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Review

Pitfalls and Practice of Luria-Delbrück Fluctuation Analysis: A Review¹

Wayne S. Kendal² and Philip Frost³

Departments of Cell Biology [W. S. K., P. F.] and Medicine [P. F.], The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77030

Abstract

Luria-Delbrück fluctuation analysis provides a method to estimate mutation rates in cell populations. Originally designed for bacterial populations, the method now is widely applied in somatic cell genetics and in cancer biology. However, there are fundamental genetic differences between bacteria and somatic cells, and this together with the inherent mathematical complexity of fluctuation analysis can lead to many pitfalls in its application. In addition there is considerable statistical error associated with the method. The use, misuse, and limitations of fluctuation analysis are reviewed here with the hope that such problems may be avoided.

Introduction

A decade before the advent of modern molecular genetics there was a controversy regarding how variant bacteria, resistant to viral lysis, arose in a much larger nonresistant population. Two contrasting hypotheses lay at the root of the controversy, spontaneous mutation *versus* acquired hereditary immunity. In an attempt to resolve this seminal issue Luria and Delbrück developed and applied the method of fluctuation analysis (1). Their studies were consistent with the hypothesis of spontaneous mutation; by means of statistical arguments the variant bacteria were shown to originate spontaneously and randomly with heritable transmission of their resistance. Fluctuation analysis has prevailed beyond this original application as an important method in somatic cell genetics and cancer biology, particularly for the measurement of spontaneous mutation rates. It is the current applications (or misapplications) of fluctuation analysis that are the subject of this review.

Before we discuss the methods and intent of fluctuation analysis let us define several terms. First, we use the term variation to denote changes in DNA base sequences, and also any other heritable change. We will use this more general term rather than the term mutation because of the fact that fluctuation analysis cannot distinguish between heritable changes occurring in the nucleus, mitochondria, or other cytoplasmic site, nor can it distinguish epigenetic changes (*i.e.*, heritable phenotypic changes unassociated with alterations in DNA sequences) from orthodox mutations. Second, variants are cells that result from either the variation of wild-type cells or the replication of existing variants. Third, we make an important distinction between variation rate and variant frequency (prevalence). In the context of this review, variation rate refers to the number of new variants that develop from wild-type cells per cell division. In contrast, frequency refers to the proportion of variants already existent in the whole cell population.

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² Fellow of the Alberta Heritage Foundation for Medical Research.

³ To whom requests for reprints should be addressed, at the Department of Cell Biology, Box 173, University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, 1515 Holcombe Boulevard, Houston, TX 77030.

The distinction between rate and frequency is of significant biological importance. For example, if one is interested in genomic instability at the level of gene control and expression (the importance of which will become clear in the next section), the rate of variation rather than frequency is a more relevant parameter to measure. Vastly different frequencies may be associated with similar variation rates, depending upon how early in the history of a cell population that variations occur, how well the variants reproduce, and the effects of selective factors. Simply to assess frequency as a measure of genomic instability would obscure the process of variant generation with that of cellular replication.

With these distinctions in mind we would like to describe why Luria-Delbrück fluctuation analysis remains important to somatic cell genetics, particularly in its applications to cancer biology. We emphasize the Luria and Delbrück methods because, while other ways to assess variation rates are available (2-6), the two methods of Luria and Delbrück remain the most popular.

Fluctuation Analysis and Cancer Biology

A fundamental area of investigation in cancer biology is that of tumor progression. Foulds (7) defined tumor progression as the stepwise acquisition of one or more traits in the development of a neoplasm. Although there is abundant evidence for the existence of tumor progression, its underlying mechanisms remain a matter for conjecture. Foulds considered tumor progression analogous to disordered embryological development (7). A contemporary model, proposed by Nowell (8, 9), considers tumor progression to result from an interplay between the generation of variant cells (a result of genomic instability) and the selection of variants with a survival advantage (a consequence of host factors). As a result of progression tumors become increasingly heterogeneous, being composed of variant cells with considerably different phenotypes (10).

An elegant demonstration of how tumor heterogeneity might develop within a tumor cell line was produced by Fidler and Kripke (11). In their study a modified form of fluctuation analysis was used to demonstrate that preexistent and highly metastatic variant cells developed spontaneously within a tumor cell population. This experiment provided early support for Nowell's model.

An additional prediction of Nowell's hypothesis was that increased genetic instability of tumor cells should be associated with increased malignant potential (8). This conclusion was based largely upon the association of an increased prevalence of cytogenetic abnormalities in tumors with increased malignant behavior (reviewed in Refs. 9 and 12). However, since the prevalence of genetic abnormalities is not as accurate a measurement of genomic instability as is the rate of their production, more stringent tests were needed to compare the spontaneous variation rates of tumor cells with different malignant potentials. It is at this point that fluctuation analysis was applied. Early experiments using fluctuation analysis indicated that the

rates of generation of drug-resistant variants for tumor cells *in vitro* were 3- to 7-fold greater in more highly malignant cells (13). Later studies using fluctuation analysis failed to demonstrate any such correlation (14-16). The reasons for these differences probably lie with the method of fluctuation analysis itself, and hence the relevance of this review.

Fluctuation analysis has found additional applications in cancer biology. For example, rates of spontaneous variation in fibroblasts from people with Bloom's syndrome (an autosomal recessive disorder that often leads to cancer at an early age) were found elevated relative to fibroblasts from normal individuals (17). In another series of experiments, using murine tumors, spontaneous generation rates of metastatic variants were purportedly much higher than the usually observed range for variation rates at loci involving drug resistance (18-21). These latter findings led to the "dynamic heterogeneity" model of malignant progression, a subject we will discuss below. Other applications of fluctuation analysis have included measurement of the rate of reexpression of metastatic potential in a non-metastatic tumor variant (22), the correlation of spontaneous variation rate with degree of viral transformation in Chinese hamster cells (23), and the rate of development of anchorage-independent variants *in vitro* (24, 25). These represent but a few examples of the ways fluctuation analysis have been applied in cancer biology.

Principles and Practice of Fluctuation Analysis

In order to intuitively understand fluctuation analysis let us turn to Fig. 1. The figure demonstrates that the prevalence of variant cells in a large population of cells derived by the expansion of a small cell number can vary widely because of both the variation rate and the time of appearance of any variation. Clearly, if similar cultures are expanded from equal aliquots of the same parental cells, the number of variant cells within each population will vary considerably as a consequence of the random appearance in time of each variant clone (Fig. 2). In contrast, if samples are taken from the larger parental culture and placed directly into selective medium, the number of variant

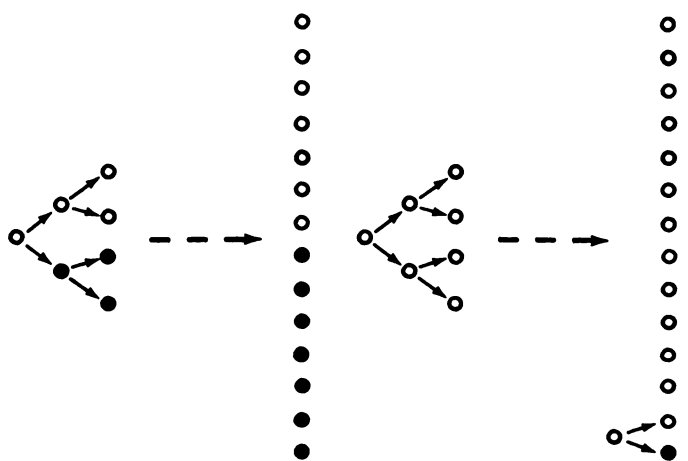


Fig. 1. Schematic diagram of the propagation of variant cells. We present here two hypothetical examples of variation and the propagation of variant cells. The first case, outlined on the left of the figure, shows that if during the expansion of a clone of cells a variation occurs on the first division and no further variations follow, then the prevalence of variant cells in the clone will approach 50% (assuming equal growth rates of variant and wild-type cells and minimal if any back variation). The second case, on the right of the figure, outlines the expansion of a clone where one variation has occurred, but during the last division. Both clones have been expanded to the same number of cells and the variation rate in each was the same; however, the prevalence of variants in each final population is vastly different.

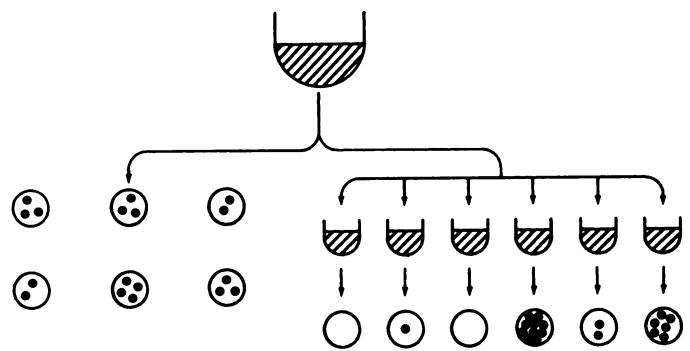


Fig. 2. Basis of fluctuation analysis. In this schematic diagram of a fluctuation experiment, initially a large population of parental cells is obtained. If in the first arm of the experiment (on the left) samples of the parental population are plated into selective medium, a random number of colonies will grow as a result of the survival of rare resistant variants. The number of colonies on each plate should follow a Poisson distribution (i.e., the variance of the number of resistant colonies should approximate the mean). The mean number of colonies per plate will reflect the prevalence of resistant variants in the parental population. In the second arm of the experiment (on the right) six small samples of cells are removed from the bulk parental culture and each is seeded into separate parallel cultures. The initial number of cells seeded is small enough so that we are assured that no preexistent variants have been included. As each parallel culture expands, variations may occur. When each of the parallel cultures has reached a set final number of cells, samples are taken from each and plated in selective medium. The number of surviving colonies reflects the prevalence of variant cells within the respective parallel culture. The number of resistant colonies ranges considerably between each parallel culture and follows a statistical distribution with much greater variance than a Poisson distribution (i.e., the variance of the number of resistant colonies should be much greater than the mean). In fact, before one may legitimately apply the Luria-Delbrück equations one must confirm that the statistical distribution is non-Poissonian in this manner. The wide variance is characteristic of the process of variation and it is the rate of these variations that is estimated by Luria-Delbrück fluctuation analysis.

cells per sample will follow a Poisson distribution and will express less intrinsic variability. The number of variant colonies from a direct sampling of the parental culture will reflect the prevalence of variants within the parental population, whereas the wide fluctuation in the number of variant colonies in parallel cultures reflects variation. The quantitative analysis of these fluctuations, fluctuation analysis, yields an estimate for variation rate.

A typical fluctuation analysis of somatic cells *in vitro* may be performed as follows. A group of parallel cultures are seeded with a predetermined small number of cells and allowed to expand to a larger fixed number. Because somatic cells are usually adherent to culture dishes (as opposed to bacterial cultures which propagate in suspension culture) it is necessary to detach and disperse the cells at some point after seeding the cultures in order to obtain adequate cell expansion without overgrowth or contact inhibition. Once the predetermined number of cells has been reached in each of the parallel cultures the prevalence of variant cells in each culture can be assessed. This requires that the cells be dispersed yet again and then plated in a selective medium. The number of resultant colonies that subsequently form in selective medium reflects the number of resistant cells present in the parallel cultures at the end of the expansion phase. Simultaneous with the plating of cells in selective medium, a small proportion of cells is also plated in regular medium so as to provide an estimate of the inherent plating efficiency of the particular cell population.

Luria and Delbrück provided two methods for estimating variation rates (1). The first method (the P_0 method) is based on the assumption that the fraction of parallel cultures with no variant cells, P_0 , can be used to characterize the Poisson distribution of the number of variations that have occurred during the expansion of each culture. This fraction can be related to

the variation rate, a . In mathematical terms (1),

$$a = \frac{-\ln(P_0)}{N},$$

where N is the final number of cells per parallel culture at the time of application of the selective agent. N is obtained by averaging the final number of cells from each parallel culture.

The second method (the method of means) uses the mean number of variant cells per parallel culture, r , present at the time of selection to characterize the different statistical distribution of the number of variants within parallel cultures. This second distribution is much more complicated since, as noted previously, variants arise both by variation of parental cells and replication of existing variants.

The value r is related to the variation rate (1):

$$r = aN \ln(NCa),$$

where C is the number of parallel cultures. The methods of means thus requires the solution of a transcendental equation, which can be easily achieved with the aid of an electronic calculator, and iterative trials with guesstimates of the variation rate.

Each of these methods possesses inherent theoretical advantages and disadvantages. The method of means allows for the correction of r with the plating efficiency; the P_0 method requires that only the presence or absence of variants in each parallel culture be known. The method of means may overestimate the variation rate, whereas the P_0 method may underestimate it (4). In practical usage, however, neither method is clearly superior and both are inefficient estimators for the variation rate (3, 5).

Considerations in Fluctuation Analysis

The rather complicated derivation of the formulae of Luria and Delbrück rests upon certain explicit and implicit mathematical assumptions. There are also specific biological considerations that are important to the design of fluctuation experiments. A lack of knowledge of these assumptions and considerations can lead the unwary investigator to inadvertently err in experimental design. In this section we review these considerations.

Process of Cellular Variation. Luria and Delbrück worked with bacteria. Their concept of cellular variation was simple and they could not have anticipated the more recently described myriad of mechanisms for heritable variation (26). They therefore made several assumptions regarding the process of variation. Foremost of these assumptions was that each cell in a population was considered to have an equal and constant probability to undergo a variation per unit time (1). This remains a reasonable first approximation, but it must now be tempered by experimental evidence that variation rates may periodically change (27). Perturbations in the rate, which could occur during the cell cycle, were considered to be averaged over the time of the cell cycle by the methods of Luria and Delbrück. While theoretical modifications do exist to account for these latter changes (without the assumption of a constant probability), these modifications have not been widely applied (4).

In order to account for any changes in the probability of variation that might occur in response to environmental conditions, Luria and Delbrück assumed that the probability of variation for an individual cell was directly related to its growth rate (1). This too was a reasonable assumption.

The requirement for a constant probability of variation limits the methods of Luria and Delbrück to the measurement of spontaneous variation rates. Any chemical, biological, or physical agent that might induce changes in the probability of an individual cell undergoing a variation during the period of culture expansion would make these methods inapplicable. For example, the prevalence of variants in a cell population immediately after chemical mutagenization will change as variants become expressed (28, 29), and there is no way that the requirement for constant probability can be assured. We also add parenthetically that after chemical mutagenization, changes in growth rate and growth advantage of selected cells may complicate any attempted application of fluctuation analysis.

Another consideration stems from the fact that in fluctuation analysis cells are classed as either variant or nonvariant (1, 4). That is to say there can be no intermediate states that would obscure the distinction between variant and wild-type cells. It is difficult to apply the methods of Luria and Delbrück to situations involving intermediate states without some additional assumptions or modifications of their procedures (30). Intermediate states do exist as in the case of gene amplification, *i.e.*, the development of methotrexate resistance (30–32), and have been demonstrated for other phenotypic properties such as growth rate and colony-forming ability in agar (33–35). Similarly, the methods of Luria and Delbrück are difficult to apply when more than one distinct population of cells coexist and undergo variation at different rates.

One last consideration regarding the process of variation is that Luria and Delbrück assumed that rates for back variation were negligible. While theoretical modifications have been proposed to take back variation into account, most notably in association with the “dynamic heterogeneity” hypothesis (21), for most loci their assumption is quite reasonable and should cause little difficulty.

Exponential Growth. Luria and Delbrück explicitly required that the growth of both variant and wild-type cell populations be exponential and have equal rates (1). This requirement may be relaxed without modification of their equations provided that the ratio of variant to wild-type growth rates remains constant (3, 4). Methods also exist to calculate the variation rate when the variant cells grow slower than the parental cells (6, 36).

A related consideration is that the Luria and Delbrück model does not account for cell death during the expansion phase of the parallel cultures (4). This is not a problem when dealing with bacterial cultures; however, in somatic cell culture problems do arise. As noted previously, most parallel cultures require the dispersal of the cells at some point during the expansion phase; it is often required at the time of variant selection. With cell detachment and dispersal a significant fraction of cells die, as reflected by plating efficiencies of much less than 100%. We are not aware of any modifications of fluctuation analysis that take this into account. The method of means, however, does allow a correction for plating efficiency at the time of variant selection. In addition to the consideration of cell death, it is assumed that with replating of the cells there is no selection of variant cells compared with wild-type cells, for any significant selection would result in an artifactually altered variation rate.

Requirements of Initial Seeding. Another critical requirement of fluctuation analysis is that one must be reasonably certain that no variant cells are seeded at the initiation of the parallel cultures (37). Luria and Delbrück assumed that any variants found at the time of selection arose during the expansion of the

parallel cultures. If, by chance, variant cells are seeded into the initial cultures, the rate estimates will be falsely elevated. Failure to provide for this critical requirement can introduce significant error in rate estimates. Fortunately the prevalence of most variants in parental populations is quite low and cultures initiated with a few hundred cells or less have an acceptably low risk of containing a variant.

Experiments designed to test the dynamic heterogeneity hypothesis of metastatic variation illustrate the critical nature of the initial seeding requirements. Briefly, this hypothesis supposes a very high rate of forward and back variation in the development of tumor cells with the capacity to metastasize (20). Much experimental work has been published to support this hypothesis (18–21). The assay used to select variant cells, capable of forming metastases, requires that the cells be injected i.v. into mice and the subsequent number of pulmonary metastases be counted. Unfortunately there is no known way to assess the fraction of cells within the initial population that have the inherent potential to form metastases and that can ultimately form such metastases after injection. The rate calculations in the dynamic heterogeneity model require the key assumption that cells with the capacity to metastasize do so with 100% efficiency. However, it is well known that as few as 0.1% of i.v. injected cells may survive the trauma of i.v. passage to form metastases (38), and even with the most highly metastatic cells only 2% form metastases (39). This is due to a variety of factors which randomly affect the survival of i.v. injected cells, including the stress of trypsinization, the trauma of i.v. injection, and host defense factors (39). This issue is further emphasized by experiments demonstrating that the coinjection of plastic microspheres with tumor cells can increase the resultant number of metastases by factors of up to 100-fold relative to injection of cells without microspheres (40). If all the cells with the metastatic phenotype had formed metastases in the animals given injections without microspheres, then the potentiation with microspheres should not have occurred. The assumption of 100% efficiency for the formation of metastases is therefore incorrect.

Since for the above reasons it is difficult, if not impossible, to assess the prevalence of cells with the capacity to metastasize within the parental population, there is no way to ensure that metastatic variants are not seeded in the initial parallel cultures. Because the variation rates estimated in such a situation could be spuriously elevated, cautious interpretation of such experiments is needed.

Phenotypic Lag. The term “phenotypic lag” denotes the putative delay between the occurrence of a variation and its expression. There is good evidence that some delay exists as a result of the time required for DNA strand segregation and the time for the wild-type gene messenger or its products to be diluted out by cell division (28, 41). The model of Luria and Delbrück does not account for this possibility (2), and its influence would be to artifactually lower the rate estimates obtained from their method (5). The effect of phenotypic delay has been the subject of considerable theoretical analysis and methods exist to correct for it (2, 4, 5, 41). However, in practice most investigators assume that its influence is negligible in relation to the other sources of error.

Metabolic Cooperation. When cells are plated at high density in selective medium it is possible that direct contact between resistant and nonresistant cells could influence the prevalence of surviving cells. This phenomenon is called metabolic cooperation and has been best studied at the hypoxanthine phosphoribosyltransferase locus (42–44). In this case if hypoxan-

thine phosphoribosyltransferase-deficient variants contact wild-type cells, the variants can express the wild phenotype, thus falsely lowering the observed prevalence of variants. Dominant markers such as ouabain resistance probably are not influenced by metabolic cooperation (45). Besides drug resistance loci, growth factors may give rise to metabolic cooperation (*i.e.*, the feeder layer effect). In practice most investigators provide suitable controls to rule out the possibility of metabolic cooperation irrespective of which marker is being used or which mechanism may be involved.

Variant Selection. The concentration of the selective agent used to identify variants may influence the prevalence of surviving colonies (28). In addition, it is also necessary to choose a drug concentration high enough to assure that only mutant cells are recovered. The performance of dose-response experiments on parental cell populations serves to resolve this issue. Generally there is sufficient flattening of the dose-response curve at high dosages to clearly demarcate variants from parental cells. However, as we have noted previously, in cases such as methotrexate resistance, a wide spectrum of resistance may exist within cell populations (31). It then becomes difficult to distinguish variant cells resistant to a given drug concentration from variants with different sensitivities. The methods of Luria and Delbrück do not account for this consideration.

Additional Biological Considerations. In addition to the points mentioned above, we list several other considerations. First, there is the matter of cell ploidy (46, 47) and gene copy number (14). Both of these factors could influence the prevalence of a variant phenotype. For this reason, especially when comparing cell lines, karyotypic analysis should be performed to assess these features. Second, as we have previously noted, fluctuation analysis does not distinguish between variants caused by epigenetic changes and those caused by nucleotide base changes, such as deletions or rearrangements.

In addition to these straightforward considerations, the factors that influence the production and expression of variant phenotypes in somatic cells are much more complicated than those in bacterial systems. For example, isolated clones can be more phenotypically unstable than polyclonal populations (48, 49). We have previously alluded to problems caused by the graded nature of certain phenotypic characteristics such as growth rate or colony-forming ability in agar (33–35). We would add here the difficulty of distinguishing between stable variants and physiological changes. Beyond these problems certain phenotypic characteristics may vary greatly from generation to generation as a result of mechanisms independent of variation. Differences stemming from variation may be only demonstrable after averaging repeated assays (35). A further difficulty arises when certain parameters are measured, *i.e.*, proliferative potential in fibroblasts or activity of 5 α -reductase in genital fibroblasts, when the rate of heritable change may be on the order of 1/cell generation (50, 51). With such high rates the methodological premises of fluctuation analysis are violated. All of these considerations indicate the need for much care in the application of fluctuation analysis to somatic cell genetics. It is important to recognize that there are biological situations where fluctuation analysis is not appropriate.

Statistical Error in Fluctuation Analysis. An important consideration in fluctuation analysis is the statistical error associated with the rate estimates. Luria and Delbrück recognized that the statistical distribution of the number of variants in parallel cultures was one with an abnormally high variance and extreme skewness (1). In practical terms this means that the mean and variance of the distribution are very inefficient statis-

tics for use in the estimation of variation rates (3). For that matter, the fraction of cultures with no variants, P_0 , is similarly unreliable (3). Considerable statistical error may thus be associated with fluctuation measurements (52). In fact, rate measurements performed with the same cell line and at the same locus but by different investigators have differed by as much as a factor of 1000 (53). The statistical problems associated with fluctuation analysis become unfortunately exemplified by one recent study where a significantly higher variation rate was claimed in neoplastic lymphocytes compared with normal lymphocytes (54). When a t test was applied to these data the difference proved to be not significant ($P = 0.3$).

A rigorous statistical analysis of the rate estimates is far from trivial and is beyond the scope of this review. We will, however, present an approximate analysis that serves to illustrate the significant statistical problems associated with fluctuation measurements.

Li *et al.* (52) provided an approximate method to predict the statistical errors associated with fluctuation analysis when the P_0 method was to be used. By means of a Taylor series expansion and by treating N as a constant,⁴ they showed the variance of the rate $[\text{var}(a)]$ to be approximated by:

$$\text{var}(a) = \frac{1-P_0}{P_0 C N^2}$$

Prior to the application of this equation one should confirm that P_0 is above about 0.2 and that N is indeed uniform between the parallel cultures, otherwise this approximation could be misleading.

Using similar mathematical methods and assumptions it is possible to calculate the error associated with the method of means:

$$\text{var}(a) = \frac{\text{var}(r)}{[N \ln(NCa) + N]^2}$$

The variance of r , $\text{var}(r)$, may be estimated from the numbers of variant colonies in the parallel cultures. The relative error of r should be small (<25%) for the approximation to be reasonably accurate.

Both of these error formulae probably underestimate the error associated with the rate measurements, since neither accounts for potential error in the measurement of N , nor do they account for biological factors overlooked in the Luria-Delbrück analysis. The error analysis itself may be misleading since an assessment based only on variance may not be adequate for the distributions characteristic of fluctuation analysis. Although the formulae are imperfect (as is fluctuation analysis itself), they provide a first approximation for the associated error. Alternatively, one may repeat a fluctuation analysis several times to assess empirically the statistical dispersion associated with the rate measurement.

Li *et al.* (52) provided an example that illustrated the significance of the statistical error in rate measurements; we will reproduce this example here. We would like to know how many parallel cultures are required in order that the rate measurements will differ between repeated experiments by a factor of no more than 2 to 3. For a cell line with an anticipated mutation rate (a) of 1×10^{-7} variants per cell generation, one can expand a series of parallel cultures to 1×10^7 cells each (N). The expected P_0 equals e^{-1} , or 0.368. In order to obtain a 95%

confidence interval for the measurement of the rate, a , such that repeated values will be in a range of 2- to 3-fold, the coefficient of variation for the rate should be about 20% (52),

$$\frac{[\text{var}(a)]^{1/2}}{a} = 0.2.$$

When these parameters are substituted into the above formula, the value for C becomes 43. For this anticipated level of experimental accuracy one thus must expand 43 parallel cultures to 1×10^7 cells each. If the consideration of metabolic cooperation allows one to plate cells in selective medium at a density of 1×10^6 cells in each 100-mm tissue culture dish this would require 430 dishes for a single fluctuation analysis. Such an experiment requires considerable resources. If still further accuracy or lower seeding densities are required the experiments would become prohibitively large.

Conclusion

Luria and Delbrück designed the method of fluctuation analysis to answer a specific qualitative question in bacterial genetics. The present day usage of this method goes far beyond what was originally intended. If the method is to be applied to somatic cells in culture, then considerable care must be taken in the design and analysis of each experiment so that meaningful data may be produced. Possibly the most appropriate application of fluctuation analysis to somatic cell genetics is in the qualitative demonstration of the consequences of variation. For example, preexistent variants within a cell population may be demonstrated (11), or the statistical distribution of variants in parallel cultures may be shown to have characteristics resembling the distribution expected with mutation (1). Certainly the quantitative application of fluctuation analysis to measure variation rates leaves much to be desired.

The problems with the interpretation of fluctuation analysis are most apparent when experiments to compare variation rates between different cell lines are considered. The statistical errors associated with rate measurements are considerable. When variation rates are compared the statistical errors become compounded; the variance associated with the difference between two rates is the sum of the variances from each individual rate measurement. Augmenting the statistical errors there are also the uncertainties introduced by the many biological variables. Taking these factors into account we would suggest that it would be difficult to detect differences in rates of less than one order of magnitude between different cell lines. The method of fluctuation analysis is much too inaccurate to satisfy the stringent requirements of these modern biological questions.

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⁴ N may be treated as a constant rather than as a random variable provided that there is minimal variation between the final numbers of cells in each parallel culture.

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