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Review

Microbial heterogeneity affects bioprocess robustness: Dynamic single-cell analysis contributes to understanding of microbial populations

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Heterogeneity or segregation of microbial populations has been the subject of much research, but the real impact of this phenomenon on bioprocesses remains poorly understood. The main reason for this lack of knowledge is the difficulty in monitoring microbial population heterogeneity under dynamic process conditions. The main concepts resulting in microbial population heterogeneity in the context of bioprocesses have been summarized by two distinct hypotheses. The first involves the individual history of microbial cells or the "path" followed during their residence time inside the process equipment. The second hypothesis involves a coordinated response by the microbial population as a bet-hedging strategy, in order to cope with process-related stresses. The respective contribution of each hypothesis to microbial heterogeneity in bioprocesses is still unclear. This illustrates the fact that, although microbial phenotypic heterogeneity has been thoroughly investigated at a fundamental level, the implications of this phenomenon in the context of microbial bioprocesses are still subject to debate. At this time, automated flow cytometry is the best technique for investigating microbial heterogeneity under process conditions. However, dedicated software and relevant biomarkers are needed for the proper integration of flow cytometry as a bioprocess control tool.

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1 Introduction

It is now well established that clonal populations of microbial cells exhibit phenotypic heterogeneities due to environmental factors, even in homogenous environments, due to stochastic fluctuations at the level of biochemical reactions. Although heterogeneity or segregation of microbial populations has received a lot of attention in the past few years resulting in many high level papers, the real impact of this phenomenon on bioprocesses remains poorly understood. The main reason behind this lack of knowledge is the difficulty in monitoring microbial population heterogeneity under dynamic process conditions. The present review focuses on the different concepts and experimental apparatus specifically developed to address the question of microbial heterogeneity from a process improvement perspective.

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Abbreviations: CTD, circulation time distribution; **FC,** flow cytometry; **FP**, fluorescent protein; **GFP**, green fluorescent protein; **LOC**, lab on a chip

2 Mechanisms leading to microbial heterogeneity in bioprocesses

2.1 Fundamental causes of microbial population heterogeneity

Microbial heterogeneity is governed by a complex set of intrinsic and extrinsic noise that has been thoroughly studied at the fundamental level [1–3] but only partially applied in the field of bioprocess engineering [4]. Intrinsic noise comes from the stochastic nature of gene expres-

sion. Indeed, considering the small number of molecular species involved at the single-cell level, gene expression is naturally subject to stochasticity [2, 5]. Extrinsic noise is linked to variations in the amounts of intracellular compounds, such as regulatory proteins and transcription factors. Both intrinsic and extrinsic components of noise contribute to microbial heterogeneity [6]. From a bioprocess perspective, it would be interesting to distinguish the impact of the normal biological processes (i.e. cell cycle and basal genetic noise resulting from extrinsic and intrinsic sources of noise) from the impact of environmental factors affecting cellular physiology. These aspects will be discussed further. Bistability is one of the most impressive phenomena resulting from noise in gene expression. In this case, an isogenic microbial population splits into two distinct subpopulations, exhibiting drastically different stress-specific protein contents [7, 8]. The resulting population contains a subpopulation with a low protein content (also called the OFF state) and a subpopulation with a high protein content (also called the ON state), with cells continuously shifting from the one state to another [8]. The time spent by a cell in a given state is called memory. In this way, if a population is exposed to recurrent stress, it will be able to switch more rapidly to the ON state, and bistability and its resulting cellular memory can be viewed as a bet-hedging strategy developed by microorganisms in a fluctuating environment in order to improve their fitness [9–11]. Fitness can be estimated in different ways (the term robustness being generally synonymous with fitness in bioprocess applications) but the basic principle involves the cultivation of microbial cells before and after a stress. In this context, fitness is defined as the ratio between the number of viable cells before and after the experiments. Variations of the test implying the measurement of the growth rate can also be found and categorized as a comparative fitness test [12]. Two main observations can be highlighted from these tests. First, pre-conditioned microbial cells exposed to a sub-lethal stress exhibit higher fitness than non-stressed cells. Secondly, in most cases, microbial cells from the same population exhibit various levels of resistance, implying that heterogeneity is linked to fitness. Fitness is important from a biotechnological point of view as it has been demonstrated that exposure of microbial cells to a sub-lethal stress improves survival, but at the expense of growth rate [13, 14]. More importantly, it has been demonstrated that segregated microbial populations showed improved fitness in a fluctuating environment [15, 16], suggesting that phenotypic diversity could be an asset under large-scale bioprocessing conditions (for this purpose, strain robustness must be distinguished from cellular productivity, their interrelation depending on the biotechnological application as discussed below). Genetic constructs integrating the level of noise have been designed as a metabolic engineering strategy for the improvement of bioprocesses [1], but their behavior in a real, i.e. industrial, environment has not been evaluated. However, recombineering strategies are now available not only to enhance the productivity of recombinant strains after the first screening steps, but also in order to reduce the product titer variations from cell to cell [17]. Impressive results have been gained by integrating noise-related phenomena for the design of synthetic circuits inside microbial cells. Such systems can be used to tune the noise of a given gene, with repercussion on fitness [18]. In addition, it is technically feasible to maintain the expression of a bistable system in a given state by tuning cellular memory [19]. This can be achieved by genetically engineered feedback circuits. Such manipulations have potential applications in bioprocesses by maintaining the cell population into the most productive state.

2.2 Implication of microbial heterogeneity in bioprocesses

There is a scientific gap between the fundamental efforts made to decipher microbial heterogeneity and their implication for biotechnological processes. Indeed, we are able to describe the molecular mechanisms at the single-cell level and their inherent stochasticity leading to population segregation, but the induction of these mechanisms under bioprocess conditions is not well understood. From a biotechnological point of view, it would be important to determine the factors affecting the degree of heterogeneity of a microbial population. At this level, the external factors, more particularly the environmental fluctuations generated in the bioreactor, are of primary importance since they can be directly exploited by the process engineer (external factors must be considered separately from the intrinsic and extrinsic sources of noise defined in Section 2.1). The main reason for the above-mentioned scientific gap is the actual lack of experimental tools available for the study of microbial population heterogeneity on the basis of process-relevant parameters in process equipment (i.e. on-line or in situ). The degree of microbial population heterogeneity is strongly correlated wih the micro-environmental conditions met in bioreactors. This statement has been reported in numerous studies (see [20–23] for the most recent reviews). One of the most interesting perspectives involving microbial heterogeneity in process equipment involves the use of bioreactor heterogeneities (or environmental noise) in order to improve bioreactor performances [24]. Indeed, as stated before, microbial cells are able to cope with a wide range of stresses and the initial phenotypic states and heterogeneity are two important factors for these adaptation mechanisms [25]. The idea behind this approach is to exploit the amplitude and frequencies of the environmental, external noise present in the bioreactor in order to improve microbial fitness [24]. This concept is at the basis of an interdisciplinary research project aiming at linking the impact of bioreactor mixing efficiency to the ampli-

tude of the stress response and its impact at the single-cell level [26]. It has been recently proposed as a new strategy for exploring the effect of environmental imperfections on the robustness of a bioprocess [27, 28]. As a first attempt, it would be straightforward to link phenotypic heterogeneity of a microbial population with the individual profile of environmental conditions experienced by each cell during the course of the bioprocess. However, this has not been done yet even if some very relevant work exists, both on the modeling of concentration time profile experienced by microbial cells during their revolution in a bioreactor [29, 30] and on the heterogeneity of microbial populations as a function of bioreactor mixing performance [31, 32]. Considering the complexity of both the numerical and the experimental methods needed to study the physico-biological interactions, only a few studies have considered both aspects together. A very interesting attempt has been made to describe the metabolic switch of *Escherichia coli* in a concentration field by using a computational fluid dynamic method to describe the displacement of microbial cells along these gradients [33]. However, this kind of model is computationally intensive and difficult to apply at the level of the microbial population. Simplified approaches have been proposed, i.e. a deterministic-stochastic compartment model allowing the simulation of individual microenvironmental profiles experienced by 30 000 microbial cells [34–36]. This approach could greatly reduce the computational cost and save resources for the integration of complex biological mechanisms. At this level, some recent efforts have been made for the design of effective population balance models able to simulate the heterogeneity of a microbial population in bioreactors [37–39]. Information about environmental fluctuations is of primary importance for population dynamics [40]. Indeed, as shown in Fig. 1, there are several overlaps between noise in gene expression and the main physico-biological mechanisms involved at the process level. Therefore, the influence of the individual paths followed by each microbial cell in the bioreactor is relevant for the generation of phenotypic heterogeneity. However, population segregation linked to environmental fluctuations is not only influenced by the individual concentration profiles experienced at the single-cell level but also by several other factors involving the history of the population (microbial memory), or the density of the population (quorum sensing). A second hypothesis, that will be called "coordinated response," considers a coordinated behavior of the microbial population in stressful environment with a degree of heterogeneity depending on the environmental conditions experienced. Several reports have shown that, in the case of unicellular organisms, phenotypic heterogeneity is a strategy used to improve the survival of a population in a fluctuating environment [41, 42]. Quorum sensing is another well known regulatory mechanism involved in the coordinated response of microbial populations and has been previously proposed as a molecular strategy for controlling microorganisms at the population level [43, 44]. The occurrence of quorum sensing at the single-cell level has been demonstrated by microfluidic technology. In this work, one to three individual cells were entrapped in a 100-fL microdroplet [45]. The authors showed that even single cells entrapped in a microdroplet were able to induce a quorum-sensing mechanism, considering the small reaction volume (corresponding to a cell density of 10^{10} cells/mL). The authors also reported a strong heterogeneity at the level of the

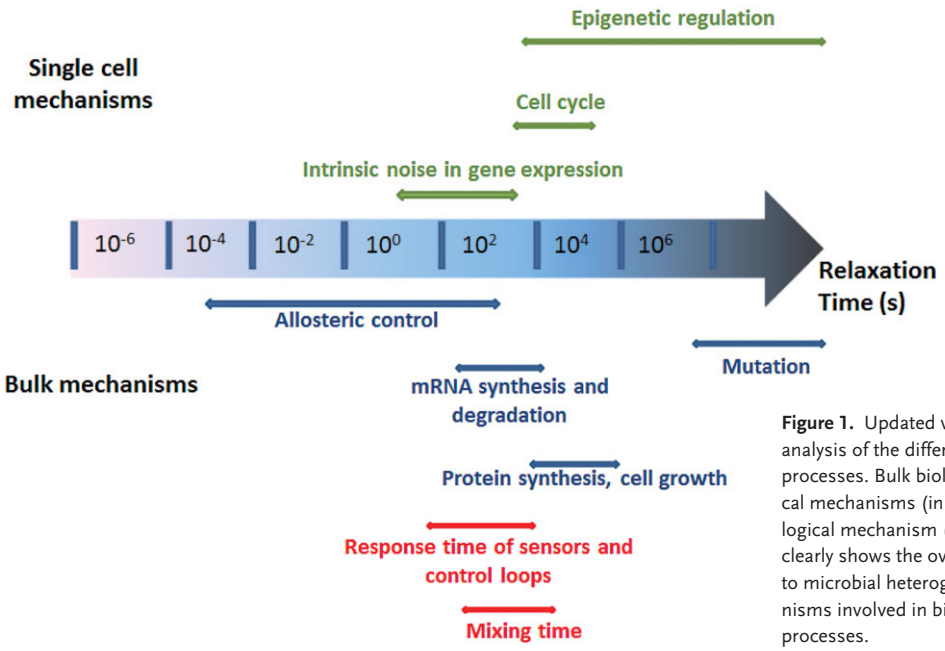

Figure 1. Updated version of the chart used for comparative analysis of the different relaxation times involved in bioprocesses. Bulk biological mechanisms (in blue) and physical mechanisms (in red) adapted from [109]. Single-cell biological mechanism (in green) adapted from [110]. This figure clearly shows the overlap between the mechanisms leading to microbial heterogeneity and the physico-chemical mechanisms involved in bioreactors and, more generally, in bioprocesses.

individual lag time before induction of the quorum-sensing mechanism. This kind of work highlights the importance of considering both hypotheses (i.e. individual trajectory or coordinated segregation) when interpreting single-cell data, microfluidic entrapment being recently proposed as a state-of-the-art technology for studying protein excretion at the single-cell level for recombinant *Pichia pastoris* [46]. This hypothesis regarding the non-coordinated (every cell responds independently to environmental changes) or coordinated (microbial population responds with a quorum sensing-like phenomenon and adjusts phenotypic heterogeneity accordingly) degree of heterogeneity could be the basis for further interesting studies. Currently, there are four experimental limits to the assessment of the real contribution of process imperfections/microbial stressors to microbial population heterogeneity:

i) In the context of the individual path hypothesis, the experimental validation of the individual circulation paths followed by individual cells is difficult to obtain. During scale-up, there is an increase of the standard deviation of the circulation time distribution (CTD), representing the diversity of the circulation flow paths that can be followed by a single cell freely flowing inside the bioreactor [47]. Quantification of these CTDs is very scarce in the literature considering the technical difficulty associated with the implementation of a flow following technique inside process equipment. Consequently, CTD studies are mainly based on computational fluid dynamics approaches [48]. A very promising alternative is the development of free-flowing probes able to record the physico-chemical path followed, alleviating the need for considering the gradient field and the circulation path separately [49].
ii) In a fluctuating environment, what are the amplitudes and frequencies actually perceived at the microbial cell level? Several fundamental works have addressed this question and have shown that these processes are dependent on the characteristic times involved in the physiological processes as well as on more complex accumulative processes, such as cellular memory [11, 50].
iii) Currently, flow cytometry (FC) is the only single-cell technology available for the at-line or in situ monitoring of microbial segregation inside bioreactors [21, 51] and efforts are required for developing single-cell technologies for bioreactor control purposes.
iv) The coordinated segregation hypothesis is well documented both by experimental and numerical approaches for different fields in which microbial heterogeneity is considered relevant (environmental studies, biomedical application, biofilms etc.). For biotechnological applications, however, this kind of phenomenon has been rarely studied and exploited [52]. However, it has been reported that the quorum-sensing regulon exhibits overlap with stress response circuitry in industrially relevant strains such as *E. coli*. It is thus of paramount importance to determine whether and how the coordinated response leading to phenotypic heterogeneity affects process productivity [53].

3 Perception of microbial heterogeneity as a function of the biotechnological application

3.1 Microbial starter and probiotics: Robustness

Biotechnological applications based on the production of microbial starters and probiotics have long incorporated the notion of microbial population heterogeneity. Microbial starters and probiotics are two biotechnological applications involving a maximal culturability at the end of the process. In this context, it is recognized that binary (i.e. leading to either a culturable or non-culturable state), cultivation-based techniques are not suitable for measuring viability. Viability must be rather regarded as a continuum of states and determining a threshold between live and dead cells is not straightforward [54]. For this purpose, a wide range of fluorescent markers are available which, in combination with FC, can be used to decipher membrane integrity, membrane potential and internal enzyme activities on an analog basis [55, 56]. This approach has led to the identification of "intermediate" phenotypes exhibiting metabolic activity but no growth on agar plates ("viable but non culturable", VBNC). The VBNC phenotype is generally observed at the same time as viable and culturable as well as non-viable cells, highlighting the importance of considering population heterogeneity in this kind of application. A shift between subpopulations during cultivation, and more particularly between partially injured cells and viable cells is possible, as demonstrated recently for the yeast *Saccharomyces cerevisiae* [57]. The response of microbial cells to stress and the resulting phenotypic heterogeneity has been the subject of many studies in the food and feed industries [58]. Consequently, much work has been devoted to studying the effect of exposing microbial cells to sublethal stresses in order to improve the recovery rate after downstream processing operations [59].

3.2 Production of metabolites and recombinant systems: Robustness and productivity

Production of metabolites and recombinant proteins are more difficult to characterize in term of phenotypic heterogeneity since at least two phenotypic traits are targeted: viability/robustness and specific productivity of the cells. Recombinant protein production is a complex biotechnological process, but it is relatively well document-

ed compared with metabolites-oriented bioprocesses, due to the fact that reporter molecules, such as green fluorescent protein (GFP), can be fused to the protein of interest, providing a direct read-out system. Population heterogeneity was found to affect microbial recombinant protein production processes at both the levels of cell viability and productivity [60–62]. Microbial stress in this case is strongly dependent on the solubility of the target protein and on the extent of inclusion body formation. Sundström et al. [60] studied the effects of inclusion body formation on cell physiology during the production of promegapoietin by *E. coli*. Similar observations were made using the same microbial chassis but with another recombinant protein (Fab antibody fragment) leading to the formation of inclusion bodies [63]. In related studies, it was demonstrated that the microbial chassis influences the heterogeneity of recombinant protein production. Lee et al. [64] studied the expression of GFP in *S. cerevisiae* under the control of four constitutive promoters and observed a strong segregation of the population in non-producing, weakly producing and highly producing cells. Surprisingly, when expression cassettes were moved to *Kluyveromyces marxianus*, the segregation disappeared. These observations point out the fact that microbial heterogeneity must be analyzed on a case-by-case basis and therefore should be incorporated in the initial screening phases for the selection of the best microbial host. An important aspect that has also been addressed is the heterogeneity of the microbial population for recombinant product secretion. The classical single-cell technique used for analyzing recombinant product outside the cells involves the cell surface display [65, 66]. In this case, the protein of interest can be tagged by immunofluorescence staining directly at the cell surface and easily detected by FC. David et al. [67] used FC for analyzing cell viability and secretory capacity for the production of antibody fragment by *Bacillus megaterium*. Protein secretion was followed by staining the products attached to the cells when exported by membrane-coupled secretory (Sec) pathway. However, there are other secretory pathways in prokaryotes and this technique cannot be generalized. In order to address this very important question, dedicated single-cell tools have been developed, such as the microengraving methods allowing the cultivation of microbial cells in microcavities, the protein secretion being monitored by ELISA [46, 68]. Whereas these conditions can be far from those encountered in large-scale bioreactor (notably because this system is limited to batch cultivation and enhances quorum-sensing effects), these works demonstrated that heterologous protein secretion is subjected to stochastic switching, bridging the gap between fundamental studies and real applications. The last class of bioprocesses is oriented towards the production of bulk or fine metabolites. As for the production of recombinant protein, both cell viability and productivity are important. Metabolite productivity cannot be determined at the single-cell level since no specific fluorescent labels are available for this purpose. Recently, fluorescent biosensors based on transcription factors have been successfully used in the case of the production of lysine [69], but this principle is not applicable to all kinds of metabolites. However, several studies have shown that metabolites productivity is often strongly correlated to cell viability. Alonso et al. [70] have investigated the robustness of *Pseudomonas taetrolens* during the production of lactobionic acid. By using a double staining combined with FC, they demonstrated that lactobionic acid production is strongly correlated to the physiological status of the cells. Tracy et al. [71] established the same kind of correlation by using the same technical strategy in the case of butanol production by *Clostridium acetobutylicum*. However, by combining different FC parameters, these authors were able to identify the different clostridial forms and assessed that only the vegetative, rod-shaped population accounted for butanol production.

4 Bioprocess monitoring with high-throughput single-cell technologies

4.1 Flow cytometry for on-line bioprocess monitoring

Flow cytometry is the workhorse of microbial single-cell analysis and its applicability to industrial bioprocesses has been previously demonstrated [51, 72]. However, if many techniques are available for the determination of cell physiology at a given moment of culture, dynamic evolution of microbial resistance to stress and adaptation is still poorly described. For this purpose, an automated version of FC has been proposed for the on-line monitoring of cell population heterogeneity under process-related conditions [73, 74]. Specific interfaces comprising a mixing chamber where microbial cells are diluted prior to analysis and stained (if required) have been developed and commercialized. In this way, FC can be used for feedback control at the level of a bioreactor control. The cytostat is a good illustration of this approach where an interfaced on-line flow cytometer is used as a sensor in a feedback loop for the control of cell density in a chemostat [75]. Until this first integration in bioprocess control, the cytostat has been used for directed evolution of yeast for the resistance to inhibitors present in cellulosic hydrolyzates [76, 77]. It must be noticed, that in the case of the initial cytostat concept, FC measurements were made on basic optical parameters without the need for fluorescent biomarkers. However, this concept of experimental evolution can easily be expanded to more complex phenotype by the use of genetically encoded fluorescent biosensors, such as in visualizing evolution in real time (VERT) experiments [78–80]. In this kind of experiments, identical microbial strains tagged with biomarkers exhibiting dif-

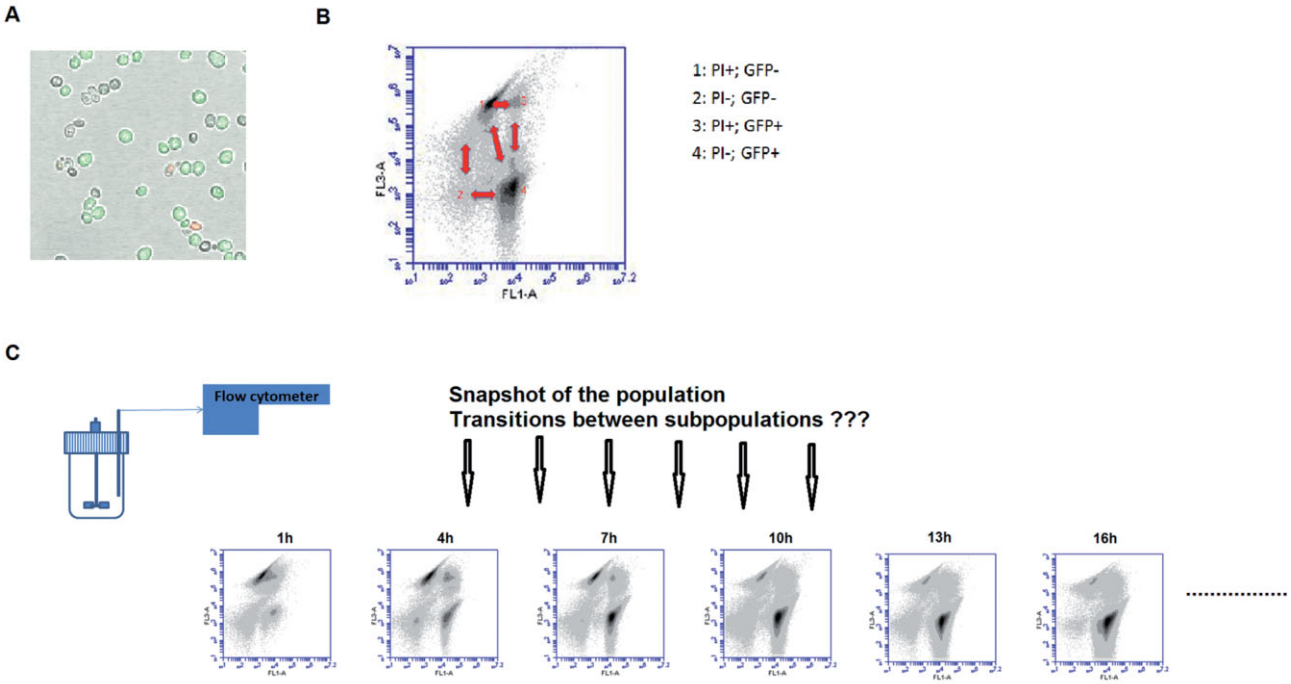

Figure 2. Set of experimental results gained from the culture of a *S. cerevisiae* GFP reporter strain developed in [14]. This strain was cultivated in a 2-L stirred bioreactor connected to a flow cytometer. (**A**) Confocal microscope imaging of the strain exhibiting GFP expression under the control of a ribosomal promoter (cells stained in green) or exhibiting propidium iodide uptake when membrane is compromised during the process (cells stained in red). (**B**) Flow cytometry analysis reveals four subpopulations according to the two markers used to decipher the phenotype. (**C**) Automated FC allows for a dynamic representation of the four subpopulations during the process, but the transition between sub-populations cannot be assessed because FC does not take into account the history of the cells (cells analyzed are withdrawn from the bioreactor and sent to the waste after analysis).

ferent fluorescence are cultivated in the same bioreactor under selective pressure (i.e. the presence of a solvent or an inhibitor found in the biofuel industry). Resistant mutants can be easily selected in this manner based on their fluorescence after the competitive fitness test. These examples illustrate the fact that FC could be implemented for process control, but until now no attempts have been made at this level except for fundamental studies. One of the main drawbacks of actual on-line FC is its inability to follow the history of given cells inside the population (which makes it difficult to experimentally validate the two hypotheses behind heterogeneity in bioprocesses evoked in Section 2). Some attempts have been made for the adaptation of a conventional FC for the following of certain cells by recirculation of the sheath fluid [81], but this kind of system is difficult to interface with a bioreactor. At this time, the most efficient way to monitor the dynamics of a single cell is by the use of time-lapse fluorescence [82]. Whereas this technique can be automated for image capture and analysis, it is not applicable for routine analysis of bioprocesses. However, automated FC could be applied for the estimation of the transient dynamics between subpopulation by the application of efficient mathematical algorithm, such as population balance models that have been discussed previously [38, 39]. This concept is illustrated in Fig. 2.

4.2 Alternatives to flow cytometry for monitoring microbial heterogeneity

An interesting perspective for the development of single-cell technologies applied to microbial bioprocesses is the lab on a chip approach (LOC). These devices comprise microfluidics for the sampling, handling (i.e. staining, dilution, etc.) and analysis at the single-cell level [83, 84] and open the way for the application of "omics" technologies at the single-cell level. However, it is quite difficult to apply these approaches to microbial cells considering their relatively small size and the limited amount of intracellular compounds to be analyzed. As an example, a mammalian cell contains approximately 10^9 protein molecules in comparison to bacteria, which contain only 4×10^6 of these molecules, illustrating the technical challenge when proteomic analysis must be performed on a single bacterial cell [83]. These kinds of experiments are more complicated when applied to transcriptomics, considering not only the small amount, but also the short half-life of bacterial mRNA molecules. These considerations lead to the conclusion that the LOC technologies are actually relatively limited in their usefulness for analyzing microbial cells and are decoupled from bioprocesses [85]. Moreover, the limited amount of intracellular molecules restricts the application of cutting-edge technologies,

such as mass cytometry, for microbial cells [86]. However, some very interesting results have been obtained with LOC systems as microbial cells can be monitored in real time. Various LOC systems have been previously reviewed, but the envirostat can be considered a benchmark in the context of the influence of the extracellular conditions on the phenotypic heterogeneity of microbial cells [87]. In this system, a single cell is immobilized by negative dielectrophoresis and can be maintained in a chemically defined environment such as in a chemostat, but without the limitation induced by the wash-out phenomenon as the cell is trapped in the microfluidics. Some very interesting results describing the effect of micro-environmental condition on the specific growth rate have been gained this way. However, it must be pointed out that the coordinated segregation hypothesis mentioned previously (Section 2.2) could not be investigated since only an isolated single cell is considered. In a bioprocess optimization perspective, it is important to have access to on-line single-cell techniques, such as on-line or automated FC. However, the interfacing of a flow cytometer with a bioreactor is not straightforward and in situ probing methods are generally preferred. At this time, most of the research is focused on two probing technologies, i.e. optical analysis [88–90] and spectroscopy [91]. Spectroscopy-based methods are mainly focused on the excretion of metabolites in the extracellular medium and do not take into account physiology-related parameters. On the other hand, significant advances have been achieved for the development of optical-based probing method in order to increase both the throughput (i.e. number of events analyzed per second) and the representativeness of these methods (i.e. possibility for the simultaneous measurement of cell concentration and viability). Among these optical methods, the focused beam reflectance method (FBRM) allows for the analysis of a large amount of particles within a second [88]. A three-dimensional version of this method has recently been applied for the investigation of the scale-down effect on *E. coli* fed-batch cultures [92]. A good correlation between flow cytometry parameters and the FBRM probe was obtained but the processing unit is not configured for the recovery of the initial single-cell data. Automated FC remains thus actually the best single-cell method that can be interfaced with bioprocesses. However, the attractiveness of this technique is strongly dependent on the development of relevant fluorescent biosensors.

5 Biosensors in bioprocess monitoring

In the context of bioprocess monitoring and control, single-cell analyses should ideally require no or very limited sample pre-treatment, in order to be amenable to automation while providing near real-time results. In this respect, genetically encoded sensors are thus preferable to techniques requiring the addition of exogenous chemicals (such as propidium iodide, which is widely used as an indicator of cell "integrity" [93]). Biosensors are typically composed of an input sensory module, which detects an intracellular event of interest, and a transduction module, which converts this event into a signal (output) that can be detected by the equipment. The use of FC as a single-cell analysis technique, mostly limits the choice of output signals to fluorescent proteins (FPs). Nevertheless, recent advances in the engineering of FPs (mostly based on the GFP from the jellyfish *Aequorea victoria*) has provided a large array of possible outputs with various spectral properties, maturation or degradation kinetics [94]. Traditionally, biosensors are designed in such a way that the expression of the FP is under transcriptional control of a promoter whose activity is regulated by the signal of interest (promoter-based sensors). Potential marker genes and promoters for bioprocess monitoring have been extensively reviewed elsewhere [95] and will not be addressed here. Although successful applications of such sensors have been reported for the study of population heterogeneity under bioprocess-relevant conditions – for instance using stress- or growth rate-responsive promoters [32, 96], such a design bears several limitations. First, these sensors rely on species-specific transcription factors; their transposition to other species (or even strains of the same species) is thus not straightforward. Secondly, because they require the de novo synthesis and maturation of the FP, they are intrinsically slow-responsive (the fastest-maturing GFP variants can only be detected about 5 min after transcription initiation [97]). Similarly, the shutdown of the response is also relatively slow, even for unstable variants such as GFP variant with reduced half-life tagged to be recognized by protease (GFP-LVA, half-life of 60 min [98]). This slow dynamics is well adapted for monitoring long-term adaptive responses, and can even be advantageous since it reflects the cell's history over several minutes or hours. The slow kinetics of these sensors can actually be used to develop so-called FP timers, which can provide information on the turnover of proteins [99]. It also has advantages from a robustness perspective: the slow dynamics is an intrinsic noise filter. However, it prevents the use of such sensors for monitoring fast cellular processes that occur at the post-translational level (metabolome, interactome, etc.), at a time-scale of a few seconds or less. In order to monitor fast cellular processes in real time at the single-cell level, alternative sensors must be used, which directly modulate fluorescence intensity and/or spectrum in response to the concentration of target molecules, without the need for de novo FP synthesis. Such sensors are typically based on constitutively expressed FP variants linked to ligand-binding domains (including Förster resonance energy transfer (FRET)-based sensors). There is an increasing number of FP sensors that allow monitoring the intracellular levels of small molecules (cyclic adenosine mono phosphate

Table 1. Characteristics of promoter-based, riboswitch-based, and FP-based biosensors

Sensor type	Maturity	Response dynamics	Signals that can be detected	Cofactor requirement	Potential for transposition to other species
Promoter-based	Numerous examples, including use in bioprocess monitoring at the single-cell level using flow cytometry	Slow	Detection of complex signals resulting from the integration of several intracellular signals at the level of a single promoter via one or more transcription factors	Host-specific transcription factor(s)	Low (species-specific)
Riboswitch-based	Recently discovered Few examples of their use for biosensor design No reported application for bioprocess monitoring	Slow	Specific metabolites (or ratio between metabolites) Limited range of metabolites can be detected with known natural riboswitches, but synthetic riobswitches can be designed with new ligand specificity	No host cofactor required	High
FP sensors	Numerous examples, mainly in the field of single-cell imaging Few examples of applications in bioprocess monitoring No reported application for bioprocess monitoring at the single-cell level	Fast	Specific metabolites (or ratio between metabolites), intracellular parameters (pH, redox status), protein-protein interactions FP sensors can be designed for the detection of virtually any molecule, provided binding modules are available	No host cofactor required	High

(cAMP), phosphate, sugars, amino acids, inorganic ions, adenosine tri phosphate (ATP), citrate, lactate, cyclic di-guanosine mono phosphate (c-di-GMP), etc.), intracellular parameters (pH, redox status), or protein-protein interactions (see [100] for a recent review of FP sensors of small molecules). Tailor-made FP sensors can be designed relatively easily for the detection of virtually any molecule, provided binding modules are available [101], further expanding the range of signals that can possibly be monitored. They can be combined to monitor several parameters in a single cell [102], and are compatible with FC [103]. Despite these advantages, there has been no report to date of the use of such genetically encoded biosensors for the real-time monitoring of microbial bioprocesses at the single-cell level. The other limitation of promoter-based sensors (dependence on species-specific host transcription factors) can also be overcome by the FP sensors described above, as they are able to sense ubiquitous molecules and require no additional protein cofactor. In this context, riboswitches – specific regions of mRNAs that directly sense cellular metabolites and modulate transcription or translation – also hold promise as regulatory elements for the design of biosensors, although there are very few reports of such an application of riboswitches [104]. Riboswitch-based sensors combine the advantages of a slow response – because they require de novo FP synthesis – with the ability to sense ubiquitous metabolites (amino acids, vitamins, c-di-GMP, etc.) without cofactor requirement [105]. Although the set of ligands that are recognized by currently known natural riboswitches is relatively limited, synthetic riboswitches with targeted ligand specificities have been successfully designed, paving the way to the design of riboswitch-based sensors that could monitor virtually any cellular metabolite. In conclusion, several types of sensors have the potential to be used for real-time analysis of bioprocesses at the single-cell level, with complementary characteristics in terms of dynamics, targeted signals, and host range (Table 1), and are thus potentially suited for the monitoring of different types of host responses. However, most of the constructs mentioned above involve strong genetic modification impairing the physiology of the host and the intensity of the signaling molecule itself. These technical limitations explain the lack of studies under real bioprocessing conditions involving these constructs. However, with the advances made in synthetic biology these tools will be optimized and available for microbial single-cell studies under bioprocessing conditions.

6 Conclusions and perspectives

The origin of phenotypic heterogeneity and its implication for microbial bioprocesses are still a subject of debate. The contribution of microbial heterogeneity to

processes in biotechnological applications must be considered carefully. Whatever the application is, the scale-up procedures induce a loss of mixing capacity and trigger a stress response at the level of the microbial population. Several authors have reported that this stress response can be beneficial and lead to substantial bioprocess improvement, the origin of which origin remains unclear [26, 32, 106, 107]. In this context, it is important to identify the respective part taken by the individual path and the coordinated heterogeneity hypotheses that have been put forward to explain the appearance of phenotypic heterogeneity in bioprocesses. Indeed, if heterogeneity depends only on the individual environmental profiles experienced at the single-cell level, it will be difficult to exploit external noise generated at the process level. However, there is a lot of experimental evidence suggesting that microbial population heterogeneity can be the result of a coordinated physiological response in order to better cope with stress [25, 42, 108]. In order to assess this hypothesis, there is an urgent need for relevant biomarkers of stress heterogeneity among the population (not only based on cell viability, but also on cell secretory capacity and productivity) in combination with on-line single-cell technologies. Flow cytometry is actually the best choice for automated, high-throughput, single-cell analysis under process-related conditions. Actually, very few examples of interfacing FC with a bioreactor can be found in the literature and this method is rarely effectively used in a feedback control loop for bioprocess control. Additionally, dynamic FC data must be better exploited in order to gain understanding about the population dynamics and the transition of cells from a subpopulation to another (as in the bistability phenomenon).

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The authors declare no conflict of interest.

7 References

[1] Rao, C. V., Wolf, D. M., Arkin, A. P., Control, exploitation and tolerance of intracellular noise. *Nature* 2002, *420*, 231–237.
[2] Mettetal, J. T., Muzzey, D., Pedraza, J. M., Ozbudak, E. M., van Oudenaarden, A., Predicting stochastic gene expression dynamics in single cells. *PNAS* 2006, *103*, 7304–7309.
[3] Thattai, M., van Oudenaarden, A., Stochastic gene expression in fluctuating environments. *Genetics* 2004, *167*, 523–530.
[4] Patnaik, P. R., External, extrinsic and intrinsic noise in cellular systems: analogies and implications for protein synthesis. *Biotechnol. Mol. Biol. Rev.* 2006, *1*, 121–127.
[5] Kaufmann, B. B., van Oudenaarden, A., Stochastic gene expression: from single molecules to the proteome. *Curr. Opin. Genet. Dev.* 2007, *17*, 107–112.
[6] Rosenfeld, N., Young, J. W., Alon, U., Swain, P. S., Elowitz, M. B., Gene regulation at the single cell level. *Science* 2005, *307*, 1962–1965.
[7] Veening, J., Smits, W. K., Hamoen, L. W., Kuipers, O. P., Single cell analysis of gene expression patterns of competence development and initiation of sporulation in *Bacillus subtilis* grown on chemically defined media. *J. Appl. Microbiol.* 2006, *101*, 531–541.
[8] Nevozhay, D., Adams, R. M., Van Itallie, E., Bennett, M. R., Balázsi, G., Mapping the environmental fitness landscape of a synthetic gene circuit. *PLoS Comput. Biol.* 2012, *8*, e1002480.
[9] Wolf, D. M., Vazirani, V. V., Arkin, A. P., Diversity in times of adversity : probabilistic strategies in microbial survival games. *J. Theor. Biol.* 2005, *234*, 227–253.
[10] Veening, J., Smits, W. K., Kuipers, O. P., Bistability, epigenetics, and bet-hedging in bacteria. *Annu. Rev. Microbiol.* 2008, *62*, 193–210.
[11] Wolf, D. M., Fontaine-Bodin, L., Bischofs, I. et al., Memory in microbes: quantifying history-dependent behavior in a bacterium. *Plos One* 2008, *3*, e1700.
[12] Molenaar, D., van Berlo, R., de Ridder, D.,Teusink, B., Shifts in growth strategies reflect tradeoffs in cellular economics. *Mol. Sys. Biol.* 2009, *5*, 1–10.

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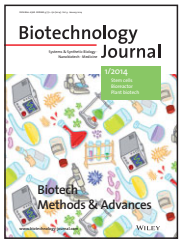
[13] Zakrzewska, A., van Eikenhorst, G., Burggraaff, J. E., Vis, D. J. et al., Genome-wide analysis of yeast stress survival and tolerance acquisition to analyze the central trade-off between growth rate and cellular robustness. *Mol. Biol. Cell* 2011, *22*, 4435–4446.
[14] Carlquist, M., Fernandes, R. L., Helmark, S., Heins, A. L. et al., Physiological heterogeneities in microbial populations and implications for physical stress tolerance. *Microb. Cell Fact.* 2012, *11*, 94.
[15] Acar, M., Mettetal, J. T, van Oudenaarden, A., Stochastic switching as a survival strategy in fluctuating environments. *Nat. Genet.* 2008, *40*, 471–475.
[16] Bishop, A., Rab, F. A., Sumner, E. R., Avery, S.V., Phenotypic heterogeneity can enhance rare-cell survival in 'stress-sensitive' yeast populations. *Mol. Microbiol.* 2007, *63*, 507–520.
[17] Boyle, N., Reynolds, T. S., Evans, R., Lynch, M., Gill, R. T., Recombineering to homogeneity: extension of multiplex recombineering to large-scale genome editing. *Biotechnol. J.* 2013, *8*, 515–522.
[18] Bayer, T., Hoff, K. G., Beisel, C. L., Lee, J. J., Smolke, C.D., Synthetic control of a fitness tradeoff in yeast nitrogen metabolism. *J. Biol. Eng.* 2009, *3*, 1.
[19] Acar, M., Becskei, A, van Oudenaarden, A., Enhancement of cellular memory by reducing stochastic transitions. *Nature* 2005, *435*, 228–232.
[20] Lencastre Fernandes, R., Nierychlo, M., Lundin, L., Pedersen, A. E. et al., Experimental methods and modeling techniques for description of cell population heterogeneity. *Biotechnol. Adv.* 2011, *29*, 575–599.
[21] Müller, S., Harms, H., Bley, T., Origin and analysis of microbial population heterogeneity in bioprocesses. *Curr. Opin. Biotechnol.* 2010, *21*, 100–113.
[22] Lara, A. R., Galindo, E., Ramirez, O. T., Palomares, L. A., Living with heterogeneities in bioreactors - Understanding the effects of environmental gradients on cells. *Mol. Biotechnol.* 2006, *34*, 355–381.
[23] Müller, S., Nebe-von-Caron, G., Functional single-cell analyses : flow cytometry and cell sorting of microbial populations and communities. *FEMS Microbiol. Rev.* 2010, *34*, 554–587.
[24] Patnaik, P. R., Can imperfections help to improve bioreactor performance ? *Trends Biotechnol.* 2002, *20*, 135–137.
[25] Ryall, B., Eydallin, G., Ferenci, T., Culture history and population heterogeneity as determinants of bacterial adaptation: the adaptomics of a single environmental transition. *Microbiol. Mol. Biol. Rev.* 2011, *76*, 597–625.
[26] Enfors, S. O., Jahic, M., Rozkov, A., Xu, B. et al., Physiological responses to mixing in large scale bioreactors. *J. Biotechnol.* 2001, *85*, 175–185.
[27] Jazini, M., Herwig, C., Effect of post-induction substrate oscillation on recombinant alkaline phosphatase production expressed in *Escherichia coli*. *J. Biosci. Bioeng.* 2011, *112*, 606–610.
[28] Jazini, M., Herwig, C., Effects of temperature shifts and oscillations on recombinant protein production expressed in *Escherichia coli*. *Bioprocess Biosyst. Eng*. In press.
[29] Lapin, A., Müller, D., Reuss, M., Dynamic behavior of microbial populations in stirred bioreactors simulated with Euler-Lagrange methods: traveling along the lifelines of single cells. *Ind. Eng. Chem. Res.* 2004, *43*, 4647–4656.
[30] Lapin, A., Schmid, J., Reuss, M., Modeling the dynamics of *E. coli* populations in the three-dimensional turbulent field of a stirred bioreactor - A structured-segregated approach. *Chem. Eng. Sci.* 2006, *61*, 4783–4797.
[31] Hewitt, C. J., Nienow, A. W., The scale-up of microbial batch and fed-batch fermentation processes. *Adv. Appl. Microbiol.*, 2007, *62*, 105–135.
[32] Delvigne, F., Boxus, M., Ingels, S., Thonart, P., Bioreactor mixing efficiency modulates the activity of a prpoS: GFP reporter gene in *E. coli*. *Microb. Cell Fact.* 2009, *8*, 15.
[33] Schmalzriedt, S., Jenne, M., Mauch, K., Reuss, M., Integration of physiology and fluid dynamics. *Adv. Biochem. Eng.* 2003, *80*, 19–68.
[34] Delvigne, F., Destain, J., Thonart, P., Bioreactor hydrodynamic effect on *Escherichia coli* physiology: experimental results and stochastic simulations. *Bioprocess Biosyst. Eng.* 2005, *28*, 131–137.
[35] Delvigne, F., Lejeune, A., Destain, J., Thonart, P., Modelling of the substrate heterogeneities experienced by a limited microbial population in scale-down and in large-scale bioreactors. *Chem. Eng. J.* 2006, *120*, 157–167.
[36] Zahradnik, J., Mann, R., Fialova, M., Vlaev, D. et al., A network-of-zones analysis of mixing and mass transfer in three industrial bioreactors. *Chem. Eng. Sci.* 2001, *56*, 485–492.
[37] Morchain, J., Gabelle, J. C., Cockx, A., Coupling of biokinetic and population balance models to account for biological heterogeneity in bioreactors. *AIChE J.* 2012, *59*, 369–379.
[38] Lencastre Fernandes, R., Carlquist, M, Lundin, L, Heins, A. L. et al., Cell mass and cell cycle dynamics of an asynchronous budding yeast population: experimental observations, flow cytometry data analysis, and multi-scale modeling. *Biotechnol. Bioeng.* 2012, *110*, 812–826.
[39] Porro, D., Vai, M., Vanoni, M., Alberghina, L., Hatzis, C., Analysis and modeling of growing budding yeast populations at the single cell level. *Cytometry Part A* 2009, *75*, 114–120.
[40] Kussell, E., Leibler, S., Phenotypic diversity, population growth and information in fluctuating environments. *Science* 2005, *309*, 2075–2078.
[41] Dubnau, D., Losick, R., Bistability in bacteria. *Mol. Microbiol.* 2006, *61*, 564–572.
[42] Lidstrom, M., Konopka, M. C., The role of physiological heterogeneity in microbial population behavior. *Nat. Chem. Biol.* 2010, *6*, 705–712.
[43] March, J. C., Bentley, W. E., Quorum sensing and bacterial cross-talk in biotechnology. *Curr. Opin. Biotechnol.* 2004, *15*, 495–502.
[44] Song, H., Payne, S., Tan, C., You, L., Programming microbial population dynamics by engineered cell-cell communication. *Biotechnol. J.* 2011, *6*, 837–849.
[45] Boedicker, J., Vincent, M. E., Ismagilov, R. F., Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behavior of quorum sensing and growth and reveals its variability. *Angewandte Chemie* 2009, *48*, 5908–5911.
[46] Love, K. R., Politano, T. J., Panagiotou, V., Jiang, B. et al., Systematic single-cell analysis of *Pichia pastoris* reveals secretory capacity limits productivity. *Plos one* 2012, *7*, e37915.
[47] Delvigne, F., Destain, J., Thonart, P., A methodology for the design of scale-down bioreactors by the use of mixing and circulation stochastic models. *Biochem. Eng. J.* 2006, *28*, 256–268.
[48] Davidson, K. M., Sushil, S., Eggleton, C. D., Marten, M. R., Using computational fluid dynamics software to estimate circulation time distribution in bioreactors. *Biotechnol. Prog.* 2003, *19*, 1480–1486.
[49] Reinecke, S., Deutschmann, A., Jobst, K., Kryk, H. et al., Flow following sensor particles-validation and macro-mixing analysis in a stirred fermentation vessel with a highly viscous substrate. *Biochem. Eng. J.* 2012, *69*, 159–171.
[50] Wolf, D. M., Arkin, A. P., Motifs, modules and games in bacteria. *Curr. Opin. Microbiol.* 2003, *6*, 125–134.
[51] Diaz, M., Herrero, M., Garcia, L. A., Quiros, C., Application of flow cytometry to industrial microbial bioprocesses. *Biochem. Eng. J.* 2010, *48*, 385–407.
[52] DeLisa, M. P., Bentley, W. E., Bacterial autoinduction : looking outside the cell for new metabolic engineering targets. *Microb. Cell Fact.* 2002, *1*, 5.
[53] Gill, R. T., D. M. P., Valdes, J. J., Bentley, W. E., Genomic analysis of high-cell-density recombinant *Escherichia coli* fermentation and cell conditioning for improved recombinant protein yield. *Biotechnol. Bioeng.* 2001, *72*, 85–95.

[54] Davey, H. M., Life, death, and in-between: Meanings and methods in microbiology. *Appl. Environm. Microbiol.* 2011, *77*, 5571–5576.
[55] Strauber, H., Muller, S., Viability states of bacteria-specific mechanisms of selected probes. *Cytometry Part A* 2009, *77A*, 623–634.
[56] Joux, F., Lebaron, P., Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes Infect.s* 2000, *2*, 1523–1535.
[57] Davey, H. M., Hexley, P., Red but not dead ? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. *Environm. Microbiol.* 2011, *13*, 163–171.
[58] Booth, I. R., Stress and the single cell: Intrapopulation diversity is a mechanism to ensure survival upon exposure to stress. *Int. J. Food Microbiol.* 2002, *78*, 19–30.
[59] Lacroix, C., Yildirim, S., Fermentation technologies for the production of probiotics with high viability and functionality. *Curr. Opin. Biotechnol.* 2007, *18*, 176–183.
[60] Sundstrom, H., Wallberg, F., Ledung, E., Norrman, B., Hewitt, C. J., Enfors, S. O., Segregation to non-dividing cells in recombinant *Escherichia coli* fed-batch fermentation processes. *Biotechnol. Lett.* 2004, *26*, 1533–1539.
[61] Hewitt, C. J., Onyeaka, H., Lewis, G., Taylor, I. W., Nienow, A. W., A comparison of high cell density fed-batch fermentations involving both induced and non-induced recombinant *Escherichia coli* under well-mixed small-scale and simulated poorly mixed large-scale conditions. *Biotechnol. Bioeng.* 2007, *96*, 495–505.
[62] Sevastsyanovich, Y., Alfasi, S., Overton, T., Hall, R. et al., Exploitation of GFP fusion proteins and stress avoidance as a generic strategy for the production of high-quality recombinant proteins. *FEMS Microbiol. Lett.* 2009, *299*, 86–94.
[63] Want, A., Thomas, O. R., Kara, B., Liddell, J., Hewitt, C. J., Studies related to antibody fragment (Fab) production in *Escherichia coli* W3110 fed-batch fermentation processes using multiparameter flow cytometry. *Cytometry Part A* 2009, *75*, 148–154.
[64] Lee, K., Kim, J. S., Heo, P., Yang, T. J. et al., Characterization of *Saccharomyces cerevisiae* promoters for heterologous gene expression in *Kluyveromyces marxianus*. *Appl. Microbiol. Biotechnol.* 2013, *97*, 2029–2041.
[65] Stadlmayr, G., Benakovitsch, K., Gasser, B., Mattanovich, D., Sauer, M., Genome-scale analysis of library sorting (GALibSo): Isolation of secretion enhancing factors for recombinant protein production in *Pichia pastoris*. *Biotechnol. Bioeng.* 2010, *105*, 543–555.
[66] Hohenblum, H., Borth, N., Mattanovich, D., Assessing viability and cell-associated product of recombinant protein producing *Pichia pastoris* with flow cytometry. *J. Biotechnol.* 2003, *102*, 281–290.
[67] David, F., Berger, A., Hänsch, R., Rohde, M., Franco-Lara, E., Single cell analysis applied to antibody fragment production with *Bacillus megaterium*: Development of advanced physiology and bioprocess state estimation tools. *Microb. Cell Fact.* 2011, *10*, 23.
[68] Love, K., Panagiotou, V., Jiang, B., Stadheim, T. A., Love, J. C., Integrated single-cell analysis shows *Pichia pastoris* secretes protein stochastically. *Biotechnol. Bioeng.* 2010, *106*, 319–325.
[69] Binder, S., Schendzielorz, G., Stäbler, N., Krumbach, K. et al., A high-throughput approach to identify genomic variants of bacterial metabolite producers at the single-cell level. *Genome Biol.* 2012, *13*, R40.
[70] Alonso, S., Rendueles, M., Díaz, M.., Physiological heterogeneity of *Pseudomonas taetrolens* during lactobionic acid production. *Appl. Microbiol. Biotechnol.* 2012, *96*, 1465–1477.
[71] Tracy, B., Gaida, S. M., Papoutsakis, E. T., Development and application of flow-cytometric techniques for analyzing and sorting endospore-forming clostridia. *Appl. Environm. Microbiol.* 2008, *74*, 7497–7506.
[72] Hewitt, C., Nebe-Von-Caron, G., An industrial application of multiparameter flow cytometry: Assessment of cell physiological state and its application to the study of microbial fermentations. *Cytometry Part A* 2001, *44*, 179–187.
[73] Zhao, R., Natarajan, A., Srienc, F., A flow injection flow cytometry system for on-line monitoring of bioreactors. *Biotechnol. Bioeng.* 1999, *62*, 609–617.
[74] Abu-Absi, N. R., Zamamiri, A., Kacmar, J., Balogh, S. J., Srienc, F., Automated flow cytometry for acquisition of time-dependent population data. *Cytometry Part A* 2003, *51A*, 87–96.
[75] 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.
[76] Pe-a, P., Glasker, S., Srienc, F., Genome-wide overexpression screen for sodium acetate resistance in *Saccharomyces cerevisiae*. *J. Biotechnol.* 2013, *164*, 26–33.
[77] Gilbert, A., Sangurdekar, D. P., Srienc, F., Rapid strain improvement through optimized evolution in the cytostat. *Biotechnol. Bioeng.* 2009, *103*, 500–512.
[78] Reyes, L., Winkler, J., Kao, K. C., Visualizing evolution in real-time method for strain engineering. *Frontiers Microbiol.* 2012, *3*, 198.
[79] Reyes, L. H., Almario, M. P., Winkler, J., Orozco, M. M., Kao, K. C., Visualizing evolution in real time to determine the molecular mechanisms of n-butanol tolerance in *Escherichia coli*. *Metabolic Eng.* 2012, *14*, 579–590.
[80] Almario, M., Reyes, L H, Kao, K. C., Evolutionary engineering of *Saccharomyces cerevisiae* for enhanced tolerance to hydrolysates of lignocellulosic biomass. *Biotechnol. Bioeng.* 2013, *110*, 2616–2623.
[81] Sitton, G., Srienc, F., Single-cell tracking with a reversing flow cytometer. *Cytometry Part A* 2011, *79*, 66–76.
[82] Muzzey, D., van Oudenaarden, A., Quantitative time-lapse fluorescence microscopy in single cells. *Annu. Rev. Cell Dev. Biol.* 2009, *25*, 301–327.
[83] Schmid, A., Kortmann, H., Dittrich, P. S., Blank, L. M., Chemical and biological single cell analysis. *Trends Biotechnol.* 2010, *21*, 12–20.
[84] Fritzsch, F. S. O., Dusny, C., Frick, O., Schmid, A., Single-cell analysis in biotechnology, systems biology, and biocatalysis. *Annu. Rev. Chem. Biomol. Eng.* 2012, *3*, 129–155.
[85] Love, K., Bagh, S., Choi, J., Love, J. C., Microtools for single-cell analysis in biopharmaceutical development and manufacturing. *Trends Biotechnol.* 2013, *31*, 280–286.
[86] Wu, M., Singh, A. K., Single-cell protein analysis. *Curr. Opin. Biotechnol.* 2012, *23*, 83–88.
[87] Dusny, C., Fritzsch, F. S. O., Frick, O., Schmid, A., Isolated microbial single cells and resulting micropopulations grow faster in controlled environments. *App.l Environ. Microbiol.* 2012, *78*, 7132–7136.
[88] Höpfner, T., Bluma, A., Rudolph, G., Lindner, P., Scheper, T., A review of non-invasive optical-based image analysis systems for continuous bioprocess monitoring. *Bioprocess Biosyst. Eng.* 2010, *33*, 247–256.
[89] Rudolph, G., Lindner, P., Bluma, A., Joeris, K. et al., Optical inline measurement procedures for counting and sizing cells in bioprocess technology. *Adv. Biochem. Eng. Biotechnol.* 2010, *116*, 125–142.
[90] Sonnleitner, B., Automated measurement and monitoring of bioprocesses: key elements of the M(3)C strategy. *Adv. Biochem. Eng. Biotechnol.* 2013, *132*, 1–33.
[91] Lourenço, N. D., Lopes, J. A., Almeida, C. F., Sarraguça, M. C., Pinheiro, H. M., Bioreactor monitoring with spectroscopy and chemometrics: a review. *Anal. Bioanal. Chem.* 2012, *404*, 1211–1237.
[92] Brognaux, A., Bugge, J., Schwartz, F. H., Thonart, P. et al., Real-time monitoring of cell viability and cell density on the basis of a three dimensional optical reflectance method (3D-ORM): investigation of the effect of sub-lethal and lethal injuries. *J. Ind. Microbiol. Biotechnol.* 2013, *40*, 679–686.
[93] Lehtinen, J., Nuutila, J., Lilius E. M., Green fluorescent protein – propidium iodide (GFP-PI) based assay for flow cytometric measurement of bacterial viability *Cytometry Part A* 2004, *60*, 165–172.

[94] Day, R. N., Davidson, M. W., The fluorescent protein palette: tools for cellular imaging. *Chem. Soc. Rev.* 2009, *38*, 2887–2921.
[95] Schweder, T., Bioprocess monitoring by marker gene analysis. *Biotechnol. J.* 2011, *6*, 926–933.
[96] Han, S., Delvigne, F., Brognaux, A., Gitte, C., Sorensen, J. S., Design of growth-dependent biosensors based on destabilized GFP for the detection of physiological behavior of *Escherichia coli* in heterogeneous bioreactors. *Biotechnol. Prog.* 2013, *29*, 553–563.
[97] Iizuka, R., Yamagishi-Shirasaki, M., Funatsu, T., Kinetic study of de novo chromophore maturation of fluorescent proteins. *Anal. Chem.* 2011, *414*, 173–178.
[98] Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjorn, S. P. et al., New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl. Environ. Microbiol.* 1998, *64*, 2240–2246.
[99] Khmelinskii, A., Keller, P. J., Bartosik, A., Meurer, M. et al., Tandem fluorescent protein timers for in vivo analysis of protein dynamics. *Nat. Biotechnol.* 2012, *30*, 708–774.
[100] Okumoto, S., Quantitative imaging using genetically encoded sensors for small molecules in plants. *Plant J.* 2012, *70*, 108–117.
[101] Tolosa, L., On the design of low-cost fluorescent protein biosensors. *Adv. Biochem. Eng. Biotechnol.* 2009, *116*, 143–157.
[102] Woehler, A., Simultaneous quantitative live cell imaging of multiple FRET-based biosensors. *Plos One* 2013, *8*, e61096.
[103] Banning, C., Votteler, J., Hoffmann, D., Koppensteiner, H. et al., A flow cytometry-based FRET assay to identify and analyze protein-protein interactions in living cells. *Plos One* 2010, *5*, e9344.
[104] Ogawa, A., Maeda, M., An artificial aptazym-based riboswitch and its cascading system in *E. coli*. *ChemBioChem* 2008, *9*, 206–209.
[105] Serganov, A., Nudler, E., A decade of riboswitches. *Cell* 2013, *152*, 17–24.
[106] Hewitt, C. J., Nebe-Von Caron, G., Axelsson, B., Mc Farlane, C. M., Nienow, A. W., Studies related to the scale-up of high-cell-density *E. coli* fed-batch fermentations using multiparameter flow cytometry : effect of a changing microenvironment with respect to glucose and dissolved oxygen concentration. *Biotechnol. Bioeng.* 2000, *70*, 381–390.
[107] Delvigne, F., Brognaux, A., Francis, F., Twizere, J. C. et al., Green fluorescent protein (GFP) leakage from microbial biosensors provides useful information for the estimation of the scale-down effect. *Biotechnol. J.* 2011, *6*, 968–978.
[108] Sumner, E., Avery, S. V., Phenotypic heterogeneity: differential stress resistance among individual cells of the yeast *Saccharomyces cerevisiae*. *Microbiology* 2002, *148*, 345–351.
[109] Stephanopoulos, G. N., Aristidou, A. A., Nielsen, J., *Metabolic engineering : principles and methodologies*, Academic Press, San Diego, California, USA 1998.
[110] Avery, S., Microbial cell individuality and the underlying sources of heterogeneity. *Nat. Rev. Microbiol.* 2006, *4*, 577–587.



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