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The Details in the Distributions: Why and how to study phenotypic variability

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Abstract

Phenotypic variability is present even when genetic and environmental differences between cells are reduced to the greatest possible extent. For example, genetically identical bacteria display differing levels of resistance to antibiotics, clonal yeast populations demonstrate morphological and growth-rate heterogeneity, and mouse blastomeres from the same embryo have stochastic differences in gene expression. However, the distributions of phenotypes present among isogenic organisms are often overlooked; instead, many studies focus on population aggregates such as the mean. The details of these distributions are relevant to major questions in diverse fields, including the evolution of antimicrobial-drug and chemotherapy resistance. We review emerging experimental and statistical techniques that allow rigorous analysis of phenotypic variability and thereby may lead to advances across the biological sciences.

Introduction

Although biologists are accustomed to thinking about the phenotypic variation that results from genetic or environmental diversity, even genetically identical individuals raised in nominally identical environments can display heterogeneity. We refer to this residual variation as “phenotypic variability.” Phenotypic variability among clonal cells can be an advantageous and even necessary feature of biological systems [1, 2]. For example, tri-chromatic vision, as found in humans, depends upon stochastic processes that underlie the photoreceptor choice of individual cone cells [3]. On the other hand, phenotypic variability can be highly undesirable and even buffered during development [4]; for example, numerous polymorphisms interact to promote invariant heart formation [5]. Mechanisms that buffer phenotypic variability may degrade with age, as evidenced by several studies that find phenotypic variability correlates with age in mice [6], yeast [7], rats and humans [8]. Phenotypic variability is also relevant to drug resistance. In microorganisms, noisy gene expression creates heterogeneous growth strategies within clonal populations that allow some cells to survive antibiotic treatment [9]. Growth heterogeneity also contributes to chemioresistance in tumors [10]; a recent study identified an epigenetic basis for growth heterogeneity that allows some cancer cells to survive chemotherapy [11]. Understanding the causes of phenotypic variability could reveal treatment strategies that minimize drug resistance [12] or could elucidate the genetic bases of congenital diseases (like heart disease).

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Despite tremendous potential gain from an improved understanding of phenotypic variability, few research programs focus on variance, while studies of trait averages abound. Consequently, important phenomena go unstudied. As Islam et al. (2012) captured with a pithy analogy: "… analyzing gene expression in a tissue sample is a lot like measuring the average personal income throughout Europe—many interesting and important phenomena are simply invisible at the aggregate level [13]." Even when phenotypic measurements have been meticulously obtained from single cells or individual organisms, countless studies ignore the rich information in these distributions, studying the averages alone.

As a result, the mechanistic basis of phenotypic variability is only beginning to be understood. Phenotypic variability may result from environmental differences that are difficult to measure, such as unevenness in nutrient concentrations or unequal numbers of adjacent cells [14–16]. Alternatively, phenotypic variability may result from stochastic differences in gene expression that stem from the non-deterministic nature of molecular kinetics [17, 18]. Such differences can propagate; for example, a difference in the concentration of a single transcription factor can lead to different levels of transcription for many downstream genes [19]. Therefore, phenotypic variability is present at many levels of biological organization (Fig 1).

Understanding the causes of phenotypic variability will not only inform medical questions, but is also important to evolutionary biology, the agricultural industry and other branches of biological science. Recent evolutionary studies suggest that phenotypic variability may allow rapid adaptation to new conditions [20], or may represent a bet-hedging strategy that enhances fitness in fluctuating environments [7, 21]. Theoretical studies also suggest that phenotypic variability can be adaptive [22–26]. A critical challenge for evolutionary biologists is to understand how often phenotypic variability influences evolutionary trajectories [27]. In agriculture, variability is largely a nuisance, as uniformity in crop size, shape and ripeness increase harvesting efficiency and overall crop yield. However, selection for uniformity has its downside, as exemplified by the unfortunate loss of flavor in most supermarket tomatoes [28], not to mention the risks of monoculture [29]. Further work is needed to understand and ultimately control the degree of variability in crop production, as well as in industrial-production cell cultures [16].

In order to encourage greater attention to variability phenotypes, we discuss emerging experimental and statistical methods that allow rigorous study of phenotypic distributions. We then conclude by discussing the major open questions and the opportunities to make advances of intellectual and practical importance.

Experimental methods for studying phenotypic variability

The study of phenotypic variability presents three unique experimental challenges. Firstly, it requires measurements from single cells or individual organisms rather than measurements of population averages. This precludes many standard techniques from being used to quantify phenotypic variability, such as growth-rate measurements that rely on increases in cell density over time, or gene-expression measurements from bulk culture, as measured by microarrays or RNA-seq. Secondly, larger numbers of observations are required to accurately estimate phenotypic variability because the sampling error on variance is greater than on mean [30]. Thirdly, it requires an experimental design that enables separation of multiple factors that can affect phenotypic variation (*e.g.* measurement error and environmental differences) (Fig 2). We describe three methodologies – flow cytometry, high-content imaging, and single-cell RNA sequencing – that address the above challenges.

Flow cytometry allows large-scale measurements (millions of cells) of single-cell phenotypes making it an ideal technique to study phenotypic variability. Flow cytometry is

often used to study phenotypic variability within tumors [31]; for example, recent flowcytometry experiments demonstrated that initially homogeneous breast cancer [32] as well as melanoma [10] tumors become heterogeneous as cells switch between different functional states. Several recent experiments used flow cytometry to survey expressionlevel variability in yeast. Two revealed higher than expected expression-level variability for stress-responsive proteins, suggesting a diverse response to stress maximizes survival in harsh or unpredictable environments [33, 34]. Another experiment identified naturally occurring polymorphisms that affect expression-level variability [35]. Still another used flow-cytometry data from a previous study [36] to identify gene deletions that affect expression-level variability in yeast [37] (Table 1). In all four studies, the experimental design incorporated replicates or controls in order to separate variability differences caused by genetic perturbation from technical variation among experiments.

Although flow cytometry almost always reports data on single cells, thus providing information about variance, many researchers ignore these data, opting instead to focus on means or on proportions of cells surpassing an arbitrary threshold for whatever trait is being measured. The overlooked, but information-rich, data from previous studies can be used to answer questions about phenotypic variability without ever picking up a pipette [37].

High-content imaging (HCI) is another technique that overcomes the aforementioned three challenges associated with the study of phenotypic variability. It presents an advantage over flow cytometry in that it allows observation of more diverse phenotypes, including: 1) subcellular protein localization and cell morphology in fixed cells (reviewed in [38]), 2) protein translocation and dynamic gene expression in live cells (reviewed in [39]), and 3) single-organism phenotypes like behavior in *C. elegans* and leaf shape in *A. thaliana* (reviewed in [40]). Another advantage of HCI is that it allows many phenotypes to be measured simultaneously, and a growing number of opensource software projects automate analysis of the resulting data-rich image files (reviewed in [41]). Although HCI provides single-cell, high-throughput data that are ideal for studying phenotypic variation, most studies do not analyze phenotypic distributions, and instead focus on measures of central tendency (*e.g.,* means). Our recent study revisited these distributions, using previously collected HCI data [42] to identify candidate genes that buffer morphological variability within genetically identical yeast populations, then validating these candidates by collecting additional HCI data [43].

Relatively few other HCI studies have focused on variability phenotypes. In a landmark study in *E. coli* researchers designed a now widely used [33, 36,37] dual reporter system that quantifies stochastic expression-level variability [44]. Recent work in *C. elegans* used HCI to count individual mRNA molecules in intestinal precursor cells, demonstrating that stochastic gene expression variability underlies incomplete penetrance of a mutant phenotype [45]. We recently used HCI to study variation in single-cell growth-rate in yeast, revealing a correlation between slow growth and survival of acute stress [7] (Table 1). Another recent HCI experiment quantified the responses of singlecells to a signaling molecule, revealing variability in binary phenotypes (whether to activate the transcription factor NF-κB) and continuous phenotypes (activation time) [46]. HCI studies in human cells have shown that stochastic differences in gene expression underlie variability in apoptosis rates [47], proliferation rates [10], and drug survival [48], whereas a deterministic response to micro-environmental differences affects virus susceptibility [14]. Given its wide range of measureable phenotypes, HCI has the potential to provide broad insights about phenotypic variability.

Single-cell RNA-seq is an emerging technology that is poised to yield insights about expression-level variability between genetically identical cells, but that currently is not high-

throughput. It is limited (by cost and time) to analysis of ~100–200 single cells per experiment [13, 49]. Additionally, single-cell RNA-seq can only reliably quantify expression of medium- to high-abundance transcripts [50]. Despite these limitations, a few studies have used single-cell RNA-seq to answer questions about phenotypic variability. One such study developed a kinetic model of transcription that explains stochastic differences in gene expression [50] among mouse embryonic stem cells (single-cell RNA-seq data from [51]) (Table 1). Another study found that individual blastomeres from the same early mouse embryo have stochastic, allele-specific expression differences for 6% of heterozygous genes [52]. Rapidly advancing sequencing technology may soon overcome the limitations of single-cell RNA-seq to allow a new wave of genome-wide studies focused on expression variability at the RNA level.

The aforementioned technologies — flow cytometry, HCI, and single-cell RNA-seq — are primarily used to study phenotypes in single cells. Study of phenotypic variability in larger multicellular animals and plants may be enabled by agricultural data [15, 53]. The agriculture industry performs many phenotypic measurements of individual organisms, uses large sample sizes, and has increasingly turned to monoculture, thus minimizing genetic sources of variation. Nevertheless, these data remain largely unexploited by variability studies.

Statistical methods for studying phenotypic variability

Experiments that quantify phenotypic variability will produce distributions representing the phenotypes of hundreds to millions of single cells or organisms. Analyzing these distributions is challenging because they are influenced by multiple factors. For example, expression-level variability depends on stochastic processes, cell size, and mRNA abundance [33]. A clever experimental design can facilitate separation of these factors during downstream statistical analysis, for example, by simultaneously measuring single-cell expression level, size, and mRNA abundance, as well as the technical variation unique to the experimental methodology. Statistical modeling allows estimation of the relative contribution from each factor to the observed phenotypic distribution. However, classic statistical models make many assumptions, including that the variance is normally distributed, equal across all samples, and independent of the mean [54]. We first discuss how paying attention to these often-violated assumptions can provide insight about phenotypic variability, and then discuss how this guides selection of a statistical model.

The shape of a phenotypic distribution depends strongly on the phenotype of interest, and can provide clues about the mechanistic underpinnings of phenotypic variability. For example, our recent imaging study found that the distribution of growth rates among genetically identical yeast microcolonies is strongly left-tailed; this prompted experiments revealing a subpopulation of slow-growing cells that over-express a stress tolerance protein, TSL1, and that have enhanced survival of high heat [7]. Although consideration of a distribution's shape can yield valuable insights, sometimes a transformation such as taking the logarithm [53, 55] or the more general Box-Cox power transformation [43, 56] can make data sufficiently approximate a normal distribution. This could allow analysis via a more common or more powerful statistical model than would be possible for non-normally distributed data. Such transformations should be applied with caution, as they may change the scale of the resulting data [54, 57], or may be inappropriate if the data are known to follow a particular non-normal distribution, as is the case for count data (*e.g.*, RNA-seq) [50, 58].

Another property of biological data that may provide insight about phenotypic variability is the presence of unequal variances among samples (heteroscedasticity). The common view that heteroscedasticity is simply an obstacle to overcome completely misses the main point

of this review: that differences in variance between groups may be the most interesting aspect of a dataset [59]. Indeed, screens for genes that modulate phenotypic variability explicitly search for significant differences in variance between populations, for example, between different single-gene knockout strains [43]. To test for unequal variance, previous phenotypic variability studies have used Levene's test [60, 61], Bartlett's test [55] or the Fligner-Killeen test [62]. These tests look for variance differences between discrete, researcher-defined groups yet ignore continuous covariates like age or measurement timing that create differences within groups; this may reduce the power of these tests [30]. Alternate approaches to model heteroscedasticity are discussed below.

A special case of heteroscedasticity occurs when variance depends upon the mean. Mean-variance relationships, although potentially informative, can be problematic for studies of phenotypic variability because the effect of interest (variance) is confounded by another effect (mean). Mean-variance relationships might also be a symptom of improper transformation [30, 56]. A favored way to deal with mean-variance relationships (and with heteroscedasticity in general) is to extract variances that are independent of the mean and to model these separately. This extraction can be done by using non-linear regression of standard deviation on mean to estimate the mean-variance relationship, and then using the residuals from such a regression as mean-corrected measures of variation [30, 37,43,53]. The first step in this approach, plotting standard deviation versus mean, is a very useful diagnostic for whether and how variation depends on mean, and it should be standard procedure in all studies of phenotypic variability. Simple linear corrections for mean-variance dependence, such as the coefficient of variation (CV, standard deviation divided by mean), are occasionally used without first assessing whether such a correction makes any sense, and indeed biological traits often show unpredicted, non-linear mean-variance relationships [43]. Such non-trivial dependencies might be informative, perhaps suggesting a mechanistic link between the molecular machineries that affect mean and variance, but at the very least they must be taken into consideration in analyses of variation.

Many sources of variation contribute to phenotypic distributions. These sources are often mixed, which means some are "fixed" effects whereas others are "random" effects. Fixed effects take values that are repeatable and of inherent interest (*e.g.*, genotype), whereas random effects take values that are sampled from a potentially infinite set (*e.g.* measurement error). Two commonly used statistical approaches to separate mixed effects that contribute to trait variation are analysis of variance (ANOVA), which works best with balanced experimental designs, and linear mixed modeling (LMM), which is more flexible and can handle unequal sample sizes in both nested and crossed (Fig 2) experimental designs [54, 57]. The *lme4* package in R provides a free, open-source implementation of LMM [63]. LMM- and ANOVA-based methods can yield biased results when applied to data derived from non-normal distributions [30]. An extension of LMM, Generalized linear mixed modeling (GLMM) accommodates nonnormal distributions and is implemented in R through the *lme4* package [54]. However, GLMM has some drawbacks [57] that can be avoided if a log or Box-Cox transformation makes data approximate a normal distribution allowing analysis via LMM.

These methods model trait averages and can be extended to model trait variability. One such extension is to apply LMM or GLMM to model sample means and then to model the residuals from the first model as a measure of variance as described above. One drawback of this two-step approach is that mean effects are estimated assuming equal variances because heteroscedasticity is not modeled until step two, which may bias estimates [30, 53]. An alternate approach, Double Generalized Linear Modeling (DGLM), iteratively estimates sample means and residuals until estimates converge [56, 64]. Although estimates do not always converge [53, 65], DGLM has been successfully used to detect polymorphisms that

contribute to phenotypic variability [56, 65]. For large, balanced, normally distributed datasets, two-step models and DGLM perform with similar power and false-positive rates on simulated data [30]. A well-documented extension of DGLM that allows mixed modeling is implemented in the *hglm* package in R [66].

Conclusion

Previous studies of genetically identical individuals have provided the first clues about the phenotypic variability present in nature, illuminating cases where it is influenced by a genetic component, and assessing – for particular phenotypes – which genes have the greatest effect on variance. Three fundamental questions about phenotypic variability should be the focus of current research efforts:

1) How many genes modulate phenotypic variability?

Comprehensive studies have found that disruption of almost any gene in *S. cerevisiae* 67] and *C. elegans* 68] reduces average fitness (population growth rate) in at least one condition. Yet these studies are unable to determine which genes influence variance in fitness because, like most growth assays, they measure the average fitnesses of bulk cultures [69]. Very few genome-wide studies have sought to identify genes that modulate fitness variability. Our recent study developed new tools to quantify growth-rate variability among thousands of yeast microcolonies and identified mutations that alter the extent of microcolony growth-rate variation [7]. Only three genome-wide screens have identified genes or genomic regions that modulate variability in other phenotypes including gene expression (yeast) [37], morphology (yeast) [43] and sensory bristle traits (Drosophila) [70]. The results from these studies suggest that genes contributing to phenotypic variability are common, with hundreds of genes identified in the two yeast studies, and 28 genomic regions (on average encompassing ~40 genes each) identified in the Drosophila study. Identifying genes that influence variability is a first step toward controlling the degree of variability in agricultural populations, cell-production cultures, and even among tumor cells.

2) How common is natural genetic variation that modifies phenotypic variability, and is such variation adaptive?

Countless screens have identified polymorphisms that alter the mean values of fitness-related traits ranging from flowering time in plants to disease susceptibility in humans. Yet only a few studies have identified polymorphisms that alter the variances of such traits [35, 55,56,60,62,65,71–74]; many of these identified polymorphisms affect trait variance while also altering trait mean, which might imply that mean and variance effects are confounded. More studies are needed to illuminate the prevalence and adaptive values of polymorphisms affecting trait variability in nature.

3) What is the mechanistic basis of phenotypic variability?

The mechanistic basis of most phenotypic variability present in nature is not understood, with a few exceptions. Differences in gene expression can arise from asymmetric cell division, as in the bacteria *Sinorhizobium meliloti* 75] and *Mycobacterium* 76], and from chromatin-mediated switching in lung cancer cells [11]. Stochastic differences in gene expression can be transiently heritable [47] and are sometimes reinforced by dedicated regulatory circuits (reviewed in [2]). Genome-wide studies suggest that stochastic gene expression is common [33, 34]. Other studies suggest that stochastic gene expression may drive the evolution of mechanisms that buffer its phenotypic effects [45]; a breakdown of this buffering can reveal mutant phenotypes [45] and may underlie many human diseases [4]. Some mechanisms that buffer or otherwise modulate phenotypic variability may do so for multiple phenotypes at once. For example, impairment of a protein chaperone increases

variability for many leaf and root phenotypes within inbred *A.thaliana* lines [20] and deletions of particular genes, including some encoding chromatin regulators, increase variability of many morphological phenotypes within clonal yeast populations [43]. How variability at different levels (*i.e.* variability in chaperone levels, chromatin structure, transcript abundance) interacts to manifest in higher-order phenotypes is not well understood.

Many questions remain, but the experimental and statistical tools are now available to facilitate rigorous analysis of phenotypic variability. Such analyses promise to reveal ecologically, evolutionarily and physiologically important phenomena that had been obscured in the aggregate. The practical benefits of understanding phenotypic variability could be great. For example, a study using flow cytometry showed that maximum antibody fragment production in *E. coli* industrial cultures occurs not when total biomass is at its maximum value, but when the subpopulation of metabolically active cells is greatest [77]. Understanding the range of phenotypes present within cell populations also has practical medical applications. For example, in a tumor or a population of pathogenic bacteria, it might be possible to develop drugs that attack various population subsets rather than targeting the population average. An alternative approach might be to target a gene or protein that promotes variability, and then to treat the population with an anti-cancer drug or antibiotic effective against the cell type that dominates the resulting, more-uniform population. The ability to modify phenotypic heterogeneity remains to be realized, but existing evidence suggests it is a worthy — and attainable — goal.

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Highlights

- Variation exists within genetically identical populations raised in nominally identical environments
- This variation, called “phenotypic variability,” is relevant in medicine and agriculture
- The frequency, adaptive value and mechanistic basis of phenotypic variability in nature are unknown.
- New experimental and statistical methods allow rigorous analysis of phenotypic variability.

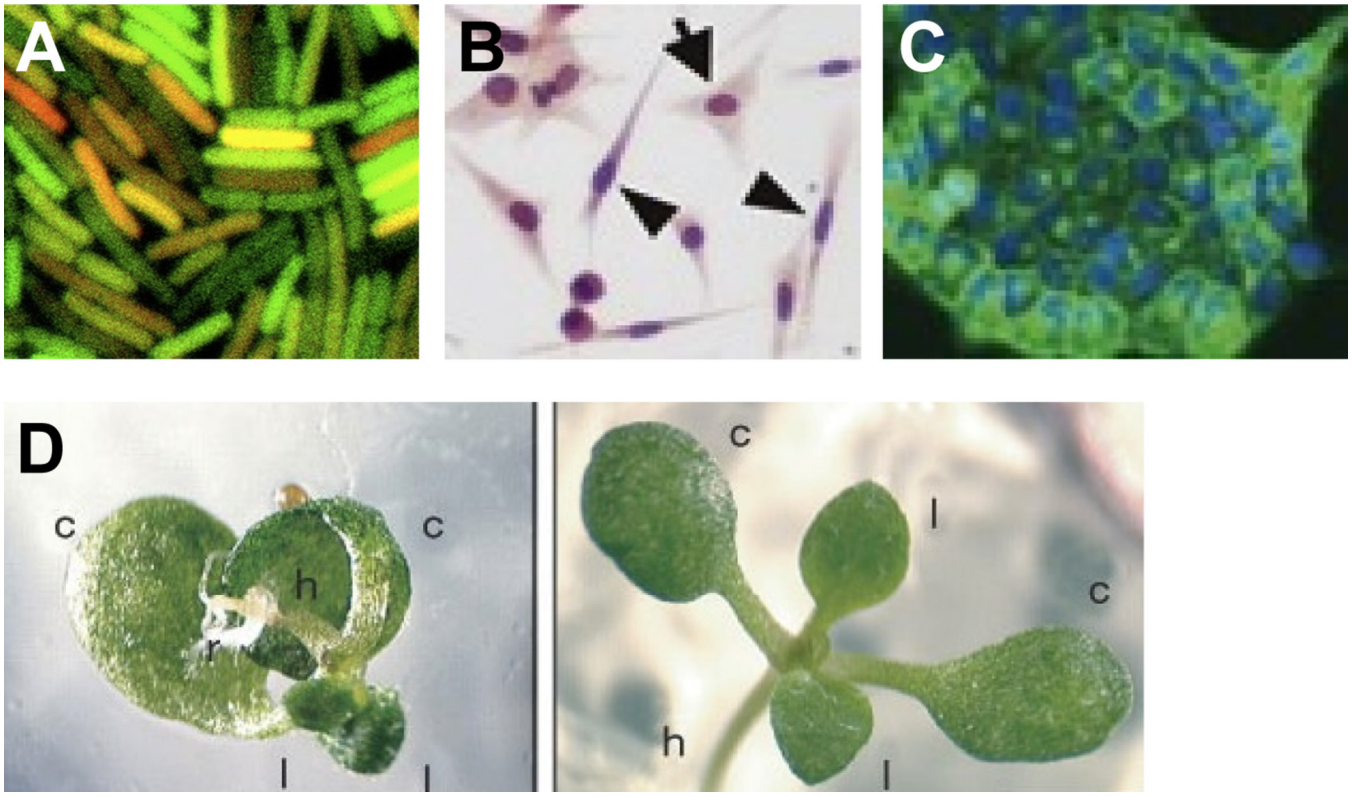

Figure 1.

Phenotypic variability is present at many levels of biological organization. (A) A within-cell difference in abundance between two fluorescent proteins expressed by the same promoter. This difference is not deterministic as different cells have different relative levels of each fluoresecent protein; reproduced with permission from [44]. (B) A between-cell difference in JARID1B expression gives some melanoma cells larger, rounder nuclei and slower doubling times than others from the same cell line; reproduced with permission from [10]. (C) A difference within structured, clonal populations of HeLa cells. Those at the islet edges (greener) are more susceptible to dengue virus; reproduced with permission from [14]. (D) A difference between multicellular organisms from the same inbred line. Morphological abnormalities in *A. thaliana* are revealed after drug treatment; reproduced with permission from [20].

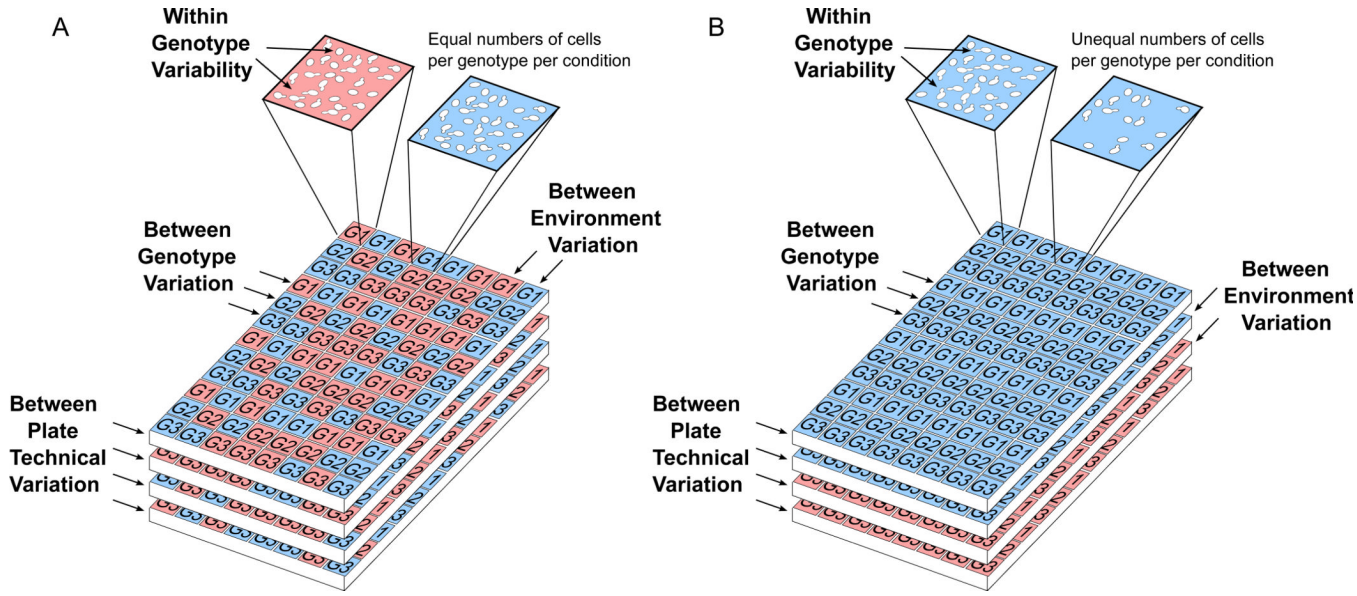

Figure 2.

Example experimental designs using 96 well plates. (A) A fully crossed design where multiple effects, both fixed (*e.g.* genotype, environment) and random (*e.g.*, technical error), contribute to yeast phenotypes. Any residual variance not explained by these factors may result from within-genotype variability. The experiment shown is balanced, meaning that equal numbers of cells are assayed for each genotype in each condition. (B) Design "B" is not fully crossed. Instead, technical errors specific to each plate are confounded with the environmental effect. Additionally, this experiment is unbalanced, which will influence statistical modeling. Although confounding effects can often be avoided by strategic experimental design, HCI experiments almost always produce unbalanced sample sizes between groups.

Table 1

Example studies combining experimental and statistical techniques to isolate phenotypic variability from other sources of variation.

Reference	Major Question	Experimental platform	How did the experimental design isolate sources of variation?	How did the statistical methods isolate sources of variation?
Rinnot *et al.PNAS*2011[37]	How much cell-to-cell variability in protein levels is due to stochastic events?	Flow cytometry	A fluorescent 2-reporter system distinguishes global variability, which coordinately affects reporters, from stochastic variability, which independently affects reporters (data from [36]).	The residuals from plots of fluorescence mean vs. CV are utilized to distinguish effects on variability from effects on mean.
Levy *et al.PloS Biology*2012 [7]	Does variation in singlecell growth and gene expression correlate with survival of acute stress?	High content imaging (HCI)	An experimental design similar to that in Figure 2 quantifies effects on growth variability from instrument error, genotypic differences, and clonal heterogeneity.	GLM is used to estimate the relative effect on heat-shock survival from clonal heterogeneity in growth rates vs. genotypic differences.
Kim *et al. Genome Biology*2013 [50]	Can expression-level variability present in mouse embryonic stem (ES) cells be explained by a kinetic model for transcriptional bursting?	Single-cell RNA-seq	Correlations between expression-level variability (data from [51]) and histone modifications (data from [78]) suggest a biological basis for cell-to-cell variation in gene expression.	A Poisson-Beta distribution is used to model the kinetics of stochastic gene expression caused by transcriptional bursting. Single-cell RNA-seq data from mouse ES cells fits this model.