# Cellular Heterogeneity: When differences matter.

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## Abstract

A major challenge for cells lies in their ability to detect, respond and adapt to changing environments that may threaten their survival. Among the numerous evolutionary strategies, cell-to-cell heterogeneity allows the emergence of different phenotypes within a population. This variability in cellular behaviors can be essential for a small fraction of cells to adapt and survive in various environments. Analyses at the single-cell level have allowed to highlight the great variability that is present between cells within an isogenic population. Numerous molecular mechanisms have been uncovered, allowing to understand the emergence and the role of cellular heterogeneity. These attempts at identifying the source of cellular noise have also provided clues for strategies needed to control heterogeneity. In this review, the different factors leading to cell heterogeneity are presented, ranging from intracellular processes to environmental constraints. In addition, some recent strategies developed to modulate cell-to-cell variability are discussed.

## Keywords

Single cell analysis; Intrinsic and extrinsic noise; Bet-hedging; Feedback control; Budding yeast *S. cerevisiae*

## Highlights

- Single-cell analyses provide insights into the variability between cells.
- Isogenic cells can differ due to intracellular and/or extracellular factors.
- Bet-hedging strategies can provide some explanation for phenotypic diversity.
- Feedback control strategies can be set in place to decrease population heterogeneity.

## 1. Introduction

Although originating from a single founder cell, the billions of cells that can constitute a multicellular eukaryote are all different. Via division and differentiation, every cell in the organism becomes unique with an identity defined by its location, shape and protein content which together govern its function. In contrast to this marked diversity, liquid cultures of unicellular microorganisms appear more uniform. However, even in this highly simplified context, every cell will be different, despite having an identical genetic background and sharing the same liquid environment. This difference is associated to a unique combination of shape, cell cycle stage, epigenetic marks and protein content.

To achieve essential tasks ranging from gene expression to cellular division, cells rely on precise molecular machines formed by multimeric protein complexes. Their function requires the presence of each member to accomplish its vital task[1]. If the level of one element of the complex varies significantly, it might hinder the function of the whole complex. Therefore, the proper function of the cell depends on its ability to regulate precisely the expression level of key proteins.

However, cellular adaptation and evolution can benefit from some form of heterogeneity and diversity in a population. For instance, cells expressing a higher level of stress response proteins might survive an environmental perturbation better than others[2,3]. One drawback is that the high expression of these protecting proteins can be associated with a fitness cost under normal growth conditions. In order to strike the correct balance between the two conflicting requirements: fast growth versus stress survival, one strategy is to generate variability in gene expression such that both low- and high-expressing cells are present in the population. Heterogeneity is an adaptive trait in a similar way as protein expression level. As a proof of this, housekeeping proteins are expressed with low noise, while stress response proteins display a larger expression noise[4].

Unicellular fungi display complex lifestyles, where cell-to-cell differences will inevitably play an important role[5–8]. As a model eukaryote, *S. cerevisiae* has been extensively used to gain a better understanding of the different sources of heterogeneity in biology. The single-cell measurements performed in budding yeast have largely contributed to the characterization of cellular noise in biological responses. In the last decades, dynamic microscopy measurements have allowed to follow the fate of individual cells during many diverse processes[9–13]. More recently, single-cell mRNA sequencing datasets have provided a new approach to investigate these questions[14–17]. This review presents different mechanisms that contribute to the generation of heterogeneity in a population of cells via the transcriptional machinery, the signaling network and the environment. In the last part, experimental strategies set in place to control or modulate cellular heterogeneity will be presented.

## 2. Intracellular noise

Protein expression is a major source of heterogeneity in all cellular systems. Cells rely on one or two copies of a gene to produce the entire pool of a protein, therefore this low copy-number entails a stochastic transcriptional process which will have repercussions

at the protein level. Evidence of such stochasticity in gene expression has been first demonstrated in *E. coli* [18].The quantification of two fluorescent reporters expressed from identical promoters showed that some part of the noise was arising from difference between cells (extrinsic) while another noise component was modulating the expression of one reporter relative to the other in the same cell (intrinsic) (Figure 1). These studies have been extended to eukaryotic models such as *S. cerevisiae* to understand the underlying molecular mechanisms responsible for intrinsic and extrinsic noise[10,11,19]. Random binding of elements acting specifically on one DNA locus such as transcription factors and chromatin remodelers together with stochastic mRNA production contribute to the intrinsic noise[19].

### *2.1. Transcriptional bursting*

In an attempt to monitor the dynamics of transcription in living cells, technologies such as the MS2 or PP7 systems have been developed[20,21]. Derived from bacteriophages, these coat proteins bind specific RNA hairpin loops. The addition of a fluorescent protein to the coat protein and the insertion of multiple stem loops on the RNA of interest allows to monitor the dynamics of mRNA production. These dynamic measurements along with RNA FISH experiments have demonstrated that genes can be transcribed in bursts[21,22]. Groups of polymerases are simultaneously transcribing a locus, generating a burst in the mRNA production. These collective transcription events are followed by pauses of irregular duration. While stress responsive genes have long been recognized as being produced in burst, it is nowadays established that housekeeping genes are subject to a similar type of regulation[21]. This behavior will produce fluctuations in the mRNA levels, which can be amplified by the translation machinery. Interestingly, it has been shown that in higher eukaryotes, export of the mRNA through the nuclear pore might act as a temporal filter that dampens the fluctuations generated during transcription[23]. While this process has not been identified in yeast, this might be a universal mechanism present in all eukaryotes to reduce the noise in the protein expression process.

### *2.2. Transcription factors abundance*

Transcriptional bursts can find some explanation from the fact that promoter activation is regulated by the transient binding of transcription factors or other DNA associating proteins to cis-regulatory elements[24]. The low copy number of transcription factors and other transcriptional regulators can limit the efficiency of the transcription process. In bacteria, the small cell size is associated with a low protein number. In *E. coli*, quantitative analysis of the proteome has found that half of the genes are expressed at 10 copies per cell or lower[25]. The limited number of some these key components has been linked to stochastic switches in gene expression and can enhance population heterogeneity[26].

While protein abundance in budding yeast is roughly 100-fold larger than in *E. coli*, transcription factors are clearly expressed at lower levels than the average protein with roughly 10% of the transcription factors being present at around 100 copies per cell[27]. For instance, the stress responsive transcription factor Msn2 is present at 125 copies per cell[27]. Nonetheless, this transcription factor is responsible for the expression of more than 150 genes upon hyper-osmotic, heat or oxidative stresses[28,29]. Moreover, the promoters

of these genes typically bear multiple consensus binding sites[30–32]. This low copy number of transcription factors relative to the number of inducible genes and putative binding sites will contribute to the heterogeneity in the induction of stress response genes. Interestingly, Msn4, the paralog of Msn2, recognizes the same DNA binding site. However, it is not expressed under log-phase conditions[27,33] and becomes induced upon stress in an Msn2-dependent manner. The noisy induction of Msn4 will further increase the heterogeneity in the population[33].

### *2.3. Chromatin state of the locus*

In addition to the low abundance of transcription factors, in eukaryotes, the chromatin state of a DNA locus also contributes to the stochasticity of gene expression. The presence of nucleosomes on a promoter creates a compacted DNA region thereby preventing transcription factors from accessing their binding sites[34]. A switch from the close to the open chromatin state induced by chromatin remodelers can temporarily modulate the level of gene expression[11,31] . Constitutively expressed genes have a permissive chromatin state[35] which contributes to their low noise expression[4].

In contrast, stress-response genes are typically repressed under favorable growth conditions. Their activation requires an important chromatin remodeling for the transcription machinery to access the promoter and induce gene expression. One well-studied example is the hyper-osmotic stress response pathway controlled by the mitogen-activated protein kinase (MAPK) Hog1. The mechanisms required to open the chromatin and induce gene expression have been extensively studied[36,37]. While the activity of the MAPK is uniform within the population, the stochastic activation of the promoter controlled by the slow chromatin remodeling step induces a strong noise in gene expression[11,38]. As a large fraction of these stress-induced promoters are activated in a stochastic fashion, this will generate a large phenotypic diversity following a hyper-osmotic stress. Other stress-response pathways have been shown to display a similar noise[11].

Interestingly, not all the repressed genes are induced with a large noise. In the mating pathway, genes are induced with high fidelity[10]. As an example, two copies of the FIG1 promoter in the same cell will be induced to the same level despite their requirement for chromatin remodeling[39]. It is intriguing to note that, while the HOG and the mating pathway are both MAPK signaling cascades, they display this striking difference in transcription outcome. One possible reason for this difference in expression noise between the hyper-osmotic stress and the mating responses could be the large difference in the dynamics of the process. While stress responses are transient, the mating response which can be associated to a cell-fate decision process leads to a sustained MAPK activity over multiple hours. As a consequence, the timing for gene induction is very different in the two pathways (2-5 min after stress[40] vs. 5 min to 1 hour for mating genes[39]). Owing to the rapid and transient induction of the stress response genes, the slow stochastic activation of the promoter has a more profound impact on the final outcome of gene expression.

### *2.4. Coding versus non-coding transcripts*

In addition to the production of coding transcripts, there is a large quantity of non-coding RNAs (ncRNA) that are transcribed by the cell[41]. Given the magnitude and variety of ncRNAs expressed, their functions remain to a large extent elusive. However, these molecules have been shown to have a direct influence on the expression of neighboring genes. It has been shown using genome-wide transcripts analysis that the presence of anti-sense transcripts on coding genes can be associated with a larger noise in expression[42]. One model explaining this phenomenon is that the active production of an anti-sense transcript over the transcription start site (TSS) of a gene represses the sense transcription in a toggle-switch manner.

Such a toggle-switch between two ncRNA has been uncovered in the regulation of the flocculin Flo11. Two long non-coding RNAs have been discovered on the particularly long FLO11 promoter: a sense transcript ICR1 3 kB upstream from the FLO11 start site and an anti-sense transcript PWR1, which starts 2.3 kB from the FLO11 start site[43]. When ICR1 is transcribed, the transcription factor Flo8 cannot bind to the FLO11 promoter thereby inhibiting FLO11 gene expression. However, the PWR1 lncRNA can block the production of the ICR1 transcript, thereby allowing the FLO11 gene to be expressed[44]. This toggle-switch mechanism generates a bimodal distribution of Flo11 expression in a population of log-phase growing cells where a fraction of the cells expresses the flocculin while the other does not. The switch between the two states is controlled in part by the histone deacetylase complex Rpd3L. The daughter specific repressor ASH1 recruits the chromatin remodeler Rpd3L to the FLO11 promoter to block ICR1 transcription and favor FLO11 induction[45–47]. This allows the production of Flo11 and the activation of filamentation in the daughter cells, while the mother continues to grow vegetatively.

Overall, a combination of multiple stochastic processes can result in the noisy activation of a gene. Some of these processes are highly dynamic such as the transcriptional bursts or the translocation of transcription factors. Other processes like epigenetic mechanisms can be maintained over multiple generations, ultimately contributing to the development of different cellular states within the population.

## 3. Cell-to-cell variability

In addition to the intrinsic noise generated at each locus by the elements required to set in place the transcriptional machinery, cells have to cope with extrinsic noise. When looking at a population of log-phase growing cells, some of this diversity is apparent as cells have different sizes or are in different stages of the cell cycle (Figure 2A). When following the growth of cells over a long time, one can notice that mother and daughter cells spend different amounts of time from one division to the next[48,49]. Furthermore, prolonged observation of micro-colonies shows that they grow at different rates[3].

At the molecular level, noise is associated to stochastic variation in the synthesis of mRNA. Then, the overall protein level in a cell will strongly depend on its expression capacity which is associated to the number of ribosomes[10]. A glimpse into the variability in protein content can be obtained using fluorescent protein tags (Figure 2B).

Independently of their mean expression in the population, some proteins will display a normal distribution of expression levels, while other proteins have clearly deviated from this standard distribution and are substantially more heterogeneous (Figure 2C). In addition, the set of proteins expressed within a cell can vary. For instance, the cell cycle regulated genes (roughly 10% of the transcripts) vary according to the cell cycle stage[50,51]. In parallel, specific pools of genes can be co-regulated. This has been suggested to be linked to the differential activation of the growth-promoting pathways TOR and PKA and the stress-response transcription factor Msn2 between individual cells[52]. Single-cell mRNA sequencing measurements allow to study this co-regulation with unprecedented resolution[42].

### *3.1. Variations in metabolic activity*

Alongside proteins, the metabolites within the cells can also fluctuate. Cell growth and division are affected by environmental conditions and specifically the level and the quality of nutrients available. This variability may affect the activity of the metabolic pathways resulting in a variation in gene expression between cells. In continuous cultures with limited nutrients, the whole population of cells alternates between respiratory and fermentative phases known as the Yeast Metabolic Cycles[53]. Linked to these oscillations are major changes in the expression profiles of the cells[54]. In batch cultures, where nutrients are plentiful, similar oscillations are present in individual cells. However, they occur in an asynchronous fashion in the population and are partially synchronized with the cell cycle[55,56].

Switching the quality of the carbon source can lead to striking differences in cellular adaptation and result into strong heterogeneity. The GAL regulatory network has been the subject of intense studies[57]. As an example, shifting cells grown in glucose into a mixture with higher concentration of galactose relative to glucose, leads to a rapid switch on the expression of the GAL genes in a fraction of the population, while in other cells the induction is delayed by multiple hours[58].

Additionally, complex memory phenomena can be observed when alternating sugar sources[59]. Cells pre-grown in galactose and exposed to glucose for a few hours will resume division faster than cells which were not exposed to galactose previously. Interestingly, this memory is a combination of epigenetic modifications at the GAL1 locus providing a short-term memory and expression of proteins during the galactose pre-exposure conferred a long-term memory [60,61]. A comparison of the galactose/glucose or maltose/glucose shifts demonstrated that it is a slow decrease in the capacity of the cell to activate respiration that can best explain this memory[16,62]. The single-cell experiments performed in these studies also highlight the large diversity in growth resumption accompanying this memory effect. Overall, the metabolic state of the cell will determine when a cell grows and divides, but also its capacity to respond to extracellular stimuli and stresses[63,64].

### *3.2. Cell cycle state*

As mentioned above, the cell cycle stage is a major parameter that differentiates individual cells. Single-cell sequencing data demonstrate the clustering of the gene

expression pattern of individual cells as function of their position in the cycle[14,15,17]. These differences can lead to various abilities to respond to extracellular cues. One well-documented example is the response to mating pheromone. Cells in G1 will strongly activate the MAPKs Fus3 and Kss1, unlike the S-phase cells which cannot respond to the pheromone stimulus[65,66]. Thus, the inherent variability existing in the population is further exacerbated by the extracellular input which generates an asynchronous activation of the mating program in the population.

Importantly, not all perturbations lead to this increased variability between cells in different cell cycle stages. When stressed by an increase in osmolarity in the medium, all the cells respond to the stimulus by activating the MAPK Hog1 which blocks transiently the cell cycle and the stress response take precedent over the cell cycle progression during the time needed for the adaptation[67–69]. Therefore, irrespective of their cell cycle stage, all the cells activate the HOG cascade and adapt in a synchronous fashion.

These two examples of how MAPK pathways interact with the cell cycle represent well two scenarios related to biological noise. On one side, cell-to-cell variability is inherent to the biological function and the cell has implemented a robust system to cope with this variability. Interestingly, the mechanisms set in place to allow Hog1 to block the cell cycle progression differ for each cell cycle stage[67,68]. On the other side, a pre-existing variability between the cell is enhanced by an external stimulus and further increases the heterogeneity in the population. In the example of the mating pathway, this variability is detrimental to the desired outcome as cells should not mate with two copies of their chromosomes. Thus, safeguards are set in place to restrict the pheromone signaling to the desired cell cycle stage.

### *3.3. Bet-hedging strategies*

The cell cycle state is one example of a cell-to-cell variability that can bias the response of a cell to an incoming stimulus. Other more subtle differences, present in a population of cells, can allow them to respond differently to a change in growth medium or to a stress. Whether these differences are due to a random process or are part of an evolved strategy to better survive in fluctuating conditions remains under debate.

Single-cell sequencing data in budding yeast have shown that in addition to cell cycle genes, metabolic genes are enriched among transcripts that are highly variable within a population[15]. One tentative explanation for this variability is the capacity of the cells that express some of these genes to adapt more rapidly in case of changes in nutrients. As an example, GAL3 has been identified to be heterogeneously expressed in the population. While 80% of a population of cells growing in glucose displays no expression of this gene, 1.5% of the population possesses more than 10 mRNA transcripts of GAL3. These high GAL3 expressing cells were shown to adapt more rapidly when transferred from glucose into a galactose medium[15]. The uncorrelated expression of these metabolic genes can be rationalized as a bet-hedging strategy of the population which prepares sub-populations of cells for a change in nutrient availability.

Stress-response genes were also shown to be expressed in a highly variable fashion within a population of log-phase growing cells[4]. It is well appreciated that induction of

stress response genes by a mild stress prepares the cell for surviving a severe stress[70]. Single-cell mRNA sequencing has shown that a small fraction of a population of unstressed cells display a higher expression of stress proteins[14]. The expression of these stress response genes can provide a protection against heat, oxidative or antibiotic stresses[2,3]. Tsl1 or Hsp12 tagged with a fluorescent protein have been used to identify and sort 0.1% of the high-expressing cells in the population (Figure 3). These cells display a better resistance to various stresses, whereas, under normal growth conditions, they proliferate more slowly. This phenotype (slow or no growth, expression of stress response genes and stress survival capacity) is reminiscent of the persistence state in bacteria[71,72]. While in bacteria, persistence represents a phenotypic switch[73,74], in yeast, there is a continuum of states from the bulk of the population that is fast-growing and expresses protection genes at low levels and the slow-dividing cells with high levels of stress response genes. Given this graded distribution, it is more difficult to characterize a persister state in yeast. Nonetheless, this heterogeneity in the population which provides a fitness advantage to a sub population of cells can be considered as a bet-hedging strategy for stress survival.

### *3.4. Influence of cellular age*

In a log phase culture, the vast majority of cells are young, either being newborn or having undergone a few divisions. However, some older cells can be present and display an aberrant shape with a large vacuole[75]. These cells accumulate damaged proteins, reactive oxygen species and DNA damage[76–78] which makes them react differently from their younger siblings to growth cues.

In budding yeast, replicative aging, defined as the number of daughter cells generated by a mother cell has been extensively studied[79,80]. One major challenge when investigating replicative aging in yeast is the ability to separate daughter cells from their mother. Different strategies have been established, among them, biotinylation has served as a method to separate daughters from biotinylated mother cells[81]. More recently, microfluidic systems have been engineered to trap mother cells and flush away their daughters, allowing to record biological changes over generations at the single-cell level[75,82]. Using these techniques, it was observed that the transcriptomic and proteomic profiles are perturbed during cell aging. Glucose uptake decreases resulting in a reduction of growth rate[83]. Transcription silencing is lost, resulting among other phenotypes in sterility[81]. Additionally, the ability to adapt and survive to fluctuating environments is decreasing with age[84].

### *3.5. Diversity of prions*

Another form of cell-to-cell variability that is under appreciated is the presence of prions. These molecular aggregates that form an epigenetic memory can confer specific properties to the cells that harbor them[85]. While it has been initially thought that only a limited number of proteins can form these aggregates, a recent overexpression screen has revealed that at least 50 proteins can maintain some form of epigenetic memory that last over hundred generation[86]. Because the presence of these aggregates can have an important impact on the cellular growth in specific conditions, prions have also been

considered as participating in a bet-hedging strategy of the cells. Stochastic switching to the prion state can happen with frequencies varying from $10^{-4}$ to $10^{-7}$and clearing of the prion probably happens with similar frequencies[85]. Moreover, environmental stimuli can also induce these transitions into and out of the prion state[87]. With the larger than expected number of proteins that can contribute to these epigenetic memories and the switching rate which are much higher than genetic mutations, already a few milliliters of culture are likely to harbor individual cells in a different prionic state.

The attributes of a cell are constantly evolving. Metabolic waves and progression in the cell cycle can modify the characteristics of the cell within a few minutes. These asynchronous changes represent a large fraction of the diversity in the population. Other characteristics, like the presence of prions, are much more stable and directly transferred to all the progeny of a cell. Between the fast metabolic oscillations and the long-lasting prionic state, the expression capacity and the palette of expressed proteins are fluctuating slowly. The transmission of these cellular features to daughter cells remains to be better understood.

## 4. Influence of the environment

Beyond the variability that exists or can emerge between individual cells, the surroundings of the cells can be a source of heterogeneity within a population. In the planktonic state, cells are surrounded by a well-mixed medium and the entire population will experience the same nutrient concentrations and will be subjected to nutrient exhaustion synchronously. However, in the lab as well as in the wild, yeast cells spend much of their time on surfaces in close proximity to neighboring cells where nutrients access may be spatially constrained.

One simple example of this environmental variability is a mating mixture where MATa and MAT$\alpha$ cells are mixed together on an agarose pad to monitor the mating process (Figure 4A). Depending on the distribution of potential mating partner in their vicinity, some cells will successfully mate to form a diploid or abandon the mating response early on. In addition, even two mating partners which are in close proximity can fail to mate if their cell cycles are not well coordinated. Therefore, on top of the cell-to-cell variability that exists in the population, the interaction with neighboring cells can add an additional level of heterogeneity.

### *4.1. Yeast colonies*

Cells within a colony are the progeny of a single founder cell and share the same genetic material. However, the emergence of heterogeneous sub-populations with distinct metabolic states may occur as an inherent consequence of access to resources. Such heterogeneity in a colony is then maintained thanks to different metabolic strategies adopted by cells. In a vertical slice of a colony, two types of cells have been described. Cells from the lower part of the colony experience a higher concentration of nutriment and are mostly relying on respiratory metabolism, while in the upper part, cells are fermenting[88]. Also, the emergence and maintenance of these sub-populations in the colony are modulated by the environment. Indeed, under limited glucose conditions, small

patches of cells perform gluconeogenesis and release metabolites in the extracellular environment. Another fraction of the population feeds on these secreted nutrients, resulting in metabolic and phenotypic diversity[89].The capabilities of cells to specialize in nutriment production and profit from shared resources within a colony has been demonstrated by the work of Campbell et al.[90]. Starting from a prototrophic founder cell, which harbors multiple plasmids compensating for deficiencies in the histidine, leucine, methionine and uracil synthesis present in the genome, a colony will develop. However, as the colony grows and cells divide, the plasmids are lost exposing the metabolic deficiencies of the cells. Thanks to a specialization of the cells which will produce and secrete some metabolites required by other cells, the colony can sustain its growth. This highlights how the differentiation and the exchange of nutriments among cells of the same colony is important for its expansion.

Common yeast lab strains such as S288c or W303 form smooth round colonies while, wild yeast strains form structured colonies[91]. Interestingly, these structures may arise from the variegated expression of the flocculin FLO11[43,44], which plays an important role in this process by aggregating neighboring cells together. Because of the bi-stable expression of FLO11, some cells will form tight clusters which will contribute to the three-dimensional structure of the colony[92]. An additional capacity of wild yeast strain compared to lab strains is their ability to filament. This differentiation will allow cells to invade into the agar or to form pseudohyphae that extend outside of the colony (Figure 4B and C). The directed division of these elongated cells will allow them to reach new sources of nutrients. Cells will undertake this transition based on their surroundings. They will probe the availability of nutrients and maybe also the presence of other cells via a quorum sensing mechanism[7,93]. Thus, only cells at the periphery of the colony commit to this new filamentation cell fate, which differentiates them from the rest of the colony.

For simplicity, most biochemical experiments are typically performed in liquid cultures, where all cell share a common environment. On solid medium, an additional level of complexity is present. Access to nutriment is limiting, which will lead to the diversification of the yeast population. In addition, cell-cell contacts can influence the fate of the cell by promoting mating or filamentation.

## 5. Controlling heterogeneity

Heterogeneity is an integral part of any biological system. However, to understand fundamental biological processes or to reliably use cells for bioproduction, it could be desirable to decrease or control cellular noise. Multiple studies have been undertaken to understand how the promoter architecture of a gene controls the level and the noise of the expression output. In the core sequence, the absence of a strong TATA motif favors a less bursty expression[19,94]. In the upstream activating sequence, nucleosome disfavoring sequences leads to lower noise while strong or multiple transcription factor binding sites increase transcription noise[95,96].

While optimizing the promoter sequence of a gene can limit the noise in the mRNA production, the protein levels will remain subject to the extrinsic noise component of the cell and primarily its expression capacity. Interestingly, some single gene deletions were

shown to display a smaller variability in growth rates than wild-type cells suggesting a lower cell-to-cell heterogeneity[3]. While the mechanism underlying this behavior remains to be identified, these mutants can offer a potential solution to reduce the extrinsic noise component in a population. One extrinsic factor that can be controlled precisely in budding yeast is the cell cycle progression, either by blocking cells in a specific cell cycle stage or by inhibiting the activity of the Cyclin dependent kinase Cdc28[10]. Alternatively, cell cycle stage markers can be used to synchronize *a posteriori* single-cell measurements using bioinformatic analysis[97]

### *5.1. Feedback control*

Instead of designing a biological specimen with reduced noise, an alternative strategy is to define a control strategy to ensure that the cell behaves in a predetermined manner (Figure 5). With either opto-genetic systems or microfluidic flow chambers, the stimulus applied to the cells can be adjusted in a continuous manner. The real-time quantification of the protein output allows to determine the level of the stimulus needed to reach a predefined set point[98,99]. This strategy can be used to set precisely the expression level of the whole population. However, due to the noisy nature of gene expression, individual cells will express at various levels and the variability will persist within the population. In order to circumvent this problem, one strategy put forward is to apply the control at the individual cell level. Using digital micro-mirror, it becomes possible to specifically activate by light individual cells in the field of view of a microscope. Therefore, the stimulus can be tailored precisely to compensate for the propensity of each cell to express more or less proteins[100].

Although this approach is very powerful, its capabilities remain limited because the number of cells that can be controlled simultaneously cannot be increased readily. An alternative technique that has been demonstrated to reduce noise in gene expression is regulation based on pulsatile activation. It has been shown that using the frequency of light pulses to control the activity of an opto-genetic promoter instead of tuning the intensity of the light allows to decouple the expression level from the expression noise[101]. This approach is interesting for two main reasons. First, while it has been demonstrated with light pulses, the system could be expanded to other types of activation methods, for instance flow channels. Second, it has been demonstrated that many endogenous transcription factors are displaying pulsatile activity[102–104]. One potential reason for this natural behavior could be to decrease the noise in endogenous protein expression.

### *5.2. Microfluidic devices*

In parallel to these methods which aim at controlling the cellular output, microfluidic devices have gained in importance by providing new ways to control the extracellular environment of the cell. Constantly refreshing the medium allows to keep cells dividing until they age[75,78,82] or to acutely perturb the environment with highly controlled temporal stimuli[105–107]. Moreover, one attractive application of microfluidic devices is the ability to mimic with well-controlled conditions some complex natural environment encountered by the cells. One example is the development of gradient chambers to study polarity site selection and mating projection orientation[108–110]. While in mating mixtures, it is difficult to

predict the concentration of the extracellular pheromones, in microfluidic chips, cells can be subjected to specific and reproducible conditions. Another example is the development of devices to study the yeast colony architecture, by engineering chambers where nutrients diffuse into a layer of cells[111]. These microfluidic devices thus provide the opportunity to simplify the natural environment thereby providing deeper insight into the interaction of the cells with their surroundings.

## 6. Conclusion

Multiple progresses have been made in the last two decades in the understanding of cellular heterogeneity thanks to the development of novel techniques. Fluorescent proteins and live-cell microscopy have enabled to monitor the emergence of noise within a population. More recently developed in yeast, single-cell sequencing is promising to open a new dimension in the observation of the variability among cells. Although challenging, the combination of these two techniques can provide important biological insights by connecting dynamic information obtained by microscopy with the genome-level information from the sequencing data[112,113].

An important challenge for the coming years is to identify the purpose of noise in a given biological context. In this review, a few examples were presented where the heterogeneity observed seems to possess a specific function and thus might be an adaptive behavior. However, in many situations, processes are designated as stochastic or random due to a lack of understanding of the underlying mechanism that generates this behavior. Even when the mechanism at the source of the heterogeneity is identified, the biological purpose of this noisy behavior remains enigmatic. Therefore, beyond the characterization of noise in biological systems, the objective is to uncover hidden biological behaviors, which are often masked by static or population averaged measurements.

## Figure legends

*Figure 1. Contribution of intrinsic and extrinsic noise to cellular heterogeneity.*

A. and B. Characterization and analysis of intrinsic and extrinsic noise in cells carrying a red and a green expression reporter under identical promoters. Diverse sources of intrinsic noise (A) responsible for a differential expression of the red versus the green reporter. Sources of extrinsic noise (B) resulting in a correlated expression of the two reporters. C. Correlation between the fluorescent signal from the two reporters in absence of noise (left), in presence of extrinsic noise only (middle) or with both intrinsic and extrinsic noise (right).

*Figure 2. Visualization of cell-to-cell variability.*

A. A bright field image illustrates differences in size or cell cycle stage observed in a population of log-phase growing cells. B. Visualization of the variability in the expression of a stress response protein (Hsp104 tagged with YFP) within this population of vegetatively growing cells. C. Histograms of the fluorescence intensity of Hog1-YFP, Hsp104-YFP and Car1-YFP. Despite their large differences in expression levels, Hog1 and Car1 display a normal distribution, unlike Hsp104 which displays a more heterogenous expression pattern.

*Figure 3. Scheme of the bet-hedging strategy for stress response proteins.*

This scheme represents an illustration of the bet-hedging strategy as demonstrated by Levy et al. 2012[3] and Yaakov et al. 2017[2]. The properties of three sub-populations sorted from the distribution of expression of a stress response protein. The growth rate of the high-expressing cells is lower compared to the bulk of the population. However, this fraction of the population displays a higher resistance to stress.

*Figure 4. Heterogeneity arising from the environment.*

A. *MATa* (blue) and *MAT*$\alpha$ (red) cells mixed on an agarose pad to monitor mating over time. The probability of the mating will be strongly influenced by the proximity of a cell of opposing mating type. Fusion events are marked by a star (*). Even cells that are touching each other can fail to fuse if their cell cycle is not in synchrony. B. Image of two colonies of $\Sigma$1278b cells grown on low ammonium medium for three days. This medium promotes the switch from vegetative growth (VG in the colony center) to pseudohyphal growth. The phenotype is represented by a few individual cells escaping the colony to forage for nutrients (FG, indicated by arrows). C. Colonies of $\Sigma$1278b cells after five days on low ammonium medium. The colony formed by vegetatively growing cells (VG) is surrounded by numerous filaments of pseudohyphal cells (FG).

*Figure 5. Scheme of feedback control in cell induction experiment.*

An experimental set-up where the sample can be stimulated in a dynamic manner either by light control of by a fluidic system allows to set in place a feedback control strategy. After sample induction (1), the image of the cells is acquired (2). The image is segmented to identify individual cells (3) and the behavior of each cell is monitored (4). Based on these new set of measurements, a new stimulus is predicted (5) and the stimulus level is modified accordingly (6).

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## Competing interests

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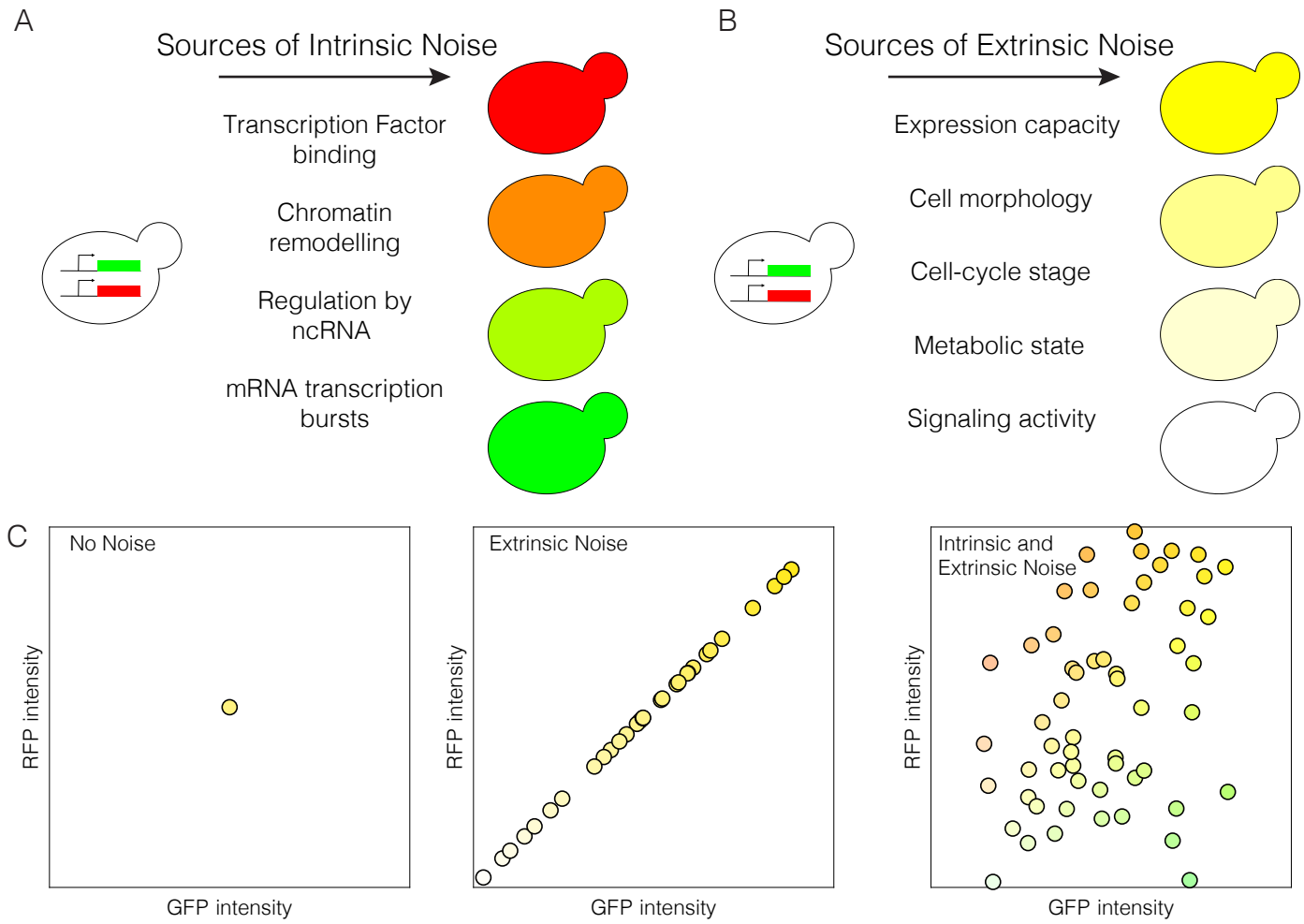
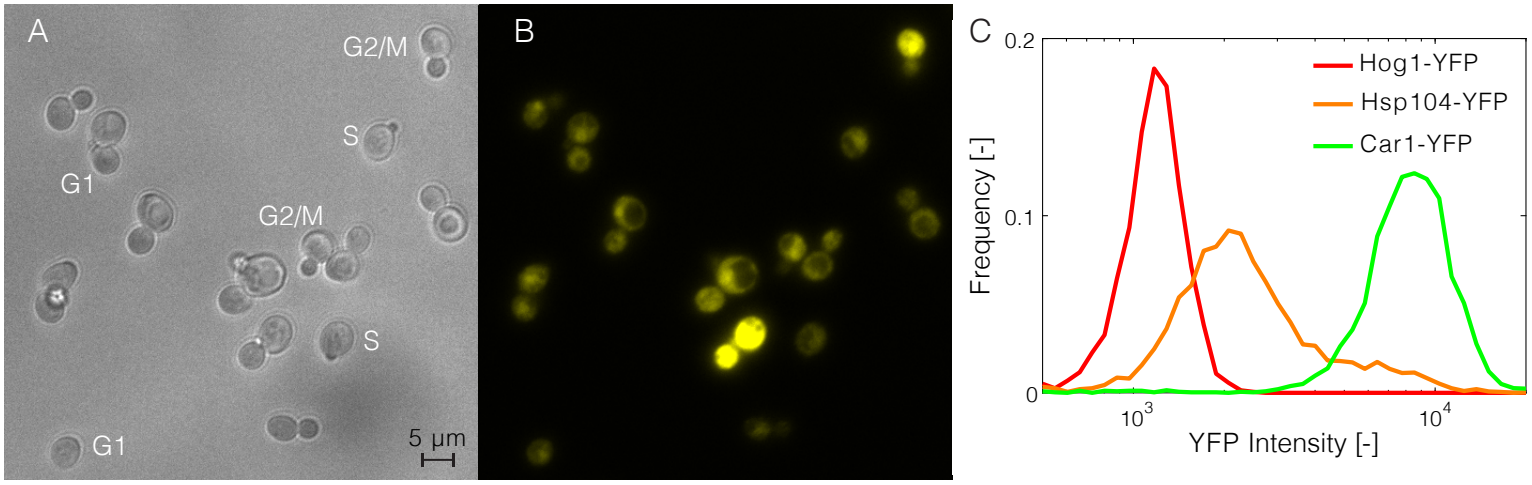

A
Sources of Intrinsic Noise
Transcription Factor binding
Chromatin remodelling
Regulation by ncRNA
mRNA transcription bursts
B
Sources of Extrinsic Noise
Expression capacity
Cell morphology
Cell-cycle stage
Metabolic state
Signaling activity
C
No Noise
Extrinsic Noise
Intrinsic and Extrinsic Noise
RFP intensity
GFP intensity

**Figure 1**


A
G2/M
S
G1
G2/M
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G1
5 µm
B
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0.2
0.1
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Hsp104-YFP
Car1-YFP
$10^3$
$10^4$
YFP Intensity [-]

**Figure 2**

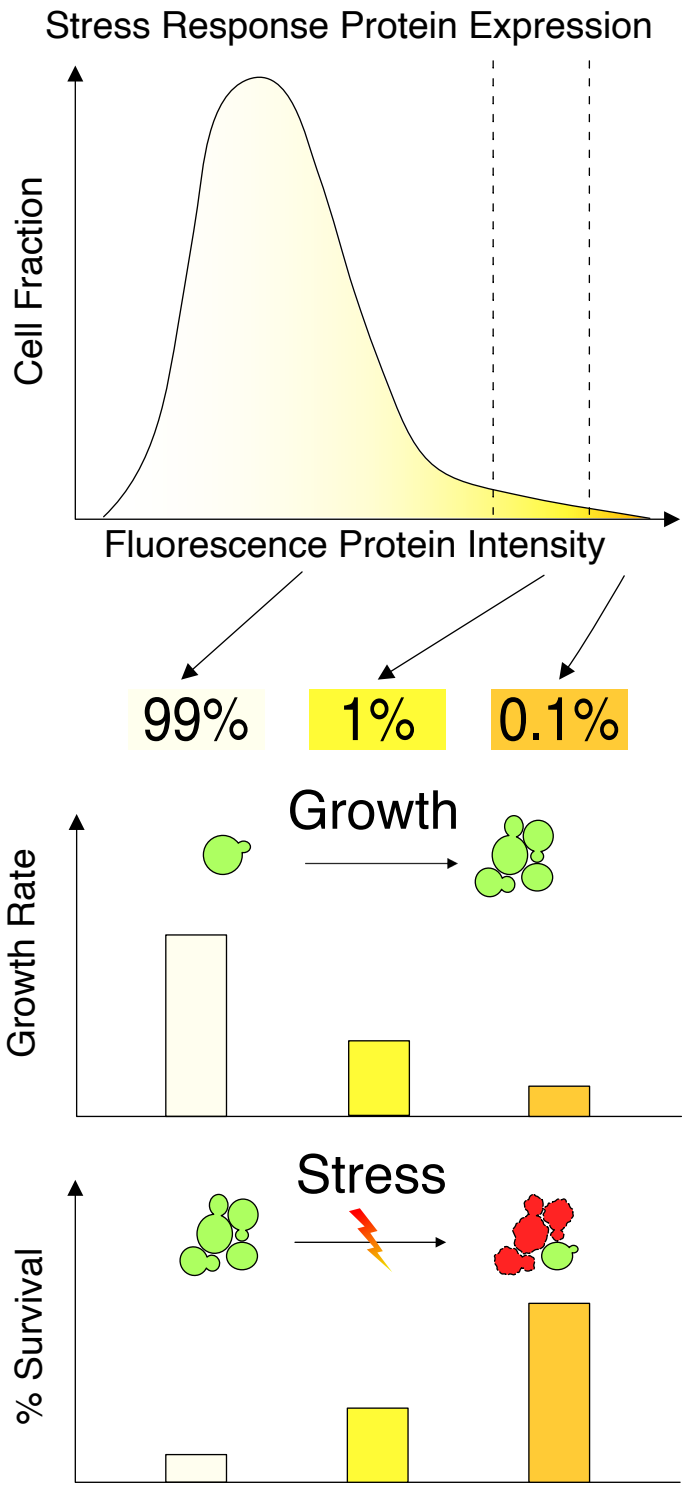

Stress Response Protein Expression
Cell Fraction
Fluorescence Protein Intensity
99%
1%
0.1%
Growth
Growth Rate
Stress
% Survival

**Figure 3**

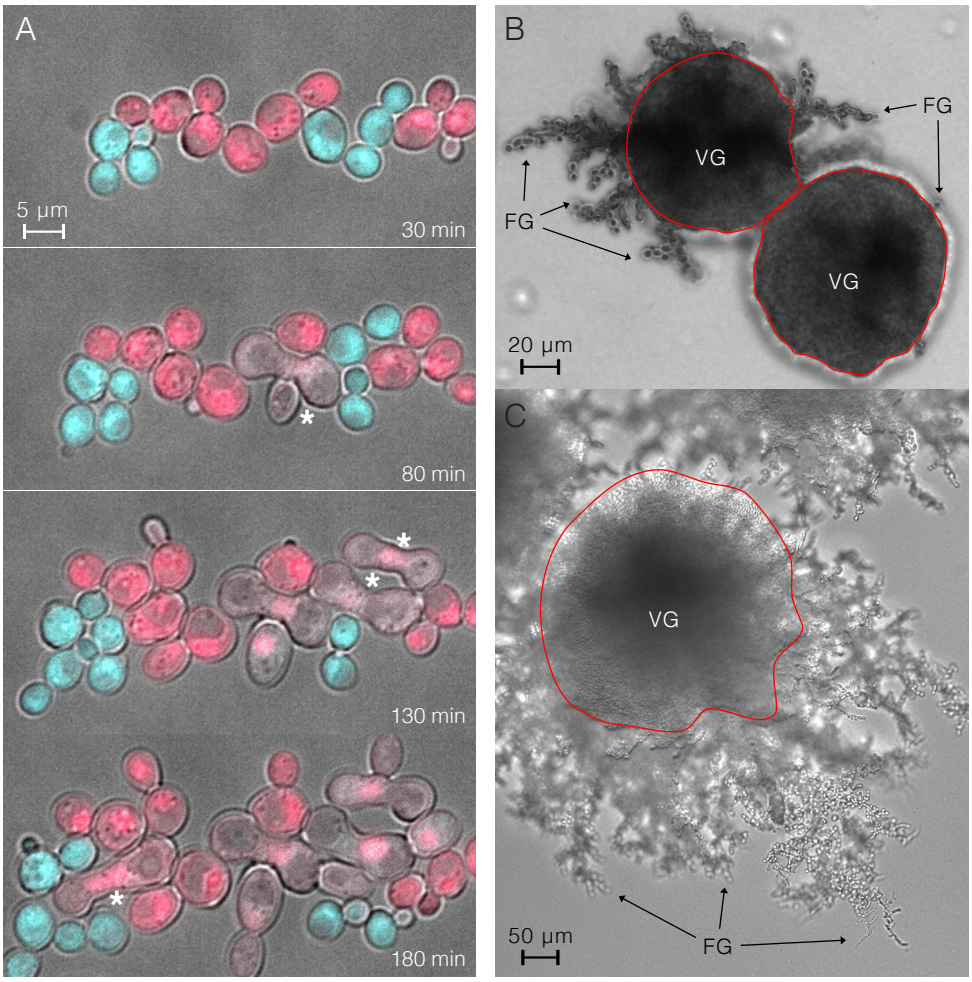
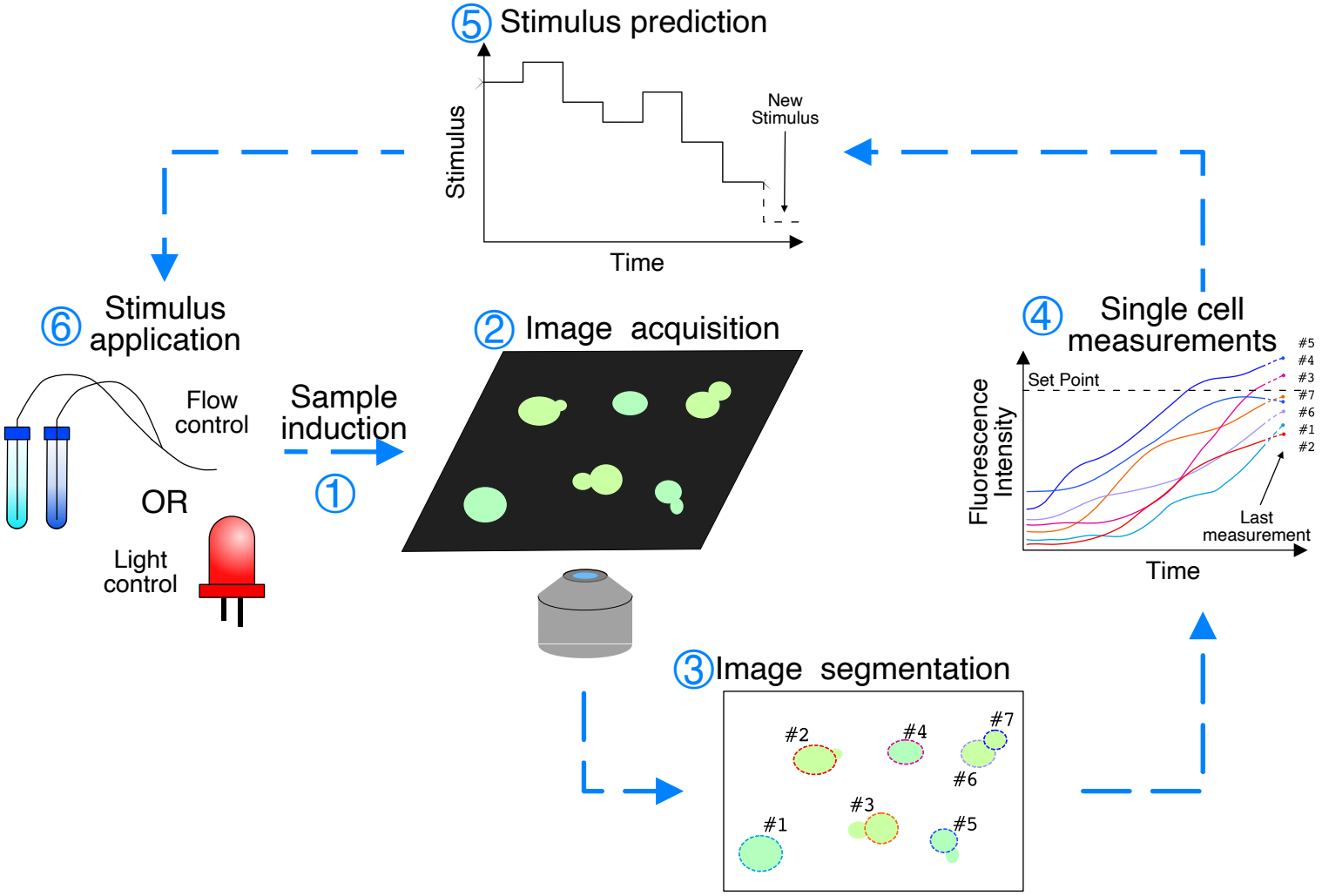



**Figure 4**


⑤ Stimulus prediction
Stimulus
New Stimulus
Time
⑥ Stimulus application
Flow control
OR
Light control
Sample induction
①
② Image acquisition
④ Single cell measurements
Set Point
Fluorescence Intensity
Last measurement
Time
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#3
#7
#6
#1
#2
③ Image segmentation
#2
#4
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**Figure 5**