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A COMPARISON OF THREE ESTIMATORS OF THE COEFFICIENT OF ERROR OF OPTICAL FRACTIONATOR CELL COUNT ESTIMATES

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ABSTRACT

The optical fractionator is a design-based two-stage systematic sampling method that is used to estimate the number of cells in populations that are too large to count exhaustively. It counts the cells found in optical disectors that have been systematically sampled in serial sections. Computer simulation was used to investigate three methods for estimating the coefficient of error (CE), the precision of a population size estimate, obtained from a single optical fractionator sample. The methods were: the original estimation equation of Gundersen and Jensen (1987), its nugget effect modification (West et al., 1996), and the method of Scheaffer, Mendenhall, and Ott (1996), which has not been used in stereology. It is difficult to evaluate the estimated precision of population cell count estimates by using biological tissue samples. They do not permit a comparison of an estimated CE with the true CE. Computer simulation does permit such comparisons while avoiding the observational biases inherent in working with biological tissue. The estimated CE's were evaluated in tests of three types of non-random cell population distribution and one random cell population distribution. The non-random population distributions varied according to both section and disector location within the section. Two were sinusoidal and one was linearly increasing; in all three there was a 6-fold difference between the high and low intensities, i.e., expected cell counts per disector. The sinusoidal distributions produced either a peak or a depression of cell intensity at the center of the simulated region. The linear cell intensity gradually increased from the beginning to the end of the region that contained the cells. The random population distribution had constant cell intensity over the region. A test condition was defined by its population distribution, the period between consecutive sampled sections and the spacing between consecutive sampled disectors. There were 1,000 trials of each test condition. In each trial were calculated the true CE of the expected cell count estimate and the three CE estimates obtained by applying the SMO and both GJ equations to single two-stage systematic samples. The estimated CE's were compared with the true CE's for all the population distributions. The CE estimates obtained by the SMO estimator were found to be closer to the true CE's and had less scatter than those of the nugget-modified GJ estimator. Both had small positive bias and comparable scatter. The CE estimates obtained by the unmodified GJ estimator exhibited

large negative bias and large scatter. In all the population distributions tested, the average true CE was very nearly proportional to $1/\sqrt{\overline{Q}_T}$, where $\overline{Q}_T$ is the average number of sampled cells.

Key words: computer simulation, optical fractionator, nugget effect, optical disector, cell count estimate precision, systematic sampling.

INTRODUCTION

In biological tissue, such as brain, cell populations are often large and dispersed through a region of interest that must be viewed in serial sections. A determination of the number of cells within the region is not practical by exhaustive counting throughout the sections. Instead, the proper approach is to estimate the population cell count by stereological statistical sampling techniques. Among them are random and systematic sampling (Scheaffer et. al., 1996). Of the two, systematic sampling is often more appropriate. It is unbiased and in this setting it can often yield more precise population cell count estimates for a given amount of sampling. Because of this it can reduce, relative to simple random sampling, the number of samples required to achieve a desired precision in the population cell count estimate.

In design based stereology the systematic sampling procedure is referred to as the fractionator (Gundersen, 1986, Gundersen et. al., 1988). It takes place in two stages and is therefore called two-stage systematic sampling. The first stage consists of a systematic sample of the K serial sections that contain the cell population whose count is being estimated. The sequence is subdivided into subsequences of k sections. Systematic sampling begins at a randomly selected initial section within the first subsequence. It continues by sampling every kth until there are no more sections left to sample. The second stage is carried out within each of the sections that were selected for sampling in the first stage. It consists of counting the cells within a systematic sample of equally sized optical disectors that are systematically spaced over the section's area (West and Gundersen, 1990). Sampling begins at a randomly selected initial location of the plane that defines the location of the first disector and continues by sampling consecutive, evenly spaced disectors within the region of interest.

The population cell count in the region of interest is estimated by multiplying the total disector cell count by a scale factor (West et al., 1991, Equation 1). The scale factor takes into account the fraction of a section that a set of disectors has sampled and the fraction of sections that have been so sampled. In our simulation, each section was constructed to contain an integral number, J, of contiguous disectors, which were then subdivided into subsequences of j disectors each. Sampling begins at a randomly selected disector within the first subsequence and continues with a period of j until all selected disectors are sampled.

It is crucial to the estimation of population cell count that the precision of the estimate be known or estimated with acceptable accuracy. In stereology, the precision of a fractionator-based estimate of a population cell count is gauged by the estimate's coefficient of error, CE. The defining equation for the true CE is then (Cruz-Orive, 1990):

$$CE(\hat{N}) = [Var(\hat{N})]^{1/2} / N \qquad (1)$$

Here $\hat{N}$ is the estimate of N, the true population cell count. In statistical theory the CE is known as the "coefficient of variation". In practical situations N will usually not be known exactly because of its large size. In simulation studies, such as the one conducted here, it can be specified exactly.

The true CE of the estimator of N can be calculated by obtaining $\hat{N}$ for each of the possible systematic samples that arise from starting at one of K sections and one of J disectors within the starting section. The $\mathrm{Var}(\hat{N})$ above is the variance of this set of all possible $\hat{N}$'s. In biological tissue this is comparable to the work of exhaustively counting cells, but in simulated cell populations it is easy to do.

It is desirable, however, to estimate the efficiency of a population cell count estimate using only the data obtained from a single two-stage systematic sample. In this paper we are concerned with three methods that attempt to do this. In general, the estimated CE can be obtained by using the estimates for the unknown parameters in the defining equation:

$$\mathrm{estCE}(\hat{N}) = [\mathrm{est}(\mathrm{Var}(\hat{N})^{1/2}]/\hat{N} \qquad (2)$$

All three methods that we have studied here yield the same estimate of N. Since these population count estimates are unbiased, the entire problem in estimating the true $\mathrm{CE}(\hat{N})$ is that of estimating $\mathrm{Var}(\hat{N})$, which was defined above.

Gundersen and Jensen (1987) proposed one such method that is based upon the work of Matheron (1971). That method (hereafter GJ) uses one-stage systematic sampling. It has been applied to tissue volume estimation obtained from cross sectional areas seen in systematically sampled serial sections. It has also been applied to data from two-stage systematic sampling; West and Gundersen (1990) applied it to optical fractionator estimates of neuron populations in the hippocampus.

Recently, the GJ method has been recognized as not providing reliable CE's of population size estimates. To improve its performance a "nugget effect" correction term has been added to the original estimator (West et al., 1996). This improves the performance of the estimator although the extent of the improvement has not been fully evaluated, and that evaluation is one of the goals of this study. We will refer to the nugget effect variant of the GJ estimator as nugGJ.

A third method for estimating the CE of a population cell count estimate is a one-stage systematic sampling procedure that has been described by Schaeffer, Mendenhall, and Ott, (1996). We refer to it as the SMO method. The SMO method uses simple random sampling theory as applied to one-stage systematic samples to estimate $\mathrm{Var}(\hat{N})$. It has not been applied to stereology. We use it with data from a two-stage systematic sample and compare its performance to the two GJ methods.

It is not known how well either of the GJ estimators or the SMO CE estimator behaves in estimating the variability of population cell count estimates, nor which of the three is superior. The performance of the GJ CE estimators has been evaluated only in limited studies of biological tissue. As used in those studies the fractionator does not permit obtaining an exhaustive set of systematic samples. These studies also did not permit extensive examinations of how the estimator is affected by different cell population distributions, differently shaped regions, or different periods for sampling sections and disectors within the sections. Likewise, the SMO CE estimator has not been evaluated in applications related to biological tissue. Since the quality of a population cell count estimate depends upon its precision, it is important that the behavior of these CE estimators be better understood. In order to do this, we have evaluated them using computer simulations of a few particular cell population distributions.

METHODS

Computer simulation bypasses the practical problem of counting real cells in real tissue, e.g., by an optical disector (hereafter disector). The nature of the simulation of a cell population is such that it can be easily modified to consider various types of cell distributions, non-random or random, within and across sections. It is a simple matter to define a region (or volume) of interest by the number of sections and the number of disectors within a section. It is equally simple to vary the cell population distributions and the fractionator sampling parameters that determine the number of sampled sections and the number of disectors that are sampled within a section. We use the term *population distribution* to refer to how the expected cell count per disector (hereafter cell *intensity*) varies across sections and disectors. The cell counts generated in the simulation are of those cells that have been specified, without error, to fall within a given disector of a given section. The total cell count of a simulated population is known and can be compared to the estimates of it obtained by the fractionator sampling procedures. Most important is the fact that the true CE of a population cell count estimate in a particular *trial*, which we define as a single realization of a cell population distribution. To do this we first calculate the true CE as described in Eq.(1). Then we compare this true CE with the estimates of it obtained from the formulae of GJ and SMO. We repeat this for each of 1,000 test cell populations and average the results for performance evaluation.

The simulation program was written in Quick Basic and consisted of the following steps. A copy of the program is available from EG upon request.

1. Generate a sequence of sections that will contain the population. We chose 48 sections (a number divisible by 12, 8, 6, 4, 3, 2 for flexibility in setting the section sampling period).
2. Partition each section into 48 (chosen for the reasons stated above) equally sized contiguous disectors (the simulated equivalent of optical disectors). Note that neither the sections nor the disectors have physical dimensions. The dimensions are unimportant since it is only the number of cells within a disector that matters.
3. Populate each disector with a number of cells. The number of cells within a disector was generated by a pseudo-random realization of a Poisson random variable whose cell intensity depended upon the section as follows: we used five different cell population distributions to specify a disector's cell intensity.

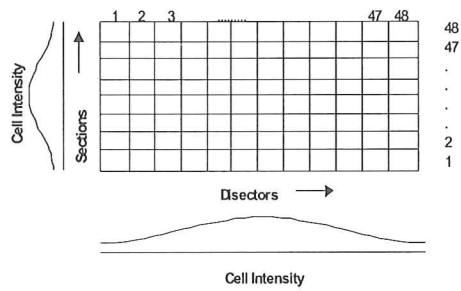

Fig. 1. Schematic diagram of the arrangement of sections and disectors. There are 48 sections, each containing 48 disectors. The curves along the axes illustrate a single cycle sinusoidal variation of expected cell count across sections and disectors. The number of disectors in a section is the product of the disector sampling period and the number of disector sampling periods per section. The same applies to the number of sections.

a) Positive single cycle sinusoid. The minimum densities occurred at the first and last disectors of the first and last sections of the sequence. The maximum cell intensity occurred at the center disectors of the center sections. The cell intensity varied from .333 to 2.0. The single cycle variation represents a narrow section containing only a single row of disectors.

b) Positive single cycle sinusoid with respect to disector number and a positive 2.5 cycle sinusoid with respect to disectors within a section. This simulates a section in which there were between two and three rows of disectors within a section.

c) Negative single cycle sinusoid. Here the minimum cell intensity of .333 per disector occurred at the center sections while the maximum of 2.0 cells per disector occurred at the first and last disectors of the first and last sections.

d) Linear ramp. The initial cell intensity per disector was 0.333 at the first disector of the first section. It increased linearly with the section number and disector number to its final cell intensity of 2.0 cells per disector at the last disector of the last section.

e) Constant cell intensity of 1.0 cells per disector in all sections. This defines a purely random population distribution.

4. Select a systematic sampling protocol. The possible section sampling periods and disector sampling periods are stated in (1) and (2) above. A section sampling period, k, was specified as an integer between 1 (every section) and 12 (only 4 sections sampled). Section sampling always began at a randomly chosen section within the first sampling period. Similarly, a disector sampling period, j, was specified and could be assigned integer values from 1 (every disector) to 12. Disector sampling began at the first randomly chosen disector within the first disector sampling period. Thereafter the initial sampled disector was the same in each sampled section.
5. Count the exact number of cells comprising the population in all disectors of all the sections. This value is N.
6. Estimate the population cell count from each of the possible systematic samples.
7. Calculate the true CE from the population estimates obtained from this exhaustive set of systematic samples. To do this one starts at each possible starting disector of each possible starting section. The population cell count estimate is obtained from each member of the set of all possible systematic samples. From this set the mean and the variance of the population cell count estimates are obtained from all the systematic samples. Then one calculates the true CE according to Equation (1). The true CE forms the basis for evaluating the goodness of the estimated CE's of the population cell count estimate.
8. Calculate the estimated CE for each of the possible systematic samples as obtained from the GJ, nugGJ, and SMO estimators separately. The GJ and the nugGJ equations are based upon Gundersen and Jensen's (1987) Equation (6). All three methods are given in the Appendix.
9. Calculate for all three estimators the mean values and variances of their estimated CE's over the set of all possible systematic samples.
10. Repeat the procedure for the 1,000 trials having the same set of simulation parameters as defined in (2), (3), and (4) above.
11. Average the 1,000 true CE's and the 1,000 mean values and variances of the estimated CE's. Compare the true and predicted CE's.

12. Calculate the CE's of the GJ, nugGJ and SMO CE estimates. This permits examining their variability.
13. Perform the foregoing steps for each of the selected population distributions and systematic sampling parameters.

RESULTS

We investigated the effect of the systematic sampling parameters upon the CE, true and estimated, for each of the population distributions. The systematic sampling parameters were the section sampling period and the disector sampling period. We selected three section sampling periods: 4, 6, and 8 and four disector sampling periods: 1, (all disectors), 2, 4, and 8. These 12 different parameter sets and the population densities yielded a total of 60 *test conditions* of 1,000 repetitions (trials) each. When we analyzed the data, we found no simple relationship between the CE and either the section or the disector sampling periods. On the other hand, it became clear that when we combined the results obtained from the separate population distributions, some general results become apparent. Plots made from these data demonstrated (1) a general relationship between the average number of sampled cells in a test condition and the true CE and (2) how well the different estimated CE's were in accord with the true CE.

Relationship between the average true CE and the average number of sampled cells

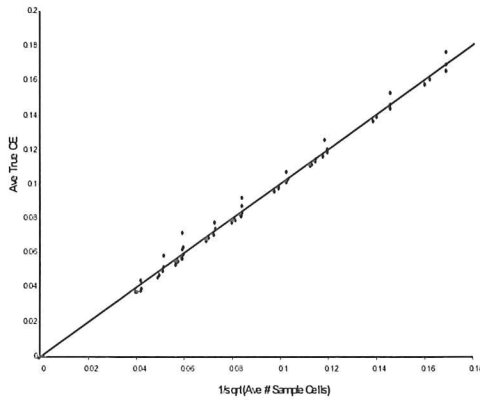

Fig. 2. The average true CE values are plotted as a function of $1/\sqrt{\overline{Q}_T}$. *In this and subsequent figures the plotted points represent the average values from 1,000 trials of each sampled test population.*

The nearly linear relationship between the true CE and the quantity $1/\sqrt{\overline{Q}_T}$, where $\overline{Q}_T$ is the average number of sampled cells, is shown in Figure 2. Elementary statistical theory yields a linear relationship for systematic or random sampling of random distributions, i.e., those in which cells are randomly distributed throughout the region of interest. This follows from the fact that the number of cells in any random sample of a random population follows a Poisson distribution. However, it is not immediately apparent that this would also apply to non-random population densities. Note that if a particular sampling design yields an average

sample count of 100, the true CE is nearly 0.1. Note that the population intensities are based upon Poisson statistics whose cell intensity varies from disector to disector.

Comparison of the average original GJ predicted CE's with the average true CE

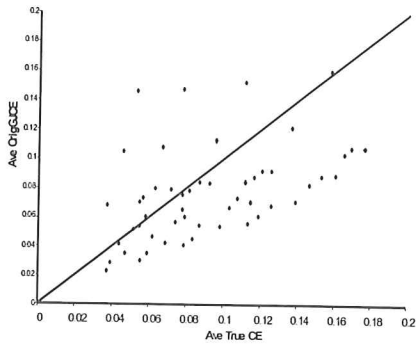

Fig. 3. The performance of the average original GJ CE estimator is plotted as a function of the average true CE. Each point represents the results for a single population distribution sampled in a particular manner (see text). The identity line appears here and in all subsequent figures.

It can be seen that there is a large scatter of predicted CE's and that this scatter yields values that can either underestimate or overestimate the true CE. In general the predictor tends to be biased high for CE's less than about 0.07 and biased low for values above that. Most of the predicted CE's are less than the true CE; it is the few highly discrepant outliers that mask the tendency of the predictor to underestimate the true CE.

Comparison of the average nugGJ predicted CE's with the average true CE

When the nugGJ estimator is used, the estimated results show a significant improvement. The results are plotted in Figure 4.

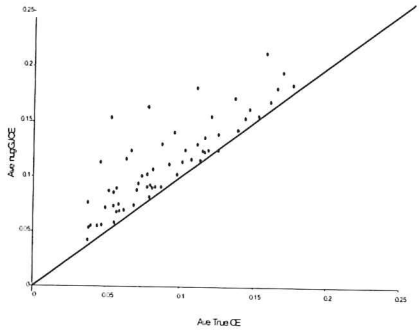

Fig. 4. The performance of the average nugGJ CE estimator is plotted as a function of the average true CE. The data points and straight line are explained in Figure 1 caption.

The scatter in the predicted values is greatly reduced as compared to the original GJ. The estimated CE's are biased high and are al-most always higher than the true CE. The bias yields predicted CE's that are about somewhat higher than the true CE when its value is about 0.1.

Comparison of average SMO predicted CE's with the average true CE

The values obtained from the SMO CE are shown in Figure 5.

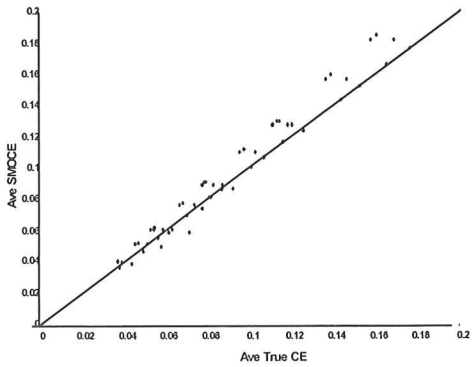

Fig. 5. The performance of the average SMO CE estimator is plotted as a function of the average true CE.

The relationship between the SMO CE and the true CE is nearly a linear one, although biased high for CE's greater than about 0.06. Unlike nugGJ, the SMO estimator can yield predicted CE's that are less than the true CE.

DISCUSSION

This is the first study to employ simulation to evaluate the performance of estimated CE's in fractionator-based population cell count estimation and to compare estimated with true CE's. Previous studies have dealt only with estimated CE's obtained from very limited amounts of biological data. They have not been able to evaluate the estimated CE's with respect to true CE's.

Simulation idealizes and simplifies the systematic sampling paradigm while preserving its essential features. Simulation avoids the tedious, error prone procedures that occur when counting cells in optical disectors. Such errors obscure the issue. Because the disectors occupied contiguous, integrally spaced locations within the region, we could perform exhaustive systematic sampling and thereby determine the true CE of a fractionator population estimate. This permitted making direct comparison between the estimated CE's and their true values for all the different population distributions. It is unlikely that this special property of our simulation materially affected the results. It should be clear that if the CE estimators do not perform well in this idealized situation, they would be even less successful in a real situation where departures from theoretical models are even greater.

The variation in population distribution within a section was such that each section could be thought of as a narrow slab of tissue sampled by a single row of disectors. To be sure this did not influence our results, we also conducted tests with a 2-cycle and a 2.5 cycle variation in cell intensity. These could be considered to represent a section with two or two

and a half rows of disectors. The results were not different from those obtained with the single cycle or linear ramp variations in cell intensity.

The population distributions we employed provided a systematic variation in cell intensity from section to section and disector to disector. The number of cells within a disector followed a Poisson distribution. It is not clear whether the choice of a Poisson distribution is a major factor that influenced our results. However, the Poisson distribution is a reasonable approximation of the kind of variability that occurs in real cell populations.

A major finding of this study is that a fractionator-based estimation of population cell count yields an average CE that is nearly proportional to $1/\sqrt{\bar{Q}_T}$. The number of sampled sections and the number of disectors within a section are important only to the extent that they influence the number of sample counts obtained.

All three CE estimators are based upon one-stage systematic sampling theory and to that extent are not fully appropriate for application to the fractionator's two-stage systematic sampling. None of the estimators take into account any cell intensity changes within individual sections. Both the SMO CE estimator and the nugGJ CE estimator yielded estimates close to the true CE. However, the SMO estimator was more precise and had less scatter. The nugGJ estimator has been used by West et al. (1996). The sampled cell count is the predominant contributor to the estimated variance of the nugGJ CE estimator. This means that the variance increases approximately as the sampled cell count and that the CE of the population size estimate is therefore nearly in inverse proportion to the square root of the sampled cell count. This behavior is close to that of the SMO CE estimates and with the behavior of the true CE itself.

In contrast, the unmodified GJ CE estimator did not perform well; it exhibited a large scatter and usually significantly underestimated the CE. Although this population size estimator has been previously acknowledged to have shortcomings, we have been unable to find published information as to why this is so. This simulation therefore has in part documented its shortcomings.

Although the nugGJ estimator did not perform poorly, the SMO estimator performed noticeably better. Also, the SMO estimator is conceptually simple, easy to implement, and it offers the possibility that its theory can be extended to two-stage systematic sampling.

We do not claim that the SMO estimator is optimal, but in the settings we examined it is better than the others. Our purpose was to compare the three methods but not to analyze the reasons for their differences.

None of the CE estimators we tested is able to estimate how precise its CE estimate is. This is a matter of some interest since a grossly incorrect CE estimate can badly influence the conduct of an experiment. Our results, not included here, indicate that the magnitude of the CE's of the CE's are about equal to the CE's themselves.

We realize that there may be some problems in applying a simple ransom sampling error predictor in a systematic sampling context, even though the 1/Q term dominates the simulated models. The problem is non-trivial and can hardly be fully explored by way of simulation alone. Nonetheless, it is interesting to see that in the settings we studied the SMO estimator is superior to the others.

CONCLUSION

Simulation studies reveal that the CE of a fractionator-based population cell count estimate is well estimated by the CE estimator described by Scheaffer et al. (1996) and almost as well by the nugget-corrected estimator of Gundersen and Jensen (1987). These results apply to both non-random and random cell populations.

The original CE estimator of Gundersen and Jensen (1987) yields values that fluctuate greatly and tend to be substantially lower than the true CE. This erratic behavior indicates that that prediction equation is inappropriate for population cell count estimation.

The CE of a fractionator estimation of population size, for nonrandom and random distributed cell population, is nearly inversely proportional to the square root of the average number of cells sampled. This empirical result is an extension of a well-known finding that applies to a random distribution of cells.

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APPENDIX

The Gundersen and Jensen CE estimator

The Gundersen and Jensen estimator of the CE of the population cell count estimate for an individual subject is given by (for clarity we have modified the notation slightly):

$$\text{GJ CE} = \sqrt{[3A + C - 4B]/12}/Q_T \qquad \text{(A1)}$$

where Q_i is the number of cells counted within the optical disectors of the ith sampled section, I is the total number of sampled sections, and Q_T is the number of sampled cells. When i+1 or i+2 exceeds I, the corresponding value of Q_i is 0.

$$Q_T = \sum_{i=1}^{I} Q_i\,,\quad A = \sum_{i=1}^{I} Q_i^2\,,\quad B = \sum_{i=1}^{I} Q_i Q_{i+1}\,,\quad C = \sum_{i+2}^{I} Q_i Q_{i+2} \qquad \text{(A2)}$$

The nugget correction alters the equation, to the form:

$$\text{nugGJ CE} = \{Q_T + [3(A - Q_T) + C - 4B]/12\}^{1/2}/Q_T \qquad \text{(A3)}$$

Note that neither the original GJ equation nor the nugGJ version are concerned with the population of cells within individual disectors and are insensitive to any spatial ordering of cell counts from disector to disector.

Scheaffer et al. CE estimator

From Scheaffer et al (Chap. 4, 1996) for the estimated population cell count that results from a single one-stage systematic sample. The estimated mean cell count per disector and the estimated variance of cell counts among disectors are:

$$\text{mean:}\quad \bar{Q} = \frac{\sum_{m=1}^{f} Q_m}{f} \qquad \text{variance:}\quad s^2 = \frac{\sum_{m=1}^{f}\left(Q_m - \bar{Q}\right)^2}{f-1} \qquad \text{(A4a\&b)}$$

where Q_m is the cell count in the mth sampled disector and f is the total number of sampled disectors in a trial.. In our usage, the sampling units are the disectors. Note that the locations of the disectors within the sections are irrelevant.

The estimated variance of the mean cell count per disector is: $fpc*s^2/f$, where fpc is the finite population correction that is given by = [(F-f)/F]. F is the total number of disectors in all the sections of the trial and is known in our simulations. It will usually be unknown in actual stereological usage.

The estimated cell population cell count, given F is $F\bar{Q}$. The estimated variance of $F\bar{Q}$ when F is known is: $fpc*F^2*s^2/f$. From this, it is easy to show that SMO $\text{CE} = \sqrt{[1/f - 1/F]} \times \left(s/\bar{Q}\right)$. We used the fpc in our simulation, but if F is much larger than f, 1/F can be neglected. Then, for random populations, $\text{SMO CE} \approx (s/\bar{Q}) \times (1/\sqrt{f})$. When the population statistics are Poisson, this simplifies to:

$$\text{SMO CE} \approx 1/\sqrt{Q_T}\,. \qquad \text{(A5)}$$

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