no single stain, and certainly none discussed here, is likely alone to be an unimpeachable or "gold standard" indicator of viability, especially if we require that its interpretation tally with the ability of cells subsequently to undergo division. How a cell dies depends on what kills it, and (fortunately for those designing novel antibiotics) there are many more ways of dying than there are different types of stain or of detectors in our flow cytometers. Indeed, insufficient consideration seems to have been given to the (anecdotally many) cases in which cells can form microcolonies but not macrocolonies (483, 692) and whether this phenomenon constitutes some kind of programmed cell death or apoptosis (133, 654, 1051), an exceptionally important feature of the biology of higher organisms. Lastly, here, and although they were not themselves concerned with microorganisms, we would thus draw attention to the elegant flow cytometric studies of Al-Rubeai, Emery, and colleagues on animal cells in suspension culture (see, e.g., references 18, 19, 21, and 22), including in particular the evidence that the low viability often seen is such cultures is likely to be caused by apoptosis rather than necrosis (23, 190, 875). In a similar vein, we would mention that a decline in mitochondrial activity is an early event in apoptosis (735, 973, 1066, 1067) and that mitochondria (given their evolutionary origin as prokaryotes) have also enjoyed fully fledged status as objects of study by flow cytometrists, with many of the same activity probes that are being applied to microbes (see, e.g., references 74, 344, 731, 732, and 736). Overall, we conclude that although there are no perfect stains as yet, substantial progress is being made toward the rapid and routine flow cytometric analysis of something reflecting microbial viability or vitality.

Natural samples. The problems of the microbial ecologist are inestimably greater than those of the microbial physiologist, who studies only laboratory strains under conditions over which the investigator can exert some measure of control. This stems largely from the fact that "in most natural microbial environments only a very small fraction of the microbes present can be enumerated using agar plate techniques" (610) (and are thus, by our definition, not viable). This discrepancy (between viable and total counts) has been found widely for bacteria in soil (55, 647) and water (647, 820) and, reflecting the fact that most natural environments are nutrient poor, may be obtained under laboratory conditions during progressive starvation of bacteria (62, 220, 480, 481, 517, 646, 648). Amann et al. (26) refer to it as the "great plate count anomaly" and provide an excellent review both of the exceptional diversity of presently noncultured organisms (see, e.g., references 134, 235, 359, 945, and 946) and of the utility of rRNA probes, together with flow cytometry for planktonic cells, in their analysis. They point out that not only do rRNA-targeted whole-cell hybridizations yield data on cell morphology, specific cell counts, and in situ distributions of defined phylogenetic groups, but also the strength of the hybridization signal reflects the cellular rRNA content of individual cells. Given the well-known relationships between rRNA content and growth rate in laboratory cultures, it is plausible that the signal strength conferred by a specific probe might allow one to estimate the in situ growth rates and activities of individual cells. They recognize, however, that in many ecosystems, low cellular rRNA content and/or limited cell permeability, combined with background fluorescence at the wavelengths presently used, does rather hinder in situ identification of autochthonous populations.

As a consequence of the high species diversity in natural ecosystems, the technical demands on flow cytometers are substantially greater than those normally needed for the study of axenic laboratory cultures; this arises in particular because of the great range of particle sizes and (auto)fluorescence which may be anticipated therefrom (274, 724, 1053), which not only require improvements in the flow system of the cytometer but also imply a substantially greater dynamic range than the four decades normally available in commercial instruments.

If starvation and predation (as well as physical and chemical stresses) are major causes of the loss of microbial viability in nature and if nonselective predation will not cause any discrepancy between viable and total counts, it is to be hoped that the same general sorts of approach that are proving fruitful in the analysis of laboratory cultures may also be applicable to environmental samples. Since a number of recent reviews attest nicely to this fact (26, 140, 765, 981) and some examples have already been given in the section on microbial discrimination, we shall be rather selective in our choice of examples, concentrating, largely because of our own interests, on bacteria. Our theme is that improved approaches and techniques have allowed a substantial increase in the ability to analyze "difficult" samples.

Because of their autofluorescence, algae and phytoplankton have long been studied by flow cytometry (3, 4, 59, 80, 102, 108, 137, 163, 201, 203–206, 236, 237, 286, 323, 333, 336, 397, 466, 551, 554, 559, 560, 562, 563, 600, 702–705, 718, 726, 748, 805, 870, 871, 931, 948, 969, 975, 1030, 1052, 1053). A particular highlight of this research was the discovery (165) and subsequent analysis, both aided by flow cytometry, of the genus *Prochlorococcus* as a major component of the marine microbiota (147, 148, 164, 970–972) and of the alga *Ostreococcus tauri* as the smallest eukaryote (167, 191).

Possibly in part because of the oligotrophic nature of the habitats and the consequently small size of many of the organisms (142, 853), the flow cytometric analysis of heterotrophic aquatic bacteria in situ is relatively in its infancy (140, 141, 168, 273, 311, 640–643, 716, 807, 939, 948, 949). Typical applications include not only the identification of microbes but also the determination of their biomasses, growth, and metabolism, factors of obvious importance to our understanding of biogeochemical dynamics, and their effects on global warming, and, as in the study of Button et al. (141) following the grounding of the *Exxon Valdez*, in assessing the ability of microbes to metabolize pollutants. Shapiro (865) illustrates some extremely elegant work by these authors and makes the very important point that their axes are calibrated to read in biological units such as femtograms of DNA rather than "channel number", something we may expect to be increasingly required.

Average specific growth rates are typically measured by a dilution technique (561) designed to restrict the activity of predators.

Thorsen et al. (939) studied three strains of the fish-pathogenic bacterium *Yersinia ruckeri*, which was found to survive starvation in unsupplemented water for more than 4 months. At salinities of 0 to 20%, there were no detectable changes in CFU during the first 3 days of starvation and only a small decrease during the following 4 months, whereas at 35% salinity, the survival potential of the cultures was markedly reduced. The authors also examined survival by using the direct viable-count method, which they combined with flow cytometry for detecting such viable bacteria after staining with mithramycin and ethidium bromide in the presence of nalidixic acid. It was found that replication initiated before the onset of starvation was completed during the initial phase of starvation and that starved cells could contain up to six genomes per cell.

Firth et al. (311) studied the survival and viability of *Vibrio vulnificus*, a pathogen of shellfish and (thence) humans which often enters a nonculturable state (see, e.g.; references 683, 700, 1016, and 1041) in which it nonetheless remains virulent (699). Since this organism causes 95% of the shellfish-related deaths in the United States (699), methods for the analysis of such cells that do not depend on culturability are clearly of substantial importance. Firth et al. (311) studied the loss of culturability and the percentage of cells which stained with ChemChrome B and with acridine orange during starvation at 4°C in a filtered-seawater microcosm. Culturable cells declined from 10^7 ml^{-1} to an undetectable number in 7 days under conditions in which acridine orange counts were unchanged and ChemChrome B counts decreased to some 10^4 ml^{-1}. The same Liverpool group, in collaboration with the Windermere laboratory (767), studied the use of fluorescence-activated cell sorting to obtain highly enriched populations of viable target bacteria by using antibody labelling of the target organisms. As in the *Cryptosporidium* studies described above, it was necessary to overcome nonspecific binding of the antibody by using blocking agents, of which 15% bovine serum albumin was the least imperfect. Sorting was shown not to kill the cells, and individual strains of *S. aureus* and *E. coli* could be selectively recovered from mixtures at a purity in excess of 90%, even when the former made up only some 0.4% of the total cells. Cell sorting was also tested for the ability to recover *E. coli* from natural lake water populations and sewage. The environmental samples were challenged with fluorescently labelled *E. coli*-specific antibodies prior to cell sorting. Final sample purities of greater than 70% were routinely achieved, and populations of *E. coli* released into environmental samples were recovered at greater than 90% purity.

One of the chief problems with environmental samples is the high incidence of noncellular particulate matter, which, despite the use of suitable gating, might be expected to prove an insuperable problem for flow cytometry. This turns out not to be so. Thus, Diaper and Edwards (246) were able to monitor the colonization of sterile compost by *Bacillus subtilis* after filtering the samples through 25-μm-pore-size filters, although the number of observable bacteria as seen flow cytometrically was, as usual, significantly greater than the number capable of forming colonies. Under oligotrophic conditions in particular, bacteria in soil tend to hide in the interstices of soil particles; for this reason, those brave souls who study bacterial processes in soil by flow cytometry tend to preprocess their samples extensively, for instance by repeated washing and centrifugation followed by density gradient centrifugation, so as to remove enough soil particles to allow the flow cytometric study of individual cells (see, e.g., reference 169).

As if soil and compost were not sufficiently demanding matrices, van der Waaij et al. (961) described a flow cytometry method for the analysis of noncultured anaerobic bacteria present in human fecal suspensions. Nonbacterial fecal compounds, bacterial fragments, and large aggregates could be discriminated from bacteria by staining with propidium iodide and gating on propidium iodide fluorescence and by exclusion of events with large forward scatter. Immunoglobulin A-coated bacteria in suspensions of fecal samples from 22 human volunteers were labelled with FITC–anti-human immunoglobulin A and could be discriminated without being cultured. To quote Shapiro (865), "the subject matter may stink, but the methodology was superb," as it was too in the study by Wallner et al. (999), who used fluorescent oligonucleotides to analyze microbial communities in activated sludge, and in the study by Porter et al., who used immunofluorescent flow cytometry and cell sorting (769).

Flow Cytometric Analysis of the Physiological State of Individual Cells—the Physiological Patterns of Microbial Cultures

While flow cytometry may be exploited to distinguish between microorganisms in artificial and natural mixtures (see above), perhaps the majority of flow cytometric applications in microbiology have entailed the study of axenic cultures of

organisms. In microbiology, the organism of interest is frequently grown in a batch culture, or for so-called (operationally) steady-state measurements, continuous-culture methods may be employed (272, 752, 935). The role of the microbial physiologist is to attempt to understand the processes occurring during growth, and while measurements on the whole population are frequently made to determine, for example, the biomass concentration, pH, DNA content, etc., of the culture, we have continued to stress that the special power of flow cytometric techniques enables one to make measurements on individual cells and thus to build a population distribution for each of the characters of interest and, indeed, by multiparameter measurements, to determine relationships among these measured parameters. Inevitably, many of the earlier studies were devoted to an analysis of the cell cycle.

Since yeasts are generally larger than prokaryotes and consequently have a higher level of many of the cellular constituents of interest to the microbial physiologist, historically they have been more amenable to study by flow cytometric methods. Consequently, they were among the first microorganisms to be studied (439, 883) and so will be considered first here.

Yeasts. Yeasts have been used for the production of bread, wine, and beer for many thousands of years, although on this timescale it is only relatively recently that their role in these processes has been appreciated and investigated. In 1984, the world market for baker's yeast alone was estimated to be 1 million tonnes per annum (83), a figure which adequately explains the need for a full understanding of the physiological processes involved in the growth of this organism, particularly when, as nowadays, it is producing heterologous proteins (412, 629, 977).

In an industrial setting, the growth of yeast cells is observed closely in order that maximum yields of biomass or product are achieved according to the type of fermentation involved. It is often desirable, therefore, that measurements such as the biomass content and so on are made on-line (178, 216, 308, 394, 474, 500, 502, 573, 574, 729, 756, 891), although presently flow cytometry is not normally implemented on-line, so that samples must be withdrawn from the fermentation vessel for further analysis (225). In the laboratory setting, flow cytometric methods have been applied to the analysis of yeast cells during their growth in both batch and continuous cultures. Bailey and colleagues carried out a number of pioneering studies designed to exploit the power of flow cytometry to help them account for the dynamics of yeast (and other) fermentation processes (see, e.g., references 419–423, 898, and 899), while Scheper et al. (844) used dual-laser flow cytometry to analyze *S. cerevisiae* samples taken from batch culture and continuous-culture fermentations. Measurements were made on cell size and protein, DNA, and RNA content. Flow cytometric cell size measurements gave a clear indication of budding, with the frequency of budding cells (determined microscopically) being closely correlated with the frequency of cells larger than 6 μm. The histograms of cell size and protein and RNA content were clearly related to the stage in the batch culture, with distributions being wider when the culture was growing exponentially. Similar results were obtained when continuous-culture techniques were employed, with the highest dilution rates giving rise to the most heterogeneous cultures.

Flow cytometric methods were exploited for the phenotypic characterization of yeast autolytic mutants and for the analysis of the formation and regeneration of yeast protoplasts, since the expression of lytic mutations led to uptake of propidium iodide. Protoplast formation and regeneration could also be monitored by analyzing relative cell sizes (228).

Studies of synchronous and asynchronous cultures of three different yeasts with respect to DNA content have also been performed (451). In the exponential phase of the batch culture of asynchronous cells, DNA histograms revealed two peaks, reflecting cells which either had or had not replicated their DNA. Cells taken from stationary phase had a single peak, corresponding to cells with a DNA content typical of a single chromosome set. With synchronized cultures, progression through the cell cycle could be monitored by movements of the cell distribution along the fluorescence axis.

Flow cytometric analysis of the cell cycle of *S. cerevisiae* in populations growing at different rates has also been used to demonstrate that variation in the growth rate of the population depends mainly on variation in the G_1 phase of the cell cycle (883). It was also shown that at all growth rates tested, the initiation of DNA synthesis occurred concomitantly with initiation of budding. Alberghina and colleagues (11, 12, 604) have used flow cytometry to study changes in the protein content of budding yeast and have shown that there is a good correlation between such distributions in protein content and the growth rate. The relationship between the growth rates of cells at different points in the cell cycle and the steady-state distribution of cell sizes is not easy to distinguish (52, 188, 217, 225, 395), but it was considered that the behavior of the cell size distributions could be interpreted in terms of a two-threshold cell cycle model in which both the critical protein content at budding and the critical protein content for cell division were differentially modulated by the growth rate. They and others also showed that the extent of uptake of Rh123 gave a good measure of respiratory activity (763, 882), as do other cationic dyes such as carbocyanines (136, 527, 734, 778). Degelau et al. (225) also studied the variation of cell size and protein and lipid content (using light scattering and the fluorescence of FITC and Nile red, respectively) during yeast batch growth; at the onset of ethanol utilization, a smaller subpopulation was observed and was presumed to be the main fraction capable of respiring the glycolytically generated ethanol (although the bivariate plots which might have supported that conclusion more fully were not shown). The recent finding (219) that such Crabtree-positive cells can exhibit deterministically chaotic growth rates may also be taken to reflect the complex and heterogeneous dynamics of baker's yeast cultures. At all events, flow cytometry clearly showed a profound differentiation of the culture (225), which would not have been seen by conventional methods.

Porro and Srienc (764) devised a flow cytometric procedure which aimed to measure the growth properties in asynchronous culture of individual *S. cerevisiae* cells, whose cell surface was labelled with FITC-conjugated concanavalin A. Because the formation of new cell wall material in budded cells is restricted to the bud tip, exposure of the stained cells to growth conditions resulted in three cell types, i.e., cells stained to the original degree, partially stained cells, and unstained cells. Analysis of the time-dependent staining pattern theoretically permits the determination of the specific growth rate of the cell population, the length of the budded cell cycle phase, and the growth pattern during the cell cycle of newly formed, partially stained daughter cells. However, the apparent single-cell-specific cell size growth rates, determined by forward-angle light-scattering intensity, were significantly lower than the specific growth rates of the overall population. This could indicate that the normal behaviors of cell cohorts was perturbed by the staining procedure and (as we have seen) that forward-angle light scattering may not always be a good indicator of the cell size. On this basis, the lipophilic tracking dye PKH26 may be better suited to this type of analysis (40). Finally, a double staining method was used to assess both the cell age and the

cell protein content of a cohort of daughter cells at the different cell cycle set points in *S. cerevisiae* cultures and to indicate in this case that the average rate of increase of the size of each individual cell is almost identical to the specific growth rate of the overall population (762).

Lastly, yeasts are also popular organisms for more basic and molecular studies of the control of the eukaryotic cell cycle, in which flow cytometric procedures are increasingly being deployed to great advantage (106, 250, 251, 277, 319, 334, 435, 515, 583, 603, 656, 663, 672, 681, 710, 802, 967, 976, 1014, 1048).

Sterols seem to be involved in the initiation of DNA replication in yeast cells (578). Flow cytometric analysis of the sterol content of yeast cells has revealed that cells with low levels of membrane sterols have suppressed proliferation activity but high fermentation activity (659). This measurement was made most cleverly via the binding of fluorescein-labelled nystatin (662), an antibiotic that binds to sterols, and one might comment that the cognate exploitation of subinhibitory concentrations of other fluorescently labelled antibiotics is an underutilized and worthwhile pursuit. Importantly for the yeast production industry, yeasts with a high content of membrane sterols were better able to survive the drying process. Flow cytometric analysis of yeasts during growth could therefore provide a valuable indication of the optimum time for harvesting.

Budding yeasts such as *S. cerevisiae* (and indeed many higher cells [402]) possess a finite replicative lifespan; each cell is capable of only a limited number of divisions before the onset of senescence and cell death, and mortality increases with each generation (43, 457, 458, 471, 472, 508, 509, 652). This simple fact necessarily contributes enormously to the heterogeneity of yeast cell cultures. Within the brewing industry, it is normal to harvest cells from each run and use a substantial fraction of them as the "pitch" (inoculum) for the next brew (432); clearly, this will tend to be accompanied by ageing and a loss in viability and performance if care is not taken (109). Sterols cannot be synthesized anaerobically, and their initial levels are probably of great importance to brewing performance (781), especially since almost nothing except temperature is usually controlled following the pitch. Since many of the senescence markers are accompanied by changes in the cell surface (65, 885), it seems likely that flow cytometric procedures might similarly be used with great advantage, especially in combination with on-line probes (42, 110), to assess the optimum pitch for the brewers' yeast fermentation and, indeed, its physiological state generally (437, 438, 441–443).

Srienc et al. have reported a flow cytometric method for detecting cloned β-galactosidase activity in *S. cerevisiae* (899). *lacZ*, the gene encoding β-galactosidase, is often used as a reporter gene to study levels of expression because if substrate analogs are used, its activity may easily be assayed by spectrophotometric techniques. However, the ability of flow cytometry to make measurements on single cells means that individual cells with high levels of expression (e.g., due to gene amplification or higher plasmid copy number) could be detected. In the method reported, a nonfluorescent compound (α-naphthol-β-D-galactopyranoside) is cleaved by β-galactosidase and the liberated naphthol is trapped to form an insoluble fluorescent product. The insolubility of the fluorescent product is of great importance here to prevent its diffusion from the cell. Such diffusion would not only lead to an underestimation of β-galactosidase activity in highly active cells but would also lead to an overestimation of enzyme activity in inactive cells or those with low activity, as they may take up the leaked fluorescent compound, thus reducing the apparent heterogeneity of the population. A lipophilic derivative of fluorescein-β-digalactoside is now perhaps most commonly used in animal cells (see, e.g., references 754 and 1070), but persuading these derivatives to enter cells (such as yeast cells) which cannot grow on lactose is often difficult. That fluorescein-β-digalactoside enters only nonviable yeast cells was nicely demonstrated by dual staining with propidium iodide (754). As well as the problems of ensuring entry of the probes, our own experience (unpublished) is that fluorescein leaks rather rapidly from bacterial cells that are not in very good condition; the analysis of the kinetics of (de)staining makes it evident when the cells begin to lose accumulated stain.

2′,7′-Dichlorofluorescein diacetate (DCFH-DA) is a membrane-permeant, nonfluorescent dye which is concentrated in esterase-positive cells. It is oxidized by H_2O_2 (and by other peroxides) to a green fluorescent fluorescein derivative, which effectively allows the monitoring of H_2O_2 production in individual cells. Yurkow and Mckenzie (1062) performed a most interesting study with this dye and *S. cerevisiae*. Aeration of yeast cultures exposed to a period of hypoxia was found to induce levels of peroxide that were 100-fold higher than the levels observed in cultures maintained under well-aerated or hypoxic conditions, and a multiparameter kinetic analysis, including simultaneous viability measurements with propidium iodide, showed nicely and unequivocally how the increase in peroxide generation preceded cell damage and death (Fig. 14). The heterogeneity in peroxide production between individual cells was itself in excess of 100-fold, and it is not plausible that a correct analysis would have been possible had macroscopic measurements of H_2O_2 production and viability alone been used. Dihydro-Rh123 could probably also have been used (410).

Prokaryotes.

> "Flow cytometry has revolutionized the study of the cell cycle of eukaryotes. It is also possible to apply the flow cytometry principles to bacteria. . . . The importance of the flow cytometry results should not be underestimated. They provide a crucial link in the analysis of the division cycle. . . . While other experiments have substantially supported the initial membrane-elution results, the flow cytometry results determine the pattern of DNA replication without any perturbations of the cell."
>
> Cooper (188)

> "The success of molecular genetics in the study of the bacterial cell cycle has been so great that we find ourselves, armed with much greater knowledge of detail, confronted once again with the same naive questions that we set out to answer in the first place."
>
> Donachie (259)

Until quite recently, much of our understanding of the way in which bacteria replicate their genetic material prior to cell division was a consequence of the study of populations (188). So that the results obtained from such studies can be interpreted on the single-cell basis, one usually synchronizes the cells prior to making measurements. There are a variety of techniques for obtaining synchronized cultures (1069), but however carefully they are prepared, there is a chance of perturbing normal growth and division (17, 115, 571).

An alternative is to study single cells, and this is possible to some extent by light or electron microscopy, but the data obtained are largely qualitative. In addition, conventional cy-

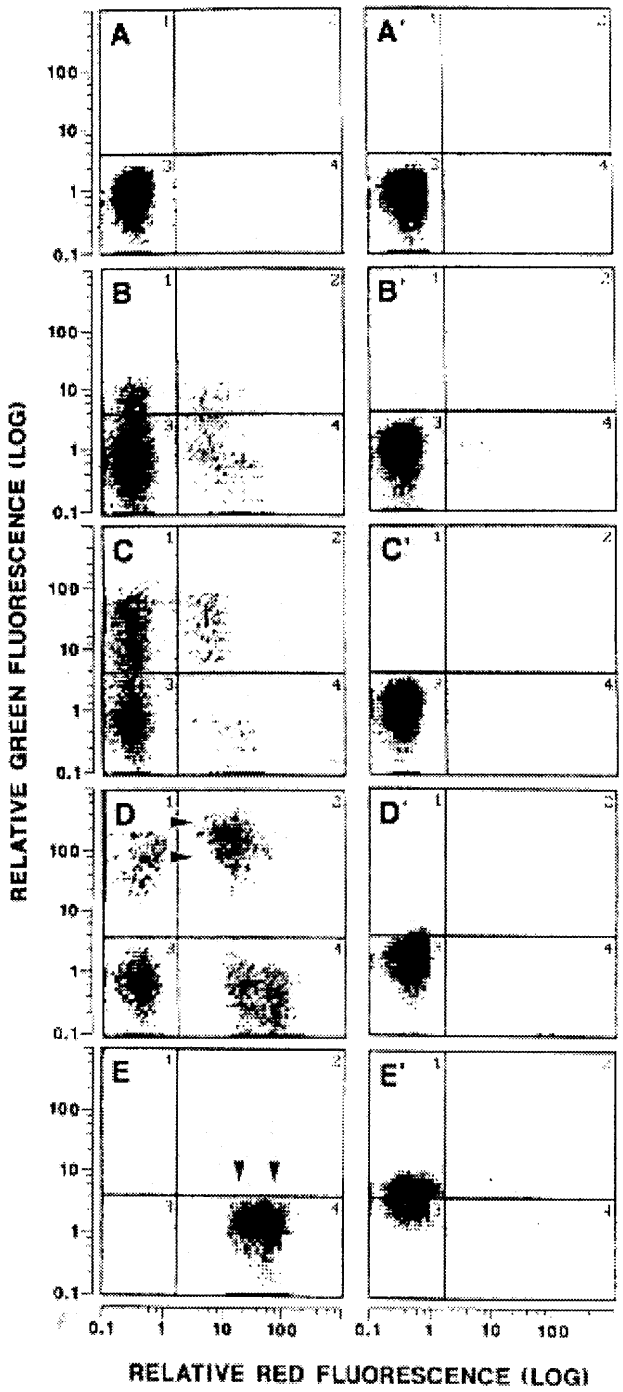

FIG. 14. Peroxide production and cell viability in hypoxic/reaerated and aerated cultures of *S. cerevisiae*. DCFH-DA-stained stationary-phase cultures of *S. cerevisiae* were either maintained under normal, aerated conditions or exposed to a 16-h period of hypoxia and then returned to normal aerated conditions. At the indicated times following reaeration of the hypoxic cultures, aliquots from each culture were removed, stained with propidium iodide, and analyzed by flow cytometry. Peroxide production (green fluorescence) and cell viability (red fluorescence) of hypoxic cultures are shown at 0, 3, 10, 30, and 50 h postaeration as A to E (left-hand column), respectively, while continuously aerated cultures are shown as A′ to E′ (right-hand column). Note how the cells clearly move clockwise in the left-hand column, albeit far from uniformly, as a function of time. Reproduced from reference 1062 with permission.

tometry is very time-consuming; examination of 100 or so cells may take several hours. This would result in generalizations being drawn from small samples and would therefore make the work prone to sampling error.

Since flow cytometry offers the advantage of making measurements on the DNA content of individual cells, the use of this method removes the need for synchronization, and since measurements can be made rapidly on several thousands of cells, the sampling error is eliminated. Indeed, one of the most abundant areas of flow cytometric research is in the analysis of DNA, with the cell cycle analysis of eukaryotic cells benefiting greatly from advances in flow cytometry (214, 942). Analysis of the cell cycle of prokaryotes is complicated not only by the problems of sensitivity discussed above but also by the possibility of more than one round of cell division in progress within a given cell. Notwithstanding this, it has been shown that in rapidly growing *E. coli* cells (906), the results of flow cytometric DNA analysis were largely in agreement with earlier models of the bacterial cell cycle that had been formulated from studies of synchronized populations. Meanwhile, studies of slowly growing chemostat cultures of *E. coli* have shown that the cell cycle periods (B, C, and D in the Cooper-Helmstetter model [188, 409]) increase with decreasing growth rate and that the B period, i.e., the time between cell division and the initiation of a new round of chromosome replication, occupies an increasing fraction (in some cases up to 80%) of the cell cycle (878). The results obtained in these studies have been compared with theoretical computer simulation routines based on the Cooper-Helmstetter model and are generally in agreement (879). Flow cytometric analysis of temperature-sensitive mutants of *E. coli* has also allowed the role of various genes involved in the control of the bacterial cell division cycle to be established (526). With regard to multiple rounds of chromosome replication, flow cytometry enables the experimenter to determine whether chromosome replication is initiated synchronously or asynchronously across all origins (877) and, where present, to determine the frequency of asynchronous initiation.

Flow cytometry of mammalian cells has proved effective for the detection of altered ploidy, a factor which is important in cancer diagnosis (358). In comparison, ploidy determination in bacteria is complicated in prokaryotes by the multiple rounds of replication that may be under way within a single cell. Nevertheless, flow cytometric methods have been used to determine ploidy in cultures of *E. coli* (280). Rifampin, a protein synthesis inhibitor, prevents the initiation of new rounds of chromosome replication while allowing those already begun to continue until completion. In this manner, it was shown that the broad peak of propidium iodide (DNA) fluorescence observed for untreated cells was due to a mixture of cells with DNA contents between n and $2n$ or $4n$ or even higher, depending on the growth rate. Durodie and colleagues used this approach to study the effects of subinhibitory concentrations of antibiotics (amoxicillin and mezlocillin) on bacterial ploidy (see the section on antibiotics, below).

DNA staining of bacteria coupled with flow cytometric analysis can also be used as an aid in determining the effects of cytotoxic drugs (347, 612, 758, 907, 912), the role of different genes and the effects of mutations in them (455, 526, 774, 876, 987, 1036), the quantitative presence of plasmids (842, 856, 857), and the presence of viral DNA within the bacterial cell (837). As a consequence of this broad range of applications, a wide variety of DNA stains have been used in conjunction with flow cytometry (Table 2).

Flow cytometric methods have also been used in the assessment of the physiology of prokaryotes during batch and continuous culture. In view of the evident ability of *B. subtilis* to

differentiate by forming spores, one of the first studies of its kind was in *B. subtilis*, in which simultaneous measurements of the double-stranded nucleic acid (propidium iodide staining) and protein content (FITC staining) of samples removed from different stages of a batch culture were made (306). Microscopic determinations of the frequencies of spores, single rods, and chains of cells were also made, and it was shown that changes in these were reflected in the flow cytometric plots. The initiation of sporulation of bacteria is a complex cellular event, controlled by an extensive network of regulatory proteins that are intended to ensure that a cell embarks on this differentiation process only when appropriate conditions are met. The major signal transduction pathway for the initiation of sporulation is known to be a phosphorelay, which responds to a variety of environmental, cell cycle, and metabolic signals to activate the Spo0A transcription factor by phosphorylation (424). Chung et al. (170, 171, 173) have carried out a series of studies with *B. subtilis* which serve nicely to show the power of flow cytometry in discriminating the differential gene expression of subpopulations of a nominally homogeneous, well-stirred culture. They measured the expression of developmental (*spo*) genes in single cells of *B. subtilis* by using *spo-lacZ* fusions, and, after staining with a fluorogenic galactoside, found by using a flow cytometer that in a culture of sporulating cells, there were two subpopulations: one that has initiated the developmental program and activated the expression of early developmental genes and one in which early developmental gene expression remained uninduced. Studies with mutant strains suggested that a threshold level of phosphorylated Spo0A (Spo0A~P) (or of a component of the phosphorylation pathway) must accumulate to induce sporulation gene expression and demonstrated that most of the cells which are able to induce the expression of early genes that are directly activated by Spo0A~P do go on to produce mature spores. It is also worth noting in this context that the initiation of sporulation also requires extracellular communication between organisms (380), another example of the increasingly widely recognized role of pheromones as agents for signalling differentiation in prokaryotes (see, e.g., references 332, 375, 476, 483, 499, 921, 926, and 927), which leads us to stress the importance of methods such as flow cytometry for the discrimination of such differentiated cells (499).

Monitoring of *E. coli* strains during their continuous culture has revealed, as is well known for a variety of organisms (449), that the cell size and protein content of the cells are related to the growth rate of the culture (843). Scheper et al. have also demonstrated an elegant method for distinguishing between plasmid-containing and plasmid-free cells. The plasmid in question carried a marker gene encoding penicillin G acylase, which confers ampicillin resistance. When ampicillin was added to the medium, it caused an elongation of the plasmid-free cells, thus allowing them to be identified by light-scattering measurements (842). By using this method, plasmid loss could be monitored rapidly and effectively during batch or continuous culture. In the later work (843), they exploited a fluorogenic analog of penicillin for the direct estimation of penicillin G acylase activity, noting an inverse correlation of enzyme activity with dilution rate.

Methods involving fluorogenic substrates are common in flow cytoenzymology (253, 254), and enzyme activity has also been used as a measure of cellular activity. For example, the protease activity in viable leukocytes following the phagocytosis of *E. coli* has been studied by using the fluorogenic substrate Z-Arg-Arg-4-trifluoromethylcoumarinyl-7-amide, which is cleaved intracellularly to the green-fluorescent 7-amino-4-trifluoromethylcoumarin (821). Enzyme activity has also been used as an indication of cellular viability measurable by flow cytometry, particularly for dehydrogenases (625). Although concentrating mainly on spectrophotometric methods, van Noorden and Jonges (965) give a useful account of histochemical approaches to the assessment of enzymatic activity in situ and of the heterogeneity thereby revealed (966). The motivation for such studies and for the simultaneous multiparameter measurements which may be deployed to advantage maps very closely onto our own, and cognate analyses might profitably be exploited in microbial flow cytometry.

A particularly powerful feature of flow cytometric procedures is that they permit, in a facile manner, analysis of the covariance of different determinands. While it is better when possible to analyze cause-effect relationships directly, rather than the covariance of what are in fact culture variables (502), biplots of two variables against each other can give useful indications of the relationships between different segments of metabolism. To date, however, these have barely been forthcoming, and it is certain that an increasing recognition of the power of these multiparameter flow cytometric methods and displays will lead to a substantial enhancement of our understanding of the overall structure of metabolic processes and of the transition times between different metabolic states following a change in a parameter (944).

Flow Cytometric Analysis of the Interaction of Drugs and Antibiotics with Microbial Cells

Despite the widespread existence in nature of microbially produced antibiotic molecules that are active against other microbes, a phenomenon recognized long before Fleming's celebrated observations (312) on *Penicillium* (see, e.g., reference 717), the modern antibiotics industry still exploits as few as six main biochemical targets (1065). In an era of worrying and increasing levels of resistance of pathogens to antibiotics (99, 221, 665, 666, 682, 896) and given the interest in flow cytometric methods for assessing viability (described above), it is not surprising that understanding the interactions between microorganisms and the drugs intended to kill them provides another fruitful area for flow cytometric methods (761, 906). The first major attempt in this direction for microorganisms occurred in 1982, when Steen et al. (907) demonstrated distinctive two-parameter LS/fluorescence histograms when bacteria were stained with mithramycin and treated with a variety of antibiotics. In the same year, Martinez et al. (609) demonstrated by flow cytometry the filamentation induced by β-lactam treatment of *E. coli*. Apart from the continuing pioneering studies by Steen and his collaborators (115, 526, 877, 904, 912), Shapiro (862, 865) stressed the potential utility of methods for the flow cytometric analysis of antibiotics based upon membrane damage (regardless of the primary target of the drugs), and Boye and Løbner-Olesen (113), using a flow cytometric method capable of adequate cell number assessment, were able to demonstrate the inhibition of *E. coli* growth within minutes (although simple optical density measurements can often do the same [503]), while Gant et al. (347) showed that a modern laser-based flow cytometer was sensitive enough to detect changes in bacterial morphology on entry into the growth cycle and after exposure to a variety of bactericidal (as opposed to bacteriostatic) antibiotics (as judged by forward- and side-scatter measurements). Antibiotic-induced morphological changes affecting subpopulations of bacteria were sufficiently specific to allow differentiation between antibiotics with different cell wall enzyme targets. The effect of such antibiotics on the integrity of the outer cell membrane of *E. coli* was also assessed by measurement of the ability of propidium

iodide to stain bacterial nucleic acids. These nice experiments demonstrated complex but reproducible patterns of cell wall leakage, related to the modes of action of the antibiotics, and in particular (again) that flow cytometric assays based on the assessment of inner membrane damage were of value for these purposes (i.e., such effects occurred sufficiently rapidly following the antibiotic challenge to be useful indicators of antibiotic susceptibility), even though the primary targets of these (and indeed most) clinical antibiotics lie elsewhere; in the studies by Gant et al. (347), these drugs (at concentrations equivalent to those estimated by conventional means to be at the level of the MIC) included gentamicin, a variety of β-lactams, and ciprofloxacin, whose targets are, respectively (825), the 30S ribosomal initiation complex, the outer membrane, and DNA gyrase.

Shapiro (862) had stressed the potential utility of so-called membrane potential dyes such as the carbocyanines for the assessment of cell viability (and depending on the effects of Tris-EDTA for Gram staining), since actively respiring cells accumulate these dyes whereas "dead" cells are usually much less highly stained. Therefore, Ordóñez and Wehmann (711) developed a flow cytometric technique for assessing antibiotic effects on *Staphylococcus aureus* in which the antibiotic action was determined by monitoring fluorescence changes of 3,3′-dipentyloxacarbocyanine iodide. Three ATCC reference strains of *S. aureus* and 13 unknown strains of the same microorganism were tested for susceptibility to penicillin G and oxacillin, and it was found that the susceptibility of *S. aureus* to these antibiotics could be measured reliably 90 min after addition of the antibiotic whereas the results were comparable to those obtained with conventional susceptibility tests. The same authors (712) applied their method to the analysis of the interaction of the antifungal compound amphotericin B with *Candida albicans* and *C. tropicalis*. Sensitivity or resistance was easily determined after 30 min of incubation in the presence of the antibiotic. Deenergization by uncoupler of *Acinetobacter calcoaceticus* stained with 3,3′-dihexyloxacarbocyanine iodide could be observed flow cytometrically within 3 min (658), a timescale similar to that for Rh123 efflux observed macroscopically (480).

Pore (758) had developed a flow cytometric susceptibility test for antifungal agents based on membrane damage-induced staining by propidium iodide or rose bengal of the same yeasts. The test was both much more rapid (9 versus 24 to 48 h) and reliable than the broth dilution test and could detect subpopulations of different resistance, and with these and other yeasts it also allowed accurate assessment of the dose-response curve (759) and the synergistic antifungal effect of pairs of compounds (760). Flow cytometric susceptibility tests have also been reported for amphotericin B with *Candida* spp. by using ethidium bromide (695), propidium iodide (608), fluorescein diacetate, and propidium iodide (153, 374) and with *Eimeria tenella* sporozoites by using propidium iodide and FDA (790).

Durodie et al. (280) used a laser-based flow cytometer and observed very rapid changes in the DNA ploidy of *E. coli* treated with amoxicillin and mezlocillin; after a 1-h treatment with mezlocillin at 0.25 times the MIC, the ploidy was ca. 15 to 20 genomes. They also found that the ratio of protein content (by FITC fluorescence) to forward-angle light scattering could provide a rapid indication of antimicrobial activity (279). Mason et al. (612) evaluated flow cytometry with oxonol probes for estimating MICs of antibacterial agents against *E. coli* and *S. aureus*, showing the flow cytometric assay to be fast (1 to 5 h), and again stressed the advantage that resistant subpopulations could in principle be detected, while Jepras et al. (460) also found the oxonol dye $DiBAC_4(3)$ to be the viability stain of choice, because no incubation with Tris-EDTA was required for it to be an effective discriminator between viable and gramicidin-treated cells. The Cardiff group showed that the same dye could also be used to assess the viability of *Trichomonas vaginalis* (436), whilst Dybowski et al. (283) used it, together with an arc lamp-based cytometer and a decision tree method, to assess antibiotic susceptibility within 5 h. Rather high concentrations of oxonol are, however, necessary to effect good discrimination between sensitive and resistant strains in some cases (611), and sensitive bacteria can continue to give an oxonol response in the presence of some antibiotics (such as the DNA gyrase inhibitor ciprofloxacin) for up to 5 h postexposure (614). Walberg et al. (998) found that DNA fluorescence and light scattering were sufficient to allow them to detect antibiotic susceptibility of *E. coli* within 40 min of drug exposure and that a staining procedure involving cold shock and EDTA-azide treatment eliminated the need for a centrifugation step and significantly improved the accuracy of the cell counts.

Shapiro (865) makes the point that any "routine" flow cytometric assessment of antibiotic action must compete with the various assays available in 96-well microtiter format; these can be run in parallel while flow cytometry assays are essentially serial. Although one flow cytometry assay may be quicker, 96 will normally not be. Where flow cytometry comes especially into its own for the analysis of antimicrobial activity is therefore under conditions in which growth of the pathogen in liquid culture is either impossible, slow, or hard to monitor. The eukaryotic microbe *Pneumocystis carinii* has been increasingly recognized as an important cause of opportunistic infection, especially in the lungs of immunodeficient hosts (384) and can only be cultured well in vivo, or at least in the presence of host cells. Conventional assays for detecting drug effects on *P. carinii* are time-consuming, give little information on the variability of the organisms, and are often affected by yeast contamination of primary isolates. However, Morley et al. (649) showed that flow cytometry with FDA staining provided a reliable indicator for drug effects on *P. carinii*. Norden et al. (689) used a similar approach with *Mycobacterium tuberculosis*, and the same group (112) showed that suspensions of untreated and drug-treated cells of a variety of mycobacteria could easily be differentiated at 6 and 24 h after the initiation of the susceptibility assays by using the same approach.

Although a number of studies have shown that antibiotics with different (known) primary modes of action lead to differently shaped flow cytometric histograms, we are not aware of any study that has yet addressed the inverse problem, i.e., that of seeking to determine the mode of action of a drug from its effects upon the flow cytometric behavior of a cell suspension. However, in a somewhat related approach, Weinstein et al. (1017) studied the pattern of activity of 134 agents (141 formulations) against a panel of 60 malignant cell lines in the National Cancer Institute drug-screening program and trained artificial neural networks to use the quantitative inhibitory data for the 60 lines to predict the mechanism of action of a drug (from six possible classes of mechanism). The principle behind this is that each of the lines will express different amounts of the various target enzymes and that cells with important targets which are the least expressed tend to prove the most sensitive to drugs which bind that target. The network missed the "correct" category for only 12 of 141 agents (8.5%), whereas linear discriminant analysis missed 20 (14.2%), and those that it got "wrong" may in fact have been misclassified by the "gold standard" method anyway. The trained networks are being used to classify prospectively the 10,000 or more agents per year tested by the screening program (531, 1018), and progress toward

detecting the site of action of novel compounds is being demonstrated (180). We may expect a better discrimination in flow cytometry experiments, because the effective readout is based not on 60 cell lines but on the potentially thousands of heterogeneous cells analyzed in one run. Because there are so few existing targets exploited by commercial antibiotics (1065), it is of great importance to be able to screen for new ones, yet in vitro (cell-free) assays are imperfect since (i) the (probably) cloned target enzyme probably does not come from the target organism(s) and (ii) a wonderful inhibitor may be a hopeless drug because of permeability problems, inactivation, or other effects that show up only in vivo. It is evident that a comparable approach with multiparametric flow cytometric data as the inputs and the mode of action as the outputs could be of great utility in the screening of antimicrobial compounds. It is also worth mentioning that the use of flow cytometry in toxicity analysis has also been outlined (209) and exploited via the ability of known mutagens and toxins to alter the DNA profile of the target cells (89, 208, 539).

Pore (761), in his thoughtful review, raises the question of how the flow cytometric susceptibility test (FCST) might be standardized against existing antibiotic susceptibility tests, since in many cases the utility of such in vitro tests for predicting clinical outcome for yeasts is very questionable, and "the suggestion that they should be viewed as the 'gold standards' with which the flow cytometric susceptibility test must be compared will be challenged, with justification." Similar comments apply to almost any instrumented method for biomass estimation (501, 503). In particular, the availability of much more extensive data from FCST means that any such comparison is likely to be questionable in principle, since the differential resistance and heterogeneity observable in flow cytometric analyses has no comparable readout in a typical antibiotic susceptibility test.

Lastly, it is worth stressing the utility of inhibitors as modulators of cellular function for physiological and metabolic studies, particularly in a bioenergetic context (see, e.g., references 377, 416, 417, 495, 496, 503, and 1022), in which the ability to inhibit metabolism at known places allows the most illuminating study of its organization at a global level. From this point of view, it is easy to predict that "flow pharmacology," the analysis of the interactions of cells and drugs by flow cytometry, is likely to become an increasingly important area of microbiology, particularly by using fluorescent derivatives of substances with known binding sites, as in the experiments of Müller et al. (661) described above and in a number of studies with fluorescent methotrexate derivatives (see, e.g., references 41, 348, 350, and 818).

Gel Microbead Method for Signal Amplification

It might be thought that the flow cytometry–fluorescence-activated cell sorter approach could be of benefit only for the analysis of cells that contain intracellularly, or are normally physically associated with, the enzymatic activity or small molecule of interest; on this basis, one could use only fluorogenic reagents which can penetrate the cell and which are thus potentially cytotoxic. To avoid clumping of heterogeneous cells, it is of course usually considered desirable in flow cytometry to analyze only individual cells, and this necessarily limits the sensitivity or concentration of target molecules that can be sensed. This restriction if not valid, however. Weaver and his colleagues at the Massachusetts Institute of Technology (352, 775, 1010–1012) and others (588, 684, 831, 1039) have developed the use of gel microdroplets containing (typically) single cells which can take up nutrients, secrete products and, indeed, grow to form colonies. The diffusional properties of the gel microdroplets may be made such that sufficient extracellular product remains associated with each individual gel microdroplet, so as to permit flow cytometric analysis and cell sorting on the basis of the concentration of secreted molecule within each gel microdroplet. (A related strategy, based again on restricting the volume containing the determinand to be analyzed, has in fact permitted the fluorimetric detection and resolution of individual fluorescent molecules [676], while the [nonflow] cytometric assessment of β-galactosidase activity in single bacterial cells was performed more than 30 years ago [822].) In the studies by Nir et al. (684), the beads were used to isolate yeast mutants growing at different rates, while the Massachusetts Institute of Technology group have analyzed antibody secretion by hybridoma cells (775), drug-resistant subpopulations and the nutrient sensitivity of hybridoma cells (1011), and chromosome hybridization (677). Tuberculosis is a reemergent infection of great concern (94, 99, 827, 923, 1056), and its analysis and control are greatly hampered by the time necessary (as a result of the slow growth of pathogenic mycobacteria) for identification and susceptibility testing. To this end, the gel microdroplet method has also been applied successfully (826) to the (relatively) rapid analysis of mycobacterial growth and its inhibition by antibiotics. While this type of technology has significance in amplifying the signals available in flow cytometric analyses, its importance is arguably greater in permitting the screening of microbial strains in strain improvement programs for biotechnology. Thus, Wittrup et al. (1039) developed a microencapsulation selection method which allowed the rapid and quantitative screening of $>10^6$ yeast cells for enhanced secretion of *Aspergillus awamori* glucoamylase. The method provided a 400-fold single-pass enrichment for high-secretion mutants. Indeed, even without the gel microbeads, it is in biotechnology that cell-sorting procedures are of especial interest and promise.

Cell Sorting and the Isolation of High-Yielding Strains for Biotechnology

Thus far, we have merely pointed out the ability of flow cytometric procedures to discriminate cells on the basis of various properties such as enzymatic activities and so on. However, as with almost any pure studies of this type (494), the analysis of heterogeneity may be carried over to applied fields, where it assumes particular importance when one is interested in the isolation of high-yielding strains for biotechnology. To this end, and as well as the increasing use of combinatorial chemical libraries (98, 244, 285, 345, 369, 453, 936), there is a large and growing interest in the screening of microbial cultures for the production of biologically active metabolites (see, e.g., references 88, 135, 197, 199, 292, 321, 540, 565, 686, 687, 707, 770, 929, 958, 978), which can provide structural templates for synthetic programmes by using rational methods of drug design. Methods based on synthetic oligonucleotides (566, 671), phage display (138, 176, 349, 579), and DNA shuffling (193, 918, 919) can provide further levels of diversity from biological starting points. Modern screens for such metabolites are targeted on the modulation of particular biochemical steps in the disease process and show a high degree of both specificity and sensitivity. This sensitivity means that metabolites showing activity during screening may be produced only in very small amounts by the organism. In such cases, increasing the titer of the metabolite is vital to provide enough material for further biological evaluation and chemical characterization and, eventually, for commercial production and consequent strain improvement programs.

Although metabolic engineering is assuming increasing importance (49, 53, 54, 144, 305, 430, 445, 464, 503–505, 512, 664, 749, 880, 881, 920), the process of titer improvement will normally involve the search for overproducing mutants derived by mutagenesis from the original producing organism (see, e.g., references 30, 45, 86, 87, 199, 444, 503, and 540). Titer-improving mutants are rare, being found typically at frequencies of 10^{-4} or less (823), and therefore many thousands of mutants need to be screened in searches for an overproducing strain. Previous methods of high-throughput mutant screening have relied on assessing the antibiotic activity of the metabolites (see, e.g., reference 60) or use rapid chromatographic methods such as thin-layer chromatography (see, e.g., reference 895) or fluorescence and luminescence methods such as the scintillation proximity assay (107, 185, 674, 952, 953). Such methods can typically accommodate 10,000 to 50,000 isolates per month. Clearly, the ability to analyze thousands of cells or beads per second by flow cytometric cell-sorting procedures opens up the possibility of increasing the rate of screening by orders of magnitude (87). A number of examples exist in which this potential has already been demonstrated to great effect.

Betz et al. (87) studied lipase production by *Rhizopus arrhizus*. They found that spore suspensions of the mold were heterogeneous as judged by light-scattering data obtained with excitation at 633 nm, and they sorted clones of the subpopulations into the wells of microtiter plates. After germination and growth, lipase production was automatically assayed (turbidimetrically) in the microtiter plates, and a representative set of the most active were reisolated, cultured, and assayed conventionally. These produced some 2,050 $U \cdot ml$ of $lipase^{-1}$ compared with 360 $U \cdot ml^{-1}$ for the parental strain, a most worthwhile increase of some sixfold. The authors also outlined possible assays for rapid sugar utilization based on changes in internal pH, as realized experimentally by Molenaar et al. (639), and their study stressed the important point that the flow cytometric assay of the activity of interest does not have to be direct but merely correlated with that which is of interest; in general, these proposals do not seem to have been followed up in any detail. Related experiments with brewing yeast have been reported by Imai and colleagues (446–448), who found a striking heterogeneity in internal pH as measured by image cytometry. In another study with yeast cells, the distribution of internal pH between individual cells (as measured by ratiometric image cytometry) was well fitted by a Gaussian distribution with a spread of ca 1 pH unit and a half-width of 0.4 pH (175).

Researchers in yeast genetics have long used the adenine biosynthetic pathway as a means of analysis of segregational and recombination events, the readout being whether colonies are white or accumulate a red pigment (813). However, Bruschi and Chuba (131) noted that the red pigment was actually fluorescent and that mutant cells could be isolated with high efficiency via fluorescence-activated cell sorting, gating on light scattering but with two-color fluorescence (excited at 488 nm) as the sorting criterion. In favorable cases, discrimination was >99%, although they noted a substantial phenotypic heterogeneity in the cell size and amount of fluorescence displayed by cells in a given colony.

Dunaliella salina is a green alga that grows in hypersaline waters and produces a variety of substances from biosolar conversion that may prove to have economic potential (72); these include β-carotene (73). Dual-parameter (light scattering and autofluorescence excited at 488 nm, measured at >600 nm) flow cytometric cell sorting was used to enrich for strains capable of producing more than twice the amount of β-carotene than the wild type.

The carotenoid pigment astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is an important component in the feeds of a variety of salmonid species grown in aquaculture (467). It is produced as a secondary metabolite by the yeast *Phaffia rhodozyma* (467, 468), which has a unique habitat in the slime fluxes of deciduous trees at high altitudes. The role of the pigment appears to be in protecting the cells from photogenerated singlet oxygen (850, 851), and the isolation of rare mutants that produce increased quantities is limited by the lack of genetic selection. In an elegant study, An et al. (30; also see reference 467) used quantitative flow cytometric cell sorting to isolate astaxanthin-hyperproducing mutants of the yeast. After *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) mutagenesis, some 6×10^{-5} of the clones produced more carotenoid than did the parent. The authors established conditions under which the carotenoid content was quantitatively related to the autofluorescence and achieved a 10,000-fold enrichment (relative to random plating) of carotenoid-overproducing strains in mutated populations. The differential fluorescence of wild-type and mutant clones was confirmed by fluorescence confocal laser microscopy, and the best strains produced some 15% more carotenoid than did the parental strain. Although not concentrating on biotechnological applications, Sensen et al. (855) recognized that microalgae fluoresce when excited with red light and stressed the utility of fluorescence-activated cell sorting for the isolation of pure strains of microalgae and their separation from contaminating bacteria. The variables used were chlorophyll autofluorescence and forward and side scatter of the laser beam. To produce clonal cultures, a single cell was sorted into each culture flask. Depending on the species, about 20 to 30% of the sorted cultures grew successfully and at least 20% of these were axenic, even when the numerical ratio between bacteria and algae in the original cultures had been as high as 300:1.

A general alternative (or complement) that may be used to improve the purity of sorted cells of interest, which may then be grown on as axenic cultures, is to simply kill the cells which are not required as they pass through the flow cytometer. This may be done with a high-power laser, which is trained on the unwanted cells as they pass. Photodynamic inactivation via sensitizers seems not to be optimal, because insufficient oxygen radicals (the ultimate agents of killing) may be generated in the short time of illumination (490); however, direct irradiation with 20 to 100 mW of a focused, frequency-doubled argon ion laser at a wavelength (257 nm) which damages cellular DNA was sufficient to induce a 4.5 log cell kill after the cells were passed through such a beam (487–489). High electric fields can induce cell membrane breakdown and cell death, and such an electroporation approach can also be used to kill unwanted cells as they pass through a flow cytometer (56). Similarly, flow cytometric and sorting procedures can be of great value in the detection of the heterogeneous, electroporation-mediated uptake of molecules into cells (93, 97, 126, 353, 779, 780, 798) and the assay of electrofusion products (57, 454, 956, 957).

Although plant protoplasts are, of course, not microorganisms, flow cytometric cell sorting procedures have been widely applied to the selection of plant protoplasts with desirable characteristics (see, e.g., references 7, 13, 82, 127–129, 227, 255, 256, 337–341, 388, 391, 392, 580, 832, 833, 964). While this historical preeminence of cell sorting in plant protoplasts is due to their very much greater size and DNA content than those of microorganisms (sometimes necessitating modifications to standard cell sorters as a result), it is more than likely that slight modifications of some of the protocols devised by these researchers might usefully be exploited for microbial selections. Thus, many alkaloids are accumulated in the plant cell vacuole, and Brown et al. (129) noted a relationship be-

tween the vacuolar pH and the accumulation of the fluorescent alkaloid serpentine (among others). The exogenous fluorescent dye 9-aminoacridine, a lipophilic base, was also accumulated in the vacuole, driven by the pH gradient between the vacuole and the cytoplasm, and a bivariate plot showed a generally striking correlation between the accumulation of serpentine and of 9-aminoacridine. Sorting on blue (serpentine) fluorescence allowed the selection of higher-yielding cultures. The chlorophyll content (from high-pressure liquid chromatography) and the (auto)fluorescence at appropriate wavelengths observed flow cytometrically were also well correlated (340). Another approach, thus far apparently unique to the plant cell world, was taken by Sakamoto et al. (833), who were interested in the single-cell flow cytometric assay of anthocyanin pigments which are not autofluorescent. To this end, they stained plant cell protoplasts (*Aralia cordata*) with a low concentration of FITC, whose fluorescence (at 525 nm) could be excited by an argon ion laser at 488 nm. FITC-stained protoplasts containing high levels of anthocyanin pigments, however, could perform resonance energy transfer from the excited state of the fluorescein molecule and exhibited a much lower 525-nm fluorescence, permitting their discrimination and sorting in a cell sorter. Sorted cells contained three times as much anthocyanin pigment as did the unsorted protoplasts, and they could be regenerated to a viable and stable cell line in one-half to one-third the time required by a previous manual method.

Tunichrome is a group of highly reducing, metal-sequestering, low-molecular-weight compounds, possessing two or three catecholate rings attached to a central enamide group, that have been isolated from blood cells of several North American ascidian species (tunicates or sea squirts) (630, 888, 889). Cell sorting of fluorescence-activated intact blood cells (using two-color red-green autofluorescence excited at 488 nm) allowed the demonstration of which of the several types of blood cells held most of the free tunichrome and which contained the vanadium, thereby providing the first unambiguous demonstration of the coexistence of tunichrome and vanadium in the same intracellular compartment (706).

Autofluorescence and light scattering are obviously the most noninvasive methods of analyzing microbial and other cells by flow cytometry. However, Azuma et al. (45) isolated a gramicidin S-hyperproducing mutant of *Bacillus brevis* by using the protein-staining fluorescent dye FITC and a fluorescence-activated cell sorter. It was established that after staining with FITC, higher-producing cells of the wild type had higher fluorescence signals than did cells with low productivity or cells from a gramicidin S-nonproducing mutant. It was found that gentle staining with FITC did not affect the viability of the cells, permitting the authors to recover viable cells after sorting. After wild-type cells were mutagenized with NTG, mutants with higher fluorescence than the parental strain were obtained by cell sorting, including a gramicidin S-hyperproducing strain, which was studied further and shown to produce 590 μg of gramicidin S per ml compared with 350 μg/ml by the wild-type strain. The authors stressed that the method has the advantage of being able to screen large numbers of cells in a short time and that the use of fluorescent dyes allows the use of cell sorting for the improvement of other cultures that produce metabolites which do not autofluoresce specifically.

Of course, dyes such as FITC are rather nonselective (in that essentially all proteins will be stained), and there are other determinands in which the relative lack of specificity of added dyes for particular structures still permits excellent discrimination. In early work, it was found that the deposition of poly-β-hydroxyalkanoates (897) (and of inclusion bodies [1038]) may be detected simply via light scattering, but more recently, the lipophilic nature of the poly-β-hydroxyalkanoate has meant that it is easily stained with very hydrophobic fluorescent dyes such as Nile red (2, 226, 660, 661).

Although the converse is not true (890), high-yielding strains must necessarily have higher enzymatic activities in the metabolic pathways leading to the flux of interest (53, 503–505, 920). Unfortunately, not all strains of interest possess relevant enzyme activities which may be assessed flow cytometrically. Sorting on the basis of the expression of particular surface antigens is straightforward (270, 635, 930, 957), however, and Francisco et al. (320) achieved a 10^5-fold enrichment of strains of *E. coli* producing a digoxin-binding single-chain antibody fragment by using only two steps. For cell surface antigens, however, magnetic labelling and magnetic cell sorting (634, 787) may have a greater throughput than conventional fluorescence-activated cell sorting (930). As well as cell surface antigens, it is always possible to use reporter gene technology to isolate strains with particularly high levels of expression of a protein of interest. Thus, Cid et al. (174) used secretable yeast exo-1,3-β-glucanases as reporter genes. The relevant open reading frames were coupled to different promoters in multicopy plasmids, and the exoglucanase activity was quantified by flow cytometry. This system was also shown to allow the recovery of viable cells.

While this does not necessarily depend upon the analysis of microbial cells, there has been a surge of interest in the use of combinatorial libraries (both biological and chemical) for the production of novel pharmaceuticals (see, e.g., references 98, 240, 244, 285, 345, 369, 453, and 936). These rely largely on the ability to synthesize thousands and even billions of related but different molecular species in the same test tube under controlled conditions, to arrange that one or more molecules of a given type are attached to an insoluble substrate such as a bead or a pin within the suspension, and to label each bead or pin in a way which enables its extraction and the identification of the molecules that it had bound. If the molecules had been shown to have had high biological activity in an assay of interest, one can then identify them and synthesize large quantities of them (and related compounds) specifically. While it is possible to deconvolute such libraries "mathematically," by screening a variety of overlapping subsets (299, 325, 453, 1071), direct physical screening and sorting are very important in this field.

The progenitor of this type of approach is arguably the phage display method (176, 349, 579, 709, 887), in which molecules are displayed in a monovalent fashion from filamentous phage particles as fusions to a gene product of M13 packaged within each particle. In the early work, phage particles were sorted by binding to beads containing solely the target of interest, but it is evident that flow cytometric procedures may also have a role to play here.

Thus, Needels et al. (671) prepared a library of some 10^6 different peptide sequences on 10-μm beads by combinatorial chemical coupling of both D- and L-amino acid building blocks. To each bead was covalently attached many copies of a single peptide sequence, together with copies of a unique single-stranded oligonucleotide that codes for that peptide sequence. The oligonucleotide tags were synthesized through a parallel combinatorial procedure that effectively recorded the process by which the encoded peptide sequence was assembled. The collection of beads was screened for binding to a fluorescently labelled antipeptide antibody by fluorescence-activated cell sorting, and the oligonucleotides identifiers attached to individual sorted beads were amplified by PCR. Sequences of the amplified DNAs were determined to reveal the identity of peptide sequences that bound to the antibody with high affinity. Fluorescence-activated cell sorting was found to permit the

TABLE 4. Some desirable properties of any method of cellular analysis

Specific or highly selective (signals due only to determinand of interest)
Precise
Accurate
Reproducible (does not drift)
Rapid
Sensitive
Probes biologically inert
Nondestructive assay
Robust equipment
Easy to set up and calibrate
Low cost
Capable of axenic operation
Signals linear with determinand concentration
User-intelligible output

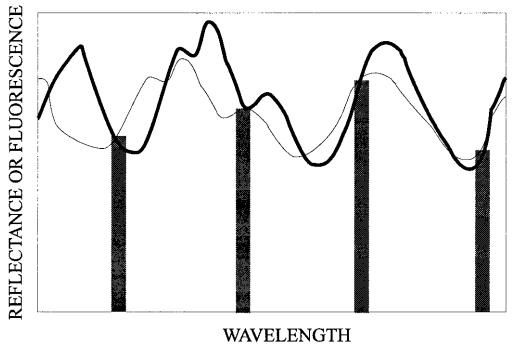

FIG. 15. Multispectral versus hyperspectral analysis. When spectra are collected solely as a small number of bands, two spectra (and thus the cells from which they originate) which are very different can appear the same; only by collecting many wavelengths can the true discrimination be effected.

facile isolation of individual beads that bear high-affinity ligands for biological receptors.

Vetter et al. (984) produced a variety of glycosylamides by reacting readily available glycosylamines with homobifunctional *N*-hydroxysuccinimidyl esters. It was arranged that the products carried a spacer group equipped with one active ester functionality. This route provided well-defined glycoconjugates, which were cross-linked to various amino-functionalized resins. Recognition of the resulting sugar-bead conjugates was successfully probed by flow cytometry with a fluorescently labelled lectin.

Muller et al. (657) described a method for the identification of high-affinity ligands to SH2 domains by fluorescence-activated bead sorting in which recombinant SH2 domains, expressed as glutathione *S*-transferase fusion proteins, were incubated with a phosphotyrosine-containing peptide library. A total of 640,000 individual nonapeptides were each displayed on beads, and phosphopeptide interaction of a given SH2 domain was monitored by binding FITC-labelled antibodies directed against glutathione *S*-transferase. High-fluorescence beads were successfully isolated by flow cytometric sorting, and subsequent pool sequencing of the selected beads revealed a distinct pattern of phosphotyrosine-containing motifs for each individual SH2 domain.

SOME PROSPECTS

As we have seen, the typical steps in flow cytometry involve interrogating a cell optically, generating one or more electronic signals reflecting the magnitude of various cell properties, typically using light scattering or via added fluorescent probes, and extracting biologically relevant information by the appropriate mathematical or other multivariate methods. Any discussion of prospects may assume that advances in each of these areas will contribute to continuing improvements in flow cytometric methods. To this end, a list of desiderata that any method of cellular analysis should possess is given in Table 4 (500).

Since flow cytometry is predominantly an optical technique and statistical arguments mean that more photons mean more signal per unit noise, major improvements will come from better optics, better (possibly pulsed [914]) and more stable light sources and flow hydrodynamics, and lower backgrounds. In particular, we may look forward to more efficient detectors such as charge-coupled device arrays, as are widely exploited in modern dispersive Raman instrumentation (see, e.g., references 32, 61, 156, 514, 615, 675, 1028, and 1031), which will allow the collection of whole spectra (981).

As currently practised, flow fluorimetric measurements tend to use only one signal channel per fluorophore, collecting what can be a substantial range of wavelengths simultaneously so as to maximize the number of photons detected in the channel of interest. Of course, the potentially available wavelength discrimination between possibly different dyes (or even the same dye in different environments [39]) is then lost, and the use of fewer relevant (632, 854) variables is necessarily accompanied by a loss in the ability to discriminate closely related cells (366). In other words, it would be highly desirable, were it possible, to increase the number of channels of scattering or fluorescence data obtained by decreasing, respectively, the scattering angle collected or the band of emission wavelengths collected by each of an array of detectors. In particular, the greatly increased number of fluorescence channels then available would allow the use of multiple, overlapping fluorophores whose contribution to each channel and hence overall staining could readily be discriminated by multivariate techniques.

This type of approach, in which many channels (often hundreds), typically of reflectance spectra, are collected simultaneously has been developed in particular by the remote-sensing community, where, when coupled increasingly to advanced data reduction and visualization algorithms and combined with spatial discrimination, it is known as imaging spectrometry (207, 360, 361) or hyperspectral imaging (1, 91, 162, 310, 335, 361, 549, 812, 1034). The discrimination afforded by the hyperspectral approach over the more traditional multispectral approach in which only a few wavelength channels are collected is predictably (Fig. 15) extremely impressive (541). It is obvious that such a generalized approach and such technology could readily be adopted for microbial analysis in flow cytometry, and it has indeed been championed at the level of the colony by Youvan and colleagues (36, 37, 364, 1050, 1057–1059). While there is clearly a trade-off between the size of the signal (number of photons) and the wavelength resolution of the detector, similarly substantial advances are currently taking place in the area of wavelength-resolved fluorescence detection for capillary electrophoresis (940, 941), in which the limit of detection for fluorophores is presently equivalent to some 50 molecules, a number not grossly dissimilar to those currently detectable by the better flow cytometers (and significantly more than the known limit [678]). Under these circumstances, as the signal per channel becomes smaller, the use of postprocessing methods (in hardware or software) to remove detector and other noise will assume greater importance, a

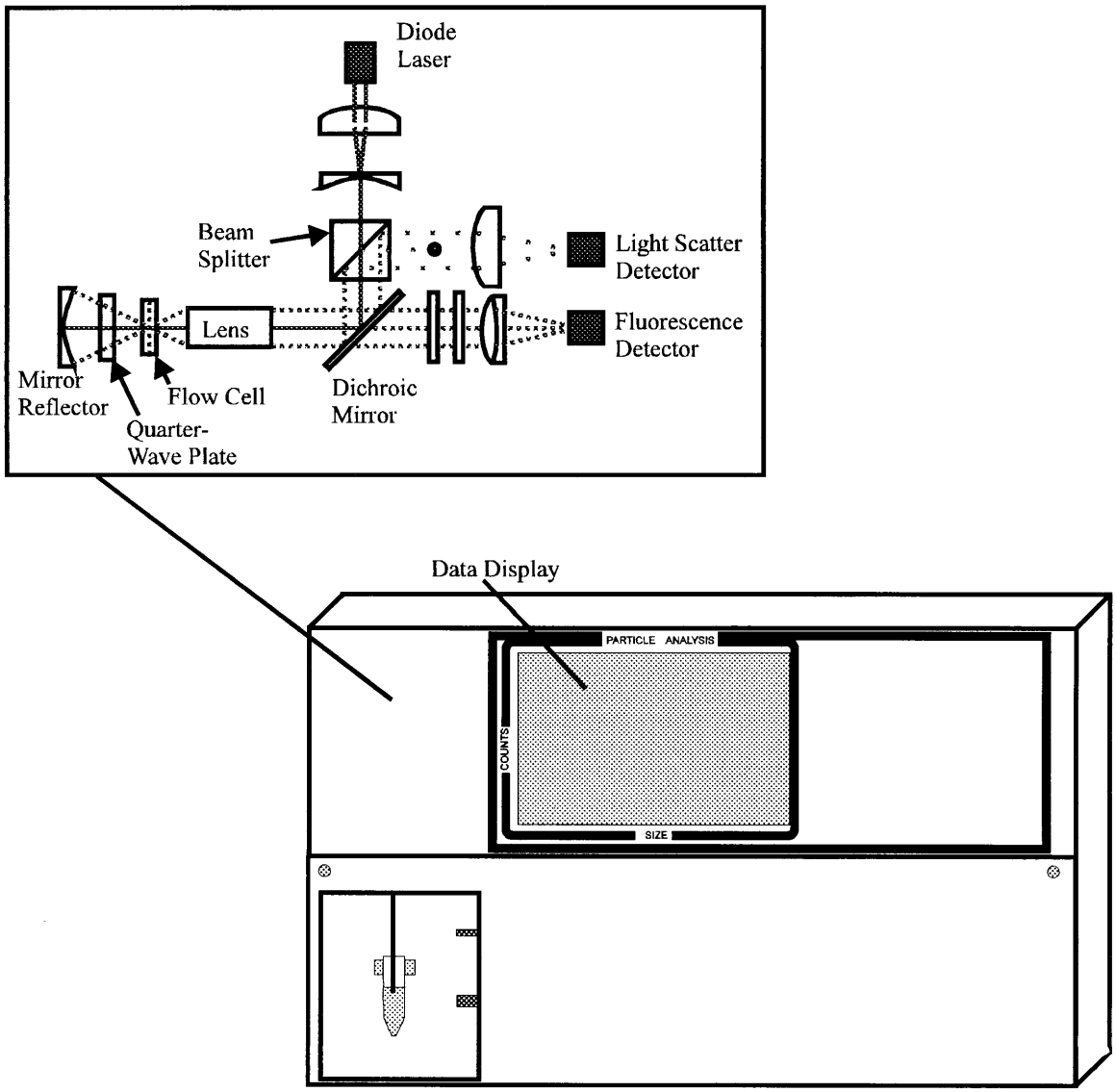

FIG. 16. Schematic representation of the Microcyte flow cytometer. The optical components (laser diode, flow cell, and detectors) are fixed within an aluminium block to avoid the necessity for daily alignment of the flow cytometer, while the use of a stabilized light source and solid-state photodetectors means that no sensitivity adjustments need be made. The instrument has a built-in display and can be powered from its own internal batteries. The option of recirculating the sheath fluid (via a filter) makes the instrument fully portable, opening up the possibilities of flow cytometric analysis of samples that are not easily transported to the laboratory.

procedure usually referred to as denoising (78, 182, 265, 266, 669, 986).

The development of low-cost light sources such as the laser diode (see, e.g., references 69, 152, 177, 267, 268, 500, and 839) and detectors such as the avalanche photodiode (see, e.g., references 486 and 925) may be expected to make the technique of flow cytometry much cheaper and thus more accessible. Such solid-state systems may be expected to be much smaller and more robust and thus portable for field analysis.

Indeed, a portable (10-kg) laser diode-based flow cytometer (the Microcyte) has recently been developed by Gjelsnes and Tangen (356). The instrument has been designed primarily for the analysis of microorganisms and can detect and provide accurate absolute counts (i.e., without an internal standard) of particles as small as 0.4 μm or lower. The instrument employs a 635-nm laser diode as the light source and has two solid-state photodiode detectors, one for light scatter and one for fluorescence (Fig. 16). Although flow cytometrists are more familiar with fluorescent stains that can be excited at 488 nm (e.g., FITC, propidium iodide, and Nile red), excitation at higher wavelengths reduces the problem of background autofluorescence for most microbes. Reduced autofluorescence, together with the smaller size and weight of diode light sources (and their power supplies) compared with even air-cooled lasers, may be expected to encourage the development and improvement of fluorescent stains for these longer wavelengths.

The problem of rapid and accurate enumeration of microorganisms has been described in detail above. In the Microcyte, a pressure regulator ensures that the sample is analyzed at a constant rate, so that accurate counting can be performed. Some recent data illustrating the use of the Microcyte for monitoring cell numbers in laboratory cultures are shown in Fig. 17. In Fig. 17A, the Microcyte counts of yeast cells are compared with direct counting by hemocytometry. Hemocy-

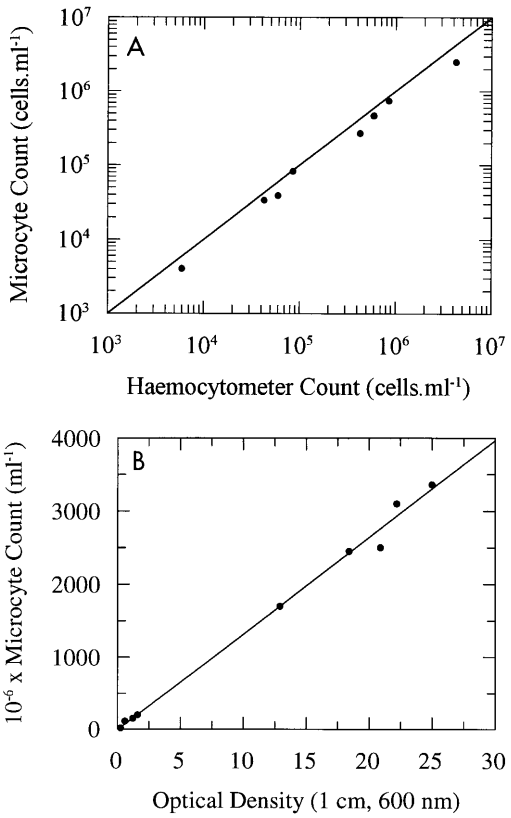

FIG. 17. Comparison of the Microcyte with other methods of biomass determination. (A) Comparison of hemocytometer and Microcyte counts for yeast cells. Only the two most concentrated samples could be enumerated by hemocytometry, the lower dilutions being calculated from their dilution factors. Hemocytometry took approximately 15 min per replicate, while three counts were done on the Microcyte in less than 30 s total. The best-fit line by linear regression has a slope of 0.9873, an intercept of −0.0704, and a correlation coefficient of 0.997. (B) *Alcaligenes eutrophus* was grown in a fermentor, and samples were taken periodically during the growth phase. The samples were diluted as appropriate for optical density measurements and for enumeration by the Microcyte. A K-HEPES buffer that had been filtered to 0.2 μm pore size was used to dilute the samples and also to provide a background reading for optical density measurements. The correlation coefficient of the line of best fit is 0.997.

tometer counts are laborious to perform, lead to operator fatigue, and are prone to error; for a count of n organisms, the percent error may be represented as $\pm \frac{100}{\sqrt{n}}$ %, i.e. ±1% for a count of 10,000 cells. Rapid sample analysis by flow cytometry permits analysis of more than 10,000 cells in a few minutes, even at a fairly conservative data rate. In most situations, it is unlikely that more than a few hundred cells would be counted by hemocytometry (5 to 10% error). A further problem with hemocytometer counts is that a minimum concentration of about 10^6 cells · ml^{-1} is required to give 100 cells in the counting chamber (the number will vary according to the design of the counting chamber used). The Microcyte, by contrast, can enumerate cells at much lower concentrations (Fig. 17A). Another common method of enumeration in the microbiological laboratory is the measurement of optical density (Fig. 17B). However, as with hemocytometer measurements, the limit of detection for conventional turbidimetry has been quoted as 2.4×10^6 cells · ml^{-1} for *E. coli* (593), with lower concentrations having an optical density not appreciably different from that of the background medium. Even with the more sensitive nephelometry measurements, the lower limit of detection is 1.6×10^5 cells · ml^{-1}, and while these levels of microbial concentration are common in laboratory studies, under environmental conditions cell concentrations may be much lower. The lower limit of detection for the Microcyte is $<10^3$ cells · ml^{-1}.

The potential of the Microcyte flow cytometer for routine analyses in the food industry has been demonstrated by the SINTEF Industrial Chemistry Group (Norway) (79). In quality control applications, it is often necessary to determine not only the microbial load but also the viable microbial load. While total counts based on the light-scattering data may be appropriate for laboratory cultures in defined media, the presence of background particulates present in foods often means that a fluorescent label must be added prior to analysis. The work of the SINTEF group showed that total counts and viable counts of bacteria and yeasts could be performed readily against a complex background of particles such as those found in pasteurized whole milk. Carboxynaphthofluorescein diacetate (which works in a similar way to the more familiar fluorescein diacetate) was shown to be a suitable probe for 635-nm excitation in the Microcyte, in which the nonfluorescent precursor is cleaved to fluorescent carboxynaphthofluorescein by esterases and retained by viable cells. A total cell count was obtained by using the nucleic acid stain TO-PRO-3 after permeabilization of the cells.

Coupled to the wavelengths at which present laser diodes tend to emit, and (because of the lowered autofluorescence) paralleling the developments in Raman spectroscopy (see above), it is easy to predict a continuing move toward the red and near-infrared and the continuing development and exploitation of red- and near-infrared-excited fluorophores, although Shapiro (865) rightly points out that this may also be a rocky road. In addition, solid-state lasers emitting in the blue are beginning to become commercially available for use in compact disc players (382) and will undoubtedly be exploited in flow cytometry.

Improvements in the design of the flow chamber of modern flow cytometers have already led to a reduction in the level of background noise, and this may be expected to improve still further. The range of available fluorescent stains continues to expand (398, 400) and will inevitably lead to more parameters being measured simultaneously; therefore, the requirement for suitable data processing techniques will also increase. While many useful studies may be carried out with fixed cells, noninvasive (and preferably online) methods are always to be preferred (493, 852); the ideal stain, which is membrane permeant, is specific for its target, has a high quantum yield and Stokes shift, and is nontoxic, is rarely encountered, and we are far from an age of rational drug design in which we might create one. This, however, remains the goal.

The tremendous interest in oligonucleotide probe technology (26) will surely lead to the continuing development and exploitation of cocktails of such stains for the analysis of complex microbial samples, and it seems that there remains much room for improvement in the generation, characterization, and specification of fluorescent antibodies against specific microbial cells. Interlaboratory studies will assist the development of fluorescence standards with known numbers of fluorophores, suitable for the absolute calibration of microbial flow cytometers.

An important means of acquiring specificity in a fluorogenic assay is to use reporter gene fusions, whose expression is governed by an appropriate promoter, and a number of examples of their flow cytometric exploitation have been described above. Aequorin is a calcium-sensitive luminescent protein from the coelenterate *Aequorea victoria* (*A. forskalea*), which

has been widely exploited by Campbell and colleagues in their study of the role of Ca^{2+} signalling in biology (146), most recently in *E. coli* chemotaxis (1003). The so-called green fluorescent protein is the Forster-type energy transfer protein in *A. victoria*, the actual emitter in vivo, and shifts the emission from 460 to 508 nm. It has recently been cloned and exploited as a fluorescent reporter gene (see, e.g., references 70, 155, 193, 241, 546, 564, 776, and 806), and mutant derivatives which fluoresce at different wavelengths have been developed (192, 200, 229, 406); given its existing exploitation in the analysis of cell-specific gene expression and subcellular protein localization during bacterial sporulation (1013), one may anticipate its future widespread exploitation in flow cytometric procedures (231, 245, 342, 534, 815, 985). Of course, oligonucleotides selected for binding to any ligand (typically proteinaceous) (184, 293) may also be made fluorescent and used as probes for the expression of that ligand (222).

Lastly, improved electronic hardware will continue to increase the speed, accuracy, and precision of flow cytometric analysis and sorting, with a move from analog to digital instrumentation (865, 1072), while continuing developments in the methods of artificial intelligence and the continuing fall in computer price/performance ratios will ensure that the multivariate nature of the flow cytometric approach is exploited to the full. It is thus easy to anticipate a continuing rise in both the number and percentage of flow cytometric studies which use microorganisms as their subject, a process which we trust that we may here have served to assist.

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