DOMAIN 7 GENETICS AND GENETIC TOOLS

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Identifying Bacterial Lineages in *Salmonella* by Flow Cytometry

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ABSTRACT Advances in technologies that permit high-resolution analysis of events in single cells have revealed that phenotypic heterogeneity is a widespread phenomenon in bacteria. Flow cytometry has the potential to describe the distribution of cellular properties within a population of bacterial cells and has yielded invaluable information about the ability of isogenic cells to diversify into phenotypic subpopulations. This review will discuss several single-cell approaches that have recently been applied to define phenotypic heterogeneity in populations of *Salmonella enterica*.

KEYWORDS phenotypic heterogeneity, Salmonella, single-cell analysis, flow cytometry

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The existence of bacterial subpopulations exhibiting different phenotypes has been known for decades (1). More recent studies have described heterogeneous responses to antibiotics in a bacterial population (2), differential expression of flagellin in *Salmonella* (3), differential production of cytotoxin K in *Bacillus cereus* (4), or bistable expression of the *Salmonella* invasion system (5). Formation of bacterial subpopulations occurs both in the laboratory and in natural environments. Recent advances in single-cell technologies have stimulated studies aimed at uncovering the mechanisms responsible for such variability (6, 7). Indeed, one of the challenges faced by microbiologists is to develop tools to identify, measure, and quantify phenotypic subpopulations in order to be able to understand the biological consequences of bacterial heterogeneity.

Phenotypic heterogeneity involves phenotypic differences between genetically identical cells grown in homogeneous environments. In certain cases, this phenomenon reflects the occurrence of bistability, the formation of two subpopulations with distinct patterns of gene expression or phenotypic states (8). Bistable subpopulations can be considered reversible. When reversion is a programmed event, bistability is known as phase variation (9, 10).

Cell-to-cell phenotypic differences can be caused by nonuniform responses to environmental signals or be independent of the environment (11). The generation of distinct phenotypes within a clonal population of cells can occur by either

programmed epigenetic mechanisms (10, 12) or randomly, due to noisy gene expression (13, 14). Programmed genetic rearrangement (15), duplication of specific genome regions (16), and phase variation of contingency loci (contraction or expansion of DNA repeats) (16–18) can be another source of heterogeneity.

Here, we review the use of single-cell analysis in the characterization of bacterial populations. We describe how flow cytometry, a powerful single-cell technology, has yielded invaluable information about the heterogeneous composition of bacterial populations. The article is focused on the formation of bacterial subpopulations by *Salmonella enterica*, a pathogen of human and livestock animals and a model organism in bacterial genetics and pathogenesis. After reviewing some technical aspects of flow cytometry, we will discuss several single-cell approaches that have recently been applied to detect phenotypic heterogeneity within *Salmonella* populations.

FLOW CYTOMETRY AS A TOOL TO STUDY BACTERIAL LINEAGE FORMATION

Batch cultures and individual bacterial colonies have traditionally been employed by microbiologists to study cellular responses. Even though these classic assays can be very informative, they are unable to determine whether a response measured in a bulk experiment is representative for each individual cell. Single-cell phenotyping in bacteria faces the challenge posed by the small size of bacterial cells (bacteria are only 1/10 of the diameter and 1/1,000 of the volume of an average mammalian cell). Small size is thus a hurdle due to the limits of resolution of single-cell technologies (19, 20).

The simplest and most popular way to analyze single cells is by fixation on a glass slide, followed by visualization using light microscopy; nevertheless, the amount of information that can be collected with this procedure is limited compared to the possibilities using other techniques. In the last few decades, flow cytometry has become an especially powerful high-throughput technique that can detect and measure the properties of thousands of bacterial cells in a short time. Figure 1 shows a guideline on how to perform single-cell analysis in bacterial populations using flow cytometry, taking into account certain considerations and questions.

To perform an assay in a flow cytometer, a sample containing cells or particles is suspended in a fluid and injected into the instrument. In nature, the majority of bacteria grow in biofilms and colonies, and only a minor fraction is found as planktonic cells. However, single-cell analysis by flow cytometry requires suspensions of single cells. This may require the disintegration of aggregates and/or the detachment of cells from surfaces as a previous step before running the sample in a flow cytometer (21, 22). The sample must be well organized into a stream of single particles so that they can be individually interrogated by the detection system to collect information on the scattering of laser light (forward scatter [FSC] and side scatter [SSC]) and on fluorescent emissions from cells harboring specific fluorochromes (23).

Flow cytometry data analysis is set up upon the principle of gating. Although it can be a complex process, the process of gating in flow cytometry is simply selecting a region of interest on the plot generated during the flow experiment that decides which cells you continue to analyze and which cells you do not. Gating does not need to be a scary process, and by following just a few simple steps, you can quickly begin to analyze specific cell populations. Before starting flow cytometry experiments, if possible, it is a good idea to find out as much as possible about the cells and include the right controls. The controls will depend on the type of samples, but they can be simple things like having an estimation of the size of the cells or having a known marker in your cells, as it will help identify the cells of interest. A known negative can also help by allowing you to set negative gates and determine specific cell populations.

Types of cytometers: not only analysis but also sorting.

Flow cytometry analyzers, the cytometers most commonly found in research laboratories, are user-friendly and are able to monitor and quantify cellular properties (cell size, relative granularity or internal complexity, and fluorescence intensity). Even though analytical cytometers can identify different phenotypes in bacterial populations, understanding the function of each subpopulation often requires the isolation and collection of a subset of cells (Fig. 1). For this purpose, the application of cell sorting acquires great relevance (Fig. 2). The gold standard for cell separation is fluorescence-activated cell sorting (FACS) (24). The acronym FACS is the trade name used by Becton Dickinson for a specialized type of flow cytometer that provides a method for sorting a heterogeneous mixture of biological cells based upon the specific light-scattering and fluorescence characteristics of each

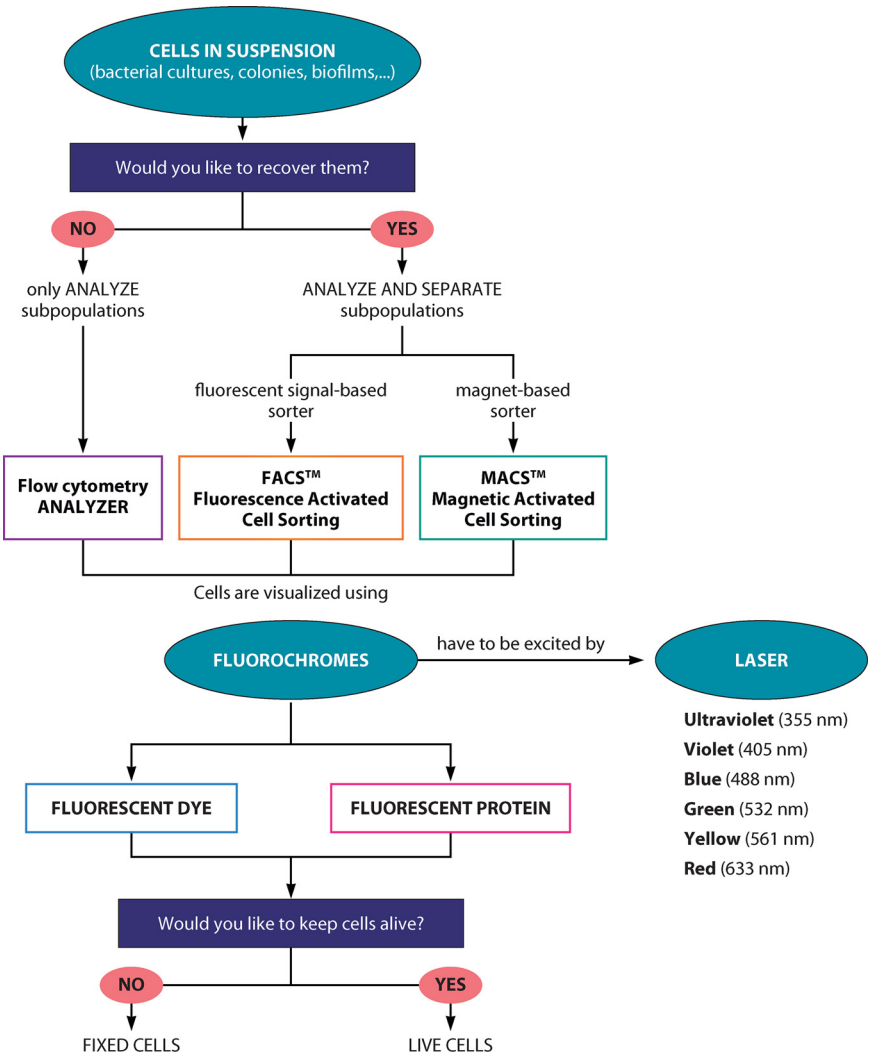


FIG 1 General workflow for analyzing single cells by flow cytometry. This guideline shows considerations to be taken into account to perform single-cell analysis in bacterial populations using flow cytometry. Analysis and/or separation of individual cells requires the use of cell suspensions. When the aim is to isolate and collect a group of cells with specific characteristics, it is necessary to employ a specialized flow cytometry instrument called a cell sorter. The type of cell sorter used will depend on the labeling of cells. If cells are labeled with fluorochromes, the fluorescence-activated cell sorter (FACS) should be used, whereas if the procedure includes a magnetic labeling of cells, the separation of cells must be done with a magnetically activated cell sorter (MACS). In order to visualize the properties of cells by flow cytometry, fluorochromes are used. Fluorochromes are chemical molecules that have the ability to absorb light of a certain wavelength and then reemit light at a longer wavelength and are used to visualize cells by flow cytometry. There is an extensive catalog of fluorescent products, and the fluorophore to be chosen depends on the experiment and on the instrument used to measure the signal. Fluorescent proteins are useful and versatile bioreporter systems to visualize cells *in vivo*. Fluorescent dyes require staining procedures and are usually targeted to proteins of interest by antibody conjugates. Fluorescent dyes have higher photostability and brightness than fluorescent proteins and do not require a maturation time. The choice of the type of fluorophore will depend on the experiment and the necessity of keeping cells alive. The most common excitation light sources in flow cytometry instruments are lasers. Flow cytometers typically contain one or more laser lines. The figure lists the most common laser lines available on today's flow cytometers (wavelengths are shown in parentheses).

cell. Other efficient sorters are the EPICS (electronically programmable individual cell sorter) from Coulter and the PAS (Particle Analysis System) from Partec. A related technology, known as virtual cell sorting, uses the force of magnetism to sort out cells, as in the case of the Miltenyi MACS (Magnetically Activated Cell Sorting) (25). This system relies on magnetic labeling of cells prior to separation in a magnetic field (Fig. 2). MACS is less time consuming

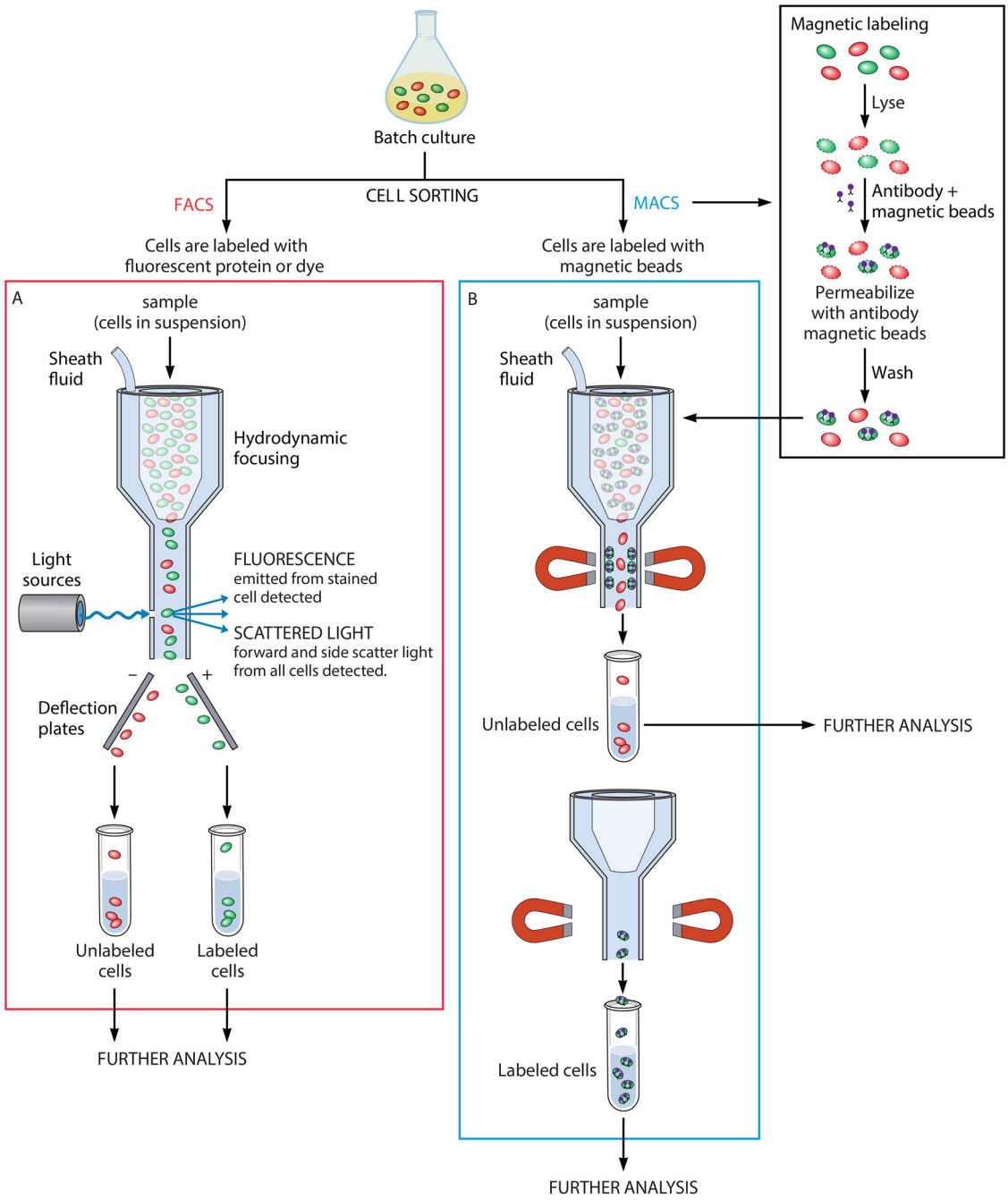


FIG 2 Flow chart of typical procedures for bacterial cell handling during separation by cell sorting prior to further analysis. Samples are grown in a batch culture to obtain a cell suspension. Subsequently, the cells are treated according to the respective protocols related to the type of cell sorting to be performed. (A) FACS requires that cells are stained with either a dye(s) or fluorescent protein(s), as fluorescence emission is essential for cell detection. (B) In MACS, cells are magnetically labeled with microbeads. The scheme shows an instrument equipped with a stream in an airflow chamber and hydrodynamic focusing. (A) Cell sorting of fluorescent cells is performed using a two-way-sort option. The positive and negative cells for fluorescence are separated simultaneously. (B) Cell sorting of magnetic labeled cells is executed using a magnetic separation procedure. First, cells labeled with magnetic beads are retained in the system, while unlabeled cells pass through (negative cell fraction). Second, the magnet is removed from the sample and the target magnetic-bead-labeled cells are eluted as the enriched, positively selected cell fraction. Eluted cells from FACS or MACS can be analyzed directly.

and requires less expensive equipment than FACS. However, it lacks the sensitivity and the cell specificity provided by a fluorescence-based system and is not easily compatible withs several simultaneous markers (26). Cell sorting can be used to isolate a large number of cells in a batch mode (often as a preenrichment step), rare cells from a complicated mixture, or even a single cell. All of these procedures use fast and real-time measurements to discern cell subsets, classify them, and make a decision on a cell-by-cell basis about which cells to collect. Cells can be collected into a tube, a well in a microtiter plate, or a chamber in a microfluidic device.

Fluorescence is one key to success in flow cytometry. A basic and useful procedure to characterize subpopulations in a bacterial culture is the use of single-cell reporters that rely on fluorescence (23). The ability of fluorochromes to absorb light of a certain wavelength and then reemit light of a longer wavelength is used to visualize cells by flow cytometry. The most common excitation light sources are lasers. Laser lights ensure that cells are illuminated with uniform light of a specific wavelength so that fluorochromes can absorb light and be excited. Flow cytometry typically employs one or more laser lines with different excitation lines across the spectrum.

An extensive catalog of fluorescent products exists, and the choice of fluorochrome depends on both the experiment to be done and the instrument used to measure the signal (27). In general, there are two classes of fluorochromes used in flow cytometry: those that bind to structures within the cell (fluorescent dyes) and those used as reporter molecules (fluorescent proteins) (Fig. 1). Visualization of biochemical or physiological differences from cell to cell by introducing a fluorescent probe requires complex staining procedures, a problem not found in bioreporter systems that use fluorescent proteins.

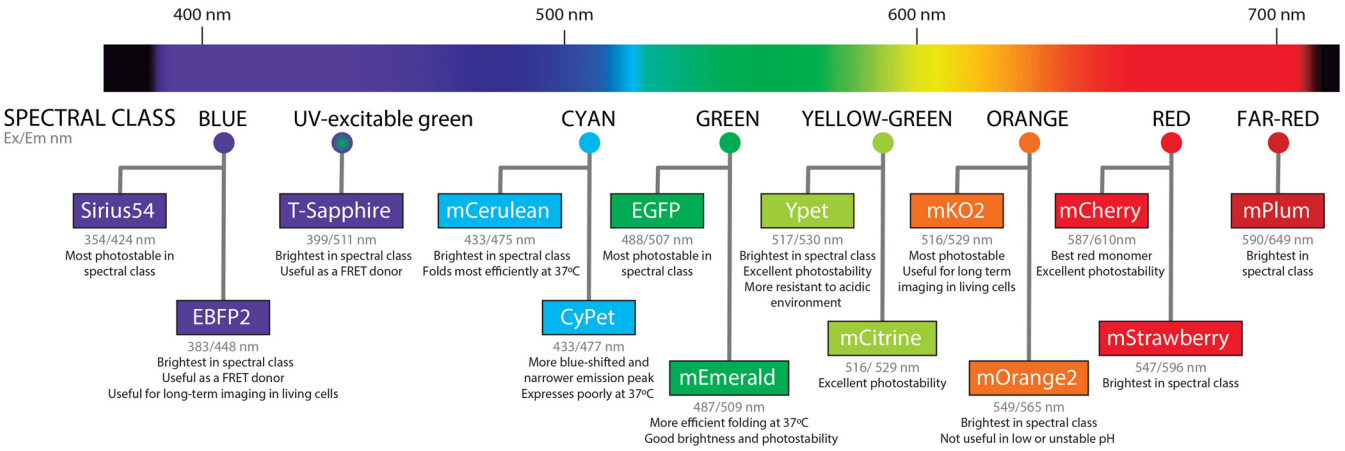
A wide variety of fluorescent dyes are commercially available (27, 28). Such dyes nonspecifically stain DNA (e.g., DAPI [4′,6-diamidino-2-phenylindole], ethidium bromide, and Hoechst stain), membranes/phospholipids (e.g., FM 4-64 and Nile red), or the cell wall (e.g., Grain staining or fluorescently labeled vancomycin) (29). Macromolecules, such as antibodies or nucleic acids, can be conjugated to a fluorescent dye to visualize protein localization (immunofluorescence) or to detect the presence of specific RNA or DNA species (fluorescence *in situ* hybridization [FISH]) (30). It is possible to modify specific properties (e.g., magnetism or density) using binding reagents (e.g., antibodies) for specific cell types to achieve antigen-specific bulk sorting. For example, antibody conjugates with magnetic particles are used in cell sorting with MACS (25).

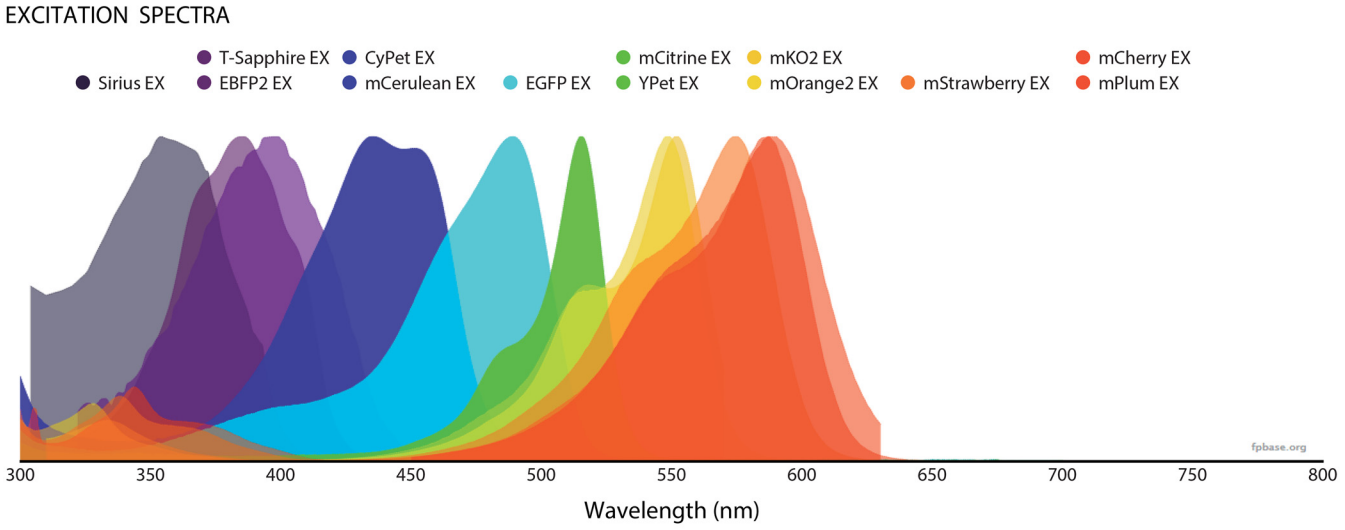
Fluorescent proteins are a versatile tool for *in vivo* visualization of protein expression and subcellular localization. Although the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* is versatile and widely used (31), the range of applications of fluorescent proteins has been expanded with the introduction of variants with different emission and excitation fluorescence spectra, fluorescence intensities, and stability characteristics (32). Figure 3 summarizes the characteristics of fluorescent proteins belonging to different spectral classes.

When designing multicolor staining panels for flow cytometry, one is limited to the use of fluorochromes compatible with the available flow cytometers. Therefore, the technical specificities of the cytometers will define the fluorochromes to be used.

Look now, analyze later. Characterization of bacterial-cell parameters at the single-cell level often requires fixation to store the cells and to keep them in a state as nearly life-like as possible for subsequent analysis (33). Procedures for fixation of bacteria to perform flow cytometry analysis should ensure morphological stability, counteract the increase of autofluorescence, and prevent cell aggregation. The most widely used fixatives for cells in suspension are alcohols and aldehydes (Table 1) (32, 33). Each fixative agent can have a different impact on the cellular content; therefore, the fixation method chosen may depend on the experiment and the information sought (Table 1).

Go one step further: IFC. The qualities of flow cytometry include high-speed analysis (3,000 to 20,000 events per second), multiparameter data acquisition and multivariate data analysis (up to millions of events), and cell-sorting ability. Therefore, the main advantage of flow cytometry compared to other techniques, such as fluorescence microscopy, consists in performing a large number of measures in an objective, rapid, and reproducible manner. In addition, the duration of analysis is shorter than that of





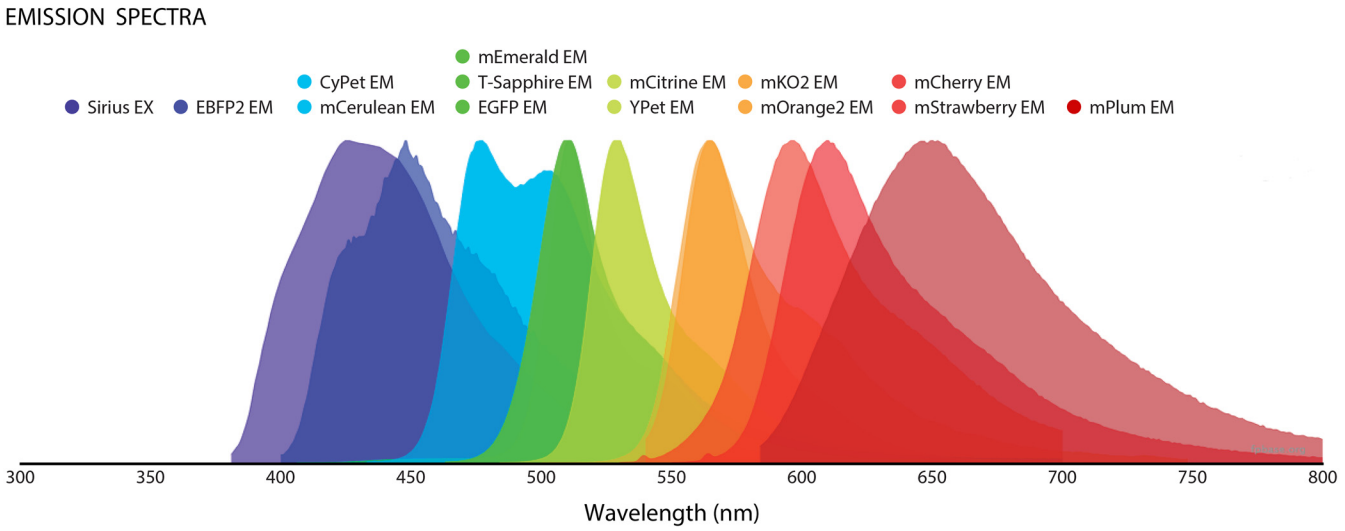


FIG 3 Properties of selected fluorescent protein variants by spectral class. Characteristics by spectral class related to brightness, photostability, oligomerization, and maturation that can help determine the optimal set of fluorescent proteins in each spectral class (blue,

(Continued on next page)

TABLE 1 Characteristics of the most common fixative agents used for bacterial cell fixation

Type of fixative agent	Permeabilization of cells	Compatible with fluorescent proteins	Stability	Most used	Undesired effects
Alcohol	Yes	No	Several weeks at 4°C	Ethanol	Increase cell aggregation
Aldehydes	No	Yes	2 to 3 days	Paraformaldehyde, formaldehyde	Increase autofluorescence

other techniques, thus contributing to avoiding the fluorescence bleaching effect. Its main limitation is lack of spatial information on the distribution of fluorescence in cells; that is, it cannot directly recognize the structures emitting fluorescence, unlike in optical microscopy. In this sense, imaging flow cytometry (IFC), a hybrid technique that combines the high speed and data acquisition of flow cytometry and features of fluorescence microscopy with data-processing algorithms, overcomes the limitations of conventional flow cytometry technology and complements its strengths. Imaging flow cytometry can quantify the spatial resolution of multiple cellular parameters (such as size, volume, shape contrast, spot count, and texture) in up to millions of events (34–36). As a representative example, Bisha and Brehm-Stecher analyzed the presence of *Salmonella enterica* subsp. *enterica* serovar Typhimurium in alfalfa sprouts, which are complex microbiological niches, to demonstrate the ability of IFC to unambiguously identify cells and cell aggregates within subpopulations (37).

APPLICATIONS OF FLOW CYTOMETRY TO CHARACTERIZE BACTERIAL SUBPOPULATIONS

Flow cytometry has the potential to resolve the properties of single cells and to characterize bacterial subpopulations. In this section, we will briefly describe how flow cytometry can help to unravel the structure of bacterial populations, using examples previously documented in *Salmonella enterica* (Fig. 4).

Flow cytometric assessment of bacterial viability.

Flow cytometric assessment of bacterial cell viability is widely used in the laboratory, with diverse applications, from assays that verify the "quality" of cells before starting an experiment to trials in which the response of the cells to antimicrobial agents is monitored.

The viability of bacterial populations can be assessed using fluorescent dyes that discriminate living and dead cells. Commonly used procedures are based on the assessment of membrane integrity; in addition, reagents that detect the activity of bacterial oxidases and reductases or the membrane potential are also employed (38, 39).

Functional live/dead cell trials are based on four main classes of dyes that detect loss of integrity of the cell membrane: (i) those than penetrate both intact and damaged cells (e.g., SYTO-9, Hoechst 33342, and acridine orange) (40, 41); (ii) those that are excluded by an intact membrane and only penetrate dead cells (e.g., Sytox blue, Sytox green, YOYO-1, TOTO-1, TOPRO-3, ethidium homidimer-1 [EthD-1], and propidium iodide [PI]) (42–44); (iii) nonfluorescent probes that are retained in the cell only if the membrane is intact and are converted into fluorescent compounds by enzymatic activities inside cells (e.g., fluorescein diacetate [FDA]); and (iv) those that label cells under specific physiological conditions with different charge-dependent distributions [e.g., rhodamine 123, membrane-potential indicator dye $DiOC_2(3)$, bis-oxonols, and carbocyanines] (45, 46). These compounds, which are commercially available as kits, can be used in combination and compared with each other.

Flow cytometric analysis of gene expression.

Gene expression of bacterial genes has traditionally been monitored using *lacZ* fusions (47–49). Nevertheless, *lacZ* reporters do not provide information at the single-cell level. The ability

FIG 3 Legend (Continued)
UV-excitable green, cyan, green, yellow-green, orange, red, and far-red). Excitation (Ex) and emission (Em) wavelengths are shown in gray below each fluorescent protein. Excitation and emission spectra of selected fluorescent proteins are displayed. FRET, fluorescence resonance energy transfer.

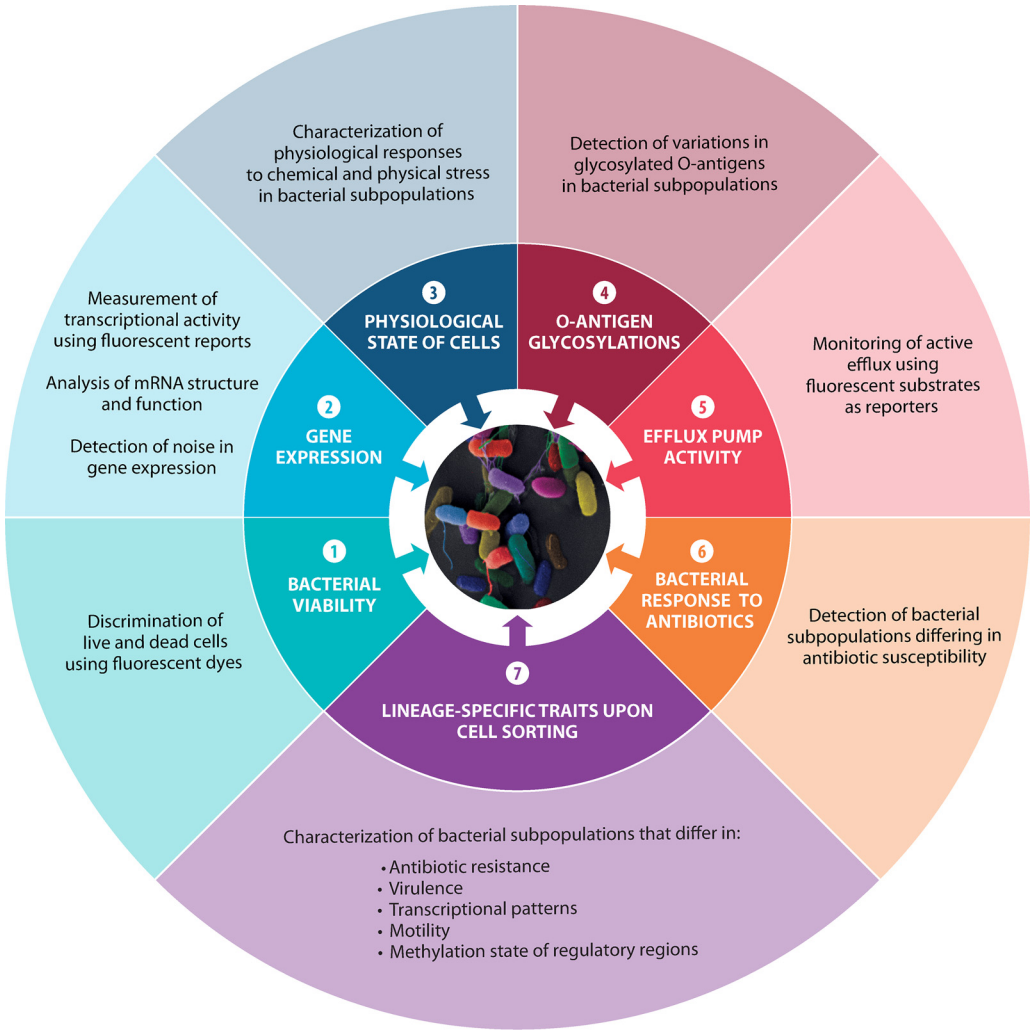


FIG 4 General overview of potential applications for resolving the properties of single cells and for characterizing bacterial subpopulations using flow cytometry. Center, colored scanning electron microscopy (SEM) images of a culture of *Salmonella enterica* cells growing under invasive conditions.

of flow cytometry to measure fluorescence has stimulated the use of fluorescent-reporter genes to analyze gene expression in individual bacterial cells within a population (50, 51).

Plasmid-borne *gfp* fusions have been widely used for monitoring gene expression. The method certainly permits easy detection of gene expression, but variation of the plasmid copy number is a potential problem. For this reason, integration of single-copy gene fusions into the bacterial chromosome was a relevant technological achievement that revolutionized monitoring of the activity of promoters (50). Indeed, fluorescent protein-based single-copy gene fusions can be used to determine quantitative expression in a more reliable manner than plasmid-borne fusions. The GFP variant harboring GFP_{uv} (GFP variant optimized for excitation by UV light) mutations F99S, M153T, and V163A along with the enhanced GFP [EGFP] mutations F64L and S65T is a fluorescent protein especially suitable for assessing bacterial gene expression because of efficient and enhanced brightness (52, 53).

GFP transcriptional fusions integrated into the chromosome of *Salmonella enterica* serovar Typhimurium have been extensively used for monitoring the regulation of virulence genes (5, 50, 54, 55). *Salmonella enterica* pathogenicity island 1 (SPI-1) and *Salmonella enterica* pathogenicity island 2 (SPI-2) are gene clusters that encode type III secretion systems and effectors involved in different stages of interaction with the animal host. Flow cytometry analysis of virulence genes revealed that both SPI-1 and SPI-2 undergo bistable expression, with concomitant formation of ON and OFF expression lineages (5, 50, 54–56). Bistable expression of

SPI-1 is likewise detected using plasmid-borne *gfp* fusions (56–58).

Dual fluorescence reporter plasmids are widely used to measure intracellular bacterial replication and growth during cell infection at the single-cell level using fluorescence dilution (59) and TIMER^{bac} approaches (60).

For decades, the *S. enterica* flagellar network had been studied from a variety of perspectives. Flow cytometry has helped to identify flagellated and nonflagellated subpopulations under the control of multiple regulators and has contributed to understanding the physiological significance of flagellar bimodal expression (54, 61–65).

Flow cytometry is also suitable for the study of bacterial interactions with host cells. As an example, Hara-Kaonga and Pistole studied the involvement of porins OmpC and OmpD in the adherence of *S. enterica* to human cells. For that purpose, they exploited a flow cytometry-based method to measure bacterial adherence to host cells based on direct fluorescent labeling of both bacteria and host cells (66). Flow cytometry was likewise useful in studying the involvement of fimbriae in the *Salmonella*-host interaction. Fimbriae are expressed in the mammalian intestine (67) and are involved in adhesion to animal tissues (68). However, fimbrial expression is often bimodal and only a fraction of cells is fimbriated in a bacterial population. Klasa et al. investigated the role of type 1 fimbriae (T1F) in the initial stages of *Salmonella* infection. Flow cytometry was used to measure the levels of T1F expression, confirming the existence of fimbriated and nonfimbriated cells in a clonal population and the correlation of increased levels of T1F expression with higher levels of *Salmonella* cell adherence to intestinal epithelial cells (69).

Many bacteria live in nature as aggregates inside the extracellular matrix of a biofilm (70). Biofilms are composed of specialized subpopulations with different characteristics (71), and flow cytometry has been successfully utilized to analyze phenotypic heterogeneity during biofilm formation. For instance, Grantcharova et al. detected bistable expression of the biofilm master regulator, CsgD, during biofilm development (72). This bistability leads to a task distribution, as CsgD^{ON} and CsgD^{OFF} subpopulations show distinct physiological characteristics (73).

A quality of flow cytometry is the ability to detect small subpopulations, and the *S. enterica opvAB* and *std* operons provide two examples. The *std* locus of *S. enterica* encodes fimbriae that permit adhesion to epithelial cells in the large intestine. The expression of the *std* operon is bistable, yielding a major subpopulation of Std^{OFF} cells (99.7%) and a minor subpopulation of Std^{ON} cells (0.3%) under laboratory conditions (74). In turn, *opvAB* encodes two proteins involved in the control of O antigen chain length (75). Phase variation of *opvAB* splits *Salmonella* populations into two phenotypic variants: OpvAB^{OFF} cells (99.8%) with a full-length O antigen and OpvAB^{ON} cells (0.2%) with a shorter O antigen (76). Note that the existence of a subpopulation with such a small size would be completely overlooked in bulk culture studies and difficult to detect by microscopy.

Monitoring *porin* gene expression by flow cytometry in individual *S. enterica* cells revealed a wide range of fluorescence intensities (77) and correlated heterogeneous *ompC* porin expression with nongenetic antibiotic resistance (see details below).

Flow cytometry has also been used to investigate mRNA structure and function. For instance, López-Garrido et al. used flow cytometry and a *gfp* reporter to investigate the role in gene expression of an mRNA 3′ untranslated region (UTR) (78). For many years, the existence of 3′ UTRs in bacterial transcripts had been ignored, and the study provided an example indicating that 3′ UTRs can be functional. In the case of the *hilD* 3′ UTR, a role in mRNA stability and turnover was shown (78).

Flow cytometry has also contributed to understanding transcriptional control by DNA adenine methylation. The expression patterns of loci harboring undermethylated GATC sites potentially involved in transcriptional control have been investigated using transcriptional fusions with the *gfp* gene. The trials were carried out in several genetic backgrounds (the wild-type strain, a *dam* mutant, and a strain carrying the *dam* gene on a multicopy plasmid) (79), and single-cell analysis helped to weigh the contribution of the DNA adenine methylome to phenotypic heterogeneity.

A major manifestation of the power of GFP and other fluorescent proteins is the possibility of using them as reporters of dynamic events occurring in living cells. GFP labeling and flow cytometry enable direct observation of microorganisms and monitoring of bacterial subpopulation variations over time. For instance, flow

cytometry analysis has been employed to observe how a *Salmonella* population containing a subpopulation of phage-resistant OpvAB^{ON} cells varies in the presence of phages. Indeed, phage challenge yielded mostly *S. enterica* cells with high levels of *opvAB* expression (76). Another application of flow cytometry to the study of living cells was the analysis of patterns of SPI-1 expression along the growth cycle. The use of *sipB*::GFP, *prgH*::GFP, and *hilA*::GFP fusions permitted quantification of the subpopulation of SPI-1^{ON} cells and its correlation with the ability to invade epithelial cells (5).

Stochasticity ("noise") in gene expression can be a source of phenotypic variation among individual cells within a clonal population. Flow cytometry has helped to identify and understand this phenomenon. Freed et al. developed a method to identify genes with high levels of phenotypic noise in *S. enterica* serovar Typhimurium (80). A genomic plasmid library fused to a GFP reporter and fluorescence-activated cell sorting (FACS) were used to analyze the levels of phenotypic variation of multiple promoters of *S. enterica*. Their observations indicated that promoters associated with synthesis of flagella, virulence, and host-pathogen interactions showed the highest levels of noise in the *Salmonella* genome (80, 81). An advantage of using fluorescent protein markers is the possibility of performing more than one test simultaneously (for example, using green and red fluorescent proteins). For instance, Elowitz et al. undertook this approach to distinguish between two sources of noise that contribute to the generation of phenotypic diversity among individual cells (82). Single-cell analyses based on flow cytometry and reporters expressed at different levels were used to discriminate intrinsic noise (from events directly related to expression of a gene) and extrinsic noise (from fluctuations in the activity of proteins/enzymes required for gene expression) (82).

Flow cytometric analysis of the physiological state of cells. Flow cytometry can be useful to characterize different physiological responses to chemical and physical stresses in bacterial subpopulations. As an example, Schulte et al. described a set of fluorescence protein-based reporter strains that were used to monitor the expression of cytoplasmic or periplasmic stress response systems in single *Salmonella* cells in the cytoplasm of epithelial cells (83). This study revealed distinct intracellular subpopulations with different levels of stress responses and proliferation.

A study that aimed to investigate the physiological functions of individual *Salmonella* cells exposed to *Mentha arvensis* and *Mentha piperita* essential oils revealed a heterogenous response. Monitoring of membrane integrity, membrane potential, efflux activity, and respiratory activity by flow cytometry revealed that *Mentha* essential oils disturb respiration and efflux pump activity and damage the bacterial cytoplasmic membrane (84).

Flow cytometry has been also employed to characterize the physiological response of bacteria to simulated gastric acid fluid. Cultures of *Salmonella* and *Shigella* exhibited physiologically heterogeneous responses under such conditions. Intact, stressed, and damaged cells were stained with viability dyes and analyzed by flow cytometry (85).

Flow cytometry has also been applied to investigate the role of regulatory networks in coping with bacterial stress and in detecting and responding to ecological competition. A screening designed to search for loci with differential fluorescence induction within a biofilm with competing strains of *Salmonella* and *Escherichia coli* identified genes showing bimodal expression. A tentative explication is that bimodality allows bacteria to respond quickly to competitors as a bet-hedging strategy (86).

Single-cell approaches based on flow cytometry have been used to determine the impact of oxidative and nitrosative stresses on individual *Salmonella* cells during cell infection (87).

Flow cytometric analysis of O antigen glycosylation. The O antigen of lipopolysaccharide (LPS) and the flagella are major antigenic factors of Gram-negative pathogens. O antigen diversity, which is used to classify *Salmonella* bacteria into serovars as part of the Kaufmann-White scheme, derives from differences in the carbohydrate composition and structure of the basal units of the O antigen (88). Variations in glycosylated O antigens are related to virulence, immune evasion, persistence, and immunogenicity. Therefore, O serotyping is a tool for monitoring the epidemiology and spread of the disease. Multiple genes have been described to be involved in the biosynthesis of O antigen (76, 89–92), providing structural heterogeneity by adding additional sugars, modifying units, or altering linkages on the O antigen component of the lipopolysaccharide. Schmidt et al. used flow cytometry to detect variations in glycosylated O antigens and to distinguish different phenotypes

in a *Salmonella* population. *Salmonella* phage tail spike protein-based assays that measured glucose 1-6 linked to the galactose of O polysaccharide were employed for discriminating multiphasic cultures differing in their glycosylation state (93).

Assessment of efflux pump activity by flow cytometry. Bacteria employ active and passive mechanisms to protect themselves against toxic and hazardous compounds. Cell membranes and cells walls act as passive barriers, while efflux pumps provide an active defense mechanism. Bacterial efflux pumps can extrude a wide range of compounds, such as antibiotics, antiseptics, heavy metals, solvents, detergents, animal hormones, and host defense molecules. Even virulence factors can be exported by efflux pumps (94–96). Flow cytometry has been adapted to monitor the activity of efflux pumps in bacterial cells, using molecules that are a substrate of the efflux pump and whose concentration is detected by measuring fluorescence. Efflux activity can be detected directly or be measured indirectly upon the intracellular accumulation of a substrate that acts as a reporter of efflux activity. The dyes most commonly used as substrates are Hoechst H33342 and ethidium bromide (77, 94, 97–100). Single-cell analyses have revealed heterogeneous efflux pump activity in adaptation to bile (99) and in antibiotic resistance (77). Monitoring either efflux or accumulation by flow cytometry can also be employed to screen for molecules that act as efflux inhibitors as potential future antimicrobials (77, 101).

Flow cytometric analysis of bacterial response to antibiotics. Advances in single-cell analyses have inspired novel approaches to the study of antibiotic resistance. Bacterial subpopulations can exhibit different susceptibilities to a particular antibiotic. Indeed, cellular responses to an antimicrobial agent are often heterogenous, with the formation of subpopulations that survive exposure to potentially lethal antibiotic treatments by mechanisms such as persistence, heteroresistance, and/or tolerance (102–106). The formation of heteroresistant, persistent, and/or tolerant bacterial cells can contribute to failure of antibiotic treatment in hospitals. In this context, the traditional bulk assays to determine antibiotic susceptibility are outdated, and single-cell studies are required to examine the response to antibiotic agents. Survival of antibiotic challenge in bacteria can be studied from different points of view at the single-cell level, including (i) monitoring antibiotic susceptibility by measuring membrane permeability to dyes or changes in the membrane potential or in the metabolic activity upon antibiotic exposure (107, 108), (ii) tracking bacterial proliferation and activity by using a single-cell growth rate reporter for pathogens in infected host tissues (60, 109, 110), (iii) analyzing the expression levels of critical genes (77, 111), and/or (iv) measuring efflux activity as a nongenetic component that may increase the MIC (Minimum Inhibitory Concentration) of antibiotics in bacterial subpopulations (77).

Analysis of lineage-specific traits upon cell sorting. As described above, flow cytometry has the potential to delineate the distribution of cellular properties within a clonal population. Flow cytometry coupled to cell sorting can also be applied to the detection and isolation of bacterial subpopulations with different cellular properties. Table 2 shows several *Salmonella* loci in which bistable expression produces phenotypic lineages (Table 2).

In this section, we describe the contribution of flow cytometric cell sorting to characterizing *Salmonella* bacterial subpopulations that differ in antibiotic resistance, virulence, motility, and other phenotypic features.

(i) Antibiotic resistance. Flow cytometry combined with cell sorting was used to assess the susceptibility of subpopulations with different *ompC* porin expression levels to kanamycin. FACS was used to sort fractions of the whole population with low and high fluorescence intensities (low and high porin expression), and the cells were plated on medium containing kanamycin. With this simple assay, an increase in antibiotic resistance was found to be correlated with a decrease in *ompC* expression, confirming the existence of a kanamycin-resistant subpopulation produced by a nonmutational mechanism (77).

(ii) Virulence assays. As described above, SPI-1 shows bistable expression with concomitant formation of SPI-1^{ON} and SPI-1^{OFF} lineages. Sánchez-Romero and Casadesús employed the FACS technology to separate SPI-1^{OFF} and SPI-1^{ON} cells and performed epithelial cell invasion assays with the separated subpopulations (5). This study revealed the unsuspected feature that both SPI-1^{OFF} and SPI-1^{ON} *Salmonella* cells are necessary for optimal invasion. Another surprising observation was made when GFP and mCherry

TABLE 2 Loci showing phenotypic diversity in *Salmonella* bacteria and the roles associated with lineage formation

Role	Locus	Function	Mechanism for lineage formation	Role of ON and OFF subpopulations	Reference(s)
Bacteriophage resistance	*gtr*	Lipopolysaccharide O antigen glycosylation	Dam methylation dependent	Example of trade-off: the Gtr^{ON} state is crucial for intestinal persistence and fecal shedding of *Salmonella* cells but reduces invasion of both epithelial cells and macrophages	116
	opvAB	Modification of lipopolysaccharide O antigen chain length	Dam methylation dependent	Example of trade-off between bacteriophage resistance and virulence: the OpvAB^{OFF} subpopulation is phage sensitive and the OpvAB^{ON} lineage is phage resistant; however, OpvAB^{ON} cells are sensitive to killing by serum, unable to proliferate in macrophages, and show reduced virulence in the mouse infection model	76
Host colonization	*pef*	Adhesion to the intestinal epithelium	Dam methylation dependent	No single-cell analysis has been performed	
	std	Adhesion to the intestinal epithelium	Dam methylation dependent	The nonfimbriated lineage contributes to acute infection and the fimbriated lineage to chronic infection	74
	fim	Adhesion to the intestinal epithelium	10-bp inverted repeat sequence in the *fimA* promoter	Cross talk between *fim*, flagellar genes, and SPI-1	63, 117
	lpf	Invasion of the intestinal epithelial mucosa at Peyer's patches	Unknown	No single-cell analysis has been performed	
	SPI-1	Type III secretion system and effectors involved in human epithelial cell invasion	Unknown	SPI-1^{ON} subpopulation synthesizes the machinery for epithelial cell invasion and shows retarded growth but higher resistance to antibiotics; SPI-1^{OFF} is required for a successful infection	
Biofilm formation	*csgD*	Biofilm master regulator	Unknown	The CsgD^{ON} lineage is made of aggregated cells, whereas the CsgD^{OFF} is made of planktonic cells; CsgD^{OFF} cells are more invasive and motile than CsgD^{ON} cells, but	118, 119

(Continued)

TABLE 2 Loci showing phenotypic diversity in Salmonella bacteria and the roles associated with lineage formation *(Continued)*

Role	Locus	Function	Mechanism for lineage formation	Role of ON and OFF subpopulations	Reference(s)
				$CsgD^{ON}$ cells express extracellular matrix components and curli fimbriae, are more resistant to desiccation, and have high levels of cyclic di-GMP which represses virulence and motility	
Motility	*fliC*	Flagellin	Regulatory proteins FliZ and YdiV are required for bistable expression	Motile and nonmotile subpopulations	54, 61

fluorescent proteins were used to simultaneously monitor OFF and ON states in SPI-1 and in the flagellar (Flag) regulon (54). Independent switching was detected, and the ability of FACS to sort four subpopulations (SPI-1^{OFF} $Flag^{OFF}$, SPI-1^{OFF} $Flag^{ON}$, SPI-1^{ON} $Flag^{OFF}$, and SPI-1^{ON} $Flag^{ON}$) allowed the conclusion that $Flag^{OFF}$ cells might contribute to optimal invasion, as previously proposed for SPI-1^{OFF} cells (54).

(iii) Host immune responses. One of the best-studied cellular models of the host-pathogen interaction is the infection of macrophages with *S. enterica*. FACS technology combined with single-cell RNA-sequencing analysis has helped to characterize the gene expression variation that triggers diverse infections and monitor infection phenotypes. These studies demonstrated a link between host and bacterial variability that allows different host immune responses and the establishment of a long-term niche (111, 112).

(iv) Transcriptional patterns of small subpopulations. When certain genes are only expressed in a minor subpopulation of cells, the phenotypic traits of this gene expression can be difficult to evaluate. Cell sorting based on flow cytometry can be used to isolate and enrich very rare cells from a mixture for further analysis. For instance, MACS technology was employed for magnetic separation of Std^{ON} and Std^{OFF} cell lineages (the Std^{OFF} subpopulation represents about 0.3% of cells). The levels of transcription were then monitored by quantitative real time-PCR, confirming the occurrence of lineage-specific transcriptional patterns (113).

(v) Analysis of methylation state of regulatory regions. Flow cytometry can help to investigate the methylation state of promoters and regulatory regions and its effect on gene expression. For instance, the methylation state of the upstream activating sequence (UAS) of the *std* fimbrial operon was analyzed *in vivo*. As previously described, the expression of the *std* operon is bistable and *Salmonella* populations split into two phenotypic variants, Std^{ON} and Std^{OFF} subpopulations. After separation of Std^{ON} and Std^{OFF} lineages by MACS, their genomic DNAs were isolated and digested with enzymes that cut GATC sequences depending on their methylation state (MboI and DpnI). The methylation state of the *std* UAS was then inferred from quantitative real time PCR analysis after digestion with MboI and DpnI enzymes (113).

(vi) Motility assays. The occurrence of lineage-specific traits of the *std* operon was also analyzed to study whether the fimbriated and nonfimbriated *Salmonella* lineages may differ in motility. Std^{ON} and Std^{OFF} cell lineages were separated by FACS, and the sorted Std^{OFF} and Std^{ON} cell subpopulations were used to perform motility assays on soft agar plates. These assays revealed that Std^{ON} cells showed reduced motility, as predicted by transcriptomic analysis (74).

CONCLUSIONS AND FUTURE PERSPECTIVES

Single-cell analysis technologies have radically changed how we conceive of the structure of bacterial populations. Numerous studies have described bacterial heterogeneity in

diverse environments and how microbial populations exploit this biological diversity to expand their phenotypic landscape. Efforts from the fields of synthetic biology, engineering, and molecular biology nowadays allow researchers to not only monitor but also manipulate the properties and behaviors of individual cells, with a variety of purposes. In this regard, in conjunction with its high sensitivity and reproducibility, the ability of flow cytometry to monitor and characterize bacterial subpopulations makes it a powerful tool in the biosensor field. For instance, a portable epigenetic switch based on *opvAB*, a *Salmonella enterica* operon that undergoes bistable expression under Dam methylation control (described previously), can be functional in a heterologous host and has potential application in the detection of bacteriophages with different types of receptors (114) and in the study of heteroresistance to antibiotics (115). Flow cytometry-based assays have thus demonstrated in recent years their potential to expand our understanding of the behavior of bacterial populations and have provided insights into the functional roles of microbial cell-to-cell heterogeneity.

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