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# Leveraging and coping with uncertainty in the response of individual cells to therapy

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Non-genetic heterogeneity fluctuates over diverse timescales, ranging from hours to months. In specific cases, such variability can profoundly impact the response of cell populations to therapy, in both antibiotic treatments in bacteria and chemotherapy in cancer. It is thus critical to understand the way phenotypes fluctuate in cell populations and the molecular sources of phenotypic diversity. Technical and analytical breakthroughs in the study of single cells have leveraged cellular heterogeneity to gain phenomenological and mechanistic insights of the phenotypic transitions that occur within isogenic cell populations over time. Such an understanding moves forward our ability to design therapeutic strategies with the explicit goal of preventing and controlling the selective expansion and stabilization of drug-tolerant phenotypic states.

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## Introduction

Surveys of molecular and phenotypic states of single cells have revealed pervasive heterogeneity, both between isogenic cells in a population and within the same cell over time [1•]. One potential source of such cell-to-cell variability is at the level of the signals themselves. For instance, individual cells exposed to the same dose of ionizing radiation will effectively receive a variable number of double-stranded DNA breaks [2]. However, even when environmental inputs remain constant, variability in responses can arise as a result of differences in initial states, such as cell cycle or differentiation stage [3]. In addition, cells that populate the same initial state, and are exposed to exactly the same signal, can exhibit different responses due to the stochastic nature of the biochemical reactions that govern the production and degradation of individual molecules [4]. Variability is therefore an intrinsic property of signaling systems that cells (and more recently researchers) have evolved to harness and cope with.

Non-genetic heterogeneity has been identified as a source of molecular and phenotypic diversity that leads to variable responses to treatment within isogenic cell populations. This plasticity has emerged as a predecessor and mediator of the evolution of genetic resistance, which ultimately leads to therapeutic failure [5,6]. Here, we summarize recent evidence of the way non-genetic phenotypic variation contributes to fractional killing in specific experimental systems, from bacteria to cancer cells. Recent technical and analytical developments provide the opportunity to quantitatively understand the dynamics of interconversion between cellular states, both from phenotypic and molecular perspectives. Such an understanding will be critical for designing strategies to optimize therapeutic outcomes that account for the presence of phenotypic heterogeneity and timescales of fluctuations in cellular states.

## Cell-to-cell variability limits the success of therapy

It has long been recognized that a small subpopulation of bacterial cells called 'persisters' can survive antibiotic treatment under drug concentrations that kill the vast majority of bacteria [7]. Bacterial populations that emerge from the expansion of persister cells after antibiotic removal exhibit similar drug sensitivity to that of the original cell population, arguing that persistence represents a reversible state that is maintained at low frequencies in bacterial populations [8]. Using timelapse microscopy to follow single bacteria over time, Balaban *et al.* showed that persister cells existed as a small fraction of growth arrested cells in unperturbed bacterial populations [9]. Notably, environmental and mutational perturbations can increase the fraction of persister cells in a population by engaging the stress response pathways that are stochastically activated in exponentially growing populations due to natural variability [10,11]. This suggested that the frequency of phenotypic switching and/or the lifetime of the persister state, could be subject to environmental or evolutionary modulation [12,13].

In striking resemblance to bacterial persistence, drug tolerant phenotypic states have been shown to mediate

fractional killing in cancer cell populations in response to targeted therapies. Sharma *et al.* showed that while the vast majority of lung cancer cells harboring oncogenic EGFR died within a few days upon exposure to the EGFR inhibitor gefitinib, ~0.3% of the cells survived treatment and remained in a quiescent state. Eventually, ~20% of drug tolerant cells were able to resume proliferation and could be propagated in the presence of drug concentrations that would be lethal to the drug naïve cell population. The transition from a quiescent to proliferative drug tolerant state was attributed to global changes in histone post-translational modifications and could be blocked by co-treatment with a histone demethylase inhibitor [14••]. Interestingly, such transition brought about concomitant changes in the average lifetime of the drug tolerant phenotype: while surviving quiescent cells re-gained sensitivity within ~9 doublings upon growth in the absence of drug, it took ~90 doublings for the proliferative drug tolerant cells to restore sensitivity [14••]. Thus phenotypic heterogeneity was not only present at the level of drug sensitivity versus tolerance, but also in the relative stability of the tolerant state (Figure 1a).

Similar results have been reported in the context of $BRAF^{V600E}$ mutant melanoma cells treated with BRAF inhibitor [15••]. Shaffer *et al.* investigated the origin of rare drug resistant colonies that emerged in the presence of $BRAF^{V600E}$ inhibitor [15••]. Using long-term live cell imaging they showed that these resulted from the expansion of a small subpopulation of cells that continued cycling normally in the presence of drug. Single molecule RNA fluorescent *in situ* hybridization (FISH) revealed that heterogeneity in the expression of resistance-associated transcripts preceded drug exposure. In addition, selective expansion of these pre-resistant cells upon treatment was accompanied by a gradual epigenetic reprogramming that transformed transient transcriptional variation in drug naïve cell populations into stable resistance in the course of ~4 weeks (Figure 1a). In an independent study, Fallahi-Sichani *et al.* discovered a subpopulation of melanoma cells that cycled slowly in the presence of BRAF inhibitor. Drug tolerant cells exhibited a de-differentiated molecular profile, which could be reverted by passaging in the absence of drug and blocked by inhibition of histone modifiers [16]. The activation of proteins involved in developmental plasticity has been linked to resistance in other models [17–19], suggesting that lineage switching could be a widespread mechanism to attain resistance.

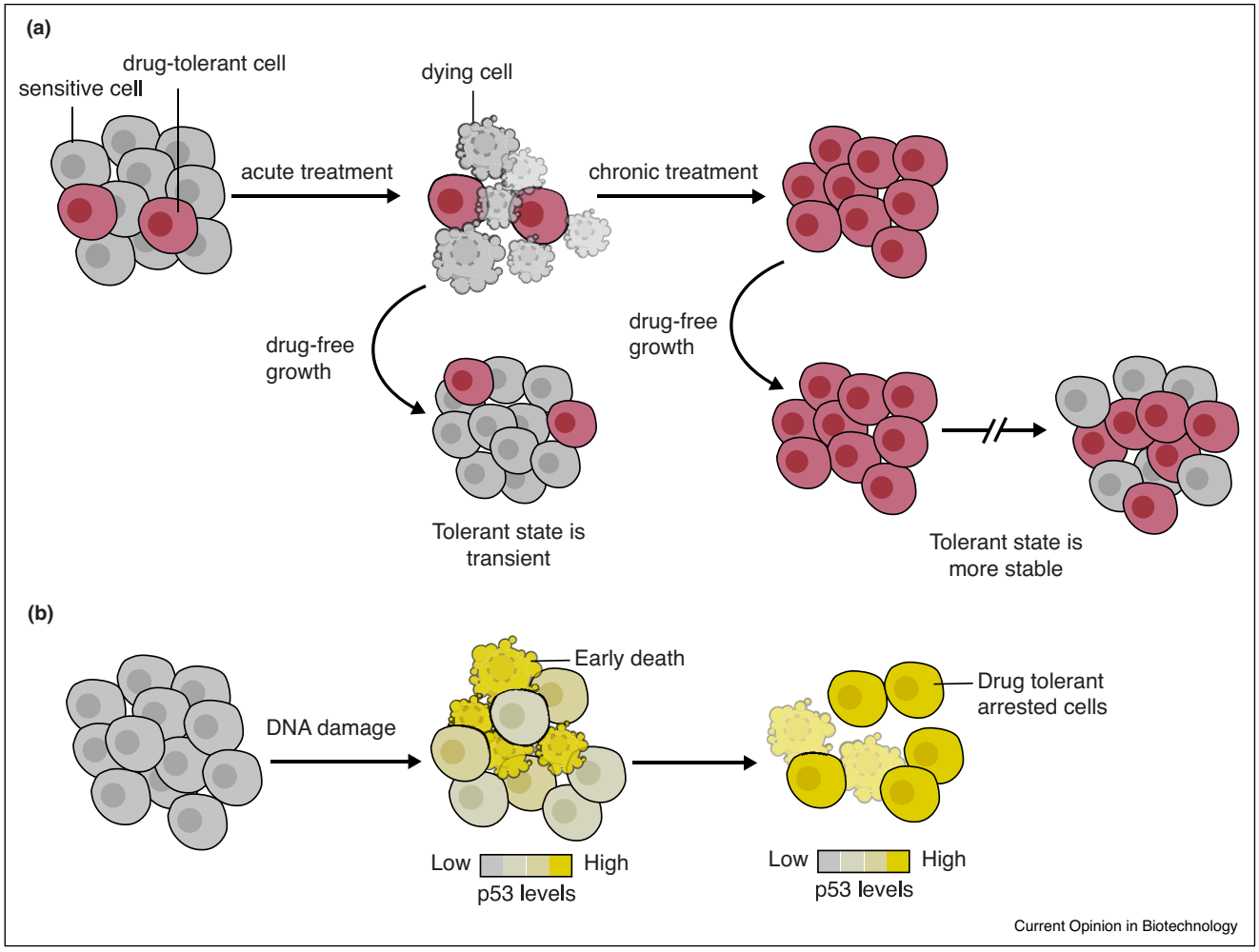
In addition to pre-existing stochastic phenotypic variation as a source of drug tolerance [14••,15••,20], cell-to-cell phenotypic variation can emerge as a direct consequence of the way individual cells respond to treatments. Paek *et al.* showed that exposure of colon cancer cells to chemotherapy led to heterogeneous activation of the pro-apoptotic tumor suppressor protein p53 and anti-apoptotic proteins. Fractional killing emerged as the result of competition between these antagonistic cellular programs: progressive accumulation of anti-apoptotic proteins gradually increased the threshold in p53 levels required to trigger cell death, limiting apoptosis to cells with early and high rate of p53 induction [21••] (Figure 1b). A similar mechanism was identified in the context of TRAIL induced apoptosis, in which the initial rate of caspase-8 activity was shown to distinguish whether a cell underwent apoptosis or survived chronic exposure to the ligand [22]. Thus, heterogeneity in signaling upon perturbations does not only contribute to variability in immediate cellular outcomes, but can also reshape the distribution of phenotypic states in the population and render cells transiently refractory to subsequent treatments.

Collectively, these and other studies have revealed a wide diversity in the timescales within which drug tolerant phenotypes fluctuate, ranging from a couple cell generations, when phenotypic variation is due to fluctuations in the levels of proteins directly involved in the response [23], to weeks or months, when phenotypic states become stabilized through engagement of self-reinforcing feedback loops or epigenomic reprogramming [14••,15••]. Short-lived states can precede the establishment of longer-lived drug tolerant states, with genetic variation ultimately conferring stable resistance [6]. It is thus critical to gain a phenomenological and mechanistic understanding of the rates at which cells enter, exit and stabilize drug tolerant states.

## Leveraging uncertainty to advance mechanistic understanding of the transitions between phenotypic states

The recognition of the presence of phenotypic heterogeneity in cell populations prompted the development of experimental paradigms to unmask such variability and to understand the dynamics of diversity-generating processes. Going back to the classical fluctuation analysis developed by Luria and Delbrück's [24], clonal expansions are powerful tools to unmask heterogeneity that is otherwise missed by population averaging. Similarly to clonal expansions, population bottlenecks such as the selective growth of cells expressing specific markers [25] or fractional killing after treatments [14••,21••,23] generate homogenized cell populations. The dynamics of phenotypic diversification after population bottlenecks hold information about the stability of cellular states even when specific details of the underlying molecular circuits are unknown. While stable phenotypic states persist after prolonged culture, transient states are expected to reconstitute the phenotypic diversity of the original population with a timescale that is defined by the dynamics of phenotype interconversion (Figure 2a). However, care should be taken when using this rationale to make inferences about the genetic and non-genetic character of

**Figure 1**

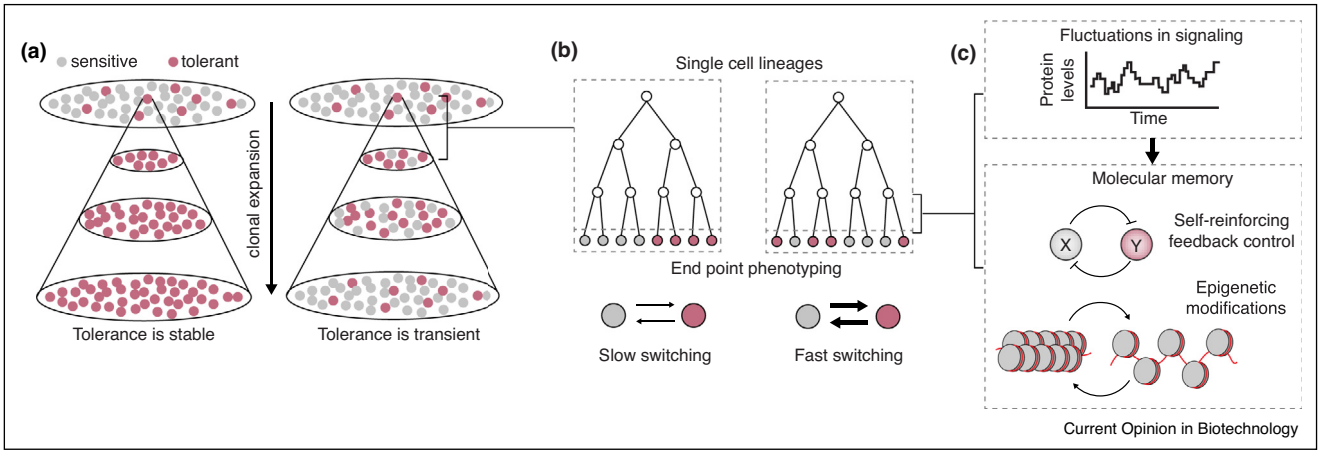



Non-genetic heterogeneity can pre-exist before, or arise as a result of, treatment leading to fractional killing. **(a)** Pre-treatment heterogeneity — Drug tolerant cells exist in a small fraction of drug-naïve populations. Upon treatment, these cells mediate fractional killing. Population heterogeneity is reconstituted rapidly upon drug removal, but recovery of sensitivity takes longer after chronic treatment. This suggests that persistent drug exposure engages a mechanism for the stabilization of drug tolerant states [14••,15••]. **(b)** Non-genetic heterogeneity in response to the treatment — Cells exhibit heterogeneous activation of DNA damage signaling upon chemotherapy. Cells with early and fast rate of p53 activation undergo apoptosis. Cells with late and slow p53 activation enter cell cycle arrest and survive even in the context of chronic drug exposure.

phenotypic variation. Non-genetic phenotypes that appear highly stable over limited experimental timescales could in principle be reversible over longer timescales. In addition, differences in growth rates between phenotypic states can contribute to the reconstitution of phenotypic diversity upon drug removal. For instance, 'drug addiction' [26,27] — which describes the genetically acquired dependency on drug exposure for proliferation — leads to population-level re-sensitization after drug removal due to competition between resistant and non-resistant cells, and not due to the interconversion between transient phenotypic states. Thus, researchers should be careful when making inferences about *single-cell level processes* from temporal changes in the diversity of *cell populations*.

Recent developments in our ability to observe individual cells over time provide the opportunity to characterize, with high temporal resolution, the unfolding of phenotypic diversity in single cells. Using microfluidics and live-cell imaging, Norman and colleagues recorded rare phenotypic switching in individual *B. subtilis* cells. The waiting-time distributions between switching events revealed insightful features of the mechanisms driving phenotypic transitions: exponential waiting-time distributions indicated memoryless switching where transitions occurred at a constant rate, while non-exponential waiting-time distributions were interpreted to result from the contribution of individual rates in a multistep process [28,29]. Recently, Hormoz and colleagues developed an

**Figure 2**




Towards a phenomenological and mechanistic understanding of the dynamics of drug tolerant phenotypic states. **(a)** The reconstitution of phenotypic diversity after clonal expansion can provide insights into the stability of drug tolerant states. **(b)** Live-cell imaging provides the opportunity to zoom into the process of diversity generation from a single cell. By combining information about lineage relationships and end-point single cell phenotype, it is possible to estimate the rates of phenotypic transitions. Adapted from Hormoz *et al.* [30••]. **(c)** Understanding the way fluctuations in signaling molecules interact with stabilization mechanisms, such as self-reinforcing feedback loops and epigenetic modifications, is critical for controlling the emergence of drug-tolerant states before and after treatment.

elegant experimental and analytical strategy that combines end-point phenotyping and single cell lineage relationships to infer transition rates between defined functional states [30••,31]. Intuitively, the correlation in cellular states between cells that diverged recently in the lineage are expected to be strong when phenotypes are stable in relation to the cell cycle length, and to decay as state transitions become faster (Figure 2b). Inferences based on kin correlations will be powered by recently developments to infer lineage relationships from fixed cells [32], allowing access to experimental systems that are difficult to address using live imaging. Lastly, synthetic memory devices can be used to record histories of phenotypic transitions within cell populations and to estimate the cumulative probability of switching over time, a valuable approach to understand the dynamics of switching over timescales that are prohibitive for live-cell imaging [33].

The elucidation of molecular mechanisms that mediate the stochastic interconversion between phenotypic states will be critical to account for non-genetic heterogeneity in a clinical setting. Single isogenic cells exposed to the same environment are independent realizations of very similar dynamical processes. Therefore, the analysis of single cells offers the opportunity to dissect, in an internally controlled setting, the way variation in specific molecules contributes to phenotypic heterogeneity. As a special case, comparisons between sister cells are controlled not only for shared environment, but also for the entire cellular history prior to division [30••,34].

Live-fluorescent reporters, recently powered by the development of novel genome engineering technologies [35], allow the detailed quantification of the temporal fluctuations in key signaling proteins before and after treatments, revealing patterns that are hard to infer from fixed timepoint assays [21••,22,36]. Complementing this approach, high-throughput assays such as CyTOF [37,38] and single-cell RNA sequencing [39,40] allow the simultaneous profiling multiple molecular species in the same cell. The analysis of these single cell profiles has led to the identification of substructure in cell populations, providing the opportunity to delve deeper into the functional differences of molecularly defined subpopulations of cells. In addition, the density of cells within particular cellular states has been used to estimate the average time cells dwell in such state [41], providing insights into the relative stability of distinct phenotypes. Together, these technological and analytical developments are important milestones towards the overarching goal of understanding the way fluctuations that occur within the timescales of production and degradation of mRNAs and proteins can bring about large phenotypic changes, such as drug tolerance or sensitivity (Figure 2c). Such insights will be pivotal in efforts to predict and control the emergence of phenotypic diversity in pathological cell populations.

## Coping with the uncertainty in the response of single cells to therapy

Therapeutic strategies that take into account diversity in drug tolerance within cell populations hold the potential to reduce or forestall the emergence of resistant clones. One promising approach is to reduce the burden of

phenotypic heterogeneity in the population through sequential treatments, as has been proposed to cope with karyotype heterogeneity in yeast and cancer cells [42]. For instance, since cancer cells that are quiescent or in early G1 are more likely to survive chemotherapy [43], synchronized cell cycle re-entry in the population could paradoxically improve the efficacy of acute DNA damaging agents [44]. Targeting global epigenetic modifiers is a promising strategy to prevent the progressive stabilization of drug tolerance [14••,15••] and revert such phenotypic states [20]. The identification of vulnerabilities specific to drug tolerant phenotypes could be used to prevent the selective expansion of these subpopulations during therapy. Moreover, genetic alterations can limit the ability of cells to establish drug tolerant quiescent states. For instance, p53 mutations result in impaired entry into cell cycle arrest after DNA damage [45]. Targeting cells that fail to establish a transient drug-tolerant state could selectively ablate the expansion of mutants while sparing their wild-type counterparts, as has been proposed in the context of cyclotherapy [45].

Complementing strategies to reduce heterogeneity in populations, therapies can be designed to account for the dynamics of phenotypic interconversion within cell populations [46]. Resistant states are frequently associated with quiescent or slowly dividing behaviors [14••,16,47,48], which stand in contrast to the actively diving cell populations from which drug tolerant cells originate. Theory is particularly suited to optimize therapeutic regimes that take into account tradeoffs in proliferation rates as well as the timescales of transitions between cellular states to control the overall growth of cell populations [49,50•,51]. Efforts to fine-tune the frequency and duration of drug exposure are expected to improve the outcomes of therapy by preventing adaptation and minimizing off-target effects that result from chronic drug exposure.

Fractional killing arises from heterogeneity at both the genetic and non-genetic levels [52]. Within non-genetic heterogeneity, different mechanisms can generate drug-tolerant states of different lifetimes. An emerging picture from the study of transient drug-tolerant phenotypic states is that short-lived states can be important for the establishment of longer-lived states. Therefore, a phenomenological and mechanistic understanding of timescales of phenotypic fluctuations is necessary to control the progressive stabilization of drug tolerance and resistance. As considerations of non-genetic heterogeneity are incorporated in the rational design of therapies, it will also be important to understand the extent to which cells can modulate the dynamics of phenotypic interconversion to adapt to treatment schedules.

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