Hemacytometer Cell Count Distributions: Implications of Non-Poisson Behavior

ARTICLE in BIOTECHNOLOGY PROGRESS · SEPTEMBER 2008
Impact Factor: 2.15 · DOI: 10.1021/bp00012a600

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Hemacytometer Cell Count Distributions: Implications of Non-Poisson Behavior

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The variance of observed cell counts using a hemacytometer was analyzed. The variance was found to be greater than expected under the normally assumed Poisson distribution because of variations in the volume of the counting area. Furthermore, counts from the two sides of the hemacytometer were found to be correlated. Total cell count on a hemacytometer was better described using a normal distribution with quadratic variance, $N(\lambda, \lambda + \sigma \lambda^2)$, with $\sigma = 0.012$. Because hemacytometer cell counts are not Poisson distributed, modified counting protocols are recommended.

Introduction

Enumeration of cells via a hemacytometer is a standard technique used in many laboratories. Particularly in mammalian systems, where the biomass concentration is relatively low, cell counts generally represent the preferred measures of growth and viability. Accurate estimates of cell numbers are essential for developing and validating quantitative models of cell growth and product expression. The two main parameters determining the accuracy of cell enumeration using the hemacytometer technique are the number of hemacytometers that are counted (i.e., number of fillings) and the number of cells counted within each hemacytometer. A priori knowledge of the underlying distribution of measured cell counts can be used (a) to determine how many cells and sides have to be counted to obtain a particular level of reliability and (b) to increase the reliability for a given number of sides and cells counted. The latter is due to the fact that if the variance is known, no reliability is lost through estimation of this parameter.

Under the assumptions that samples are fully suspended, that the volume over the counting area on the hemacytometer is constant, and that cells are distributed randomly over the hemacytometer, cell counts can be described by (Rohatgi, 1984)

$$X = \text{cell count} \sim \text{Poisson} (VN)$$

where $V$ is the volume over the counting area and $N$ is the cell concentration in the sample. If the cell counts are distributed according to a Poisson distribution, the variance is equal to the mean. In our laboratory, however, we have generally observed a variance significantly higher than the mean, suggesting that cell counts do not follow a Poisson distribution. This has occurred despite the application of very careful analytical procedures. This paper investigates possible reasons for the higher variance and the use of alternative statistical distributions to describe hemacytometer cell counts more accurately. Implications for experimental protocols are assessed.

Materials and Methods

The two sets of data used in the analysis were collected over a 2-year period from viable cell counts in laboratory-scale cultures of a murine:murine hybridoma. All samples were diluted 1:1 with trypan blue to distinguish between viable and dead cells. Both sides of an improved Neubauer hemacytometer (Weber, England) were filled with diluted sample using a micropipette. The first data set consists of 360 observations from 20 samples, where the two sides of a hemacytometer have been counted and the cell count has been recorded for each of the nine major squares in the grid on the hemacytometer. All observations were collected by an experienced research assistant. The observations were in the range of 8–102 cells/major square. The second data set consists of 1364 observations from 290 samples, where the cell counts from the two sides of 1–5 hemacytometers were recorded. The majority of observations in this data set were collected by an experienced research assistant. Approximately 300 observations collected by another person were pooled into this data set after homogeneity had been assured graphically. The observed counts fell in the range of 25–650 cells/side.

Results and Discussion

Nonrandom Sources of Variation. There are several possible sources of variance in cell counts apart from randomness: dilution, mixing, hemacytometer preparation and filling, and clustering. Variance introduced during dilution with trypan blue is generally invisible as only one dilution is made. We diluted 200 $\mu$L of sample 1–5 times depending on the expected cell concentration. This dilution method does not introduce significant additional variance in much more accurate assays and dilution variance can, therefore, be regarded as negligible.

The diluted sample is mixed with a micropipette or a whirl mixer (this does not affect viability for our cell line). We believe the samples are fully mixed, but variance from mixing cannot be distinguished from variance introduced from volume variations.

The preparation and filling of the hemacytometer might introduce significant variation. First, the volume (approximately 10 $\mu$L) mounted on each side between the cover slip and the hemacytometer might vary significantly. Second, the two sides within one hemacytometer could be positively correlated (i.e., "overfilling" of one side is likely to cause overfilling of the other side). Finally, the cells might cluster on the hemacytometer. Clustering here should be understood as nonrandom spreading of the cells over the hemacytometer. Hybridomas rarely form clusters of cells sticking together, but the dynamics of capillary filling could introduce an uneven distribution of the cells over the hemacytometer, i.e., the distribution might vary with the speed of filling.
ANOVA test for a nested classification and the results are value added for the known difference between samples, contributes significantly to the total variance. This is the variance measure between sides will only represent the hypothesis that the sample volume used for the counts is constant. If the volume is distributed rather than ANOVA with the model each side is equal to 0.25 and whether shown in Table I. variation in volume between sides of hemacytometer, and between sides, is significant. This test is performed as an Table I. Analysis of Variance of Cell Counts

<table>
<thead>
<tr>
<th>source of variation</th>
<th>SS</th>
<th>df</th>
<th>$s^2$</th>
<th>test</th>
</tr>
</thead>
<tbody>
<tr>
<td>between sides</td>
<td>10.30</td>
<td>20</td>
<td>0.50</td>
<td>0.50/0.27 = 0.84 = $F(20,320)_{0.88}$</td>
</tr>
<tr>
<td>within sides</td>
<td>87.35</td>
<td>320</td>
<td>0.27</td>
<td>87.35/0.25 = 349.4 = $x^2(320)_{0.88}$</td>
</tr>
<tr>
<td>theoretical</td>
<td></td>
<td></td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

a SS = sum of squares, df = degrees of freedom, $s^2 = SS/df$.

Cell counts for each of the nine major squares on two sides of one hemacytometer were registered for the first data set. As described previously, up to 102 cells/square were counted, i.e., a level where one normally would have diluted the sample further with trypan blue. If clustering does occur during normal cell counting, therefore, one would expect to see non-Poisson behavior for data collected within a particular side for this data set. If the cells do not cluster, one would expect the observations to be Poisson-distributed within each side of the hemacytometer, i.e.

$$X_{ijk} = \text{cell count sample } i, \text{ side } j, \text{ square } k$$

where $i = 1-20$, $j = 1\text{ or } 2$, $k = 1\text{ or } 9$, and $\lambda_{ij}$ is expected number of cells in a square. The data may be transformed to obtain variance homogeneity (half power transformation). It can be shown that (Anscombe, 1948)

$$Y_{ijk} = \left(\frac{X_{ijk}}{\lambda_{ij}}\right)^{1/2} \approx N\left(\sqrt{\lambda_{ij}}, 0.25\right)$$

when $\lambda_{ij} > 10$ (3)

where $N = \text{the normal distribution with mean } \lambda_{ij}^{1/2}$ and variance 0.25.

Consequently, we can test whether the cells cluster by testing whether the variance of the counts of the square root transformed data from each side is significantly different from 0.25. Simultaneously, it is possible to test the hypothesis that the sample volume used for the counts is constant. If the volume is distributed rather than constant, the variance between the two sides will be significantly larger than the variance within one side. As only one hemacytometer was counted for each sample, the variance measure between sides will only represent variation in volume between sides of hemacytometer, and a possible correlation between sides of a hemacytometer cannot be discerned. Both hypotheses were tested using ANOVA with the model

$$Y_{ij} = \mu + \alpha_i + S_{j(i)} + E_{k(i)}$$

where $\mu$ is the overall mean for all samples, $\alpha_i$ is a fixed value added for the known difference between samples, $S_{j(i)}$ is a random addition to the $j$th side of the $i$th sample \(\{\epsilon N(0, \sigma^2)\}\), and $E_{k(i)}$ is a random addition to the $k$th square \(\{\epsilon N(0, \sigma^2)\}\). We want to test whether the variance of the counts of the square root transformed data from each side is equal to 0.25 and whether $\sigma^2$, the variance between sides, is significant. This test is performed as an ANOVA test for a nested classification and the results are shown in Table I.

The first null hypothesis (i.e., the variance within a side being equal to 0.25) is accepted at all levels of significance less than 12%. We conclude that there is no significant evidence for clustering. The hypothesis $\sigma^2 = 0$ is rejected at all levels of significance higher than 2% and we conclude that the variance between counts from different sides contributes significantly to the total variance. This is strong evidence for variable sample volumes within one hemacytometer.

The second data set was used to test whether there is

Table II. Analysis of Variance of Cell Counts

<table>
<thead>
<tr>
<th>source of variation</th>
<th>SS</th>
<th>df</th>
<th>$s^2$</th>
<th>test</th>
</tr>
</thead>
<tbody>
<tr>
<td>between hemacytometers</td>
<td>590</td>
<td>392</td>
<td>1.50</td>
<td>1.50/0.43 = 3.83 $F(592,682)_{0.9995}$</td>
</tr>
<tr>
<td>within hemacytometers</td>
<td>429</td>
<td>682</td>
<td>0.63</td>
<td>429/0.25 = 1716 $x^2(682)_{0.9995}$</td>
</tr>
<tr>
<td>within sides</td>
<td></td>
<td></td>
<td>0.25</td>
<td>(theoretical)</td>
</tr>
</tbody>
</table>

a SS = sum of squares, df = degrees of freedom, $s^2 = SS/df$.

A Modified Counting Protocol. The second data set was further analyzed to establish the number of cells and the number of hemacytometers to obtain a required level of accuracy. It is assumed that both sides of the hemacytometer were filled every time. Hereby, the nontrivial problems of accounting for correlation between sides can be avoided by looking at the total cell count for both sides of the hemacytometer.

$$Y_{ij} = X_{ij1} + X_{ij2}$$

= total cell count for sample $i$, hemacytometer $j$ (7)

where $i = 1-290$, $j = 1-n_j$, $k = 1$ or 2, and $\lambda_{ijk}$ is expected number of cells in a side. Again, the half power transformation is used to obtain variance homogeneity.

Distinguishing between variance between sides within the hemacytometer and variance between hemacytometers leads to the following ANOVA model:

$$Y_{ijk} = \mu + \alpha_i + H_{j(i)} + S_{k(i)} + E$$

where $\mu$ is the overall mean for all samples, $\alpha_i$ is a fixed value added for the known difference between samples, $H_{j(i)}$ is a random addition to the $j$th hemacytometer of the $i$th sample \(\{\epsilon N(0, \sigma^2)\}\), $S_{k(i)}$ is a random addition to the $k$th side \(\{\epsilon N(0, \sigma^2)\}\) and $E$ is the (unobservable) random Poisson error for each side \(\{\epsilon N(0, 0.25)\}\). We want to confirm that $\sigma^2 \neq 0$ (i.e., the volume varies significantly between sides) and test whether $\sigma^2 \neq 0$ (i.e., whether the variance between hemacytometers is greater than the variance within a hemacytometer). This test is performed as an ANOVA test for a nested classification and the results are shown in Table II. The variance within sides is significantly greater than 0.25, confirming the observation that the volume of sample varies between sides of the hemacytometer. The variance between hemacytometers is significantly greater than within hemacytometers and we conclude that there is a significant positive correlation between the two sides of the hemacytometer.
Table III. Parameter Estimate for the Model

<table>
<thead>
<tr>
<th>Parameter Estimate for the Model</th>
<th>α</th>
<th>goodness of fit</th>
<th>degrees of freedom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y_{ij} \sim N(\lambda_j, \alpha \lambda_j^2)</td>
<td>α = 0.0137</td>
<td>326</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>α = 0</td>
<td>1326</td>
<td>236</td>
</tr>
</tbody>
</table>

* Estimated standard deviation is in parentheses.

statistical distribution, i.e.

Y_{ij} \sim \text{Poisson}(VN_j) \tag{8}

where V is stochastic and N_j is deterministic. In statistical terminology, Y_{ij} has a compound Poisson distribution. A short calculation shows that

\[ \text{var}(Y_{ij}) = E(Y_{ij}) + \alpha E(Y_{ij})^2 \tag{9} \]

\[ \alpha = \text{var}(V)/E(V)^2 = CV(V)^2 \]

where E refers to the expected value and CV to the coefficient of variation.

In eq 9 constant sample volume (i.e., Poisson behavior) corresponds to \( \alpha = 0 \). Thus, approximating the compound Poisson distribution with a normal distribution yields

\[ Y_{ij} \sim N(\lambda_j, \lambda_j + \alpha \lambda_j^2) \tag{10} \]

where \( \lambda_j = E(V)N_j \) = expected number of cells over the total counting area. The approximation was used to determine a maximum likelihood estimate for \( \alpha \) from the second data set. The resulting estimate and standard deviation are given in Table III. The second column of Table III gives approximate \( \chi^2 \) goodness-of-fit statistics that measure quantitatively how well the quadratic variance function tracks the observed sample variance (McCullagh and Nelder, 1983). The dramatic increase in goodness of fit, 1326 - 326 = 1000, on 1 degree of freedom illustrates how significant the variation in volume is.

The suggested model still shows significant lack of fit, namely, 326 on 235 degrees of freedom, which is significant at 0.5%. The lack of fit, however, is no more than could be expected from the approximation in eq 10 of a compound Poisson distribution with a normal distribution.

Maximum likelihood estimators are relatively sensitive to the error distribution, and it was felt that the least-squares estimation method should be tried as well. In the least-squares method, the parameters are selected to minimize the weighted sum of squares mean deviation, where the weights are the reciprocal expected variance of the mean, i.e.

\[ \min F = \sum \frac{(Z - E(Z))^2}{V(Z)} \tag{11} \]

We estimate \( \alpha \) from the sample variance. If the observations follow the distribution in eq 10, then the sample variance, \( S_i^2 \), is distributed as

\[ S_i^2 = \sum \frac{(Y_{ij} - \hat{\lambda}_i)^2}{f_i} = (\hat{\lambda}_i + \alpha \hat{\lambda}_i^2) / f_i \tag{12} \]

where \( f_i = n_i - 1 \) and \( \hat{\lambda}_i = \sum Y_{ij}/n_i \). To obtain weights independent of \( \alpha \), the second data set was logarithmically transformed. It can be shown (Johnson and Kotz, 1970) that if \( S_i^2 \) is given by eq 12, then

\[ E(\log S^2) = \log (\hat{\lambda}_i + \alpha \hat{\lambda}_i^2) - \log (f_i/2) + \Psi (f_i/2) \tag{13} \]

and

\[ V(\log S^2) = \Psi' (f_i/2) \tag{14} \]

where \( \Psi \) and \( \Psi' \) denote the di- and tri-\( \gamma \) functions.

![Figure 1](image.png)

Figure 1. Goodness of fit for model \( Y_{ij} \sim N(\lambda_j, \lambda_j + \alpha \lambda_j^2) \). Data were transformed, \( Y_{obs} = \log \left( \frac{\alpha}{2} + \log (f_i/2) - \Psi (f_i/2) \right) \) and \( X_i = \log \lambda_i \) to obtain variance stability according to eq 13 (apart from variation in degrees of freedom). Goodness of fit is shown for the model with \( \alpha = 0 \) (Poisson), \( \alpha = 0.0101 \) (least-squares estimator), and \( \alpha = 0.0137 \) (maximum likelihood estimator), respectively. The value of \( \alpha \) was estimated by inserting eqs 13 and 14 into eq 11, giving a value of 0.0101 with a standard deviation of 0.0013.

Data were plotted to show the goodness of fit (Figure 1). The skewness observed in Figure 1 is expected for a log-transformed \( \chi^2 \) distribution. The variation around the mean, however, is greater than expected and, with the skewness, suggests that the maximum likelihood estimate is too high. The least-squares method, on the other hand, will give an absolute minimum for \( \alpha \) due to the skewness. We conclude that a better estimate for \( \alpha \) is between 0.0101 and 0.0137, presumably toward the upper limit. In the remainder of this paper we will work with the model \( N(\lambda_j, \lambda_j + \alpha \lambda_j^2) \) with \( \alpha = 0.012 \).

The reliability of an observed mean of cell counts can be calculated and expressed as the 95% confidence interval. An approximate 95% confidence interval for the true mean can be calculated from the observed mean, \( m \) (for both sides of the hemacytometer), and the number of hemacytometers counted, \( n \), as \( n = (2(m + \alpha \lambda^2)/2)/n \).

The number of sides required to obtain \( p \) 100% accuracy (i.e., to obtain a 95% confidence interval which can be written as \( m \pm \sigma \)) is \( n = (2/p)^2(4/m + \alpha) \), which is that for the Poisson distribution plus \( \alpha^2/p^2 \).

The addition of a term independent of the number of cells counted implies that one might count 2000 cells on one hemacytometer and still only have a good estimate of the cell number for the given volume on the hemacytometer. This volume might be more or less close to the average volume used when calculating the cell density. In fact, it is not possible to obtain greater than 22% accuracy when counting only one hemacytometer, 16% for two hemacytometers, and 13% for three hemacytometers. In publications where higher accuracy is claimed for duplicate measurements, it is likely that the two observations have been obtained on one hemacytometer, thereby hiding the variance introduced between hemacytometers.

Table IV shows different protocols leading to 10%, 15%, and 20% accuracy. There is a trade-off between the total number of cells counted and the number of hemacytometers counted. We prefer counting 200-300 additional cells rather than an extra hemacytometer, and, therefore, we count seven hemacytometers of 180 cells for 10% accuracy (modeling work), three hemacytometers of 200 cells for 15% accuracy (qualitative work), and two hemacytometers of 125 cells for 20% accuracy (routine passing).

We earlier assumed that both sides of the hemacytometer were counted. As the two sides are correlated, one
Table IV. Protocols for Obtaining 10%, 15%, and 20% Accuracy

<table>
<thead>
<tr>
<th>accuracy</th>
<th>no. of hemacytometers</th>
<th>cells per hemacytometer</th>
<th>total cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>5</td>
<td>2000</td>
<td>10000</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>333</td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>182</td>
<td>1273</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>125</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>95</td>
<td>857</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>77</td>
<td>769</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>65</td>
<td>710</td>
</tr>
<tr>
<td>15%</td>
<td>3</td>
<td>205</td>
<td>615</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>96</td>
<td>381</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>62</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>46</td>
<td>276</td>
</tr>
<tr>
<td>20%</td>
<td>2</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>56</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>36</td>
<td>143</td>
</tr>
</tbody>
</table>

obtains higher accuracy by counting one side on twice as many hemacytometers. On the basis of variance estimates extracted from the ANOVA of Table II, we found that the same accuracy is obtained when counting one side on 1.42 times the number of hemacytometers with both sides counted (e.g., 15% accuracy can be obtained by counting two sides of a total of 205 cells on three hemacytometers or one side of 103 cells on 4.26 hemacytometers). The 30% reduction in total number of cells counted would generally not compensate for the extra number of fillings. If two samples are to be enumerated, however, one might consider doing them simultaneously, filling one side with one sample and the other side with the other sample.

Conclusions

We have illustrated that cell counts from one side of a hemacytometer are not Poisson distributed but compound Poisson distributed; i.e., the sample volume mounted over the counting area of the hemacytometer is distributed rather than constant. Furthermore, a strong positive correlation was found between the two sides of a hemacytometer. Improved description of the total cell counts on a hemacytometer was obtained using a normal distribution with quadratic variance function, \( X = \text{cell count} [e^\lambda(\frac{\lambda}{\alpha} + a\lambda^2)] \) with \( \alpha = 0.012 \).

Table V summarizes the protocols suggested when both sides of the hemacytometer are filled. If only one side is counted, 1.4 times as many hemacytometers should be counted.

Table V. Protocols for Obtaining 10%, 15%, and 20% Accuracy

<table>
<thead>
<tr>
<th>desired accuracy</th>
<th>no. of hemacytometers</th>
<th>cells per hemacytometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>7</td>
<td>180</td>
</tr>
<tr>
<td>15%</td>
<td>3</td>
<td>200</td>
</tr>
<tr>
<td>20%</td>
<td>2</td>
<td>125</td>
</tr>
</tbody>
</table>

Literature Cited


Accepted August 22, 1991.