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Perspective

What are we missing in teaching the Luria-Delbrück experiment?

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ABSTRACT The importance of teaching the Luria-Delbrück experiment to biology students is increasingly recognized by educators, and improved pedagogical methods for teaching the classic experiment have been proposed and tested in the classroom. However, there are still obstacles that impede the proper teaching of the classic experiment. This note proposes two strategies to further improve the teaching of the classic experiment. The first strategy is to be frank with an inherent limitation of the classic experiment, and instructors should explain from a logical point of view why the classic experiment cannot be used to refute the possibility of directed mutation. The second strategy is to emphasize the pioneering work of Delbrück on developing the mutant distribution that enables researchers to estimate microbial mutation rates using data generated by fluctuation experiments, and instructors should shift their attention to the overlooked essential role of the mutant distribution.

KEYWORDS random mutation, fluctuation experiment, mutation rate, partial plating

The fluctuation experiment, also called the fluctuation test, was conceived by Luria and Delbrück 80 years ago (1) and is now regarded as a landmark experiment in the history of microbiology. A steady stream of innovative pedagogical research on teaching the fluctuation experiment (2–4) attests the experiment's lasting value in shaping students' thinking about evolution and about experimental design. In this note, I furnish some information that is overlooked in current teaching practice but that would help instructors explore new ways of teaching the topic to better meet the needs of 21st century biology students. Here, I address two shortcomings common in today's teaching of the fluctuation experiment. First, the historical pictures painted to students by instructors are largely incomplete and lopsided because instructors are often willing to dispel students' preexisting misconceptions at the cost of skipping a discussion about an important limitation of the fluctuation experiment. Second, the teaching of mutation rate and its computation using data from fluctuation experiments has been dominated by incorrect or obsolete methods, partly due to instructors' misunderstanding of the role of the Poisson distribution in the original mathematical treatment by Delbrück.

There are excellent descriptions of the classic experiment written for classroom use (2–4). Here, I offer a simplified version to highlight an important experimental detail that has been ignored by most instructors. A fluctuation experiment starts with a number (n) of liquid cultures residing in test tubes. [The value of n ranges from 10 to 27 in recent classroom examples (2, 4).] The experimenter inoculates each culture with a small number of wild-type bacterial cells and then incubates the liquid cultures. At the end of the incubation period, the experimenter transfers either the entirety or a portion of each culture onto a solid medium sitting in a Petri dish. This process is called plating. If only a portion of a liquid culture is plated, the plating is deemed partial or incomplete. When partial plating is adopted, the fraction of each culture plated is called the plating efficiency of the experiment, which is often denoted by ϵ. In the classic paper of Luria and Delbrück (1), partial plating was employed in all experiments except experiment 23, possibly for logistical convenience. Note that the solid cultures are appropriately coated

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with a selective agent that would eliminate all wide-type cells but that would allow each mutant cell to divide and form a visible colony. The experimenter counts the colonies in each dish as the experimental data to be analyzed.

The mutation-mutant principle

Proposed in 2003 (5) as a mnemonic, the mutation-mutant (M-M) principle can effectively help students grasp the essence of the fluctuation experiment's underlying statistical idea, and it can also help students acquire a balanced understanding of the role of the legendary slot machine at the Bloomington country club that enabled Luria to invent the classic experimental protocol. The M-M principle is easy for students to understand. If mutations occur only after the wild-type cells come into contact with the selective agent, then mutations can only occur after plating, on a solid culture. As a result, all mutants spawned by a single mutation will clump together forming a single colony because these mutants, being unable to move around on a solid culture, will not disperse to form separate colonies. Hence, one mutation gives one colony. In striking contrast, if mutations can occur without interacting with the selective agent, then mutations will occur before plating, in a liquid culture. As a result, mutants spawned by a single mutation will separate easily in a liquid culture. Therefore, each of these mutants will form its own colony after plating. Hence, one mutation may give one, several, or numerous colonies.

Students can benefit from assimilating the M-M principle in two important ways. First, the Poisson distribution serves merely as a reference point. Mutation, which can accompany each cell division with a small probability, should occur according to the Poisson distribution in view of well-known statistical theory. Therefore, whether the number of colonies obeys a Poisson distribution in a fluctuation experiment indicates merely whether each mutation contributes exactly one separate colony. As a corollary, a Poisson pattern of mutant colonies does not necessarily constitute evidence of directed mutation. This lack of an inherent link between the Poisson distribution and the directed mutation hypothesis is now easy for students to understand with the aid of the M-M principle, but this point escaped the attention of some early investigators. Second, despite its importance in the intuitive understanding of the fluctuation experiment, the Poisson distribution did not propel Luria to the discovery of the fluctuation test protocol. As research by Summers (6, p. 187) indicates, Luria was familiar with the Poisson distribution at least 3 or 4 years before he discovered the fluctuation experiment. What initially eluded Luria for a long time was a helpful characterization of the distribution of the number of mutants, not the number of mutations. The slot machine at the Bloomington country club offered Luria a vivid analogy between the number of mutants and the highly fluctuating slot machine returns. The M-M principle helps students see why Luria must consider the two kinds of distributions together before he could devise an experiment to show the possibility of random mutation.

An inherent limitation of the fluctuation experiment

The fluctuation experiment has an important limitation: it cannot be employed to establish the impossibility of mutations that are directed by the environment. Some instructors are unwilling to discuss this important limitation with students perhaps because they fear that such a discussion might hamper their efforts to dispel students' preexisting Lamarkian misconceptions. However, concealing this limitation of the fluctuation experiment from students may have an opposite effect on students who eventually will have access to the rich literature on past and current efforts to search for alternative mutational mechanisms called by some directed mutations. Students can develop a better understanding of the fluctuation experiment if instructors also help them see what the classic experiment cannot accomplish in addition to what the classic experiment has already accomplished. Instructors can facilitate this task by discussing all possible experimental outcomes from a logical point of view as suggested by Zheng (7).

TABLE 1 Possible experimental outcomes and their interpretations

Post-plating	Pre-plating	
	No	Yes
No	No conclusions	Random mutation only
Yes	Directed mutation only	Multiple possibilities

An outcome of a fluctuation experiment is a combination of the occurrence or nonoccurrence of pre-plating and post-plating mutations. Therefore, an ordered pair of yes/no logical values can be used to refer to any experimental outcomes. For example, the symbol (yes, no) refers to the outcome where mutations occur before plating but where no mutations occur after plating. Table 1 gives all four possible outcomes and their interpretations.

In all experiments reported by Luria and Delbrück (1), the variance to mean ratio far exceeded unity. In view of the M-M principle, these non-Poisson patterns pointed to the occurrence of pre-plating mutations. Because the phages were highly lethal to the nonmutants, the possibility of post-plating mutations was excluded. As a result, it was believed that the outcomes of these experiments were of the (yes, no) type. Therefore, these experiments allowed Luria and Delbrück to demonstrate the occurrence of random mutations elegantly.

However, the fluctuation experiment is no longer helpful when the experimental outcome is of the (yes, yes) type, as a (yes, yes) outcome can imply three possibilities. First, the post-plating mutations are just continuation of the random mutations occurring before plating, as there is no reason for the random mutations to cease to happen once the bacteria are exposed to a nonlethal selective agent (5). Second, part of the post-plating mutation is induced by the selective agent. That is not a new idea, as many chemical and physical mutagens have been discovered. However, mutagens may enhance mutation rates indiscriminately, regardless whether the mutation is beneficial to the bacteria. Third, rates to certain beneficial mutations have been preferentially enhanced by the selective agent. The last possibility could suggest directed mutation (8). In view of the M-M principle, all three possibilities should yield Poisson patterns of the number of mutant colonies. Hence, the fluctuation experiment cannot be used to distinguish the three subtly distinct possibilities.

Students will benefit from learning how Luria and Delbrück viewed this inherent limitation of their experiments. After asserting that their experimental results constituted proof of random mutations occurring in some special cases, Luria and Delbrück added this cautionary note: "It remains to be seen whether or not this is the general case" (1, p. 509). Furthermore, instructors can instill an effective dose of sound scientific attitude in students by relating how Delbrück responded to a report of Lwoff (9), who employed succinic acid as the selective agent, and in whose experiments, it took days for mutant colonies to appear. Lwoff used the term spontaneous mutation for those mutations that would confer ability to utilize succinic acid as the sole carbon source. Delbrück's response to that report, published along with the Lwoff paper, exemplified a good scientist's open-mindedness, objectivity, and honesty. Delbrück was unequivocal about the possibility of succinic acid having an influence on the mutation rates and, hence, about the possible occurrence of specifically induced adaptive mutations. Delbrück, therefore, deemed it improper to designate those mutations as spontaneous without further investigation. Delbrück's comments on the Lwoff report are an important addendum to the classic paper of Luria and Delbrück, and instructors should not shield that information from students.

Furthermore, a growing appreciation of the fluctuation test's limitation eventually gave strong impetus to seek new methods to understand some perplexing mutational phenomena. For example, experiments similar to the Lwoff experiment that intrigued and perhaps slightly alarmed Delbrück were later performed and scrutinized from multiple angles (10, 11). These meticulous experimental and theoretical efforts led to

the discovery that some seemingly nonrandom mutational phenomena were explicable as random mutation in disguise.

Too much math?

The argument of Luria and Delbrück in favor of the random mutation hypothesis relies solely on the M-M principle, which students can understand without having to understand any complicated mathematics. Students only need a rudimentary statistical knowledge of the sample mean and variance to appreciate why the experimental data led to the conclusion that mutations conveying resistance to phage occurred before the nonmutants were exposed to the phages. However, the classic paper of Luria and Delbrück involves an overwhelming amount of difficult mathematics, which can easily frustrate even a most mathematically minded biologist. The degree of this kind of frustration is reflected in the terse titular claim of a thought-provoking essay: "Look, Max—No Math Required!" (12). However, this protestation is largely unjustified, as the author of the essay, like some other readers of the classic paper, failed to see a groundbreaking contribution of Delbrück buried in involved mathematics. The often overlooked contribution was a theoretical framework for the estimation of microbial mutation rates, which was based on work that Delbrück started in 1939 or earlier according to a report by Delbrück to the Rockefeller Foundation (13, p. 143). Before the publication of the classic paper, microbiologists did not know how to define a microbial mutation rate, let alone estimate it. Today, researchers continue to use the definition given by Delbrück in the classic paper, and they increasingly rely on methods developed on the foundation laid by Delbrück to estimate microbial mutation rates. The topic of mutation rate estimation has been the most challenging for instructors, partly because some instructors are under the misconception that the Poisson distribution is the key tool for estimating microbial mutation rates. In fact, statistical inference about microbial mutation rates depends crucially on a new type of distribution that Delbrück pioneered and that we now call the mutant distribution. The reason for needing this distinction is easy for students to understand. Biologists can count mutants, but not mutations. As the M-M principle connects the Poisson distribution with the number of mutations, not the number of mutants, students would expect the Poisson distribution to play only a limited role in estimating microbial mutation rates using mutant count data.

Instructors should, therefore, not overemphasize the role of the Poisson distribution. In particular, instructors should skip a lengthy discussion of the general equation for the Poisson distribution, although the general equation has recently been advocated by some instructors (4, 14). It suffices to discuss only the zeroth term of the Poisson distribution:

$$P_0 = e^{-m} \tag{1}$$

which is equation 5 in the classic paper (1, p. 496) and in which m is the expected number of mutations per culture. The zeroth term of the Poisson probability is sometimes helpful because an absence of mutations occurring in a culture is the same as an absence of mutants in that culture. Therefore, P_0 , the probability of no mutations, can be estimated by the proportion of cultures containing no mutants. This unique role of P_0 should help explain why all other terms of the Poisson probability are irrelevant in estimating mutation rates. Furthermore, there are three important drawbacks to equation 1. First, as Hutchison et al. (4) noticed, an experiment may oftentimes produce no culture plates with zero colonies, rendering the estimation of P_0 by equation 1 impossible. To circumvent this vexing problem, Hutchison et al. (4) asked students to examine mutant frequency instead. This is a counterproductive solution to an inherent shortcoming of the method based on the Poisson formula because an important contribution of the classic paper is to help researchers shift from mutant frequency to mutation rate. Students should be cautioned that, despite its relatively popular use in research, the mutant frequency is an unreliable yardstick for measuring how often a

mutation of interest occurs [see references (15, p. 125) and (16)]. Second, as Henkin and Peters (17, p. 134) put it, this method is wasteful, as it ignores the actual mutant counts and relies only on the proportion of cultures with zero mutants. Third, unbeknownst to most instructors, equation 1 is not applicable to experiments where partial plating is employed. In experiment 16 of Luria and Delbrück, a subculture of 0.08 mL was sampled from each of 20 cultures of 0.2 mL. Therefore, the plating efficiency was $\varepsilon = 0.4$.

When plating is partial ($\varepsilon < 1$), the probability of no mutations is no longer the same as the probability of no mutants being observed. Even when mutations occur in a culture, the (partial) plating process may miss all the mutants in that culture. To extend the idea of equation 1 to partial plating, one should apply the formula for the probability of no mutants:

$$P_0 = \exp\left(m\frac{\epsilon\log\epsilon}{1-\epsilon}\right), \tag{2}$$

which was first derived by Stewart et al. (18). Solving equation 2 for m, one finds that the expected number of mutations in the whole culture was

$$m = \frac{(1-\epsilon)\log P_0}{\epsilon\log\epsilon} = \frac{(1-0.4)\times\log 0.55}{0.4\times\log 0.4} = 0.9787$$

in experiment 16 of Luria and Delbrück. Because the number of bacteria in the whole culture was estimated at 5.6×10^8 cells, the estimated mutation rate would be 1.75×10^{-9} mutations per cell division. A more accurate approach is to apply the maximum likelihood (ML) method, and students can easily accomplish this task by using existing software tools. For example, the webtool webSalvador (19) gives an ML estimate of 2.2×10^{-9} mutations per cell division with a 95% confidence interval (1.04×10^{-9}, 3.73×10^{-9}). (See Fig. 1.) Direct application of the Poisson formula would lead to an erroneous estimate of 1.07×10^{-9} (17, p. 134).

Thus, the approach by Meneely (14) is conceptually incorrect, and so is the approach by Griffiths et al. (20, p. 481). The Poisson formula appeals to students and instructors alike partly because it allows swift manual computation of the mutation rate. In general, calculating mutation rates by hand is impractical, and instructors should encourage students to use modern software tools. Interested instructors may consult the recent comprehensive review of existing software tools by Łazowski (21). Instructors wishing to discuss the Poisson formula may reanalyze experiment 23 in the classroom, as this is the only experiment in the classic paper in which Luria adopted complete plating ($\varepsilon = 1$). Experiment 23 comprised 87 cultures, 29 of which contained no mutants. Thus, $m = -\log(29/87) = 1.10$. Because each culture had 2.4×10^8 cells, the mutation rate was 4.58×10^{-9} mutations per cell division.

Concluding remarks

The fluctuation experiment poses challenges to both students and instructors. Hutchison et al. (4) rightly encouraged instructors to read the original Luria-Delbrück paper, and this note is intended to help instructors better appreciate the classic paper. This note draws instructors' attention to two persistent misconceptions that can impede an instructor's ability to teach the fluctuation experiment.

First, instructors should not eschew discussing the inherent limitation of the fluctuation experiment. Acknowledging the fluctuation experiment's unsuitability to refute the directed mutation hypothesis in no way implies the instructor's acceptance of that hypothesis, and instructors should not allow their personal conviction to color their view of the actual accomplishment by Luria and Delbrück. Being vague about the inherent limitation of the fluctuation experiment can confuse students. For example, Green and Bozzone (2), on one hand, stated that the results of Luria and Delbrück did not rule out the possibility of directed mutation for all cases. However, on the other hand, they claimed that Luria and Delbrück's results "proved" that such notions had no validity. An overemphasis on instilling the notion of random mutation in students may

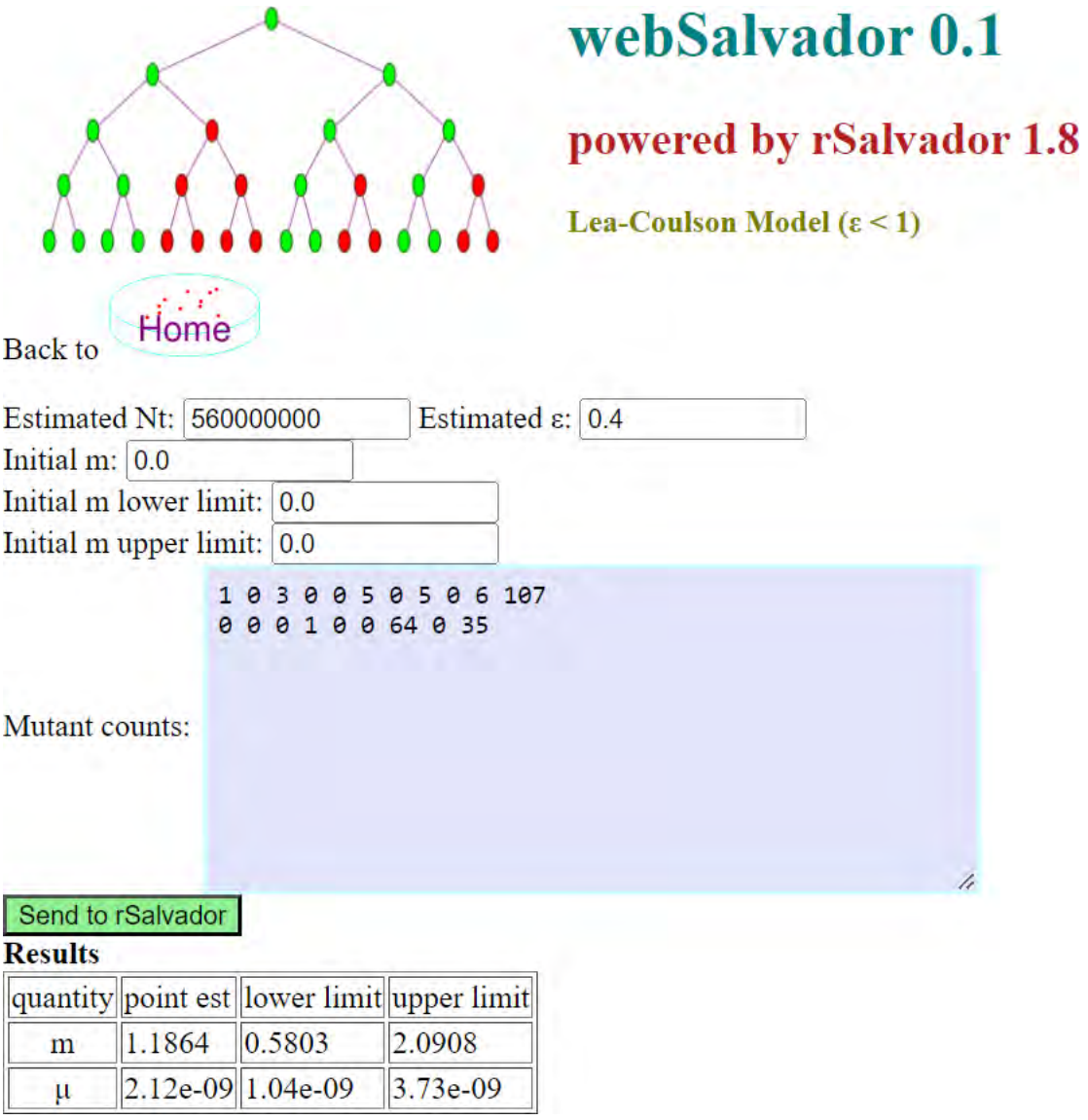

FIG 1 One can apply the maximum likelihood method via webSalvador to estimate the mutation rate in experiment 16 of Luria and Delbrück (1).

have a detrimental effect on students because being reticent about the limitation of the fluctuation experiment can cause students to question the value of objectivity and open-mindedness in scientific research.

Second, instructors should highlight the groundbreaking work of Delbrück on the estimation of microbial mutation rates. It was Delbrück who gave us the definition of the mutation rate that we still use today. Equally important is Delbrück's work on a prototype of the mutant distribution that served as a theoretical framework for further fruitful investigations starting with the influential work of Lea and Coulson (22). Current overemphasis on the Poisson distribution obscures Delbrück's pioneering work and gives students a distorted sense of how microbial mutation rates are estimated in real-world research. Drawing inferences about mutation rates requires a new type of distribution to bridge the gap between mutation and mutant—the mutant distribution, to which Delbruck made seminal contributions in the classic paper. Finally, instructors are advised to refrain from stressing the importance of the classic paper's equation 8, which appeared in two prominent recent textbooks—references (23, p. 27) and (19, p. 134). This equation, based on intuition, has been shown by simulation to be inaccurate

(24) and has fallen into disuse for quite some time. Instructors should steer students toward modern estimation methods by introducing them to specialized software tools, preventing them from getting mired in outdated information.

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