# Single-Cell Microbiology: Tools, Technologies, and Applications

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## INTRODUCTION

The field of microbiology has traditionally been concerned with and focused on studies at the population level. Information on how cells respond to their environment, interact with each other, or undergo complex processes such as cellular differentiation or gene expression has been obtained mostly by inference from population-level data. New appreciation for the existence and importance of cellular heterogeneity, coupled with recent advances in technology, has driven the develop-

ment of new tools and techniques for the study of individual microbial cells. As a result, scientists have been able to characterize microorganisms and their activities at unprecedented levels of detail.

Single-cell techniques have been used to more fully describe the environmental distribution and activities of microorganisms, have been a key element in revealing otherwise invisible processes such as interspecies gene transfer and chemical communication, and have been used to detail discrete physico-chemical interactions between microbes and the surfaces they colonize (11, 31, 60, 150, 231). Single-cell methods have also been essential to our understanding of connections between cellular biochemistry and behavior and of the cellular bases of population-level phenomena (58, 126, 248). As a result, new insights into the properties of chemical signaling pathways and mechanisms behind the coordination of multicellular behav-

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iors have been possible. Single-cell methods have also enabled direct micro- or nanoscale measurements of the mechanical properties of individual cells, including turgor pressure, elasticity, and bursting force (218, 257). Microphysiological studies of metabolite, protein, or elemental localization, intracellular water dynamics, host-pathogen interactions, and surface-associated redox activity represent other areas in which single-cell techniques have been applied (12, 13, 45, 178, 183, 187).

Apart from enabling fresh perspectives on issues of concern to basic science (58, 183), the tools and technologies of single-cell microbiology have been brought to bear on problems of direct interest to researchers in applied science. Individual microorganisms, even those in "clonal" populations, may differ widely from each other in terms of their genetic composition, physiology, biochemistry, or behavior (40, 66, 75, 127, 208). This variability or heterogeneity has important practical consequences for a number of human interests, including antibiotic and biocide resistance (21, 228, 237), the productivity and stability of industrial fermentations (184, 205, 229), the efficacy of food preservatives, (17, 223, 229), and the potential of pathogens to cause disease (67). Additionally, methods for identification, characterization, and/or physical separation of individual microorganisms are needed for the detection of pathogens and for the identification and selection of strains with beneficial or improved properties (124, 224).

Because studies made at the single-cell level are not subject to the averaging effects characteristic of bulk-phase population-scale methods, they offer a level of discrete microbial observation that is unavailable with traditional microbiological methods. Single-cell techniques have been key in probing microbial viability phenomena that are beyond the resolution of culture-based approaches (22, 125, 194), in elucidating mechanisms of pathogenesis (12, 136, 227), and in measuring the motility and the invasive forces of individual cells or hyphae (26, 162, 200).

This paper reviews some of the tools and technologies available for the study of microbes at the level of the single cell. Special interest is given to methods capable of monitoring discrete and dynamic processes occurring within living microbial cells. The limitations of traditional, population-based microbiological techniques as the motivation for the development of these single cell approaches are discussed throughout. Several of the tools and technologies discussed here have themselves been the subjects of more specialized reviews, to which the reader is referred for more detailed information. Although single-cell microbial phenomena have received attention in the past, recent advances in technology have enabled unprecedented access to processes occurring at this scale. Because our primary focus is on these recent technological advances, a historical perspective is beyond the scope of this review.

## MICROBIAL HETEROGENEITY

Variability is a hallmark of biological systems. Microbial cells have a remarkable capacity for displaying a multitude of genetic and nongenetic differences from each other. This inherent genetic and phenotypic plasticity forms the basis of a successful "lifestyle strategy" that enables them to adapt to and survive adverse conditions or to persist and cause disease (36, 97, 127). The central theme driving the need for methods

capable of resolving the properties and activities of individual microbial cells is that of microbial heterogeneity (66, 208). Bulk-scale measurements made on a heterogeneous population of cells report only average values for the population and are not capable of determining the contributions of individual cells. However, properties such as viability, protein concentration, possession of a mutant allele, or the number of flagella expressed on the cell surface are discrete and intrinsic states or properties of each individual cell. Methods capable of analyzing these properties at the level of the individual cell enable a more complete understanding of phenomena that are inaccessible to researchers using population-scale approaches.

The types of individual differences contributing to heterogeneity within a microbial population can be divided into at least four general classes: genetic differences, biochemical differences, physiological differences, and behavioral differences. The lines dividing different modes of heterogeneity are often nebulous and interactive. For example, biochemical or behavioral differences might ultimately be traced back to a genetic basis. Even physiological heterogeneity, which may be driven by forces external to the cell (e.g., nutrient limitation or the presence of antibiotics), could be viewed in terms of the organism's genetic potential to respond to these forces. However, the choice of tools used to explore cellular differences often makes it operationally clear which source of heterogeneity is the subject of investigation. For example, genetic heterogeneity is addressed using methods such as single cell PCR or fluorescence in situ hybridization (FISH), biochemical heterogeneity is measured using enzyme assays or single-cell electrophoretic separations, and behavioral heterogeneity is measured through direct observation of cellular responses to various stimuli. Examples of how individual microbial cells may vary according to their genetic, biochemical, physiological, or behavioral properties are described briefly below.

### Genetic Heterogeneity

Microbial genomes can be remarkably plastic, being capable of substantial change within very short periods of time (177). Genetic heterogeneity in individual microorganisms can arise from a number of random, semirandom, or programmed events. Modes and mechanisms of genetic variability include spontaneous point mutations (40, 66); random transcription events (75, 127); phage-related phenomena (e.g., transduction and lysogeny); chromosomal duplications and gene amplification (103, 127); the presence, absence, and copy number of mobile genetic elements such as plasmids and transposons (40); flagellar or capsular phase variation (97, 127), and even intracellular genetic heterogeneity, such as that arising from transcription of multiple rRNA operons within a single cell (5, 127).

Asymmetries in the distribution of genetic material between daughter cells may be important in driving processes of differentiation, as has been suggested for the strand-specific imprinting of mating-type switching in *Schizosaccharomyces pombe* (62). Processes related to cell aging, including the accumulation of DNA damage or variability in gene expression and loss of gene silencing, may also be used to describe genetic variability between individual microbial cells (92, 184). Other sources of cellular heterogeneity are discussed briefly below.

These can be described as nongenetic or phenotypic in nature (229).

## Biochemical or Metabolic Heterogeneity

Biochemical or metabolic heterogeneity in a population is characterized by individual cellular differences in macromolecular composition or activity and may stem from cell cycle-related physiological processes such as turnover or from events related to aging (66, 184). As the phenotypic expression of genetic phenomena, biochemical heterogeneity could also stem from mutations, programmed events associated with differentiation, or random transcription events and "noise" (75, 127). As with nucleic acids, proteins may also be distributed asymmetrically between mother and daughter cells. Preferential retention of oxidatively damaged proteins within the mother cell has recently been described for *Saccharomyces cerevisiae*, suggesting a mechanism for enhancing the fitness of newborn cells (1). Quantities of certain macromolecular components such as carotenoids (10), intracellular carbohydrate, or lipid storage polymers may also vary among individual cells, contributing to their biochemical heterogeneity (44, 184, 204).

## Physiological Heterogeneity

Physiological heterogeneity stems primarily from progression through the cell cycle and describes morphological differences between individual cells, including differences in size, shape, and surface or internal characteristics (66, 92, 184, 229). Examples of physiological heterogeneity in yeast include size differences between mother and daughter cells, bud scarring, surface wrinkling, and variation in vacuole size (184). Sources of physiological variation in bacteria include differences in cell volume, cell shape, buoyant density, and nucleoid morphology (152). More pronounced examples of cell cycle-related physiological heterogeneity occur in organisms undergoing processes of differentiation, such as sporulation or the formation of fruiting bodies (237, 248). Physiological (and biochemical) heterogeneity may also be driven by microenvironmental factors acting on cells located in different strata within a colony or biofilm (42, 55).

## Behavioral Heterogeneity

Behavioral heterogeneity is the observable consequence of cell-to-cell variation in biochemical or physiological characteristics, such as the presence, number, state, or activity of components of chemotactic and other signaling pathways (142). Such variation may stem from genetic mutation or from stochastic processes affecting either gene expression or the subcellular distribution of key pathway components (142). Observation of individual cellular responses to chemotactic or phototactic stimuli, measurement of swimming speed or direction, and analysis of flagellar motor bias represent potential means through which behavioral heterogeneity can be explored (14, 58, 156, 175, 212).

## ADVANTAGES OF SINGLE-CELL APPROACHES

Plate counting and light microscopy represent the original set of tools available for single-cell analyses (168). As such, they have been remarkably useful for more than 100 years, and for many applications, they remain both adequate and appropriate (40, 168). However, the past few decades have been marked by the introduction of a number of technological and methodological innovations, including advances in computing or imaging technologies and the development of culture-independent methods such as in situ hybridization and PCR. Progress in these areas has dramatically advanced our abilities to resolve the features and activities of individual microbial cells. Examples of some of the types of information that have been made more accessible through the use of single-cell approaches are introduced below.

### Revealing Cryptic Processes

Microorganisms carry out a number of processes that may have substantial impact on human life. Without the proper set of tools, however, the details of these processes are inaccessible, or cryptic. Examples include gene transfer or distribution in the environment and biochemical interactions between microbial cells or between pathogens and their hosts (11, 60, 163, 235). The nature and operation of biochemical networks occurring within individual cells and issues surrounding the gray area between cell death and viability represent other areas in which single-cell approaches have furthered our understanding of otherwise unseen microbial phenomena (22, 58, 105, 125).

### Observing Discrete and Dynamic Events within Living Cells

Until recently, the bacterial cell was commonly thought of as an "...amorphous vessel housing a homogeneous solution of proteins..." (148). The structure of the bacterial cell, and of other microbial cells, is now recognized as being much more complex than previously imagined. Discrete subcellular domains have been observed in microbial cells in which distinct biochemical or genetic processes occur or are regulated (148, 170, 206). Additionally, certain proteins involved in control of the bacterial life cycle change their "subcellular address" over relatively short time intervals, and the activities of other proteins may be regulated according to their location within the cell (148). Other phenomena, such as actin polymerization in *Listeria monocytogenes* or protease secretion in *Vibrio cholerae*, occur only at the cell poles (151, 206). The use of single-cell techniques allows the observation of such discrete and dynamic events occurring on or within living microbial cells with high spatial and/or temporal resolution (74, 78, 187, 206, 239).

### Relating Microscopic, Mesoscopic, and Macroscopic Observations

Coordinated multicellular activities such as aggregation, development of specialized structures, and colony pattern formation are visible, population-scale manifestations of individual cellular behaviors or properties (30, 158, 248). Examples of such organized phenomena include fruiting-body development in myxobacteria, mound and slug formation in *Dictyostelium*

TABLE 1. Selected applications of single-cell microbiological methods

| Application | Methods used | References |
|---|---|---|
| Effects of antimicrobials and other stressors on individual cells | Electrorotation, flow cytometry, fluorescence ratio imaging microscopy, fluorescence video microscopy, GFP, immunofluorescence | 17, 19, 24, 42, 70, 94, 106, 108, 194, 215, 228, 229, 242, 247 |
| Metabolic or enzymatic properties of single cells | AFM, confocal LSC, flow cytometry, fluorescence microscopy, fluorescent and nonfluorescent enzyme substrates, image analysis, microcapillary electrophoresis | 25, 27, 39, 54, 68, 139, 167, 179, 230, 246, 249 |
| Growth and activity of single fungal hyphae | Fluorescence microscopy, image analysis, video-enhanced light microscopy | 23, 26, 57, 79, 87, 90 |
| Microspectroscopic analysis of individual cells or hyphae | Image analysis, fluorescence microspectroscopy, Raman microspectroscopy, microbeam analysis, NMR microscopy, scanning confocal laser microscopy | 10, 13, 44, 88, 95, 128, 135, 178, 204, 205, 252, 262 |
| Photosynthetic properties of individual cells or protoplasts | High-repetition-rate fluorometry, fluorescence microscopy, microamperometric measurements | 89, 181, 219, 258 |
| Nutritional state or elemental content of individual cells | GFP, immunofluorescence, microbeam analysis, Raman microscopy | 70, 88, 95, 120, 178, 242 |
| Measurement of intracellular pH | Fluorescence ratio imaging microscopy, GFP | 17, 38, 42, 94, 113, 172, 214, 215 |
| Host-pathogen interactions | GFP, image analysis, immunofluorescence, laser-tracking microrheology, optical tweezers | 12, 26, 122, 136, 144, 159, 227, 265 |
| Predator-prey interactions | Flow cytometry, FISH, GFP, immunofluorescence | 20, 37, 72, 96, 101, 141 |
| Separation, sorting, or manipulation of individual cells | Capillary electrophoresis, electrorotation, dielectrophoresis, flow cytometry, optical trapping, optical tweezers | 4, 9, 15, 43, 77, 81, 83, 159, 165, 170, 211 |
| Strain or process improvement | Flow cytometry, fluorescence ratio imaging microscopy, image analysis | 9, 44, 57, 124, 215 |
| Power and torque in individual cells or cellular motors | Electrorotation, force-calibrated glass microprobes, "optical funnel," optical trapping | 32, 53, 121, 162, 200 |
| Nanomechanical properties of individual cells | AFM, glass or optical fiber microprobes | 18, 154, 213, 218, 232, 233, 257 |
| Discrete cell surface properties, cell-cell interactions, cell-substrate interactions | AFM, biological force microscopy, microsphere adhesion assay, optical tweezers, SECM | 26, 45, 85, 149, 150, 216, 220, 259, 266 |
| Analysis of gene expression or transfer, detection of genetic damage, other molecular analyses of single cells | Flow cytometry, FISH, GFP, in situ PCR or reverse transcription-PCR, scanning confocal laser microscopy, single-cell DNA preparation, single-cell rDNA sequencing | 19, 51, 52, 60, 90, 144, 153, 161, 163, 186, 196, 197, 221, 234, 235 |

*discoideum*, chiral colony morphology in *Bacillus subtilis*, and coordinated movement (e.g., traveling waves, whirls, and jets) within populations of myxobacteria or *B. subtilis* (158, 248). Methods capable of single-cell resolution enable connections to be made between these mesoscopic or macroscopic phenomena and their microscopic, cellular origins (116, 126, 248).

### A Caveat: the "Uncertainty Principle"

Many of the methods reviewed here enable the observation of living cells under physiological or minimally invasive conditions. However, these observations may still involve the exposure of cells to potentially toxic fluorescent dyes (77, 207), intense light, electric or magnetic energies (50, 169, 201), or physical manipulation using mechanical, optical, or electrokinetic forces (47, 77, 111). Alternatively, cells carrying genes for reporters such as β-galactosidase or green fluorescent protein (GFP) may experience an increased metabolic load associated with the expression of these genes (234). As a result, the very process of observing a cell may affect the outcome of the observation. This, in effect, is the biological equivalent of Heisenberg's "uncertainty principle" (173). Bridson and Gould (40) have coined the term "quantal microbiology" to describe the inherent uncertainties of microbiological phenomena at the single-cell level. An individual cell (the quantal unit here) either is exposed to a measurement or is not. Because an

experiment and its control cannot be carried out on the same cell, assurances that an observation does not affect experimental results may be impossible. The inability to separate a measurement from its potential influence on an individual cell will probably be a recurrent theme in single-cell microbiology.

### TOOLS AND TECHNOLOGIES

A broad overview of the tools and technologies available for resolving the properties and activities of single microbial cells is provided below. Table 1 highlights the range of studies in which these tools and technologies have been applied. Because fluorescence is of fundamental importance to many of the approaches used to investigate single-cell microbial phenomena, additional background has been included on this concept.

### Fluorescence

Fluorescence is an extremely useful physicochemical property of certain molecules and compounds and, as a basic tool, has many applications in the study of single microbial cells. Fluorescence staining methods are generally rapid, are more sensitive than colorimetric techniques, and facilitate the staining of microbial cells within complex mixtures according to their individual biochemical, physiological, or taxonomic properties (22, 119). Multiple fluorescent stains may be used simul-

taneously, allowing the collection of more than one parameter per cell, and many fluorescent stains are compatible with living cells (66).

The fundamental principles of fluorescence have been reviewed extensively elsewhere (66, 123, 208), as have many of the staining techniques applicable to microbial cells (66, 102, 119, 208, 244). An excellent historical account of developments in fluorescent-dye technology is also given by Kasten (123).

Briefly, fluorescence occurs after photons from an incident light source raise electrons in a fluorophore (in many cases an organic molecule with multiple, conjugated double bonds) to a higher-energy or "excited" state. Return of the molecule to a lower-energy state is accompanied by the emission of light as fluorescence after some energy loss (66, 123, 208). Fluorescence is emitted at a lower energy (e.g., longer wavelength) than that of the original excitation light, and the difference in excitation and emission wavelengths is termed the "Stokes shift" (66, 123, 208). The magnitude of the Stokes shift can be critical in ensuring spectral separation of signals from more than one fluorescent stain or when dealing with cells or sample matrices having highly autofluorescent backgrounds. Variables of practical importance to fluorescence include the intrinsic properties of the fluorophore: its excitation and emission spectra, molar absorbance coefficient, quantum yield, quantum efficiency, and photostability (66, 102, 123, 208). The local chemical or electronic environment also plays a role, and factors such as pH, the physical proximity of other molecules in solution, and the presence of localized charge concentrations (e.g., the negatively charged backbone of DNA) can all affect the resulting fluorescence (123, 208, 217).

**Fluorescent dyes and stains.** Fluorescent dyes with affinities for all of the major macromolecules occurring within microbial cells are commercially available (102). These include stains that react with nucleic acids, proteins, or lipids or that stain polyester or polyphosphate inclusion bodies. Additionally, fluorescent enzyme or respiratory substrates, reporters of intracellular pH or ion concentration, and dye kits providing "fluorescent Gram staining" are available (66, 102, 244). The performance of these commercial kits is often validated using specific microorganisms grown under standardized conditions. However, if these assays are to be used with different microorganisms or natural populations, they must be revalidated under the new conditions, since basic physiological differences or increased biochemical heterogeneity within these populations may complicate data analysis (209). The difficulties in transferring multiparameter staining protocols across generic or species boundaries may be even more pronounced (210).

Macromolecules such as lectins, antibodies, and nucleic acid probes may be labeled with fluorescent dyes to create conjugates capable of reporting molecular recognition events. Other fluorescence-based molecular methods, such as in situ PCR or in situ reverse transcription, may result in the incorporation of fluorescently labeled deoxynucleoside triphosphates into reaction products as they are formed within the cell (104). Endogenous sources of fluorescence, including carotenoids, tryptophan, thiamine (after chemical derivitization), and the cell's own photopigments, may also serve as reporter molecules, for instance in industrial or environmental applications (9, 10, 117, 124).

Dynamic microbial phenomena, including protein expression and behavior (187), substrate uptake (167), binding and release of individual chemoattractant molecules to cell surface receptors (239), selective degradation of uniparental DNA within newly formed algal zygotes (170), bacterivory (96), and drug efflux (21, 118), may also be observed or measured at the single-cell level through the use of fluorescence staining techniques. Specialized techniques such as fluorescence ratio imaging microscopy may provide insights into dynamic cellular events that are important to the outcome of microbial fermentations (214), which highlight the physiological responses of spoilage organisms to chemical stresses (17), or that are related to cellular inactivation resulting from treatment with antimicrobials (42) (Fig. 1).

Staining with multiple fluorescent labels can yield detailed information on the identity and activities of individual microbial cells. For example, the combined use of FISH and the fluorescent respiratory substrate CTC can yield data on both genetic identity and respiratory activity (Fig. 2). The ability to correlate single target cells with their metabolic activities could provide greater information on which to base important decisions, such as those regarding food safety or productivity in industrial fermentations.

However, multiplex fluorescence assays may be limited by the need to balance dye properties and instrument capabilities. Incompatible spectral or chemical properties and requirements for multiple excitation sources can place practical constraints on the fluorescent dye combinations that can be used. Recently, though, a new class of compounds with promise as fluorescent labels has been introduced (41). Fluorescent semiconductor nanocrystals, or "quantum dots," have several advantages over conventional fluorescent labels, including large extinction coefficients and reduced susceptibility to photobleaching. However, the most intriguing properties of these labels are their narrow, size-dependent (and therefore "tunable") emission spectra and the fact that differently emitting nanocrystal labels may be excited with a single UV light source. Recent work has shown that fluorescent nanocrystals can be directed to specific tissues or cell types if they are coated with antibodies or homing peptides (2, 115). These fluors may also allow long-term labeling of live cells without interfering with cell growth and development (115). These studies highlight the potential of fluorescent nanocrystals for improving the performance of multicolor single-cell analyses while minimizing the requirements for specialized equipment.

**Fluorescence in situ hybridization and immunofluorescence.** The principles behind the use of FISH and immunofluorescence methods have been extensively and informatively reviewed elsewhere (6, 7, 66, 164). In the FISH technique, fluorescently labeled nucleic acid probes are hybridized to complementary rRNA targets located on ribosomes within whole, permeabilized cells. The ribosome is a naturally amplified target molecule, especially in actively growing cells, where each cell may contain several thousand ribosomes (7). The aggregate signal from multiple probe-ribosome binding events leads to the sequence-specific fluorescence of target cells. Apart from rRNA, other forms of RNA (e.g., tmRNA) can serve as a target for hybridizations, especially if a signal amplification step is used (203). Recently, FISH-based methods have also been developed to detect low-copy-number targets on plasmid ($10^1$ to $10^3$ copies/cell) or chromosomal (<10 cop-
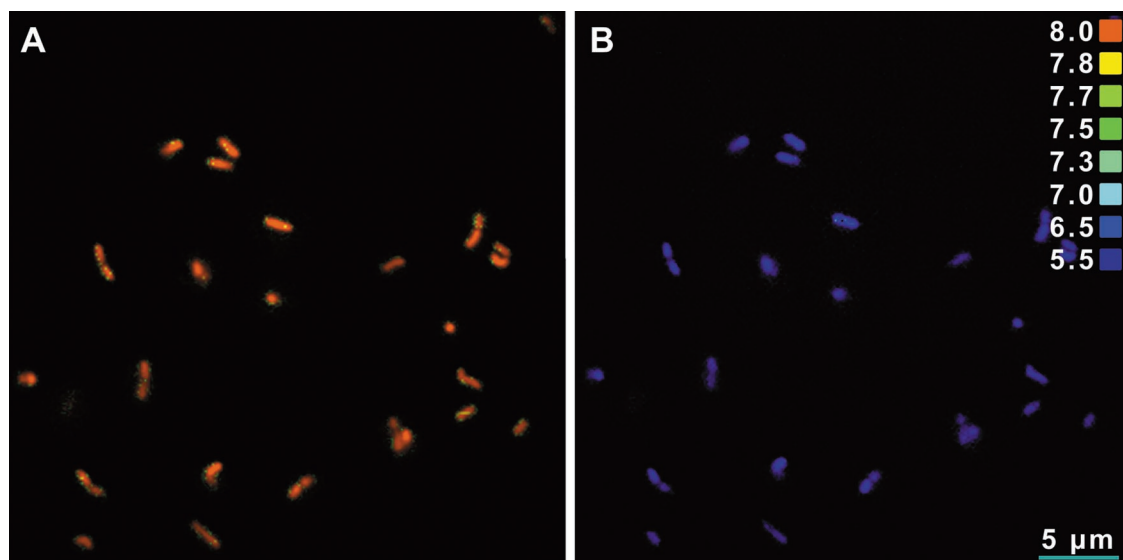
FIG. 1. Fluorescence ratio imaging of nisin-mediated dissipation of ΔpH in *Listeria monocytogenes*. Fluorescence ratio imaging microscopy was used to monitor the intracellular acidification of broth-grown cells of *L. monocytogenes* after exposure to the membrane-permeabilizing lantibiotic nisin. The pH-dependent spectral response of carboxyfluorescein diacetate succinimidyl ester (CFSE) was used as a probe of intracellular pH ($pH_i$). The ratio of CFSE fluorescence intensity at 490 nm to that at 435 nm was calibrated over a pH range of 5.0 to 9.0. (A) Live, intact cells of *L. monocytogenes* maintained $pH_i$ values between 8.0 and 8.4, even when the pH of the external medium was low (e.g. pH 5.5). (B) Nisin-mediated membrane permeabilization resulted in the equilibration of $pH_i$ with the pH of the medium after 12 min of exposure. Individual cellular responses were more heterogeneous for cells derived from colonies, suggesting the importance of microenvironmental factors in differential susceptibility to nisin (not shown). A color-coded pH scale is shown in the upper right-hand corner. Reprinted from reference 42 with permission from the publisher.

ies/cell) DNA (268). This approach differs substantially from rRNA-targeted FISH in that it utilizes polynucleotide probes (~50 to 1,200 nucleotides in length), higher (1,000-fold) probe concentrations, and much longer hybridization times (268). The resulting fluorescent signal is also qualitatively different from that achieved with classic rRNA-targeted FISH and is characterized by the formation of a fluorescent "halo" around the periphery of target cells. The technique has thus been named RING-FISH.

Fluorescently labeled antibodies also enable the detection of diagnostic molecular binding events and can be directed against surface antigens, such as capsular, flagellar, or cell wall antigens, or against internal targets, including ribosomal proteins or cell cycle-specific cytoplasmic proteins (66, 194).

FISH and immunofluorescence have substantial overlap in their applications and benefits as single-cell detection techniques. Both are whole-cell methods, and as such they can preserve a wealth of potentially valuable information that is unavailable outside the context of the intact cell. Apart from providing information on microbial identity, information about cell morphology, number, and distribution may also be collected for specific target cells. Both methods have the potential to be carried out simultaneously or in succession with other means of cell characterization, including the observation of light-scattering characteristics, staining of inclusion bodies, fluorescence-based measurements of nucleic acid or protein content, cytochemical characterization using fluorescent or colorimetric enzyme substrates, and microautoradiography (22, 39, 140, 249). The combination of FISH or fluorescent-antibody labeling with methods for high-throughput multiparametric data collection, analysis, and sorting (e.g., flow cytometry) can

be especially useful in the study of complex microbial populations (39, 66).

FISH is used primarily as a means of detecting specific microbial cells, although the intensity of staining with FISH has also been used to provide an indication of physiological activity (146). For the most part, the use of FISH for microbial detection has involved DNA-based methods, but peptide nucleic acid probes may have substantial practical and functional advantages, especially for the detection of gram-positive bacteria (39, 224).

As a means of detection, fluorescent-antibody approaches can be limited by problems with cross-reactivity, variable antigen expression under different culture conditions, or potential instability and loss of cell surface epitopes (166). However, unlike FISH, immunofluorescence-based detection does not require cell permeabilization and can therefore be used on living cells, potentially followed by isolation for culture (66, 244). Apart from their use as taxonomic probes, fluorescent antibodies may be used for fine-structure analyses, such as the discrete localization of specific proteins within individual cells.

Neither FISH nor immunofluorescence approaches require that a cell be culturable (4, 66). However, because the number of target antigens may not be as tightly coupled to the cell growth rate as is the rRNA copy number, fluorescent-antibody techniques may yield higher detection sensitivities for dormant cells than FISH does.

**Green fluorescent protein and related reporters.** GFP is a versatile tool for the in vivo visualization of protein expression, localization, and functionality. Because it retains its fluorescence after fixation with paraformaldehyde, GFP can be combined with fixation-dependent staining methods such as FISH
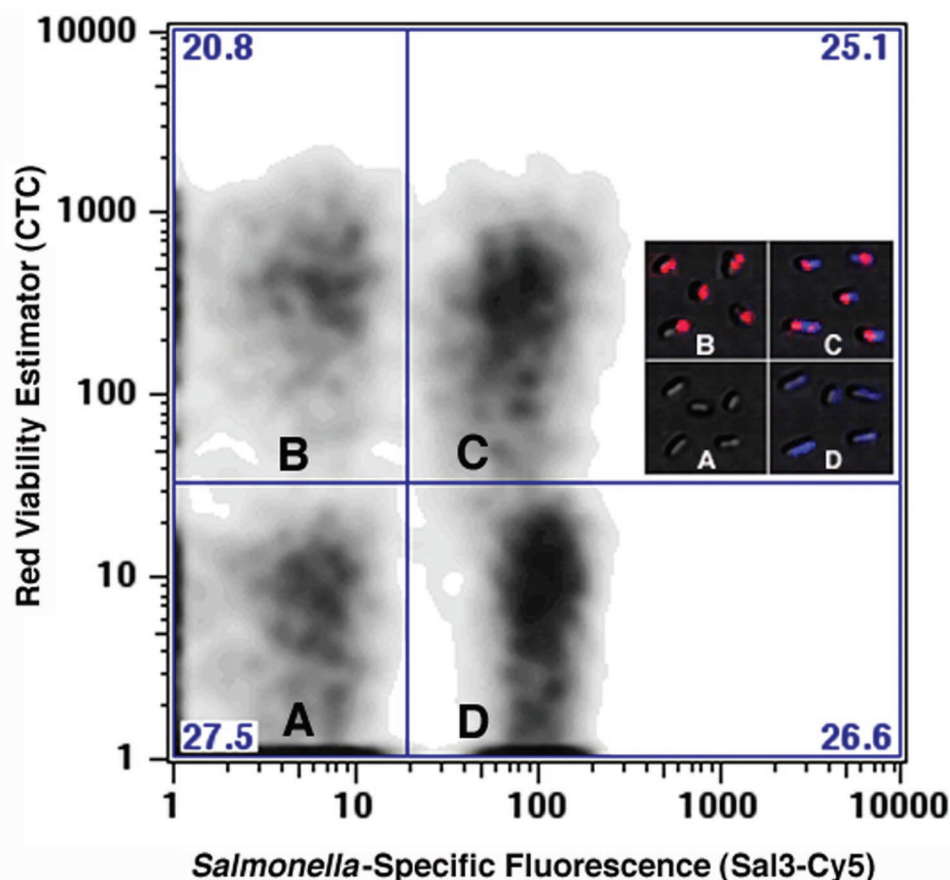
FIG. 2. Flow cytometric analysis of a genetically and metabolically complex cell mixture. This figure illustrates the power of single-cell staining methods in combination with flow cytometric analysis for the fluorescent "dissection" of complex microbial populations. Here, *Salmonella enterica* serotype Typhimurium is differentiated from a mixture of *E. coli*, *Citrobacter freundii*, *Proteus vulgaris*, and *Shigella dysenteriae* on the basis of both cytochemical activity (CTC staining) and genetic identity (*Salmonella*-specific FISH staining). A complex mixture containing both live and formalin-killed representatives of each cell type was incubated with CTC, fixed with 10% buffered formalin, hybridized with a *Salmonella*-specific DNA probe (Sal3-Cy5), and examined by flow cytometry. Four distinct populations can be seen. Clockwise from the bottom left, they are dead non-*Salmonella* members of the *Enterobacteriaceae* (A), live non-*Salmonella* members of the *Enterobacteriaceae* (B), live *Salmonella* (C), and dead *Salmonella* (D). The numbers in each quadrant represent percentages of the total population. The photographic inset provides a visual interpretation of the cytometry data. Reprinted from reference 209a with permission from the publisher.

(71). However, the true power of GFP is as a visual reporter of dynamic events occurring in living cells. For example, Raskin and de Boer (187) used GFP fusions to probe the function of proteins associated with cell division in *Escherichia coli*. They observed a regular, pole-to-pole oscillation for GFP-MinD and theorized that the cell may use this protein as a "measuring device" to continuously probe the location of the center of the cell. Cluzel et al. (58) used a *cheY-gfp* fusion, fluorescence correlation spectroscopy, and video microscopy to relate CheY-GFP expression levels to flagellar-rotation behavior in single cells of *E. coli*. These authors found that small changes in the concentration of CheY-P led to large changes in the rotational bias of the flagellar motor, suggesting that the motor itself acts as a signal amplifier and that additional cellular mechanisms exist for maintaining CheY-P concentrations within the operational range of the motor (58).

Other applications of GFP include the construction of whole-cell sensors for in situ monitoring of iron availability on leaf surfaces (120); measurement of cytoplasmic viscosity and protein diffusion rates in living cells (74, 182); investigation of quorum-based interspecies communication or coordinated, multicellular behaviors (11, 116, 126, 248); measurement of the internal pH of bacterial cells (172); and real-time reporting of fungal susceptibility to antimicrobial compounds (247).

GFP is especially well suited to in situ analyses of individual cells within complex consortia such as biofilms. Because its use does not require preparative steps such as dehydration, fixation, or application of exogenous probes or cofactors, GFP labeling enables the observation of microorganisms directly in these fragile structures (35, 67, 190). The range of applications of GFP has been further expanded with the introduction of fluorescence-shifted spectral variants. In a novel application of such variants, Fehr et al. (78) created chimeric "nanosensor" proteins based on the fusion of enhanced cyan fluorescent protein (ECFP), a bacterial maltose binding periplasmic protein, and enhanced yellow fluorescent protein (EYFP). Conformational changes of these nanosensors on binding of maltose led to more efficient fluorescence resonance energy

transfer (FRET) from ECFP to EYFP. When these nanosensors were expressed in *S. cerevisiae*, changes in ECFP/EYFP FRET ratios enabled maltose uptake and compartmentation to be monitored in individual living cells. The broad range of organic and inorganic substrates recognized by periplasmic binding proteins suggests the use of this strategy in generating fluorescent nanosensors specific for a wide variety of analytes (78).

Stochasticity, or noise, in gene expression can lead to substantial phenotypic variation among individual cells in an otherwise clonal population (75). Such noise can be either intrinsic (stemming directly from events related to the expression of a gene) or extrinsic (resulting from fluctuations in the quantities or activities of the enzymes and other cellular machinery required for gene expression). In a novel application of GFP variants, Elowitz et al. (75) constructed strains of *E. coli* capable of distinguishing between these two sources of noise in gene expression. Their results indicated that both sources of noise contribute to the generation of phenotypic heterogeneity among individual cells. Their findings also suggested that any component in a cellular biochemical network that is prone to intrinsic fluctuations in concentration can serve as a source of extrinsic noise for other components in the network (75).

## Cytometry

"Cytometry" is a general term that may apply to any technology used to measure, count, compare, or otherwise characterize biological cells. The general term has become nearly synonymous with flow cytometry, due to the popularity of this technique. However, other forms of cytometry have specialized advantages for use in single-cell microbial studies and, along with flow cytometry, are discussed below.

**Flow cytometry.** Flow cytometry is a powerful fluorescence-based diagnostic tool that enables the rapid analysis of entire cell populations on the basis of single-cell characteristics (4). Multiple characteristics, including cell count, cell size or content, and responses to fluorescent probes diagnostic of cell function may be collected simultaneously by this method (66, 244, 253). Cells in a liquid sample are passed individually in front of an intense light source (e.g., a laser, laser diode, or arc lamp), and data on light scattering and fluorescence are collected and saved as a data file. Detailed numerical analyses of populations and subpopulations of interest can then be carried out offline by using a number of analysis packages. Because of its capacity to collect information-rich data sets on thousands of cells, flow cytometry facilitates valuable insights into connections between single-cell and population-level processes not available with other techniques (66, 86, 119, 208, 243). Flow cytometers capable of sorting cells on the basis of their fluorescence characteristics or of simultaneous in-line video microscopy add to the versatility of this method (66, 250). Flow cytometry has proven to be an invaluable resource in the study of apoptosis in mammalian cells (64). Recent work has suggested that programmed cell death is not limited to eukaryotes but may also be active in prokaryotic systems (76, 191). Therefore, flow cytometry may also be a useful tool for elucidating these processes in bacteria.

**Laser scanning cytometry.** Flow cytometry collects data on single cells in a liquid sample as they stream past the illumi-

nation source. Although multiple light scatter and fluorescence parameters may be measured, cells pass only once through the system. Because of this, flow cytometry is not suited for time-resolved studies of individual cells (65, 68, 124, 208). An exception may be the microfluidic cell sorter described by Fu et al. (84), in which the fluid flow may be stopped or reversed, allowing multiple observations of the same cell, but this technology is not yet widely available.

Laser scanning cytometry (LSC) is a solid-phase cytometric technology for collecting laser-induced fluorescence from cell samples on slides or on membrane filters. At their simplest, LSC instruments provide a rapid means of counting, quantifying, and recording the distribution of fluorescent events on a filter. Microscope-based LSC instruments can provide visual information on both cell morphology and the spatial distribution of fluorescence within each cell (65). Because LSC can be used to make multiple measurements of the same cells, this technique is well suited for the observation of cellular properties as a function of time. Examples include monitoring the kinetics of fluorescence staining in living cells (e.g., substrate uptake, enzyme activity, and dynamic changes in intracellular pH) and observing interactions between neighboring cells (65, 68, 230). Spatial "addressing" of fluorescent events may facilitate the reexamination of archived samples (65). The ability to concentrate cells prior to analysis gives filter-based LSC methods definite practical advantages over fluorescence microscopy or flow cytometry when working with dilute suspensions of microorganisms in filterable liquids (146). However, because LSC may involve exposing the sample to the excitation source for relatively long periods, photobleaching of fluorescent labels could be problematic for some applications, particularly if multiple scans are required. These effects can, in part, be minimized through the use of low-intensity (microwatt versus milliwatt) illumination sources (68).

**Image cytometry.** The terms "image cytometry" and "image analysis" are used interchangeably here to describe a wide range of methods by which quantitative biological information may be extracted from microscopic images (Fig. 3). These techniques can be used to gain information on individual cell properties such as staining intensity and label specificity; cell number, size, and volume; and distribution within a field of view (73, 155, 96, 202). Advanced image analysis techniques can be used to monitor ultradiscrete physical phenomena such as the micronewton invasive forces generated by individual fungal appressoria (26).

Most image analysis methods incorporate some form of colorimetric or fluorescent cell staining (Gram staining, Lugol's solution, colorimetric enzyme substrates, 4′,6-diamidino-2-phenylindole [DAPI], FISH, etc.). This provides a means of cell identification or characterization and generation of high-contrast images suitable for further processing (44, 54, 61, 198, 202). Alternatively, intrinsic changes in the light-scattering characteristics of a microorganism as it undergoes certain physiological processes may be sufficient to allow analysis. An example is the phase-bright to phase-dark transition of bacterial spores on germination, which has been used to investigate the variability of germination among individual spores of *Clostridium botulinum* (M. W. Peck, personal communication).

Image collection is often followed by a number of processing steps designed to facilitate extraction of the desired informa-
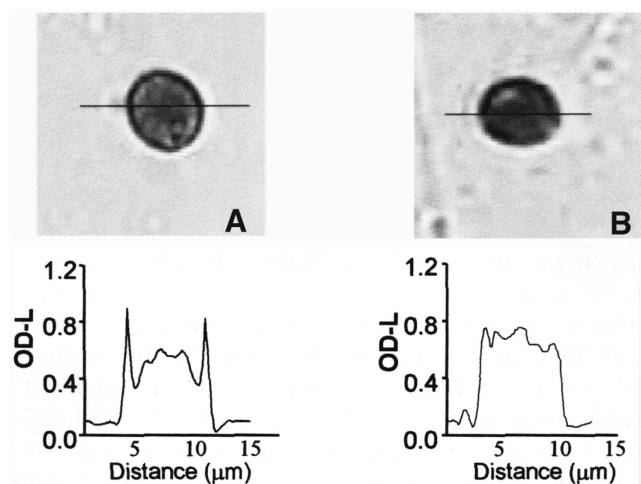
FIG. 3. Single-cell determination of yeast glycogen content by image cytometry. The glycogen content of individual *S. cerevisiae* cells was determined from their optical density (OD) values after staining with Lugol's solution ($I_2$-KI). Images were processed using a series of steps designed to extract quantitative information about cell size, shape, volume, and OD. The OD profile of cell A (glycogen poor) shows concentrated staining only on the periphery of the cell, whereas the profile of cell B (glycogen rich) shows dense staining throughout. To avoid overestimation of the mean glycogen content in glycogen-poor cells, only the central portion of each cell was used for measurement. The ability to quantitate the glycogen content in individual cells allows the determination of glycogen distribution within a population. Because the character of this distribution is related to yeast quality, image cytometry can be used as a tool for quality control. OD-L, optical density, Lugol staining. Reprinted from reference 44 with permission from the publisher.

tion. These include thresholding, filtering, edge detection, removal of optical artifacts (e.g. fluorescent "halos"), background subtraction, pixel averaging, and other transformations (34, 54, 120, 202, 231). Advantages of such thorough image processing may include the ability to differentiate target cells from background material, particularly in "difficult" sample matrices such as soil (202). Through the use of special algorithms, images may be automatically processed on the basis of user-defined criteria or artificial neural networks may be

trained for the automatic classification of objects (34, 44). Such automation can greatly aid image processing, especially where manual data extraction would be impossible, tedious, or error prone (61). Recently, a fully automated high-throughput microscopy system has been described that combines computer-controlled autofocusing and stage movement with advanced image segmentation, classification, and retrieval algorithms (185). With the ability to rapidly acquire and categorize data from large numbers of cells on slides or in microtiter plates, such high-throughput microscopy systems may eventually become competitive with flow cytometry as a method for the rapid and detailed analysis of populations on a cell-by-cell basis. However, due to the longer integration times often needed for imaging-based techniques, efforts must be made to minimize the effects of photobleaching, which are not a significant issue with flow cytometry.

In addition to single still images, multiple still images from a time series or video images may be analyzed. Video-based images, which allow the display of a time code with each frame, can provide a continuous record of a cellular measurement with high temporal resolution (158). Dynamic cellular properties associated with cell motility (cell speed, the number and duration of runs or tumbles, etc.) or changes in fluorescence related to some physiological characteristic can be resolved in terms of both space and time (Fig. 4) (38, 116, 159, 212). Movements of individual cells within a larger population can also be monitored, enabling connections to be made between cell behaviors at both microscopic (individual) and mesoscopic (population) scales (116, 126, 158, 190, 248).

### Scanning Probe Microscopies

Scanning probe microscopies (SPMs) are a related group of technologies which can yield information on both the topography and the mechanical, electrochemical, electrostatic, or magnetic properties of a sample surface (99, 145). In all SPM formats, samples are imaged by rastering a cantilever-mounted tip over the surface of the sample in the *x*-*y* plane. Direct (e.g., physical) and indirect (e.g., atomic force) interactions result in *z*-plane deviations of the cantilever. These deviations can reveal topographical details in the sample at atomic resolutions
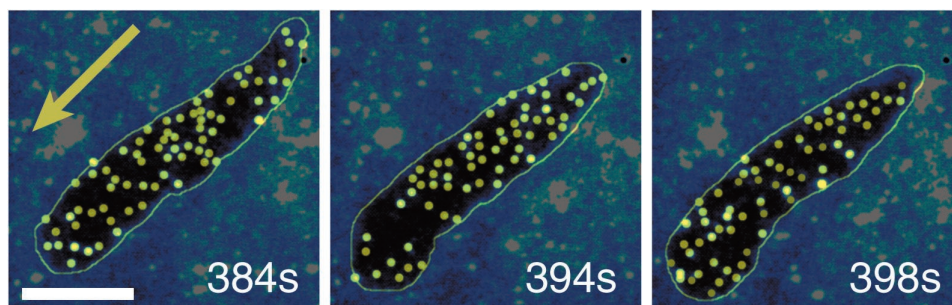


FIG. 4. Single-molecule analysis of cyclic AMP (cAMP) receptor occupancy on the surface of a *Dictyostelium discoideum* cell during chemotaxis. Cells were exposed to a gradient of Cy3-labeled cAMP (Cy3-cAMP, shown to be functional as a chemoattractant) and observed for up to 10 min. Binding of Cy3-cAMP to cell surface receptors was monitored at single-molecule resolution by using total internal reflection fluorescence microscopy. Occupied cAMP receptors appear as bright yellow dots on the surface of the cell. The arrow indicates the direction of the Cy3-cAMP source. The time for each sequential image is given in seconds. Kinetic analysis showed that Cy3-cAMP receptor complexes located on anterior pseudopods dissociated faster than those on the posterior tail (239). This work enabled the discrete characterization of receptor dynamics in single living cells, suggesting a role for cell polarity in the chemotactic process. Reprinted from reference 239 with permission from the publisher.

(99, 145). The SPM family of tools includes scanning tunneling microscopy, atomic force microscopy (AFM), scanning electrochemical microscopy (SECM), and magnetic force microscopy (99, 145, 254). SPM technologies have found widespread use in materials science applications and are fast becoming recognized for their potential to characterize biological materials, including single living cells, as described in the sections below.

**Atomic force microscopy.** AFM is a member of the SPM family of tools, the forerunner of which was the scanning tunneling microscope (STM). The central mechanism of an AFM consists of a cantilever, or "arm," to which a very sharp probe, or "tip," is attached. An often-made and apt comparison is to the arm and needle of a (very small) phonograph (138). The cantilever arm may be only 100 $\mu$m long, and, ideally, the tip, or "needle," should terminate in a single atom (138). As the tip is scanned across a surface, tip-sample interactions cause deflections of the cantilever, which are detected and amplified by a laser. These interactions may be direct (e.g., physical), or indirect (e.g., electrostatic, electrosteric, and van der Waals' forces) (18, 46, 257). Conversion of cantilever deflection data to topographical information results in both qualitative output (e.g., images) and quantitative output (e.g., measurement of interaction forces and force-distance relationships).

Several modes of imaging are used: contact, noncontact, and tapping (47, 138, 220). Contact imaging involves "dragging" the tip across the sample and may give rise to undesirable effects such as frictional forces and sample damage (138). Noncontact imaging based on electrostatic deflection of the probe tip can be used to investigate charge development or distribution on biological surfaces (220). Tapping-mode imaging was developed as an alternative method for measurements of "soft" biological surfaces likely to sustain damage during contact imaging (47, 138). In this technique, the tip does not scrape the sample but oscillates over its surface, minimizing tip-sample frictional forces (47, 99, 138).

AFM is capable of measuring discrete interaction forces in the piconewton range (149). Because little sample preparation is needed and cells may be observed in liquid environments, AFM can be used for detailed ultrastructural studies of the surfaces of living microbial cells (8, 69). Dynamic events, such as bacterium-mineral adhesion interactions and viral exocytosis, may be measured in real time, under native conditions of hydration and oxygen tension (150, 260).

AFM tips may be chemically functionalized to study properties such as cell surface hydrophobicity. Alternatively, they may be functionalized with biomolecules such as biotin, antibodies, enzymes, or even single, intact microbial cells (69, 85, 149, 179). AFM cantilevers with such functionalized tips can be used as "nanobiosensors" for the study of discrete receptor-ligand interactions or for characterization of cell-substrate interactions (85, 179).

AFM can also be used as a method for the nanomechanical manipulation of individual microbial cells. As such, it can be used to provide quantitative data on cellular physical properties such as rigidity or elasticity (see "Mechanical micromanipulation" below) (18, 257). Measurements of force-distance relationships for AFM tip indentation have also provided a direct means of measuring turgor pressure in individual bacterial cells (18, 257).

**Scanning electrochemical microscopy.** Electrochemical phenomena such as electron transfer and ion fluxes are associated with both energy production and intracellular signaling processes (258). Well-established techniques for the electrochemical characterization of single, living microbial cells include the use of microelectrodes or patch-clamping approaches (258). SECM is a recently introduced, SPM-based tool for mapping redox activity in living cells (147). In SECM, the scanning tip is an ultramicroelectrode designed for measuring charge transfer reactions (45, 147, 259). Grayscale images, or "redox maps," are generated from variations in tip current as the tip is scanned in the x-y plane above an electrochemically active cell (259). SECM has been used for the electrochemical visualization of oxygen production in single algal protoplasts on exposure to light, for assessment of the permeability of membrane to charged redox species, and for electrochemical studies of *Rhodobacter sphaeroides* cells (45, 259). SECM imaging is carried out in solutions containing hydrophilic or hydrophobic redox species which function to mediate the transfer of electrons between cellular redox centers and the SECM tip (45). Redox mediators may differ in their abilities to penetrate various cellular permeability barriers (e.g., the outer membrane versus the cytoplasmic membrane). Therefore, carefully chosen mediators may facilitate redox studies of physiologically distinct cellular structures, such as the periplasmic space (45).

## Microspectroscopic Methods

The term "spectroscopy" describes methods used to separate a light signal into its component wavelengths. More generally, the term can be used to describe the same process for other regions of the electromagnetic spectrum (emission or absorption) or for analogous processes such as mass spectrometry, where complex mixtures are separated on the basis of the molecular masses of their components.

In biology, as in astronomy, the spectral characteristics of an object can be used to provide information on its chemical or physical makeup. Spectroscopic methods have been used to monitor the presence and activities of natural microbial populations via remote-sensing techniques (176); for spectral identification of bacterial suspensions in pure culture by Fourier-transform infrared, proton nuclear magnetic resonance, or mass spectroscopic methods (171, 195, 241); for noninvasive investigations of biochemical changes in *P. mirabilis* populations during differentiation (93); and for the characterization of pigmented colonies formed by various photosynthetic bacteria (251).

However, when they are applied at the population level, spectroscopic measurements suffer from the same drawbacks as other bulk-scale approaches, and contributions from individual microorganisms cannot be assessed (88, 178, 261). Spectroscopic methods capable of single-cell resolution (e.g., microspectroscopic methods) enable the observation of target analytes or properties within specific cells at cellular or subcellular scales (Fig. 5). This can be especially important because information obtained within the context of a whole cell may reveal important clues to the role or function of the analyte within the cell (13, 135).

As an alternative to "wet-chemistry" methods, spectroscopic approaches allow target compounds to be analyzed quickly and
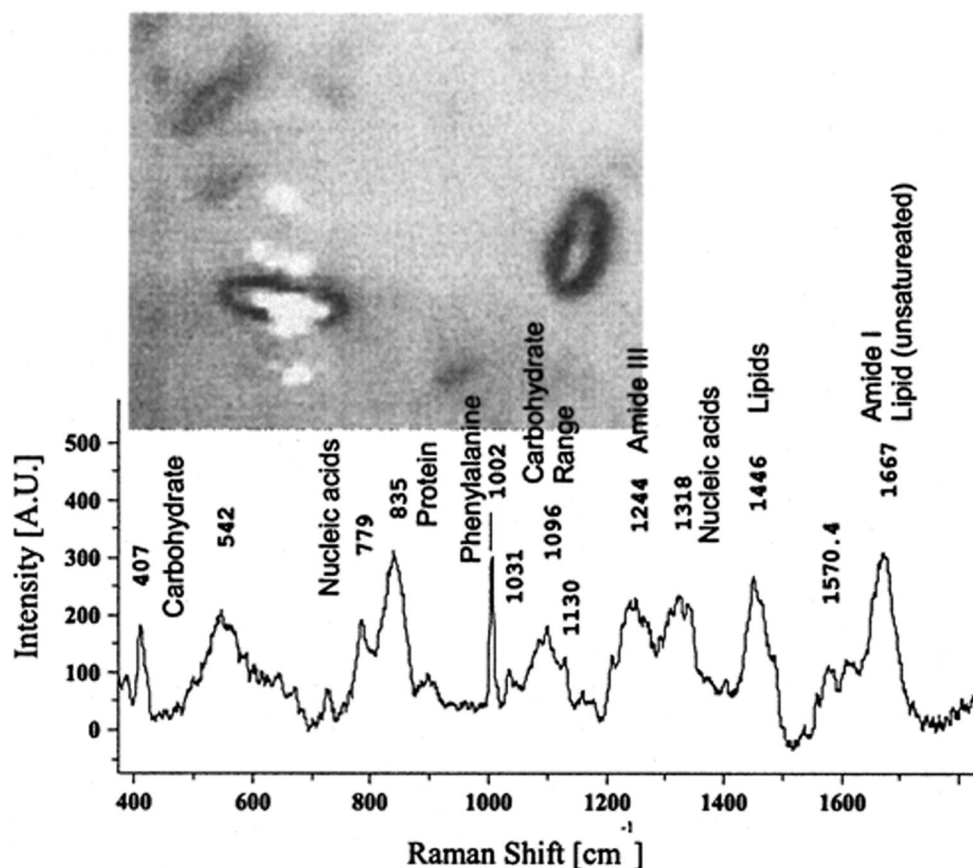
FIG. 5. Raman spectrum of a single *Clostridium beijerinckii* cell. Spectral peaks ascribed to major cellular macromolecules (e.g., nucleic acids, proteins, lipids, and carbohydrates) are shown. The video inset shows a cell illuminated in the laser focus, which is of approximately the same diameter as the cell. The laser diffraction pattern, which can serve as a visual cue for achieving the proper laser focus, can also be seen. Single-cell Raman spectroscopy represents a noninvasive means of investigating the biochemical heterogeneity of microbial populations. Reprinted from reference 204 with permission from the publisher.

without the need for extraction, which may be incomplete or may introduce artifacts, especially when dealing with potentially labile species or features (e.g., carotenoids or elemental inclusion bodies) (135, 178, 262). Additionally, many spectroscopic approaches require only minimal sample preparation and may be used for the analysis of living cells (178, 183, 262, 267).

Microspectroscopic methods can provide biochemical information on the overall macromolecular composition of cells (204) or on specific analytes at either whole-cell (88) or subcellular (135, 178) resolutions. Vibrational spectroscopy may also be used to generate images of individual cells by using data from the aliphatic C—H stretching within membrane lipids (267) or from the O—H stretching of intracellular water molecules (183).

Not all spectroscopic methods provide direct information on the chemical composition of a cell. Methods such as electrorotation can be used to determine other characteristics of single cells, including their dielectric properties (106, 131). Some of the more frequently used methods for obtaining spectroscopic data from single microbial cells are described below.

**Raman microspectroscopy.** The Raman effect is an induced emission of light resulting from the inelastic scattering of a small number of photons from a monochromatic light source (48, 205, 261). Raman spectra provide information on molecular vibrational states, which are dependent on the nature of chemical bonding within a molecule or sample (178, 261). These spectra yield clues to the types and lengths of chemical bonds present and on the molecular conformation or environment (48, 205). Microspectroscopic Raman probes capable of illuminating an area as small as 1 by 1 μm enable the characterization of individual cells and their subcellular components (135). Spectra may be collected at different points along the length of a cell or hypha or at different depths (13, 205) (Fig. 5). Spectra may also be compared among different species, between mutant strains, or at different points in the cell cycle (13, 135).

The range of energies used to generate Raman spectra includes UV (e.g., 257 nm), visible, and infrared excitation frequencies (55, 256). Common visible sources used are argon-ion lasers (~514 nm) (135, 178) and helium-neon lasers (~632 nm) (13, 178, 204, 205). When the wavelength of the incident light approaches the absorption wavelength of a chromophore within a sample, scattering efficiency is greatly increased, an advantageous effect referred to as "resonance" Raman scattering (256, 261). An example of an application where resonance
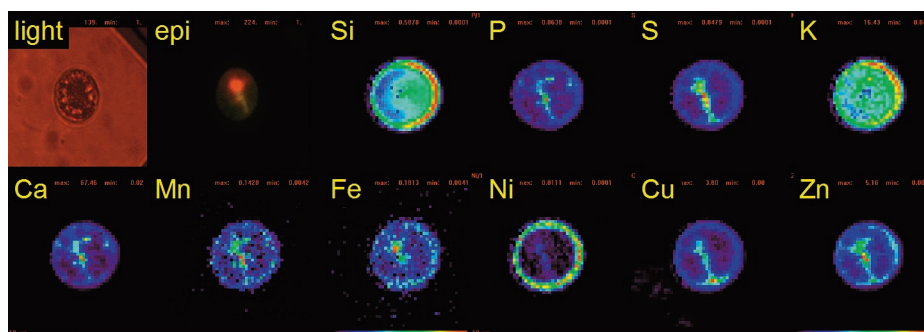
FIG. 6. Synchrotron X-ray fluorescence mapping of the relative elemental distribution in a single diatom. An X-ray microprobe was used to focus a monochromatic X-ray beam on a diatom collected from the Southern Ocean. The sample was scanned through the focused beam in pixel steps of 0.5 μm, and the full X-ray fluorescence spectrum was collected at each step. Two-dimensional elemental maps were generated from the resulting energy spectra by using element-specific filtering. Elemental concentrations were calculated from X-ray fluorescence data using National Institute of Standards and Technology thin-film or similar standards. Comparison of light and epifluorescence (epi) micrographs with elemental maps for the same diatom enabled the discrete localization of each element within the cell. Data collected for this diatom show that Si and K map onto the cell's siliceous frustule; P, S, Ca, Mn, Fe, Cu, and Zn appear to be associated with the cytoplasm; and Ni is present only on the outer membranes or frustule. This approach to characterizing elemental distributions within individual diatoms provides biologically relevant information not available from population-scale methods of elemental analysis. The resulting data may offer unique insights into the physiological state, ambient chemical environment, and role in elemental cycling of these organisms (238). Reprinted from reference 238 with permission from the publisher.

enhancement would be expected to occur is in the UV-Raman analysis of nucleic acids.

Other applications of Raman microspectroscopy include investigations of microbial carotenoid content and subcellular distribution. Kubo et al. (135) used Raman microspectroscopy to map the carotenoid content in *Euglena* and in *Chlamydomonas reinhardtii*. These authors also applied polarization techniques to demonstrate that the carotenoid molecules in the eyespot of *C. reinhardtii* are oriented parallel to the long axis of the cell. Raman microspectroscopy has also been used to investigate biochemical differences between cells in morphologically heterogeneous cultures of clostridia during solvent fermentations (205). Traditional, bulk-scale methods of analysis of these differentiated cultures do not facilitate connections between the morphological appearance of individual cells and their biochemistry or role in the fermentation. Analyzed by Raman microspectroscopy, morphologically distinct cells yielded spectra that differed in regions ascribed to proteins, lipids, or the storage polymer granulose (204, 205). Analysis of small cell clusters also showed spectral evidence for the presence of polysaccharides, suggesting the presence of aggregation-promoting extracellular polymers. The ability to correlate morphology with biochemical characteristics may provide clues to the activities of the different cell types during solvent production (204, 205). Other applications of Raman microspectroscopy include the reagentless identification of individual bacterial spores (49) and detection of the neurotoxic amino acid domoic acid in single cells of toxigenic phytoplankton (256).

In related technology, coherent anti-Stokes Raman scattering microscopy allows imaging of individual microbial cells on the basis of the vibrational spectra of specific cellular components (e.g., proteins and lipids). The vibrational signatures of these molecules provide a means of generating contrast (267). In this way, the distribution of specific molecular components in living cells can be mapped without the need for fluorescent dyes and at relatively low power levels (100, 267). Finally, time-resolved coherent anti-Stokes Raman scattering imaging can reveal dynamic processes, such as real-time changes in intracellular water concentration (183).

**Microbeam analysis.** Methods for microbeam analysis represent sensitive means of characterizing single-cell elemental composition (88, 262). These methods allow the measurement of the concentration, chemical state, or cellular location of biologically relevant inorganic nutrients, including phosphate, sulfur, potassium, calcium, iron, and zinc (238, 174, 262). Multiple elements can be measured in a single pass, resulting in an "elemental map" of an individual cell (174, 238) (Fig. 6). Available techniques include X-ray fluorescence imaging and absorption spectroscopy, as well as various ion beam-dependent methods (88, 262).

X-ray microprobe techniques may not require extensive sample preparations, allowing biological materials to be examined in their natural, hydrated states. This may be essential for ensuring the stability of the chemical (oxidation) states of elements within the sample (262). Plant roots infected with mycorrhizal fungi have been studied by X-ray fluorescence imaging at elemental sensitivities of 500 ppb. With an X-ray beam spot of 1 by 3 μm, elemental mapping at single hyphal resolution is possible (262).

In contrast to the minimal preparative requirements for X-ray microprobe analysis, samples to be studied by ion beam methods may need to be dried and vacuum compatible, constraints that could hinder the analysis of many cell types. A major disadvantage of all microbeam methods described here is that they are very time-consuming. Scanning times ranging between 30 min and 4 h can be required to generate an image (88, 262).

Still, single-cell microbeam analysis may provide useful information on the physiological states of individual microbial cells, as illustrated in the study by Gisselson et al. (88). As these authors noted, sample preparation prior to traditional measurements of algal nutrient ratios may include fractionation steps designed to isolate the subset of the planktonic

community to be studied. Despite such careful preparations, contributions to nutrient ratios from bacteria, protists, or particulate organic material may still skew the results (88). Microbeam analysis methods can be used effectively to ensure that measurements are derived from the intended cell type and to probe the nutritional heterogeneity of individual target cells within the population (88, 238).

**Electrorotation.** When a cell is exposed to an electric field, a dipole is induced, whose character is dependent on the composition of the cell, the frequency of the applied electric field, and the conductivity of the medium in which the cell is suspended (107, 111, 131). In the presence of a rotating electric field, the dipole will form across the cell in synchrony with the rotation rate of the field (110). If the field is rotating with sufficiently high frequency, though, formation of the dipole may become asynchronous with the field's rotation rate. In this case, the cell will experience a torque and begin to rotate, either in the direction of the field ("cofield rotation") or in the opposite direction ("antifield rotation"), depending on the angular difference between the field and the induced dipole (63, 110, 111).

Precise positioning of the electrodes used to generate the rotating field is used to create a dielectrophoretic trap capable of holding individual cells in place during analysis (see "Electrokinetic micromanipulation" below) (Fig. 7A). Electrorotation spectra are displayed as cellular rotation rate versus frequency of the applied field (Fig. 7B). Cell rotation rates can be automatically measured and documented using computer-interfaced video microscopy or interferometric methods (106, 188). Because the dielectric properties of a cell are responsive to mechanical or chemical perturbation, methods for dielectric spectroscopy such as electrorotation can yield information on both the integrity and the physicochemical properties of individual cells (63). Compared to other methods for investigating the electrical properties of cells (e.g., the use of microelectrodes or patch clamping), electrorotation is relatively noninvasive and does not require extensive cell preparations (106, 192, 258). Applications for electrorotation include monitoring the effects of antibiotics on single yeast cells (106) and distinguishing between nonviable and viable protozoan cysts on the basis of the direction of their rotation at specific field frequencies (63). The ability of electrorotation to distinguish between viable and nonviable protozoa is especially useful, since no direct culture-based methods are currently available (63).

## Micromanipulation

In some instances, a means of physically manipulating individual microbial cells may be needed. Examples include the isolation of cells for subsequent analyses such as single-cell PCR, the selection of cells with unique or beneficial characteristics, and the isolation of cells to obtain pure cultures of microorganisms that are difficult to purify using traditional culture-based methods (5, 81, 83, 124). This can also be extended to include the direct isolation of dormant, stressed, or otherwise unculturable cells for further study (22).

Micromanipulation can be used to address a cell to a specific position in a liquid medium and hold it there in order to examine its ability to replicate (77). Physical segregation of daughter cells may also be used to trace the pedigree of a
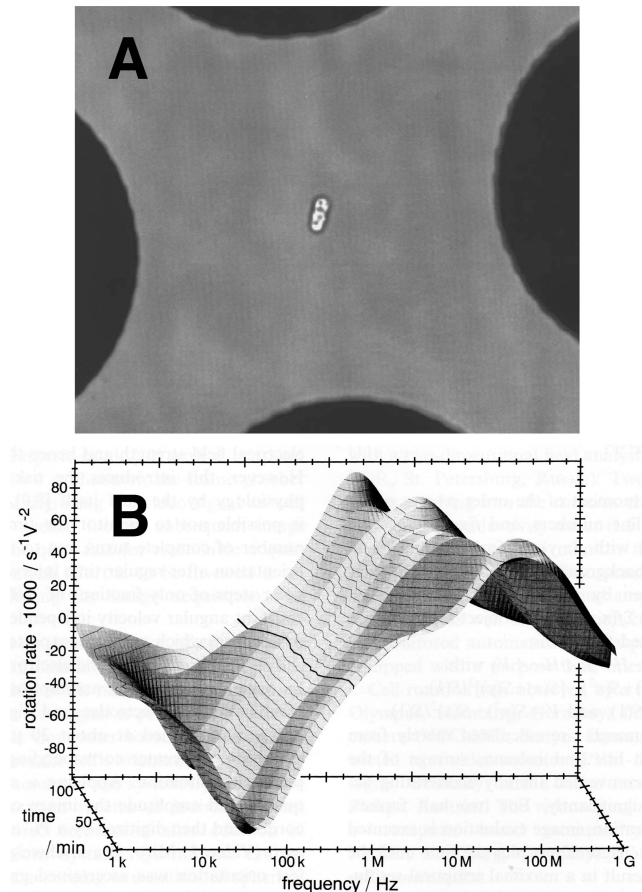


FIG. 7. Electrorotational analyses of single yeast cells. (A) Single cell of *S. pombe* during analysis in a microstructured electrorotation chamber. Four circular electrodes (dark semicircles), spaced 100 μm apart, are precisely positioned to allow dielectrophoretic trapping of individual cells. (B) Cellular dielectric properties are responsive to mechanical or chemical perturbation. This panel illustrates time-resolved changes in the electrorotation spectra of a single *S. cerevisiae* cell treated with nystatin at $t = 12$ min. Nystatin-mediated leakage of intracellular ions is expected to change the dielectric properties of the cell, leading to the frequency-dependent shifts in cell rotation rates seen for both cofield and antifield rotations. Panels A and B reprinted from references 131 and 106, respectively, with permission from the publishers.

single cell as it undergoes multiple cycles of division or to examine adaptation processes of individual cells subjected to changes in nutrient availability (240, 245, 263).

Alternatively, a cell may be positioned in close proximity to or touched against other cells, immobilized enzyme substrates, or inorganic surfaces. In this way, discrete binding, chemical, or other interaction forces may be measured (31, 46, 149, 150, 159, 179, 216). Micromanipulative techniques also allow the stable positioning of cells for observation during single-cell assays for pharmacological or biochemical activity (189, 219).

Aside from methods of physical separation or positioning, micromanipulation may permit the direct measurement of the physical or structural characteristics of an individual cell (Fig. 8). AFM and related force transduction technologies can be used to measure turgor pressure, elasticity, bursting force, and
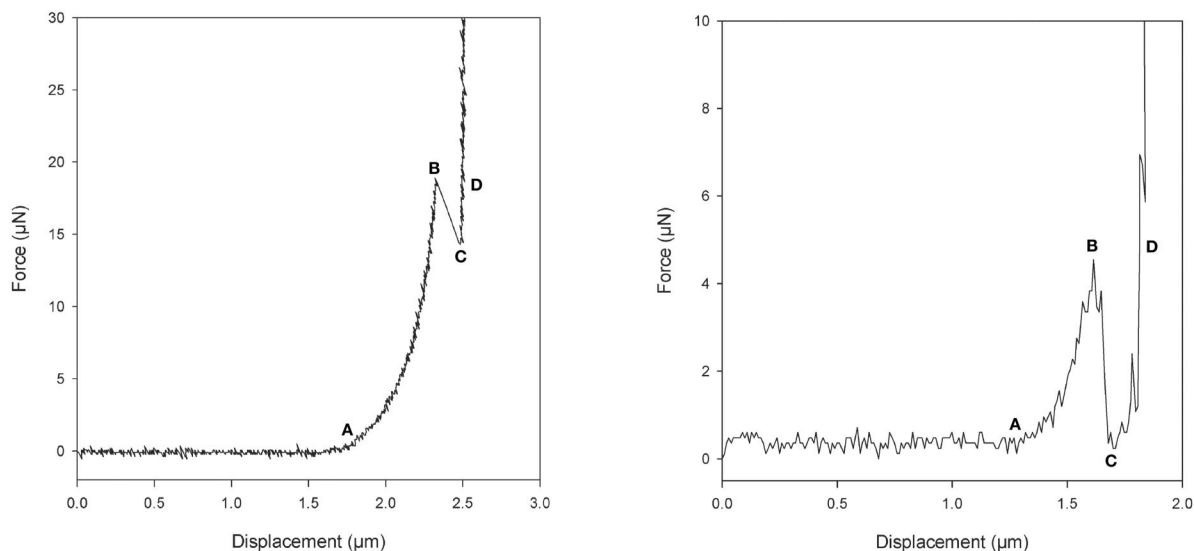
FIG. 8. Comparison of whole-cell bursting responses from *Staphylococcus epidermidis* (left) and *E. coli* (right), as determined by micromanipulation. Force diagrams for single cells compressed between the surface of a glass slide and an optical fiber are shown. In both diagrams, datum point A indicates the first contact of the microprobe with the cell and datum point B corresponds to the point at which cell rupture takes place. The microprobe continues to advance after cell bursting, eventually compressing the cellular debris (points C and D). To correlate bursting properties with cell size, a video image was taken of each cell prior to manipulation. Data kindly provided by C. Shiu, Z. Zhang, and C. R. Thomas.

other micro- or nanomechanical cellular properties (18, 213, 218, 257).

Methods of mechanical, optical, or electrokinetic micromanipulation can also be used to measure forces exerted by a microorganism on its environment, including motile power, pilus retraction forces, and the torque generated by an individual flagellar motor (53, 121, 159, 162, 200). Currently available methods for the manipulation of individual microbial cells are described briefly below.

**Mechanical micromanipulation.** The use of mechanical means of manipulation of single microbial cells is not a new concept. In 1951 Zelle (263) used a microscope-mounted mechanical micromanipulator to directly monitor the pedigrees of individual *E. coli* cells positioned on the surface of an agar-covered slide. However, the advent of computer-assisted stage or micromanipulator movement and more sensitive methods for fluid aspiration and deposition has led to improvements in the basic technology. Together, these improvements have resulted in more accurate, more accessible, and less exacting processes for the mechanical manipulation of microbial cells (81, 82, 83).

Current technologies enable the direct isolation of individual cells of interest from within complex natural populations. For example, Frölich and König (81) used a sterile capillary tube method to isolate individual cells of *Enterococcus* and *Sphingomonas* spp. from the diluted contents of a termite gut. Their procedure involved suspension and manipulation of cells in microvolume quantities of a cell transfer medium (phosphate-buffered saline). Dilution and microsuspension-based approaches to micromanipulation are probably less stressful to cells than are processes which result in the absorption of energy and heating (e.g., optical and electrokinetic methods).

Other mechanical methods, including AFM and methods for microprobe-based force transduction enable direct measurements of the physical or structural properties of individual cells at micro- and nanoscale resolutions (18, 213, 218, 233, 257). Although AFM has been used to estimate whole-cell properties such as turgor pressure (18, 257), it is particularly well suited to probing local mechanical properties such as cell stiffness and elasticity (69). Microprobe-based force transduction methods may also yield information on cell elasticity, deformability, and bursting strength, but they provide whole-cell rather than localized measurements (218). Finally, mechanical force spectroscopy can be used to characterize binding interactions between cells, shedding new light on cell-cell adhesion events important for multicellular development (31).

**Optical micromanipulation.** Although the Sun exerts a radiation pressure on the Earth's surface, its light is diffuse and the resulting pressure is negligible (129). However, highly collimated light sources, such as lasers, can exert a focused radiation pressure that is substantial enough to manipulate large particles, including microbial cells (129). As a result, optical forces can be used to trap, move, pull, twist, or cut individual cells (77, 137, 159, 192, 245). Optical manipulation may also be used to measure forces exerted by a microorganism on its environment. This is accomplished by measuring the laser power needed to displace a cell or by observing the ability of a cell to escape from a known trapping force (121, 159, 162). Because optical manipulation requires no physical contact, cells can be manipulated within enclosed glass chambers under sterile conditions (129).

Although most applications (e.g., trapping and moving) are relatively noninvasive, cell injury and death can occur from photodamage incurred during manipulation. Variables involved in photodamage include both the wavelength and power of the light source and environmental factors such as the presence or absence of oxygen (169). As a means of minimizing cell damage, optical trapping is usually carried out using wavelengths in the near-infrared (NIR) region, which do not coincide with absorption or excitation maxima for most biological

chromophores or fluorophores (129, 169, 189). Under certain circumstances, however, NIR optical sources can be used to elicit fluorescence, and NIR trapping may be used in conjunction with separate excitation sources (129). The ability to simultaneously trap or move microorganisms while monitoring their fluorescence can simplify fluorescence-based studies of otherwise motile microorganisms and can facilitate the harvesting of specific cells on the basis of their fluorescent properties (3, 77, 129). Additional applications of optical micromanipulation methods include the photorelease of caged compounds and the manipulation of specific organelles within cells (91, 129).

More invasive uses of optical micromanipulation include microsurgical applications, such as laser ablation of fungal hyphae prior to patch clamping, and insertion of individual. *Agrobacterium* cells into plant cells using a combination of "optical scissors" and "optical tweezers" techniques (43, 192).

**Electrokinetic micromanipulation.** Although electrorotation can be thought of as a microspectroscopic tool, it also can be used as a method of micromanipulation. As such, it provides a noncontact means of holding cells in place in a liquid medium. Once a cell has been immobilized in a field of view, its response to added nutrients, antibiotics, or fluorescent enzyme substrates may be monitored visually or with dielectric measurements (106, 165).

Like electrorotation, dielectrophoresis depends on the polarization of a cell exposed to an external electric field (e.g., formation of a whole-cell dipole) (111). The interaction between a polarized cell and a nonuniform electric field leads to the generation of unequal forces on opposing sides of the dipole, resulting in net movement of the cell (111). Depending on differences in polarizability between the cell and the surrounding medium, net dielectrophoretic movement may be either attractive ("positive" dielectrophoresis) or repulsive ("negative" dielectrophoresis) (111). As a micromanipulative tool, dielectrophoresis can be used to trap, move, separate, or concentrate cells based on their dielectric properties (111).

Other single-cell technologies may also incorporate methods of electrokinetic micromanipulation in order to separate or move individual cells. For example, in flow cytometry, cell sorting is accomplished through the electrostatic deflection of sheath fluid droplets containing cells of interest (4). Also, many microfluidic devices, such as the "cytometer-on-a-chip" described by Fu et al. (84), rely on electroosmotic flow to move cells during analysis. In electroosmotic flow, ionic movement in response to an electric field results in bulk fluid movement, representing an indirect means of electrokinetic manipulation of cells (264). Methods for the charge-based separation of whole microbial cells, which may also fall under the rubric of "electrokinetic manipulation," are described below.

## Microcapillary Electrophoresis

Charge-based microscale separations have long been a staple of analytical chemistry, and these methods are now being applied to the analysis of single microbial cells (15). Microcapillary methods can be used for either isoelectric focusing of whole cells or the electrophoretic separation of intracellular analytes from a single cell after lysis (15, 16, 133, 139, 211). Microcapillary separations are relatively rapid, and reactions
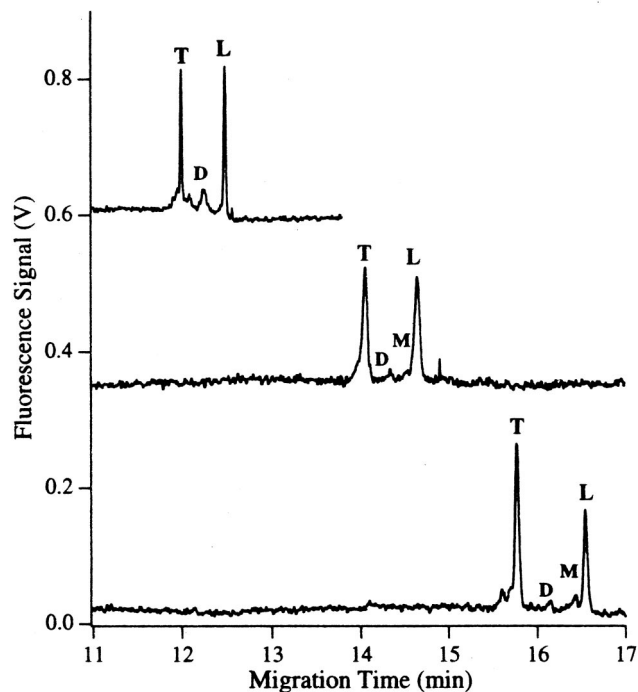


FIG. 9. Serial microcapillary electrophoresis of three individual *S. cerevisiae* spheroplasts. The labeled peaks (T, D, M, and L) represent, respectively, the original substrate and three different fluorescent hydrolysis products of a tetramethylrhodamine-labeled triglucoside. The hydrolysis products accumulated in each cell due to in vivo enzymatic activity. The larger peaks (T and L) contain between 500 and 1,000 molecules of each fluorescent analyte. To ease visual comparison, the electropherograms from the first two cells have been shifted upward on the y axis. This work demonstrates the capacity of microcapillary electrophoresis to analyze sequential metabolic reactions occurring in single microbial cells (e.g., "metabolic cascades"). Reprinted from reference 139 with permission from the publisher.

can be monitored using either UV absorption or fluorescence detection methods. Fluorescence-based monitoring can be used to observe whole-cell staining with diagnostic fluorescent dyes or to characterize metabolic transformations of fluorescent enzyme substrates in single cell lysates (Fig. 9) (16, 139, 211).

Whole-cell isoelectric focusing separates microbial cells on the basis of their surface properties (e.g., charge) (15, 211). Surface properties may vary with cell type, age, or physiological conditions and may also be altered by processes of differentiation or after exposure to antibiotics or chemical preservatives (211). Therefore, microcapillary methods may have wide-ranging applications in the study of single microbial cells.

By definition, separation methods are applied to cell populations, but the number of cells analyzed by some microcapillary methods may be very small (~3 to 15 cells per capillary) and single cells may be easily resolved (16, 211). Technical difficulties with whole-cell microcapillary separations may include the amphoteric nature of some cell types and problems with cell clumping, adhesion to capillary walls, or cell lysis (15, 134).

Microcapillary-based methods for performing biochemical separations of the contents of individual cells, including yeast,

have also been described (98, 132, 133, 134, 139). Individual cells may be delivered into a capillary tube by suction forces or electroosmotic flow, with monitoring via microscopy (134, 139). After a cell is loaded into the capillary, it is lysed to release the cell contents for analysis. The addition of surfactant may be sufficient to lyse mammalian cells, but thick-walled microorganisms such as *S. cerevisiae* may require spheroplasting first (134, 139).

Krylov et al. (133) found that if electrophoresis was begun immediately following cell lysis, enzymatic activity was effectively "quenched" and artifacts stemming from nonmetabolic enzymatic activity or other degradative processes were suppressed. Although this work was done on mammalian cells, it emphasizes the capacity of single-cell methods to provide high-resolution data and to avoid artifacts common to bulk-scale preparative methods. Individual cells and population-level extracts analyzed using the same method showed significantly different product distributions. In the population-level extracts, enzyme decompartmentalization stemming from the extraction process was responsible for nonmetabolic substrate degradation. The greater variability in peak heights seen between single-cell separations was attributed to the metabolic heterogeneity of individual, asynchronously grown cells (133).

### Biological Microelectromechanical Systems

The term "biological microelectromechanical systems" (BioMEMS) describes a family of devices that combine electrical, mechanical, chemical, and/or microfluidic approaches for the microscale analysis of biological materials (28). BioMEMS "chips" are capable of integrating several analytical steps (e.g., cell capture, concentration, addressing, and lysis, with subsequent extraction, purification, amplification, and detection of target analytes) within a single microscale device (56, 109). These devices may use pressure, acoustic energy, dielectrophoresis, or electroosmotic flow to exercise precise control over very small volumes of liquids. Steps such as the manipulation of cells, the introduction, mixing, and washing of reagents, temperature cycling, and analyte detection can be carried out sequentially within the same device (84, 109). Although the amount of analytical material handled by a single BioMEMS device is small, so are the amounts of potentially expensive reagents used (59).

Analytical chambers fabricated at near-cellular dimensions prevent the diffusive loss of analytes expressed by individual cells, allowing their measurement at low levels (59). While the need for enrichment of target cells prior to analysis is a major disadvantage of macroscale detection and diagnostic methods, BioMEMS and related microscale approaches may allow the capture and analysis of individual microbial cells, which may lessen or preclude the need for such enrichment. Multiple BioMEMS devices may be operated in parallel, and they are amenable to automation, presenting the possibility for continuous, high-throughput performance of analytical processes that once were the exclusive domain of highly trained personnel (109). Analytical methods that have been successfully translated to the microscale and could potentially be incorporated within a BioMEMS device capable of single-cell analysis include flow cytometry and cell sorting (84), PCR, various

isothermal methods of nucleic acid amplification (109), and nuclear magnetic resonance (28, 226, 236).

Microfabrication techniques commonly used for the construction of BioMEMS devices include silicon micromachining and lithography, chemical etching, laser ablation, photopolymerization, micromolding, and embossing (29, 33, 56, 84, 180, 245). These processes can be used to create the valves, channels, reservoirs, and other discrete microstructures critical to the function of BioMEMS devices and may also allow the incorporation of sensing or control elements such as microelectrodes or ion-selective field-effect transistors (59). Examples of actuators, or the "moving parts" of BioMEMS devices, include pH-responsive hydrogel valves, ferrofluidic micropumps (28), and even microrobotic "arms" fabricated from conducting polymer bilayers (114). Microrobotic devices such as these, which are capable of manipulating individual micronscale objects within an aqueous environment, could conceivably be used for the discrete positioning or transfer of individual cells between analytical stations within a BioMEMS device (114). More detailed information on BioMEMS components and their principles of action can be found in the comprehensive reviews by Beebe (28), Beebe et al. (29), and Huang et al. (109).

### CONCLUSIONS AND FUTURE PERSPECTIVES

Individual microbial cells may differ from each other in their genetic, biochemical, physiological, or behavioral properties. Recent advances in analytical methods and technologies have enabled microbiologists to resolve these individual cellular differences at unprecedented levels of detail. Methods capable of single-cell resolution have provided fundamental insights into the inner workings of microbes and their interactions with each other, with higher organisms, or with the environment.

This paper has reviewed some of the tools and technologies currently available for the study of individual microbial cells or structures, including bacteria, yeasts, protozoa, unicellular algae, and single fungal hyphae. Where applicable, we have also included relevant work on other microbiological subjects, such as mammalian sperm cells. We have sought to identify the most basic categories of instrumentation and analysis that form recurrent themes in the literature on single-cell microbiology and to group them here in a logical and accessible manner. In view of its importance to single-cell analyses, a limited amount of background theory on fluorescence has also been provided.

The availability of high-throughput sequencing methods and increased computing power has fueled a rapid pace of discovery in genomics, proteomics, and related fields. The knowledge gained in these areas holds promise for helping us control or direct the impact that microbes have on human life. Toward this end, access to genomic and proteomic data may ultimately result in a greater understanding of disease processes of microbial origin, reveal new drug targets, and provide clues to how we may maximize the biotechnological potential of industrially important bacteria and fungi. However, the ability to amass large volumes of data on selected microbes brings new challenges in ordering and understanding such information. We are almost exclusively reliant on the use of powerful computer-based methods for the collection and analysis of genomic, proteomic, and metabolomic information. It may

therefore be tempting to view these data merely as digitized abstractions to be compiled, annotated, and filed. However, the importance of the cellular context from which these data are collected is becoming increasingly apparent. The cell is the ultimate, irreducible unit of biological integration (P. J. Smith, http://www.isac-net.org/enews/Summer01/world.htm). Within the cell, information occurs and is regulated in multiple dimensions, including those of space and time (143, 148). Cell structure and informational content are intrinsically linked. The emerging field of cytomics (J. P. Robinson, http://www.cytomics.info) acknowledges this view and provides a framework for a more holistic outlook of the cell and its processes. The growth and maturation of this field depends on the continued development and application of sensitive single-cell measurement techniques, some of which are described here, as well as others not yet imagined. We are still only scratching the surface regarding the complexity of microbial cells. Therefore, we can expect that there will be much more to explore in the future of single-cell microbiology.

## AFTERWORD

Although the primary focus of this review has been on the technologies available for single-cell microbiology, we would be remiss if we did not also briefly mention some alternative approaches to this field. For example, mathematical modeling represents a powerful tool for describing single-cell processes. In particular, modeling can be used to probe the relationships between individual cellular properties and their impact on emergent macroscopic phenomena (112, 130, 199, 222). This can be of direct practical value in helping to understand, control, and improve microbial fermentations, in which individual cellular properties may be important determinants of bulk phase behaviors (222). At a more basic level, modeling can help explain how physical and chemical interactions between individual cells can give rise to complex and coordinated behaviors in populations (112, 199). Mathematical approaches cannot replace direct experimentation, but they represent an additional resource for testing hypotheses with an economy, speed, and flexibility that cannot be matched by "hands-on" biology.

Another important benefit of modeling lies in its predictive value. For example, the field of predictive microbiology uses mathematical functions to describe the fate of microorganisms in foods (157). However, most models for bacterial growth in food, as well as most experimental work in this area, are based on the use of relatively high inocula grown under homogeneous conditions (80). A more realistic scenario probably involves small numbers of contaminating bacteria that have been subjected to various physiological stresses such as starvation, heat injury, or osmotic shock (80, 160, 193, 225). At these low levels of contamination, a single cell could give rise to a population that could ultimately cause spoilage or disease. An understanding of the factors governing the recovery and growth of individual microbial cells is therefore important in more accurately describing the risks for the safety and shelf life of the food (40, 157, 225). Individually based modeling approaches, in conjunction with experimental evidence, can be useful in assessing these risks (130, 157). Although this review has focused primarily on "high-tech" methods of single-cell

analysis, much of the work done in bridging predictive modeling in food with experimental observation has been carried out using relatively "low-tech" tools such as turbidometry (80, 160, 193, 225, 255). In this approach, bacterial cultures are serially diluted to near extinction, yielding a high probability that individual wells of a microtiter plate will contain a single cell (80, 225). The microtiter plates are incubated, and optical density measurements are made automatically at regular intervals. Although the sensitivity of turbidometry is low ($\sim 10^6$ cells/ml), it is possible to derive lag times for individual bacterial cells from turbidometric detection times through mathematical extrapolation (160). This approach reveals that sublethally injured cells demonstrate a wide variability in individual cell lag times, an observation that may have important implications for our ability to detect low levels of pathogens in microbiologically heterogeneous samples by using traditional culture-based approaches (225).

## ACKNOWLEDGMENTS

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