Microbial individuality: how single-cell heterogeneity enables population level strategies

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Abstract

Much of our knowledge of microbial life is only a description of average population behaviours, but modern technologies provide a more inclusive view and reveal that microbes also have individuality. It is now acknowledged that isogenic cell-to-cell heterogeneity is common across organisms and across different biological processes. This heterogeneity can be regulated and functional, rather than just reflecting tolerance to noisy biochemistry. Here, we review recent advances in our understanding of microbial heterogeneity, with an emphasis on the pervasiveness of heterogeneity, the mechanisms that sustain it, and how heterogeneity enables collective function.

Introduction

Colonies of microbes exhibit a large degree of physiological heterogeneity at the level of individual cells. One fundamental and long acknowledged type of heterogeneity is the accumulation of genetic mutations by subgroups in the colony [1]. Another, perhaps more

subtle, type of heterogeneity is the phenotypic cell-to-cell variation observed even in small isogenic colonies (i.e., when the whole population has the same genotype) and in spatially homogeneous environments [2] (Figure 1A,B).

Technologies that deliver individual cell resolution data, such as time-lapse microscopy, flow cytometry, microfluidics and single-cell RNA-seq, are being increasingly used to precisely quantify cell-to-cell heterogeneity in isogenic populations [3]. At the same time, theoreticians have developed models of this heterogeneity to understand the principles underlying it [4]. It is now apparent that single-cell heterogeneity is a widespread phenomenon, spanning many microbial taxa. Single-cell heterogeneity can manifest itself in processes as diverse as developmental programmes [5,6], metabolism [7], or the partitioning of cytoplasmic content at cell division [8,9].

In this review we examine recent advances in characterising phenotypic heterogeneity, the regulatory mechanisms that generate it, and its functionality. Phenotypic heterogeneity may exist only as a consequence of the stochasticity inherent in biochemical interactions, or may be an adaptive trait. We must therefore test whether heterogeneity at the single-cell level provides functionality to the population (Figure 1). Only then can we properly assess phenotypic heterogeneity as a relevant microbial decision-making strategy.

Phenotypic heterogeneity is a widespread phenomenon

Cell-to-cell heterogeneity often reflects variation in the abundance of intracellular proteins. This variation can be inherited, and can be amplified by the biochemical circuitry or the cell

cycle progression. Is cell-to-cell variation in protein abundance regulated? High-throughput measurements reveal disparities in how noisy some genes are relative to others within the same organism [10-12]. These disparities are not arbitrary because essential genes are typically less noisy than genes that are associated with stress or certain metabolic functions [10,11,13]. This pattern suggests that noise can be harnessed to drive cell-to-cell heterogeneity, and that different growth conditions will promote different levels of heterogeneity.

Stressful conditions can activate cellular responses that are heterogeneous at the single-cell level. In budding yeast, Msn2 is a general stress response factor, whose activity is regulated through a phosphorylation-dephosphorylation cycle [14]. When dephosphorylated, Msn2 translocates from the cytoplasm to the nucleus and activates transcription of a number of stress response genes. Glucose limitation induces stochastic oscillations in Msn2 translocation, which are desynchronised at the single-cell level (Figure 2A-C). The frequency of the oscillations is determined by the severity of the stress [15]. Interestingly, Msn2 shows different types of dynamics when induced by other types of stress. Under osmotic stress, for example, there is a single pulse of Msn2 translocation to the nucleus. There is little cell-to-cell heterogeneity in the timing and amplitude of this single peak of activity (Figure 2D-F). Different stress inputs have also been observed to generate different single-cell activation dynamics in general stress regulators in *Bacillus subtilis* [16,17] and mammals [18]. Stress specific activation dynamics may have evolved to allow the cell to choose the appropriate single-cell response (Figure 2) while maintaining a general stress regulator [14,19].

Some microbes respond to changing conditions by activating developmental decisions heterogeneously among the population, as is the case with the soil bacterium *B. subtilis*. Under stress, most cells in a colony activate a sporulation programme driven by the transcription factor Spo0A [5,20]. However, the transcription factor ComK can drive a small number of cells to activate a state of competence instead, causing the cells to uptake extracellular DNA. Given that spores are prevented from becoming competent, suggesting cells must choose one programme or the other, how is this decision made? Kuchina *et al*. [5] followed the activity of both transcription factors using two fluorescent reporters. Surprisingly, they found that the sporulation and competence programmes may both progress independently of each other within a cell until a decision-point is reached. An alternative model has also been proposed where Spo0A can promote and repress ComK, resulting in a limited time window during which competence can be activated [20]. In both cases, the outcome of the competition is based on the relative activity of Spo0A and ComK [5,20]. Competence is effectively shut down only after sporulation emerges as the winner. Although competence is rare, the probability of an individual cell becoming competent is a stochastically regulated trait that becomes apparent at the population level.

In bacteria, the best-known type of regulated cellular heterogeneity is the phenomenon of persistence. When exposed to a severe stress, such as antibiotics, most cells in a colony perish. However, a small fraction of cells can be unaffected and resume proliferation at a later stage [21,22]. Survival of these cells has been linked to a transient state of slow or arrested growth [21,23-25]. This phenomenon is different to antibiotic resistance, where cells express factors which directly inhibit the action of antibiotics. In some cases, the transition to a state of slow growth is a regulated, rather than passive, process [25-27]. This is likely to be a general characteristic of bacterial persistence, but very different underlying principles may be

involved, ranging from stochastic pulsing of regulatory enzymes [26] to a toxin-antitoxin competition controlled by auto-regulatory feedback [25].

The mechanisms of phenotypic heterogeneity are diverse

Heterogeneity can be mechanistically driven by noisy gene circuits, such as stochastic pulses in the activity of regulatory factors [19]. These pulses have been shown to allow cells to alternate repeatedly between active and inactive states of key cellular processes [15,16,19] (Figure 3A). If stochastic pulses are not coordinated across cells, two distinct subpopulations can coexist at any given time, and there is a dynamic turnover of cells from one group to the other. For many of these circuits the source of the noise has not been determined. One potential source of heterogeneity, transcriptional noise, has been well-studied in theory and experiment [28-30]. There are multiple examples of gene circuits that can use noise to generate alternative transcriptional states [4]. Bistable circuits are often generated by positive feedbacks (such as the mutual activation or inhibition between two genes), and underlie many mechanisms that can maintain heterogeneity (Figure 4A-C) [31-33]. In bistable circuits, a threshold crossing input will fix the system into one state (Figure 4B,C). This carries risks (e.g., the energetic cost of degrading the memory of the last transition), and so organisms may favour mechanisms which allow the transient entry into an alternative transcriptional state, such as excitable circuits (Figure 4D). Excitable circuits undergo a stereotyped pulse of activation after a threshold crossing event, and, during this pulse, are insensitive to further input (Figure 4E,F) [34,35].

The mechanisms of cell-to-cell heterogeneity can be decisively influenced by extrinsic variation originating from other processes in the cell. Bulk-level studies have suggested that global physiological factors, such as growth rate and the cell cycle, affect gene expression the most [36-39]. Single-cell studies are now revealing how important the feedback of growth is to cell-to-cell heterogeneity [32,40-42] (Figure 3B). For example, fluctuations in metabolic gene expression can cause growth rate fluctuations, which can in turn perturb expression of not only metabolic genes, but also other unrelated networks [40]. A feedback loop between growth rate and gene expression can also play a role in bacterial resistance to antibiotics. Under a translation-inhibiting antibiotic, expression of resistance genes reduces the effect of the antibiotic, increasing the relative growth rate, which in turn leads to higher expression of resistance [32]. For a range of expression and antibiotic concentrations, a bistable population emerges, containing some cells in a state of growth arrest. Although a different phenomenon, this observation is reminiscent of the growth arrest state observed in a subpopulation of persistent cells [21]. In both persistence and resistance, the existence of a non growing subpopulation, involving an interplay among gene expression, growth and survival, may indicate the two phenomena are more similar than previously thought.

The dynamics that generate cell-to-cell heterogeneity can also be non-noisy (Figure 3C). In budding yeast, for example, cell division is asymmetric with a small daughter cell budding off from a larger mother cell. The replicative age of individual cells relative to one another is therefore measurable by microscopy imaging. When switched from rich media to media that is poor in metal ions, the cell cycle of daughter cells is arrested in the G1 phase, and so the population differentiates into two types: older dividing cells (the cells that were already present before the switch), and younger non-dividing cells [8]. The vacuole (which is a reservoir for metals) is kept in the mother cell: in times of scarcity, some microbes opt to

retain limiting resources in a subset of cells, rather than diluting them in the larger population [8]. Replicative age is also an important factor in predicting resistance to stress [8,9].

Organisms can take advantage of both stochastic and deterministic mechanisms to regulate their degree of heterogeneity. A noisy switch can be used to activate a phenotype and a deterministic switch to deactivate it. For example, in *B. subtilis*, the timing of the transition from a motile to a sessile state is highly variable, but the timing of the switch back to motility correlates tightly with the number of cell divisions undergone in the sessile state [6]. Similarly, in the sugar metabolism of *E. coli*, expression of the arabinose metabolic genes is heterogeneous at the single-cell level, with cells switching the arabinose system on at different times. However, once the sugar is exhausted, switching off appears to be abrupt and coordinated across the population [43].

Phenotypic heterogeneity implements population level functions

While phenotypic heterogeneity hinges on the expression of single-cell individuality, understanding whether it provides a function or fitness advantage requires a careful consideration of the environmental and population dynamics. Natural environments change in unpredictable ways, and it may be energetically costly to sense the change, or to respond to it in time. One solution is for cells to switch randomly between phenotypes appropriate to each environment at a rate that matches the historical probability of environmental change [44,45].

This behaviour forms the basis of the evolutionary strategy of bet hedging [46,47], in which different phenotypes coexist at any time, but only one phenotype is adapted to the

environmental conditions at that particular time (Figure 1C-G). The maladaptive phenotypes have lower fitness, but offer indirect fitness benefits because they free resources for their clonal sisters. More importantly, their phenotype may enable them to resist future catastrophes, thus ensuring the population survives [48]. Recent studies sought the mechanisms of stochastic phenotype switching by evolving heterogeneity-generating networks *in silico* [33,49]. Kuwahara and Soyer [33], for example, evolved bistability by selecting for non-linear protein production rates in stochastic environments. A common theme in all these models is to measure heterogeneity in relation to certain patterns of environmental variation [31].

Bet hedging has been proposed as the strategy behind bacterial persistence, and it is straightforward to see why it is a beneficial strategy [21,25]. Persistent cells pay a huge fitness cost in mild environments, because typically these cells do not grow [21], but the potential long-term payoff is survival to extinction. Presumably, the rate of antibiotic exposure in the wild is slower than the time scale of the cell cycle, and so persistent cells are usually a minority. Perhaps surprisingly, some microbes may bet hedge on their metabolic states [7]. A single-cell study of the diauxic shift from glucose to cellobiose in *Lactococcus lactis* shows this particular lag phase is not characterised by a coordinated population level acclimation to the second sugar, but by variable individual cell responses [7]. During the shift, two groups emerge: one that stops growing, and another that continues to grow by metabolising the second sugar. The level of catabolite repression and activation of stringent response factors regulate growth arrest. Critically, when transferred to a medium with galactose as the sole sugar, the cells that did not metabolise cellobiose outgrew those that did metabolise cellobiose [7]. This suggests *L. lactis* cells are bet hedging to maximise long-term population level growth based on unpredictable availability of carbon sources in the future.

Despite being unicellular, many microbes form communities and aggregates of heterogeneous cells, where subgroups of cells perform specialised roles and interact with each other to enable new population level functions. Such 'division of labour' [24] underlies the formation and maintenance of biofilms (Figure 1H-J), which are highly heterogeneous in space and time [50-52], and the dynamics of infection by *Salmonella typhimurium* (in which division of labour can, in fact, occur in conjunction with bet hedging) [24]. These examples bridge the conceptual gap between unicellular and higher organisms, and demonstrate how microbes explore cell-to-cell heterogeneity to flirt with multicellularity. The most evocative demonstration of this is the *de novo* evolution of multicellular budding yeast in the laboratory by Ratcliff *et al*. [53]. Cells were grown in liquid culture and, over many generations, repeatedly selected for their ability to sink due to gravity. Clusters of cells sink faster than single cells, and so were selected. These clusters were maintained by post-division adhesion, which assures the cluster is made up of clonal cells, and by the emergence of division of labour: the coexistence of cells committed to cell death with cells committed to division was necessary for the reproduction of the cluster and the perpetuation of the system [53].

Conclusion

Single-cell technologies have transformed our knowledge of microbial behaviour, allowing us to move beyond the limitations of bulk-level observations, and feed a number of exciting scientific propositions. First, noise is pervasive in the cellular environment, generating cell-to-cell heterogeneity [34]. Second, cells evolved genetic circuitry to regulate and use heterogeneity to implement single-cell level functions [14,16,19]. Finally, some single-cell

behaviours ought to be seen instead as demonstrations of collective functionality stemming from heterogeneity [54].

Heterogeneity spans across different scales and levels of organisation, and so a comprehensive synthesis may be elusive. It will likely require an expansion of our set of quantitative tools. Time-lapse microscopy is currently the only technique that allows examination at the single-cell level through time, but is limited to three-four simultaneous products due to spectral overlap of fluorescent proteins. However, new single-cell high-throughput techniques, such as single-cell RNA-seq [55] or super-resolution bar coding [56], could allow snapshots of single-cell expression across the genome. Presently, some of these methods remain difficult to apply to prokaryotic microbes, due to the size of these cells and their short-lived transcripts [12]. At another level, the demonstration of collective functionality will require a renewed focus on observing microbes in conditions as close as possible to their natural context. Biofilms, which incorporate single-cell behaviours, as well as cell-to-cell signalling, such as quorum sensing, offer a clear example [57]. It will also be critical to measure variation and heterogeneity away from the stereotypical, but likely rare in the wild, exponential growth phase [58]. These endeavours will likely nurture exciting new dialogues between microbial systems biology, ecology and evolutionary biology [47,59,60].

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Figure Captions

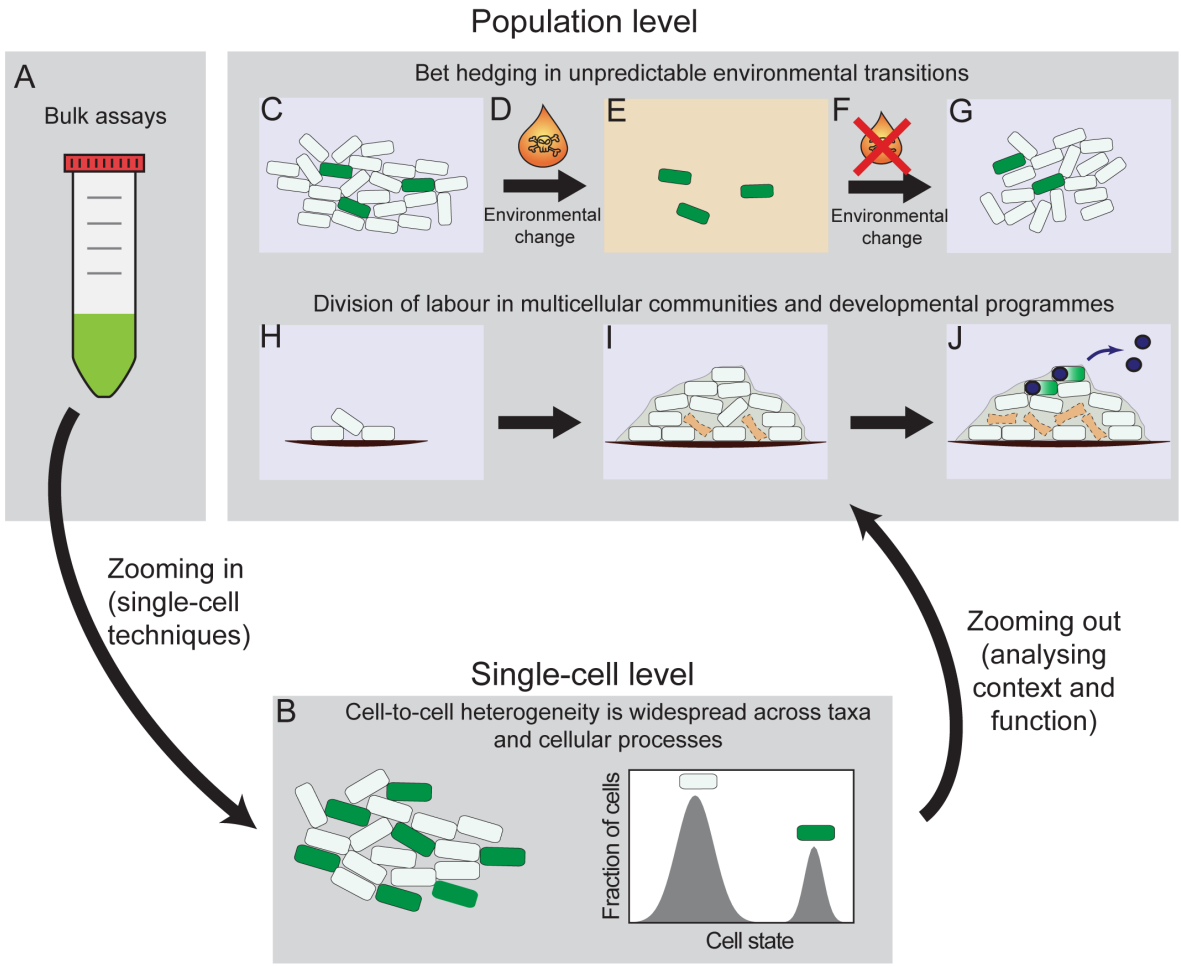

Figure 1: The loop between population level and single-cell level studies. A) Much of what we know about microbial physiology comes from bulk assays, but these fail to capture the heterogeneity that can operate at the level of isogenic single cells. Single-cell techniques can identify differences between individuals. **B)** For example, an isogenic colony may

contain cells in two distinct cell states (light grey and dark green cells). **C-J)** Cell-to-cell heterogeneity often reflects collective strategies, so one must 'zoom out' back to the level of populations to understand it. **C-G)** The strategy of bet hedging copes with unpredictable environmental change. Often, a fraction of bacterial cells growing in rich media displays a phenotype that is not adequate for that particular environment (dark green cells) (**C**). These cells can, however, survive an unpredictable stress (e.g., antibiotic exposure) (**D**,**E**), thus allowing the population to survive and thrive again in the future (**F**,**G**). **H-J)** Many microbes form multicellular aggregates and implement a strategy of division of labour, which allows the population to endure stress and activate developmental programmes. In microbial biofilms, the colony grows from a small aggregate (**H**) to a large sized community. Biofilms accommodate significant cell-to-cell heterogeneity. The growth of the structure relies on spatial and temporal regulation of apoptotic programmes (orange cells with dashed lines) (**I**), while the survival of the colony (**J**) is dependent on the successful sporulation, dispersal and future germination of a sub fraction of cells (dark blue spores).

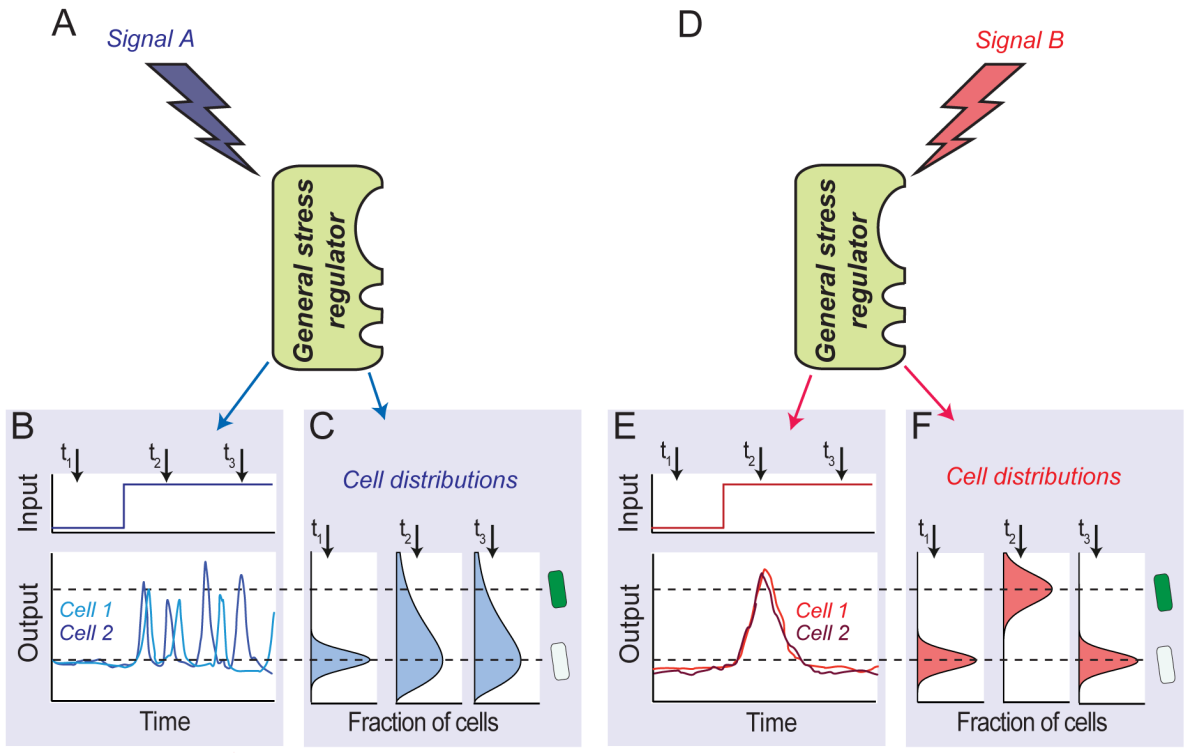

Figure 2: Heterogeneity reflects signal processing dynamics at the single-cell level. General stress regulators (e.g., Msn2 in yeast [14,15] and sigB in *B. subtilis* [16,17]) can respond to different stress inputs with different single-cell activation dynamics and distributions. In this example, Signal A (e.g., glucose limitation for Msn2 in yeast) (**A-C**) generates stochastic pulses in the activity of the general stress regulator (**B**), which results in a heterogeneous distribution of single-cell states (**C**). In contrast, Signal B (e.g., osmotic stress for Msn2 in yeast) (**D-F**) generates a single pulse of activation of the general stress regulator (**E**), which occurs in all cells and is homogeneous (**F**). Dark green cells and light grey cells represent two distinct cell states.

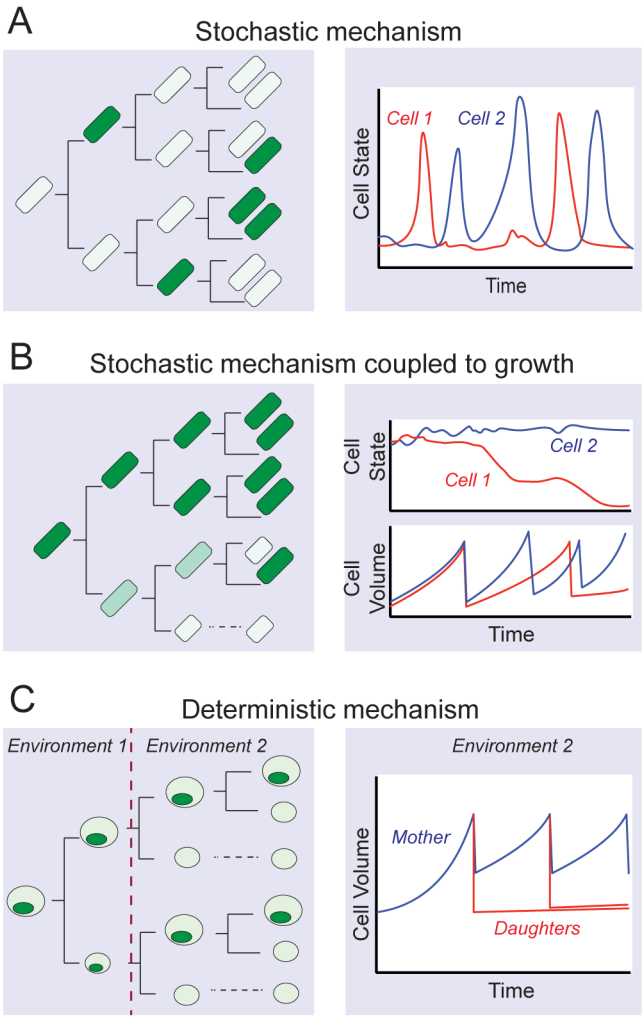

Figure 3: Mechanisms of cell-to-cell heterogeneity. A) Left: in a stochastic pulsing mechanism [15,16,19], a regulator protein switches randomly between two states of activity, generating two cellular phenotypes (dark green cells and light grey cells). The state of two sister cells need not be correlated. Right: the single-cell traces of the state of two cells reveal uncoordinated state switching. **B)** Left: growth rates can feedback on gene expression and amplify heterogeneity [32,40,42]. In this example, after the first cell division, two sister cells stochastically diverge in their expression of a particular gene (green shades). This causes a difference in growth rates (represented by cells of different lengths in subsequent divisions). Lower growth may, in turn, further downregulate gene expression, resulting in subpopulations of slow growing, low expressing cells (short light grey cells), and fast

growing, high expressing cells (dark green cells). Right: the single-cell traces of two cells detail the feedback between expression (cell state) and growth. **C)** Left: cell-to-cell heterogeneity can be driven by a deterministic mechanism that differentiates cells by their age [8]. Some microbes, such as budding yeast, divide asymmetrically producing a smaller daughter cell from a larger mother cell. In rich media (Environment 1), all cells are dividing normally. In a resource-limited environment (Environment 2, to the right of the dashed purple line), only the mother cells divide, keeping limiting resources with them (dark green organelle). Right: single-cell traces of the growth of a mother cell (blue) and its daughters (red) in a resource limited environment (Environment 2) show the daughters in a state of growth arrest.

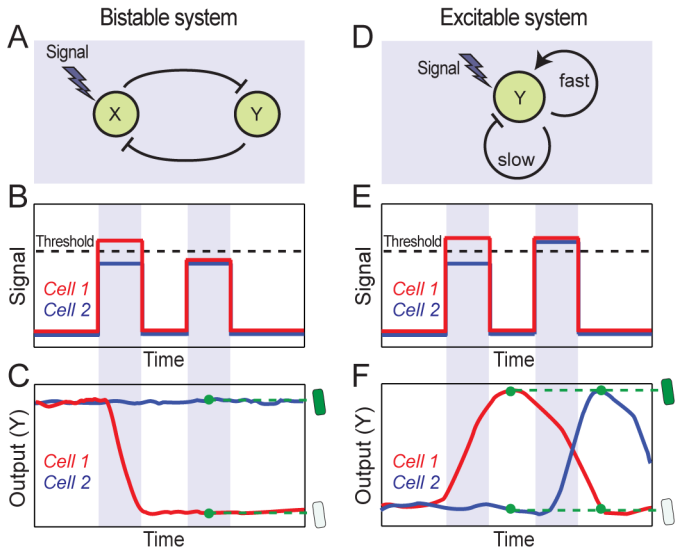

Figure 4: Bistable and excitable circuits generate cell-to-cell heterogeneity. A) Bistable circuits [31-33] are generally driven by positive feedback loops, such as a two-gene mutual inhibition circuit. An external signal acts on component X, which downregulates an output Y. Y, in turn, downregulates X. **B, C)** Two cells are repeatedly stimulated by a step-like signal that is of the order of a threshold (dashed line). Owing to stochastic fluctuations, cell 1 (red

line) senses a signal above the threshold and switches to a low output state. Cell 2 (blue line) remains in a high output state. **D)** Excitable circuits [34,35] are generally driven by positive and negative feedback loops with different time-scales. An external signal acts on an output Y. Y auto-regulates itself with a fast positive feedback loop and a slow negative feedback loop . **E, F)** Cell 1 (red line) senses a signal above the threshold and undergoes a pulse of activation. Excitable circuits are unresponsive to further stimulation during a characteristic relaxation period, and so cell 1 does not respond to the second step, whereas cell 2 (blue line) does. Dark green cells and light grey cells represent two distinct cell states.

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