

Flow cytometry for bacteria: enabling metabolic engineering, synthetic biology and the elucidation of complex phenotypes

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Flow cytometry (FC) and FC-based cell sorting have been established as critical tools in modern cell and developmental biology. Yet, their applications in bacteria, especially in the multiparametric mode, remain limited. We argue that FC technologies have the potential to greatly accelerate the analysis and development of microbial complex phenotypes through applications of metabolic engineering, synthetic biology, and evolutionary engineering. We demonstrate the importance of FC for elucidating population heterogeneity because of developmental processes or epigenetic regulation. FC can be engaged for both synthetic and analytical applications of complex phenotypes within a single species, multispecies, and microbial-library populations. Examples include methods to identify developmental microbial stages associated with productive metabolic phenotypes, select desirable promoters from a single species or metagenomic libraries, and to screen designer riboswitches for synthetic-biology applications.

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Current Opinion in Biotechnology 2010, **21**:85–99

This review comes from a themed issue on
Analytical biotechnology
Edited by Peter Neubauer and Andreas Schmid

0958-1669/\$ – see front matter
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DOI [10.1016/j.copbio.2010.02.006](https://doi.org/10.1016/j.copbio.2010.02.006)

Introduction

What is flow cytometry (FC): to measure the detailed properties of individuals in a population, and sort the individuals according to desired properties

Flow cytometry (FC) is a flow-based method that enables the simultaneous measurement of multiple physical and chemical/biological characteristics of single particles, which typically are cells. On the basis of the principle of hydrodynamic focusing, the flow cytometer fluidics system transports particles in a fluid stream, one cell at a time at high speed, to a laser beam for the interrogation of particle properties. At the laser intercept, one or multiple

laser beams illuminate the cells in order to measure their light scattering properties and to excite fluorescent molecules. Suitable lasers can excite naturally fluorescent cellular molecules and fluorescent molecules specifically employed to tag or probe (stain) various cellular components or processes. The optics system also includes lenses, beam splitters, and filters which are used to direct the incident light scattered by the cells, or the emitted fluorescent light that results from the cell illumination, to light detectors. Light signals captured by these detectors are converted into electronic signals by the cytometer's electronic system, which then filters and processes these signals using computer-based algorithms. Cell/particle properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity of the specific fluorescent molecule(s) being interrogated. Fluorescent dyes can be used to directly or indirectly (e.g. using antibodies) label cellular components such as DNA, surface proteins/receptors, intracellular structural proteins and enzymes, specific nucleic acids (NAs), membrane properties and ion fluxes, secreted proteins or small molecules, and cell organelles. While not all of these have been widely applied in bacteria, the potential of such applications is becoming increasingly apparent.

The flow cytometer can be also equipped with a cell-sorting capability for Fluorescence-Activated Cell Sorting (FACS) analysis. While the term FACS is a registered trademark of the Becton-Dickinson Company, it is widely used in a generic way by the scientific community. Sorting can be based on different methods of capturing the cells, and sorting decisions can be based on one or multiple cellular properties. Cells (single, a few, or in thousands to millions) thus collected in tubes or wells can be used for further assays, microscopy, and molecular and functional assays. If the staining method allows, cells can be cultured for further assays or enrichment.

FC and FACS: from applications in cell and developmental biology and medicine to microbial systems

Applications of FC and FACS are abundant for identifying and sorting cells with desirable properties for basic studies in cell and developmental biology, as well clinical applications [1,2]. These applications have facilitated the development of FC/FACS instruments, fluorescent probes, and methods to label cells, analyze the fluorescent signals and sort cells to achieve a desirable analytical or clinical outcome. However, this technology still remains

relatively underused for applications in microbial and especially bacterial systems. A profound review in 2000 [3**] discussed the core issues facing the application of FC/FACS to bacterial systems: small cell size, reduced per cell content of proteins, NAs and other biological molecules, and lack of experience in bacteria in applying probes developed for higher eukaryotes. Furthermore, bacteria membranes are less permeable to fluorochromes than mammalian cells, and many fluorochromes can be pumped out since many bacteria have efficient efflux pumps. Nevertheless, FC applications in bacteria systems have been developed significantly in the last few years and we argue that FC/FACS is poised to make major inroads in microbial physiology, genetics, and biotechnology.

FACS analysis for screening protein libraries to power protein engineering

The goal of this review does not include FACS applications in protein engineering and protein screening more broadly, but a brief summary of the FACS impact on this important field is warranted. Application of FACS for interrogating protein–protein interactions, selecting for ligand-binding proteins, and quantifying enzyme activities have been reviewed [4]. The advent of microbial surface display of polypeptide libraries in conjunction with FACS has greatly improved screening of ligand-binding protein libraries, and had a large impact on the discovery and characterization of immunological molecules. Microdroplet compartmentalization [5] using water–oil–water systems coupled with FACS has become an invaluable tool for screening enzyme libraries for improved activity and selectivity. By encapsulating single cells in their own microenvironment, the connection between plasmid-borne gene expression, the encoded enzyme mutant variant, and the resulting fluorescent product are preserved, and can be sorted for enrichment, further analysis or subsequent rounds of mutagenesis.

Population heterogeneity and why individuals matter: metabolism, differentiation, epigenetic regulation, and the crucial role of FC

In any cell population, large or small, even when cell-cycle synchronization (for eukaryotes) has been attained, individual cells can still exhibit heterogeneity for many reasons. For example, small differences in gene expression of core or secondary sigma and transcription factors can occur that are often caused by cell-to-cell communication by secreted small molecules (as in quorum sensing), and can cause this heterogeneity. Thus, it is possible, and in fact frequently true, that the average cell behavior is not representative of the entire population. Cell differentiation and morphogenesis are also a source of population heterogeneity, whereby small culture or environmental variations may result in large compositional changes in the differentiation stage of individuals in a population. This is well documented in bacilli,

whereby sporulation (the differentiation leading to endospore maturation) is widely accepted to be a bistable process with two distinct populations of cells (sporulating and nonsporulating) coexisting [6*]. In bacilli, this bistability is generally and simply explained by the different levels of activation of the master sporulation factor Spo0A as a result of the phosphorylation process that leads to Spo0A activation [6*]. In clostridia, the sporulation process is even less synchronous and more variable than in bacilli, and while this has been known for many years, application of FC analysis in a recent study of *Clostridium acetobutylicum* sporulation [7**] demonstrates a much more heterogeneous, differentiation-wise, population of cells. This is a form of multistability, which, in microbial systems, is defined as the simultaneous, metastable coexistence of two or more distinct cellular phenotypes arising from clonal populations [8]. Similar population heterogeneity appears in other differentiating prokaryotes (e.g. in streptomycetes and more broadly actinomycetes) and fungal systems.

Because metabolite production is frequently differentiation/morphogenic-stage dependent, differentiating-population heterogeneity can lead to variable metabolic outcomes, which has several practical and fundamental implications. First, since some metabolites may impact cell differentiation, it is possible that an initial population heterogeneity may be further amplified because of differential metabolite production. Second, for biotechnological applications, population heterogeneity needs to be eliminated or suppressed if the metabolite-producing phenotype is a relatively small fraction of the total population, which is frequently the case for both secondary and primary metabolites. Secondary metabolites are low MW organic compounds generally produced at a restricted phase of the life cycle, and often in association with differentiated morphological structures. In fungal systems, as well as in antibiotic-producing prokaryotes such as streptomycetes, the impact of the cellular state of differentiation on the formation of secondary metabolites such as mucotoxins, terpenes, alkaloids, and peptide or polyketide antibiotics, is well documented and broadly understood. Similarly, several primary metabolites are associated with a restricted differentiation state of the cell, such as in the case of solvent production by anaerobic butyric-acid clostridia [7**]. Thus, cell differentiation and morphogenesis, and the associated population heterogeneity, in both eukaryotic (e.g. fungal) and prokaryotic systems (e.g. in bacilli, clostridia, and streptomycetes) profoundly impact the formation of primary and secondary metabolites. The practical implications of this population heterogeneity are significant, and have been well documented such as in the case of the industrial erythromycin production, whereby the lack of proper differentiation and morphogenetic control of the producing actinomycete *Saccharopolyspora erythraea* may result in unproductive fermentations [9]. From the biotechnological point of view, when selecting

or engineering strains for such products, beyond pathway engineering, one needs to consider what differentiation/morphogenetic state is the most productive. Additionally, it is important to consider if that state can be autonomously sustained, if the product can be produced in a continuous or semi-continuous fashion, and if the 'unproductive' differentiation cellular states can be eliminated. In this context, FC/FACS becomes a crucial tool to identify, study and perhaps reclone the productive cellular states and phenotypes. This has been recently demonstrated in the identification of the most productive, for butanol formation, cellular phenotype in *C. acetobutylicum* [7**]. The conclusion was that the clostridial-cell forms, characterized by granulose formation, are not the solvent-producing phenotype, and rather the predominant solvent formation phenotype is a vegetative-like cell type. This analysis and approach are discussed later in more detail.

Probes and labeling techniques for FC analysis of bacteria: from viability to complex metabolic and biophysical microbial traits

FC allows for the measurement of a variety of phenotypic characteristics of individual microorganisms by measuring an array of cellular properties. These properties can be classified as intrinsic or extrinsic. Intrinsic refers to traits that can be directly measured without labeling the cells, and often include Light Scattering (LS) properties of cells. Side scatter (SSC, large angle scatter) intensity routinely relates to the internal granularity of the cell, while forward scatter (FSC, small angle scatter) often, but not always, provides information on cell size and shape. Measurements of extrinsic parameters typically use fluorescent stains or fluorescence labeled probes to assay cellular characteristics and components such as DNA content, membrane potential, and expression of surface and intracellular proteins. The following sections briefly discuss commonly employed microbial FC dyes, more of which are listed in Table 1.

Nucleic-acid assays

Many FC reports analyze the NA content of individual cells and the distribution of cells within a population in order to identify NA containing particles, to interrogate the proliferation state of cells, and to determine membrane integrity. NA dyes vary predominately in their selectivity for staining DNA, RNA or both (i.e. lack of selectivity), selectivity for A–T versus G–C rich sequences, and in their permeability to bacterial cell membranes. For example, cell membrane impermeant NA dyes include the phenanthridinium dyes [i.e., ethidium bromide (EtBr) and propidium iodide (PI)] and fluorescently labeled antibiotics chromomycin, olivomycin, and mitramycin [10]. EtBr and PI stain both DNA and RNA while chromomycin, olivomycin, and mitramycin are selective for DNA [11]. Discrimination between DNA and RNA can be performed with cell membrane permeant dyes of the Syto family, which stain both DNA

and RNA, and Hoeschst family dyes, which are selective for DNA and are membrane permeant [10]. Additionally, chromomycin, olivomycin, and mitramycin exhibit increased affinity for the 2-amino group of guanine, while Hoeschst dyes and DAPI bind preferentially to repetitive A–T regions of DNA, thus distinguishing between G–C and A–T rich DNA [11], respectively. On the basis of these properties, NA dyes can be used in many applications. Membrane permeant dyes can be used to discriminate NA containing particles from nonbiological material. DNA specific, cell membrane permeant dyes such as Hoeschst dyes can be used to assess DNA content, which suggests ploidy and cellular division [12]. G–C versus A–T preferential binding NA dyes can distinguish between G–C rich and A–T rich genomes in a mixed species sample or culture. Lastly, NA staining by cell-impermeant dyes, commonly PI, suggests a compromised cell membrane, which is often inferred to be a nonviable cell.

Membrane functions, intracellular pH, and general metabolic activities

Beyond NA staining, an assortment of functional probes exists for assaying microbial membrane potential, intracellular pH, and metabolic activity within the cell. In order to generate ATP and operate the large repertoire of membrane functions, cells must maintain a membrane potential, which results in polarized cells that exhibit a positive charge on the outside and a negative charge on the inside of the cytoplasmic membrane. Lipophilic, cationic dyes can be used as estimators of cell membrane potential because they can readily cross the lipid portion of the cell membrane, and cationic properties attract them to the negatively charged cytoplasm. Thus, single-cell fluorescence intensity either increases with uptake of the dye, or a spectral shift occurs as dye aggregates form because of increased concentration in the energized cell cytoplasm. It is important to note that Gram[−] bacteria outer membranes can present a barrier to lipophilic dye uptake, but this can be overcome and cell viability can be maintained with mild treatment of a chelating agent such as Tris–EDTA [10]. Available membrane potential dyes are well reviewed by Shapiro [10], and a few important ones are listed and discussed in Table 1.

The intracellular pH and membrane Δ pH are also central to cellular homeostasis, and desirable traits to measure by FC on an individual cell basis. Dyes such as carboxyfluorescein diacetate (cFDA), biscarboxyethyl carboxyfluorescein (BCECF), and SNARF dyes exhibit pH-dependent spectral shifts in excitation, emission or both, thus ratiometric analysis of fluorescence between the spectral shift renders an estimate of intracellular pH [10]. Many of these dyes and in particular their ester forms, such as carboxyfluorescein diacetate succinimidyl ester (cFDA-SE), are cell permeant. Upon entering the cytoplasm, the dye is converted into an impermeant form

Table 1

Overview of commonly used fluorescent probes to assay structural as well as functional characteristics of bacteria

Physiological characteristic	Probes (examples)	Mode of action	Applications or examples	Reference
Functional parameter assaying viability or more accurately the physiological state of the cell				
Nucleic-acid content, base ratio and membrane integrity	<p>Permeant dyes</p> <ul style="list-style-type: none"> - Syto family (e.g., Syto9) - Thiazole orange (TO) - Hoechst dyes (e.g., Hoechst 33342) <p>Impermeant dyes</p> <ul style="list-style-type: none"> - Rhodamine 123 - Ethidium bromide - Cell-impermeant Sytox - Propidium iodide (PI) 	<p>The dyes intercalate with double-stranded nucleic acids and can be detected based on fluorescence. Some dyes are sensitive to A + T/G + C ratio of DNA. For example, DAPI and Hoechst bis-benzimidazole dyes 33258 and 33342 increase their fluorescent intensity while binding to A-T triplets.</p> <p>Integrity is assayed by the uptake of an impermeant dye. In combination with a permeant dye, they can be used to counter stain intact cells.</p> <p>See also enzyme activity for assaying membrane integrity.</p>	In principle these are used to test for cell viability, but this does not work well for all prokaryotes. They can still be used to distinguish various cellular states by differential staining.	[57-59]
Membrane potential	<p>Cationic cyanine dyes</p> <ul style="list-style-type: none"> - DiOC₂(3); DiOC₆(3) <p>Anionic oxonol dyes</p> <ul style="list-style-type: none"> - DiBAC₄(3) - Rhodamine 123 (cationic) 	Depending on the charge of the probe and the potential of the cell membrane, the probe (a charged lipophilic molecule) accumulates to a larger or smaller extent or not at all in the cell thus emitting a different fluorescent signal. Cationic dyes accumulate in polarized cells, anionic dyes accumulate in depolarized cells.	Membrane potential is used as a test of viability, since viable cells should have an intact electrochemical membrane potential. Measurement of the membrane potential is an important assay as well.	[60]
Membrane fluidity	1,6-Diphenyl-1,3,5-hexatriene (DPH)	Higher amounts of DPH intercalate into membranes with higher fluidity, leading to increased fluorescence.	Measuring membrane composition changes with changes in the physiological state of the cell, with or without stresses.	[61]
Enzyme activity	<p>Dehydrogenase activity</p> <ul style="list-style-type: none"> - Tetrazolium dye, like 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) <p>Esterase activity</p> <ul style="list-style-type: none"> - Fluorescein diacetate (FDA) - Acetoxymethyl ester (calcein-AM) - Carboxyfluorescein diacetate (CFDA) - Carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) - 2,7-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy methyl ester (BCECF-AM) 	<p>In general, a nonfluorescent permeant substrate is taken up by the cell and converted inside the cell to a fluorescent substance (ideally impermeant). For example, CTC is converted by dehydrogenases to the insoluble fluorescent substrate formazan (impermeant).</p> <p>Testing for enzyme activity is also used to assay membrane integrity. When cells have a compromised membrane, impermeant end products will be quickly lost to the extracellular space, thus resulting to a decreased fluorescent signal.</p>	<p>Viability of endospores</p> <p>Malolactic activity of individual <i>Oenococcus oeni</i> cells</p> <p>Evolutionary engineering of enzymes</p> <p>Test and assess acid stress</p>	<p>[57]</p> <p>[44]</p> <p>[13]</p> <p>[14**]</p>

Table 1 (Continued)

Physiological characteristic	Probes (examples)	Mode of action	Applications or examples	Reference
	Phosphatase activity - 6,8-Difluoro-4-methylumbelliferyl phosphate (DiFMUP)	Test of dehydrogenase activity in principle correlates with respiratory activity. Test for esterase activity is a more generalized assay for viability.		
	Sugar metabolizing enzyme activity - Fluorescein di- β -D-galactopyranoside (FDG), - Fluorescein di- β -D-glucopyranoside (FDGlu), - Fluorescein di- β -D-glucuronic (FDGluU)			
Pump activity	- Rhodamine 123 - Ethidium bromide - Biscarboxyethyl carboxyfluorescein (BCECF) - Carboxyfluorescein (CF)	Different dyes are loaded into bacteria cells. Cells that can actively pump out these (and other) 'drugs' will display decreased fluorescence from these dyes.	Test of viability and also general or specific pump activity.	[43]
Intracellular pH (pH _i)	- Carboxyfluorescein diacetate (CFDA) - Carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) - Biscarboxyethyl carboxyfluorescein (BCECF) - BCECF acetoxymethyl ester (BCECF, AM) - SNARF dyes	The fluorescent characteristics of this probe change depending on the pH value. In order to measure the intracellular pH, the probe is loaded (e.g. with the permeant BCECF, AM ester) and then exposed to altered extracellular conditions. The fluorescent signal from the probe inside is proportional to the internal pH. Some organism can have active pumps to exclude Carboxyfluorescein; see pump activity.	Test of viability. Also, measurement of internal pH and how it changes with culture changes.	[62,63]
Measurements of physical or structural parameters of a cell, including surface proteins				
Morphology	Forward (FSC) and side scatter (SSC)	Analyze the shape as well as the granularity of the cell.	Follow the sporulation/ differentiation process of endospore-forming organisms like <i>C. acetobutylicum</i> .	[7**,64,65]
Gram staining	- Hexidium iodine (Gram ⁻ impermeable) - Oregon green (with germ agglutinin) stains Gram ⁺	-	Discriminate mixed populations of Gram ⁺ and Gram ⁻ cells.	[66]
Cell surface and antibiotic-binding sites	Antibiotic-binding sites (e.g., penicillin-binding proteins) - Labeled antibiotics (e.g., Bocillin labeled penicillin, BODIPY labeled polymyxin) Surface polysaccharides/glycosylation: - Labeled lectins (e.g., Concanavalin A)	Antibiotic-binding proteins, like penicillin-binding proteins, can effectively bind antibiotics, which can be detected if they are conjugated to a fluorophore.	Screening of enhanced PHB-producing bacterial strains.	[19]
	Membrane lipids - Nile red - Styryl dyes (e.g., FM 4-64 or FM 1-43 from Molecular probes)	Lectins are oligomeric proteins with saccharide-binding sites, which can selectively bind to polysaccharides present as lipopolysaccharide on the surface of Gram ⁻ bacteria. These proteins can be conjugated to fluorophores and therefore used as a specific probe for surface polysaccharides of bacteria.	Population analysis of mixed bacterial populations. Labeling of active cell-wall biosynthesis sites with BODIPY FL vancomycin.	[61] [14**]
			Detection of penicillin resistance in bacterial cells.	[17]

Table 1 (Continued)

Physiological characteristic	Probes (examples)	Mode of action	Applications or examples	Reference
Immunofluorescent techniques	Primary as well as secondary fluorescent labeled antibodies (e.g., labeled with fluorescein isocyanate (FITC), phycobiliproteins (allophycocyanin (APC), phycoerythrin (PE)) or cyanine dyes like Cy3, Cy5 or Cy7, or ALEXA dyes)	Cellular compounds can be directly labeled through fluorophore conjugated primary antibodies or through the use of a nonfluorescent primary antibody and a second fluorescent antibody directed against the primary antibody.	Examination of antigenic surface components of viable but nonculturable (VBNC) cells.	[21]
			Surface localization of antigens.	[22]
Sulfhydryl groups/glutathione	Monochlorobimane (mBBR)	mBBR diffuses passively into cells and reacts with glutathione to generate a fluorescent product.	Screening of a cDNA library of glutathione transferases.	[67]
Reactive oxygen species (ROS)	- Dihydroethidium - Dihydrorhodamine 123 - 2',7'-Dichlorofluorescein diacetate (DCFH-DA)	Probes react with ROS species leading to changes in their fluorescent characteristics, which can be measured.	Detection of ROS species as stress indicators.	[68]
Transcription and protein biosynthesis				
Transcription	Fluorescent labeled RNA molecules (FLOW-FISH)	Short probes (fluorescently labeled RNA molecules) are taken up by the cells and anneal to complementary RNA molecules (rRNA or mRNA).	Quantification of distinct species in a mixed population	[31]
	Transcriptional fusions to GFP	Transcriptionally fused GFP is only expressed if the target sequence is transcribed, whereby possible influences of the translation of GFP through the target sequence are avoided.	Screening tool to find species with desired genes.	[30]
Expression	Green fluorescent protein (GFP) as well as its derivatives can be used as an expression reporter assay (only for cells growing under aerobic conditions, as it requires O ₂ to form a fluorescent signal). Evoglow [®] protein can be used as a reporter fluorescent protein for anaerobic conditions (fluorescence is not dependent on the presence of O ₂)	The gene of interest is either transcriptionally or translationally fused to the GFP, or other fluorescing protein, so that the expression level of GFP correlates either to the level of transcription or expression (transcription and translation).	Biosensors for quorum sensing Promoter library analysis.	[69,25**]
			Detecting heterologous gene expression.	[26**]
	- 5-Acetylaminofluorescein di-β-D-galactopyranoside (C ₂ FDG) - 3-Carboxyumbelliferyl-β-D-galactopyranoside (CUG)	Expression levels can be related to the amount of fluorescent signal emitted by GFP or Evoglow [®] proteins.	No flow cytometry applications have been reported so far with Evoglow [®] , but are certainly possible.	[28]
- FIAsH-EDT2 - ReAsH-EDT2	β-Galactosidase activity is assayed by measuring the fluorescent signal deriving from its action on the substrates C ₂ FDG or CUG.	Detection of heterologous gene expression.	[70]	
	Both probes (FIAsH and ReAsH) become fluorescent after binding to a specific amino acid tag (Cys-Cys-Pro-Gly-Cys-Cys) of the recombinant protein.	No application in combination with flow cytometry in bacterial systems yet, but in principle possible.	[71]	

by nonspecific esterase hydrolysis, thus capturing the dye in the cell [10]. Additional intracellular pH indicator dyes are outlined in Table 1.

As demonstrated with the pH indicator dye cFDA-SE, enzymatic activity can be assayed in single cells by FC. Several enzyme activities that have been measured by FC and the dyes involved are included in Table 1. Ideally, a nonfluorescent, permeant substrate enters the cell, is enzymatically converted into a fluorescent, impermeant substance, and accumulates within the cell where enzyme activity is present. If the product of these enzymatic reactions diffuses out of the cells, cell encapsulation can be used where the cell is captured in an agarose droplet or inside droplets using an oil–water–oil emulsion [13]. Thus, a physical link between the cell exhibiting enzyme activity and the fluorescent signature are preserved.

Multiparametric FC (MPF) to assess viability and improve the discrimination of physiological states

The combination of NA, membrane potential, intracellular pH, and enzyme-activity dyes can provide great insight into the physiology of individual and populations of bacterial cells. NA dyes are commonly used with membrane potential dyes, such as PI and DiOC₆(3), to provide insight into membrane integrity and potential. Viability, defined as cells that can form a colony on an agar plate [11], is often inferred from the exclusion of PI and maintenance of membrane potential. The combination of NA and enzyme-activity dyes allows for the discrimination of additional physiological states that cell plating cannot distinguish. For example, FC is now routinely employed to distinguish viable but not culturable cells [11], which exhibit an intact membrane and enzymatic activity, but do not form colonies on plates. As discussed through examples later, one may employ dye cocktails for MPF detection of NA staining, membrane potential, esterase activity and even cell-wall biosynthesis [14^{••}] to distinguish physiological states that provide fundamental insight into complex phenotypic traits. These MPF assays have significant potential to identify and develop strains exhibiting complex phenotypic traits, such as solvent-tolerant strains that exhibit superior biophysical membrane properties in view of the fact that a major mechanism of tolerance to solvents derives from altered membrane properties [15]. Future applications with cell sorting will provide powerful tools for industrial-strain development.

However, it should be noted that not all dyes are applicable to all species or even broad classifications, such as Gram⁺ versus Gram⁻ species. For example, bacterial efflux mechanisms can make cells appear impermeable to dyes that they are actually permeable to [11], and even commonly employed probes such as PI, can exhibit different permeabilities that are not fully

understood [16]. Thus, careful protocol development and appropriate controls are necessary for extracting reliable and biologically relevant information from microbial FC experiments.

Assays for surface and intracellular components of the cell

A wide variety of probes exist to target and assay various specific cellular components. These include surface molecules such as surface/membrane proteins and intracellular components. Antibiotics, such as penicillin and polymyxin, can be conjugated with fluorophores and used to detect antibiotic-binding sites (e.g. penicillin-binding proteins (PBP)) on the cell surface [17], and such binding could be related to antibiotic resistance and cell-wall biosynthesis [14^{••}]. Fluorescently labeled lectins, which are sugar-binding proteins with high affinity for the lipopolysaccharides (LPS) on the outer membrane of Gram⁻ bacteria, can be used to selectively label the surface of Gram⁻ versus Gram⁺ bacteria [18]. Cellular lipids can be stained with the solvatochromic dye Nile Red, whose fluorescent signal is quenched in hydrophilic environments, but exhibits a strong fluorescence in hydrophobic ones [19]. Similar fluorescence behavior is exhibited by lipophilic styryl dyes, like FM 1-43 or FM 4-64, which have been shown to preferentially stain the inner membrane of Gram⁻ bacteria [20].

Surface antigens/proteins [21,22] and inclusion bodies [23] can be stained through immunofluorescent techniques. This involves the binding of an antibody directed against the target compound or an epitope on a protein that could capture all forms of a specific protein or specific protein modifications like phosphorylation and acetylation. Labeling can be either direct using fluorescent primary antibodies, or indirect using a secondary fluorescent antibody directed against the nonfluorescent primary antibody. In order to access intracellular targets, the cells have to be gently permeabilized, such as through the use of lysozyme. Although few applications of intracellular immunostaining have been reported for bacteria cells [23], the potential is great as new small synthetic antibodies are being developed.

Fluorescent proteins for promoter and protein expression studies: aerobic and anaerobic

Noninvasive labeling techniques to assess protein expression by FC are commonly based on reporter gene assays or protein tags involving green fluorescent protein (GFP) or one of its derivatives [24]. Depending on the genetic construct used, GFP expression can reveal information about the transcriptional activity of a promoter or about the expression level of a specific protein. To assay for transcriptional activity, the GFP gene is engineered for expression as a self-contained transcriptional and translational unit with its own ribosomal binding site and the promoter sequence or library of promoter sequences

desired to be assayed [25^{••}]. When assaying expression level of a specific protein, GFP is translationally fused to the protein of interest [26^{••}], such that resulting GFP signal relates to the expression of the specific protein.

However, GFP and its derivatives cannot be used for the analysis of anaerobic organisms because of the strict requirement of molecular oxygen to form the fluorescent signal. To overcome this issue, a technique called aerobic fluorescent recovery (AFR) was developed to facilitate the use of anaerobically expressed GFP in *Enterobacter aerogenes* [27]. Plasmid-coded GFP was expressed after induction with isopropyl β -D-1-thiogalactopyranoside (IPTG) under anaerobic conditions resulting in a nonfluorescent form. GFP fluorescence was then induced by aerobic cultivation of the cells, where GFP fluorescence detection could be performed in less than 10 min. This technique can only be applied to aerotolerant or facultative anaerobic organisms. Recently, however, fluorescent proteins (the 'Evo-glow[®]' family) have been developed, which do not require oxygen to generate a fluorescent signal, and can therefore be used as reporter proteins for FC assays in anaerobic organisms [28].

FLOW-FISH: screening for nucleic acids and their impact FISH (Fluorescent *In Situ* Hybridization) enables the identification of cells containing specific nucleic-acid sequences, and can be successfully extended to FC applications, commonly referred to as FLOW-FISH. Whole cells are labeled with short nucleic-acid oligomers conjugated with a fluorescent molecule like FITC. These oligomer probes hybridize to their target RNA [29] or DNA [30] sequences in the cell and form a stable complex. Oligomers targeting the 16S-rRNA were used to enumerate cells of three closely related species of a mixed starter culture of lactic acid bacteria [29]. In another study, FLOW-FISH was used as a screening tool to find hydrogen-producing clostridial species [30]. The labeled oligomer probe was designed to target genes coding for specific clostridial hydrogenases. Thus, FLOW-FISH can be used as a rapid screening tool to identify organisms with desired mRNA characteristics [30] or to quantify distinct species in a mixed population [31]. It is possible then to use FLOW-FISH to examine the regulation or stability of specific mRNAs and the expression of noncoding RNAs, and also sort cells based on such expression characteristics. Thus, identification of cells in a heterogeneous population that are expressing specific mRNA species at different levels is in principle feasible. This might prove especially useful in the context of understanding the epigenetic regulation of heterogeneity. Moreover, it may provide a faster and more molecularly detailed means for deconvoluting such complex phenomena, and for assessing their impact on cell metabolism, persistence, and differentiation (see above).

MPF to analyze, sort, and develop microbial complex phenotype

The term complex phenotype is not only commonly used to describe traits in human and eukaryotic systems often associated with disease, but also increasingly used to describe prokaryotic phenomena such as endospore formation [32], chemical, metabolite or osmotic-stress response and tolerance [33], motility, and chemotaxis [34]. Complex phenotypes cannot often be accurately predicted from knowledge of individual effects or individual factors considered alone, regardless of how well understood each separate component may be. FC, and in particular MPF is an ideal tool for simultaneously assaying multiple phenotypic and physiological characteristics of single cells and of entire populations. Moreover, MPF can be used with FACS to isolate subpopulations or individual cells based upon an arsenal of traits for subsequent analysis and subculturing. Surprisingly, so far, MPF has not been extensively used for analyzing microbial complex phenotypes, but has tremendous potential for providing fundamental physiological insights into such phenotypes and for harnessing their medical and industrial potential. In the following sections, we review a select subset of significant contributions of MPF for analyzing and manipulating microbial complex phenotypes. Table 2 summarizes these contributions.

Bioprocess development, monitoring and control: metabolism, physiology, and identification of productive cellular states

Bioprocess monitoring is one of the earliest applications of MPF to the analysis of microbial complex phenotypes. There are a number of MPF applications for developing, monitoring, and controlling industrial-scale microbial bioprocesses. MPF assays have been developed to distinguish physiological states of cells between modes of fermentation (batch, fed-batch versus continuous) [35[•]], under conditions of substrate starvation [36], under varying bioprocessing conditions (dilution rate, agitation rate) [37[•]], and upon induction to express recombinant proteins [38]. For example, a light scattering (LS), PI, and Bis-oxonol (BOX) assay was used to assess single-cell physiology and culture heterogeneity during fed-batch, batch, and continuous culture cultivations [35[•]]. Comparison between the three modes of cultivation revealed that cells could not withstand glucose limitation in fed-batch operation because of very high cell densities, which were not encountered in batch and continuous cultures. MPF was also used to monitor the physiological effect on host cells when induced to express a model mammalian protein AP50 [38]. By using the same PI and BOX dye mixture, it was shown that the loss of cell membrane potential and cell viability correlated well with AP50 expression induction. Cells producing a protein that did not generate inclusion bodies did not exhibit viability loss, thus suggesting that loss of viability in the AP50-

Table 2

Application	Specific topic	Stains/probes	Phenotypic or physiologic trait assayed	Reference
Bioprocess development, monitoring or control	Comparing modes of fermentation	DiOC ₆ (3) and PI	Membrane potential and permeability	[36]
	Automated process control	FSC, PI, FITC and GFP	Size, membrane permeability, total protein and product	[41]
	Processing parameter effects	DiOC ₆ (3) and PI	Membrane potential and permeability	[37*]
Analysis of metabolite and general stress physiology	Substrate, product and by-product stress	Bis-oxonol and PI	Membrane potential and permeability	[42]
	Organic acid stress	Carboxyfluorescein diacetate (cFDA), PI, DiBAC ₄ (3) and BODIPY FL vancomycin	Membrane potential and permeability, esterase activity (metabolic activity) and cell-wall biosynthesis.	[14**]
	Solvent stress	Carboxyfluorescein (cF)	Efflux capability.	[44]
	Prolonged stress response	Syto9 and PI	Membrane permeability.	[47]
	Induction of recombinant protein expression	GFP-fusion and PI	Protein expression and correct folding and cell membrane permeability.	[26**]
Fundamental analysis of microbial complex phenotypes	Cell adhesion and toxin production	cFDA, PI, SYTOX Green, C ₁₂ -resazurin, Hoechst 33342, Syto9 and PI	Membrane potential and permeability, size and esterase activity.	[63]
	Endospore formation and metabolic flux correlation	Syto9, PI, FSC and SSC	Size, granularity and differential NA staining.	[7**]
	Quantify physiological states in fermentation	Chrome 6 (CV6), PI and DRAQ5	Membrane permeability and esterase activity.	[72]
	Identification of acid-inducible promoters	GFP expression	Induced expression.	[25**]
Metagenomic library screening	Substrate-induced gene expression	GFP expression	Induced expression.	[49**]

producing strain is due to inclusion body formation rather than the metabolic burden of synthesizing a foreign protein [38]. In another application, MPF analysis was used to assess population physiological heterogeneity in high cell density *E. coli* fed-batch fermentations [39]. This information was then used to design a two-component, stirred-tank, and plug-flow reactor to mimic the industrial-scale pH, glucose and dissolved oxygen micro-environments encountered in industrial-scale fed-batch fermentations. Ultimately, the advantages of MPF analysis have led to the design of MPF apparatuses that automate sampling, cell preparation, and analysis [40*], which can feed results to an automated controller in order to operate bioreactors such as a cytostat [41] upon quantitative, real-time, physiological inputs.

Analysis of metabolite and other stress responses, and their application in bioprocessing

MPF is becoming an important research tool to interrogate single-cell and population scale physiological responses to extracellular stresses. Stress responses analyzed by MPF include: substrate stress [42]; metabolite stress such as from organic acids [14**,43] and solvents

[44]; substrate starvation [45]; and temperature stress [46]. Survival of extracellular stress is often conferred by complex regulatory networks [15,33] that are not well understood, and upon selection for improvement, can resolve to be multigenic traits. Data obtained from MPF analysis provides fundamental insight into this complexity, and we believe will become commonly used to rationally engineer strains with improved tolerance phenotypes.

For example, Papadimitriou *et al.* used a carboxyfluorescein and PI viability marker set to assess the acid tolerance response of *Streptococcus macedonicus* [14**]. Cells were adapted before lethal acid stress by one of the following: an auto-acidifying approach, through exposure to nonlethal acidic pH, by an acid habituation approach, and by the reversion of acid habituation. The response to lethal levels of acid of each adapted culture was compared to nonadapted and stationary phase cultures. MPF and cell sorting made possible to detect and quantify population heterogeneity induced by acid-adapting cultures (i.e. viable, injured, and nonviable), and then to assess the population and single-cell responses and kinetics of death upon subsequent

exposure to lethal doses of lactic acid. Significantly, they used MPF in conjunction with biochemical inhibitors of *de novo* protein, fatty acid, and cell-wall biosyntheses to elucidate central cellular mechanisms of acid tolerance [14**].

During the stress response, MPF can also be employed to rapidly assess metabolic activity at the single-cell level. For example, it was shown that malolactic acid metabolism could be assessed by measuring the active extrusion of carboxy fluorescein in ethanol-stressed *Oenococcus oeni* cells [44]. Also, MPF allows for superior temporal analysis and quantification of population heterogeneity during the stress adaptation process for understanding the cellular physiology. For example, the LIVE/DEAD *BacLight* assay (Invitrogen®) was used to monitor the osmotic-stress response of *Enterobacter* sp. Strain *mcp11b* over a period of >500 hours [47]. Seven different subpopulations were identified based on intracellular DNA content, and it was shown that during osmotic stress, cells experience spontaneous metastable variations in their cellular and population characteristics before reaching an equilibrium state of survival [47].

In another application, Sevastyanovich *et al.* employed MPF to monitor both the physiological response of recombinant protein expressing *E. coli*, and for correctly folded recombinant protein [26**]. Specifically, they used a GFP-translational fusion to the *E. coli* CheY protein (CheY::GFP) as a reporter of recombinant protein induction, accumulation and correct folding, and stained dead cells with PI. By varying the concentration of IPTG for CheY::GFP induction, and by maintaining a constant growth temperature (as opposed to varying preinduction and postinduction), the authors demonstrate a reduction in PI positive (dead) cells and increase in GFP expressing cells. MPF analysis strongly suggested that expression of correctly folded recombinant protein was correlated with reducing the general stress response of individual cells and the population. Overall, the MPF analysis allowed the authors to devise a generic procedure for producing correctly folded recombinant protein at levels approaching 30% of the total protein content.

Analysis and sorting of endospore differentiation and metabolism in clostridia: relating metabolic fluxes to differentiation phenotype for improved metabolic engineering

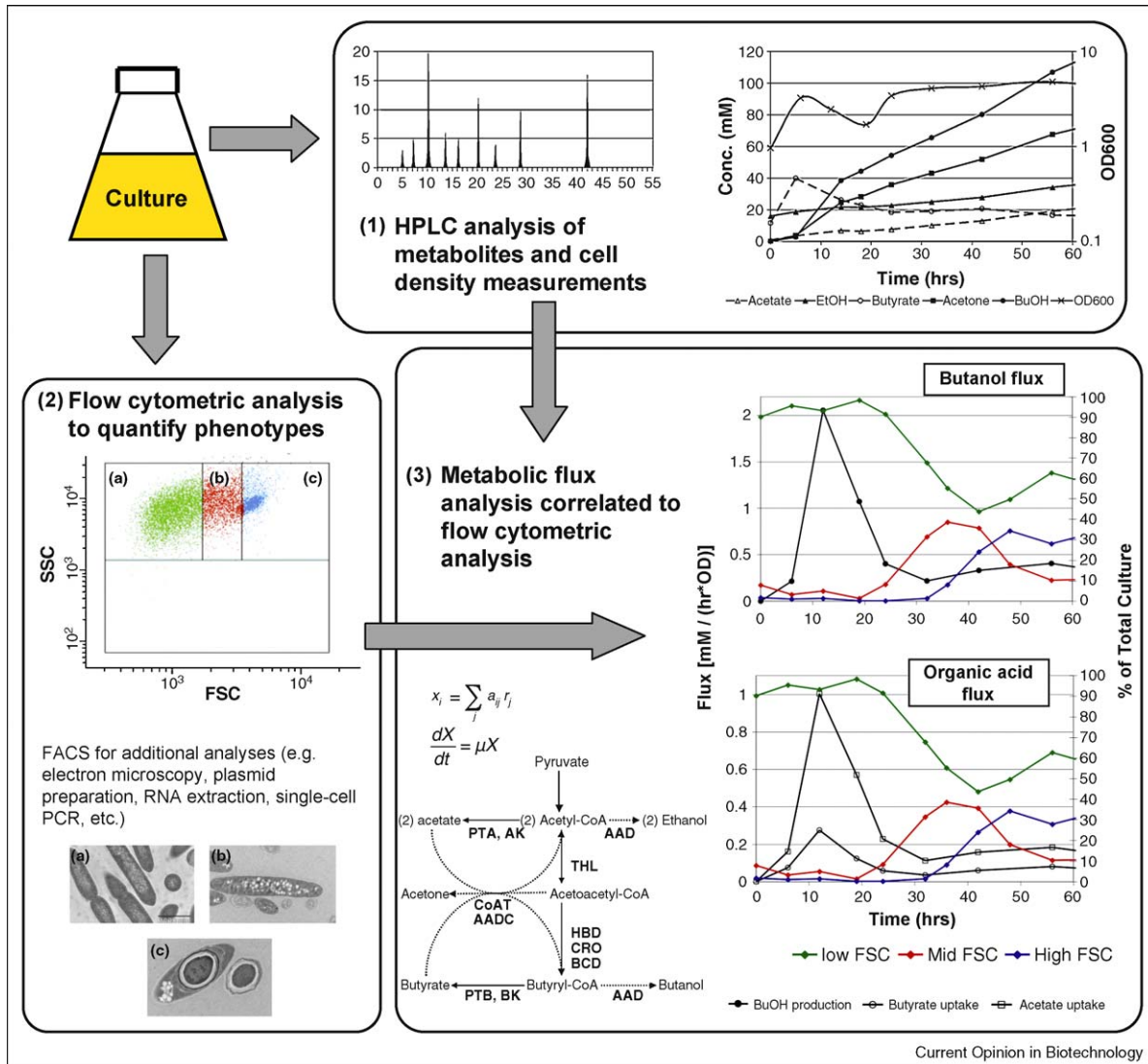
We recently demonstrated the application of MPF to discriminate, quantify and sort all major endospore-associated phenotypes in clostridia and specifically in *C. acetobutylicum* [7**]. We demonstrated that the five major clostridial morphologies (i.e. vegetative rods, swollen clostridial-form cells, forespores, endospores, and mature spores) could be identified and quantified as a % of total cell population based on light scattering (LS) and differential NA staining characteristics with

Syto9 and PI. LS alone could discriminate and quantify cells that were undergoing clostridial sporulation, and provided strong evidence that multiple rounds of sporulation and germination frequently occur in batch cultures. We also used MPF to correlate phenotypes to metabolic flux analysis in order to better hypothesize which cell morphology is mostly responsible for butanol production. The overall scheme of this analysis is shown in Figure 1. Batch cultures of the wild-type and a well-characterized plasmid control strain were analyzed every 6–12 hours for metabolite concentrations (Figure 1, box 1) and cellular morphology (Figure 1, box 2) by FC LS analysis. The LS-identified subpopulations were sorted and analyzed by electron microscopy to show that low FSC events represented mostly rod-shaped-vegetative cells, mid FSC events were clostridial-form cells and forespores, and high FSC events were endospores and mature spores. Metabolite fluxes were calculated for the primary metabolism of *C. acetobutylicum*, and then plotted on the same plots with the % of total population of each LS subpopulation, as shown in Figure 1, box 3. From this analysis we observed that as the % of rod-shaped cells decreased and the clostridial-forms increased, butanol production and organic acid uptake rates decreased, which suggests that clostridial-sporulation detracts from solvent production and that clostridial-form cells are not the major butanol-producing phenotype. In support of this hypothesis, we noticed a second burst of butanol production and organic acid uptake at ~50 hours that was concurrent to a germination event, indicated by a decrease in the endospore and mature spore population and a concurrent increase in rod-shaped-vegetative cells. Future experiments will likely analyze the single-cell metabolome of endospore differentiation phenotypes via C-13 tracers, mass spectroscopy and FACS. Such experiments have been performed in yeast [48], and should be readily transferred to analyzing bacterial metabolomes. Nonetheless, this analysis has been essential for guiding recent efforts to engineer nonsporulating, solvent-producing clostridia through a differentiation-engineering process, namely by inactivating sporulation-specific transcription factors in order to abolish sporulation without altering solvent formation.

Screening promoter libraries

Valdivia and Falkow pioneered a FACS-based approach called Differential Fluorescence Induction (DFI) to detect and select acid-inducible promoters from the *Salmonella typhimurium* genome [25**]. *S. typhimurium*'s ability to survive and replicate in low phagosomal pH environments of murine macrophages is dependent on complex, coordinated events at the transcriptional level. In order to determine the acid-inducible promoters, they created a library of random promoters fused to a mutant GFP protein and sorted cells that were highly fluorescent upon acid induction [25**]. The fluorescent population was then outgrown and sorted for the least fluorescent

Figure 1



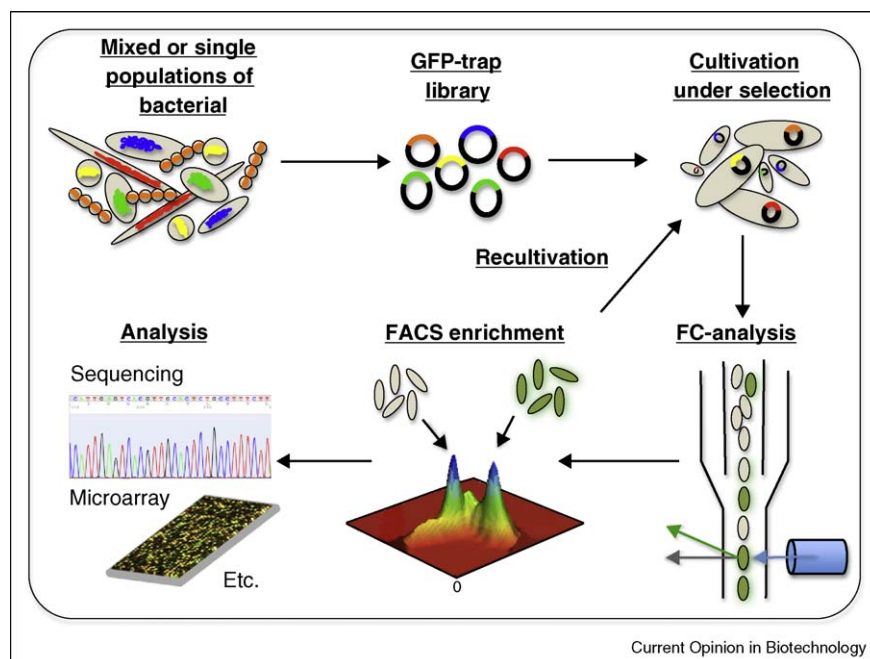
Integrating flow cytometric and metabolic flux analyses [7**]. (1) Cultures are sampled for metabolite analysis over the time course of the culture. (2) The same samples are analyzed by MPF for quantitative phenotypic and physiological characteristics. (3) Metabolite information is inputted into metabolic flux analysis to generate metabolite fluxes on a per biomass (e.g. per unit of optical density (OD), A_{600}) basis. Metabolic fluxes and MPF populations are plotted together to correlate cell populations and metabolite fluxes. For example, plots in box 3 reveal direct correlations between butanol production and organic acid (i.e. butyrate and acetate) uptake with the percentage of rod-shaped cell types (low FSC). As the percentage of rod-shaped cells decreases and of the clostridial-cell forms (mid FSC) increases, solvent production and organic acid uptake rates decrease. A second burst in solvent production and organic acid uptake occurs at ~50 hours, concurrent to a germination event indicated by a decrease in the endospore and free spore populations (high FSC) and increase in rod-shaped cell population (low FSC).

cells under nonacidic conditions in order to remove promoters that were not specifically induced by low pH stress. The general approach is illustrated in Figure 2. Eight enriched promoters were identified by sequencing, and exhibited considerable homology to promoter regions of genes encoding for cell-surface-maintenance enzymes, stress proteins, and efflux pumps. This approach has been used in a metagenomics application [49**] discussed next and holds considerable promise for engineering industrially relevant complex phenotypes.

MPF analysis and sorting to engineer complex phenotypes for industrial applications: lessons from metagenomics

There is considerable interest to accelerate the engineering of microbial complex phenotypes for industrial applications for the production of chemicals and biofuels from renewables [50*], and also for engineering improved microbial strains for bioremediation. Such applications require the development of cells with multigenic trait improvements, and may require large-scale genetic

Figure 2



General schematic for Differential Fluorescence Induction (DFI) pioneered by Valdivia and Falkow [25**] and similar adaptations since then, such as Substrate-Induced Gene Expression (SIGEX) by Uchiyama *et al.* [49**]. The general approach is to construct a metagenomic or single species GFP-trap library, which places GFP expression under the control of promoters from the library inserts, but whereby GFP is not expressed as a fusion protein. The microbial library is cultivated in a suitable host strain under selective conditions, such as organic acid stress, in order to induce GFP expression. The microbial library is analyzed by FC and is FACS selected based on GFP expression. Sorted populations are often enriched via subsequent recultivation and FACS. Enriched populations and even single cells can then be analyzed by various techniques such as sequencing of the library insert, DNA microarray analysis of the enriched population, or single-cell or few-cell RT-PCR or PCR.

modifications. Several strategies have been proposed toward this goal, such as evolutionary engineering techniques and library based strategies that result in large-scale genetic or transcriptional changes to achieve the desirable phenotype [50*]. Selection for improved complex phenotypes via whole-cell approaches is typically limited to a single parameter, such as fitness (i.e. better survival) against organic acids or other toxic chemicals. Additionally, once an improved phenotype has been selected, the analysis tools for interrogating one aspect of the biological function often requires expensive and time consuming approaches, such as determining point mutations in a bacterial genome by sequencing. FC has not been extensively applied to complex phenotype selection or characterization when engaging whole-cell engineering approaches. However, lessons could be learned from recent advances in integrating MPF sorting in microbial metagenomic studies. Similar to the Valdivia and Falkow [25**] approach discussed above, Uchiyama *et al.* generated operon-trap *gfp*-expression metagenomic libraries of catabolic genes in *E. coli* [49**]. They employed FACS to first remove self-ligated and constitutively expressed clones via GFP detection. They next used FACS to select clones expressing GFP only in the presence of various added substrates such as naphthalene

or benzoate. Not only were positive clones selected for, but the relative fold induction could also be quantified by dividing the GFP fluorescence intensity of a specific clone under substrate induction by the GFP intensity without substrate induction. Sequencing of selected clones returned ORFs that were homologous to genes in benzoate-degradative, catechol-degradative, and aromatic-hydrocarbon transformation operons. We envision similar operon-trap *gfp*-expression approaches being applied to screen multispecies libraries for catabolic genes induced when pure or mixed cell populations are exposed to biomass hydrolyzate as carbon substrates, or for interrogating the stress response induced by organic acids and/or solvents [33].

FC in synthetic biology

The engagement of molecular biology tools to engineer synthetic or biologically inspired circuits in cells in order to reprogram dominant cell processes has led to the development of synthetic biology (e.g. [51]). Engineered circuits have been developed based on the interaction of DNA, protein and/or RNA molecules. In several of these studies, a reporter gene/protein is used, frequently GFP or a derivative fluorescing protein (e.g. [51,52*]). More recent applications of FC/FACS include the ability to

screen for synthetic riboswitches [53^{*}]. Still, the capability of MPF to measure simultaneously several fluorescent molecules remains to be explored in the context of synthetic biology, but as this field is fast developing into engineering more complex circuits and microbial phenotypes [51], use of MPF is bound to prove an invaluable tool. Recent advances in optical detection strategies for reporter signal measurements [54] will greatly facilitate such MFP applications in synthetic biology.

Conclusions and future developments

We anticipate that microbial MPF techniques will have a considerable impact on fundamental and applied microbial analysis and engineering, including applications of synthetic biology. Future work should address developing novel and better probes that exhibit greater selectivity for cellular functions and structures, are capable of detecting intracellular metabolic intermediates and proteins, and are suitable for multiparametric assays. Multivariate (MV) analysis techniques will need to be more frequently applied and further developed. Would MPF find applications in intracellular signaling in microbial systems? This remains a tantalizing possibility. Lastly, we believe that there are great opportunities in combining MPF, MV, and whole-cell engineering approaches to accelerate the engineering of industrial strains exhibiting complex phenotypes.

Although not reviewed here, there are many opportunities to advance MV analysis techniques for analyzing and extracting the most biologically significant conclusions from MPF data. MV analysis has the potential to make sense out of 3–20 parameter detection on populations ranging from 100 to 1 000 000 individual cells over time courses of a culture, between culture conditions, and in complex heterogeneous populations. As mentioned by Davey *et al.*, the typical dual-parameter plot analysis increases with the number (n) of parameters according to the expression: $n(n - 1)/2$ [55^{*}]. Thus, for an experiment involving a modest five time points and comparing four culture conditions, the need for MV analysis becomes obvious. Davey *et al.* also demonstrated the utility of applying common dimension reduction techniques such as principal components regression (PCR), partial least squares (PLS) regression, and artificial neural networks (ANN), which rendered powerful predictive capabilities. We believe this will be an important area of application and development in the future.

FC is increasingly used in the analysis of signaling networks in higher eukaryotes, particularly for the analysis of the cell phosphoproteome [56^{*}]. By assessing the dynamic phosphorylation state of a suitable set of proteins, through phosphorylation-specific antibodies, it is possible to deconvolute signaling pathways that are relevant to immunology and cancer biology. In principle, this approach would be immediately applicable to eluci-

dating the complexity of protein-phosphorylation driven signaling in developmental processes of bacilli and clostridia. Such advances would greatly aid in understanding the associated bistability and multistability states exhibited in endospore differentiation [6^{*}]. Applications could be extended to understanding the dynamics and the interaction network of two-component signaling in microbial physiology, ecology, and pathogenesis.

Lastly, we see exceptional opportunities for combining microbial MPF, MV, and whole-cell engineering approaches to accelerate engineering of complex phenotypes for industrial applications. Similar to the work of Sevastyanovich *et al.* [26^{**}], MPF can be used for correlating multiple, physiological phenomena to desirable industrial phenotypes. MPF-FACS applications would not necessarily or only focus on optimizing bioprocessing schemes, but rather aim to select highly desirable mutants in large libraries of genetic diversity that would not be selected for by fitness alone.

Conflicts of interest

The authors declare that they have no conflicts of interest related to this review paper.

Acknowledgements

We acknowledge support under National Science Foundation Grants CBT-0853490 and CBT-0756451 and ONR (Office of Naval Research, USA) grant N000141010161.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Perez OD, Nolan GP: **Phospho-proteomic immune analysis by flow cytometry: from mechanism to translational medicine at the single-cell level.** *Immunol Rev* 2006, **210**:208-228.
 2. Giepmans BNG, Adams SR, Ellisman MH, Tsien RY: **Review — the fluorescent toolbox for assessing protein location and function.** *Science* 2006, **312**:217-224.
 3. Vives-Rego J, Lebaron P, Nebe-von Caron G: **Current and future •• applications of flow cytometry in aquatic microbiology.** *FEMS Microbiol Rev* 2000, **24**:429-448.
- An older but exemplary review of FC applications in aquatic microbiology and microbiology in general. It takes a didactic approach to the subject and that makes for excellent reading for both the beginner and the specialist. It has lost none of its potency in its almost 10 years of life.
4. Link AJ, Jeong KJ, Georgiou G: **Beyond toothpicks: new methods for isolating mutant bacteria.** *Nat Rev Microbiol* 2007, **5**:680-688.
 5. Tawfik DS, Griffiths AD: **Man-made cell-like compartments for molecular evolution.** *Nat Biotechnol* 1998, **16**:652-656.
 6. Veening JW, Smits WK, Kuipers OP: **Bistability, epigenetics, and • bet-hedging in bacteria.** *Annu Rev Microbiol* 2008, **62**:193-210. An exceptional recent review discussing microbial bistability and epigenetic inheritance from a network-topology and synthetic-biology point of view, and with a quantitative frame of mind.
 7. Tracy BP, Gaida SM, Papoutsakis ET: **Development and •• application of flow-cytometric techniques for analyzing and sorting endospore-forming clostridia.** *Appl Environ Microbiol* 2008, **74**:7497-7506.

This work demonstrated the ability to identify and sort all major clostridial-endospore differentiation phenotypes, and demonstrated how FC can guide future metabolic engineering efforts.

8. Dhar N, McKinney JD: **Microbial phenotypic heterogeneity and antibiotic tolerance.** *Curr Opin Microbiol* 2007, **10**:30-38.
 9. Mironov VA, Sergienko OV, Nastasyak IN, Danilenko VN: **Biogenesis and regulation of biosynthesis of erythromycins in *Saccharopolyspora erythraea*.** *Appl Biochem Microbiol* 2004, **40**:531-541.
 10. Shapiro HM: *Practical Flow Cytometry*. edn 4. New York: Wiley-Liss; 2003.
 11. Davey HM, Kell DB: **Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses.** *Microbiol Rev* 1996, **60**:641-696.
 12. Shi L, Gunther S, Hubschmann T, Wick LY, Harms H, Muller S: **Limits of propidium iodide as a cell viability indicator for environmental bacteria.** *Cytom A* 2007, **71**:592-598.
 13. Aharoni A, Amitai G, Bernath K, Magdassi S, Tawfik DS: **High-throughput screening of enzyme libraries: thiolactonases evolved by fluorescence-activated sorting of single cells in emulsion compartments.** *Chem Biol* 2005, **12**:1281-1289.
 14. Papadimitriou K, Pratsinis H, Nebe-von-Caron G, Kleetsas D, Tsakalidou E: **Acid tolerance of *Streptococcus macedonicus* as assessed by flow cytometry and single-cell sorting.** *Appl Environ Microbiol* 2007, **73**:465-476.
- Used FC to investigate the physiological response of *Streptococcus macedonicus* that was exposed to various lactic acid stress conditions. Moreover, they used biochemical inhibitors and creative MPF assays to elucidate central cellular mechanisms responsible for acid tolerance.
15. Borden JR, Papoutsakis ET: **Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*.** *Appl Environ Microbiol* 2007, **73**:3061-3068.
 16. Jones SW, Paredes CJ, Tracy B, Cheng N, Sillers R, Senger RS, Papoutsakis ET: **The transcriptional program underlying the physiology of clostridial sporulation.** *Genome Biol* 2008, **9**:R114.
 17. Jarzembowski T, Wisniewska K, Jozwik A, Bryl E, Witkowski J: **Flow cytometry as a rapid test for detection of penicillin resistance directly in bacterial cells in *Enterococcus faecalis* and *Staphylococcus aureus*.** *Curr Microbiol* 2008, **57**:167-169.
 18. Betanzos CM, Gonzalez-Moa MJ, Boltz KW, Werf BDV, Johnston SA, Svarovsky SA: **Bacterial glycoprofiling by using random sequence peptide microarrays.** *ChemBiochem* 2009, **10**:877-888.
 19. Tyo KE, Zhou H, Stephanopoulos GN: **High-throughput screen for poly-3-hydroxybutyrate in *Escherichia coli* and *Synechocystis* sp. strain PCC6803.** *Appl Environ Microbiol* 2006, **72**:3412-3417.
 20. Fishov I, Woldringh CL: **Visualization of membrane domains in *Escherichia coli*.** *Mol Microbiol* 1999, **32**:1166-1172.
 21. Falcioni T, Papa S, Campana R, Manti A, Battistelli M, Baffone W: **State transitions of *Vibrio parahaemolyticus* VBNC cells evaluated by flow cytometry.** *Cytom B Clin Cytom* 2008, **74**:272-281.
 22. Blom K, Lundin BS, Bolin I, Svennerholm AM: **Flow cytometric analysis of the localization of *Helicobacter pylori* antigens during different growth phases.** *FEMS Immunol Med Microbiol* 2001, **30**:173-179.
 23. Wallberg F, Sundstrom H, Ledung E, Hewitt CJ, Enfors SO: **Monitoring and quantification of inclusion body formation in *Escherichia coli* by multi-parameter flow cytometry.** *Biotechnol Lett* 2005, **27**:919-926.
 24. Shaner NC, Steinbach PA, Tsien RY: **A guide to choosing fluorescent proteins.** *Nat Methods* 2005, **2**:905-909.
 25. Valdivia RH, Falkow S: **Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction.** *Mol Microbiol* 1996, **22**:367-378.
- Developed differential fluorescence induction (DFI) to determine the acid-induced promoters in the *Salmonella typhimurium* genome. It is a good example of MPF for understanding complex microbial responses to stress, and DFI has since been used for many other applications.
26. Sevastyanovich Y, Alfasi S, Overton T, Hall R, Jones J, Hewitt C, Cole J: **Exploitation of GFP fusion proteins and stress avoidance as a generic strategy for the production of high-quality recombinant proteins.** *FEMS Microbiol Lett* 2009.
- Demonstrated the utility of MPF in bioprocess development, which potentially resulted in a generic protocol for any recombinant protein expression. Also demonstrates the superior ability of MPF to analyze and correlate single-cell and population physiology to recombinant protein expression.
27. Zhang C, Xing XH, Lou K: **Rapid detection of a gfp-marked *Enterobacter aerogenes* under anaerobic conditions by aerobic fluorescence recovery.** *FEMS Microbiol Lett* 2005, **249**:211-218.
 28. Drepper T, Eggert T, Circolone F, Heck A, Krauss U, Guterl JK, Wendorff M, Losi A, Gartner W, Jaeger KE: **Reporter proteins for in vivo fluorescence without oxygen.** *Nat Biotechnol* 2007, **25**:443-445.
 29. Friedrich U, Lenke J: **Improved enumeration of lactic acid bacteria in mesophilic dairy starter cultures by using multiplex quantitative real-time PCR and flow cytometry-fluorescence in situ hybridization.** *Appl Environ Microbiol* 2006, **72**:4163-4171.
 30. Jen CJ, Chou CH, Hsu PC, Yu SJ, Chen WE, Lay JJ, Huang CC, Wen FS: **Flow-FISH analysis and isolation of clostridial strains in an anaerobic semi-solid bio-hydrogen producing system by hydrogenase gene target.** *Appl Microbiol Biotechnol* 2007, **74**:1126-1134.
 31. Fornasari ME, Rossetti L, Remagni C, Giraffa G: **Quantification of *Enterococcus italicus* in traditional Italian cheeses by fluorescence whole-cell hybridization.** *Syst Appl Microbiol* 2008, **31**:223-230.
 32. Paredes CJ, Alsaker KV, Papoutsakis ET: **A comparative genomic view of clostridial sporulation and physiology.** *Nat Rev Microbiol* 2005, **3**:969-978.
 33. Alsaker KV, Paredes C, Papoutsakis ET: **Metabolite stress and tolerance in the production of biofuels and chemicals: gene-expression-based systems analysis of butanol, butyrate and acetate stresses in the anaerobe *Clostridium acetobutylicum*.** *Biotechnol Bioeng* 2010, **105**:1131-1147.
 34. Tomas CA, Alsaker KV, Bonarius HPJ, Hendriksen WT, Yang H, Beamish JA, Paredes CJ, Papoutsakis ET: **DNA array-based transcriptional analysis of asporogenous, nonsolventogenic *Clostridium acetobutylicum* strains SKO1 and M5.** *J Bacteriol* 2003, **185**:4539-4547.
 35. Hewitt CJ, Caron GNV, Nienow AW, McFarlane CM: **The use of multi-parameter flow cytometry to compare the physiological response of *Escherichia coli* W3110 to glucose limitation during batch, fed-batch and continuous culture cultivations.** *J Biotechnol* 1999, **75**:251-264.
- Nice example of using MPF to compare and understand single-cell and population level physiology between modes of microbial cultivation.
36. Reis A, da Silva TL, Kent CA, Kosseva M, Roseiro JC, Hewitt CJ: **Monitoring population dynamics of the thermophilic *Bacillus licheniformis* CCMI 1034 in batch and continuous cultures using multi-parameter flow cytometry.** *J Biotechnol* 2005, **115**:199-210.
 37. da Silva TL, Reis A, Kent CA, Roseiro JC, Hewitt CJ: **The use of multi-parameter flow cytometry to study the impact of limiting substrate, agitation intensity, and dilution rate on cell aggregation during *Bacillus licheniformis* CCMI 1034 aerobic continuous culture fermentations.** *Biotechnol Bioeng* 2005, **92**:568-578.
- Nice example of using MPF to compare and understand single-cell and population level physiology between various bioprocessing parameters such as substrate limitation, agitation rate, and dilution rate.
38. Lewis G, Taylor IW, Nienow AW, Hewitt CJ: **The application of multi-parameter flow cytometry to the study of recombinant *Escherichia coli* batch fermentation processes.** *J Ind Microbiol Biotechnol* 2004, **31**:311-322.

39. Onyeaka H, Nienow AW, Hewitt CJ: **Further studies related to the scale-up of high cell density *Escherichia coli* fed-batch fermentations: the additional effect of a changing microenvironment when using aqueous ammonia to control pH.** *Biotechnol Bioeng* 2003, **84**:474-484.
40. Kacmar J, Zamamiri A, Carlson R, Abu-Absi NR, Srienc F: **Single-cell variability in growing *Saccharomyces cerevisiae* cell populations measured with automated flow cytometry.** *J Biotechnol* 2004, **109**:239-254.
- Pioneers in the field of developing and applying automated flow cytometry for real-time and online bioprocess control.
41. Kacmar J, Gilbert A, Cockrell J, Srienc F: **The cytostat: a new way to study cell physiology in a precisely defined environment.** *J Biotechnol* 2006, **126**:163-172.
42. Amanullah A, Hewitt CJ, Nienow AW, Lee C, Chartrain M, Buckland BC, Drew SW, Woodley JM: **Measurement of strain-dependent toxicity in the indene bioconversion using multiparameter flow cytometry.** *Biotechnol Bioeng* 2003, **81**:405-420.
43. Bunthof CJ, van den Braak S, Breeuwer P, Rombouts FM, Abee T: **Rapid fluorescence assessment of the viability of stressed *Lactococcus lactis*.** *Appl Environ Microbiol* 1999, **65**:3681-3689.
44. Da Silveira MG, Abee T: **Activity of ethanol-stressed *Enterococcus oeni* cells: a flow cytometric approach.** *J Appl Microbiol* 2009, **106**:1690-1696.
45. da Silva TL, Reis A, Kent CA, Kosseva M, Roseiro JC, Hewitt CJ: **Stress-induced physiological responses to starvation periods as well as glucose and lactose pulses in *Bacillus licheniformis* CCM1 1034 continuous aerobic fermentation processes as measured by multi-parameter flow cytometry.** *Biochem Eng J* 2005, **24**:31-41.
46. Baatout S, De Boever P, Mergeay M: **Temperature-induced changes in bacterial physiology as determined by flow cytometry.** *Ann Microbiol* 2005, **55**:73-80.
47. Sachidanandham R, Gin KYH: **Flow cytometric analysis of prolonged stress-dependent heterogeneity in bacterial cells.** *FEMS Microbiol Lett* 2009, **290**:143-148.
48. Eek KM, Sessions AL, Lies DP: **Carbon-isotopic analysis of microbial cells sorted by flow cytometry.** *GeoBiol* 2007, **5**:85-95.
49. Uchiyama T, Abe T, Ikemura T, Watanabe K: **Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes.** *Nat Biotechnol* 2005, **23**:88-93.
- Applied DFI to screen and identify genes in metagenomic libraries whose expression was induced by naphthalene and benzoate. This approach has significant potential in future biotechnological applications.
50. Patnaik R: **Engineering complex phenotypes in industrial strains.** *Biotechnol Prog* 2008, **24**:38-47.
- A brief, but concise review of recent developments in generating complex microbial strains for applications in bioprocessing. It captures the essence of genome-scale cell engineering approaches very well.
51. Tanouchi Y, Pai A, You LC: **Decoding biological principles using gene circuits.** *Mol Biosyst* 2009, **5**:695-703.
52. Tan C, Marguet P, You LC: **Emergent bistability by a growth-modulating positive feedback circuit.** *Nat Chem Biol* 2009, **5**:842-848.
- A nice recent piece in synthetic biology demonstrating that the host is not an inert bystander to engineered circuits. An eye opener for those that view a cell host as a car-like, inert 'chassis'.
53. Lynch SA, Gallivan JP: **A flow cytometry-based screen for synthetic riboswitches.** *Nucleic Acids Res* 2009, **37**:184-192.
- Use of FC to screen synthetic riboswitches that induce strong gene expression in the presence of theophylline. The approach can be generalized to selecting a large spectrum of 'designer' riboswitches.
54. Wells M: **Advances in optical detection strategies for reporter signal measurements.** *Curr Opin Biotechnol* 2006, **17**:28-33.
55. Davey HM, Jones A, Shaw AD, Kell DB: **Variable selection and multivariate methods for the identification of microorganisms by flow cytometry.** *Cytometry* 1999, **35**:162-168.
- Nicely demonstrates the utility of dimension reduction techniques for analyzing and interpreting MPF data.
56. Schmezle K, White FM: **Phosphoproteomic approaches to elucidate cellular signaling networks.** *Curr Opin Biotechnol* 2006, **17**:406-414.
- Cellular signaling largely rests on post-translational modifications of transcription factors and their interplay with kinases, both in eukaryotic and prokaryotic systems. This review summarizes the approaches that make possible to analyze signaling deriving from changes in protein phosphorylation, a most important modification. FC is a key tool in this endeavor.
57. Laflamme C, Lavigne S, Ho J, Duchaine C: **Assessment of bacterial endospore viability with fluorescent dyes.** *J Appl Microbiol* 2004, **96**:684-692.
58. Kleinstaub S, Riis V, Fetzer I, Harms H, Muller S: **Population dynamics within a microbial consortium during growth on diesel fuel in saline environments.** *Appl Environ Microbiol* 2006, **72**:3531-3542.
59. Allegra S, Berger F, Berthelot P, Grattard F, Pozzetto B, Riffard S: **Use of flow cytometry to monitor *Legionella* viability.** *Appl Environ Microbiol* 2008, **74**:7813-7816.
60. Nielsen TH, Sjöholm OR, Sørensen J: **Multiple physiological states of a *Pseudomonas fluorescens* DR54 biocontrol inoculant monitored by a new flow cytometry protocol.** *FEMS Microbiol Ecol* 2009, **67**:479-490.
61. Muller S, Ullrich S, Losche A, Loffhagen N, Babel W: **Flow cytometric techniques to characterise physiological states of *Acinetobacter calcoaceticus*.** *J Microbiol Methods* 2000, **40**:67-77.
62. Rault A, Bouix M, Beal C: **Fermentation pH influences the physiological-state dynamics of *Lactobacillus bulgaricus* CFL1 during pH-controlled culture.** *Appl Environ Microbiol* 2009, **75**:4374-4381.
63. Cronin UP, Wilkinson MG: **Monitoring growth phase-related changes in phosphatidylcholine-specific phospholipase C production, adhesion properties and physiology of *Bacillus cereus* vegetative cells.** *J Ind Microbiol Biotechnol* 2008, **35**:1695-1703.
64. Stecchini ML, Spaziani M, Del Torre M, Pacor S: ***Bacillus cereus* cell and spore properties as influenced by the micro-structure of the medium.** *J Appl Microbiol* 2009, **106**:1838-1848.
65. Comas-Riu J, Vives-Rego J: **Cytometric monitoring of growth, sporogenesis and spore cell sorting in *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*).** *J Appl Microbiol* 2002, **92**:475-481.
66. Forster S, Snape JR, Lappin-Scott HM, Porter J: **Simultaneous fluorescent gram staining and activity assessment of activated sludge bacteria.** *Appl Environ Microbiol* 2002, **68**:4772-4779.
67. Eklund BI, Edalat M, Stenberg G, Mannervik B: **Screening for recombinant glutathione transferases active with monochlorobimane.** *Anal Biochem* 2002, **309**:102-108.
68. Yamada T, Shimomura Y, Hiraoka Y, Kimbara K: **Oxidative stress by biphenyl metabolites induces inhibition of bacterial cell separation.** *Appl Microbiol Biotechnol* 2006, **73**:452-457.
69. Burmölle M, Hansen LH, Sørensen SJ: **Use of a whole-cell biosensor and flow cytometry to detect AHL production by an indigenous soil community during decomposition of litter.** *Microb Ecol* 2005, **50**:221-229.
70. Chung JD, Stephanopoulos G, Ireton K, Grossman AD: **Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation.** *J Bacteriol* 1994, **176**:1977-1984.
71. Griffin BA, Adams SR, Tsien RY: **Specific covalent labeling of recombinant protein molecules inside live cells.** *Science* 1998, **281**:269-272.
72. Quiros C, Herrero M, Garcia LA, Diaz M: **Quantitative approach to determining the contribution of viable-but-nonculturable subpopulations to malolactic fermentation processes.** *Appl Environ Microbiol* 2009, **75**:2977-2981.