Response of *Escherichia coli* to nutrient availability during cultivation at single cell level
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PhD thesis
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Preface

The work presented in this thesis was conducted at the Section of Microbiology, Department of Biology, University of Copenhagen, and part of the work was done at Center of Microbial Biotechnology (CMB), Department of Systems Biology, Technical University of Denmark (DTU) and Gembloux Agro-Bio Tech, Université de Liège (ULg), Belgium. The Danish Council for Strategic Research is gratefully acknowledged for financial support in the frame of the project “Towards robust fermentation processes by targeting population heterogeneity at microscale” (project number 09-065160). ERA-IB (ERA-NET Industrial Biotechnology) is gratefully acknowledged for financial support in the frame of the project “Targeting population heterogeneity at microscale for robust fermentation processes” (project number EIB.08.031). Fond de la recherche scientifique (FRS-FNRS) in Belgium is gratefully acknowledged also for partially financial support.

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This thesis is dedicated to my family, whose support, encourage and patience have been tremendous important to me throughout these years.

Finally, I would like to quote a saying and hope it benefits people whoever read it in life and work.

‘What is of all things most yielding, can overcome that which is most hard.’----Lao Tzu

Shanshan Han

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Summary

Industrial fermentation processes comprise the potential sources of heterogeneity since the microbial cells exhibit an intrinsic cell-to-cell variability that is both affected by and influence the fermentation process itself (reactor conditions and medium composition) over time. However, population-averaged data, assuming uniform cellular behavior in the bacterial cultures, restrict our understandings of how changing environments affect population heterogeneity or how different sub-populations contribute to the fermentation process. In this thesis, the focus was given to study and quantify single cell responses to glucose perturbations in the bioreactor using *Escherichia coli* as model systems.

In the introduction chapters, the concept of microbial population heterogeneity is briefly introduced and hereafter used as a way to study the heterogeneous fermentation process. Followed by a short experimental outline, a short description of reporter strain construction is given. The reporter system using a low copy plasmid under the regulation of the *rrnB* promoter or *fis* promoter was constructed, the expression of which may provide complementary signal in responding to nutrient availability since they have similar growth related expression pattern. Then characterization of the reporter strains was performed in batch, fed batch and continuous cultivations with different environmental perturbations to simulate large-scale conditions and population heterogeneity was analyzed by flow cytometry.

Oxygen and glucose oscillation, typical for large-scale cultivation, was studied in Manuscript 1 with the two-compartment scale-down reactor consisting of a well-stirred compartment connected to a plug-flow compartment, which allows reproduction of mixing imperfections. Similar expressions of both *rrnB* and *fis* promoters were related to the cultivation mode with different glucose limitation. The sudden glucose relief elevated the ribosomal RNA synthesis as well as its transcriptional activator Fis. Heterogeneities induced by scale-down may affect protein leakage into the broth medium because of the increased membrane permeability and thus resulted in the loss of cellular fluorescence. Such an observation was further investigated in Manuscript 2 with an in-house flow cytometer and PI staining. Growth and cell permeability were monitored in real-time during the process at the single cell level and the performance of the modified on-line
analysis was demonstrated by showing similar result of glucose perturbations carried out in chemostat cultivation as shown in Manuscript 1. A “mean to median ratio” of recorded parameters was introduced to detect segregation in the population. At the mean time, the observed suggested that bacterial cells are robust cell factories that they could self-repair membrane integrity and thus contribute to the redistribution of subpopulations.

The study of response adaptation was extended in Manuscript 3. Single glucose pulse experiments were performed in aerobic glucose-limited Saccharomyces cerevisiae and Escherichia coli chemostat cultures. The physiology of S. cerevisiae and E. coli at low growth rates deserves more attention as slow-growing cells were more robust towards stress than fast-growing cells. A high GFP expression and more dynamic changes were observed in slow growing cells than fast growing cells during the glucose oscillation. Population heterogeneity was quantified by introducing new parameters to describe fluorescence distribution data.

As a complement to using reporter strains for detection of heterogeneity in the fermentation context, the use of different fluorescent stains targeting particular physiological features such as membrane integrity and metabolic activity was studied as these will provide tools that easier may be applied in an industrial setting where one maybe do not want to introduce specific reporter strains. In manuscript 4, characterization and application of the fluorescent dyes such as CTC, SYBR Green, PI, DiBAC4(3) and RSG was used in batch cultivation to assess cellular viability of bacterial cells as regards respiration activity and membrane potential. Cellular heterogeneity was reflected at the transcription level of the ribosomal genes and specific physiologic and metabolic characteristics of the bacteria pictured by the reporter strain and the fluorescence stains.
Sammendrag

I industrielle fermenteringsprocesser vil der altid være potentielt heterogenitet, idet mikrobielle celler har en indre celle-til-celle-variabilitet, der både påvirkes af og påvirker selve fermenteringsprocessen (reaktorbetingelser og mediets sammensætning) over tid. Hvis man imidlertid antager ensartet celleopførsel i en bakteriekultur, vil ”population-averaged data” begrænse vores forståelse for hvordan ændringer i miljøet påvirker populations heterogeniteten og hvordan forskellige sub-populationer bidrager til fermentations processen. Stor skala fermentering bevirker, at der vil være en rumlig heterogenitet på grund af utilstrækkelig omrøring, dvs der dannes gradierter af fx opløst ilt, substrater, temperatur og pH. Således er mikrobielle celler dyrket i store fermentorer udsat for hurtige ændringer i miljøforholdene, da de cirkulerer i hele reaktoren og heterogeniten kan have negativ effekt på både kvalitet og kvantitet af det dannede produkt. I denne phd afhandling er der blevet fokuseret på effekten af varierende glukosekonzentrationer på single cell niveau med *Escherichia coli* som model system.

I introduktions kapitlerne bliver population heterogenitets begrebet introduceret og derefter benyttet til at studere mikrobiel heterogenitet i fermentations processen. Derefter følger en kort redegørelse for den eksperimentelle outline og en kort beskrivelse af konstruktion af reporter gener. Der blev konstrueret et reporter system ved hjælp af et ”low copy” plasmid regulert af en *rrnB* eller en *fis*-promotor hvis ekspression kunne give et komplementært signal som respons på næringsstof tilgængelighed, da de har samme vækst relaterede expreesions mønstre. Karakterisering af reporter-stammer blev udført i både batch, fed-batch og kontinuerlige kulturer med forskellige ændringer i miljøet for at simulere stor skala betingelser; population ændringerne blev analyseret med flowcytometri.

Oxygen og glukose oscillation, typisk for stor skala dyrkning, blev undersøgt (Manuskript 1) i en two-compartment scale-down reaktor bestående af en grundig omrørt del forbundet til et ”plug-flow” kammer, som giver mulighed for at undersøge mangelfuld opblanding. Lignende expressioner for både *rrnB* og *fis* promotorerne var relatet til dyrkningsbetingelser med forskellig glukose begrensning. Det pludselige glukose puls eleverede både ribosomal RNA-syntesen og
dens transkriptional activator Fis. Heterogenitet induceret af skala-down kan påvirke protein udslip i vækstmediet på grund af den forøgede membran permeabilitet og resulterer således i tab af cellular fluorescens. Denne observation blev yderligere undersøgt (Manuskript 2) med et in-house flowcytometer og PI farvning. Vækst og celle permeabilitet blev målt i real-time under processen på single cell niveau, og resultatet af den modificerede on-line-analyse blev påvist ved at vise lignende resultater af glucose perturbationer udført i kemostat dyrkning som vist i tidligere forsøg (Manuscript 1). ”Mean to median ratio” af registrerede parametre blev indført for at opdage segregering i populationen. At ”the mean time” blev det observeret at bakterieceller er robuste cellefabrikker, at de kunne selvreparere membran integritet og dermed bidrage til en omfordeling af subpopulationer.


**List of publications**

This thesis is based on the following manuscripts, which are included at the end of the thesis.


2. Alison Brognaux, Shanshan Han, Søren J. Sørensen, Frank Delvigne. **A low-cost automated flow cytometry procedure for the characterization of microbial stress dynamics in bioreactors.** *In preparation*

3. Anna-Lena Heins*, Shanshan Han*, Ted Johanson, Rita Lencastre Fernandes, Luisa Lundin, Gitte E Charbon, Magnus Carlquist, Krist V. Gernaey, Søren J. Sørensen, and Anna Eliasson Lantz. **Shifts of population heterogeneity distributions in response to steady-state growth rate and glucose perturbations in *Escherichia coli* and *Saccharomyces cerevisiae*.** *Contributed equally. In preparation*

4. Luisa Lundin*, Shanshan Han*, Inês Nunes, Anna-Lena Heins, Rita Lencastre Fernandes, Gitte Charbron, Waleed Abu Al-Soud, Anna Eliasson Lantz, Krist Gernaey, Søren J. Sørensen. **Improving robustness in batch cultures by investigating heterogeneity at single cell level.** *Contributed equally. In preparation*
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Chapter 1

General Introduction
Microbial populations have been traditionally thought of as large clonal groups. In fact, seemingly identical individuals display substantial heterogeneity due to the environmental surrounding[2]. Population heterogeneity has been found previously in large-scale fermentations[3, 4]. Since then many efforts have been made into revealing the traditional averaged population structure. Evidence pointed that a microbial population in a fermenter is heterogeneous[3]. *E. coli* and *S. cerevisiae* have developed complex stress responding systems. Intensive studies of stress responses have focused primarily on physiology and regulation of gene expression in response to environmental changes of the key model microorganisms *Escherichia coli* and *Saccharomyces cerevisiae*[5]. Some nutrient and stress response in bioprocess will be detailed in chapter 2, but first in this introduction I will try to illustrate the adaptive response, normal stressors in bioprocess and a simple introduce of ways to visualize heterogeneity.

### 1.1 Nutritional and environmental factors required for growth

Cell growth (the increase in cell mass through macromolecular synthesis) requires the synthesis of cellular components, which needs proper supply of sugars, N source, sulfur, phosphate, etc, to build up the cell factory. Environmental factors, such as temperature, pH, oxygen and water availability, are determining factors that affect bacterial growth. The environment can be highly heterogeneous in terms of physical and biological parameters that affect both microbial growth and development. The nutrient availability is important for understanding bacterial growth and physiology. Glucose is the most preferred carbon source for many organisms and, accordingly, changes in glucose availability often have profound consequences in many types of cell gene expressions[6]. ‘Stress is any change in the genome, proteome or environment that imposes either reduced growth or survival potentials. Such changes lead to attempts by a cell to restore a pattern of metabolism that either fits it for survival or for faster growth.’ [7].

### 1.2 Adaptive responses to stresses/ nutrient limitation

#### 1.2.1 A joint response and nutrient sensing

*E. coli* responds to stress by reducing growth. The cell becomes smaller and rounder, accumulates storage compounds such as glycogen and polyphosphate, changes membrane composition and DNA structure and adjust the cellular activity under carbon limitation to maintain survival[8]. The tradeoff between growth and survival is the re-distribution of cellular resources to enable a higher stress resistance at the lower growth rate[9]. *E. coli* has different gene and protein profile under different growth conditions and behaves differently
according to their niche surroundings in a seemingly homogenous environment[2]. Cell-to-cell differences, including differences in cell cycle and cell aging, contribute to the generation of phenotypic heterogeneity that exist at all the time, even if the microenvironment is constant, and only become relevant or important during environmental perturbations[10]. Stress response is usually represented by a combination of specific responses, aiming at minimizing deleterious effects (e.g. catalase during oxidative stress), or repairing damage (e.g. protein chaperones under temperature stress, SOS system induced by DNA damage) and general responses such as the RpoS-regulated response aiming to produce a more resistant cell[11]. The reaction to nutrient limitation can be seen as the transition from an active metabolic state and fast cell division at maximal growth to a maintenance metabolism and the induction of many genes whose function is to provide maximal protection against a large variety of stress conditions. The stress response is coupled with cell growth phase in E. coli [12] or cell cycle phase in yeast[13]. All in all, physiological readjustments to environmental stresses involve multilayers of genes, enzymes and proteins. The joint response adopted by bacteria involves multiple layers of genes and metabolites interaction that is integrated by cross protection through multiple signaling networks, which has been observed previously between different types of stresses: exposure to stress of one kind increases the resistance to other stresses[14].

E. coli carbon stress response utilizes mechanisms which are part of the rpoS governed general stress response, and involve several changes in cellular physiology. Carbon stress responses displayed by E. coli when growing under carbon source limitation are similar to those encountered during stationary phase or glucose limited conditions in chemostats, fed batch cultures, or in strains with limited glucose transport capabilities[15]. Still there might be stress responses that are not activated when growing yeast in the chemostat but are activated in the batch cultures [16]. In the glucose-sensing pathway the large serine/threonine protein kinase (TOR) functions as a central controller of eukaryotic cell growth that senses and integrates the signal of nutritional status, the response of gene expression, including transcription, translation and protein stability into regulation of cell growth and cell cycle progression[5]. This conserved kinase for yeast is not subject to growth-rate control at the transcription level, but is dedicated to activate translation initiation in response to nutrients[5, 17].

1.2.2 Changes in cell size and morphology

Bacterial growth is reflected as an increase in cell size and number of cells. Nutritional stress can change bacterial morphology. Morphological plasticity is one of the bacterial
survival strategies. Bacterial cell morphological alteration is related to medium composition but not the temperature, which only has effect on the growth rate[18]. Cell size is strictly regulated in response to their access to nutrients, being larger when nutrient levels are higher[19]. Cell grows larger in rich medium than in minimal medium. In *E. coli* chromosomal DNA replication is not initiated until the cell reaches an appropriate size, since the cell mass (cell size)-to-DNA ratio is always constant. Fatty acid (FA) biosynthesis is argued to be involved in regulating the size of *E. coli* cells in response to nutrients availability in the environment[19]. It can be assumed of as a balance between growth and cell division. Opposite changes in cell size were observed during phosphate and sulfate batch limitations[16]. Entry into stationary phase *E. coli* cells become smaller, develop a spherical or egg shaped rather than a rod-shaped morphology[12] and preserve a tough membrane[20]. Morphogene *bolA* regulated by sigma factor S contributes to the decrease of ‘surface to volume’ ratio and modulation of outer membrane properties[21, 12]. By contrast, many gram-positive bacteria produce dormant spores in response to starvation[22]. In the budding yeast *Saccharomyces cerevisiae* nutrient limitation induces morphological changes that depend on the PKA pathway and the filamentous growth Kss1 MAPK pathway in either diploids or haploids. The protein kinase RIM15 functions in the integration of the nutrient signals, particular glucose depletion, into these pathways and thus mediates colony morphology[23]. Low levels of carbon combined with abundant nitrogen trigger colony morphology response in budding yeast[24] which refers to the formation of biofilm, multicellular stalk-like structures, etc.

### 1.2.3 Changes in membrane, DNA structure, protein production and protein composition

Membrane permeability, DNA integrity and protein folding are three classes of cell structure to be valued on the magnitude of stress and are central to bacterial survival[25]. In Gram-negative bacteria such as *Escherichia coli*, the envelope comprises three layers, an inner membrane, a periplasmic space that contains the cell wall, and an outer membrane. Being in contact with the external medium, the envelope is the initial target or boundary of physical (e.g., hyperthermia, osmolality), chemical (e.g., ethanol, pH, detergent) or biological (e.g., adhesion, infection) stresses that may alter envelope components, thus inducing an extra cytoplasmic stress response[26]. The response to most stress factors relies at least partly on the membrane integrity. An intact and robust membrane is essential to keep cytoplasmic pH rather constant; that is how a bacterial cell physiology is tightly controlled and how well cell could cope with external stimuli[27]. Confronting with acetate stress, genes belonging to or
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independent of RpoS family provide a joint protection to maintain a near neutral pH in *E. coli*, for instance, cyclopropane fatty acid synthase (coded by *cfa* gene) helps to decrease the permeability of the membrane to protons[28]. Although overall protein synthesis rate is reduced to 20% in the first hours of starvation, bacteria synthesize new survival proteins characteristic to conditions of carbon, nitrogen or phos starvation[29, 30]. Transition from glucose-excess to glucose-limited growth conditions resulted in enhanced membrane permeability of glucose in short-term and mutations of three loci (mlc, mglD/O and malT) to increase glucose affinity in long-term physiological adaptation[31]. More recently, some small (low molecular weight) neglected stress response proteins are surprisingly identified under different growth conditions and/or after exposure to stress, which might count for the inner membrane permeability[32]. Outer membrane structure and protein composition can be altered by medium composition, glucose-feeding rate in batch, continuous or fed-batch cultivations[33, 34]. In *E. coli* the synthesis of the major outer membrane proteins OmpC, OmpF and OmpA will be altered by different nutritional conditions[35]. Protein leakage seems to be a process related phenomena, it was found to be as twice as high in chemostat cultivation compared to the constant leakage level in fed-batch[34, 36]. Some subpopulations of starving bacteria tends to take up foreign DNA or have an increased mutation rate, thus altering their genomes[37].

Envelope stress is triggered by the toxic accumulation of periplasmic misfolded proteins (LamB) in the envelope under stressors like nutrient availability and heavy metals. In *E. coli* several extracytoplasmic pathways are activated in response to envelope stress to protect cells, including the σ^E_ pathway (responds to misfolded outer-membrane proteins), the Cpx pathway (monitors cell-surface-protein assembly), the Psp system (senses dissipation of the proton motive force) and the Bae pathway (responds to antimicrobial compounds). Release of outer membrane vesicles is addressed as a new and independent envelope stress response[38].

1.2.4 Changes in substrate utilization

Substrates in a rich medium (LB) were used sequentially under optimal growth and then turned over to simultaneous utilization when substrates limitation occurs [39-41]. Similar utilization of fructose, mannose, maltose and ribose in combination with glucose is found in the growth - limiting chemostat cells[42]. This uptake style through phosphotransferase sugar uptake system (PTS) is independent of dilution rate. Other sugars (e.g. glycerol, galactose, lactose) through the transcription factor pathway require a lag before being used. As undesirable by-product, growth upon gluconeogenic carbon - acetate showed a rather distinct physiology through other unclear sensing routes. The shifts of acetate growing to glucose or
broth bring up nutrient specific growth kinetics[43]. With the presence of either glucose or acetate, the cells adapt to each of these carbon sources; in the presence of both carbons, glucose will be preferably completed first then the cells turn to acetate[44]. Enhanced tolerance to oxidative stress and thermo is thought to be a positive effect of acetate, the induced genes of acetate belong to the rpoS family and some of them are acid protection genes[28].

1.2.5 Changes in metabolism and gene expression: induced/repressed

Metabolism is the totality of bacteria’s chemical processes to maintain life, including reactions in glycolysis, tricarboxylic acid cycle (TCA), oxidative phosphorylation, ATP biosynthesis and amino acid metabolism. The study of metabolism in different physiological states of cell provides useful information in understanding specific metabolic pathways, or simply provides a metabolite concentration that could be related to any gene transcription. When integrated with transcriptomics and proteomics analysis, the physiological state of the cell can be fully understood. And based on the integrated result modifications in gene could be a tool for improving strain performance, especially for those strains grown under certain stimuli in bioprocess.

Shifting energy metabolism appears to be an important strategy in cell survival and stress responses. During starvation or under exposure to stress many bacteria experience a significant turnover (metabolic breakdown, rearrangement and re-synthesis) of protein[45, 46] and RNA (carbon and energy source). There are some other secondary metabolites with unknown function in the growth and development of the bacterial cells found in the marine environment[47]. Stimulus-response or pulse experiments are a typical way of metabolic investigation. By applying a substrate pulse to a substrate-limited, steady state culture, cellular metabolism is shifted away from its regular metabolic steady state. Variation in the response of the different organisms to external stimuli is reflected in secreted ethanol in S. cerevisiae or increased storage material in P. chrysogenum, but not in E coli[48].

Comprehensive profiling of a large amount of metabolites needs a high sampling frequency for E. coli, since the new steady state is quickly achieved in a short time period of 40–60s[48]. Pathway fluxes of E. coli MG1655 have been verified for stress of carboxylic acids using $^{13}$C labeling methods[49]and transcription data of heat shock, osmotic shock, oxidative stress genes and TCA cycle genes from fed batch cultures has been captured with genome scale array technique at different specific growth rates[50]. Energy conservation is a general strategy at both transcription and metabolite level, whereas, metabolite response
reacts more specifically than response at transcriptase level under five conditions of cold, heat, oxidative stress, lactose diauxie and stationary phase[51].

Carbon and nitrogen starvation share similar induced proteins and genes[52] with more genes induced under carbon limitation[53]. RpoS and (p)ppGpp are mostly studied as major components of the general and specific response that regulate some key cellular processes ensuring global control upon perturbation. It is interesting to classify each physiological profile under different conditions since they are quite different and nutrient specific, e.g. different densities’ population has non-identical rpoS expression and membrane composition at a dilution of 0.3 h⁻¹ under a glucose range of 0.005-0.12% [54]; altered growth rates correlate a particular pattern of gene expression, enzyme production and mutation[33, 53]; RpoS synthesis is unstable during phosphorus and nitrogen limitation, but becomes stable during carbon limitation; central metabolic pathway is shut down in carbon starvation but still functioning in phosphate limitation[55]; induction in rpoS transcription is inversely correlated with the decrease of the specific growth rate[56]; cells stop growing immediately in carbon and nitrogen limited assays but slowly grow in phosphate-lacking cultures[57].

Three classes of genes encoding starvation proteins have been defined. These are the cst genes, controlled only by carbon starvation and requiring cyclic AMP/CRP; the csi genes—also induced by carbon starvation alone but independent of cAMP; and the pex genes, induced by nitrogen, or phosphorus starvation other than carbon and cAMP/CRP-independent[58]. Once any damage posed on the membrane there are five signaling pathways of Bae, Cpx, Psp, Rcs, and σE existing in E. coli that start simultaneously to detect stimulations and allocate cellular resources to recover from the damage[26]. With some overlaps, each pathway functions distinctively. All these changes imply that cells must undergo structural changes to live on the edge of survival. A summary lists of E. coli genes that response to nutrient limitation, environmental stress, or those genes indicating the physiological status of the bacteria were presented by Schweder[59], for instance, σS is required to enhance its acid tolerance when confronted with pH stress in a fermentation process.

1.3 Microbial heterogeneity

The types of heterogeneity within a microbial population can be divided into four general classes: genetic level, biochemical level, physiological level and behavioral level[60]. Biochemical or behavioral differences might ultimately be traced back to a genetic basis; physiological heterogeneity, which may be driven by forces external to the cell (e.g., nutrient limitation or the presence of antibiotics), could be viewed in terms of the organism's genetic
potential to respond to these forces. Genetic heterogeneity is addressed through disturbance on genetic material. Biochemical or metabolic heterogeneity is characterized by individual cellular differences in macromolecular composition or activity and may stem from diversities in physiological processes and genetic resources.

1.3.1 Physiological and behavior diversity: VBNC, persister, chemotaxis behavior, phenotypic modification, etc.

Sources of physiological variation in bacteria include differences in cell volume, cell shape, population density, nucleoid morphology and environmental characteristics. Assessment of physiological state of individual cells is very useful in monitoring bacterial populations in biotechnological process. The changes of bacterial cell physiological state during cell cycle and life cycle as well as interaction of cells with inhomogeneous microenvironment result in the formation of a physiologically heterogeneous cell population[61].

Behavioral heterogeneity is an observation of individual cellular responses to chemotactic or phototactic stimuli and measurement of swimming speed or direction, which is termed as ‘chemotaxis’[62]. It is measured through direct observation of cellular responses to various stimuli. This proton gradient-dependent chemotaxis system in E. coli is reported to be affected in glucose limited fed-batch cultivation, since majority of chemotaxis genes were transcribed from active to less active in order to save energy for other cellular activities[63].

1.3.2 Genetic diversity (genetic arrangement, epigenetic modification, mutations, etc)

Heterogeneity could also result from changes or rearrangements at the DNA sequence level, from diverse cell cycle phases and age distributions. Mutations occurred during storage make stored archival cultures of bacteria becoming heterogeneous, which is likely to contribute to strain variation in stocks of e.g, K-12 strains in regards of its physiology, genetics and gene regulation[64]. Epigenetic differentiation occurs stochastically but can also be triggered by external stress or changes, with a higher occurrence frequency than mutations[65], leading to differential gene expression and add complexity in an identical population. The presence, absence and the copy number of transposable genetic elements (transposons[66], insertion/IS elements) can diversify population genetic structure, as well as inversion, translocations, etc. Those genetic arrangements create a better adaption capability to the environmental conditions.

1.3.3 Biochemical or metabolic heterogeneity

Bacteria metabolic performance is largely depend on their growth rate, which is in turn controlled by their genetic material and physiological state[60, 67]. Cellular differences in
General introduction

Macromolecular composition is also contributes to biochemical heterogeneity. Metabolic heterogeneity is measured by substrate uptake or metabolic flux within cultures and is evidenced in many glucose pulse studies[68, 69].

1.4 Heterogeneity inside the fermenter

A typical microbial population in a bioreactor is heterogeneous in many aspects at single-cell level[10, 61]. Heterogeneity, to some extent, is a beneficial solution for the robustness of the fermentation process with quick adaptation to new conditions thus boosts population level fitness [10, 3]. Significant gradients of e.g. dissolved oxygen, substrates, and pH are typically observed in many industrial scale fermentation processes[70]. Cells fluctuating in large volume reactors therefore experience significant micro-environmental changes while mixing inside the reactor, especially in aerobic fermentations[71]. Consequently, diversities in gene expression and protein translation generate the presence of subpopulations. Local microenvironments that induce dynamic cellular genetic, metabolic and physiological responses have been identified as the key driver for the development of heterogeneity in the bioprocess[25]. The resulted residence time and the relevant various types of cell responses are expected to be more distinct from each other in the bioprocess based on the sizes of the microenvironments, which in turn are responsible for causing the development of heterogeneity in a pseudo homo population. The gradient formation effect is further magnified by reactor enlargement as a result of deficient mixing, which leads to zones with diverse external conditions[70]. Therefore the larger reactor is the greater possibility of finding heterogeneity (Figure 1.1, A). Population fragmentation is quite bad in a fed-batch process[72], since a highly concentrated substrate is fed through a narrow zone to avoid a significant increase in volume. The severe glucose gradients generated by improper mixing in high density fermenter were shown to be responsible for inducing four stress sensitive genes and affecting heterologous protein production, product yields and cell viability[3]. Changes in glucose availability often have profound consequences in many types of cell gene expressions (as simplified in Figure 1.1, B), which is supported by a recent study of responses of yeast to the period of nutrient excess engendered by a glucose or ammonium impulse (single pulse)[73]. Although responses have been studied, not much is known about the influence of such inhomogeneity on the process performance[61]. Understanding the dynamics of heterogeneity in bioprocesses is considered to be a valuable tool for bioprocess optimization since population heterogeneity has a significant impact on the product consistency and quality[3]. On the other hand, growing cells continuously alter their immediate environment during their lifespan[53, 16], thus, those cells with ability to adapt
smoothly and efficiently to environmental changes confers a selective advantage over other cells.

Figure 1.1 Scale up bioprocess (the generation of spatial heterogeneity, A), basic set up of perturbation studies (stimulus-response, B, adapted from Delvigne et al. 2009), and potential distribution of the cellular properties(C). (Adapted from Dhar et al. 2007[74])

1.4.1 Osmotic stress in the fermenter

Osmotic stress in industrial fermentations mostly is a direct consequence of product accumulation, it affects bacterial growth behavior and metabolic production in many bioprocess[75]. Hyperosmotic stress is a situation when the solute concentration inside the cell is lower than that of its environment, and vice versa. One of the strategies confronting higher osmotic pressure is to utilize organic intracellular solutes and/ or maintain ion concentration gradients across membrane. The immediate response of E. coli to hyperosmotic shock with NaCl in the batch fermentation is a fast taking up of K⁺ to increase the internal osmolality with a concomitant intracellular increase of the glutamate and then in the subsequent phase charged ions are exchanged by neutral solutes like proline, trehalose, glycine, betaine, etc[76]. Meghna Rajvanshi, et al. showed that at hypoosmotic condition, trehalose and glutamate are the main compatible solutes for Corynebacterium glutamicum but
at higher osmolalities proline plays a dominant role[77]. Glutamate is shown to be important in osmotic response after sudden increase in the external osmolality. Trehalose and proline are the main protecting agent in the long-term adaptation. When it moves to continuous culture, the widely used Corynebacterium glutamicum for the industrial synthesis of glutamate is confronted with osmotic stress better at higher specific growth rates than lower because of a higher energy efficiency by a rapid changing of the osmolite[78]. Frequent encounters with fluctuations in the osmolality, E. coli has developed efficient methods to cope with hypo- and hyperosmotic stress. The osmotically sensitive gene proU (uptake of protectants of glycine betaine and proline) gives a detectable response within 15 seconds after an osmotic shock[79]. The mRNA analysis of the proU expression shows that the osmotic pressure decreases in the fed-batch cultivation despite a high cell density and accumulation of byproducts, since substrate limitation in fed-batch with feeding nutrient allows elimination of osmotic effects[80]. Time dependent different adaption behavior to osmotic stress under aerobic or anaerobic conditions is published in [46]

1.4.2 Anaerobic condition in the fermenter

The oxygen transfer in particularly is a critical parameter in large-scale aerobic fermentation, which frequently has a lower yield compared to small scale. The uneven distribution of substrate and oxygen transfer, the measuring port of the probe and the quality of the probe affect dissolved oxygen concentration. Larsson et al. (1996) demonstrated that oxygen gradients vary with stirrer speed, with the time of cultivation and with the organism type and process[4]. Cells passing through the oxygen-limited zones sense and respond to oxygen depletion, leading to the activation of anaerobic metabolism. Cells are more sensitive to osmic pressure when grown under anaerobic rather than aerobic condition due to a less activation of antiporter for Na⁺ extrusion in E. coli[81].

1.4.3 Acidity condition in the fermenter

The pH gradient can lead to a reduction of cell viability. Protection provided for adapting to acid stress (pH 5.5-4.5) is controlled by a mechanism known as the acid tolerance response (ATR). Aerated E. coli cultures respond to pH changes by selective expression of numerous envelope and periplasmic stress proteins, redox modulators and catabolic enzymes. The modulation of catabolism in complex medium such as Luria-Broth medium at low pH is modulated to pathways to produce various fermentation acids. Lactate is preferable produced instead of acetate and formate, with the latter more detrimental to cells. The pH gradient may drive the fermentation acids back into the cells and the released proton after dissociation acidifies the intracellular pH[8]. When encounter anaerobic condition, the excretion of weak
acid (organic acid) from cells is increased and some other pathways for lighting the stress effect is available[82]. Inorganic acid or organic acid both disrupts some key biochemical processes through intracellular acidification. Furthermore, levels of phospholipids that contain membrane-stabilizing cyclopropane fatty acid [8] and accumulated rpoS- controlled acid shock proteins will be elevated in front of acid stress. Acid response of E. coli K12 in log phase involves different pathways when rich or minimal medium is used. The rpoS-dependent oxidative system (AR1) and two distinct fermentative acid resistance systems of glutamate decarboxylase (AR2) and arginine decarboxylase (AR3) are involved in the rich medium defense[83]. But in another research study it is reported that only AR2 system is activated in minimal medium and none of the three AR systems are activated in exponentially grown cells in rich medium[84].

1.4.4 Oxygen gradient and substrate gradient in the fermenter

Oxygen availability is very important for E. coli growth with respect to acetate production and product yield. Oxygen gradients are frequently found in large scale bioreactors due to transport limitations when the oxygen consumption rate exceeds the oxygen transport rate, which is a situation particularly obvious in fed-batch cultivations[72], since a highly concentrated substrate is fed through a narrow zone to avoid a significant increase in volume. E. coli cells are able to recognize gradients in a bioreactor within seconds[79], as exposure to transient oxygen limitations, even a few seconds (13 sec), is enough to induce genes of anaerobic pathways and divert essential carbon skeletons to undesirable by-products[85]. Accumulation of acetate and formate was detected after only 2s exposure to the glucose-rich (aerobic) region in the plug flow reactor[69].

The nutrient inside the fermenter is not equally distributed. The high concentrated feeding has shown to generate substrate gradients due to repeated stirring that creates nutrient starvation zones and oxygen limitation. More than 10,000 fold concentration difference between the feed solution and the bioreactor broth in large-scale reactor generates varied gradients depending on the location of the feed[86, 4]. Glucose gradients generated by improper mixing in a E. coli fed-batch fermentation at top, middle and bottom levels in a 22 m³ fermenter affect heterologous protein production, product yields and cell viability, induce stress responses of four stress sensitive genes and bacterial cells differentiate even morphologically near the feeding point[3].

1.5 Ways to visualize heterogeneity

Ordinary bioprocess monitoring often relies on population-based measurements of cell viability and yield production, which is unable to reflect the heterogeneity among individual
cells within a population. A number of methods have been applied to discovery and monitor the cellular mechanisms and regulatory circuits on the single-cell level.

1.5.1 Molecular characterization of genetic variability

The most common methods used for genetic fingerprinting are the ‘gold standard’ method of pulsed-field gel electrophoresis (PFGE), two PCR-based techniques of repetitive extragenic palindromic (REP) and the enterobacterial repetitive intergenic consensus (ERIC), simplified amplified fragment length polymorphism (AFLP), suppressive subtractive hybridization (SSH), etc. The detection of specific mRNA in individual cells may provide more in situ information. RT-PCR was used to specific gene induction (groEL mRNA per cell) and general growth activity (tsf mRNA per cell) The number of targeted and per cell was first announced in Kim Holmstrøm’s work[87].

1.5.2 Green fluorescent protein and related reporter proteins

The capacity of being a reporter protein has been revalued from the point of accuracy and reproducibility. Reporter proteins such as luminescence and fluorescence have served as in a variety of biological studies. Standard reporter proteins have very slow clearance rare and thus are quite stable, for instance, chloramphenicol acetyl transferase (CAT), beta galactosidase (β-gal) and enhanced green fluorescent protein (EGFP) have protein half-lives of ~50, 30 and 24 h, respectively[88]. Unlike several other commonly used reporters, the application of GFP is quite simple and does not require additional cofactors or external substrates. The GFP protein has been modified to produce fluorescent proteins with a variety of colors including blue, cyan, yellow, and red, making it flexible to use in biological studies[89].

GFP and its derivatives are widely used in biological studies[90], since it does not interfere with cell growth[91], however, release of GFP to the supernatant is regarded as a tracer of membrane integrity and cell death[92]. The evaluation of cell damage using this way could not be consistent all the time, since the release of cellular contents is a size dependent process. As the high degree of stability of the wild type GFP protein (the fluorescent signal can last for over 24 hours[91]), non-active, non-viable or dormant bacteria cells containing stable GFP would continue to be fluorescent or still retain GFP fluorescence in the absence of promoter gene expression[93] and the fluorescence will extend over many bacteria generations, it might pose a problem in experiments where transient or dynamic gene expression is concerned. Therefore, the use of a destabilized reporter system is preferred. Driven by this purpose, Andersen et al. made destabilized variants of GFP by adding a C-terminal oligopeptide extension that targets GFP for degradation through an ssrA-mediated
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degradation system[91]. SsrA, also referred as 10Sa RNA or tmRNA, tags are recognized by SmpB (small protein B) and are targeted for proteolytic degradation[94]. The half-life of tagged proteins in E. coli is dependent on the last three amino acids of the C-terminal-tail[95]. Addition of an ASV-, AAV- and LVA -tag reduces the half-life of GFP_{mut3.1} from 24 hours to 110 min, 60 min and 40 min, respectively[96]. It has been experimentally identified that the half-life of these GFPs is different between E. coli and P. putida and has more weight than mRNA half-lives in interpreting the gene expression dynamics[97]. Still, the mRNA stability of reporter protein contributes to the degradation of the reporter protein and might interfere with sensitive and fast detection of changes in gene expression. Improvement has been made in constructing both reporter protein and its RNA with short half-life in double destabilized reporters[88]. The feature of rapid degradation of these fluorescent proteins makes it very useful in in situ studies of temporal gene expression. The unstable GFPs have found multiple applications, such as dynamic monitoring of bacteria growth rate[91]. But, it is a nuisance that the expression of unstable GFPs with a weak promoter cannot be detectable due to analytical limits. And its fluorescent property in different strains is not the same[91]. Likewise, luciferase is intrinsically less stable than GFP and its half-life can be further reduced by similar tag fusion. Short half-life variants of LuxA and LuxB protein from Photorhabdus luminescens were constructed into E. coli by fusing an 11-amino acid C-terminal tag that is the target of endogenous tail-specific proteases[98]. Another, the application of using the destabilized GFP (yEGFP3 - Cln2PEST) with estimated half-life of 30 min or so has been extended to yeast to detect cell cycle-dependent gene expression[99]. To date, there are over 10 different color variants commercially available including blue, cyan, yellow and red.

GFP is the most well known fluorescent protein, however, one need to be aware that GFP signal may be interfered from sample handling, at least, population quality differs [100]. The influence of delay in measuring is not very clear and is rather personal. Except a single fluorescence gene, dual fluorescence proteins are introduced into the same plasmid to study cell-to-cell variability in protein expression [101]. A newly made flavin mononucleotide (FMN)-based oxygen-independent fluorescent protein is alternatively [102] tested in plasmid transfer feasibility together with a red fluorescent protein under both aerobic and anaerobic conditions[103]. Nevertheless, it has a potential in oxygen availability studies since GFP and other protein require oxygen in their maturation process. β-galactosidase, which is encoded by the bacterial gene lacZ, can be used as a reporter gene (e.g. the best known blue/white selection) but not an ideal choice in time scales study. As one of the first reporter gene, β-
galactosidase assay is widely used in food industry[104], in distinguishing subpopulations [105] and protein leakage study in fed-batch cultivation[106].

1.5.3 Fluorescent dyes and stains

Staining techniques allow the determination of individual cell viability, activity, surface properties, etc, which are the most relevant physiological parameters to be assessed. The study of the different physiological state of a cell has been redefined by the application of flow cytometry in the fermentation process[107] and a range of stains is available for studies of single cell characteristics by flow cytometry. The interpretation of bioprocess data often fails to reflect the actual state of individual cells within a population since, as reported by several researchers, cells are not just alive or dead, but have a range of intermitted states (Figure 1.2)[107, 108]. Therefore, more and more applications tend to rely on using the combination of fluorescent dyes and by staining with appropriate fluorochrome the physiological state of both subpopulations and individual cells can be quantified and distinguished between vital (i.e., active and viable) and compromised (or dead) cells[109, 110].

![Life and death spectrum: different levels of cell viability and vitality in the physiological assessment](image)

1.5.4 Single cell and global analysis

Complex physiological behavior emerges from complex interactions between transcription level gene expressions, metabolism and enzymes. Ordinary bioprocess monitoring often relies on population-based measurements of cell viability and yield production, which is unable to reflect the heterogeneity among individual cells within a population[74]. It is interesting to study the potential distribution of the cellular properties (Figure 1.1, C) or transient cellular responses that overlooked by measurements on population level. The ability of accurate measurement has rapidly accelerated over time with the integration of omics and single cell analysis. Properties of single cells within a population can be quantified by flow
cytometry without averaging any signal intensity, for instance, unveiling cell-to-cell differences and thereby the heterogeneity of the population[60]. Application of fluorescent stains for assessment of viability and physiological state of individual cells together with flow cytometry bring great potential that cells of interest can be sorted out and cultured afterwards. “omics” based technologies have enabled the direct measurement of internal variables within the cell and contribute to expand our understanding of stress responses in face of environmental changes. Global relative changes in gene expression are thus comparable. Using DNA arrays, the relative gene transcription can be rapidly quantified and thus overall physiological response of the cell could be captured[111-113]. Population fitness in biofilm formation has been contributed to the arcA gene in a DNA array study[112] and is visualized in Philip S and coworker’s work[114]. More yeast genes on the chromosome were induced (and to a higher levels) in the carbon-limited chemostat than in nitrogen-limited chemostat as proved by using hybridization array method[53].

1.5.5 Experimental tools

Stress responses at gene expression level are transient, leading to new steady state levels similar to the unstressed cells even in the continued presence of the stress[115]. External perturbations can prevent optimal enzyme activities, disrupt metabolic fluxes, destabilize cellular structures, perturb chemical gradients, etc., leading to overall instability[70]. Depending on the duration and magnitude of the perturbation the duration and the magnitude of transient changes in cellular RNA level varied before adapting to the new steady state expression level[116].

A bunch of experimental approaches were used to study gene expression of cells in response to external perturbations. For example, Audrey P. Gasch et al[116] allocated genes into different clusters based on types of stress suffered by the yeast cells. The authors were able to set a diverse group of stress factors (temperature shocks, hydrogen peroxide, hyper- and hypo-osmotic shock, amino acid starvation and so on) and describe stress response at genomic level. A similar experimental design was undertaken by Harald Weber[117] to identify genes whose expression were RpoS dependent in the E. coli genome: up to 10% of E. coli genes are under the control of RpoS. The global gene expression analyses under defined cultivation conditions enable the identification of genes, the expression of which is relevant and specific for processes evaluations[116, 115]. Schweder[59] recently summarized E. coli genes that respond to nutrient limitation, environmental stress, or those genes indicating the physiological status of the bacteria.
Another way of looking into stress response is to perturb the steady state system with pulses of substrates or sudden shifts of oxygen or temperature. Sirichai Sunya[118] compared dynamic cell responses under a single glucose pulse of different intensities (0.08, 0.4 and 1 g L\(^{-1}\)) in the chemostat fermentation running at a dilution rate close to 0.15 h\(^{-1}\). In their study, they found that quick response of substrate uptake or consumption rate was independent of glucose intensity, whereas, process related promoter genes showed a dose related behavior. Successive glucose pulses can be done using an on/off-feeding strategy. Stress situation is increased in the oscillation process and fast perturbation frequency makes the stress even worse[72]. However, the disadvantage of perturbing the culture is that no subsequent pulse can be applied until the new steady state is reached. Single glucose pulse, on the other hand, were normally carried out by sudden injection of a glucose solution into a glucose-limited chemostat and results in sudden relief of carbon-limited conditions and thus in a sharp increase of the oxygen uptake rate. To quantify metabolic responses to nutrient perturbations, a fast sampling procedure is desired where metabolites have to to be tightly followed with each perturbation. First significant changes in gene expression were reported only visible between 120 and 210 s in *S. cerevisiae*[119], whereas for *E. coli*[68], the nucleotides do not change significantly during the perturbation experiments. Taken all the other perturbations, like two different doses of single glucose pulse[113], or a combination of oxygen and glucose[120], all these can provide deep insight into how different environmental factors influence cell population heterogeneity and whether there exists such a thing as an optimal level of heterogeneity.

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Bioprocess consideration—A way to simulate heterogeneity in controlled fermenters
Heterogeneity results from poor mixing inside the reactor both spatially and temporally often leading to conditions in which substrate concentrations and/or physical parameters oscillate thereby continuously exposing the cells to gradients of for instance nutrients, oxygen and pH[70]. When it comes to industrial bioprocesses where a controlled cultivation of microorganism is central to maintain production efficiency and quality, understanding of the physiological property of living cells and maintaining optimal growth conditions is of crucial importance since population heterogeneity has a significant impact on the product consistency and quality. Ideally, cell heterogeneity would not be expected if the growth condition were well controlled. However, cell-to-cell differences, including differences in cell cycle and cell aging, contribute to the generation of phenotypic heterogeneity that exist at all the time, even if the microenvironment is constant, and only become relevant or important during environmental perturbations[10]. Heterogeneity, to some extent, is a beneficial solution during environmental perturbations[10], either with higher growth rate or strength competence to boost population level fitness and process robustness[3]. In the present chapter cultivation techniques will be overviewed and special consideration will be given to process related cell physiology.

2.1 Cultivation methods and process related heterogeneity

Fermentations in different reactor configurations can be designed and considered as a representative heterogeneous microenvironment. Cell population inside the well-mixed reactor is rather homogeneous, but more heterogeneity could be found in reactors with less mixing efficiency. Reasons can be that parts of the bioreactor may provide optimal growth conditions whereas cell growth condition becomes harsh in other parts. Also the inoculation and operation with regard to, for example, inoculation time and substrate concentration may have major influence on cellular metabolic and physiological states[61]. Conditions like high substrate concentrations in high-density fermentation may produce various undesired subpopulations with byproducts. This kind of side effect is magnified through reactor enlargement where subpopulation is found[70]. The heterogeneous environment in large-scale fermenters may lead to the repeated cycles of production/re-assimilation of overflow metabolites and repeated induction/relaxation of stress responses resulting in reduced biomass yield and productivity[71]. The three major and popular cultivations are batch, fed-batch and continuous, each of which has a distinct characteristic.
2.2.1 Batch cultivation

Batch culture has predominantly been used for years in the biotech industry in terms of operability, reliability and economic considerations. Batch cultivation in a shake flask is the main cultivation used at bench level experiments. It is a closed simplest process where nutrients are added at once into the initial medium. It is not ideal for characterizing cellular processes, since when growth in a batch mode the physiological state of the cells is constantly changing because of adaptation to the changing environment\[53, 16\], indicating a changing gene expression and a varied physiological response to environmental perturbation\[121\].

2.2.2 Fed batch cultivation

Fed-batch is a high-density batch process, which is based on feeding with a growth limiting substrate to a culture. Now this cultivation becomes one of the most effective and preferred techniques in industry to maximize productivity. From aspects of feeding strategy, feed components and feeding outcome, means have been developed to avoid energy overflow (side products) and oxygen limitation (which could make up to 10% of the total working volume\[3\]). Feeding can be achieved in a certain way, including feeding exponentially at a constant rate, stepwise increase and feedback control (based on pH or dissolved oxygen measurements (pH-stat or DO-stat). Surface additions, although not optimal, is still a common way used in industry. Feed doesn’t have to be only glucose, other components like amino acid\[122\] and ammonium\[123\] can also be fed into the culture when needed. Feeding profile may influence growth rate, production rate, outer membrane permeability\[3\] and protein compositions and periplasmic protein leakage\[106\]. Leakage, distinguished from cell lysis, is defined as the passive/selective passage of proteins through the outer membrane\[124\]. Protein leakage seems to be a process related phenomenon, which is found to be twice as high as in chemostat cultivation compared to the constant leakage level in fed-batch \[34, 36\] and a higher feed rate correlates with a higher protein leakage\[106\]. To minimize the influence of leakage, membrane proteins as OmpF and LamB are identified as the main contributors\[106\]. This optimization is preferable in industrial production since the periplasmic space provides a more oxidative environment than cytoplasm that facilitates disulfide bond formation and also a decreased proteolytic activity. Improvement of periplasmic production has been considered from optimizing the induction parameters in \textit{E. coli} host strain\[125\].

A stress condition usually comes with glucose starvation and the present of cell
death (≈16%) and cell lysis in the late feeding phase[126], are mostly due to the high density of the culture over a long time scale. The increase in mixing time during scale-up inevitably leads to a formation of gradients in the concentration of the limiting substrate[70]. The consumption of the residual glucose normally leads to elevated levels of ppGpp and defensive proteins in E. coli within one minute[127] and specifically, significant changes in gene expression were reported between 120 and 210 s in S. cerevisiae[119]. Based on these findings, selection and study on specific stress relevant genes open up the way to look into cell physiology, which could help to understand and circumvent some problems in the bioprocess[59]. Reactor performance is influenced on a large scale by mixing inefficiency inside the reactor both spatially and temporally[128]. Scale up level oscillating conditions includes gradients of substrates, oxygen and pH, that cells are continuously exposed to in large reactors. Such an inhomogeneity is amplified in fed-batch cultivation in terms of substrate gradient. More than 10,000 fold concentration difference between the feed solution and the bioreactor broth in large-scale reactor generates varied gradients depending on the location of the feed[86, 4]. Glucose gradients generated by improper mixing in a E. coli fed-batch fermentation at top, middle and bottom levels in a 22 m³ fermenter affect heterologous protein production, product yields and cell viability, induce stress responses of four stress sensitive genes and bacterial cells differentiate even morphologically near the feeding point[3].

### 2.2.3 Scale down devices

The scale up of bioprocesses from bench scale to large scale leads to lower yields, lower production and an elevated by-product accumulation[3]. The aim of designing a scale down method is fairly clear that potential conditions encountered in scale up level can be foreseen and considered at the lab scale. Many kinds of scale down simulators were thus invented in the lab to generate certain degree of inhomogeneous condition with respect to magnitude of the substrate gradient, the period of oscillation and the circulation time[70, 129]. Cells are either circulated between two connected well-mixed stirred tank reactors (STR+STR)[130], or a round circulation from a STR through a plug flow reactor (STR+PFR)[131, 129]. The practical and the feasibility of this plug reactor is validated with different residence time setup and compared to a well mixed 5 liter reactor and 20-m³[27]. With some biomass loss, in general, the plug reactor is a good candidate of studying large-scale heterogeneity at bench level. Of many-presented scale down studies, one or two compartments models have
been widely utilized (Figure 2.1). In this PhD study the plug flow reactor (PFR) is transformed into a 12-meter recycle loop (diameter 0.005 m) and used to simulate perturbation by adding the feed (glucose) to the entry point, with the residence time of 79.8 s aiming to prevent anaerobic fermentation at a short resident time[132]. Schweder et al. (1999) found in a similar configuration with a residence time of 10 min that only 13 sec of exposure to low oxygen at the presence of high glucose is sufficient to cause mRNA level accumulations of overflow byproducts[79]. Compared to normal fed-batch fermentation, concentration gradients of glucose and oxygen are generated at the inlet of the recycle loop. The glucose variation at different locations of the SDR, as representatively diagramed in our previous study [8], is in the range between 0.01 and 0.25 g/L. Statistic interpretation can be found in[133] and comparison was done between a 2-m³ reactor and the scale down reactor. Foaming formation is surprisingly reduced in this kind of set up with both residence time of 100s and 200s[134], which bring its potential application in foaming control to improve reactor performance. Differed with a recycle loop, the pulse can be performed through the BioScope from the outside of the reactor[69], which seems have broader application.

Scale-down effect at the level of cell growth is reflected as a decrease of the biomass yield and by-product accumulation compared to a well-mixed reactor[135]. However, positive effect has also been observed in the way that microbial cells exposed to inhomogeneous conditions tend to exhibit lower membrane permeability and a higher viability[3], which is the fact occurring in the large fermenters. Heterogeneities induced by scale-down may also affect protein balance of synthesis and degradation[86]. An increase of protein degradation upon either glucose or oxygen starvation is controlled by the stress signal guanosine tetraphosphate (ppGpp) formation[136]. For those damaged proteins, proteolysis would remove them and renew the cell body with favored built-up material.
2.2.4 Chemostate

The growth environment in batch cultures is continuously changing. Those growth rate-dependent factors tend to obscure the identification of genes that are specific to a particular experimental treatment, mutation, or physiological condition. One way to address these issues is to use chemostat cultures where the growth condition is rather stable. Chemostat fermentation is a good tool to study the physiology of cells since it creates a relative steady environment where cell growth rate is set equal to dilution rate after a certain time range, and then intrinsic variables in this open system are remaining constant.

Genes specific to the particular limitation are grouped into an induction or repression pattern[16, 137]. A uniform dividing and non-dividing manner in the exponential and stationary growth phase has been experimentally observed in batch culture and dividing and non-dividing subpopulations of different GFP expression have emerged after transition of the stationary phase cells into a fresh LB medium[138]. Steady state growth-limiting cells are not able to up-regulate their growth rate immediately upon transition to glucose non-limiting condition in batch[42]. Transcriptional follow up studies subjected to two different doses of single
glucose pulse[113], or to a combination pulse of oxygen and glucose[120] reported that there were different response patterns upon perturbation in the *Saccharomyces cerevisiae* chemostat culture due to sensitivity to the available carbon. Further, the choice of dilution rate may alter some of the gene expression pattern in either glucose or ammonia shortage[139]. An oscillation study based on a combined method of scale down setup and chemostat was initiated to study the glucose disturbing effect on *E. coli* in aerobic and anaerobic condition[69]. The proposed fast sampling procedure enables an early detection of acetate and formate within 2 seconds, which dedicates that even short exposure to glucose gradient, may cause an imbalance of *E. coli* glucose metabolism and respiration.

Continuous cultivation, on the other hand, is complicated in operation, due to the long time needed to reach steady state, high possibility of contamination, medium preparation cost, etc. High throughput cultivation therefore is initiated because of its massive information and low costs[140]. A micro-scale system named as microfluidic device recently turns up to ease a chemostat process with less medium consumption and precise control. Continuous-flow microfluidics have successfully been used in condition control in characterizing physiological behavior[141], in persistence study[142] and in tracking of single cell lineages on protein level[143]. Without sampling, online detection of fluorescent proteins in parallel small-scale continuous cultivations has been developed in microtiter plates[144, 145], real time data of growth kinetics and GFP production for different strains have been gathered without interruption of growth. Microarray, for instance, was preferable used in this kind of high throughput genome-wide experiments, since a large number of genes can be statistical analyzed simultaneously.

Chemostat is a poor but not starved process[146, 16] where cells are less stressed than in batch culture and cell viability remains quite high[147]. The interlink between chemostat and batch mode is that chemostat represents a way to maintain the cells at a particular point in the batch process[148]. However, cell status is not the same in either batch or its comparable chemostat under nutrient limitation. Evidence showed that stress responses that are not activated in chemostat are activated at the point comparable in the batch[16]. The high level of the residual nutrient allows the cells to maintain in unhealthy but not stressed condition.

Further, chemostat offers a great advantage of reproducibility and accuracy over the other techniques. One thing that needs to be kept in mind is that not a single
method is pure perfect, both the pros and cons of the current prevalence miniature bioreactors are detailed in a review[140]. The availability of those cultivation techniques offers an excellent window to study the population behavior that occurs in nature. On the other side, it is convenient to use the microbial population as physiological tracer for estimation of the bioreactor mixing and transfer efficiency. This idea is also reflected throughout the thesis.

2.2 Influence of byproduct accumulation

Most often, conditions like glucose or oxygen gradients, overflow or mixed-acid fermentation, would cause production of some highly undesirable byproducts, because of the redirection of biomass formation to useless energy consuming pathways and the inhibition on growth. Mixed acid fermentation usually occurs under anaerobic conditions and the accumulation of formic acid, acetate, lactate, ethanol, and succinate is detectable 2s after expose to glucose and O$_2$ gradients[69]. In aerobic conditions the overflow metabolism initiates when pyruvate are not oxidized through citric acid cycle and respiration. All these byproducts can easily be re-consumed during the process[85]. There is no doubt that cycles of synthesizing and consuming of these mixed acids when exposed to oxygen sufficient/oxygen deficient zones result in a lower biomass yields, especially at large scale[3, 85].

Using acetate as an example, the production and inhibition effect of acetate is specific to strains, medium composition, cultivation conditions or feeding strategy, growth rate[149-151] and pH[152]. In *E. coli*, the “acetate switch” is physiologically defined as the moment when acetate dissimilation/excretion/production equals its assimilation/utilization; experimentally it is where extracellular acetate peaks during aerobic growth in minimal medium with glucose used as the sole carbon source; transcriptionally, initiation of *acs* transcription is a primary player among many other participants[153]. The gene *acs*, encoding acetyl coenzyme A (acetyl-CoA) synthetase, is responsible for converting excreted acetate to acetyl-CoA. Interestingly, the activation *acs* is mediated through quorum sensing according to the cell nutritional condition, rather than the extracellular acetate concentration[154]. The rate at which acetate forms is directly related to the growth rate and the glucose consumption rate. *E. coli* generates acetate when glucose is the limiting substrate and cells grow above a threshold growth rate. Apart from the diversion of carbon that might be used in the synthesis of biomass or the protein product, acetate production
causes a number of other problems since it inhibits growth even at very low concentrations, e.g. 0.5 g/l[155], inhibits protein formation, affects proteins and genes involved in stress response and relation process[156]. The growth inhibition for *E. coli* expands more significant in a defined medium than in a complex medium in a shake flask experiment and more severe in recombinant strains than in wild type strains[157]. Therefore, growth on glycerol, which is known not to provoke acetate accumulation[158], is of interesting as alternative to carbon to be explored in detail. And again, not all the strains, exemplified by *E. coli* strain MC1060 and JRG1061[150], are capable of shifting to grow on acetate.

*E. coli* can utilize acetate via the glyoxylate pathway, thus the total acetate concentration in the medium is the result of both production and consumption. In the context of the mixture of glucose and acetate[159], glucose is preferably used in the first place over acetate in a batch fermentation, whereas, simultaneous uptake of both substrates is reported in chemostat at a dilution rate below 0.3 h\(^{-1}\). At a dilution higher than that, *E. coli* produce more acetate from acetyl-CoA generating huge amount of ATP and NADH\(_2\) to meet a higher energy demand in order to keep a functioning cell factory. This suggests that the acetate inhibition effect can be minimized by its consumption, besides the way of preventing overflow on a lower glucose concentration. Trials on genetic deletion of the acetate pathway minimized acetate accumulation, but elevated other byproducts were found[160]. Some other process modification approaches of reducing acetate in *E. coli* fermentation are still in progress to meet a variety of production demand. The advancement in recent developed approaches has been nicely reviewed by Mark et al[149].

By considering the metabolic routes employed by *E. coli* for the metabolism of acetate (Fig. 3.1), Harry Holms and Peter Bennett were the first to provide the evidence that *E. coli* can not use acetate as sole source and energy until it synthesize the enzyme of the glyoxylate bypass, which is repressed during the growth on glucose[158]. Surprisingly, the addition of chloramphenicol at the end of the growth inhibits synthesis of ICL (isocitrate lyase) enzyme, the existence of which is reported to be necessary when growing on acetate. The conformational changes and domain shifts of the ICDH (isocitrate dehydrogenase) in conserved TCA cycle is central to successful adaption and growth on acetate[161].
Figure 2.2 Metabolic network of *E. coli* growing on glucose or acetate as the sole carbon and energy source. Abbreviations: ICDH, isocitrate dehydrogenase; ICL, isocitrate lyase. During growth on acetate, ICL mediated glyoxylate bypass and ICDH mediated TCA cycle are in direct competition for isocitrate.

A similar toxic metabolite in cultures as acetate is formic acid with high membrane permeability, which is more toxic than acetate and inhibits DNA synthesis and cell division[162]. Both acetate and D-lactate are re-assimilated much more quickly than formate when *E. coli* cells re-enter an oxygen sufficient zone, leading to an accumulation of formate in the culture medium[85]. Therefore formic acid used to be considered as an indicator of anaerobic zones because of its slow rate of re-uptake. It has been proved by the oxygen limitation study in the STR+PFR setup that formic acid will not be accumulated once trace element solution of selenium, nickel, and molybdenum is added to *E. coli* growth medium[163]. However, instead of formic acid an elevated amount of lactate is recorded in a shake flask test.

### 2.3 Process monitoring

The tight control over the physical and chemical surrounding environment of the cultured microorganism ensures a consistent and optimal productivity, therefore, a controlled cultivation becomes a central core to bioprocesses where both online and offline monitors are highly needed. A range of parameters most often controlled is pH, dissolved oxygen tension (DOT), optical density, agitation, foaming, exhaust gas composition and substrate concentration, etc[164]. Specific fermentation strategies aiming at minimizing acetate and elevating production, like glucose-feeding approaches, controlled growth rate and alternative feeds of glycerol, have been demonstrated with certain degree of success. Although undesirable low production
and non-optimal growth always weaken the strength of these methods, focus on process monitoring still have not shifted totally. In order to get reliable online measurements detecting sensors or probes with high integrity and stability cannot be omitted. These sensors in general should be able to endure sterilization, be durable for long-term usage and easy for calibration. Polarization with electrolyte solution and calibration of the probe is needed, further, exchange of electrolyte for each new run. The DO probe's membrane must also be inspected prior to use. Similar rules apply for the pH probe. Ensure pH probe has enough electrolytes, in good condition and carefully calibrated is crucial for a successful experiment.

Indirect measurement on a representative sample of the process means a lot to get a proper estimation and evaluation of process supervision. Ideally, as many online measurement as possible is preferred, since the real time information is provided.

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for description of cell population heterogeneity.


Fernandes, R.L., Nierychlo, M., Lundin, L., Pedersen, A.E., Puentes Tellez, P.E., Dutta, A.,

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Chapter 2


Chapter 3

Short outline of Experimental design
Chapter 3

3.1 Scope

1) Demonstrating the applicability of the reporter system in addressing the physiological state of the cells in respect to substrate limitation in batch, fed batch, and chemostat.

2) Presenting a destabilized variant of GFP as reporter in addressing cellular status together with the application of fluorescence stains;

3) Transient behavior in front of nutritional disturbance. Stimulus-response experiments are performed to monitor cellular physiological responses.

4) An insight into heterogeneity in robust fermentation processes.

3.2 Experimental Outline

3.2.1 Batch fermentation

Batch processes have been intensively investigated for a long time but there are still some variations among different batches. Among others, the physiological state of the cells when grown in a closed batch-culture is constantly changing because of adaptation to the changing environment, indicating a changing gene expression and a varied physiological response to environmental perturbation. Rapid staining procedures provide quick information on cell states and therefore enable fast initiation of process control. It is important to analyze heterogeneity by distinguishing the stages of functionality of cells probed by the staining procedure such as cells with certain degrees of membrane integrity, distinct enzyme activity, or different energy status. In Manuscript 4, the combination of CTC, SYBR Green, PI, DiBAC4(3) and RSG as indicator of respiration activity and membrane potential are used to assess cellular viability of bacterial cells. The application of these staining dyes in a E. coli population gives us better insight into diverse physiological states of bacteria cells in a batch cultivation.

3.2.2 Scale down fermentation

Bioprocess scale-down simulators are used to investigate the phenomenon occurring in industrial scale cultivations. The aim is to simulate the large-scale conditions, characterized by long mixing times introducing local gradients into the bioreactor, in laboratory scale bioreactor. Typical scale down (SD) configurations has been described in chapter 2. It is a nice tool to investigate the bacterial response to spatially structured environment. Manuscript 1 deals with problems associated with the concentration gradients that exist in large-scale bioreactors. The situation was mimicked with a two-compartment bioreactor system consisting of a stirred tank reactor (STR) connected to a 12-meter recycle loop to generate the poor mixing scenarios. Oxygen was provided in the well-mixed stirred tank and
glucose is fed in the beginning of the recycle loop, thus a zone featured with glucose rich and oxygen limit in the loop is generated. The response of *E. coli* to transient exposures to glucose gradients under controlled aerobic and anaerobic conditions was investigated and analyzed in this long-term transit exposure to nutrient limitation.

### 3.2.3 Pulse oscillation

Cell physiology studies over a range of perturbations are necessary to make accurate predictions of how external variants cause cellular imbalance. The perturbing agent is generally chosen to be glucose, because glucose is the most preferred carbon source for many organisms and, accordingly, changes in glucose availability often have profound consequences in many types of cell gene expressions.

1. **Single pulse**

Some other ways that have been used to study physiology responses to substrate gradients are those in the chemostat fermentation context but perturbation with substrates pulses, followed by a fast catching up sampling. The metabolic and physiological responses to the perturbations, together with gene expression analysis are focus in this short time oscillation. This is accomplished by injecting a glucose pulse in one shot at two dilution rates of 0.1 h⁻¹ and 0.5 h⁻¹, respectively, in *Manuscript 3*.

2. **Programmed pulses with fixed frequency**

Considering the period of pulse effect in the given steady experiment, a rather longer observation window is opened for another experiment, which is programmed for long time substrate oscillation using the dedicated matlab code. The dilution rate in this study is 0.14 h⁻¹ aiming to create a severe starvation environment and minimizing side effect of byproducts such as acetate. Gene expression at the phase transition from batch to the switch of continuous cultivation and at the stabilized culture is evaluated, as well as membrane permeability (*Manuscript 1* and *Manuscript 2*).
Abstract

Attempts were made to construct *E. coli* biosensor strains carrying chromosomal inserts of GFP under the control of a ribosomal promoter and stress responsive promoters, respectively. However, constructed chromosomal reporter was proved unsuitable for this project as the GFP signal was too low to be detected by the flow cytometer, hence, it was decided to continue the work with plasmid based biosensor, that is MG1655/pGS20PrrnBGFPAAV and MG1655/pGS20PfisGFPAAV.
Previously, cell growth control mechanism has been taken into consideration concerning heterogeneity, cell behavior and bioprocess approach, this chapter will describe strain construction and its signal verification.

4.1 Biosensor construction

4.1.1 Unstable GFP amplification

The gfp(AAV) gene, encoding the unstable GFP variant GFPAAV with reported short half-life around 60 min in *Escherichia coli* [1], was PCR amplified from plasmid pBSKPsosGFPmut3 (pUC origin, ampicillin resistant marker, 50 µg/ml) (Epoch Biolabs Inc., US) by using primers GFP-up and GFP(AAV).

4.1.2 Construction of plasmid pGS20PsosGFPAAV and plasmid pGS20rpoSGFPAAV

Plasmid backbone pBSKPsosGFPmut3 carrying the *E. coli* promoter sos was digested with *Nde*I and *sgrDI* and subsequently ligated with the same sites of gfp(AAV), resulting in plasmid pBSKPsosGFP(AAV).

Later, gene cassette PsosGFP(AAV) flanked by restriction sites of *Not*I and *sgrDI* derived from this plasmid was included into a pre-digested pGS20PrrnB plasmid (colE1 replication origin, ~10 to 15 copies/cell, chloramphenicol resistant marker, 25 µg/ml) (Epoch Biolabs Inc., US), creating pGS20PsosGFPAAV. This plasmid furthermore contains a strong *rrnB T0 T1* transcription terminator to prevent readthrough from the promoter.

4.1.3 Construction of plasmid pGS20PrrnBGFPAAV, pGS20PrpoSGFPAAV and pGS20FisGFPAAV

The 760 bp PCR product of gfp(AAV) was digested with *Nde*I and *sgrDI* and subsequently inserted into the same sites of the low copy number plasmid backbone pGS20PrrnB, which is carrying the *E.coli* ribosomal promoter *rrnB P1P2*. Thus, the resulting plasmid, pGS20PrrnBGFPAAV, expresses GFPAAV under control of the *rrnB* promoter.

pGS20PrrnBAAV was digested with *Not*I and *Nde*I to replace the *rrnB* promoter region with the *E. coli* fis promoter. The fis promoter was amplified from the *E. coli* chromosome by using the primers FisP-SD (the primer contains an optimized SD sequence) and FisP-up. The 300 bp PCR product was digested with *Not*I and *Nde*I and
ligated into the vector fragment. The resulting plasmid was called pGS20FisGFPAAV.

*RpoS* promoter, similarly, was first amplified by PCR amplification using primer RpoS-FW and RpoS-RW, followed by restriction using *Not*I and *Nde*I. Then, ligation of promoter *rpoS* into vector fragment containing the same restriction sites resulted the plasmid pGS20rpoSGFPAAV.

4.1.4 Construction of plasmid pGS20PrrnBGFPmut3

A *Not*I-*Nde*I fragment containing the *rrnB* promoter from plasmid pGS20PrmBGFPAAV was cloned into the same sites of plasmid pGS20PA1O4O3GFPmut3 thereby replacing the $P_{A1O4O3}$ promoter.

4.1.5 Plasmid transformation and verification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (Relevant restriction sites are underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-up</td>
<td>5'-ATATAACATATGCACCAGCTGAGAATCTGTTTAC</td>
</tr>
<tr>
<td></td>
<td><em>Nde</em>I</td>
</tr>
<tr>
<td></td>
<td>5'-CTCTCGTCGACGATTAAAAGCGATGCTGAGCCTTTTGCGTTCGTTGC</td>
</tr>
<tr>
<td>GFP (AAV)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>FisP-SD</td>
<td>5'-CCCCCCTATGTAATCCCTCTTATTGAAATGACCATACTTGACT</td>
</tr>
<tr>
<td></td>
<td><em>Nde</em>I</td>
</tr>
<tr>
<td></td>
<td>GCAAGGC</td>
</tr>
<tr>
<td>FisP-up</td>
<td>5'-CCCCCGCCGCGCCGCTACAAAAAGGCCACGTAATTTTGCGG</td>
</tr>
<tr>
<td></td>
<td><em>Not</em>I</td>
</tr>
<tr>
<td>RpoS-FW</td>
<td>5'-GCACGTACGCGCGCTCCTCGGGTGAAACAGAGTGCTAAC</td>
</tr>
<tr>
<td></td>
<td><em>Not</em>I</td>
</tr>
<tr>
<td>RpoS-RW</td>
<td>5'-CAGTCCACCATATGCTGGCTCCTAAAAAGCCCGTGCTCCCTTG</td>
</tr>
<tr>
<td></td>
<td><em>Nde</em>I</td>
</tr>
</tbody>
</table>
The generated plasmids were transformed into Genehogs and finally to E. coli wildtype strain MG1655 by electroporation using a Bio-Rad Micropulser according to the manufacturer's instructions and stored at -80°C. All the promoters and GFP region were fitted into the same restriction frame (NotI-promoter-Ndel-GFPAAV-sgrDI-T1-SacI) for easier use in future. Plasmid verification was done by enzyme restriction and sequencing. A series of primers used for construction is listed in Table 4.1.

4.1.6 Construction of chromosomal reporter MC1000::PrrnBGFPAAV

For the plasmids constructed above, pGS20PrrnBGFPAAV, pGS20rpoSGFPAAV and pGS20PsosGFPAV, the promoter and the protein gene region of each plasmid was extracted by restriction using NotI and SacI, followed by the ligation into the template plasmid pUC18R6KT-mini-Tn7T[2] (originally carrying ampR, and the gene of KnR was extra inserted between the Tn7R and Tn7L region). This operation generated plasmids: pTn7PrrnBGFPAAV, pTn7rpoSGFPAAV and pTn7PsosGFPAV. Clones had been verified by restriction and sequencing. After confirmation of the right clones, four-parental filter mating was made to insert the target gene of promoter and fluorescence protein into the chromosome of E. coli MC1000. Relevant antibiotic resistant property is shown in Table 4.2.

### Table 4.2 Strains and plasmids used in mating

<table>
<thead>
<tr>
<th>Role</th>
<th>E. coli Strain</th>
<th>Plasmid</th>
<th>A</th>
<th>K</th>
<th>G</th>
<th>S</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient</td>
<td>MC1000</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Donor</td>
<td>MT 102::pir</td>
<td>pTn7PrrnBGFPAAV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Helper</td>
<td>HB101</td>
<td>pRK600 (mob+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Helper</td>
<td>DH5a</td>
<td>pTNS2 (TnansABCD carrying)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Resulted</td>
<td>New MC1000</td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: A=Ampicillin; K=Kanamycin; G=Gentamicin; S=Streptomycin; C=Chloramphenicol. pTNS2 was derived from reference[3].

Later, recombinant strain with the right resistant property had been selected, followed by a serial dilution procedure of five days at 42°C without antibiotics in LB medium aiming to lose the parent plasmid after several generations. Then, colonies
appearing on the selective plates were tested by PCR amplification. Results showed that there was no insertion between the \textit{phoS} and \textit{glmS} sites on the chromosome (see simplified principle in figure 4.1), indicating that random insertion had occurred during the process. One of the reasons might be that the transposon sequence was unfortunately incorrect.

To make a new functioning Tn7 transposon, a 1969bp gene fragment containing the temperature-sensitive replication and the ampicillin gene was obtained from ampicillin resistant plasmid pKD46\[4\] by using primers pKD46 oriR101 XbaI FW and pKD46 oriR101 NcoI RW. Another 2736bp Tn7 fragmentet was PCR amplified from template pUC18R6KT-mini-Tn7T[2] (amp\textsuperscript{R}) with primers PUC18T Tn7T XbaI FW and PUC18T Tn7T NcoI RW. Thus, the ligation of the two resulting fragments created a new Tn7 transposon of 4661bp (amp\textsuperscript{R}). Next, gene cassette of NotI-rrnBGFPAAV-T1-$SacI$ was moved from pTn7PrnBGFPAAV into the same sites on this new Tn7 transposon (temperature sensitive replicon, oriR101, 30°C, ampicillin resistant, 50 $\mu$g /ml), getting a new pTn7PrnBGFPAAV. A kanamycin resistant gene from pUC18R6KT-mini-Tn7T[2] was later inserted between the $Clal$ and $BamHI$ sites on the new pTn7PrnBGFPAAV. Again it had been verified by sequencing. Parallely, pTn7PrpoSGFPAAV, pTn7PsosGFPAVV were all constructed. But, from this point on, only promoter \textit{rrnB} has been chosen to proceed with by considering the promoter strength (see next section of signal verification). Primers used for amplification are listed in Table 4.3.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (restriction sites have been underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKD46 oriR101 XbaI FW</td>
<td>CGACTCCG \textbf{TCTAGA} GTCAGACCAAGTTTACTCATATATAC</td>
</tr>
<tr>
<td>pKD46 oriR101 NcoI Rw</td>
<td>CGAAGAAT CCATGG GTATGGACAG</td>
</tr>
<tr>
<td>PUC18T Tn7T XbaI FW</td>
<td>GTTCACGG \textbf{TCTAGA} CAGTTACCAATGCTTAATCATAGTGTGGG</td>
</tr>
<tr>
<td>PUC18T Tn7T NcoI RW</td>
<td>CGTACAAG CCATGG GCTCACATGTTCCTTCTTCGTCGTATC</td>
</tr>
</tbody>
</table>

Then the new Tn7 plasmid was electroporated into \textit{E. coli} DH10B, after 6 hours regrowth at 30°C in SOC medium, plating was carried out and selective plates
Strain construction

with antibiotics of ampicillin and kanamycin were kept at 42°C overnight to block plasmid replication. After this was done, three sets of primers (see Table 4.4, Figure 4.1) were used for PCR verification of the right clones (two colonies were tested, shown on Figure 4.2 left panel). After PCR verification of the right integration into DH10B, chromosomal integration of genes of interest into E. coli MC1000 was facilitated by phage P1 transduction. Sixteen colonies had been tested using primer set of phoS Fw and glmS Rw and the result is shown in Figure 4.2 right panel). Thus chromosomal reporter MC1000::PrripBGFPAAV was made.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>phoS FW</td>
<td>GGAACTGGAACAGACCTCCTCTGAG</td>
<td>None (-) 690bp (+)</td>
</tr>
<tr>
<td>glmS Rw</td>
<td>CGATCAGTACCCAATCGCGCTGGAAG</td>
<td>None (-) 540bp (+)</td>
</tr>
<tr>
<td>pTn7L</td>
<td>ATTAGCTTACGACGCTACACCC</td>
<td>1kb (-) &gt;1kb (+)</td>
</tr>
<tr>
<td>Tn7R</td>
<td>CACAGCATAACTGGACTGATTTC</td>
<td></td>
</tr>
<tr>
<td>phoS Fw-Tn7L</td>
<td>None (-)</td>
<td>690bp (+)</td>
</tr>
<tr>
<td>glmS Rw-Tn7R</td>
<td>None (-)</td>
<td>540bp (+)</td>
</tr>
<tr>
<td>phoS Fw-glmS Rw</td>
<td>1kb (-)</td>
<td>&gt;1kb (+)</td>
</tr>
</tbody>
</table>

Note: -, denotes no insertion between sites phoS and glmS, otherwise, +. The corresponding size using each primer set is writing as PCR product size.

Figure 4.1 Physical maps of gene phoS and glmS on E. coli chromosome (left panel), primer targets and physical map of the inserted genes (right panel).
4.1.7 Construction of plasmid reporter pX1.0PrrnBGFPAAV and pX1.0PrrnBGFPmut3

pX1.0PrrnBGFPAAV and pX1.0PrrnBGFPmut3 were further constructed based on a 30.2 kilobase (kb) synthesized plasmid X1.0 with approximately 3–9 copies per chromosome and stable inheritance [5]. The cloning procedure for pX1.0PrrnBGFPAAV is: The T0T1 terminator was PCR amplified from pUC18T-mini-Tn7 by using primers T0T1-FW and T0T1-BW. The PCR product was digested with NotI and BamHI. PrrnB-gfpAAV was PCR amplified from pGS20 derivatives (mentioned above) carrying this construct by using primers rnrB-FW and AAV-BW. The PCR product was digested with BamHI and ApaI. The two PCR products were ligated into pKS digested with NotI and ApaI (triple ligation). The resulting plasmid was called pKS-T0T1-PrrnBgfPAAV. This plasmid was digested with ApaI and SbfI and the T0T1-PrrnBgfPAAV fragment was purified and ligated into pX1.0 digested with the same enzymes (thereby removing the tra cassette of pX1.0). The ligation
mixture was electroporated into *E. coli* Genehogs. Subsequently the final plasmid construct was moved into *E. coli* strain MC1000.

Similarly, Gfpmut3 was PCR amplified from pUC18T-Tn7-PA1O4O3gfp by using primers gfpmut3-FW and gfpmut3-BW. The PCR product was digested with NdeI and ApaI and ligated into pKS-T0T1-PrrnBgfpmut3 digested with the same enzymes (thereby substituting the *gfpAAV* gene). The resulting plasmid was called pKS-T0T1-PrrnBgfpmut3. This plasmid was digested with ApaI and SbfI and the T0T1-PrrnBgfpmut3 fragment was purified and ligated into pX1.0 digested with the same enzymes (thereby removing the tra cassette of pX1.0). The ligation mixture was electroporated into *E. coli* Genehogs. Subsequently the final plasmid construct was moved into *E. coli* strain MC1000.

### Table 4.5 PCR verification of the chromosomal integration

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>(Relevant restriction sites are underlined or strikethrough)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0T1 Fw</td>
<td>CCCCCGCGGCCGCCTGCAAGGCGTTAATTAGCCTGAGCTTGCGACTCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NotI BamHI</td>
</tr>
<tr>
<td>T0T1 Bw</td>
<td>CCCCCCGATCCCGGAATAGGTACTTCAAGATCCCC</td>
<td></td>
</tr>
<tr>
<td>rrnB Fw</td>
<td>CCCCCCGATCCaggctgatttggtgaatgtgctgc</td>
<td></td>
</tr>
<tr>
<td>AAV Bw</td>
<td>CCCCCGGGCCCCTAAAACGTGCAGCGTAGTTTTCG</td>
<td></td>
</tr>
<tr>
<td>gfpmut3 Fw</td>
<td>CCCCCcatatgcgcaaggtgaagaactg</td>
<td></td>
</tr>
<tr>
<td>gfpmut3 Bw</td>
<td>CCCCCGTGACGGGCCTTTTTTTGCTACAGTTTTACATCCATCCATGGCG</td>
<td></td>
</tr>
</tbody>
</table>

### 4.2 Signal verification of the constructs

The different types of reporters for *E. coli* aimed to monitor different responses on population heterogeneity were constructed, namely, plasmid pGS20PsosGFPAAV, pGS20rpoSGFPAAV, pGS20PrrnBGFPAAV, pGS20FisGFPAAV,
pGS20PrnBGFPmut3, pX1.0PrnBGFPAAV, and pX1.0PrnBGFPmut3, plus chromosomal reporter MC1000::PrnBGFPAAV. The signal verification was then carried out to confirm each construct is capable of delivering detectable signal. A FACSARia™ III (Becton Dickinson, USA) flow cytometer was used to analyze the fluorescent signal and the results were analyzed by FlowJo software (TreeStar, Inc., USA). The flow system includes two lasers with minimum laser power of 10 mW with the specific wavelengths of 488 nm and 561 nm. The detection settings for the forward scatter and side scatter were 301 V and 316 V, respectively. The fluorescent detection channel of GFP (530/30 nm) was set at 520 V. Fresh samples of bacterial cultures were taken at indicated times, centrifuged, suspended in 0.9% NaCl solution and then analyzed by flow cytometry. At least 30,000 events per sample were counted to have a good view of population structure. Alternatively, CDF (Cumulative Distribution Function) plots, which displaying exactly the same information as histograms provide, were used to interpret the data. The fluorescence of a histogram distribution corresponding to any given percentile can easily be found on a CDF plot (see figure 4.3).

![CDF plot](image)

Figure 4.3 Demonstration of a CDF plot (left) and the overlay of the CDF plot and the histogram. Same sample information is used.

1-liter medium was made by mixing 100 ml BM solution, 100 ml TM solution, and distilled water, supplemented with 5 µg/ml thiamine, 50 µg/ml of isoleucine, leucine and valine. A detailed composition of the BM and TM solution is listed in Table 4.5. Agar 18 g/l was added to the medium when needed. Cells were grown aerobically at 37°C in minimum medium or in LB medium. Chloramphenicol 25 µg/ml was added.
to prevent contamination of fungal and other bacteria and bacteria that lost the plasmid will die out.

Table 4.6 Medium composition

<table>
<thead>
<tr>
<th>Stock</th>
<th>Component (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM (10X)</td>
<td>K₂HPO₄·3H₂O, 42.5; NaH₂PO₄·H₂O, 10; NH₄Cl, 20.</td>
</tr>
<tr>
<td>TM (10X)</td>
<td>MgSO₄·7H₂O, 2; FeSO₄·7H₂O, 0.12; ZnSO₄·7H₂O, 0.03; MnSO₄·H₂O, 0.03; Nitrilotriacetic acid, 1.23.</td>
</tr>
</tbody>
</table>

For plasmids pGS20PrpoSGFPAAV and pGS20SOSGFPAAV, induction signal was quite weak so we decided to continue with pGS20PrrnBGFPAAV in later on construction experiments. Unfortunately, after insertion of rrnB promoter into E. coli chromosomal, no clear induction signal of chromosomal reporter can be found in the output data when using flow cytometry. Encountered by the failure signal reading from chromosomal reporter, the big synthetic pX1.0 was considered. However, the GFP signal of pX1.0PrrnBGFPAAV was also too weak to detect with flow cytometry. One possible explanation is the very low copy of the reporter gene sitting on the chromosome or on the plasmid pX1.0. Plasmid pGS20PrrnBGFPAAV and pX1.0PrrnBGFPmut3 were proved to have a better signal performance detected in flow cytometer.

4.2.1 Result of signal induction test

E. coli MC1000 bearing either plasmid pGS20PrrnBGFPAAV and pX1.0PrrnBGFPmut3 were cultured in Luria-Bertani (LB) medium and in minimum medium aiming to provide different growth rate in order to see the fluorescent shift in growth phases. Typical data extracted from the signal testing experiment were shown in Figure 4.4. The arrow indicates the order of the displayed samples start from early exponential phase till stationary phase. Data are plotted in regards of fluorescent intensity, forward scatter, side scatter and then comparisons were made in Cumulative Distribution Function (CDF) plots. Overlaid histograms offset are properly used in order to distinguish the curves better.

In general an induced single GFP peak appeared in early exponential phase and then declined for the rest of the growth phase, showing that all the cells are
propagating at an equal rate. Greater increment of fluorescence is seen in the case of pGS20-based plasmid (Fig 4.4, A and B) than pX1.0-based plasmid (Fig 4.4, C and D). Possible reason medium is that the copy number of pGS20 per chromosome is larger than pX1.0, which is considered as 3–9 copies per chromosome[5]. Besides information obtained from fluorescent protein, the emitted light gathered from forward scatter (FSC) and side scatter (SSC) contains information on cellular property, such as cell size is reflected by FSC and cellular/particle density is by SSC. The principle is that microorganisms could scatter and bend light, thus light scatter can be used to provide information on metabolic and physiological states of cells. Compared with GFP expression, similar inducement is shown in FSC, which is an indicative of cell size changes. It infers that cell becomes bigger in early exponential phase and then after goes back to small size when growth is slowed down, since the size of E. coli depends on the growth phase and the nutrients available in the medium[6]. Cell size when grown in LB medium is limited by the scant amount of carbon and then is the mount of usable amino acid, so cell size starts to decrease even in exponential phase[7].
Strain construction

B

C

D

Alexa Fluor 488-A

SSC-A

FSC-A

$10^0, 10^1, 10^2, 10^3, 10^4$

$10^0, 10^1, 10^2, 10^3, 10^4$

$10^0, 10^1, 10^2, 10^3, 10^4$
Chapter 4

Figure 4.4 Fluorescence data derived from plasmid pGS20PrrnBGFPAAV and plasmid pX1.0 PrrnBGFPmut3 in E. coli MC1000. GFP intensity at 488nm is listed as Alexa Fluor, data on side scatter and forward scatter are shown in flow histogram format. Samples displayed order is shown by the arrow. Histogram overlay profile of pGS20PrrnBGFPAAV in glucose containing minimal medium (A) and LB medium (B). Histogram overlay profile of pX1.0 PrrnBGFPmut3 in glucose containing minimal medium (C) and LB medium (D). The Y-axis of histogram is cell count number. X-axis is fluorescent intensity. CDF (Cumulative Distribution Function) plots (E and F) of the fluorescence data derived from (A) to (D), the Y-axis is relative frequency from 0% to 100%. Subplots in (E) represent growth in glucose containing medium, left is for pGS20 plasmid, right panel for pX1.0 plasmid. Subplots in (F) represent growth in LB medium, left is for pGS20 plasmid, right panel is for pX1.0 plasmid.

A small shoulder appeared in the SSC and was more obvious in LB medium than in minimum medium, maybe because promoter *rrnB* P₁ is more active by comparison with *rrnB* P₂ promoter in rich medium[8] and as so more complexity of cell activity is seen in rich medium where population tends to be fractionated easily. LB medium provides only a scant amount of carbohydrates, and small amounts of other utilizable
Strain construction
carbon sources, whereas, for growth in glucose-containing medium, this limitation seems less enormous than in LB medium (Figure 4.4). It could also be a signature of the properties of the cell wall, ribosome content or amount of macromolecules per cell in the bacterial cell. This fraction of cells is not totally separated from the main body of the population and its size is reduced in mid exponential phase. When it turns to stationary phase, some cells remain in a different state distinct from the main population in SSC.

Degradable and stable GFP were both quickly synthesized in fast dividing cells and slowed down afterward because of the slower growth and less demanding of ribosomal proteins. The stepwise weakening of the destabilized GFP(AAV) signal intensity showed lower fluorescence concentration than GFPmut3 in late growing phase. Wild type GFPmut3 was rather stable signal with less induction and regression. Furthermore, it seems that the GFP induction correlates with forward scatter and side scatter. Similar increment and decrement trends have been found in FSC and GFP intensity.

The overview of the whole population from the Cumulative Distribution Function plots indicates that the variation in LB medium is larger than in minimal medium, and more obvious in pGS20 than in pX1.0. Probably more variation of plasmid copy number occurred in pGS20 but not pX1.0, besides the nature of different versions of GFP. The use of destabilized GFP could minimize cellular signal accumulation, thus it is interesting to see if destabilized GFP can serve as a good indication of cell divisions in the fermentation context.

4.2.2 Plasmid stability

To test the plasmid stability of pGS20PrrnBGFPAAV, a serial 100-fold dilutions of the overnight culture in fresh rich medium was carried out over 3 days at 37°C, 160 rpm, with no addition of antibiotics, followed by plating on LB plates. Then 768 colonies were restreaked on CHL-containing plates overnight. Three triplicates were used. The number of cells without plasmids was calculated as the ratio of the non-antibiotic resistant colonies to the total number of the streaked colonies. 41 out of 768 colonies did not grow on CHL plates and this corresponds to about 5% plasmid loss.

4.3 Conclusion
Growth reporters for *E. coli* having GFPAAV expressed under ribosomal promoter was constructed and its signal showed a growth dependent manner as expected. Therefore, experiments with further environmental perturbations will be carried out to investigate the effect of environmental parameters on the population heterogeneity in the cultivation process. Meanwhile, plasmid based constructs will serve as copy number reporters in experiments where effects of environmental conditions on the plasmid copy number are concerned. As a complement of using reporter strains a number of fluorescent dyes targeting particular physiological features such as membrane integrity and metabolic activity are under consideration as well since these dyes would provide a full picture of the physiological state of the cell on single cell level from different angle. It has to be mentioned that the host strain used at this time for signal test was *E. coli* MC1000 and later all plasmids were transformed to *E. coli* MG1655 in the fermentation process due to its better growth behavior than MC1000.

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**Reference**

5.1 Manuscript 1
Design of growth-dependent biosensors based on destabilized GFP for the detection of physiological behavior of *Escherichia coli* in heterogeneous bioreactors

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Abstract

In this work, we present the design and characterization of Green Fluorescent Protein (GFP)-based reporter systems designed to describe cellular activity in “complex,” heterogeneous bioreactors. The reporter systems consist of Escherichia coli strains carrying growth dependent promoters fused to genes expressing stable and unstable variants of GFP, respectively. The response of Escherichia coli cells to transient exposure to glucose was studied in a two-compartment scale down bioreactor (SDR) consisting of a well-stirred tank reactor (STR) connected to a plug-flow reactor (PFR). Such a SDR system is employed to mimic the situation of high glucose concentration and oxygen limitation that often encountered in large-scale, fed-batch bioreactors and the response of E. coli was simulated by continuously pumping microbial cells from STR to the PFR. We found that repeated addition of concentrated glucose pulses with varied frequency at the entrance of the PFR had consequences on strain physiological behavior. The GFP expressions were significantly marked after 10 h of cultivation in STR (control reactor) and SDR, whereas, growth rates were rather similar. Additional experiments in chemostat with programmed glucose perturbation suggested that the activities of the promoters were linked with the substrate limitation signal. Taken together with immunoblot analysis, we suppose protein leakage is responsible for the overexpression of fis and the related promoters, such as rrnB in this case study, but additional works are required in order to confirm this relationship. This investigation is useful for a better understanding of the fast dynamic phenomena occurring in heterogeneous large-scale bioreactors.

Keywords: Destabilized GFP, Scale down, Heterogeneity, Escherichia coli
Introduction

*Escherichia coli* is a well-studied bacterium, which is able to direct its metabolic pathways depending on the type of substrate present in the growth medium. Whenever growth conditions are not optimal, growth is challenged by stress, *i.e.*, by-products synthesis (short term response) and genetic reprogramming directing cells to a dormant state (long-term response, such as the general stress response governed by the sigma factor (*rpoS*) in the case of *E. coli*).

Physiological readjustments to environmental stresses involve multilayers of genes and proteins and are known as the cell stringent response.

In industrial bioprocesses controlled cultivation of microorganisms is essential in order to maintain production efficiency and quality. However, mixing inefficiency inside the reactor both spatially and temporally often leads to conditions in which substrate concentrations and/or physical parameters oscillates, continuously exposing the cells to gradients of for instance nutrients, oxygen and pH. This might lead to heterogeneities within the cellular population in a seeming homogeneous bioreactor where individuals behave differently according to the conditions experienced in their surroundings. Heterogeneities can be minimized but not avoided in large-scale bioreactors, particularly when operating under the fed batch mode. Among others, oxygen limitations and substrate gradients are particularly obvious in fed-batch cultivations, since a highly concentrated substrate is fed to the top surface of the culture through a narrow zone to avoid a significant increase in volume. Consequently, inhomogeneity is generated while cell circulating throughout the 22 m$^3$ reactor. Cell segregation is further magnified by reactor enlargement as a result of deficient mixing, which leads to zones with diverse environmental conditions. The glucose gradients generated by improper mixing in this high density fermenter, as the main source of failure during the cultivation scaling up, were shown to be responsible of inducing four stress sensitive genes and negatively affecting heterologous protein production, product yields and cell viability. To quantify metabolic responses to nutrient perturbations, Schweder et al. summarized *E. coli* genes that respond to nutrient limitation, environmental stress, or those genes indicating the physiological status of the bacteria. Such rapid stimulus-response experiments have been applied to *Saccharomyces cerevisiae*. However, a limited
analysis on cell response can be derived from the studies mentioned above and still less is known about the influence of such inhomogeneity on the process performance.\textsuperscript{11}

One attractive way of gaining information about potential heterogeneities within bioreactors is to use the microbial population itself as physiological tracer for cellular activity.\textsuperscript{12,13} This can be done by using transcriptional fusions of inducible promoters and genes expressing fluorescent proteins (such as green fluorescent protein, GFP). GFP is an excellent signal tracer in biological research because of its bright and stable fluorophore, low toxicity, and ease of quantitative detection without disrupting the cell.\textsuperscript{14} The first quantitative application of GFP to indicate the level of heterologous protein was provided by Albano et al.\textsuperscript{15} Till now, GFP and its variants are used in many biological studies.\textsuperscript{16} The wild type GFP with a high degree of stability accumulates to high levels that facilitate detection is suitable for analyzing the fluorescent signal over time. Unfortunately, it becomes unsuitable in the viability studies and the studies that require reporter protein turnover, such as in the analysis of transient gene expression in response to environmental signals. As the high degree of stability of the GFP protein (the fluorescent signal can last for over 24 hours\textsuperscript{17}), the non-active, non-viable or dormant bacterial cells containing stable GFP would continue to fluoresce or still retain GFP fluorescence in the absence of promoter gene expression\textsuperscript{18} and the fluorescence would extend over many bacteria generations. It might pose a problem in experiments where transient and dynamic changes in gene expression is concerned. Therefore, to answer the question of how gene expression changes in individual cells as they respond to various environmental stimuli in real time, the use of a destabilized reporter system is preferred. Driven by this purpose, Andersen et al.\textsuperscript{17} previously made destabilized variants of GFP by adding a C-terminal oligopeptide extension that targets GFP for degradation through an \textit{ssrA}-mediated degradation system. The newly created GFP is rapidly turned over by proteolysis and no fluorescence is accumulated. Depending on the last three amino acid of the C-terminal tail, the protein variants’ half-life is shortened to 110 min, 60 min and 40 min for ASV-, AAV- and LVA/LAA-tag, respectively.\textsuperscript{19} Since then, unstable GFP has enabled precise inspection in enormous studies, such as, monitoring nutrient availability\textsuperscript{20} and quantifying stress response of \textit{E. coli}.\textsuperscript{21} GFP derivatives with a broader range of stabilities have been extended to yeast to detect cell cycle-
dependent gene expression. The reported GFP half-life is as short as 30 min in bacteria and yeast and CFP protein is as short as 5 min in yeast.

Ordinary bioprocess monitoring often relies on population-based measurements of cell viability and yield production, which is unable to reflect the heterogeneity among individual cells within a population. A number of methods, such as flow cytometry and fluorescence microscopy have been applied to discover and monitor cell-to-cell differences, cellular mechanisms and regulatory circuits on the single-cell level. In front of other techniques, like RT-qPCR involving mRNA and fluorescence microscopy, flow cytometry has been extensively used to study the phenotypic diversity of cells used in industrial process. Flow cytometry can be used to analyze thousands of cells per second without the need of processing samples, although it provides a snapshot of gene expression at given time point.

In this study, each growth reporter strain contains a vector that was created by fusing gfp (stable and destabilized variant) to either the E. coli promoter rrnBP1P2 or fis. Therefore, within cells containing the reporter vector, any changes in fluorescent signal from the unstable GFP protein is seen as an indicative of promoter activity. rRNA synthesis is the first to respond to the nutrient up shift or down shift of the medium in order to meet the demand for protein synthesis and provide maximal protection for long term survival. It is known that regulation of rRNA synthesis is responsive to environmental and nutritional signals and inversely reacts to rRNA gene dosage. rRNA transcription is the rate-limiting step in ribosome synthesis and is controlled primarily at the level of initiation from rRNA promoters in E. coli. The rRNA promoters are among the strongest in E. coli and is accounting for distribution of the majority of RNA polymerase during rapid growth. Changes in nutrient availability result in almost immediate changes in rRNA promoter activity. The rrnBP1P2 promoter is sensitive to the concentrations of two small molecules, the initiating NTP (iNTP) and guanosine 5’-diphosphate 3’-diphosphate (ppGpp). FIS is a small nucleoid-associated protein involved in many cellular processes, including the activation of rRNA synthesis, and the expression of FIS has certain physiological effects on E. coli, suggesting that FIS plays a global role in the adaption of E. coli to changing environmental and nutritional conditions. For rrnBP1 promoter most of the activation by FIS is due to the three FIS-binding sites located
upstream and FIS acts as a transcription factor that \textit{trans} activates the \textit{rrnB} promoter\textsuperscript{32} (Figure 1). In addition to its role in recruiting RNA polymerase (RNAP)\textsuperscript{33} to the \textit{rrnBP1}, FIS may also affect the transcription initiation after the recruitment. The FIS expression pattern is quite similar to that of rRNA, and expression of the \textit{fis} promoter is regulated under the same conditions as rRNA promoters, as the \textit{fis} promoter responds to the same signals of nutrient availability that regulate rRNA promoters (e.g., iNTP concentration and ppGpp). So, FIS facilitates the expression of rRNA when sensing the cell demands for protein synthesis. In other words, the expression of \textit{fis} promoter is regulated by growth\textsuperscript{34} and in response to nutritional upshift.\textsuperscript{35} According to Huijun Zhi et al.,\textsuperscript{33} this promoter is activated depending on the cellular nutritional status. \textit{Fis} is highly expressed in the very early log phase of rapidly growing cells whereafter the promoter is switched off in late log phase and stationary phase. \textit{Fis} expression is furthermore regulated FIS itself (feedback control) and the level of DNA supercoiling that couples to changes in cellular physiology.\textsuperscript{36}

The effects of environmental gradients and their exposure influence on cells can be studied at the laboratory scale using scale-down experimental approaches.\textsuperscript{3} The most commonly used setup is a two-compartment scale-down bioreactor (SDR) consisting of a well-stirred tank reactor (STR) compartment connected to plug-flow reactor (PFR) compartment. Here in order to test their potential use as physiological tracer for process perturbation, the destabilized GFP reporter strains were tested both in lab-scale bioreactors and in scale-down bioreactor. This last configuration comprises a lab-scale stirred bioreactor connected to a tubular loop and is generally denominated SDR.\textsuperscript{37} The cultivation medium is continuously re-circulated into this recycle loop in which glucose is injected. By this way glucose concentration gradient can be generated and microbial cells are stochastically exposed to environmental fluctuations such as those met in large-scale bioreactor. Unexpectedly, our growth-dependent biosensors have exhibited a continuous increase of GFP synthesis when cultivated in well-mixed fed-batch bioreactors, whereas this phenomenon was not observed in the SDR. Additional experiments conducted in chemostat and perturbed chemostat have confirmed the results. Analysis of supernatants has revealed that this phenomenon could be linked with protein leakage.
Figure 1. Plasmid pGS20 is used as the common backbone for constructing reporters in this study. Fis protein has three binding sites in front of *rrnB P1* promoter. The physical location of *rrnB P1P2* promoter and *gfp* gene is illustrated. Alternatively, *fis* promoter was used to replace *rrnB P1P2* promoter to construct pGS20fisGFPAAV.

**Material and Methods**

**Construction of reporter strains**

Plasmid pGS20PrrnBGFPAAV and pGS20PrrnBGFPLAA: The *gfp*(AAV) gene, encoding the unstable GFP variant GFPAAV with a reported short half-life around 60 min in *E. coli*,\(^\text{17}\) was PCR amplified from a plasmid carrying *gfpmut3* by using primers GFP-up and GFP(AAV). The 760bp PCR product was digested with *NdeI* and *sgrDI* and subsequently inserted into the same sites of a low copy number plasmid backbone pGS20PrrnB (colE1 replication origin, ~10 to 15 copies/cell, chloramphenicol resistant marker, 25 µg/ml) (Epoch Biolabs Inc., US), which is carrying the *E. coli* ribosomal promoter *rrnBP1P2*. Thus, the resulting plasmid, pGS20PrmBGFPAAV, expresses GFPAAV under the control of the *rrnB* promoter. This plasmid furthermore contains a strong *rrnB T1* transcription terminator to prevent readthrough from the promoter. The *gfp*(LAA) gene, encoding the unstable GFP variant GFPLAA, was reported to have short half-life around 40 min in *E. coli*.\(^\text{17}\) The primer pair used for amplifying *gfp*(LAA) gene is GFP-up and GFP(LAA). Except the PCR primer, all other construction steps were the same as constructing pGS20PrmBGFPAAV.
Plasmid pGS20FisGFPAAV and pGS20FisGFPLAA: pGS20PrrnBAAV was digested with NotI and NdeI in order to replace the rrnB promoter region with the E. coli fis promoter that encodes a DNA binding protein, FIS. The fis promoter was amplified from the E. coli chromosome by using primers FisP-SD (the primer contains an optimized SD sequence) and FisP-up. The 300bp PCR product was subsequently digested with NotI and NdeI and ligated into the vector fragment. The resulting plasmid was called pGS20FisGFPAAV. Instead of pGS20PrrnBAAV, pGS20PrrnBGFPPLAA was used as the template to create pGS20FisGFPLAA. The same procedure was used to construct pGS20FisGFPAAV.

Plasmid pGS20PrrnBGFPmut3: A NotI-NdeI fragment containing the PrrnB promoter from plasmid pGS20PrrnBGFPAAV was cloned into the same sites of plasmid pGS20PA1O4O3GFPMut3 thereby replacing the PA1O4O3 promoter.

The generated plasmids were finally transformed individually into E. coli wildtype strain MG1655 by electroporation using a Bio-Rad Micropulser according to the manufacturer's instructions. Plasmid verification was done by enzyme restriction and sequencing. Primers for plasmid construction are shown in Table 1.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (Relevant restriction sites are underlined) Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-up</td>
<td>5'-ATATACATATGCGCAAGAGGTAGAAACTGTTTAC</td>
</tr>
<tr>
<td>GFP (AAV)</td>
<td>5'-CTCTCCTGAGCGATTAAGACTGCAGTCGTATGTTTTCGTCGGTGCG</td>
</tr>
<tr>
<td>GFP(LAA)</td>
<td>5'-CTCTCCTGACTGATTAAGACTGCAGTGCAGTAAAGCGTAGTTTTCGTCGGTGCG</td>
</tr>
<tr>
<td>FisP-SD</td>
<td>AGGCCTTTTTTACAGTTTTCATCCATGGCG</td>
</tr>
<tr>
<td>FisP-up</td>
<td>5'-CCCCCCTATAGGTAATCCTCCTATGAAATGACCGACCTACTGTGACTGCA</td>
</tr>
</tbody>
</table>

Table 1 Primers used in this study

Medium composition, inoculum and bioreactor operating modes

The defined minimal medium (MM) used in this study (g/L): K2HPO4 14.6, NaH2PO4·2H2O 3.6; Na2SO4 2; (NH4)2SO4 2.47, NH4Cl 0.5, (NH4)2-H-citrate 1, glucose 5, thiamine 0.01. Additionally, 3 ml/L of trace solution, 3 ml/L of a FeCl3·6H2O solution (16.7 g/L), 3 ml/L of an EDTA solution (20.1 g/L) and 2 ml/L of
a MgSO₄ solution (120 g/L) are supplemented. The trace solution contains (in g/L): CoCl₂·H₂O 0.74, ZnSO₄·7H₂O 0.18, MnSO₄·H₂O 0.1, CuSO₄·5H₂O 0.1, CoSO₄·7H₂O 0.21.

Working seeds (2 ml) of the strain were maintained at -80°C in MM with 40% of glycerol. A pre-cultivation step was performed in 100 ml of the mentioned medium (with glucose 10 g/L as an exception) in a baffled shake flask under orbital shaking at 37°C, 160 rpm. Cell growth was followed by optical density (OD) measurement at 600 nm. For cell dry weight measurement, the filtered and washed cultivation broth were dried during 24 hours at 105°C, cooled down in a desiccators and afterwards weighted on an analytical balance. Glucose concentration was measured with an electro-enzymatic system YSI (model 2700). The lab-scale stirred bioreactor (Biostat B-Twin, Sartorius) used in this study was configured with a standard Rushton turbine, and as described in detail elsewhere, the reactor is equipped with controls for dissolved oxygen concentration, pH, temperature, and aeration rate (remote control unit by the MFCS/win 3.0 software).

High cell-density experiments were carried out in either the classical stirred vessel (total volume: 3 L; initial working volume: 1 L; final working volume: 1.5 L; reference reactor configuration) or the stirred vessel connected to a 12-meter recycle loop (silicon pipe) to generate a non-well mixed condition (scale down reactor configuration). This silicon pipe (diameter 0.005 m, length 12 m) leads to a mean residence time of 79.8 s (as measured in a previous study) reproducing heterogeneities expected in large bioreactors. Dissolved oxygen concentration was monitored directly in the recycle loop by non-invasive optical sensors (flowthourgh cells – Presens). Medium was circulated between the stirred reactor and the recycle loop by a peristaltic pump (Watson Marlow 323) of 80 rpm, with the glucose feeding solution (500 ml 400 g/L glucose in minimum medium) at the inlet of the recycle loop to generate a substrate gradient. During the experiments, pH was maintained at 6.95 (regulation by ammonia and phosphoric acid) and temperature at 37°C. For reference and scale down reactor, initial agitation speed and aeration rate was set at 800 rpm, 1 vvm and 800 rpm, 0.5 vvm, respectively. Once feeding started, the agitation rate and aeration rate were progressively achieved to 1300 rpm and 2 vvm. Feeding control was based on dissolved oxygen level (setpoint fixed at 30% above saturation) in order
to prevent oxygen limitation. This control was setup on the basis of a PID regulation previously considered. Samples were collected from the STR compartment every 1.5 hours. A further addition of MgSO$_4$ and FeCl$_3$·$6\text{H}_2\text{O}$ solution was added to the culture in the beginning and middle of the fed batch process (at an optical density of 50) in order to avoid problems associated with precipitation. All the fed-batch fermentations started with batch cultures, and a dissolved oxygen control based feeding was adopted when initial glucose was depleted.

Oscillation experiments were performed in chemostat mode at a chosen dilution rate of 0.14 h$^{-1}$ and the agitation speed and aeration rate was set at 800 rpm, 0.5 vvm. Chemostat cultivation was preceded by a batch phase, which was carried out on a medium identical to the feed medium. Before starting medium feeding, cells were starved for 1 hour after glucose depletion at the end of batch phase, which was indicated by a steep rise of the dissolved oxygen, in order to see the impact of glucose pulse at the level of gfp synthesis. After reaching the steady state, perturbations were made by adding a glucose solution of 30 g/L (made up in distilled water) to the reactor following a switching profile controlled by a dedicated Matlab code. The ON/OFF sequence was 100/900 s with an intermittently stopped flow rate of 0.08 ml per second. The time interval between two pulses was predetermined by the glucose consumption speed in the current reactor setting based on the DO response. DO was measured, but not controlled, and is only used to verify the glucose depletion in batch mode and aerobic conditions were maintained at all times in the chemostat. Samples were taken every 30 min after the glucose feed start.

Antibiotics were added in the medium to prevent plasmid loss and the development of mutants and contamination during the cultivation. Foam was disrupted by addition of antifoam agent-polypropylene glycol (PPG) in demand. Independent cultivations were performed in duplicates.

**Flow cytometry**

The analysis of GFP expression level was performed with a FACscan flow cytometer (Becton Dickinson). Samples were divided in two subsamples, the untreated subsample for GFP analysis and the other for PI staining at room temperature for 10 min. Since we used a destabilized variant of GFP with a reported half-life of 1 hour,
sampling was a critical step. In order to avoid hydrolysis of GFP by intracellular proteases between sampling and analysis, samples were diluted twice in a 30% glycerol solution and directly stored at -80°C. This process was validated by comparison with fresh samples analyzed directly by flow cytometry. For each measurement, 30,000 cell events were analyzed for gfp gene evolution with the excitation and emission wavelength at 488 nm and 530 nm. The FSC, FL1, FL2 and FL3 channels are logarithmically amplified with settings of FSC E00, FL1 500, FL2 420, FL3 420. FSC and SSC channel can be used to determine cell size and shape respectively. A threshold of 52 was set upon the SSC channel. Propidium iodide (PI) stained samples were passed through flow with setup of FL1 500, FL2 420, FL3 520 to check cell membrane permeability. The results were evaluated with Flowjo (version 7.6.1) and represented as specific GFP intensity, which corresponds to GFP intensity per cell based on analyzing 30,000 cells with FACS.

**Total protein determination and supernatant fluorescence measurement**

The total amount of protein was measured with Folin Lowry method. Samples withdrawn from bioreactor were centrifuged at 12000 rpm for 3 min and filtered on 0.2 µm cellulose membrane in order to remove the cells. Fluorescence of the supernatant (samples of 200 µL on 96 wells black microtiter plate) was analyzed by spectrofluorimetry (Victor 3 V Wallac, Perkin Elmer) and shown in arbitrary units, A.U. Proteins from 7 µL of the supernatant were separated on 30% polyacrylamide gels (Biorad) by standard SDS-PAGE procedures. Immunoblot was performed to detect the band corresponding to GFP. Detailed information can be found in one of our previous work.

**Mathematical modeling of the dynamics of GFP synthesis**

A dynamic mathematical model was set up in order to predict GFP synthesis in batch and in continuous bioreactors. In order to simulate GFP synthesis, the following equation was used:

\[
\frac{d\text{GFP}}{dt} = \mu^2 - \mu \cdot \text{GFP} - r_{\text{decayAAV}} \cdot \text{GFP}
\]  
(Equation 1)
With $\mu$, being the growth rate (h$^{-1}$), GFP the concentration of GFP (arbitrary unit) and $r_{\text{decayAAV}}$, the decay rate constant of the $\text{gfpAAV}$ (h$^{-1}$). The generation term in this equation is proportional to $\mu^2$, since this kind of ribosomal promoter is activated in a way responding to the so-called ‘$\mu$ squared’ rule and the synthesis of rRNA in a cell is proportional to the square of the growth rate of the culture.$^{40}$ The value of $\mu$ is driven by the bioreactor-operating mode. For the batch mode, the following ODEs were considered:

$$\frac{dX}{dt} = \mu_{\text{max}} \frac{S}{K_S + S} \cdot X = r_X \cdot X \quad (\text{Equation 2})$$

$$\frac{dS}{dt} = - \frac{r_X}{Y_{X/S}} \quad (\text{Equation 3})$$

With $X$, $S$ and $C_L$ being respectively the biomass, substrate and dissolved oxygen concentration (g/L or mg/L). $K_S$ being the affinity constant for substrate. $Y_{X/S}$ being the coefficient yield of biomass from glucose (g/g). $\mu_{\text{max}}$ is the maximum growth rate (h$^{-1}$). The equations were simulated by using the ‘ode23s’ routine available in MatLab® R2009b (The MathWorks, Inc., Natick, MA, USA). Most of the simulation parameters were adjusted on the basis of the dynamics observed experimentally in our cultivation conditions, i.e. microbial growth and substrate consumption curve. All these parameters were kept constant during the simulation while the parameter $r_{\text{decayAAV}}$ was varied in order to show the effect of the GFP stability on the reporter behavior.

**Results and Discussion**

**Predictive modeling and experimental validation of the behavior of GFP biosensor in batch bioreactor**

Growth rate-dependent control in bacteria involves regulation at the levels of transcription and translation, and an increase in growth rate is associated with an increase in cell size and number of ribosomes.$^{28}$ In this study, reporter strains based on the expression of destabilized GFP under the control of either $\text{rrnB P1P2}$ promoter or $\text{fis}$ were employed to illustrate the dynamic growth response of bacteria population in heterogeneous bioreactors. Those two promoters may provide complementary signal in responding to nutrient availability since they have similar expression pattern. The GFP expression level is seen as the function of promoter strength. We constructed several different plasmids, which were later introduced individually into
*E. coli* strain MG1655. Due to the rapid turnover of destabilized GFPLAA (half life is about 40 min\textsuperscript{17}) and the low copy number of reporter protein, the majority of cells exhibited very low fluorescence that is not suitable in this study for detection using flow cytometry (data not shown). Therefore, we decide to continue the work with GFPAAV that has a higher fluorescence.

To predict the behavior of the constructed biosensors in function of the bioreactor operation mode a dynamic mass balance model was used. GFP synthesis was modeled by an ordinary differential equation comprising three terms, \textit{i.e.} a production term responding to the \( \mu \) squared rule\textsuperscript{41} and two distinct consumption terms taking into account GFP dilution by cell division and the GFP decay under the action of intracellular proteases. This last term is very important since most microbial GFP biosensors are based on GFP variants exhibiting a very high stability with half-life exceeding 24 h. This intrinsic stability limits the use of the GFP reporter system for the detection of microbial stress in bioreactor operations, since physiological processes involving smaller characteristic times can be hidden by this property of the reporter molecule. Several simulations were made and a special attention was given on the impact of the stability of GFP varies from 0.1 to 10 h. Figure 2A shows the GFP signal cannot be directly correlated to the growth rate in the late exponential phase of a batch culture for the stability time exceeding 2 h by considering the intrinsic stability of the reporter protein.

As shown in Figure 2B, dynamics of the *rrnB::gfpAAV* reporter system in cells grown in a batch bioreactor can be modeled with a stability half-life of GFPAAV between 45 and 60 min, which is close to the previously reported value for this parameter.\textsuperscript{17} The data shows that both the stable and the destabilized reporter systems respond quite well to the \( \mu \)-squared rule (Equation 1, Figure 2C) in batch cultivation as expected in early exponential phase, however, GFPmut3 is more stable than GFPAAV and not follows the \( \mu \)-squared rule when growth is slowed down (Figure 2B and 2C). The simulations point out the fact that the stability of GFP is of primary importance for the quality of detection of variation in growth conditions by the biosensor.
Figure 2. Comparison of simulated and experimental data for specific GFP dynamics in batch bioreactor. A: Several simulations were estimated by varying the stability time of the GFP from 0.1 to 10 h. B: Experimental data were extracted from a batch culture of *E. coli* carrying either an *rrnB::gfpAAV* or an *rrnB::gfpmut3* reporter system. GFP intensities were normalized by the maximum fluorescence of each sample. C: Experimental and simulated growth rate values.

Scale-down effect on growth dependent GFP biosensors: destabilized variant exhibits an unexpected behavior in homogeneous conditions

The objective of this work is to determine whether growth-dependent GFP biosensors are able to detect process-related perturbations. A set of fed-batch cultivations was used in order to evaluate the expression profile of our GFP biosensors in process-related stress conditions. For this purpose two kinds of promoters were used, namely, the ribosomal promoter *rrnB* and the *fis* promoter. The fed-batch operating mode is particularly relevant since it is the one most frequently used at the industrial level and is also known to promote concentration gradient at the industrial scale. A
corresponding scale-down system, that is, a well-mixed reactor equipped with a recirculation loop inducing the appearance of local heterogeneities, was also employed for each reporter strain. The two-compartment scale-down reactor (SDR) allows reproduction of mixing imperfections expected at large-scale and was used for that purpose in a number of previous studies.\textsuperscript{8,37}

From Figure 3A we can see that GFP synthesis rate seems to be correlated to the growth rate progression in the first 10 h of cultivation. Specific GFP intensity increased dramatically at the end of the cultivation for the cultures carried out in well-mixed conditions by comparison with those conducted in scale-down configuration. As a better growth is expected in well-mixed reactor, therefore, a higher amount of GFP is expected to accumulate in the well-mixed reactor than in the scale down reactor because of the increase of plasmid copy number in the early stage of slow growth.\textsuperscript{42} However, growth rate evolutions (Figure 3B) were very similar between a well mixed and a scale-down bioreactor, indicating the discrepancy in fluorescence of the two reactors was not caused by the variation of plasmid copy number but additional mechanism accounts for this process. The other reason to explain GFP accumulation might be that more cells lost their capability of hydrolyzing produced protein in the well-mixed reactor. No fluorescent difference was observed in the case of the \textit{rrnB::gfpmut3} biosensor, suggesting that the destabilized version of the biosensor is more suited to point out differences between well-mixed and scale-down modes. Glucose was the growth-limiting factor during the fed-batch phase and the amount of glucose varied between 0.01 and 0.25 g/L in the mixed compartment of the SDR, which is in accordance with our earlier reported data in the same bioreactor operating condition.\textsuperscript{21} Microbial cells in this kind of reactor are indeed exposed to stochastic environmental fluctuations mainly governed by the random passage between the well-mixed part and the tubular part, so GFP fluorescence can be impaired by dissolved oxygen deprivation and glucose fluctuations inside the tubular loop. Dissolved oxygen fluctuations were observed experimentally in the SDRs and shown in Figure 4. Clearly we can see that microbial cells were exposed to dissolved oxygen limitation when crossing the recycle loop, whereas these effects on the GFP maturation steps can be excluded by considering the relatively short residence time of the cells in the tubular loop of the SDR (around 70 s, by comparison with 6.5 min for GFPmut3 maturation\textsuperscript{43}).
Figure 3. A: Evolution of specific GFP intensity and B: growth rate in well-mixed and scale-down bioreactors operating in fed-batch mode. The subplots from top to bottom panel each represent *E. coli* strain bearing *rrnB*gfpmut3, *rrnB*gfpAAV, or *fis*gfpAAV.

To confirm that the evolution of GFP in well mixed and non well mixed reactor became distinct at the end of the fed batch process, experiments using the *fis::gfpAAV* biosensor were reproduced with a higher sampling frequency of 1 h (Figure 5). Dynamic change of *fis* activity is relatively well documented in the literature and could contribute to the understanding of the molecular processes involved in our experimental conditions.
Figure 4 Dissolved oxygen concentrations monitored at different level of the SDR. The dissolved oxygen levels have been monitored at different lengths (L) of the recycle loop by using non-invasive optical sensors.

Indeed, FIS is a small DNA-binding protein, which is significantly affected by the growth rate and the environmental conditions of the culture. Basically FIS level is known to increase in early exponential phase and then decrease progressively during late exponential and stationary phase in rich medium, or significantly reduced during growth in MM. In connection to rrnB, FIS is able to communicate with the nutritional quality of the environment and is involved in the regulation of ribosomes synthesis. An important observation that was previously made is that FIS is overexpressed after a nutrient upshift, for instance, when cells are rapidly transferred to a rich medium. This observation matches the result obtained in well-mixed fed-batch reactor in this study. The overexpression of fis promoter was significantly marked after 10 h of culturing in current experiment, however, in our case a mineral salt medium was used without any addition of “rich” component such as amino acids. It is relatively difficult to assess this particular physiological effect from experiments conducted in SDR, even though the condition in the SDR caused interesting deviations in the GFP evolution. Therefore additional experiments are needed in order to determine the source of excess expression. To this purpose, the dynamics of the fis
and \( \text{rrnB} \) promoters will be confirmed in stabilized chemostat experiments and the results are described in the next section.

![Graph showing evolution of intracellular GFP intensity](image)

**Figure 5.** Evolution of the intracellular GFP intensity for fed-batch cultures carried out with the \( \text{fis::gfpAAV} \) biosensor in well-mixed reactor and scale-down reactor. (Sampling frequency: 1 h).

**Increasing activity of growth dependent promoters is confirmed in chemostat mode and is relieved when glucose oscillations are imposed to the system**

The chemostat mode imposes a growth-limiting condition such as those experienced in prolonged fed-batch bioreactor. In chemostat cultivation cells are maintained in stabilized conditions (i.e. constant pH and dissolved oxygen), both the \( \text{rrnB} \) and the \( \text{fis} \) biosensor exhibited the same excess expression pattern as that observed in well-mixed fed-batch bioreactor (Figure 6). The GFP signal of both reporters increased to a level beyond the maximal level observed during the batch phase. This observation confirms the earlier results and suggests that the excess expression is linked with substrate limitation. Substrate limitation was partially relieved in SDRs while microbial cells crossing the tubular part where local substrate excess can be encountered. In order to simulate this kind of effect, repeated glucose pulses of fixed frequency were injected into the prior stabilized chemostat culture by using an abrupt ON/OFF DO-feed control. The time interval between two pulses was predetermined by the glucose consumption speed, that is, no glucose pulse was fed before the prior
glucose was completely consumed. During the experimental feeding process glucose concentrations were monitored to insure that no glucose accumulation occurred in the oscillating chemostat.

**Figure 6.** Evolution of the GFP signal from *rrnB::gfpAAV* (left) and *fis::gfpAAV* (right) biosensors cultivated in chemostat mode (*D*=0.14 h⁻¹). The steep rise of the dissolved oxygen indicates the end of batch phase and glucose depletion.

The result showed that glucose concentration was maintained below 0.056 g/L and a short increase of GFP signal in the beginning of feeding was observed followed by a progressive reduction of the GFP signal of the *rrnB* and *fis* biosensors after hours of oscillations (Figure 7). Repeated glucose pulses of fixed frequency were speculated to relieve the carbon limitation signal and accordingly the GFP signal decreased. This observation together with the finding of *fis* and *rrnB* excess expression in fed-batch conditions confirms the fact that the reporter behavior are linked with the substrate limitation signal. Our previous results showed that protein leakage is induced when cells are cultivated in fed-batch reactor or in chemostat mode.¹²,²¹ This leakage of protein into the extracellular medium could possibly explain the *fis* and *rrnB*
overexpression after a sudden upshift to ‘rich’ medium. To assess this hypothesis, protein leakage was investigated in our operating conditions.

Figure 7. Effect of programmed glucose pulses on the fluorescence intensity of *rrnB::gfpAAV* (left) and *fis::gfpAAV* (right) biosensors cultivated in chemostat mode at a dilution rate of $D = 0.14 \, \text{h}^{-1}$.

The effect of long-term exposure to nutrient limitation could possibly be explained by protein leakage

Microbial cells can alter their outer membrane structure and protein composition to facilitate substrate uptake under various growth conditions and glucose-feeding rate in continuous or fed-batch cultivations. Altered membrane structure might in turn facilitate protein leakage. Protein leakage is found to be twice as high in chemostat cultivation compared to the constant leakage level in fed-batch. Mechanisms associated with the increase of membrane permeability are associated with the up-regulation of porins and sugar transporters, as well as changes at the level of composition. We therefore decided to examine membrane permeability and protein leakage in our bioreactor set-up. This was achieved by looking into three parameters: the total protein content in the supernatant (Figure 8), the presence of
GFP in the supernatant (Figure 9) and the membrane permeability (Figure 10). We found that the protein concentration was significantly higher in the supernatant of well-mixed bioreactors compared to SDRs (Figure 8). This result is consistent with above results showing that microbial cells are more susceptible to protein leakage under repeated high glucose zones in well-mixed bioreactors, where a higher frequency of glucose injection was encountered. Consistent with this, Figure 9 shows that the amount of GFP protein as well as the fluorescent signal were significantly higher in the supernatant coming from a well-mixed bioreactor compared to a SDR, both harboring the fis::gfpAAV reporter. Similar results were obtained with the rrnB::gfpAAV construct (data not shown). Interestingly, total extracellular protein concentration are consistent with the induction profile of the fis::gfpAAV biosensor mention above (Figure 9C, D to be compared with the GFP induction profiles of fis::gfpAAV depicted at Figure 5).

Figure 8 Comparative evolution of the total protein concentration of supernatant collected from a well-mixed fed-batch bioreactor and from a scale down reactor.
Figure 9. Representative immunoblot analysis of GFP coming from the supernatant of a culture carrying the fis reporter in a well-mixed (A) and a scale-down bioreactor (B). Evolution of the supernatant fluorescence (in arbitrary units, A.U.) of the fis reporter culture in a well-mixed (C) and a scale-down bioreactor (D).

Figure 10. Susceptibility of the fis::gfpAAV microbial population to PI uptake in different operating conditions. Samples for the fed-batch reactor, well-mixed and scale-down reactor were taken at the end of the cultivation.
To assay membrane permeability, we used the dye exclusion test based on propidium iodide (PI) uptake for the fis reporter strain (similar observations were found with therrnB reporter strain (data not shown)). In Figure 10, an intermediate subpopulation between PI negative (intact membrane) and PI positive (damaged membrane) cells are noticed in almost all the operating conditions. This subpopulation is particularly prominent in cultures grown in a stabilized chemostat and in well-mixed fed-batch bioreactors, but is almost absent in the cultures carried out either in chemostat with glucose oscillations or in SDR (Figure 10). It can be hypothesized that this population corresponds to cells with a permeabilized outer membrane leading to protein leakage and protein leakage in turn modifies the nature of the medium, thus this medium can no longer be considered as minimal medium in which proteins and amino acids are released to. Apart from this, the accumulation of amino acid from elevated amino acid synthesis pathway was stimulated by the glucose pulse.50 Regardless of the specific reason for medium enrichment, the appearance of the permeabilized cell membrane represents a shift from minimal growth conditions to ‘rich’ growth conditions. This hypothesis is partially validated by the up-regulation of the fis promoter in stabilized chemostat and in well-mixed fed-batch reactor where protein leakage is significant, because fis promoter is indeed known to be up-regulated when cells are transferred from minimal to rich medium34 (see introduction). The potential release of proteins to the extracellular medium is interesting since this phenomenon could possibly explain the long-term effect on expression of our gfp reporters. It may also interpret that there is GFP accumulation in the well-mixed reactor because of the loss capability of cells to degrade GFP protein, however, it can be argued that all these phenomena are linked in the context of this work.

**Conclusion**

The commonly utilized fluorescent proteins such as GFP can serve as reliable reporters in gene induction studies. However, their application is limited due to the slow degradation rates. Rapid changes in gene transcription can be of interest when they occur in response to physiological signals. Typically, fluorescent reporters are used to monitor gene expression dynamics at the translational level. The advantage of using destabilized reporter is evident in comparison with the stable GFP because if the GFP takes a long time to mature and degrade, then fluorescence would stay and the
reporter system would have ‘memory’ effect. To be a good temporal marker, the destabilized fluorescent protein is expected to have a quicker turn over, therefore, gene expression can be easily analyzed in living cells in nearly real time by using fast turn over reporter proteins. However, at meantime, the rapid degradation always problematically maintains a low fluorescence signal that is maybe hardly detectable. In this study two destabilized GFP biosensors were successfully constructed and used to estimate the performances between a well-mixed reactor and a non-well mixed reactor (SDR). Unexpectedly we found a progressive increase of GFP synthesis when biosensors were cultivated in well-mixed bioreactors by comparison with SDRs. This observation was made both for the \textit{rrnB::gfpAAV} and the \textit{fis::gfpAAV} reporters. This similarity between the behaviors of the two growth-dependent promoters can be attributed to the fact that they have similar expression pattern and Fis protein is known to act as an activator of the \textit{rrnB} promoter.\textsuperscript{31} This phenomenon was confirmed in chemostat with and without controlled glucose oscillations, where increment of the GFP intensity followed by its decrease was found. Taken together with results from fed batch studies, it suggests that there may exist a relationship between the reporter behavior and the glucose limitation signal. Our previous findings have driven our attention on the fact that prolonged glucose limitation induces protein leakage.\textsuperscript{12} Since \textit{fis} is overexpressed when cells are exposed to a nutrient upshift, such as the conditions being met in well-mixed reactors when proteins are released into the extracellular medium and it might be the reason of inducing GFP over expression since the medium was turned into ‘rich’ by the released amino acid or peptide. Analysis of supernatant showed that the potential protein leakage was significantly lower in scale-down conditions, demonstrating less protein release and lower gene overexpression in SDR. From a practical perspective, our results suggest that the difference between well mixed and SDR conditions could be easily detected in the early stage of the cultivation. The results depicted at figure 5 showed that the GFP profiles exhibited in the two cultivation conditions diverge after 6 h of cultures. This divergence was correlated with major changes at the physiological level, such as membrane permeability and release of proteins into the extracellular medium.

The rapid turnover results in less accumulation in cells leading to lower toxicity in the cells. Our destabilized GFP biosensors are thus very promising in order to detect mixing imperfections but also major physiological changes of industrial interest such
as protein leakage. The potentialities of this technology would be only fully exploited if online tools are available in order to monitor fluorescence changes. For this purpose, the use of destabilized GFP biosensors in conjunction with online flow cytometers would be of great interest for the development of new single cell analytical tools.⁵¹

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Literature Cited

5.2 Manuscript 2
A low-cost automated flow cytometry procedure for the characterization of microbial stress dynamics in bioreactors

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Abstract
Actual bioreactor performances rely mainly on the possibility to gain insight about process dynamics via on-line parameters and actual Process Analytical Technologies (PAT) recommendations are focused mainly on the increases of the on-line variables available for bioprocess documentation and control. In this context, flow cytometry (FC) has been automated in order to give access to the dynamics of single-cell physiological parameters in process-related conditions. This approach is not yet widespread applied both at the academic and the industrial level, probably because the technical challenges behind the automatization of FC. In this work, we propose a simple and low cost automated FC system allowing us to monitor two distinct cell physiology related parameters, i.e. the synthesis of a destabilized Green Fluorescent Protein (GFP) that under the regulation of the fis promoter and propidium iodide (PI) uptake. The automated FC enables to compute these parameters at the single-cell level and at regular interval of 15 minutes. In addition, the mean to median ratio of these two parameters is a good indicator as a way to quantify the degree of population segregation.

Keywords: Flow cytometry, phenotypic heterogeneity, Escherichia coli, membrane permeability, green fluorescent protein
Introduction

Flow cytometry (FC) is a very powerful tool in the follow up study of physiological properties of microbial cells in process-related conditions.\textsuperscript{1} The main advantage of this methodology is that simultaneous quantification of hundreds of thousands of individual cells per minute based on cell size, granularity and fluorescence properties in different channels provides loads of information at single cell level about the phenotypic heterogeneity of a microbial population. This information is critical in a bioprocess improvement perspective since the appearance of unwanted phenotype can impair cultivation efficiency and product quality.\textsuperscript{2,3} Such phenomena has recently been pointed out during culture of recombinant \textit{Pichia pastoris} with the appearance of a non-secreting phenotype.\textsuperscript{4} In the process analytical technology (PAT) framework, FC is proposed as an efficient on-line monitoring system for bioprocesses evaluation.\textsuperscript{5} The development of the on-line FC has been used for monitoring of recombinant protein synthesis,\textsuperscript{6,7} determination of mammalian cell viability,\textsuperscript{8,9} isolating robust microbial phenotype\textsuperscript{10} and monitoring water disinfection.\textsuperscript{11} However, the use of automated FC is still largely underexploited in front of its powerfulness in bioprocess optimization. Commercial systems, such as the Flowcytoprep (MSP corp, MN) device, are also available but are generally expensive.\textsuperscript{12} In this work, we propose to use a bench BD Accuri\textsuperscript{TM} flow cytometer as a basis for the design of an automated FC that interfaces smoothly with automated bioreactor sampling systems. This apparatus is simple, easy to handle and leads to real-time, accurate data for process evaluation.\textsuperscript{13} The presence of peristaltic pumps controlled by a microcontroller facilitates the interfacing of this apparatus to a bioreactor since no pressurization of the sample is needed to ensure the fluid displacement. Sample dilution and staining is carried out in the tubing between the FC and the bioreactor.

Spatial heterogeneity occurs during the scale-up of cultivation from laboratory to production volume due to imperfect mixing, consequently cellular heterogeneity is generated from the fact that each cell behaves more or less different from the other, even in the culture that with a homogenous nature.\textsuperscript{14,15} Therefore, averaged measurement of cell characteristics from samples of millions of cells cells has to be updated with single cell analysis, such as flow cytometric analysis, to picture the heterogeneous population structure at a single-cell as well as at a population level.
Our previous study pointed out that the fis promoter is up regulated when cells are exposed to prolonged substrate limitation.\textsuperscript{16} This phenomenon could be attributed to the release of extracellular components during the cultivation that can stimulate the fis activity, which was previously shown to be up-regulated when cells shifted from a minimum to a rich medium.\textsuperscript{17,18} However, additional information about cell physiology is required in order to confirm this hypothesis. This automated FC system will be used in the follow up study of \textit{Escherichia coli} MG1655/pGS20PfisGFPAAV fluorescent biosensor in the cultivation context, due to the fact that the current destabilized GFP variant exhibited a half-life less than one hour.\textsuperscript{19} Distribution of cellular responses to glucose oscillation and the temporal evolution of a single cell by using fluorescent reporters will be recorded in a time dependent manner.

\textbf{Material and methods}

\textbf{Reporter strain, medium and pre-cultivation}

\textit{Escherichia coli} MG1655 bearing a pGS20 plasmid with a cassette of fis::gfpAAV gene and a chloramphenicol resistance gene was used in this work. Detail information about strain construction and pre-cultivation condition can be found in our prior study.\textsuperscript{16} During the cultivation, 25 \textmu{g}/mL of chloramphenicol (stock concentration: 50 mg/ml in 96\% ethanol) was added to medium to keep the selection pressure.

\textbf{Automated FC platform and sample analysis}

The interfacing system between the bioreactor and the BD Accuri C6 has been performed on the basis of in-line mixing principle (figure 1A).
A first peristaltic pump (Watson Marlow 040.DP1D.N2R operating at a rate of 32 mL/min), as the sampling pump, is set to extract cell suspension (sample) directly out of the bioreactor and inject it in a flow of dilution water (milli Q water as the sheath fluid for Accuri C6). The dilution fluid is carried by a sequential peristaltic pump (Watson Marlow 040.DP1D.N2R operating at a rate of 32 mL/min), denoted as dilution pump, and mixed with the sampling line. In line mixing is achieved by a polypropylene T-mixer. Silicon pipes (internal diameter ID: 1 mm) are mainly used to build up the system, with an exception for the peristaltic pump where a long life Marprene tubing is used. All the connections are locked by Male Polypropylene Luer plug (ID: 1.6 mm). The activation or deactivation sequence of these two peristaltic pumps is controlled by a PIC18F microcontroller embedded in a full PICPLC16.
control card provided by mikroElektronika supplier (the different sequences are illustrated at figure 1B).

This automated FC platform was preliminary adjusted to ensure the full cleaning of sampling lines to avoid cross-contamination between consecutive samples. A linear and efficient range of fluorescent analysis was found for samples with less than $3 \times 10^6$ events per mL (figure 1), therefore, before FC analyzing appropriate sample dilution was made and the dilution efficiency is validated (data not shown). Following inoculation, each sample at an interval of 15 min is pumped through the FC analyzing station at a flow rate of 33 $\mu$L/min and approximately 200,000 events is thus recorded within 1 minute recording time. The recorded mean and median fluorescence signal in the FL1 and FL3 channel are corresponding to the GFP signal and the membrane permeability that measured by propidium iodide (PI) staining, respectively. Membrane permeability by PI measurement was done by its direct addition into the milliQ reservoir at a concentration of 10 $\mu$g/mL. The staining procedure is validated and it proved that the incubation for 2 minutes is sufficient for appropriate PI staining (data not shown). Processing and analysis of the flow cytometry raw data was performed using MatLab ® R2010b (The MathWorks, Inc., Natick, MA, USA).

![Figure 2. Determination of the linear range for fluorescent analysis using accuri C6 flow cytometer.](image)

Calibration was made by using 1 $\mu$m green fluorescent microsphere (Fluorosphere, Invitrogen) and the linear range of particles and fluorescence intensity can be found up to $3 \times 10^6$ events per mL.

**Cultivation conditions**
Chemostats were carried out in lab-scale stirred bioreactors (Biostat B-Twin, Sartorius with a total volume of 3 L; working volume of 1 L; mixing provided by a standard Rushton turbine) controlled by the remote control unit with the MFCS/win 3.0 software. During the experiments, pH was maintained at 6.9 (regulation by ammonia and phosphoric acid) and the culture was grown at 37°C and stirred at 1000 rpm with air flow rate at 1 L/min. Chemostat with a feeding solution containing 5 g/L of glucose solution (with the same minimal medium as previously described) at a dilution rate of 0.14 h\(^{-1}\). Glucose oscillations were performed by pulsing 8 mL of a concentrated glucose solution (30 g/L made up in distilled water) in each 15 minutes cycle.

**Results and discussion**

Automated online sampling of the culture using flow cytometry provides high-resolution analysis for process monitoring. This platform consisting of sampling from bioreactor, sample preparation including staining with fluorescent dye PI, and sample injection into Accuri C6 flow cytometer, is a rapid online measurement of single cell properties. The application of this platform is extended in a biological study where heterogeneous population response and distribution was investigated over time by perturbing the steady state *E. coli* culture with a concentrated glucose pulse at growth rate of D=0.14 h\(^{-1}\).

**Follow-up investigation of the *fis::gfpAAV* activity by automated FC**

In order to test the efficiency of the automated FC platform, validation test was carried out on the basis of our previous results, where the *fis::gfpAAV* reporter system is shown strongly sensitive to substrate limitation and fluctuations.\(^{16}\)
Figure 3. Direct comparisons of on-line variables with GFP evolution (A) acquired by automated FC. Dissolved oxygen profile is shown in order to highlight the three distinct phases: batch, chemostat mode at $D = 0.14 \, \text{h}^{-1}$ and chemostat mode $D = 0.14 \, \text{h}^{-1}$ with glucose oscillations (B) as well as pH profile (C) and addition of acid (dotted line) and base (solid line) profiles (D). Representative result of three independent cultivations was shown.

After an initial batch phase, a chemostat mode was initiated at a dilution rate ($D$) of $0.14 \, \text{h}^{-1}$ and glucose pulses was performed at a given frequency to the stabilized chemostat culture. Further assessment of this sensitivity in the chemostat experiment was thus conducted and the response of the GFP biosensor was followed by automated FC (Figure 3A). The oscillated condition can be visualized in the evolution profile of dissolved oxygen (Figure 3B) and pH (Figure 3C), which fluctuated corresponding to the glucose pulses period.

During the batch phase, GFP synthesis is seen to be correlated directly to growth rate, the correspondence of which is known as the “μ squared” rule. This observation is consistent with off-line GFP analysis and can be efficiently validated with a higher number of sampling points with the automated FC. When shifted to the chemostat...
mode, GFP level is stabilized for a very short period of time (around 2 hours) and then the signal increased and continued throughout the stabilizing. This phenomenon is unexpected since GFP would keep constant level if the activation of the fis promoter were proportional to the growth rate. In our previous studies, we have shown that proteins are secreted into the extracellular medium when microbial cells are exposed to substrate limitation.\textsuperscript{21,22} This phenomenon is reported by several authors and is generally termed as protein leakage.\textsuperscript{23,24} Since the fis promoter is up-regulated when cells are shifted from minimal to rich medium conditions, this release of proteins could possibly contributes to the increases of the pool of available amino acids (AA). This trend can be visualized on the basis of the pH regulation profile (Figure 3D), implying mainly the addition of acids in the first phases of the culture (from 3 to 12 hours). A significant advantage of the automated FC is that parameters related to cell physiology (such as GFP synthesis) could match on-line process variables in real time. In our case, this phenomenon is related to a slight increase of the pH above set point and a continuous activation of the acid pump, which can be the basis of designing new control loops for bioprocess monitoring. By using automated FC, these on-line parameters can be directly correlated to the up-regulation of the fis promoter and the accumulation of GFP. However, this tendency was reversed after approximately 15 hours of cultivation where the acid addition stopped, the pH dropped slightly and the accumulation speed of GFP signal ceased. This tendency is later confirmed over the course of glucose pulsing and the GFP signal even dropped back to the level lower than its starting point in the continuous culturing. Cell growth during this phase is mainly based on glucose consumption with a global acidification of the medium and the activation of the base pump.

**Substrate limitation induces a significant segregation of the population according to PI uptake**

The automated FC system used in this work allows for the determination of two physiologically related parameters, i.e. the intracellular GFP recorded in the FL1 channel and the PI uptake intensity recorded in the FL3 channel. One of the advantage of FC is the possibility to quantify single-cell parameters and thus to determine whether the distribution of this parameter is segregated or not in the microbial population. This segregation is critical since it becomes clear now that for several
recombinant systems, the productivity is generally related to a fraction of the population but not to the whole population. The availability of single-cell analytical tool is thus of importance at the process control level. In our previous operating conditions (Figure 3), no segregation was observed at the level of GFP synthesis. GFP is generally considered as a poor molecular tracer of physiological activity considering its slow degradation rate\textsuperscript{25,26} and even the destabilized version of the GFP used\textsuperscript{19} in our study cannot explain the appearance of segregation that not recorded in the FL1 channel, due to the fact that a significant segregation of the population can be seen at the level of the PI uptake intensity (Figure 4).

**Figure 4.** Flow cytometric dot plot for the evolution of subpopulations according to the FL1 (GFP synthesis) and FL3 (PI uptake) parameters in the batch and the chemostat phase (A) and the chemostat phase with additional glucose pulses (B).
The appearance of the subpopulations can be clearly visualized in the FL3 channel and is displayed as the subpopulation distribution.

When the cultivation shifted from the batch to the chemostat mode, the fractioning of microbial population appeared and can be clearly visualized in PI profile in Figure 4A, resulting a progressive drop in the events of R1 region from 81% to 45.9% of gated *E. coli* cells in the first 21 hours’ cultivation. The appearance of a R3 subpopulation exhibiting a higher red fluorescence that attributed to the uptake of PI corresponds to a fraction of cells with damaged membrane. It is interesting to see that microbial cells are capable of adapting themselves to the limiting conditions. The observation of an “intermediate” subpopulation (R2) appeared and the R3 subpopulation progressively disappeared along the cultivation. Clearly, two subpopulations were present in the glucose oscillation condition (figure 4B). This observation is important since the R2 subpopulation could be responsible for the protein leakage effect noticed at the level of the GFP synthesis. The intensity of segregation could not be quantified by looking at conventional flow cytogram that displayed in figure 4, the quantification of this segregation will be addressed in the next section.

**Segregation intensity can be quantified by using the mean to median ratio**

In a process control perspective and considering its biological implications, it is important to parameterize the segregation level of a microbial population by automated FC. The mean to median ratio (MMR) can be used to quantify the segregation since the median value is less affected by extreme values than the mean value in a population with the normal or non-normal distribution. As so, for our results of the non-segregated parameter, GFP, similar trends of inducement and decrease was shown for the median and mean values, whereas for the segregated parameter, PI, median value and mean value tended to be very different with mean PI intensity lower than its medium, suggesting a dramatic variation in the population structure (Figure 5). With some discrepancy, the mean and the median value of PI intensity kept rather constant after 10 hours culturing with a disturbance from glucose oscillation, which contributed to the observation of downshifts in both parameters.
Figure 5. Time profile of the mean and the median value for both GFP and PI intensity. Similar evolution profile of both mean and median is shown for the GFP fluorescence, but not for the PI measurement. A disturbance is seen in the PI intensity plot, corresponding to the glucose pulsing.

Figure 6. The mean to median ratio (MMR) of the GFP signal and PI uptake intensity is shown over time (A), corresponding with the fractioning of population (B) distribution in three regions, namely, R1 (healthy cells), R2 (intermediate population) and R3 (damaged cells).

In figure 6, the initial phase of the segregation involves the distribution of the cells between a R1 and a R3 subpopulation, corresponding respectively to healthy and
membrane-compromised cells. As the cells approached the continuous cultivation phase, a significant increase of the MMR is seen, suggesting the elevated separation of R1 and R3. After this initial increment, however, cells adapt themselves to limitation and an intermediate R2 subpopulation appeared, which mirrored as the decrease of MMR with about 4.9% of cells falling into the R3 region, 33.6% in R2 region and 45.1% in R1 region. Glucose pulsing seemed contribute negatively to this adaption process supported by the jump of MMR (Figure 6A) and a larger population in R3 region (Figure 6B). It appears that the intensity of the segregation can be quantified by introducing the MMR parameter and on the other hand, the loss of GFP after pulsing may ascribe to the appearance of subpopulations.

**Conclusion**

The interest of the automated FC is to gather on-line physiologically related parameters and to allow direct comparison of these parameters with process variables. The algorithm integrating the residence time of microbial cells inside the sampling loop and the incubation time with the fluorescent stain can be easily modified in order to meet the requirement of other types of stains. The mean to median ratio tends to be another parameter to quantify population segregation. In conclusion, our low-cost automated flow-cytometry protocol is very efficient tool for tracking dynamic cellular response in the cultivation process and it also provides great potential of designing new feedback control system based on such as cell density, base or acid addition.

**References**


5.3 Manuscript 3
Shifts of population heterogeneity distributions in response to steady-state growth rate and glucose perturbations in *Escherichia coli* and *Saccharomyces cerevisiae*

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Abstract

Two growth rate reporters of *S. cerevisiae* and *E. coli* based on Green Fluorescent Protein (GFP), was characterized and used to investigate the effects of glucose pulses at single-cell level. A direct positive correlation between growth rate and GFP fluorescence could not be seen for either growth reporter. The *E. coli* cells and slow growing yeast reacted to the glucose pulses with slight up-shifts in fluorescence, indicating that the gene regulations of the promoters primarily responded to environmental shifts. The effect of freezing on cell before sample analysis was also investigated. The yeast cells were seen to be heavily influenced by the freezing procedure at high dilution rates, whereas they were more resilient at low dilution rates. In addition, the glucose pulses had an instant effect in protecting parts of the cell population of the rapidly growing cells (D=0.3 h⁻¹) against freeze-thaw damage. This was not seen for the slower growing cells (0.05 h⁻¹). Novel methods of quantifying population heterogeneity were also developed. Using peak width and coefficient of variation (CV) of the population distributions, information on the spread of the population was obtained in a quantitative manner. The slope of cumulative distribution functions (CDF) plots was used to quantify shifts over time and differences in peak shape. Together these methods offered a less arbitrary way of quantifying flow cytometry data and population heterogeneity. Using these methods it was seen that both faster growing yeast and *E. coli* had a significant shift in heterogeneity in response to the glucose pulse, whereas the heterogeneity of the slower growing cells remained largely unaffected.

Keywords: Population heterogeneity, glucose pulse, reporter strain, freezing effect, flow cytometry
Introduction

Optimization of industrial cultivation processes requires a comprehensive analysis and understanding of the physiology of the host strain throughout the cultivation. Traditionally, this is performed on averaged cell characteristics from samples containing millions of cells. However, microbial cells exhibit an intrinsic cell-to-cell variability that is both influenced by and affect the cultivation process. In addition, large scale cultivations introduce spatial heterogeneity through gradients of e.g. dissolved oxygen, substrate, temperature and pH exposing the microbial cells to rapid changes in environmental conditions as they circulate throughout the reactor. These dynamic conditions pose a stress on the cells, which affects their metabolism and the inherent population heterogeneity. Although this heterogeneity has been found to be harmful for cultivation processes by reducing yields and increasing byproduct formation, there are also indications that it may be beneficial as it facilitates a quick adaption to conditions. Transient metabolic responses of microorganisms to similar rapid changes in nutrient availability have been addressed in several studies. It has been shown that external perturbations can disturb optimal enzyme activity levels, disrupt metabolic fluxes, destabilize cellular structures, affect chemical gradients, influence the intracellular pH etc., leading to overall instability. The concomitant stress responses are transient at gene expression level, leading to new steady state levels similar to the unstressed cells even in the presence of a persistent stress. The magnitude and duration of such transcriptional responses corresponds with the duration and magnitude of the perturbation. Although stress responses have been studied, little is known about how changing environments effects population heterogeneity or how different sub-populations contribute to the cultivation process.

The aim of the present study is to gain deeper insight into how microbial population heterogeneity is influenced by growth rate and changing nutrient availability. To enable this, reporter strains for the industrially relevant model organisms *Saccharomyces cerevisiae* and *Escherichia coli* were employed and the cell responses were analyzed at single cell resolution. The reporter systems are based on expression of green fluorescent protein (GFP) coupled with ribosomal or ribosomal related promoters. The state of the individual cells is then reflected in the expression of the
reporter protein, which can be followed by its fluorescence by using flow cytometry. In addition, information about changes in cell size is obtained from the flow cytometer forward scatter (FSC) signal, which normally correlates with particle size.

The integrated *S. cerevisiae* reporter system (FE440) is based on expression of the stable eGFP under the control of the ribosomal RPL22a promoter. This promoter was chosen as its expression has been shown to be growth correlated in a transcriptome analysis study. In addition, the reporter system allows the analysis of cell membrane robustness as a loss of GFP signal in cells has been seen to be coupled with permeabilised cell membranes. These two abilities make the reporter strain an interesting tool for further investigations of how cell robustness and growth rate population distributions respond to changes in nutrient availability.

The *E. coli* reporter system is of a slightly different nature expressing an unstable fluorescent protein from a low copy number plasmid. The promoter reported on, the so-called *fis* promoter, controls the expression of a transcriptional activator that in turn activates the bacteria ribosomal promoter *rrnB* P1. The reporter strain MG1655/pGS20fisGFPAAV was selected based on its ability to rapidly respond to changing environmental conditions and its high fluorescence signal as seen in a prior trial in a heterogeneous reactor. It has earlier been demonstrated that the behaviour of the reporter system depends on the operating mode of the bioreactor and that the reporter gives different responses when exposed to either short-term or long-term nutrient limitation and may hence be used to evaluate cellular behaviour in heterogeneous bioreactors, where cells experience dynamic environmental conditions.

Using the described reporter systems in continuous cultivations growth rate related effects on population heterogeneity were investigated. Glucose perturbations mimicking the conditions cells experience in industrial scale bioreactors were introduced to steady-state cultivations operating at different dilution rates and the concomitant cellular responses studied. The aim was to increase the understanding of microbial behavior at the single-cell level, which might provide important insight for the generation of novel strategies to improve the performance and robustness of industrial scale cultivations.
Materials and Methods

*S. cerevisiae* reporter strain FE440\(^6\) and *E. coli* MG1655/pGS20PfisGFPAAV\(^{20}\) was used throughout this study. In *E. coli* cultivation, 25 µg/mL of chloramphenicol (stock concentration: 50 mg/ml in 96% ethanol) was added to medium to keep the selection pressure.

Cultivation conditions

**Pre-culture.** *Saccharomyces cerevisiae*: A single colony of the growth reporter strain FE440 was picked from a plate with minimal medium and used to inoculate a 0.5 L baffled shake flask with 100 ml of defined mineral medium containing 7.5 g/L (NH\(_4\))\(_2\)SO\(_4\), 14.4 g/L KH\(_2\)PO\(_4\), 0.5 g/L MgSO\(_4\).\(_{7\text{H}_2\text{O}}\), 2 ml/L trace metal solution, 1 ml/L vitamin solution and 10 g/L glucose.\(^{21}\) The pre-culture was incubated in an orbital shaker set to 150 rpm at 30°C until mid-exponential phase (approximately 10 h) and directly used for inoculation.

*Escherichia coli*: The *E. coli* inoculum was obtained by a two-step procedure. First, a single colony of MG1655/pGS20PfisGFPAAV from a fresh LB plate was used to inoculate a 100 mL Luria Broth containing shake flask at 37°C and grown overnight. A dilution series with 10-fold steps was prepared from the overnight culture and incubated at 37°C for 6 to 8 hours on an orbital shaker (180 rpm). The optical density from the dilution series was subsequently measured and the one with an OD\(_{600}\) between 0.4 and 0.6 was used to inoculate the bioreactors.

**Chemostats.** *S. cerevisiae*: Aerobic level-based chemostats with 5 different dilution rates (D= 0.05/0.1/0.2/0.25/0.3 h\(^{-1}\)) were performed in duplicates using 1L bioreactors (Sartorius, B. Braun Biotech International, GmbH, Melsungen, Germany) with a working volume of 1L. pH and DOT electrodes (Mettler Toledo, OH, USA) were calibrated according to standard procedures provided by the manufacturer using two point calibration (pH 4 and 7, gassing with oxygen (100%) and nitrogen (0%), respectively). The growth medium was a defined mineral medium according to Verduyn *et al.* (1992)\(^{21}\) with 5 g/L glucose. The OD\(_{600}\) for inoculation was 0.001. The pH was adjusted and kept constant at 5.0 using 2M NaOH. Temperature, aeration and stirring were kept constant at 30°C, 1 v/v/m and 600 rpm.
*E. coli*: chemostat cultures were inoculated with an OD<sub>600</sub> of 0.01 and operated at 37 °C in the same bioreactor setup but with a working volume of 0.6 liter. The growth medium was a defined mineral medium containing 4.5 g/L glucose, 2 g/L Na<sub>2</sub>SO<sub>4</sub>, 2.468 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g/L NH<sub>4</sub>Cl, 14.6 g/L K<sub>2</sub>HPO<sub>4</sub>, 3.6 g/L NaH<sub>2</sub>PO<sub>4</sub>*H<sub>2</sub>O, 1 g/L (NH<sub>4</sub>)<sub>2</sub>-H-Citrate, 50 µL Sigma 204 Antifoam and 0.1 g/L thiamine. After autoclaving, 3 mL MgSO<sub>4</sub>, 1 mL trace metal solution was sterile filtered (0.2 µm) into the medium. The trace metal solution contained 0.5 g/L CaCl<sub>2</sub>*H<sub>2</sub>O, 0.18 g/L ZnSO<sub>4</sub>*7H<sub>2</sub>O, 0.1 g/L MnSO<sub>4</sub>*H<sub>2</sub>O, 20.1 g/L Na<sub>2</sub>EDTA, 16.7 g/L FeCl<sub>3</sub>*6H<sub>2</sub>O, 0.16 g/L CuSO<sub>4</sub>*5H<sub>2</sub>O and 0.18 g/L CoCl<sub>2</sub>*6H<sub>2</sub>O. Aeration and agitation speed were kept constant at 1 v/v/m and 1000 rpm to avoid oxygen limitation.

The batch phase of both yeast and *E. coli* culture was followed by OD<sub>600</sub> measurement and continuous analysis of the off-gas composition by a Mass spectrometer (Prima Pro Process MS, Thermo Fisher Scientific, Winsford UK). Steady state was considered established when dry weight, dissolved oxygen tension (DOT), HPLC metabolites and exhaust gas concentration (CO<sub>2</sub>) had remained constant for at least three residence times. After glucose depletion, detected as a rapid drop in the CO<sub>2</sub> content of the off gas, the cultures were switched to chemostat by applying a feed with the same medium as used for the batch with the desired dilution rate. The volume was kept constant by a level based outlet. After confirming steady state all cultures were perturbed with a concentrated glucose solution corresponding to a final bioreactor concentration of 1 g/L for *S. cerevisiae* and 0.45 g/L for *E. coli* using a sterile syringe and followed by frequent sampling (time depending on the organism, see result section).

Samples were withdrawn for OD<sub>600</sub>, high performance liquid chromatography (HPLC), dry weight (DW) and flow cytometry analysis. Samples for OD<sub>600</sub> and DW were analyzed directly, HPLC samples were sterile filtered and stored at -20 °C. Samples for flow cytometry were mixed with glycerol to a final concentration of 15% and frozen and stored in a -80 °C freezer. For yeast, flow cytometry analysis was also performed on fresh samples for two of the dilution rates (D= 0.05 h<sup>-1</sup> and 0.3 h<sup>-1</sup>), where broth was mixed with 15% glycerol and kept on ice until analysed (maximum an hour).
Freezing test of *E. coli* samples

To investigate the physical effect of different sample treatments on GFP detection, a simple freezing test was performed in shake flask using same minimum medium as in chemostat cultivation. Triplicate samples were collected from each growth phase and each sample was divided into three sub-samples, one was measured directly, one instantaneously frozen in liquid nitrogen with 15% glycerol and one more slowly frozen in a -80°C freezer, also with 15% glycerol. To avoid non-desirable degradation during the sample treatment, 10 samples were thawed at a time, then centrifuged, and re-suspended in 0.9% NaCl. The maximum lag time between the first and the 10th analyzed sample was 10 min, during which we assume that the intracellular GFP concentration did not influenced by the sample preparation.

Sample analysis

**OD, DW and HPLC.** Growth was monitored by measuring OD$_{600}$ with a Shimadzu UV mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan). Dry weight measurements were performed on 5 ml cultivation broth according to Olsson and Nielsen (1997). The concentration of glucose, acetate, ethanol, glycerol and pyruvate in the broth of the *Saccharomyces cerevisiae* cultivation was determined by HPLC as earlier described by Carlquist *et al.* (2012). The *E. coli* cultivations were in addition analyzed for lactate and formate, and the glucose concentration was measured by enzymatic colorimetric procedure (ABX Pentra Glucose HK CP).

**Flow cytometry.** A FACSARia™ III (Becton-Dickinson, NJ, USA) flow cytometer was used for single-cell analysis of both yeast and bacteria. Excitation wavelength for the laser was set to 488 nm. Two scattering channels (FSC and SSC) and two fluorescent detection channels were used in the analysis. Fluorescence emission levels were measured using a band pass filter at 530±30 nm and 610±20 nm. Light scattering and fluorescence levels were standardized using 2.5 µm fluorescent polystyrene beads. Samples for flow cytometry were centrifuged for 1 min at 3000 g and 4 °C, resuspended in 0.9 % saline solution and directly analysed. 20,000 and 30,000 events were recorded for yeast and *E. coli* respectively. CS&T beads (Cytometer Setup and Tracking beads) (Becton Dickinson, USA) were used for the automated QA/QC of the machine performance.
**Data analysis.** Processing and analysis of the flow cytometry raw data was performed using MatLab® R2010b (The MathWorks, Inc., Natick, MA, USA). The raw data was extracted as fcs files and loaded in MatLab with the help of the readfsc function (by L. Balkay, University of Debrecen, Hungary, available on matlab central file sharing). The HPLC data was collected in an excel file and imported into MatLab. The data from the fcs files was extracted and saved into mat files including the recorded GFP fluorescence and the FSC for each steady state and pulse experiment. The cumulative cell distribution from the 1024 recording channels of the flow cytometer was fitted to the data of the GFP fluorescence using the hist function and afterwards applying the cdfplot function. By applying the hist function to the 1024 recording channels cell count was saved for all channels and plotted as channel number fluorescence for the GFP detector. Biplots for FSC and GFP were created using this function. For better quantitative data for the distributions of GFP, the peakwidth at baseline level was calculated by searching for the borders of the peak, which were considered as 25 cell counts per channel number to disregard the noise level of the flow cytometer. By substracting the channel numbers for the higher and lower border the peakwidth was determined. Furthermore, the mean function was used to calculate the Mean FSC and Mean GFP fluorescence. The normalized GFP was estimated by dividing the mean GFP by the mean FSC. By dividing the peakwidth by the Mean GFP the coefficient of variance of the distribution was generated. Finally the slope of the cdfplot was estimated by fitting a line to the exponential part of the cumulative distribution of the GFP fluorescence histograms by using the polyfit function with a degree of one. For frozen pulse samples the sub-population percentage was computed by dividing the histogram plot into three areas, a low, middle and a high fluorescent. The areas were set in relation to the local minimum in the histogram between two sub-populations, which was calculating using the min function. The middle fluorescence range was excluded to avoid biased data and then the sub-population percentage in the low and high range was calculated by dividing the cell amount in the sub-population by the total cell amount found in the two fluorescence ranges. Due to having duplicate datasets for all samples, all values and estimated parameters were stated showing replicates.
Table 1. List of abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
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</thead>
<tbody>
<tr>
<td>cdf</td>
<td>Cumulative distribution function</td>
<td>-</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
<td>[%]</td>
</tr>
<tr>
<td>D</td>
<td>Dilution rate</td>
<td>h⁻¹</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
<td>-</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
<td>-</td>
</tr>
<tr>
<td>rₛ</td>
<td>Specific substrate uptake rate</td>
<td>[g glucose/g cells /h]</td>
</tr>
<tr>
<td>rₑ₆₇ₙₐ₇</td>
<td>Specific ethanol production rate</td>
<td>[g ethanol/g cells /h]</td>
</tr>
<tr>
<td>RT</td>
<td>Residence time</td>
<td>[h]</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scatter</td>
<td>-</td>
</tr>
<tr>
<td>Yₛₓₐ</td>
<td>Growth yield on the fedded substrate</td>
<td>[c mole/c mole]</td>
</tr>
<tr>
<td>Yₛ₃₇ₒ₂</td>
<td>Yield of CO₂ on the fedded substrate</td>
<td>[c mole/c mole]</td>
</tr>
<tr>
<td>Yₛₑ₇₈₇</td>
<td>Yield of ethanol on the fedded substrate</td>
<td>[c mole/c mole]</td>
</tr>
<tr>
<td>Yₛ₉₇₈₇</td>
<td>Yield of glycerol on the fedded substrate</td>
<td>[c mole/c mole]</td>
</tr>
<tr>
<td>Yₛ₇₈₇₂</td>
<td>Yield of acetate on the fedded substrate</td>
<td>[c mole/c mole]</td>
</tr>
<tr>
<td>Yₛₗ₇₈₇</td>
<td>Yield of pyruvate on the fedded substrate</td>
<td>[c mole/c mole]</td>
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</tbody>
</table>

Results

To investigate how microbial population heterogeneity is influenced by growth rate and glucose excess, glucose-limited continuous cultivations with glucose pulses where performed using reporter strains of *S. cerevisiae* reporter strain FE440⁶ and *E. coli* MG1655/pGS20PfisGFPAAV.²⁰ Based on that we wanted to compare the population response for cells in fully respiratory versus respiro-fermentative growth, continuous cultivations at low and high dilution rates were performed for both yeast and *E. coli*. The low dilution rates used correspond to approximately 13% of the maximum specific growth rate, i.e. 0.05 and 0.09 h⁻¹ for *S. cerevisiae* and *E. coli* respectively, and the high dilution rates correspond to approximately 77% of the
maximum specific growth rate, being 0.3 and 0.53 h\(^{-1}\) for *S. cerevisiae* and *E. coli* respectively. In addition, for *E. coli* one additional dilution rate, 0.44 h\(^{-1}\), just around the rate where overflow metabolism is believed to set in,\(^{24}\) was also included and for yeast three dilution rates in between those chosen as low and high were included in the evaluation of influence of growth rate on level of heterogeneity.

**Physiology of cultivations for *S. cerevisiae* and *E. coli***

Yields on glucose in steady state yeast cultivation were calculated for cell mass, CO\(_2\), ethanol and acetate as well as the uptake and production rate of glucose and ethanol was shown in table 2. In Table 3, yields on glucose in steady state for *E. coli* cultivation for cell mass, CO\(_2\) and all metabolites produced in significant amounts, as well as the uptake rate of glucose were calculated. Carbon balances were calculated from the yield coefficients to test data consistency.

**Table 2. Yields (ratios of cell mass, CO\(_2\), ethanol and acetate produced on amount of consumed substrate) and carbon balance including standard deviation, as well as consumption respectively production rates for glucose and ethanol for aerobic glucose-limited continuous cultivation of *S. cerevisiae* FE440 performed with different dilution rates (D= 0.05/0.1/0.2/0.25/0.3 h\(^{-1}\)).** All data are presented as mean values including standard deviations and originate from two independent cultivations and, for each cultivation samples were taken over a minimum of three different residence times.

<table>
<thead>
<tr>
<th>Chemostat parameter</th>
<th>D=0.05 h(^{-1})</th>
<th>D=0.1 h(^{-1})</th>
<th>D=0.2 h(^{-1})</th>
<th>D=0.25 h(^{-1})</th>
<th>D=0.3 h(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Y_{sx}) [cmole/cmole]</td>
<td>0.58+/−0.02</td>
<td>0.56+/−0.06</td>
<td>0.60+/−0.01</td>
<td>0.35+/−0.02</td>
<td>0.22+/−0.01</td>
</tr>
<tr>
<td>(Y_{sc02}) [cmole/cmole]</td>
<td>0.53+/−0.02</td>
<td>0.40+/−0.04</td>
<td>0.38+/−0.01</td>
<td>0.35+/−0.01</td>
<td>0.27+/−0.01</td>
</tr>
<tr>
<td>(Y_{sen}) [cmole/cmole]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.22+/−0.01</td>
<td>0.42+/−0.01</td>
</tr>
<tr>
<td>(Y_{sace}) [cmole/cmole]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.01+/−0.00</td>
</tr>
<tr>
<td>(r_{G}) [g/g]</td>
<td>0.10+/−0.01</td>
<td>0.20+/−0.02</td>
<td>0.38+/−0.00</td>
<td>0.82+/−0.05</td>
<td>1.61+/−0.05</td>
</tr>
<tr>
<td>(r_{Ethanol}) [g/g]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.16+/−0.00</td>
<td>0.52+/−0.00</td>
</tr>
<tr>
<td>Carbon balance</td>
<td>1.11+/−0.05</td>
<td>0.96+/−0.10</td>
<td>0.98+/−0.01</td>
<td>0.94+/−0.03</td>
<td>0.92+/−0.01</td>
</tr>
</tbody>
</table>
For yeast, the dilution rates below $D=0.25\ h^{-1}$ all showed similar yield on biomass, $Y_{SX}$, around 55-60%, which is consistent with literature data for respiratory metabolism.\textsuperscript{25,26} Also, no accumulation of metabolites was detected, indicating an absence of overflow metabolism, which is in accordance with earlier observations.\textsuperscript{27} For the two higher dilution rates ($D=0.25\ h^{-1}$ and $0.3\ h^{-1}$), $Y_{SX}$ was significantly lower and was accompanied by accumulation of ethanol as well as small amounts of acetate for the highest dilution rate ($D=0.3\ h^{-1}$). This is in agreement with results found in another study where a critical dilution rate for onset of respiro-fermentative metabolism has been found to be $0.28\ h^{-1}$ in CEN.PK113-7D.\textsuperscript{27} The $Y_{SCO2}$ decreased with increasing dilution rate, whereas $r_S$ as well as $r_{\text{Ethanol}}$ increased, which also are in agreement with Postma \textit{et al.} (1989).\textsuperscript{27} In general, all yields showed low standard deviation and thereby confirmed steady state on the physiological level and high reproducibility between replicates. Carbon balances for all the cultivations closed with a maximum error of 11%.

For \textit{E. coli}, biomass yields on glucose were about 0.6 cmole/cmole for the low dilution rate and 0.65 cmole/cmole for the higher ones. These results correspond well with literature data on \textit{E. coli} K12 where an $Y_{SX}$ of about 0.6 cmole/cmole for lower dilution rates, which then increased with increasing dilution rate to reach a maximum of 0.69 cmole/cmole before tapering off at even higher rates.\textsuperscript{28} Since ethanol was present in the medium (added as a solvent for chloramphenicol) negative yields on ethanol was obtained when it was consumed. Similarly to \textit{S. cerevisiae}, at the low growth rate ($D=0.09\ h^{-1}$) \textit{E. coli} cultivations a strictly respiratory metabolism with no acetate formation was observed. Literature data suggests a shift to respiro-fermentative growth at a dilution rate above about $0.4\ h^{-1}$ with concomitant acetate production.\textsuperscript{24} However, this was not seen in the present study as only small amounts of acetate were observed for the higher growth rates, instead pyruvate accumulated. This observation is consistent with Qiang Hua \textit{et al} (2004)\textsuperscript{29} where no acetate excretion was found at detectable levels, conversely their biomass yields were found to be lower and CO$_2$ yields higher compared to our data. Lactate and formate were only seen in very low concentrations in the medium (data not shown). The low standard deviations for the calculated yields, in addition to that carbon balances closed with a maximum error of 5%, demonstrate high data consistency for the present study.
Table 3. Yield coefficients and carbon balance as well as glucose consumption rate at different dilution rates for aerobic glucose-limited chemostats of *E. coli* MG1655/pGS20PfisGFPAAV (+/-standard deviation). Data are presented as mean values and originate from samples taken over at least three different residence times. Each dilution rate was carried out as a biological duplicate (D=0.09 h$^{-1}$) or as a single measurement (D=0.44 h$^{-1}$ and D=0.53 h$^{-1}$).

<table>
<thead>
<tr>
<th>Chemostat parameter</th>
<th>$D=0.09$ h$^{-1}$</th>
<th>$D=0.44$ h$^{-1}$</th>
<th>$D=0.53$ h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y_{SX}$ [cmole/cmole]</td>
<td>0.59+/-0.02</td>
<td>0.65+/-0.01</td>
<td>0.65+/-0.01</td>
</tr>
<tr>
<td>$Y_{SCO2}$ [cmole/cmole]</td>
<td>0.38+/-0.009</td>
<td>0.31+/-0.002</td>
<td>0.30 +/-0.003</td>
</tr>
<tr>
<td>$Y_{SEth}$ [cmole/cmole]</td>
<td>-0.01+/-0.009</td>
<td>-0.03+/-0.02</td>
<td>-0.03+/-0.02</td>
</tr>
<tr>
<td>$Y_{SAce}$ [cmole/cmole]</td>
<td>-</td>
<td>0.02+/-0.04</td>
<td>0.017+/-0.004</td>
</tr>
<tr>
<td>$Y_{SFor}$ [cmole/cmole]</td>
<td>0.020+/-0.002</td>
<td>0.029+/-0.001</td>
<td>0.022+/-0.005</td>
</tr>
<tr>
<td>$Y_{spyr}$ [cmole/cmole]</td>
<td>0.012+/-0.004</td>
<td>0.063+/-0.009</td>
<td>0.083+/-0.009</td>
</tr>
<tr>
<td>$r_S$ [g/gh]</td>
<td>0.18+/-0.09</td>
<td>0.78+/-0.04</td>
<td>0.94+/-0.00</td>
</tr>
<tr>
<td><strong>Carbon balance</strong></td>
<td><strong>0.98+/-0.02</strong></td>
<td><strong>1.05+/-0.03</strong></td>
<td><strong>1.04+/-0.02</strong></td>
</tr>
</tbody>
</table>

For all cultivations a pulse of glucose was added after steady state had been reached. In order to investigate population heterogeneity in steady state and after glucose perturbation to *S. cerevisiae* and *E. coli* cultures, single cell analysis using flow cytometry was applied. The distribution of individual cell responses is presented in the following sections.

**GFP fluorescence distribution for *S. cerevisiae* cells at steady-state conditions**

GFP fluorescence versus forward scatter (FSC) distributions (figure 1 A and B) for the two dilution rates, D= 0.05 h$^{-1}$ and D= 0.3 h$^{-1}$, showed resemblance, but a slightly different subpopulation pattern was seen. For both dilution rates population fluorescence varied within an interval between 100-600 fluorescence channel numbers and the cells grouped into two subpopulations, one with high fluorescence and one with low. However, for the low dilution rate cultivation, the high fluorescence subpopulation was covering a broader fluorescence range (approx. 300-600 fluorescence channel numbers) compared to the high dilution rate cultivation (approx. 400-600 fluorescence channel numbers). The FSC, which usually correlates with particle size, was seen to be within a range of 800-1000 for the majority of cells.
at both dilution rates. However, at D=0.05 h\(^{-1}\) a minor subpopulation with smaller cell size, FSC=300-600, was observed.

Additionally to the above discussed characteristics of the GFP expression and distribution, quantitative and more descriptive parameters can be generated to facilitate comparison of flow cytometry data obtained for cells from different conditions. We have chosen to introduce coefficient of variation (CV) for mean GFP, peakwidth and the slope of the cumulative distribution curve as parameters describing population heterogeneity (table 4).

In general the fluorescence distribution for cells grown at a high dilution rate is more narrow and distinct, compared to the distribution for cells grown at a low dilution rate (figure 1E and F), which can also be confirmed by the peakwidth (table 4). Additionally, more noise in the distribution was seen for the low dilution rate. This indicates a higher degree of population heterogeneity for growth at low rates. Another way to look at the cell fluorescence distribution is to plot the cumulative distribution and by calculating its slope, which can be used to quantify heterogeneity (cdf-plot, figure 1G and H and table 4). The lower slope seen for D=0.05 h\(^{-1}\) (0.0072 +/- 2.24·10\(^{-4}\) compared to 0.0101+/- 5.20·10\(^{-4}\) for 0.3 h\(^{-1}\)) indicates a broader distribution for the low dilution rate. Mean GFP fluorescence was rather similar for the two dilution rates, 456.9 +/- 28.3 and 464.6 +/- 20.3 fluorescence channel numbers, for D=0.05 and 0.3 h\(^{-1}\) respectively, however, the slightly higher CV of the GFP distribution for the low dilution rate further confirms a higher level of heterogeneity at D=0.05 h\(^{-1}\). After normalizing mean GFP fluorescence with mean cell size, i.e. fluorescence per cell size unit, the value obtained for D= 0.05 h\(^{-1}\) (0.49) and D= 0.3 h\(^{-1}\) (0.50), is almost the same, which also proves to be the origin of the almost same mean GFP fluorescence as they have almost identical mean cell sizes.
Figure 1. Fluorescence and size distributions for fresh and frozen yeast cells at steady-state conditions: Biplots for FSC against GFP for fresh cells grown at $D=0.05$ h$^{-1}$ (A) and $D=0.3$ h$^{-1}$ (B) as well as for comparison between fresh and frozen cells for both dilution rates (C and D). Also GFP fluorescence represented as cell count against channel number fluorescence (E and F) and cdfplots of cumulative cell number against channel number fluorescence (G and H) for $D=0.05$ h$^{-1}$ and $D=0.3$ h$^{-1}$ for fresh and frozen $S.\textit{cerevisiae}$ FE440 cells in steady state in aerobic glucose-limited chemostat. The plots represent average values from samples taken after three different residence times.

Table 4. Heterogeneity at steady-state conditions for fresh yeast cells: quantitative values for measure of GFP fluorescence heterogeneity for $D=0.05$ h$^{-1}$ and $D=0.3$ h$^{-1}$ represented as Mean GFP with the belonging CV, the slope of cdf plots and peakwidth

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<td>$227+/-42.4$</td>
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Cell robustness to freeze-thaw stress for cells from steady state conditions

The *S. cerevisiae* reporter allows the identification of subpopulations with high and low cell membrane robustness to freeze-thaw stress as an inverse correlation of GFP signal and membrane integrity has been found. The underlying reason might be a reduction in fluorescence due to a shift towards lower intracellular pH due to leaking membranes and an extracellular pH around 5.5. We investigated this phenomenon by permeabilizing yeast cells with Amphotericin B and incubating in buffers with different pH before analysis on the flow cytometer. A linear decrease in fluorescence was observed as the external pH dropped below 7 (data not shown). To investigate how growth rate affects membrane integrity, samples from the five dilution rates $D=0.05/0.1/0.2/0.25/0.3$ h$^{-1}$ were frozen in 15% glycerol at -80°C before flow cytometry analysis and data compared to data from fresh cells.

Cells growing at a high and low dilution rate, respectively, are differently affected by freezing, as can be seen from the FSC-GFP biplots and fluorescence histograms for fresh and frozen cells in figure 1C to F. For $D=0.05$ h$^{-1}$ the shape of the distribution as well as the fluorescence range remained the same after freezing and only a slight increase in the lower fluorescence population could be seen. However, for $D=0.3$ h$^{-1}$ the fluorescence distribution got broader and the fluorescence was also considerably shifted towards lower values. As a result, the mean GFP fluorescence remained almost the same with freezing for $D=0.05$ h$^{-1}$ (409.0 +/- 14.3) but decreased for the high dilution rate to 295.4 +/- 6.9 (see also figure 2, indicated by red circles). This leads to the conclusion that cells growing with a low growth rate ($D=0.05$ h$^{-1}$) are more robust towards freeze-thaw stress.

To further investigate how growth rate influences freeze-thaw stress tolerance and the level of population heterogeneity; mean GFP, mean GFP normalized by cell size as well as CV of mean GFP, slope of the cdf plot and peakwidth were calculated for all 5 dilution rates and plotted in figure 2. A similar mean GFP fluorescence (figure 2A) was found for cells from all dilution rates, except for $D=0.05$ h$^{-1}$, where the mean fluorescence was higher. From FSC data it was seen that the cell size decreased with increase in growth rate until the dilution rate where respiro-fermentative metabolism sets in, and then sharply increased afterwards (data not shown). To remove the effect of cell size change on mean fluorescence, normalization with cell size was performed.
A steep increase in the normalized mean GFP was seen when going from 0.05 to 0.1 h\(^{-1}\). Thereafter, the value remained almost constant for growth rates up to the dilution rate where respiro-fermentative metabolism sets in (0.25 h\(^{-1}\)). Interestingly, above this growth rate, the normalized GFP fluorescence decreased sharply.

The CV of the GFP distribution, giving indication of the level of heterogeneity for the population, showed a similar trend as for the normalized mean GFP, though in the opposite matter, suggesting a higher level of heterogeneity for D=0.05 and D=0.3 h\(^{-1}\) than for the in between dilution rates. In general the GFP fluorescence distribution for cells grown at 0.1 h\(^{-1}\) and 0.2 h\(^{-1}\) was more narrow (peakwidth, figure 2B). This can also be seen in the cdfplot which has the highest slope for D= 0.1 h\(^{-1}\) and a bit lower value for 0.2 and 0.25 h\(^{-1}\) (figure 2B). For D= 0.05 h\(^{-1}\) and 0.3 h\(^{-1}\) the GFP fluorescence distribution is broader, resulting in bigger peakwidth compared to the other dilution rates.

Though the peak characteristics are different for D= 0.05 h\(^{-1}\) and 0.3 h\(^{-1}\), the slope of the cdfplot is similar for these two growth rates (figure 2B). Compared to fresh cells (figure 1E), the distribution for frozen cells grown at 0.05 h\(^{-1}\) was broader with a more distinct low fluorescent subpopulation, though keeping the same shape, resulting in a similar value of the slope of the cdfplot. For 0.3 h\(^{-1}\) the slope of the cdfplot for frozen cells was considerably lower as the distribution shape changed with the big tailing towards higher fluorescence and a division of the peak into two subpopulations (figure 1F).
Figure 2. The effect of growth rate on fluorescence and heterogeneity for yeast cells exposed to freezing: Mean GFP fluorescence distribution as well as GFP fluorescence normalized by cell size together with the belonging coefficient of variance (A) as well as the peakwidth and the slope of the cdfplot (B) for *S. cerevisiae* cells at steady-state conditions in aerobic glucose-limited chemostat for D= 0.05 h$^{-1}$, 0.1 h$^{-1}$, 0.2 h$^{-1}$, 0.25 h$^{-1}$ and D= 0.3 h$^{-1}$. The plots represent average values from samples taken after three different residence times. The blue line in both plots is indicating the dilution rate for which, as shown by the data, respiro-fermentative metabolism sets in.

**Physiological response of *S. cerevisiae* to glucose perturbation**

A glucose pulse of 1 g/L was introduced to the steady states at the different dilution rates and FSC, GFP fluorescence and HPLC data recorded over the duration of each perturbation.
Figure 3. **Time profiles of physiological response to** a 1 g/L glucose pulse in aerobic glucose-limited chemostats of *S. cerevisiae* growing with a dilution rate of 0.05 h\(^{-1}\) (top) and 0.3 h\(^{-1}\) (bottom). Glucose (g/L), CO\(_2\) (v/v %), ethanol (g/L) and acetate (g/L) concentrations are shown during the duration of the perturbation.

The lower dilution rates, 0.05 h\(^{-1}\) (figure 4) as well as 0.1 h\(^{-1}\) and 0.2 h\(^{-1}\) (data not shown) displayed a batch like behavior with overflow metabolism, concomitant ethanol and acetate accumulation, as also earlier seen by Visser et al. (2004),\(^3\) and a sharp increase in CO\(_2\) production when perturbed with a 1 g/L glucose pulse. In contrast, the respiro-fermentative chemostats (0.25 and 0.3 h\(^{-1}\)), growing above the critical dilution rate, consumed the extra glucose at a much slower rate, did not react with an increased ethanol or acetate production and only showed a very slight increase in CO\(_2\) generation.

**Influence of glucose perturbation on *S. cerevisiae* GFP fluorescence**

Figure 4 shows the responses of the D= 0.05 h\(^{-1}\) and 0.3 h\(^{-1}\) steady states to a 1 g/L glucose pulse with regards to GFP fluorescence. From the fluorescence histogram data for fresh cells (figure 4 top) it can be seen that the pulse caused a slight shift towards higher fluorescence for cells grown at 0.05 h\(^{-1}\), whereas cells grown at 0.3 h\(^{-1}\) where not influenced. This is also seen in the mean fluorescence, which remained
constant for the higher rate and increased for cells grown at the lower rate after around 30-40 min after the pulse (figure 5A fresh). CV and peakwidth (figure 5B and C fresh) indicate a somewhat higher heterogeneity for the low growth rate, however, no influence of the pulse was observed, as the values were constant over time. On the contrary, the slope of the cdfplot (figure 5D fresh) increased as a consequence of glucose perturbation for $D=0.3 \text{ h}^{-1}$ and decreased for $D=0.05 \text{ h}^{-1}$. The change in the slope of the cdf plot may be attributed to a decline in the low fluorescence subpopulation for cells grown at $D=0.3 \text{ h}^{-1}$ and an increase for cells grown at $D=0.05 \text{ h}^{-1}$ (see figure 4), whereas the main subpopulation remained constant. Coinciding with glucose depletion (compare with figure 3) the slope returned to the steady state value for both dilution rates.

Figure 4. Time profiles of GFP fluorescence distribution for *S. cerevisiae* fresh cells and cells exposed to freezing: GFP fluorescence represented as cell count against channelnumber fluorescence of *S. cerevisiae* FE440 before, during and after a 1 g/L glucose pulse in aerobic glucose-limited chemostats for $D= 0.05 \text{ h}^{-1}$ (left) and $D= 0.3 \text{ h}^{-1}$ (right) shown as comparison for fresh (top) and frozen cells (bottom). The plots each represent time points between -60 min to +150 min after the pulse.
Figure 5. Heterogeneity dynamics following a glucose perturbation for fresh and frozen yeast cells: Mean GFP fluorescence (A) as well as CV of GFP fluorescence (B), peakwidth (C) and slope of the cdfplots (D) plotted against time following a 1 g/L glucose pulse for S. cerevisiae FE440 in aerobic glucose-limited chemostats for D= 0.05 h⁻¹ and D= 0.3 h⁻¹ for fresh (red and blue) and frozen cells (magenta and cyan). The red (respectively magenta) and blue (respectively cyan) colored vertical lines indicate the time when the pulsed glucose is consumed for 0.05 h⁻¹ respectively 0.3 h⁻¹, whereas the black line is indicating the time of the glucose pulse. Dotted lines show values for replicate cultivation.

Influence on cell robustness by glucose perturbation of S. cerevisiae cultivations

Comparing fluorescence histograms for fresh and frozen cells for D=0.05 h⁻¹ and D=0.3 h⁻¹ a distinct difference could be seen for the two dilution rates. For cells grown at the low dilution rate, GFP fluorescence distributions for cells exposed to a glucose pulse and frozen before analysis, were similar to those of cells from steady-state conditions. Hence, glucose perturbation did not influence the membrane integrity of cells grown at 0.05 h⁻¹ (figure 4 and 5). On the contrary, the pulse significantly affected cells grown at 0.3 h⁻¹. The high fluorescence subpopulation
increased as a response to the pulse (figure 4), which also was reflected in the increased mean GFP value (figure 5A frozen). Even though the mean GFP still was more reduced compared to fresh cells for D=0.3 h⁻¹ than for D=0.05 h⁻¹, addition of glucose seemed to some extent protect fast growing cells towards freeze-thaw stress.

To further elucidate the effect of the pulse on subpopulation distribution, the relative fraction of the low and high fluorescence sub-population was calculated at the different time points (figure 6). The low fluorescence sub-population contributed with 80% and the high fluorescence sub-population with 20% during steady-state conditions for cells grown at 0.3 h⁻¹. When glucose was added, this changed to an approximately equal distribution between the two sub-populations. For cells grown at 0.05 h⁻¹, the sub-population distribution was unaffected by the pulse and stayed at being approx. 20% of the low fluorescence population and approx. 80% of the high fluorescence sub-population.

**Figure 6. Subpopulation distribution dynamics following a glucose perturbation of yeast cells frozen before analysis:** percentage for low (red) and high (blue) fluorescence subpopulation plotted against the time following a 1g/L glucose pulse for *S. cerevisiae* FE440 in aerobic glucose-limited chemostats for frozen cells grown with D= 0.3 h⁻¹ and D= 0.05 h⁻¹. The red and blue colored vertical lines are indicating the time when the pulsed glucose is consumed for 0.05 h⁻¹ and 0.3 h⁻¹, respectively, whereas the black line is indicating the time of the glucose pulse.

Comparing with a distinct and constant distribution of fresh cells, particularly for cells growing at a dilution rate above the dilution rate where respiro-fermentative
metabolism sets in, a much broader population distribution was seen for frozen cells, which got even more pronounced after the glucose pulse as seen from values for CV, peakwidth and slope of the cdf plot (figure 4 and 5).

**Effect of freeze-thaw stress on *E. coli* cells**

Due to the significant influence of freeze-thaw stress on yeast cells, the freezing effect was investigated in *E. coli* as well. Shake-flasks cultivations were performed and two samples withdrawn for each growth phase resulting in a total of 6 samples throughout the cultivation (sampling time points in relation to growth can be seen in figure 7A). Each sample was divided into three aliquots, one for direct analysis and the other two frozen, one flash-frozen in liquid nitrogen and one more slowly at -80°C. The GFP signal intensity of each sample was subsequently analyzed by flow cytometry.

The fresh cells behaved as expected with a short increase of the GFP signal in early exponential phase followed by a stepwise decrease (figure 7 A and B). After 12 h of growth when the cells had reached stationary phase, the GFP signal decreased further to values only slightly higher than the background level. Subpopulation in terms of GFP expression was not observed during the cultivation (data not shown). It was concluded that freeze-thaw stress only had a minor influence on the GFP intensity in *E. coli*, in contrast to what was observed for yeast. One probable reason being the higher pH used for the *E. coli* cultivations, which would negate the negative effect a low extracellular pH has on the fluorescence of cells with damaged cell membranes from the freeze-thaw cycle. The GFP synthesis time profile in the batch culture is in agreement with our previous findings that FIS is abundant in the very early log phase of rapidly growing cells, whereafter the promoter is switched off in late log phase and stationary phase. In contrast, the corresponding profile of the cdf slope (figure 7 C) showed that there was less influence on the fluorescence distribution for cells in the early and mid exponential phase than in the late exponential and the stationary phases. However, the lower slope may be a consequence of loss of GFP since the fluorescence had fallen back to background levels at this time point. The slope curve peaked in the mid exponential phase, which is in line with the highest growth rate. This can also be seen in the cdfplot that started with a low slope and end with a high slope (figure 7 C). Fluorescence distributions of cells that were frozen at -80°C before
analysis, resembled those of the fresh cells as seen from the mean GFP and cdf slope values obtained (figure 7 B, C). Hence, all data presented in the following sections were obtained from samples frozen at -80 °C.

**Figure 7. Effects of freeze-thaw stress on GFP signal distribution of bacterial cells.** From left to right: growth curve (A), mean GFP (B) and slope of the cdf plot (C). The decrease of GFP fluorescence is connected with increased cdf plot slope. The plots represent average values from two biological replicates.

**GFP fluorescence distribution of E. coli cells at steady-state conditions**

The *E. coli* reporter strain was subsequently cultivated in continuous mode to investigate the effect of growth rate on GFP expression and population heterogeneity. From the FSC-GFP biplots (figure 8) it can be seen that the population spanned over a large range of sizes, which shifted towards higher values with increasing growth rate. An increase of 100 and 200 channel numbers was seen for the dilution rates of 0.44 h⁻¹ and 0.53 h⁻¹, respectively. A similar population distribution was seen for the GFP fluorescence at the different dilution rates (figure 8 and 9). However, a slight shift towards lower fluorescence was observed for the higher growth rates. The mean fluorescence for cells grown at D=0.09 h⁻¹ was 446.4+/−10.8 fluorescence channel numbers, whereas cells grown at D=0.44 h⁻¹ and D=0.53 h⁻¹ had a mean fluorescence of 411.3+/−1.0 and 410.9+/−1.5, respectively (figure 9, table 5). The low GFP levels found in cells with relatively high FSC values, might be an effect of these cells undergoing binary fission and hence are diverting cellular resources towards this process. The slope of cdf plots, coefficient of variance (CV) of GFP and the width of the peak for the three dilution rates (figure 9, table 5), showed a somewhat broader population distribution and higher relative standard deviation for the low dilution rate,
indicating a higher degree of population heterogeneity at this growth rate. The similar peakwidth for cell grown at the higher growth rates of 0.44 h\(^{-1}\) and 0.53 h\(^{-1}\) is lower than D=0.09 h\(^{-1}\), indicating a narrower GFP peak in rapidly growing cells.

Figure 8. FSC against GFP biplots for *E. coli* aerobic glucose limited chemostats. From left to right: D=0.09 h\(^{-1}\), 0.44 h\(^{-1}\), 0.53 h\(^{-1}\).

Figure 9. Steady state GFP distributions for *E. coli* aerobic glucose limited chemostats. Cdf plot (A) and histogram (B) of D=0.09 h\(^{-1}\), 0.44 h\(^{-1}\), 0.53 h\(^{-1}\) were shown.
Table 5. Quantitative values for measurement of heterogeneity of GFP fluorescence represented as CV of the mean GFP signal, the peakwidth and the slope of the cdf plots including standard deviation at steady state for D=0.09, 0.44 and 0.53 h⁻¹.

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Physiological responses of *E. coli* cells to glucose perturbations

The responses of aerobic, glucose-limited *E. coli* cultures to sudden glucose excess was investigated by perturbing the steady-state system with 0.45 g/L glucose pulses. At time point zero, glucose was added and the dynamics of the extracellular metabolite and intracellular fluorescence levels followed over 3 hours. The glucose pulse was depleted in 25 min for D=0.09 h⁻¹, 35 min for D=0.44 h⁻¹ and 45 min for D=0.53 h⁻¹ (figure 10). No acetate formation was detected at 0.09 h⁻¹, which is consistent with earlier findings.²⁸ Acetate and pyruvate were already present in the cultivation broth before the perturbation for D=0.53 h⁻¹ due to the overflow metabolism.

However, for both the high dilution rates acetate concentration increased as a consequence of the pulse. The reduction of the acetate level was very slow at D= 0.53 h⁻¹, as the cells were already growing with overflow metabolism and unable to re-assimilate the produced acetate. Acetate in this case was probably just diluted out by the feeding rather than consumed by the cells. Formic acid levels were very low and did not seem to respond to the pulse (data not shown). The CO₂ evolution at D= 0.53 h⁻¹ and D=0.44 h⁻¹ increased slightly after the pulse and a glucose and acetate utilization phase, respectively, could be distinguished from this data.
Figure 10. Perturbation profiles of *E. coli* chemostats after a 0.45 g/L glucose pulse. Profiles for aerobic glucose-limited chemostats, D=0.09 h⁻¹ (A), 0.44 h⁻¹ (B), 0.53 h⁻¹ (C) (from left to right). Glucose (g/L, blue), acetate (g/L, red), pyruvate (g/L, pink), CO₂ (v/v%, black) and the mean GFP intensity (green).

**Influence of the glucose pulse on GFP distribution in *E. coli***

The pulse seemed to cause a slight shift towards higher fluorescence for all three growth rates, though it could be argued for D=0.09 h⁻¹ as the fluorescence was fluctuating quite a bit in steady state (figure 10). As the pulses abated, the fluorescence returned to lower levels again. No population segregation was found in either of the three dilution rates and the peak shapes remained constant during the pulses (data not shown).

A second distinct acetate re-assimilation phase was seen for D=0.44 h⁻¹, where the mean GFP followed the overall trend of the CO₂ evolution. Thus indicating that the reporter strain may have a fast enough response to capture both the glucose and acetate growth phases. Cells growing at the high dilution rate of 0.44 h⁻¹ and 0.53 h⁻¹, showed a more dynamic response to the glucose addition than the low dilution rate. This can be seen in the slight increase in peakwidth for the faster growing cells, whereas the peakwidth remained almost constant for the low growth condition (figure 11 B). No change in peakwidth or CV of the GFP for D=0.09 h⁻¹, could be seen after the pulse, indicating that the level of heterogeneity was not affected by the glucose addition. The constant high value of peak width at low dilution rate indicated a
broader distribution than the other two dilution rates during the pulse. The generally higher GFP intensity in the continuous culture (figure 10) compared to the batch (figure 7 B) is in agreement with our previous study. The underlying cause might be related to the effect of the metabolic state at exponential growth on the fis promoter or to the fact that environmental conditions change over time during a batch. In particular the decrease in pH and possibly oxygen limitation might affect the overall fluorescence.

Figure 11. Glucose pulse effects on heterogeneity measured by CV (A), peak width (B), and CDF plot slope (C) of E. coli chemostats at D= 0.09 h⁻¹ (green), 0.44 h⁻¹ (red), 0.53 h⁻¹ (pink).
Discussion

Cellular heterogeneity is present in all living things from simple bacteria, to eukaryotic and mammalian cells. To explore how cell-to-cell variability correlates with growth rate and glucose limitation, growth reporter strains of *S. cerevisiae* and *E. coli* were grown in steady state at different dilution rates and had their respective population distributions followed over time after the introduction of a glucose pulse.

As suggested earlier, destabilized GFPAAV with a shorter half-life was used in the *E. coli* reporter as the employment of stable GFP may have a ‘memory’ effect on the evolution of gene expression. The rapid turnover of the destabilized GFP facilitates detection of gene down regulations after brief exposures to environmental stimuli as it extinguishes the fluorescent signal when the promoter becomes inactive. Stable GFP on the other hand produces a much brighter signal as it accumulates in the cell, which is an advantage when measuring low expressed genes. In consideration of the different properties and response times in yeast and *E. coli*, we constructed a yeast reporter with a stable GFP integrated into a chromosome and an *E. coli* reporter with an unstable GFP expressed by a low copy number plasmid.

Quantification of heterogeneity

As unsynchronized microbial cultures are heterogeneous, averaged response values fail to describe the influence of different sub-populations. The currently most used method for single cell analysis is flow cytometry, which allows simultaneous quantification of thousands of individual cells per second. The collected data is generally illustrated as single parameter histograms or in plots where two parameters are correlated, such as biplots, scatterplots or contourplots. Here we present new tools to quantify population heterogeneity using a combination of growth reporter strains and mathematical methods. By using classical histograms and biplots (figure 1 as well as figure 4, 8 and 9) and by calculating mean GFP fluorescence, general traits of the GFP distribution can be observed. However, the selection of parameters and divisions of subpopulations are based on subjective interpretations, which points to the need for more objective standards for the quantification of population heterogeneity. To achieve this the shape of the distribution values have been enumerated using simple statistical and mathematical functions, which permits the
peakwidth and the CV to be used to describe population heterogeneity. The wider the peak the bigger the CV and the more heterogeneous the population. The appearance of sub-populations, shifts over time and differences in peak shape can be illustrated by using a cumulative distribution, which can be quantified via its slope. In conclusion, by using the newly developed parameters a cell population can be objectively described using conventional trend plots over time. These are also easier to comprehend than rows of distribution plots. The challenge, depending on the study, now lies in determining which or what combinations of parameter/s that can reveal relevant information from the torrent of data.

**Freezing effect**

For practical reasons, it is often convenient to freeze cells before analysis. However, the freeze-thawing procedure is not without consequence and the effects changes with cell type, growth phase, growth rate and medium composition. In addition, the cooling and thawing rate has a significant influence on the amount of damage inflicted. In general, faster growing cells are more susceptible to freeze-thaw stress, whereas slower growing cells tend to redistribute cellular resources towards stress tolerance functions. We have previously looked into freezing effects in yeast on fluorescence distributions and shape of cells by comparing cells kept on ice versus frozen cells. Whereas there was no observable effect on cell morphology, clear differences were seen for the GFP fluorescence including the formation of sub-populations at different fluorescence levels. The faster growing cells in the glucose phase of a batch were most susceptible, whereas the slower growing cells in the ethanol phase were far more resilient. The underlying cause of the decreasing fluorescence can be two-fold. The loss of GFP molecules through a damaged cell envelope or a decreased fluorescence is probably due to a pH drop caused by an equalization of the external and internal pH via membrane pores/holes. Fluorescent proteins, especially the green and yellow variants, are severely affected by acid shifts close to physiological pH. The latter being mostly an issue for the yeast cultivations, which were run at the lower pH of 5.5 compared to pH of 7.0 used for the *E. coli* cultivations. We have previously observed this phenomenon with both freeze-thawed yeast cells and yeast cells permeabilized with Amphotericin B, both displaying a linearly decreasing fluorescence as the external pH dropped below 7. In contrast,
fresh cells did not respond to the pH shifts. In the current study the influence of growth rate on freeze-thaw stress in yeast can be clearly seen in Fig. 2. The slower growing cells at D=0.05 h\(^{-1}\) were unaffected by the freezing procedure whereas the rapidly growing D=0.3 h\(^{-1}\) cells showed a clear drop in fluorescence. In contrast to yeast, we did not see any freeze-thaw effect on the GFP fluorescence in *E. coli* during the different phases of batch growth when comparing fresh cells to cells frozen in either liquid nitrogen or a -80ºC freezer. These results indicate pH being the dominant factor in this study.

**Influence of steady-state growth rate**

The ribosomal RPL22a promoter was initially selected as an indicator of the general growth rate for the construction of the yeast growth reporter strain. Also this growth reporter strain had previously been shown to be growth related in a batch study.\(^6\) In the current study, we did not observe an increase in GFP fluorescence with increasing dilution rate as expected (Fig. 2). Instead, the mean fluorescence level was fairly constant with the exception of an increase at the very lowest dilution rate (0.05 h\(^{-1}\)). However, after normalizing with FSC, which is correlated with cell size in yeast, a clear increasing trend was observed up until D=0.25 h\(^{-1}\), whereafter the fluorescence/FSC sharply declined. This would indicate that the “expression per cell volume” increased with growth rate in the respiratory phase. Also, as observed previously in the shift from respiro-fermentative growth on glucose to respirative growth on ethanol,\(^6\) the particular type of metabolism seems to have a strong influence on fluorescence as indicated by the sharp decline above 0.25 h\(^{-1}\), which marks the onset of respiro-fermentative growth.

In this study we employed the *fis* promoter as a reporter to visualize shifts in growth conditions of *E. coli*. The Fis protein, as an activator of ribosomal promoter, is responsive to nutrient availability and is believed to play a global role when *E. coli* cells adapt to changes in environmental conditions.\(^{38}\) Its rapid response to nutrient upshifts and downshifts, as well as the fact that its general molecular mechanisms are known,\(^{39-41}\) makes it an attractive option as a reporter for the study of transient responses in bacterial cultivation processes. The destabilized GFPAAV variant of GFP was chosen as it permits the observation of rapid downshifts, however, a plasmid based expression system was deemed necessary to achieve a sufficiently high GFP
fluorescence. Here, the steady-state GFP fluorescence was significantly higher at the low dilution rate (0.09 h\(^{-1}\)) (figure 10), which challenged the usefulness of the pFis-GFP strain as a pure growth rate reporter. One contributing factor might be that the GFP expression was plasmid based in contrast to the chromosomally integrated yeast reporter, as plasmid counts have previously been shown to decrease at high dilution rates in \textit{E. coli} chemostats.\textsuperscript{42}

**Pulse response comparison between yeast and \textit{E. coli}**

To follow the dynamics and responses of the reporters to transient changes from glucose limitation to glucose excess and back, 1 g/L (yeast) and 0.45 g/L (\textit{E. coli}) pulses of glucose were introduced to the continuous cultures. Fresh samples from the yeast cultivations showed a slightly shifting fluorescence intensity at the lower dilution rate (D=0.05 h\(^{-1}\)), but a constant intensity at the higher dilution rate (0.30 h\(^{-1}\)). This seems intuitive, as any shift in metabolism after a relief of glucose limitation must be greater for slower growing cells. The frozen cell samples from the low dilution rate cultivations showed comparable shifts to the fresh samples. However, in stark contrast to the fresh cells, the frozen samples from the high dilution rate cultivations displayed significant shifts in response to the pulses (Fig. 5 and 6). This behavior was mirrored by the analysis of the frozen \textit{E. coli} cells. At the low dilution rate the \textit{E. coli} GFP fluorescence barely shifted with the pulse, whereas as the dilution rate increased, the shifts became more pronounced (Fig. 11). Figure 4 shows how the yeast population distribution changed after the freeze-thaw cycle. At the low dilution rate there was a slight shift to lower fluorescence and a widening of the peak. In contrast, the high dilution rate population showed a dramatic peak widening and a shift to a lower fluorescence for a large part of the population. Interestingly, after the introduction of the pulse a larger part of the population maintained the fluorescence. Thus a second mechanism influencing the fluorescence must have been in action for the frozen cells. As discussed above, it is well established that faster growing cells are much more susceptible to freeze-thaw damage than slower growing ones. This can also be readily observed here by comparing the fluorescence levels of the frozen and fresh yeast cells (Fig. 5A fresh and frozen), where the frozen cell samples from the high dilution rate cultivations had significantly lower fluorescence than the fresh counterparts. However, why the pulse seems to protect some of the faster growing
cells from freeze-thaw damage is harder to explain. The only thing connected to the physiology of the cells that can react on the time-scales of the almost instantaneous changes observed is the intra-cellular metabolite levels. One could surmise that the fast growing cells, already primed for over-flow metabolism, quickly absorbed the glucose and perhaps even produced some storage carbohydrates. An intracellular shift in metabolite concentrations might very well affect the physics of the freezing process offering additional protection to the cell membrane. Our results indicate that while the growth rate did not contribute to the expression of our reporter genes in neither yeast nor E. coli, clear up-regulations after the introduction of the glucose pulses were observed. This indicates that the gene regulations of the promoters are primarily responding to environmental shifts rather than growth rate at steady state conditions.

**Potential with the reporter strains and future outlook**

High throughput single-cell gene expression studies are vital for elucidating the dynamics and contributions of heterogeneous cell populations. In general, the new tools presented here combines growth reporter strains and mathematical methods to explore sub-population dynamics in changing environments. It is difficult to select parameters for the description of fluorescence distribution data. However, changes that previously were only subjectively quantified by comparing classical histograms can be now be objectively quantified and analyzed using this newly developed method.

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**References**


5.4 Manuscript 4
Studying heterogeneity at single cell level as a way to develop robustness in batch cultures

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Abstract

Microbial cells in batch cultures have traditionally been considered to be uniform populations. However, bacterial heterogeneity occurs and it leads to reduced productivity as suggested in many studies. Our aim is to develop a simple toolbox of combined single cell techniques that will allow rapid assessment of cellular heterogeneity in batch cultures.

Time-resolved cellular heterogeneity profile of *Escherichia coli* strain MG1655 during batch growth with two carbon sources (glucose and acetate) was studied by the evolution of the destabilized GFP expression with different fluorescent activity stains targeting particular features, using flow cytometric methods (FCM). Obtained results suggested that the growth depended GFP expression is consistent with the observation of metabolic activity. The protein synthesis (GFP expression) was higher in the acetate growing culture than in the glucose growing culture, suggesting that cells were undergoing distinctive changes trying to cope with harsh conditions. The Redox Sensor Green (RSG) and Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC_4(3)) are more reliable as indicators of metabolic activity than other stains used in the study.

Cellular heterogeneity was evident in all the measured parameters, and the developed approach enabled a deeper study of heterogeneity in bacterial batch cultures. Also, the combination of fluorescent probes proved to be an accurate and fast alternative to the employment of biosensors. We would like to highlight the tremendous potential that flow cytometry has for fast and accurate monitoring of heterogeneity in bioreactors, and eventually showing us new pathways to enhanced robustness in bioprocesses.

**Keywords:** Flow cytometry, Biosensor, Heterogeneity, Robustness, Activity Stains
Introduction

The use of living cells for the making of biomass or product formation is the cornerstone of enormously profitable industries. As most cell cultures used in batch cultures are isogenic, these populations have traditionally being regarded as homogenous. However, studies found that local microenvironments induce dynamic genetic, metabolic and physiological cell responses and have been identified as key drivers for the development of cell heterogeneity in bioprocesses.\textsuperscript{1,2} Such an environmental gradient formation effect is unavoidable in large-scale bioreactors and is further magnified by reactor enlargement as a result of deficient mixing, which leads to zones with diverse environmental conditions.\textsuperscript{2} Understanding the dynamics of heterogeneity in bioprocess is therefore considered to be a valuable tool for bioprocess optimization since population heterogeneity has a significant impact on product yields, quality and cell viability.\textsuperscript{3} Batch culture is predominantly used in many industrial processes due to the simplicity, operability and less contamination of the operation. However, not much is known precisely about the influence of heterogeneity on the process performance.\textsuperscript{4}

The different bacterial cell physiological states arise when cells are grown in a closed batch-culture due to constant environment fluctuations,\textsuperscript{5,6} which indicates changes in gene expression and diverse physiological responses to environmental perturbations\textsuperscript{7} leading to a physiologically heterogeneous cell population,\textsuperscript{8,9} and consequently, to reduced production productivity as suggested in other studies.\textsuperscript{3}

Ordinary bioprocess monitoring often relies on population-based measurements of cell viability and yield production, which is unable to reflect the heterogeneity among individual cells within a population.\textsuperscript{10} Flow cytometry (FC), has been applied to discover and monitor cell-to-cell differences, cellular mechanisms and regulatory circuits at the single-cell level.\textsuperscript{7} With the application of flow cytometry to microorganisms since late 1970s,\textsuperscript{11} labeling fluorescently cells, e.g. by staining, has enabled the study of the physiologic and the activity state of bacteria with a higher resolution and these methodologies have been frequently applied to study heterogeneity in batch cultures.\textsuperscript{8,12}
The application of different fluorescent stains synchronically unveils the population heterogeneity and the functional differences between bacterial cells. The tetrazolium dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), is reduced by respiring cells to a water insoluble intracellular red fluorescent formazan and has been commonly used for monitoring the respiratory activity of cells. The still relatively unused stain, the redox sensor green (RSG) is a vitality stain that becomes fluorescent (green) when altered by bacterial reductases and as many of these are part of the electron transport system, this stain has also been employed as an indicator of the respiratory ability of bacteria. As a counterpart to activity measurements the propidium iodide (PI) dye is commonly used as a membrane integrity indicator. It is a nucleic acid stain that is also membrane impermeable and therefore commonly used to detect cells with disrupted membranes in bacterial populations. Cells that are not metabolic active and not dead, lack the ability to exchange ions through the membrane, that they have no membrane potential. The bis-(1,3-dibarbituric acid)-trimethine oxanol (DiBAC₄(3)) dye has the ability to enter depolarized cells and while retained inside turns green.

FC also permits to collect information about potential heterogeneities within bioreactors using the microbial population itself. The green fluorescent protein gene (gfp) is one of the most well-known and used reporter genes. GFP is a small bioluminescent protein that is used as promoter activity reporter in many bacteria and yeasts cells, not interfering with cell growth. The high degree of stability of the wild type GFP (the fluorescent signal can last for over 24 h might pose a problem in some experiments where transient gene expression is concerned. Non-active, non-viable or dormant bacterial cells containing stable GFP would continue to fluoresce or still express the fluorescent signal in the absence of promoter gene expression and the fluorescence would extend over many bacterial generations. Nevertheless, the addition of an AAV-tag to the C-terminal-tail of stable GFP reduces the half-life of GFPmut3.1 from 24 h to 60 min, forming a destabilized form of GFP. In the present study growth reporter was created by fusing destabilized gfp variant (AVV) to the Escherichia coli ribosomal promoter rrnB P1P2. The best-studied rrnB P1P2 promoter when inserted in the plasmid is known to be growth rate regulated and responsive to nutrient starvation and inversely reacts to rRNA gene dosage.
The physiological adaptation of microorganisms to changing environments in batch cultivation is quite distinctive as the degree of heterogeneity in a bioreactor is also dependent on the growth stage of the cells in a batch process. Metabolic versatile E. coli cells can adapt quickly to the available carbon source such as glucose or acetate, but in the presence of both, cells will preferably consume glucose over acetate. Studies have also shown that the stress response is coupled with cell growth phase in E. coli, therefore, in this present study we combined the use of CTC, RSG, DiBAC₄ and PI and FC to investigate heterogeneity in the growth phase of an E. coli strain with the destabilized GFP reporter system driven by ribosomal promoter rrnB P1P2 growing in a batch bioreactor with either glucose or acetate as carbon source. The state of the individual cells is then reflected in the expression of the reporter protein and fluorescent probes employed and can be followed by flow cytometry. We hypothesize that this combined approach will reflect the tradeoff between growth and survival along the growth curve. This study aims to cast some light into the intricate relations of single cells and its immediate surrounding environment in a real time basis in bioreactors, which can lead to better monitoring of heterogeneity and ultimately became a useful tool in attaining better optimization and robustness of industrial batch cultivations.

Methods

Strains

A reporter strain based on the expression of destabilized GFP under the control of rrnB P1P2 promoter, namely, MG1655/pGS20PrtnBGFPAAV, was constructed earlier to illustrate the growth dynamic of a bacterial population in batch cultures. Likewise, the recombinant strain MG1655 bearing pGS20PrrnB was used as the control strain as it did not express GFP. The low copy plasmid pGS20 served as the vector backbone.

Staining procedure and validation

Five activity stains were used in this study: 5-cyano-2,3-ditolyltetrazolium chloride (CTC) (Sigma-Aldrich, Germany), SYBR Green (SYBR) (Invitrogen, USA), Propidium Iodide (PI) (Sigma-Aldrich, Germany), Bis-(1,3-dibutylbarbituric acid)
trimethine oxonol (DiBAC$_4$(3)) (Molecular probes, USA) and Redox Sensor Green (RSG) (Invitrogen, USA). For each staining procedure, 1 µL of stain was added to 100 µL of sample suspended in 0.9% of NaCl saline solution. The final stain’s concentrations were 1x for SYBR, 0.05 mg/mL for PI, 0.5 mM for CTC and 0.001 mM for both DiBAC$_4$(3) and RSG. The CTC staining was incubated during 1 hour at 37ºC. The SYBR staining was incubated during 20 min and DiBAC$_4$(3), PI and RSG for 10 min at room temperature. The stains were protected from the light during all the procedure.

With the aim to find if staining results vary during sample handling, a simple validation procedure was done. The validation of the staining procedure allowed the optimization of the samples’ handling prior to staining and subsequent flow cytometric analysis. The biosensor MG1655/pGS20PrrnBGFPAAV was inoculated in shake flasks using 100 mL of a defined minimal media with two different carbon sources, glucose (5 g/L) or acetate (2 g/L). The cultures were grown at 37ºC with a continuous stirring of 140 rpm. Samples were taken every hour until the cultures reached the stationary phase for both carbon sources, although the acetate culture sampling started only after 12 h of incubation (after the start of the lag phase). The optical density of the cultures was measured at each time point and the staining procedure was applied to samples immediately, after 30 and 60 min of ice incubation and after 30 and 60 min of room temperature incubation. Flow cytometric methods (FCM) were used to detect the growth dependent GFP expression of the biosensor and the different fluorescent activity stains.

Two-way analysis of variances (ANOVA) was applied to the obtained mean fluorescence signals, using the GraphPad Prism 5 (GraphPad, Inc, USA). A significance level of 0.05 was employed. Thus, adequate incubation times and temperatures were chosen when no significant differences (p>0.05) were observed between the results obtained immediately after sampling and after the applied incubation conditions. As so, samples for staining with RSG, SYBR green, and PI and) should be kept on ice until flow cytometric measurements. The samples to be stained with DiBAC$_4$(3) were placed at room temperature and after the tenth hour of growth in batch, on ice. The samples to be stained with CTC were kept at room temperature. Proper dilution increases staining efficiency and accuracy, so based on
calibration curves relating the $\text{OD}_{600}$ and number of events recorded in the flow cytometer, samples with an $\text{OD}_{600}$ above 0.2 should be diluted 10 times before staining and further analyzed in the flow cytometer.

**Flow cytometry**

All bacterial cells were analyzed using a FACSria™ III (Becton Dickinson, USA) flow cytometer. The system includes two lasers with minimum laser power of 10 mW with the specific wavelengths of 488 nm and 561 nm. Two scattering channels (FSC and SSC) and two fluorescent detection channels (530/30 nm and 610/20 nm) were used in the analysis. The amplification voltages on the detectors were set based on negative and positive controls (stained, non-stained, viable and non-viable bacterial populations) in a way to better visualize the populations and were as followed: FSC–301V; SSC –316 V; 530/30 nm –520 V and 610/20 –582 V. The detection thresholds were set in FSC and SSC in order to eliminate background noise.

The flow cytometer (FC) has specific software used in combination with CS&T beads (Cytometer Setup and Tracking beads) (Becton Dickinson, USA) for the automated QA/QC of the machine performance. All results obtained with the FCM were saved as FCS files and analyzed by FlowJo software (Tree star Inc., USA).

**Preculture preparation**

The inoculums used to start the batch culture were obtained by a two-steps preculturing. In the first step, a 100 mL overnight culture of the biosensor MG1655/pGS20PrrnBGFPAAV was obtained, in LB medium, from a fresh culture plate. In the second step, a 10-fold dilution series was made in LB medium from the overnight culture and grown at 37ºC for 6 to 8 h on an orbital shaker (160 rpm). The $\text{OD}_{600}$ was measure for all the shake flasks and the one with $0.4< \text{OD}_{600}< 0.6$ was picked to inoculate the bioreactors. The medium used in the pre-cultures was supplemented with 25 µg/mL of chloramphenicol (dissolved in 96% ethanol, stock concentration: 10 mg/ml). Similar procedure was used for preparing the inoculum of the control strain.

**Batch cultivation**
Batch cultivations of the biosensor MG1655/pGS20PrrnBGFPAAV strain were grown with 5 g/L glucose or 2 g/L acetate as carbon source. Also the control strain MG1655/pGS20PrrnB was grown in parallel with 5 g/L glucose as carbon source. The growth medium used was a defined mineral medium containing 2 g/L Na₂SO₄, 2.468 g/L (NH₄)₂SO₄, 0.5 g/L NH₄Cl, 14.6 g/L K₂HPO₄, 3.6 g/L NaH₂PO₄*H₂O, 1 g/L (NH₄)₂-H-Citrate, 50 µL Sigma 204 Antifoam and 0.1 g/L thiamin. After autoclaving, pre-filtrated (0.2 µm) 3 mL MgSO₄, 1 mL trace metal solution and 25 µg/ml chloramphenicol were added into the sterilized medium. The trace metal solution contained 0.5 g/L CaCl₂*H₂O, 0.18 g/L ZnSO₄*7H₂O, 0.1 g/L MnSO₄*H₂O, 20.1 g/L Na₂EDTA, 16.7 g/L FeCl₃*6H₂O, 0.16 g/L CuSO₄*5H₂O and 0.18 g/L CoCl₂*6H₂O. All cultivations were performed using 1-liter bioreactors (Sartorius, B. Braun Biotech International, GmbH, Melsungen, Germany) with a working volume of 1 L. The pH and DOT electrodes (Mettler Toledo, OH, USA) were calibrated according to standard procedures provided by the manufacturer using a two-point calibration (pH 4 and 7 respectively gassing with Oxygen (100%) and with Nitrogen (0%)).

The inoculum (0.4<OD₆₀₀<0.6) from pre-cultivation was used to inoculate the growth medium to a final OD₆₀₀ of 0.005 for both carbon sources. The pH was adjusted and afterwards controlled to pH 7 using 2M NaOH and 2M HCl. Temperature, aeration and stirring were kept constant at 37°C, 1v/v/m and 1000 rpm respectively to avoid oxygen limitation.

Samples for OD₆₀₀ measurements, high performance liquid chromatography (HPLC), dry weight (DW) and flow cytometry analysis were withdrawn periodically with intervals between 1.5 and 2 h. Samples for OD₆₀₀ and DW were analyzed directly, whereas filtrate samples for HPLC were kept at -20 °C until measurement. Samples for flow cytometry were kept on ice or at room temperature until analysis (please see “Staining procedure and validation”).

OD, DW and HPLC

Growth was monitored at OD₆₀₀ with a Shimadzu UV mini 1240 spectrophotometer (Shimidzu, Kyoto, Japan). For dry weight measurements 5 ml of the cultivation broth was filtered, washed and dried for 20 min at 150W in a microwave, cooled down in
desiccators and afterwards weighted on an analytical balance. The concentration of glucose, lactose, acetate, ethanol, glycerol and pyruvate was determined by HPLC (Agilent 1100, Agilent Technologies, CA, USA) with a 300 mm × 7.8 mm Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, CA, USA), refractive index detector (RID Agilent 1200, Agilent Technologies, CA, USA) and UV detector (Agilent 1100, Agilent Technologies, CA, USA) set to 210 nm. The mobile phase was 5 mM H$_2$SO$_4$ (aq.), temperature 60°C and flow rate 0.6 mL/min. A MS gas analyzer with multiplexer (Thermo Prima SigmaV) was used to monitor the composition of the outgoing gas from the reactors.

**Results**

In order to study the onset of heterogeneity in axenic batch cultures, a reporter strain (biosensor) based on the expression of destabilized GFP (AAV) under control of the *rrnB* P1P2 promoter was employed in this study. The use of destabilized gfp gene allows a close follow up monitoring of gene expression by flow cytometry at either population level or individual cell level. A homologue strain not carrying the gfp gene was also constructed as a control strain, in order to assess the biological burden of producing a GFP signal. Both strains were grown in bioreactors with glucose as carbon source and the biosensor reliability was further tested with acetate as a carbon source. The bacterial activity trade-off between cellular growth and survival was examined in both strains by application of various stains targeting the basic metabolic needs of cells.

**Cellular growth**

The following parameters: glycerol, acetate and glucose, consumed during growth were measured by HPLC. Also cell mass was measured so that the final carbon balance of the batch cultivations could be calculated in order to test data consistency. The data originated from three different samples, one per reactor (n=3), of which the mean was estimated as well as the standard error. The final summary of yields including standard error showed that the cultivations where glucose was used as carbon source were in balance as shown in table 1. The growth rate and biomass yield of the reporter strain did not differ from the control strain with no GFP expression, indicating that GFP expression was not a burden to the cell if using these parameters.
The calculated carbon balance based on the average value of biomass and main produced metabolite concentration was around 1, providing confidence in the accuracy of data as the carbon balance is nearly closed. The data obtained from growth of the biosensor in bioreactors with acetate as carbon source is shown in figure 3. The collected data from all batches performed (n=3) presented a high degree of heterogeneity and for exemplification purposes a set of the most representative data was chosen to be depicted here. When calculating the carbon balance for the reporter strain growing in acetate, around 60-70% of the carbon is missing (data not shown).

Table 1. Yields and carbon balance in the batch cultivation with the respective standard error for biosensor and the control strain with glucose as carbon source (n=3). YSX, YSG, YSAC and YSC are respectively the yields of biomass, glycerol, acetate and carbon dioxide produced.

<table>
<thead>
<tr>
<th>Yield</th>
<th>Control</th>
<th>Reporter</th>
</tr>
</thead>
<tbody>
<tr>
<td>YSX</td>
<td>0.654575±0.01867</td>
<td>0.695433±0.07329</td>
</tr>
<tr>
<td>YSG</td>
<td>0.000457±0.00003</td>
<td>0.000490±0.00006</td>
</tr>
<tr>
<td>YSAC</td>
<td>0.313612±0.02335</td>
<td>0.340141±0.01975</td>
</tr>
<tr>
<td>YSC</td>
<td>0.082129±0.01133</td>
<td>0.083616±0.01701</td>
</tr>
<tr>
<td>Carbon Balance</td>
<td>1.050773±0.00363</td>
<td>1.119679±0.03738</td>
</tr>
</tbody>
</table>

Growth studies in glucose

The control strain showed a typical growth behavior as demonstrated by the measurements of the concentrations of the substrates and OD (fig. 1A). The glucose was depleted at t=15 h and the acetate switch occurred at 11.5 h of growth. The CO₂ consumption increased along the exponential phase and a peak was registered, as expected, just as the strain reached their maximum growth rate (fig. 1A). The metabolic activity demonstrated by the different staining procedures employed showed an increase of nucleic acids in cells (SYBR green), of respiratory activity (RSG and CTC) and of the number of cells with a compromised cell membrane (PI) immediately before and during the beginning of the exponential phase (fig. 1B). The staining with DiBAC₄(3) shows that the number of cells with a depolarized cell
membrane increases during the exponential phase and reached a peak at the beginning of the stationary phase. In the present study, a peak of the respiratory activity was observed at the beginning of the exponential phase after which it decreases for the rest of the cultivation period. The measured parameters and data obtained from the activity stains showed larger standard error during the exponential phase as can be observed on figure 1, indicating a high degree of cellular heterogeneity. All the activity indicators depicted slower growth in the late exponential and stationary phases.

Figure 1. Batch cultivation and metabolic activity data along time for the strain MG1655/pGS20PrrnB (control strain) growing with glucose as carbon source (n=3). The OD<sub>600</sub>, metabolites concentration (g/L) and CO₂ % is depicted in (A) the intensities of CTC, RSG, and SYBR green and the percentage of PI and DiBAC4(3) positives cells in (B). Error bars represent standard deviation.

Comparably, the biosensor strain exhibited expected growth behavior in defined mineral media with glucose (fig. 2A). Growth was first supported by glucose, which was exhausted after 10 h and then the acetate switch occurred around the same time.
Stationary phase was reached approximately after 12-14 h. The control strain has a longer lag phase but presents a faster average growth rate ($\mu_{\text{max}} = 0.72 \text{ h}^{-1}$) if compared with the biosensor strain ($\mu_{\text{max}} = 0.70 \text{ h}^{-1}$). The earlier appearance of acetate switch in the control strain (11.5 h) is a reflection of the faster glucose consumption rate and slightly higher growth rate in control strain than the biosensor strain. The CO$_2$ production increased along with the exponential phase and a peak was registered in end of the exponential growth phase (fig. 2A). The peaks of CO$_2$ production occurred in different stages of the growth curve for both strains. Likewise, the metabolic activity data registered for PI, RSG and SYBR green also differed in the location of their peaks analogously with the respective growth curve.

**Figure 2.** Batch cultivation and metabolic activity data along time for the strain MG1655/pGS20PrnBGFPAAV (biosensor strain) growing with glucose as carbon source ($n=3$). The OD$_{600}$, metabolites concentration (g/L) and CO$_2$% is depicted in (A) the intensities of CTC, RSG, and SYBR green and the percentage of PI and DiBAC4(3) positives cells in (B). Error bars represent standard deviation.
The fraction of PI and CTC positive cells was immediately increased before and at the very beginning of the exponential phase. Whereas data obtained from the SYBR green and DiBAC₄(3) showed a peak during the beginning of the exponential phase and a subsequent peak at the onset of the stationary phase, together with the RSG (fig. 2B).

By comparing both strains’ growth in glucose (fig. 1 and 2) and the location of the stain peaks in relation with the growth curve, it was noticed that the biosensor strain had, to a degree, delayed physiological responses when compared with the non-expressing GFP strain. The observed GFP signal obtained via the biosensor correlates well with the exponential phase, indicating that the strain is growing more actively in that phase.

**Growth studies in acetate**

![Figure 3. Batch cultivation and metabolic activity data along time for the strain MG1655/pGS20PrrnBGFPAAV (biosensor strain) growing with acetate as](image-url)
carbon source (n=1). The OD600, metabolites concentration (g/L) and CO2 % is depicted in (A) the intensities of CTC, RSG, and SYBR green and the percentage of PI and DiBAC4(3) positives cells in (B).

Comparatively with having glucose as a carbon source, the biosensor strain when growing in acetate has a longer lag phase, a minor average growth rate ($\mu_{\text{max}} = 0.20 \ h^{-1}$), a lower biomass production (OD values) and the CO2 production is very low all the time with a little peak at the beginning of the exponential phase (fig. 3A). The metabolic activity data retrieved from the staining (fig. 3B) shows that the strain is more active during the very beginning of the exponential phase, as the SYBR green values are higher at that point and the PI, DiBAC4(3) and RSG values peak at the same time. The PI and the DiBAC4(3) have each two coincidental peaks: on the early and on the later exponential phase, after which the percentage of positive bacteria for both stains diminishes. The respiratory activity highlighted by the CTC staining appears to be relatively constant throughout the whole growth. The sub-standard growth conditions of the strain in acetate leads to an increasing number of bacteria, which are unable to cope with these conditions and become not viable or non-growing (dormant).

Figure 4. GFP evolution profile of the strain MG1655/pGS20PrrnPrrnBGFPAAV (biosensor strain) in two carbon sources of glucose and acetate (n=3).

It is known that bacterial populations under stress became asynchronous and contains organisms at all stages of the division cycle33, leading to a higher level of heterogeneity. The onset of the GFP shows that the strain is actively synthetizing
ribosomal RNA during the whole exponential phase as the signal is slightly higher at that point and the signal was approximately 2 times more intense in the acetate medium (fig.4) during the exponential phase indicating a higher production of ribosomal RNA.

**Discussion**

Our data corroborates earlier studies where heterogeneity in axenic cultures growing in batch is much higher than generally supposed which has significant repercussions for the microbial process analysis (quantitatively and qualitatively). The microbes that were actively metabolizing were detected by flow cytometry-based redox sensing (FCRS) methods, specifically; two redox probes were employed in this study, the CTC and the RSG. In glucose, the control strain appeared to accumulate nucleic acids (SYBR green data) and respire more actively (RSG and CTC data) immediately before the exponential growth phase, showing the cells preparing in high gear for growth. The same parameters for the biosensor strain, with exception of CTC, seem to peak later during growth. Discrepancies were found between the results obtained with these two stains; the CTC curve is more even out and the signal is less bright when compared with the RSG results. CTC has frequently been used to define the amount of “active” of bacteria in diverse samples, however, there has never been demonstrated a direct correlation between the amount of cells that are actively respiring, growing and dividing and the amount of cells that reduced enough dye to be detected. These facts and the nonexistence of a unified protocol for the application of CTC created a consensus among several researchers that this method lacks great sensibility, it only detects the minimum number of viable cells and obtained results reported in different published studies are hard to compare. RSG, on the other hand, is altered by the active reductases in the cells and as these are not exclusively present in the electron transport system, we conclude based on these facts and on our results, that not only is RSG a more reliable but as well is a more sensitive viability and vitality indicator than the CTC.

In contrast to metabolic activity, bacteria with compromised or depolarized membrane are respectively regarded as non-viable or dormant as negatively charged DiBAC₄(3) cannot penetrate into active cells due to their interior negative charge. In order to access membrane status, PI and DiBAC₄(3) were employed in turn to target
compromised and depolarized membranes. It was noticed that the percentage of PI positive cells increases slightly along the growth curve, reaching a peak for the control strain at the beginning of the exponential phase, coinciding with when the cells are accumulating more nucleic acids (SYGR green data). These observations appear to corroborate the existence of false positives as PI intercalates with any double stranded nucleic acids (DNA and dsRNA). The percentage of DiBAC4(3) positive cells, after an initial stabilization of the culture, are similar in both strains and because the shape of the curves are similar in both cases, we agree that the DiBAC₄(3) is more suitable as a viable indicator than PI.

The physiological adaptation of E. coli growing on acetate in batch cultivation is quite distinctive. Growth is inhibited even at very low concentrations, e.g. 0.5 g/l, and this inhibition becomes very significant in a defined medium. The cellular growth in the acetate batch culture revealed that most of the carbon was missing (data not shown). By looking at the histograms of the substrate and metabolites obtained by HPLC, an unidentified peak was found, which could be oxaloacetate. A possible reason is that the over produced oxaloacetate through the bypass of isocitrate dehydrogenase in acetate grown cultures is central to successful adaption and growth on acetate. Apart from that, the yields of YSX and YSC (biomass and carbon dioxide, respectively) produced and the lower growth rate are found consistent with data retrieved from E. coli growing on acetate reported previously.

The onset of the GFP signal is coincidental with the exponential phase in both carbon sources and the SYBR green data reveals that in both carbon sources the cells increase the amount of nucleic acids in the beginning of that phase, indicating that cells are preparing to divide. Homologically, the decrease of SYBR intensity is related to lower nucleic acid concentration, since the DNA and RNA concentration on a single cell level basis is lowered after cell division. The measured GFP intensity corresponding to the biosensor on switch indicates that the cells growing in glucose were actively synthesizing ribosomal RNA during the beginning of the exponential phase and that the fraction of active cells was kept at the same level until the end of the exponential phase where the GFP signal was switched off. However when growing on acetate, the GFP expression data shows that the cells are more actively producing proteins even though the growth rate is substantial lower than when
growing in glucose. It is known that although starving bacteria have resistance to autolysis, there are mechanisms that overcome this resistance and it has been observed that bacteria are able to disassemble and mark dying cells as response to adverse environmental conditions. As so, the number of inactive cells diminishing at the beginning of the exponential phase for the cultures growing in acetate, lead us to speculate that the harsh conditions select not only for bacteria that grows on acetate but as well for bacteria that are scavenging of cytoplasmic proteins released through autolysis or leakage of not viable or non-growing bacteria, as these ready-made products are more attractive than the acetate. The same thought path can be applied when the cells are growing in glucose to explain why the percentage of PI positive cells decreases in the end of the exponential phase as this decrease coincides with the drop of the carbon source concentration.

Even if the overall metabolic activity of the strain in all measured parameters conveys the impression of lower growth in acetate, the GFP signal has a higher intensity growing in acetate than when the strain is grown in glucose (fig. 4). This fact is contradictory to the general accepted believe that fast growing cells have a higher number of ribosomal operons resulting in higher synthesis rate of rRNA than cells growing at a slower growth rate. Potentially because the strain’s machinery is working harder to cope with the harsh conditions in acetate, growing cells in acetate influences the growth but it enhances bacterial activity, as reflected in more ribosomal activity (fig. 4) and the RSG data (fig. 3). Further investigations are required to fully understand the mechanisms involved in these phenomena.

The physiological state of microorganisms can be affected by a number of environmental factors and consequently tampering with the robustness of bioprocesses. An understanding of the state of the individual cells is thus critical in achieving high process efficiency. Traditional cultivation data collecting reflects the general growth ability of the strains but do not illustrate single cell differences. The developed tool box for this study, using rapid fluorescent staining procedures combined with FCM provided quick information on cellular physiological status changes by crosslinking the data obtained by the measurements of standard growth parameters, the biosensor GFP expression and the metabolic and physiologic information. This methodology enabled a fast distinction of functionality and
metabolic status of cells in the batch cultures during growth. Although this approach was comprehensive, we realized that the single use of the combination of RSG and DiBAC4(3) stains would already reveal more about population heterogeneity than the traditional methods used in the industry to monitor batch cultures. With the given carbon sources, both strains seem to have a period immediately before or after the beginning of the exponential phase where the cells are the most active as if preparing for the growth boom recorded on the exponential phase. This is true for all tested scenarios, except for the biosensor strain in glucose, where this reaction is somewhat delayed when comparing with the growth curve. Leading to the conclusion that biosensor strain pays an energy cost of expressing a gene translated into an alteration of its regular metabolism and physiology, leading to a paradox: The reporter strain is used to monitor growth physiology but at the same time it changes growth physiology.

It is clear that the tested strains prefer glucose as a carbon source to acetate, and that enhanced stress increases the heterogeneity of the population as cells adopt different strategies, exchanging continuously between growth and survival mode, presenting different metabolic and physiological status in each phase of the growth curve.

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References

and modeling techniques for description of cell population heterogeneity. 


Chapter 6

Future respective
*Escherichia coli* is a versatile bacterium that experiences transitions between aerobic and anaerobic metabolism and between nutrient excess and stress as part of its lifestyle. Therefore, it is an ideal system for exploring this capability. Glucose is the most preferred carbon source for bacteria and accordingly changes in glucose availability often have profound consequences in many types of cell gene expression and it ensures an immediate response in central metabolism. The advantage of using flow cytometry analysis is that it efficiently collects data from loads of cells without any averaging of the signal intensity, therefore, this method was employed throughout this thesis to quantify microbial heterogeneity. The main results were obtained in glucose perturbation experiments followed by the evolution of GFP in high-density cultivation and in chemostat, by physiological correlation between staining parameters and cell growth status. As so, averaged cellular response is re-evaluated by taking the microbial heterogeneity into consideration on single cell level, which enables an accurate behavior assessment of the cells in the population and crucial for the bioprocess control.

Microbial cells have developed mechanisms to respond to environmental changes in a rapid and effective manner, including the rapid reprogramming on the transcription and translation level. Later on the system could be pertubated in an alternative way, e.g. using a different substrate such as acetate, which will yield different responses and therefore gives more important information to capture the distinct stimuli response. On the other hand, the expression of the reporter gene could be examined by RNA-qPCR on the promoter to verify that the GFP level is correlated to the expression or see if there are other things that influence the GFP expression. Further, due to the fact that the plasmid copy number evolves with time and depends on growth phase and growth rate, the variation of copy number might have some effect on the bacterial physiology once the balanced growth is disturbed.

In addition, oxygen limitation is quite often linked with substrate limitation and thus has profound effects on *E. coli* physiology. Similar experimental concept can be expanded from glucose to the study of oxygen availability. Perturbation of anaerobic steady-state cultures by providing a limited supply of O$_2$ or the aerobic steady state with perturbation of differed O$_2$ supply might also be interesting. Another, since lower temperature in industrial process is usually used to prevent protein aggregation and maintain the balance between oxygen solubility and growth rate, the heat shock response of cell is thus worth to study during temperature up or down shift.
Since a bulk of cells were analyzed simultaneously with flow cytometer, distributions of individual cell behavior within the population (population heterogeneity) can be quantified via the introduced new parameters (manuscript 3) on all the flow data obtained from glucose oscillation process.