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Functional roles of microbial cell-to-cell heterogeneity and emerging technologies for analysis and control

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Abstract

Clonal cell populations often display significant cell-to-cell phenotypic heterogeneity, even when maintained under constant external conditions. This variability can result from the inherently stochastic nature of transcription and translation processes, which leads to varying numbers of transcripts and proteins per cell. Here, we showcase studies that reveal links between stochastic cellular events and biological functions in isogenic microbial populations. Then, we highlight emerging tools from engineering, computation, and synthetic and molecular biology that enable precise measurement, control, and analysis of gene expression noise in microorganisms. The capabilities offered by this sophisticated toolbox will shape future directions in the field and generate insight into the behavior of living systems at the single-cell level.

Introduction

The behavior of individual microbial cells in isogenic populations can significantly deviate from the population average. With the development of single-cell technologies has come both recognition of the widespread nature of this effect and the drive to uncover the mechanisms underlying such variability. From these studies, researchers have demonstrated that fluctuations in gene expression are an inevitable consequence of transcription and translation and a major contributor to phenotypic heterogeneity [1,2]. Such stochastic fluctuations in gene expression can be further amplified or attenuated by circuit architecture and propagation of these signals through the downstream regulatory network can significantly impact cell-to-cell variation [3–6].

The combination of high-throughput flow cytometry, single-molecule imaging, and libraries of transcriptional or translational fluorescent reporters have also enabled insights into the pervasiveness of noise across thousands of genes [7–9]. Collectively, results from these Herculean studies have revealed overarching patterns of gene expression noise, hinting that evolutionary forces may have tuned the noise levels associated with different sets of genes, and suggesting a potential functional role for this stochastic process. A recent systematic investigation of approximately 100 genes in two growth conditions reinforced this idea by

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revealing an anti-correlation between expression noise and a gene's sensitivity to changes in expression [10]. The potential implications of stochastic variation for many cellular processes have provoked questions, such as: What functional capabilities do these phenotypic distributions provide for populations of genetically identical cells? What are the temporal dynamics associated with the heterogeneity? And, what features of genetic circuits contribute to the generation and regulation of this variability?

In this review, we discuss the utility that stochastic gene expression provides, with a focus on outcomes related to the behavior of clonal microbial populations, as there have been significant recent advances in this area. Complementary research on the origins of noise and its functions across prokaryotes and eukaryotes have been nicely reviewed by Sanchez *et al.* [11], Eling *et al.* [12], Raj and van Oudenaarden [2], and Engl [13], and we point the reader to these sources for background and additional context. Next, we discuss the increasingly sophisticated experimental and computational tools that are opening new possibilities for live single-cell assays with microbes. These technologies enable researchers to not only monitor noise levels with accuracy, but also to precisely manipulate them while tracking cellular responses in real time.

Functional roles of cell-to-cell heterogeneity

Transient stress survival

In the past two decades, many studies have provided experimental evidence of functional roles enabled by phenotypic heterogeneity. A popular example is associated with the implementation of 'bet-hedging,' in which a subpopulation of individuals with suboptimal fitness, but superior stress response, is maintained across the population as a strategy for survival upon sudden environmental changes. One of the first illustrations of this mechanism was provided by the characterization of a small subpopulation of cells called 'persisters' that can survive antibiotic treatment without intrinsic resistance mechanisms [14,15]. Many mechanisms have been implicated in the formation of persister cells, including cell dormancy, variations in the expression of tricarboxylic acid cycle enzymes, toxin-antitoxin levels, among others [14,16–19]. Interestingly, a recent study that tracked single cells in exponential growth during transient antibiotic treatment demonstrated that DNA content and SOS response induction are heterogeneous among persister cells and even metabolically active cells can generate persisters, highlighting the multiple routes that microorganisms might leverage for survival [20].

Beyond persister formation, other mechanisms that allow transient tolerance to stress have been identified. For example, at the single-cell level and in the absence of stress, higher expression of the multiple antibiotic resistance activator MarA correlates with survival during carbenicillin treatment (Figure 1a) [21]. Other genes with diverse functions have also been shown to serve as good predictors of cell survival, and underlying phenotypic heterogeneity results in distributed survival times [22]. Noise that originates from variable induction of a key regulator of acid stress resistance genes during antibiotic treatment has also been shown to cross-protect surviving cells against subsequent exposure to acid stress [23]. In addition, mechanisms of noisy growth rate have been discovered in cells growing in steady-state conditions and appear to function as a survival strategy. For example, Patange *et*

al. [24••] demonstrated that RpoS, a key stress response regulator, is heterogeneously expressed in clonal *Escherichia coli* populations. This heterogeneity stems from pulsatile activity of RpoS, which is inversely correlated with the growth rate and allows cells to survive transient oxidative stress. Asymmetric cell division has also been recently linked to the emergence of cell-to-cell heterogeneity. By tracking cells for many generations, Bergmiller *et al.* [25••] uncovered a partitioning bias of the multi-drug efflux pump AcrAB-TolC to the old cell pole in *E. coli*. This process leads to long-lived phenotypic differences between mother and daughter cells, where the aging mother displays more pump complexes and increased tolerance to subinhibitory antibiotic concentrations. This finding highlights the potential for further investigations on the role of asymmetric divisions in the generation of phenotypic heterogeneity during aging, a behavior that could be reproduced with emerging tools for programmable asymmetric cell division [26,27].

Variation in mutation rates

Mounting evidence is also indicating that gene expression noise can lead to variability in mutation rates across a cell population, thus contributing to the acquisition of permanent genetic changes and microbial evolution (Figure 1 b). For example, fluctuations in the levels of the DNA repair protein Ada correlate with variability in mutation rates during treatment with a toxic and mutagenic alkylating DNA damaging agent in *E. coli* [28,29••]. In addition, the expression of MutS, a protein involved in DNA mismatch repair, has been shown to be heterogeneous and inversely correlated with the expression of the multidrug efflux pump AcrAB-TolC, suggesting that cells which export antibiotics may also be more mutation prone [30]. Although mutations originating from spontaneous DNA replication errors occur at a constant rate with Poisson statistics [31], a higher frequency of replication errors were found in subpopulations undergoing endogenous stress [32•]. Future studies combining microfluidics with fluorescent reporters for mutations and cellular state could provide additional information about subpopulations of phenotypic mutators, such as quantifying the window of opportunity that these cells have to accumulate permanent genetic changes.

Temporal organization of regulatory dynamics

Heterogeneous expression of key transcriptional regulators can serve as an important layer of regulation in gene networks. For example, in *Bacillus subtilis*, several sigma factors have been shown to activate repetitive pulses and compete for binding to core RNA polymerase complexes, allowing a population of clonal cells to access a range of transcriptional programs, even in a homogeneous environment (Figure 1c) [33,34••]. Whether the pulsatile dynamics of key transcription factors propagate all the way to downstream genes resulting in phenotypic consequences is a critical question. One recent example of this link was demonstrated in *E. coli* populations in which a mutual inhibition feedback loop between the sigma factor RpoS and growth rate resulted in pulses of RpoS expression that correlated with transient survival of oxidative stress [24••]. Additionally, fluctuations in the expression of the transcription factor ComK have been shown to play an important role in enabling subpopulations of *B. subtilis* cells to enter a transient state of competence, in which cells can take up extracellular DNA. When ComK levels surpass a threshold, a positive feedback loop is activated, increasing its levels, and upregulating downstream genes involved in DNA uptake. Thus, lowering ComK noise levels effectively reduces the fraction of the population

entering the competent state [35,36]. For an excellent review on how pulsing dynamics are generated and controlled and how they enable biological functions, we refer the reader to Levine *et al.* [37].

Cell fate and metabolic switching

Other biological processes with origins in stochastic gene expression include the basis for cell differentiation and metabolic specialization [35,36,38–42]. For instance, the pulsatile phenotypic transition between the motile and sessile states in *B. subtilis* cells is associated with stochastic fluctuations of SinI and controlled by a double negative feedback loop, which creates long pulses of high SinR interspersed with short pulses of high SlrR expression (Figure 1d) [43]. Metabolic diversity also exists among individual cells. For example, heterogeneity in the ability to metabolize lactose is associated with stochastic dissociation of the lac repressor from the operator sites in intermediate inducer concentrations [41]. Metabolic switching during diaxic shift is also heterogeneous due to variations in the levels of catabolite repression and stringent response; these factors lead a subpopulation of cells to halt growth [42]. For a comprehensive review on the role of noise in cell fate decisions, we refer the reader to Norman *et al.* [44].

Emerging technologies for measuring, controlling, and analyzing gene expression noise

The emergence of precise single-cell methods has allowed scientists to gather an astounding wealth of information about bacterial phenotypic heterogeneity and its functional consequences. Technologies that allow long-term single-cell tracking, increased throughput and spatio-temporal control of gene expression are evolving at a fast pace and are laying the groundwork for future discoveries on the mechanistic origins of noise and its phenotypic consequences. Here, we highlight examples of these advances, with an emphasis on tools for measuring and controlling gene expression dynamics.

Measuring and monitoring gene expression noise

Accurate reporters coupled with the capabilities offered by microfluidic devices and time-lapse fluorescence microscopy enable measurements of noise and the temporal dynamics of gene regulatory networks [45–49]. Microfluidic devices allow researchers to monitor single cells dividing for thousands of generations in precisely controlled conditions (Figure 2a). A design that has become particularly popular in recent years is commonly referred to as the ‘mother machine’ [46,50]. In this device, thousands of cell lineages grow in independent chambers, where each mother cell is trapped at the dead-end of the chamber and offspring are washed away from the opposite end. This configuration allows for tracking of hundreds of lineages for hours to days. Technical advancements to allow on-chip control of different parameters, such as pressure, oxygenation, and media switching, also offer exciting potential for improved experimental control [51].

The combination of automated time-lapse microscopy and microfluidic devices has resulted in increasingly larger and complex image datasets. Thus, advancements in accuracy and speed of single-cell image analyses have been essential. Significant progress has been made

on this front, including software compatible with agarose pads and mother machine images [52–56]. The implementation of machine learning approaches is also a promising tool [57,58], as exemplified by the incorporation of deep learning strategies into an image processing pipeline, which resulted in a significant increase in accuracy and processing speed [57].

Spatio-temporal control of gene expression

The technologies described thus far have improved our ability to quantify gene expression noise, as reflected in variations in the levels of single-cell mRNA or protein abundance. Novel synthetic biology and engineering tools are now enabling researchers to precisely manipulate noise levels while tracking cellular responses in real time. For instance, input media switching is a straightforward capability when applying microfluidics, which facilitates the application of regimens of small molecule inducers for the temporal control expression. As a recent example, Perrino *et al.* [59] implemented dynamic regulation of galactose-inducible protein aggregation levels in a yeast cell model of Parkinson's disease, effectively decreasing variability between single cells and controlling aggregation timing. Such precise time-varying control was achieved by implementing a feedback loop, in which a computer simultaneously processed real-time protein expression levels measured via time-lapse fluorescence and computed the duration of galactose pulses necessary to maintain the desired pre-set values. Additionally, a 'noise tuner' system was engineered in yeast through orthogonal control of transcription and mRNA degradation rates using two different inducer molecules [60]. The control capabilities offered by small molecules can further benefit from recent improvements in precision, orthogonality, and increased dynamic range of the sensors [61].

Light-based systems for manipulating gene expression in bacteria and yeast also offer advantages for probing the impact of noise, including rapid induction and easily programmable control (Figure 2b). A recent application combined single-cell optogenetic regulation of a transcription factor, real-time nascent RNA readout, and automated feedback to dynamically control transcriptional bursting in yeast [62•]. Using this platform to tune transcription to pre-specified setpoints significantly reduced cell-to-cell variability over time. Similar tools and strategies have also been applied to control population variability in *E. coli* during antibiotic treatment, a perturbation that leads to complex changes in cell physiology. Chait *et al.* [63] showed that closed-loop control of a light-responsive promoter is only slightly impacted during treatment with subinhibitory concentrations of doxycycline. Using this platform, reporter levels before and during drug treatment remained largely stable around a pre-defined target, significantly minimizing reporter noise in the population. These results highlight the potential applications of optogenetic approaches for investigating the role of noise in overcoming sudden stress. Recently, an optogenetic system has also been ported and optimized in the Gram positive bacterium *B. subtilis*, opening up exciting opportunities for characterization of noise and dynamics in this model system for cellular commitment and decision making [64•]. For example, optogenetically controlled components could be used for precise manipulation of the temporal dynamics associated with sporulation, competence, and timesharing of sigma factors [34•,35,36,38,40]. Optogenetic methods also offer excellent potential for manipulating dynamics to reveal

underlying properties of regulatory networks via comprehensive system identification studies; we point the reader to two recent reviews for further discussion on these opportunities [65,66].

Other emerging tools have the potential for refined time-varying control of gene expression in microbes. For example, inducible CRISPR activation/inhibition systems [67,68], photoactivatable recombinases [69,70], and electronic-based control [71–73] can be applied to perturb endogenous pathways within physiologically relevant ranges. Additionally, inducible asymmetric cell division systems offer unique capabilities for investigating mechanisms linked to phenotypic variation, such as the recently reported unequal partitioning of efflux pumps in bacteria [25••]. Tools for generating asymmetric cell division in a prescribed fashion [26,27] could help to reveal the role and impact of these partitioning events. In combination, these approaches have the potential to greatly expand our ability to probe the functional roles of phenotypic variability by allowing researchers to precisely control a population's environment while also tuning its phenotypic distribution.

Emerging technologies for single-cell isolation and 'omics'

The ability to quantify heterogeneity across expression of many of genes simultaneously and to connect phenotypes of interest to genotype has historically posed a major technical challenge. However, technologies to fill this gap are coming online. For instance, the recently developed methods PETRI-Seq [74] and microSPLiT [75] have achieved high-throughput bacterial single-cell transcriptomics for thousands of individual cells, unravelling hidden transcriptional states masked at the population level. In addition, two recent studies provide methods for tracking and then genotyping single cells. First, SIFT (single-cell isolation following time-lapse) allows the retrieval of single cells of interest after long-term imaging [76••]. The approach uses a remodeled mother machine device containing a series of valves in tandem with an optical trap to isolate individual bacteria after imaging (Figure 2c). Without the use of barcodes or any genetic modifications, single cells displaying complex and dynamic phenotypes can be collected alive and free from genetic and physiological perturbations and used in downstream analyses, such as for clonal expansion and 'omics' characterization. A second method, DuMPLING (dynamic μ -fluidic microscopy phenotyping of a library), performs *in situ* genotyping of strain libraries after time-lapse imaging in a microfluidic device [77,78]. With this approach, Camsund *et al.* [78] tracked the phenotypes of individual lineages derived from a CRISPRi library in a time-lapse movie. Then, after chemically fixing the cells in the device, the genotypes within the library could be optically inferred by sequential fluorescence *in situ* hybridization to a barcode. Although this approach requires barcoding cells before the experiment, it offers the capability to link every genotype to a specific phenotype. Together, these approaches offer the unique potential to connect phenotypes that are time-varying, single-cell, or subcellular to genotypic information, opening new possibilities for live single-cell microscopy assays.

Conclusions

The emergence of single-cell technologies has enabled long-term monitoring of gene expression and cell growth under carefully controlled and time-varying environmental

conditions. As a result, numerous studies have uncovered important biological consequences of gene expression noise, including many examples of functional roles for clonal microbial populations. Currently, joint efforts from the fields of engineering, computation, synthetic biology, and molecular biology are allowing researchers to not only monitor, but also accurately manipulate gene expression at the single-cell level while precisely controlling the environment and tracking real-time cellular responses. These technologies will fuel new discoveries on how cells control noise to produce reliable behavior, and also how microbial populations exploit noise to diversify their phenotypic landscape.

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Figure 1.

Functional roles of cell-to-cell heterogeneity in microorganisms. (a) Clonal populations of bacteria displaying varying expression levels of a gene associated with stress resistance show heterogeneity in survival outcomes upon exposure to transient stress. (b) Fluctuations in the expression of genes related to DNA repair and maintenance result in subpopulations of cells with increased mutation rates. Simultaneous tracking of reporters for mutations and genes of interest can reveal important information about the window of time within which cells are more prone to mutation. (c) Pulsatile dynamics of key transcriptional regulators may allow clonal cell populations to diversify their phenotypic landscape in constant environment conditions. (d) Genes involved in different phenotypic states can be activated in response to stochastic fluctuations in gene expression.

Figure 2.

Technologies for measuring and controlling gene expression at the single-cell level. **(a)** Microfluidic devices combined with time-lapse microscopy enable monitoring of single-cell growth and fluorescent reporters over many generations in precisely controlled conditions. Time trace representation of reporter expression for one single mother cell during growth in two different types of media. **(b)** Schematic of spatio-temporal control of gene expression in cell lineages growing in a mother machine device. Green light activates a light-responsive promoter, while red light represses transcription. Time trace representation of reporter expression for one single mother cell under a time-varying light regimen. **(c)** Schematic of mother machine device and single-cell isolation via an optical trap.