Luria-Delbruck Fluctuation Test

April 20, 2013

0.1 Pre-Lab

We want to introduce you to one of the most clever and intriguing experiments performed in the last century in the field of biology, by Luria and Delbruck. It is a demonstration of how potent the combination of theoretical and experimental approaches can be in the study of living organisms. Also, this experiment is centered around a unifying theme in biology, namely evolution. It is a test of two theories of genetic inheritance and it offers clever methods for calculating the mutation rates of microorganisms. After many decades, this experiment is still used in molecular biology labs.

To fully appreciate this experiment, you will need to spend some time to read the **Appendix** and to look up the following molecular biology terms and techniques if you are not familiar with them:

1) Bacteria or yeast culturing methods, including plating techniques (selective plates, agar plates, phage-agar plates, etc), cell counting (hemocytometers).

2) Biological Machineries involved in transcription, the cause of mutations, and mutation correction mechanisms.

3) Molecular evolution. Jot down mutation rates for different organisms including bacteria, different viruses, yeast and other eukaryotes.

0.2 Background

When mutations occur in nature they are often deleterious to the organism. However, mutations are a critical part of the genetic heritage of living organisms, arising in every type of organism and allowing life to evolve and adapt to new environments. In 1943, the question of how microorganisms acquire mutations was described in a famous article by Salvador Luria and Max Delbruck [1]. At the time, two theories of genetic inheritance existed. Scientists did not know if mutations arose randomly in the absence of an environmental cue, the"mutation hypothesis", or whether they occur as an adaptive response to an environmental stimulus, the"acquired immunity hypothesis". See Figure 1.

To test these two hypotheses, Luria and Delbruck grew many parallel cultures of bacteria and then plated each culture on phage agar containing viruses known as phages (which infect and kill nearly all of the bacteria). Although most bacteria are unable to survive in the presence of phages, often mutations could enable a few survivors to give rise to resistant mutant colonies. If the acquired immunity hypothesis is correct, mutations occur **only** after bacteria come in contact with phages, thus only after plating the bacteria on phage-agar plates. Under this hypothesis, we would expect a low variance in the number of resistant colonies that appear on each plate.

However, if the mutations arose randomly prior to phage exposure as bacteria were growing in the liquid culture, the number of mutations in each culture would vary wildly as mutations could occur at any time during the liquid culture phase and accumulate exponentially. Mutations that arise early in the culture will give rise to an exponentially growing population of mutant cells, which will result in large number of resistant colonies after plating. In contrast, mutations that occur at later times will result in fewer

colony counts after plating. Hence, the mutation hypothesis makes the prediction that there will be a large variance in the number of resistant colonies coming from different cultures.

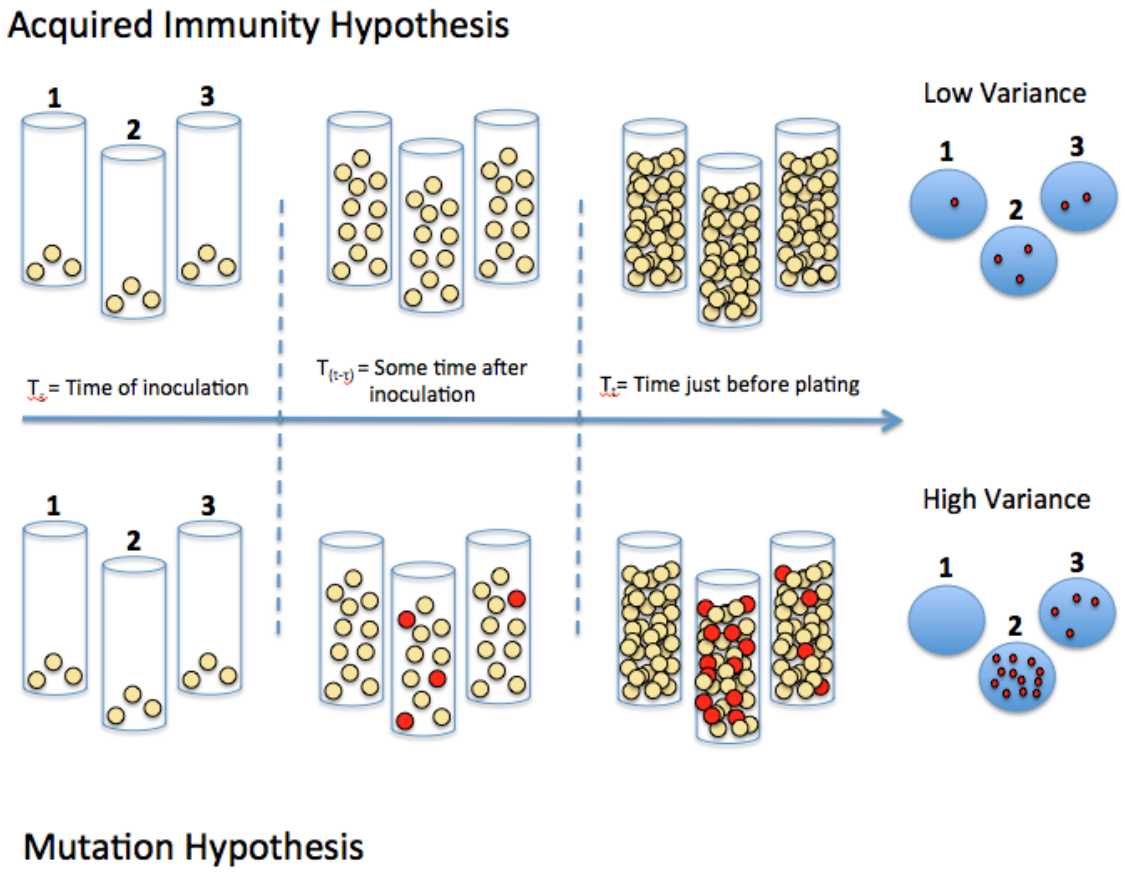

Figure 1: Luria-Delbruck fluctuation experiment schematic.

0.3 Your Goal

Your task will be to test the two hypotheses of inheritance on cultures of the yeast *S. cerevisiae*. Using the mean and variance obtained from number of mutant colonies in each culture, you will be able to deduce which one of the two hypotheses more accurately describes the mechanism underlying the rise of mutations. Additionally, we will calculate the mutation rates in two types of yeast strains, Wild Type and mutator strain. The mutator strain contains a deletion in one of its DNA repair genes. The logic is that the mutator strain should have a higher mutation rate and hence should result in more colonies reflecting a higher proportion of resistant cells.

However, before we delve into methods for calculating mutation rates, let's take a theoretical look at the two hypotheses (See Figure 2), adapted from *Physical Biology of the Cell* by Rob Phillips, et al. The beauty of this approach is that using just three generations of bacteria, we will be able to distinguish between the variance to mean ratio of mutation events under these two different hypotheses. To calculate the expected value of mutations, you simply have to note that there are a limited number of outcomes (5 outcomes

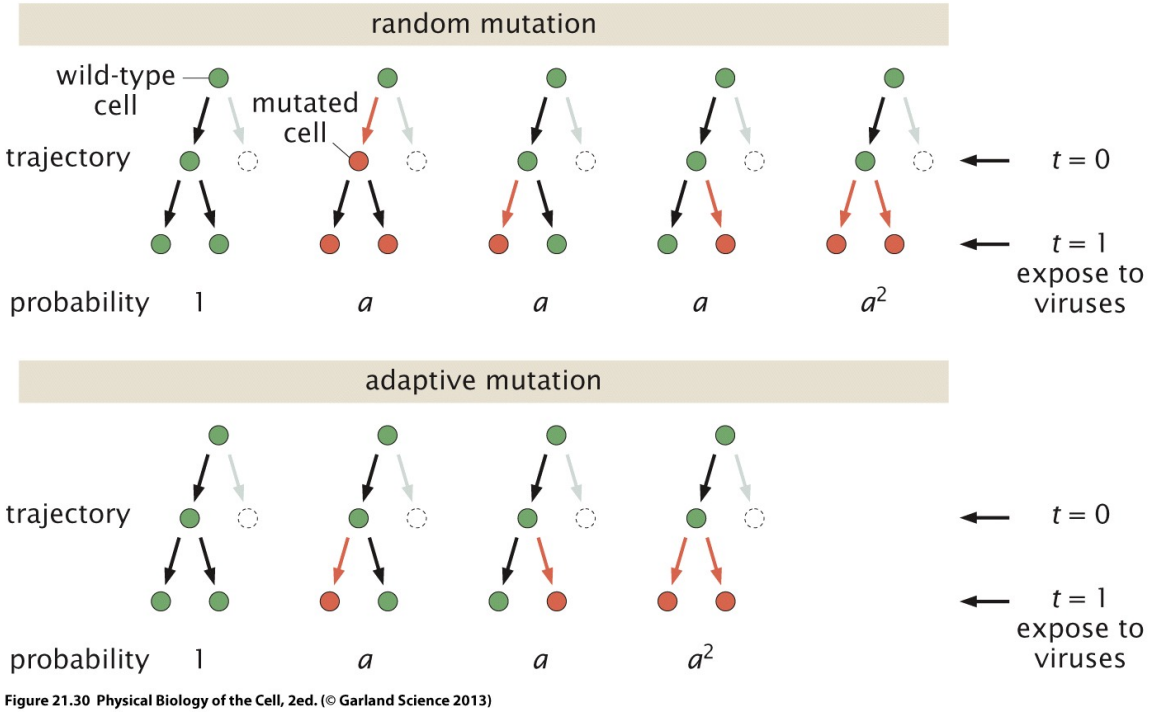

Figure 2: A theoretical approach to testing Luria-Delbruck mutation hypotheses, adapted from *Physical Biology of the Cell* by Rob Phillips, et al.

for the mutation hypothesis, for example, looking at three generations of bacteria). You also have to note that each outcome has a particular probability. In fact, the probability of each event will be its weight in the calculation for the expected value (take a look at the Appendix). So under the mutation hypothesis, the expected number of mutations, $M_{mutation}$, is

$$M_{mutation} = \frac{3a + 2a + 2a^2}{1 + 3a + a^2}, \tag{1}$$

where a is the probability of a mutation (and is very small). Moreover, the denominator represents the summation of all the weights, and the numerator is the the number of mutant cells in each outcome multiplied by the weight of each outcome. Looking at the acquired immunity hypothesis, you can similarly calculate $M_{aq.immunity}$, the expected number of mutations, as

$$M_{aq.immunity} = \frac{2a + 2a^2}{1 + 2a + a^2}. \tag{2}$$

At this point, you can use what you learned about variance from the appendix to calculate the variance. If you calculate the Fano Factor, the ratio of variance to mean, under both hypotheses you will see that the mutation hypothesis yields a non-Poissonian Fano-Factor with its variance greater than the mean whereas the Fano Factor under the opposing hypothesis will be 1. In the following section, you will learn two different ways in which Luria and Delbruck were able to obtain the mean, variance, and also the mutation rate of *E. coli*. In the lab, you will be able to reproduce their results and test the two hypotheses using your own data. You will also simulate this experiment using MATLAB as part of your homework.

0.4 Mutation Rate: Method 1

Bacteria divide into two at every cell division, which for *E. coli* takes roughly 20 minutes. Hence, their growth is exponential. Convince yourself that their growth rate at time t is proportional to their population at time t by a constant, k. Thus, we have

$$\frac{dN_t}{dt} = kN_t. \tag{3}$$

Now, integrate the above equation to arrive at

$$N_t = \int_{t_s}^{t} \frac{dN_t}{dt} dt = N_s e^{(t-t_s)}, \tag{4}$$

an expression for the total number of bacteria as a function of time. Your integral should go from t_s, the time at which the cultures were inoculated with bacteria to t, any arbitrary time after that. You should obtain an integration constant which you can determine by noting that at t_s, the cultures are each inoculated with N_s bacteria.

Let's now talk about mutations. Let's say that a is the mutation rate of a bacterium during a small time element dt, such that the number of mutations that arise in one cell during dt is equal to adt. Note a is an unknown. There are two methods by which a can be derived. In this section we're covering the first method that Luria and Delbruck used, which is different from the method you will use in class.

You can calculate the total number of mutations dm in dt that come about in a population of cells by

$$dm = adtN_t. \tag{5}$$

The number of mutations that arise in a culture of bacteria over longer periods of time can be obtained by integrating the above expression using t_s and t as your lower bound and upper bound, respectively, such that you obtain

$$m_t = a(N_t - N_s). \tag{6}$$

The above expression should make intuitive sense: as time passes and the population of bacteria grows, there will be a greater chance that mutations arise both within a cell and within a culture of cells.

As you may recall from the appendix, the Poisson distribution is used for characterizing the expectation and variance of rare events in large populations. Naturally, it can be used here for modeling the number of mutations that arise in large population of cells, since probability of a mutation is very small (as we will find out in this experiment!). Hence, our observations of the number of mutations immediately after inoculation could vary greatly from culture to culture. So Luria and Delbruck came up with a cool way of excluding the early growth phase from their calculations.

They defined a time, t_o, which occurs some time after t_s at which the culture is large enough but still not overwhelmed by mutations. Hence the average number of mutations

they expect to arise during the duration of $t - t_o$ is given by

$$r = (t - t_o)aN_t. \tag{7}$$

So, what is that magic time, t_o? Luria and Delbruck defined the time before which only 1 mutation on average has occurred in a group of C similar cultures, and using

$$1 = aC(N_{t_o} - N_s), \tag{8}$$

and the idea that N_s is negligible compared to N_{t_o} to arrive at

$$N_{t_o} = \frac{1}{aC}. \tag{9}$$

We can also express N_{t_o} in terms of N_t by

$$N_{t_o} = N_t e^{-(t-t_o)}, \tag{10}$$

where N_t is the final number of cells immediately before plating. When solving this equation, we can substitute N_{t_o} with the right hand side of Equation 9, such that

$$\ln(N_t Ca) = t - t_o. \tag{11}$$

So, we can substitute $t - t_o$ back into Equation 7 to obtain

$$r = aN_t \ln(N_t Ca). \tag{12}$$

Note that r, C, and N_t are all observables from the experiment. Hence, the value of a can be calculated using the above equation. This is the first method of calculating the mutation rate, a.

0.4.1 Mutation Rate: Method 2 (What is used in class)

The second method of obtaining a is equally clever. Luria and Delbruck used the fact that the number of mutations in a series of C similar cultures will be distributed according to the Poisson distribution (See Appendix).

Hence, they used the proportion of plates bearing zero mutations, P_o, to solve for λ as

$$P_o = \frac{\text{zero-colony plates}}{\text{total number of plates}} = \frac{\lambda^0}{0!}e^{-\lambda}. \tag{13}$$

In summary, using this second method, they solved for the mutation rate per basepair per bacterium, $\lambda = a$.

Could they have used proportion of plates containing 4 or 5 or 100 colonies, for example? The answer is no. This is because when you spot 100 colonies on a plate, you don't know whether that is due to 100 mutations or 1 that just gave rise to 100 daughter cells. It's ambiguous. However, when there are no colonies, we are sure that there were zero mutations.

0.5 Materials and Methods

Parallel cultures of Wild Type and mutator strains of *S. cerevisiae* have already been started for you. To begin the cultures the TAs grew each strain in 96-well plates and incubated them. During this lab period, you will plate these cultures, and after two or three days you will be able to count the number of mutant colonies that arise from each culture. The culture conditions have already been optimized to ensure that a portion of the plated cultures bear zero mutations, since this information will be used for calculating mutation rates.

Day 1

You will be given a 96-well plate containing saturated cultures. In order to calculate the mutation rate of each strain, you will need to determine the total number of cells. You will count the yeast cells using a *hemacytometer*. This microscopy device contains a specialized cell counting chamber which has a grid of known area and depth. Three separate cultures of each strain of yeast will be counted to determine reproducibility of the counting method.

Counting Yeast Cells:

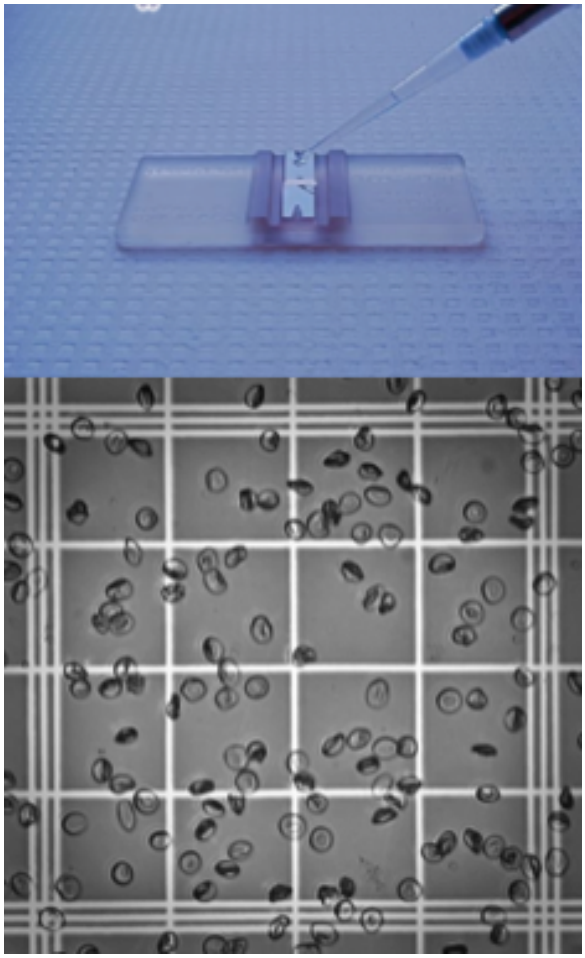

Figure 3: Hemocytometer.

1. Sample preparation: Choose a culture and resuspend cells thoroughly by pipetting

up and down. Your cells will be too concentrated for counting so you will need to dilute them. Your TAs will tell how much to dilute and what media to dilute with. Yeast cells tend to clump together so you will have to be diligent with your mixing. Once you are confident that you have a homogenous suspension, you are ready to inject the cells into your hemacytometer (See Figure 3).

2. Sample injection: Have ready a hemacytometer and your diluted sample. Your goal is to fill the counting chamber. With a steady hand, pipette 10 μL culture in the injection area making sure to fill the chamber. This injection technique will be familiar to you from last week's single molecule digest when you pipetted fluids into a flow chamber.

3. Cell counting: When you are ready to view your yeast cells place the hemacytometer in a microscope. Use the Brightfield setting with a 40X objective lens and appropriate phase contrast. Adjust the focus so that you view a portion of the center square containing 100 small squares. Count cells in the small squares and calculate cells/mL for all 6 cultures. Using the group data, determine an average cell count for each strain of *S. cerevisiae*. The hemacytometer has a depth of 0.02mm. A small square measures 0.1mm x 0.1mm. The large center square measures 1.0mm x 1.0mm.

Plating Cells: Once the yeast cells are counted, the next step will be to plate 27 cultures of each strain on dry agar plates containing *canavanine*, a selective agent that is toxic to yeast. The dry plates have a textured surface and reduced moisture content that will provide a good surface for pipetting large-volume spots onto a plate. Begin by pipetting all 100 μL of culture from a chamber onto a spot on one of the plates. Repeat for another 8 cultures to make a 3x3 grid on the plate. Do this again for 2 more plates, then repeat for the other strain. You will have spotted a total of 6 plates. Allow the plates to dry before inverting and placing in the 30 C incubator to grow overnight. Your plates will be removed from the incubator after 2- 3 days and saved for future analysis.

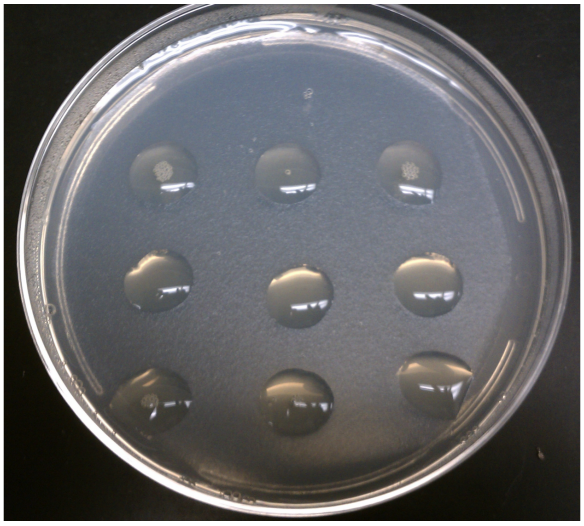

Figure 4: Plating yeast cultures.

Day 2

Obtain your plates and count colonies for each culture. Record in your lab notebook. Record the proportion of zero-colony spots, P_o.

Time permitting:

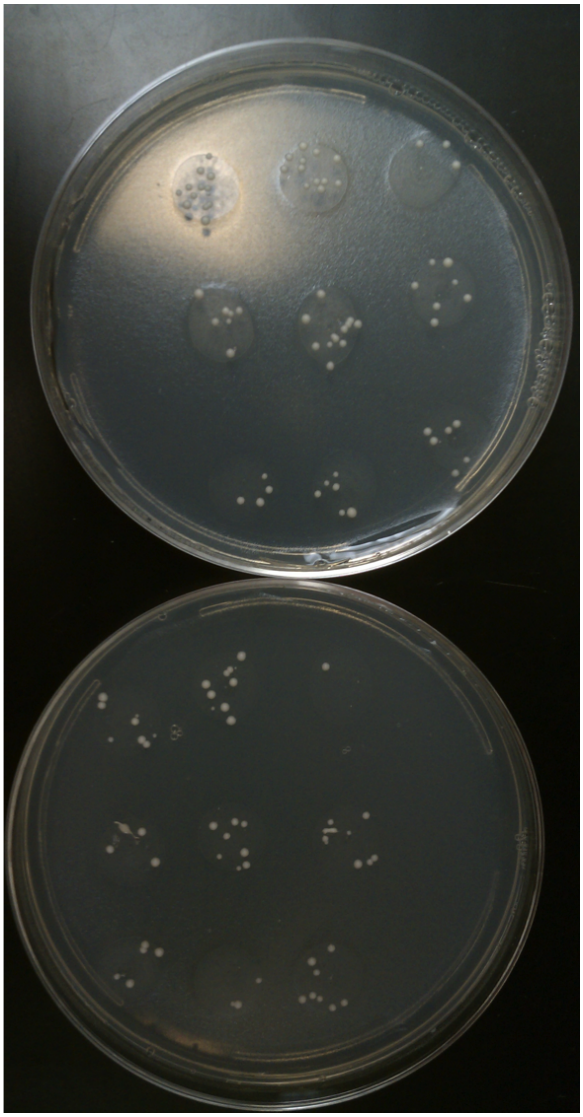

Figure 5: Mutator yeast colonies after 17 hours of incubation. *Note there are no zero-colony spots in this figure, which means that this set of data could not be used for calculations.*

Later analysis will include sequencing the CAN1 region of your mutated yeast colonies. The CAN1 gene produces a protein that carries canavanine across the cell barrier. Mutations in this gene are what allow yeast to survive on the selective media. The TAs will isolate this gene from your colonies and send the CAN1 PCR product (amplified region of DNA) for sequencing. You will prepare 3 colonies for the TAs to run PCR on. Label 3 eppendorf tubes and pipette 100 μL of sterile water into each. Using a culture loop, carefully pick a single colony and suspend in the water. Give your suspensions to your TA. Sequence results will be emailed to you. Save for future analysis.

0.6 Post Lab Questions and Data Interpretation

1. Calculate P_o and mutation rate for the two types of yeast strains.

2. Build a histogram of your data (number of colonies per spot) and calculate the mean and variance of the distribution. Is it a Poissonian distribution?

3. What are some of the sources of error in this experiment?

0.6.1 Matlab Questions

In this section, you will explore the difference between two possible models of genetic mutation: 1) mutations arise as a response to selective pressure (in our case, plating on selective media), and 2) mutations occur spontaneously during cell division.

For case 2, your TAs have written a Matlab function to simulate a population of cells starting from 1000 cells and undergoing 7 cell divisions. The function returns the number of mutants in the population of cells after 7 divisions. Run the simulation 100 times and compute the mean and variance of the number of mutants observed from each simulation (hint: a For Loop will be useful here). Next, compute the Fano factor, which is equal to the variance divided by the mean. This quantity gives a measure of how dispersed or spread out a probability distribution is. Note that for a Poisson distribution, the variance is equal to the mean, and thus the Fano factor equals 1 identically.

For case 1, you will write the function to simulate the number of mutants yourself. Say you have 128000 cells ($128000 = 1000 * 2^7$ if you're wondering where that number came from). Each cell undergoes a mutation with probability 10^{-5}. We aren't dealing with cell division or anything like that. For each cell in the population of 128000, there is a 10^{-5} probability it will mutate, and a $1 - 10^{-5}$ probability that it won't mutate. Your function should return the number of cells that mutated. Run this simulation 100 times and compute the mean and variance of the number of mutants. Finally, compute the Fano factor (variance divided by mean).

How does this Fano factor compare with what you calculated for case 2? Of the two cases considered here, which is more similar to what your observed experimentally?