The 400 microsphere per piece “rule” does not apply to all blood flow studies

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Polissar, Nayak L., Derek C. Stanford, and Robb W. Glenny. The 400 microsphere per piece “rule” does not apply to all blood flow studies. Am. J. Physiol. Heart Circ. Physiol. 278: H16–H25, 2000.—Microsphere experiments are useful in measuring regional organ perfusion as well as heterogeneity of blood flow within organs and correlation of perfusion between organ pieces at different time points. A 400 microspheres/piece “rule” is often used in planning experiments or to determine whether experiments are valid. This rule is based on the statement that 400 microspheres must lodge in a region for 95% confidence that the observed flow in the region is within 10% of the true flow. The 400 microspheres precision rule, however, only applies to measurements of perfusion to a single region or organ piece. Examples, simulations, and an animal experiment were carried out to show that good precision for measurements of heterogeneity and correlation can be obtained from many experiments with <400 microspheres/piece. Furthermore, methods were developed and tested for correcting the observed heterogeneity and correlation to remove the Poisson “noise” due to discrete microsphere measurements. The animal experiment shows adjusted values of heterogeneity and correlation that are in close agreement for measurements made with many or few microspheres/piece. Simulations demonstrate that the adjusted values are accurate for a variety of experiments with far fewer than 400 microspheres/piece. Thus the 400 microspheres rule does not apply to many experiments. A “rule of thumb” is that experiments with a total of at least 15,000 microspheres, for all pieces combined, are very likely to yield accurate estimates of heterogeneity. Experiments with a total of at least 25,000 microspheres are very likely to yield accurate estimates of correlation coefficients.

organ perfusion; heterogeneity; correlation; modeling; statistics; Poisson noise

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The hypothetical example presented in Table 1 motivates the methods developed later and shows that some firm conclusions can be made even when all sample pieces have <400 microspheres. The first column shows the expected number of microspheres for each of 10 pieces based on the true flow to each piece, and the next two columns show “observations” on these pieces, generated by random sampling from the Poisson distribution with the expected number of microspheres for each piece. As predicted from Buckberg’s work, the percent error for some pieces is unacceptably high. However, it should be noted that when the mean and heterogeneity (coefficient of variation) are calculated for these three columns, they are very similar, differing by <2% out of 58%. Note also that the correlation between the two observations, \( r = 0.985 \), is close to 1.0, which is the correlation that would be observed in the absence of any random variation (the correlation of column 1 with itself). Thus the experimental observations are very similar to the “truth” (column 1), even though not one of the pieces has an expected value or an observed value >400 microspheres. As a further example, it seems clear from the data in experiment 1 that the piece with 6 microspheres and the piece with 101 microspheres represent very different flows. This is formally confirmed by a \( \chi^2 \) test (1 degree of freedom), which yields \( P < 0.0001 \). Thus one can conclude with a great deal of confidence that there is a different flow to these two pieces, even though both pieces have many fewer than 400 observed microspheres. A number of other differences in observed microsphere counts between pairs of pieces in Table 1 are also statistically significant. The table also includes adjusted values for the coefficient of variation (CV) and correlation, using the methods developed later in this paper. In each case, the adjusted values are closer to the true values than the values based on observed results. The adjustments are quite minor.

We show that quite good estimates of heterogeneity and correlations can be calculated even when many regions (pieces) have relatively low microsphere counts. Furthermore, heterogeneity and correlation can also be adjusted to take account of the Poisson noise.

**METHODS**

**Theory**

For any given piece or region, there is a certain probability that a microsphere will lodge in the region at the time of injection. If microspheres act independently, every microsphere has the same probability of reaching a specified region. The expected number of microspheres reaching a region will be \( N \times p \), where \( N \) is the number of microspheres injected and \( p \) is the proportion of total flow to the organ, which perfuses the region. Thus \( p \) is also the expected proportion of microspheres captured by the specified region. Because \( N \) is large and \( p \) is small, the distribution of \( n \), the number of microspheres actually reaching the region, can be well approximated by the Poisson distribution (12). Previous studies have attempted to correct for Poisson noise using the total microsphere count for the entire organ (5, 7), but the methodology presented here is more realistic and precise in its use of microsphere data for individual pieces of an organ.

Notation. We use the subscript “true” to indicate the unobservable true value of a quantity, e.g., \( CV_{true} \) represents the coefficient of variation of true flow, measured without error. We use the subscript “obs” to denote a quantity calculated by conventional formulas from the observed data from an experiment or from randomly simulated data that are intended to mimic observed data from a real experiment. We use the subscripts or notation “adj,” “adjusted,” or “corrected” to indicate an estimate, based on the equations developed in this paper, that attempts to remove the effect of random error due to Poisson noise. We also use the hat symbol “\(^\hat{\}\)” to indicate these adjusted estimates. Thus \( CV_{true} = CV_{adj} \) is an estimate of the true CV. Similarly, \( r_{true} \) is the true unobservable correlation, \( r_{obs} \) is the observed correlation, and both \( r_{adj} \) and \( r_{true} \) are estimates of the true correlation incorporating the adjustments described in this paper.

Adjusting estimates of heterogeneity for noise. When heterogeneity is expressed as the CV, then, with the use of the observed number of microspheres per piece (\( X_i \) for piece \( i \)), the observed CV is calculated as

\[
CV_{obs} = \frac{S}{\bar{X}} = \sqrt{\frac{\sum_{i=1}^{N} (X_i - \bar{X})^2}{\sum_{i=1}^{N} X_i}}
\]

where \( S \) is the standard deviation of \( X_i \) (calculated with \( N \), number of pieces, in denominator) and \( \bar{X} \) is the mean of \( X_i \). Random errors will tend to cancel in the denominator when the mean is taken across all pieces, but the numerator, the standard deviation, will be inflated when Poisson variation is added to the true variation in flow.

The Poisson noise can be adjusted “out” of the CV estimate by using a relatively simple equation that is derived in
APPENDIX 1. Equation 2 provides an estimate of the true CV

\[ \hat{CV}_{true} = \sqrt{CV_{obs}^2 - \frac{1}{N}} \]  

(2)

where \( N \) is the number of pieces in the analysis. We note that if the computation leads to the square root of a negative number, the CV should be set to zero and this value indicates small heterogeneity.

If \( N \) and \( X \) are large, this formula simplifies to

\[ \hat{CV}_{true} = \sqrt{CV_{obs}^2 - \frac{1}{X}} \]  

(3)

Because the distribution of microspheres is Poisson, it can be shown that \( CV_{noise}^2 \), the contribution of Poisson noise variance to the overall CV, can be written as \( CV_{noise}^2 = 1/N \). Equation 3 is then equivalent to that proposed by Iversen et al. (7), which is \( CV_{true}^2 = CV_{obs}^2 - CV_{true}^2 \).

Adjusting estimates of correlation for noise. Pearson’s correlation \( r \) measures the tendency of flows to vary together (e.g., before and after an experimental change, or time 1 vs. time 2, etc.). The Pearson correlation \( r_{obs} \) is calculated from the observed data, pairs of values \((X_i, Y_i)\) for \( N \) pieces, as

\[ r_{obs} = \frac{\sum_{i=1}^{N} (Y_i - \bar{Y})(X_i - \bar{X})}{\sqrt{\sum_{i=1}^{N} (Y_i - \bar{Y})^2 \sum_{i=1}^{N} (X_i - \bar{X})^2}} \]  

(4)

The value of \( r_{obs} \) is a biased estimate of the true correlation, \( r_{true} \), because of the inflation of the denominator of \( r_{obs} \) by Poisson noise, an effect noted earlier for the CV. As was true for the CV, the Poisson noise can also be adjusted out of the correlation using an equation that is derived in APPENDIX 2. The estimate that corrects for Poisson noise is

\[ r_{true} = \frac{r_{obs} \cdot S_X \cdot S_Y}{\sqrt{S_{X}^2 - \frac{N-1}{N} \bar{X} - \frac{N-1}{N} \bar{Y}} \cdot \sqrt{S_{Y}^2 - \frac{N-1}{N} \bar{Y}}} \]  

(5)

where \( S_X \) and \( S_Y \) are the population standard deviations for the \( X \) and \( Y \) observations, respectively. If this formula leads to a number > 1, the correlation estimate should be set to 1.

Using Eqs. 2 and 5, an investigator can derive estimates of the true CV and true correlation for an experiment. The resulting values of \( CV_{true} \) and \( r_{true} \), however, estimate heterogeneity and correlation of true absolute flow or true relative flow. It is important in using Eqs. 2 and 5 that microsphere counts be used in calculation of all means and standard deviations. Absolute blood flow or relative blood flow should not be used in the calculations.

Simulation Tests

The applicability and robustness of the derived formulas, Eqs. 2 and 5, were explored through computer simulations of organ blood flow and microsphere injections. Organ blood flow distributions were generated with the fractal model of van Beek et al. (11). This model produces heterogeneous flows that are skewed to the right (toward higher flows), similar to experimental observations of perfusion distributions to myocardium (11), lung (4), and skeletal muscle (6). The details of the construction of simulated organs can be found in APPENDIX 3.

Single- and paired flows for true organs. “True” organs were generated by specifying the perfusion heterogeneity and the number of pieces in the manner described in APPENDIX 3, and paired true sets of piece flows were generated with specified correlations, representing paired observations on pieces. Every generated true organ, then, had a paired value of \((X_{true}, Y_{true})\) for each piece. A variety of true organs were generated by varying the nominal perfusion heterogeneity (CV = 25, 50, 100%), the nominal correlation \( r = -0.7, -0.3, 0.0, 0.3, 0.7, 0.98 \), and the number of pieces \( N = 8, 16, 32, 64, 128, 256 \). Because of the numerical procedures used in generating true organs, the CVs and correlations can differ slightly from the specified target values. The true CV values for generated organs ranged from 24.8 to 100.2%, and the true correlations ranged from \(-0.742\) to 0.995.

Simulation of observed organs. The microsphere data for simulated observed organs were generated by randomly drawing from the Poisson distribution to yield an observed value or pair of values for each piece. The Poisson distribution used for each piece had an expected value of \( X_{true} \) (or \( Y_{true} \)). The overall noise level of an experiment was specified by the expected number of microspheres per piece, which varied across 14 levels: 3, 6, 12, 25, 50, 100, 200, 400, 800, 1,600, 3,200, 6,400, 12,800, and 25,600 microspheres. For each combination of perfusion heterogeneity, number of pieces, number of microspheres/piece, and correlation, we generated observed organs and paired organs with the same mean number of microspheres/piece. Altogether, 1,512 combinations of these parameters were used to test the CV correction (Eq. 2) and the correlation coefficient (Eq. 5). We simulated 500 observed organs per combination. For each of the 500 simulations, the value of \( CV_{obs} \) was corrected to \( CV_{true} \) using Eq. 2 and the value of \( r_{obs} \) was corrected to \( r_{true} \) using Eq. 5. For each set of 500, we calculated the mean, variance, and standard deviation of \( CV_{obs} \), \( CV_{true} \), \( r_{obs} \), and \( r_{true} \).

Experimental Protocol

We carried out an animal experiment to illustrate the use of our adjusted estimates with biological data. We also wanted to demonstrate that relatively small numbers of microspheres per sample piece could still yield quite accurate estimates of the CV and correlations.

The study was approved by the University of Washington Animal Care Committee. A single 3-kg New Zealand White rabbit was chemically restrained with intramuscular ketamine, anesthetized with thiopental, and allowed to breathe spontaneously. An internal jugular catheter was placed. Fifteen-micrometer-diameter microspheres with four different radionuclides (\(^{153}\)Gd, \(^{113}\)Sn, \(^{103}\)Ru, and \(^{95}\)Nb; NEN) were used to determine regional pulmonary blood flow. Radioactivity per microsphere for each radionuclide was determined by visually counting microspheres on filters and then determining the radioactive counts from each filter. The numbers of microspheres injected were intentionally chosen to provide large numbers of gadolinium and tin with small numbers of rubidium and niobium. The microspheres were sonicated, vortexed, mixed in a single syringe, and then injected via the internal jugular catheter over 30 s. The animal was killed by an intravenous overdose, and the lungs were removed, air dried, inflated, and diced into 100 pieces. Each piece was placed in an individual scintillation vial, and the radioactivity of each radionuclide was determined in a scintillation counter (Packard Minaxi gamma counting system, model 5550). Each sample was read for 20 min or until sufficient counts were obtained to provide a counting error < 0.5% for each radionuclide. The radioactive counts for each radionuclide in each
piece were corrected for spillover using a matrix inversion method and were also corrected for background and decay. The numbers of each microsphere in each piece were calculated from radioactive counts and the measured value of radioactive counts/microsphere.

RESULTS

Experimental Results

Our data consist of 100 pieces of lung, each with 4 radioactive microsphere measurements (\(^{153}\)Gd, \(^{113}\)Sn, \(^{103}\)Ru, and \(^{95}\)Nb). On average, there are 1,599 \(^{153}\)Gd, 880 \(^{113}\)Sn, 131 \(^{103}\)Ru, and 80 \(^{95}\)Nb microspheres per organ piece. We examine the CVs and correlations in these data, as well as our assumption that the noise can be modeled by a Poisson distribution.

Table 2 shows the conventional observed CV for each microsphere measurement computed from Eq. 1, as well as the adjusted CV computed from Eq. 2. With these data, our adjustment changes the CV only slightly. The adjusted CV values for all four measurements show extremely good agreement, differing by \(\leq 0.008\). The observed values of CV are all also quite similar (differing by \(\leq 0.01\)) even though two of the injections have quite low mean counts of microspheres/piece (\(^{103}\)Ru and \(^{95}\)Nb), well below 400 microspheres/piece.

Table 3 shows the Pearson correlation between each pair of microsphere measurements; for each correlation, we show both the conventional observed value computed from Eq. 4 and the adjusted value computed from Eq. 5. The true correlation in the absence of all noise should be 1, because the measurements are made at the same time on the same pieces. The correction brings each correlation closer to the true value of 1.0; however, all correlations are very similar and very close to 1.0, even though some are based on low microsphere counts (\(^{103}\)Ru and \(^{95}\)Nb). Figure 1 shows the strong correlation between \(^{103}\)Ru- and \(^{95}\)Nb-based microsphere counts, with little scatter around the least-squares line. The observed correlations differ from 1.0 by \(\leq 0.03\), and the adjusted correlations differ from 1.0 by \(\leq 0.006\). Clearly, Poisson noise has had little influence on these correlations.

A further examination of the microsphere counts provides evidence in favor of the Poisson noise assumption. True Poisson noise has a piece variance equal to the piece mean. To check this assumption, we estimated the variance and mean for each piece. We regressed each of the low signal measurements (\(^{103}\)Ru and \(^{95}\)Nb) on the sum of the other three measurements with a zero-intercept model. For each piece the squared residual from the regression model is an estimate of the piece variance; this can be compared with the regression-fitted expected microsphere count for the same piece, which is an estimate of the true piece mean. (A single outlier in the entire data set was excluded from the analysis of \(^{103}\)Ru because its residual was >4 standard deviations from the regression line.) In a second analysis, we regressed the estimated piece variance on the estimated mean. We found that the regression slope of variance versus mean was close to 1.0, the value expected if the microsphere counts follow the Poisson distribution. This supports (but does not prove) the assumption of Poisson noise. The increment in slope above 1.0 is most likely caused by variation not related to the distribution of microspheres. Such variation would include random variation in radioactive counting and laboratory error.

Definitively showing that the Poisson distribution fits microsphere count data would require an experiment that is currently impossible. One would have to carry out a large number of simultaneous microsphere injections, each with a unique radioisotope or other tag, and then compare the resulting microsphere count distribution to the Poisson distribution. Constraints on tagging microspheres and on body burden render this experiment impossible with current technology.

Simulation Tests

When the number of microspheres in an experiment is large, the unadjusted CV and correlation differ little from the true CV and correlation and either can be

<table>
<thead>
<tr>
<th>Mean Microspheres per Piece</th>
<th>Observed CV</th>
<th>Adjusted CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{153})Gd</td>
<td>1.599</td>
<td>0.6474</td>
</tr>
<tr>
<td>(^{113})Sn</td>
<td>880</td>
<td>0.6471</td>
</tr>
<tr>
<td>(^{103})Ru</td>
<td>131</td>
<td>0.6601</td>
</tr>
<tr>
<td>(^{95})Nb</td>
<td>80</td>
<td>0.6583</td>
</tr>
</tbody>
</table>

Table 3. Observed and adjusted correlation from rabbit data

<table>
<thead>
<tr>
<th></th>
<th>(^{113})Sn</th>
<th>(^{103})Ru</th>
<th>(^{95})Nb</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{153})Gd</td>
<td>0.9975</td>
<td>0.9941</td>
<td>0.9803</td>
</tr>
<tr>
<td>(^{113})Sn</td>
<td>0.9996</td>
<td>0.9967</td>
<td>0.9935</td>
</tr>
<tr>
<td>(^{103})Ru</td>
<td>0.9867</td>
<td>0.9867</td>
<td>0.9992</td>
</tr>
<tr>
<td>(^{95})Nb</td>
<td>0.9739</td>
<td>0.9739</td>
<td>0.9967</td>
</tr>
</tbody>
</table>

Fig. 1. Scatterplot of microspheres per piece based on \(^{103}\)Ru and \(^{95}\)Nb. N, no. of pieces; CV, coefficient of variation; R, Pearson correlation.
used. However, as the number of microspheres in an experiment is reduced, the adjusted CV and correlation are approximately unbiased and are accurate for much smaller numbers of microspheres than the unadjusted values, as shown by the simulation results below.

We initially illustrate the simulation results with some examples and then present the overall results. The relative performance of the observed and adjusted CV are displayed for four true organs in Fig. 2, which shows simulation results for small (CV = 25%) and large (CV = 100%) heterogeneity and for small (n = 16) and large (n = 256) numbers of pieces. Note in all plots that the mean CV_{true} (noted as “corrected”) is closer to the true CV for considerably smaller numbers of microspheres than the mean CV_{obs} (noted as “observed”). Also note that both CV_{true} and CV_{obs} are quite close to the true CV, with rather small standard deviations, for a number of the points (observed organs) <400 microspheres/piece. Also, in the top panels of Fig. 2, where the true CV is larger than in the bottom panels, CV_{obs} diverges less rapidly from the true CV as microspheres/piece decreases. The larger true CV (the “signal”) is much more prominent than the Poisson error (the “noise”), and the noise has relatively less influence.

Although the observed CV of organ perfusion is dependent on the number of pieces into which the organ is dissected (1), these simulations show that unbiased estimates of the observed perfusion heterogeneity can be obtained from an organ with as few as 16 pieces and an average of only 4 microspheres per piece by using Eq. 2 for CV_{true}. The primary consequence of using such small numbers of organ pieces and microspheres is a large variance in the estimate of heterogeneity and hence a loss of confidence in the observation from a single experiment. Because the corrected estimates are unbiased, observed CV values averaged over a number of experiments will produce quite accurate estimates of the true CV.

The simulation results for correlations show features similar to those for the CV. Figure 3 illustrates performance for low (true \( r = 0.27–0.34 \)) and strong (true \( r = 0.98–0.99 \)) correlations and for many (n = 256) and few (n = 16) pieces. The CV for all of the simulations in these figures is \( \approx 50\% \). Again, both \( r_{obs} \) (noted as “observed”) and \( r_{true} \) (noted as “corrected”) are close to \( r_{true} \) with small standard deviations for a number of the points below 400 microspheres/piece. The mean \( r_{true} \) stays closer to the true \( r \) than \( r_{obs} \) for much smaller numbers of microspheres per piece. Also, \( r_{obs} \) diverges from the true correlation much more rapidly with decreasing microspheres/piece when the correlation is large.

These examples illustrate that the observed CVs and correlations can be quite accurate for many experiments with <400 microspheres/piece and that use of corrected CVs and correlations extends accurate results into experiments with considerably smaller numbers of microspheres/piece.

Table 4. Regression results from rabbit data

<table>
<thead>
<tr>
<th></th>
<th>Squared Residual</th>
<th>Error</th>
<th>Microsphere Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>X</td>
<td>N</td>
<td>Slope SE</td>
</tr>
<tr>
<td>99Nb</td>
<td>153Gd + 113Sn + 103Ru</td>
<td>100</td>
<td>1.1493 0.1869 &lt;0.001</td>
</tr>
<tr>
<td>103Ru</td>
<td>153Gd + 113Sn + 99Nb</td>
<td>99</td>
<td>1.0442 0.1290 &lt;0.001</td>
</tr>
</tbody>
</table>

X and Y, injections; N, no. of microspheres injected.

Fig. 2. Examples of observed and corrected CV based on simulation for 16 (A and C) or 256 (B and D) pieces. True CV = 99.8 (A), 100.1 (B), 24.9 (C), and 25.2 (D). Each point represents mean of 500 simulated values. Vertical lines at bottom of each plot represent standard deviations.
We can summarize the accuracy of the corrected and uncorrected CV and correlation estimates using two key measures: bias (systematic error, e.g., expected value of \( CV_{\text{obs}} - CV_{\text{true}} \)) and root mean square error (RMSE = (bias\(^2 + \) variance\(^{1/2} \)). The RMSE incorporates both bias and variability. A small value of RMSE ensures that both bias and variability are small and that the estimated quantity is accurate. For completeness, we also present results on the standard deviation of the CV and of the correlation. The standard deviation is a concept of precision (not accuracy), but good precision is not helpful if bias is large.

Table 5, based on the simulations, shows the minimum size of experiments, expressed as the total number of microspheres in the organ, that yielded an estimated bias, standard deviation, and RMSE at or less than the amount shown, for all sets of simulation parameters (number of pieces, \( CV_{\text{true}} \) and \( r_{\text{true}} \)). The 1,512 simulated experiments used to construct the table were described in Simulation Tests and include a wide range of numbers of pieces, true CV, microspheres per piece, and correlation. The corrected estimates have a clear advantage in bias and RMSE; the uncorrected estimates require many more microspheres to obtain levels of bias comparable to those of the uncorrected estimates. For example, to have a quite accurate estimate of the CV, as indicated by an RMSE of \( \pm 2\% \), at least 25,260 microspheres are needed (all pieces combined) when the conventional \( CV_{\text{obs}} \) is used. However, if \( CV_{\text{true}} \) is used, only 12,803 microspheres are needed, about one-half as many. In an experiment involving correlations, the conventional \( r_{\text{obs}} \) would require \( >400,000 \) microspheres and \( r_{\text{true}} \) would require \( >100,000 \) microspheres to yield very good accuracy with a small RMSE of 0.02. The standard deviation results are about the same for both corrected and uncorrected estimates. Thus the smaller values of RMSE for the corrected estimates compared with the conventional observed estimates are primarily caused by the smaller values of bias for the corrected estimates.

The blank cells in Table 5 indicate that there were some parameter sets for which the standard deviation or RMSE never reached the minimum level indicated in the table. The maximum number of microspheres (organ total) in the simulations was 6,553,600; using more microspheres would provide lower levels of bias, standard deviation, and RMSE, even in the extreme cases.
Effect on Experimental Design

We developed approximate equations for the variance of $\hat{CV}_{true}$ and $\hat{r}_{true}$ that can be used in planning the number of microspheres to be used in experiments to achieve a specified level of accuracy. The exact variance of $\hat{CV}_{true}$ and $\hat{r}_{true}$ cannot be written in closed form, and the asymptotic variances are extremely complex. Thus we fit approximate models for these variances using linear regression with the observed variances as dependent variables and combinations of powers, products, and other functions of simulation parameters (number of pieces, heterogeneity, microspheres per piece, correlation) as independent variables. All of the simulations were pooled for this modeling.

The fitted models for the variance of $\hat{CV}_{true}$ expressed as a percentage (Eq. 6), and the variance of $\hat{r}_{true}$ (Eq. 7) can be used to estimate the precision of an experiment. The model for the variance of $\hat{CV}_{true}$ fit well for experiments with at least 1,500 total microspheres, yielding $R^2 = 0.89$. We obtained $R^2 = 0.61$ from the model for the variance of $\hat{r}_{true}$ for experiments with at least 3,300 total microspheres.

\[
\text{Var}(\hat{CV}_{true}) = \frac{15,330}{\text{Total}} \quad (6)
\]

In Eq. 6, Total is the total number of microspheres in the entire organ. The Total value in an organ is the number of pieces in the organ, $N$, times the average number of microspheres per organ piece, $X$. The variance of $\hat{CV}_{true}$ will, therefore, be approximately the same for two experiments, one with 10 pieces and an average of 800 microspheres/piece and a second with 100 pieces and an average of 80 microspheres/piece.

\[
\text{Var}(\hat{r}_{true}) = \frac{15.46 - 0.00159\hat{CV}_{true,X} \hat{CV}_{true,T}}{\text{Total}} \quad (7)
\]

In Eq. 7, CV is expressed as a percentage and Total is the total number of microspheres in the entire organ for each injection (the same for X or Y). If the number of microspheres differs between the X and Y injections, then setting Total to the minimum of X and Y would yield a conservatively large variance of $\hat{r}_{true}$.

To illustrate the use of the variance equations, if we consider an experiment with $N = 200$ pieces, an average of 200 microspheres/piece for each of the two separate microsphere injections, and $\hat{CV}_{true} = 50\%$ for both of our paired measurements, then the estimated standard error (SE) of $\hat{CV}_{true}$ (square root of the variance) would be 0.62%, and the estimated SE of $\hat{r}_{true}$ would be 0.017. This would lead to approximate 95% confidence intervals of $\pm 1.2\%$ for the adjusted CV estimate and $\pm 0.033$ for the adjusted correlation estimate. This clearly indicates quite small uncertainty in the estimates and, overall, a very accurate experiment.

Now consider an example with $N = 100$ pieces, an average of only 50 microspheres/piece for each of the two separate microsphere injections, and true heterogeneity ($\hat{CV}_{true} = 50\%$) for both paired measurements. In this case, the estimated SE of $\hat{CV}_{true}$ is 1.75%, and the estimated SE of $\hat{r}_{true}$ is 0.048. This leads to approximate 95% confidence intervals of $\pm 3.4\%$ for the corrected CV estimate and $\pm 0.094$ for the corrected correlation estimate. Although the variability here is larger than the previous example, it is still small enough to be useful if there is a large effect or if a rough estimate is sufficient. Note that Eqs. 6 and 7 should be used only for planning experiments and not for estimation of actual standard deviations for completed experiments.

We can use Eqs. 6 and 7 to come up with a “rule of thumb” for the number of microspheres needed to provide estimates of the CV and correlation that are relatively free of Poisson noise and are reasonably precise. Although it may be somewhat arbitrary, let us consider that a relatively small amount of Poisson noise is acceptable if we are 95% confident that an estimated CV is within $\pm 2\%$ of the true value. These 95% confidence bounds imply a standard error of the CV of $\pm 1\%$ and a variance of $(1%)^2 = 1.0$. Using this value for the variance in Eq. 6 and solving for Total, we get $15,330 \pm 0.094$ rounded, 15,000 microspheres needed to provide 95% confidence of good precision of the CV. Note that this estimate is independent of the number of pieces.

We can provide a similar rule of thumb for the correlation coefficient. Correlations range from $-1.0$ to $+1.0$. Let us consider that Poisson noise is acceptable if we are 95% confident that the estimated correlation coefficient is within $\pm 0.05$ of the true value. These 95% confidence bounds imply a standard error of 0.025 and a variance of $(0.025)^2 = 0.000625$. We can use Eq. 7 for a “worst case” scenario by setting each $CV = 10\%$. This CV would imply an extraordinarily small level of variability for a biological system, a level that would rarely be encountered in practice, and thus, a highly conservative numerical choice. Solving Eq. 7 for Total then yields $Total = 24,481$, which is rounded up to an easily remembered value of 25,000. Again, this estimate is independent of the number of pieces.

DISCUSSION

The 400 microsphere rule for $\pm 10\%$ precision of flow to a single region or piece is certainly a useful and important guideline for investigators for whom that is the single goal. We have shown both experimentally and by simulation that experiments that use <400 microspheres/piece can yield valid and accurate estimates of heterogeneity and of correlation after adjustment using Eqs. 2, 3, and 5. Often, adjustments are so small that no correction will be needed for heterogeneity and correlation estimates. This methodology can be extended to include other types of microsphere analyses as well, such as regression slopes expressing the gradient in flow versus distance within an organ. When measurements of slope, heterogeneity, or correlation are made for several animals, hypotheses about this sample of statistics can be made using a single sample t-test comparing the mean heterogeneity, mean correla-
tion, or mean slope to a specified value, such as zero. If a statistic, such as CV<sub>true</sub>, is unbiased for each animal, then the mean for the collection of animals is unbiased. Naturally, the use of more microspheres per animal will increase the precision of the statistic within each animal and allow a greater power to detect effects in the collection of animals. However, if a statistically significant result is obtained for a mean (e.g., mean CV) from experiments involving several animals with small numbers of microspheres, then the hypothesis test, the P-value, and the confidence intervals are all valid, regardless of the number of microspheres per piece.

The driving force behind precision and bias is the magnitude of Poisson noise in relation to the size of the effect being considered. Thus, if true heterogeneity is large, Poisson noise may be relatively unimportant. If correlations are very strong, they are likely to be quite evident, even without correction. Correction will usually give a more precise estimate of heterogeneity and correlation.

A rule of thumb for the number of microspheres to use in an experiment is 15,000 if an accurate coefficient of variation is of interest and 25,000 if the correlation coefficient is of interest. An accurate correlation coefficient requires more microspheres (almost twice as many) than the number required for the CV, because two sets of microspheres are involved.

Our methodology does not need to take account of reference flows. Reference flow microspheres play no role when heterogeneity and correlation are being estimated using the methods developed in this article or when microspheres are individually counted with histological methods. Reference flows also play no role when the mean flow or mean normalized flow to pieces is adjusted to a fixed value, such as 1.0, that is used for all animals in an experiment. Reference flows will be important, however, when the absolute flow (e.g., ml/min) to a region is of interest. In this case, the methods developed by Buckberg et al. (2) and Nose et al. (9) can be used to achieve a satisfactory level of precision.

In conclusion, measurements of heterogeneity and correlation can be corrected for Poisson noise. The 400 microspheres/piece rule does not apply to many analyses. At least 15,000 microspheres (total) should be used for accurate estimates of heterogeneity and at least 25,000 microspheres for accurate estimates of correlation coefficients.

**APPENDIX 1**

Derivation of Adjusted CV

Let X<sub>i</sub> represent the number of microspheres counted in the ith piece for i = 1, . . . , N pieces.

The observed coefficient of variation is

\[ CV_{obs} = \frac{S}{\overline{X}} \]

where S is the standard deviation of X<sub>i</sub> and \( \overline{X} \) is the mean of X<sub>i</sub>.

For the model

\[ X_i \sim \text{Poisson} \left( \lambda_i \right) \]

\[ \lambda_i = \text{true flow to piece i} \]

\[ \sigma^2 = \text{variance} \left( \lambda_i \right) \]

\[ \Lambda = \text{expected value} (\lambda_i) = \text{true mean of piece flows} \]

We want

\[ CV_{true} = \frac{\sigma}{\Lambda} \]

The method is to obtain an expression for E(CV<sub>obs</sub><sup>2</sup>) in terms of \( \sigma^2, \Lambda, \) and N, solve for \( \sigma^2 \), and then express CV<sub>true</sub> in terms of E(CV<sub>adj</sub>), \( \Lambda, \) \( \sigma^2 \), and N.

First

\[ E(CV_{obs}^2) = E \left( \frac{S^2}{\overline{X}^2} \right) = \frac{E(S^2)}{E(\overline{X}^2)} \]

\[ E(S^2) = E \left( \frac{1}{N} \sum_{i=1}^{N} (X_i - \overline{X})^2 \right) = \frac{1}{N} \left( \sum_{i=1}^{N} E[X_i^2] - N \cdot E(\overline{X}^2) \right) = \frac{1}{N} \sum_{i=1}^{N} (\lambda_i + \lambda^2) - N \cdot \left( \frac{1}{N} \cdot \Lambda + \lambda^2 \right) = \frac{N - 1}{N} \cdot \Lambda + \sigma^2 \]

\[ E(\overline{X}^2) = E \left( \left[ \frac{1}{N} \sum_{i=1}^{N} X_i \right]^2 \right) = \frac{1}{N^2} \left[ \sum_{i=1}^{N} E(X_i^2) + \sum_{i=1}^{N} \sum_{j=1, j \neq i}^{N} E(X_i \cdot X_j) \right] = \frac{1}{N^2} \left[ \sum_{i=1}^{N} (\lambda_i + \lambda^2) + \sum_{i=1}^{N} \sum_{j=1, j \neq i}^{N} (\lambda_i \cdot \lambda_j) \right] = \frac{1}{N} \cdot \Lambda + \lambda^2 \]

We separately adjust the estimates for \( S^2 \) and \( \overline{X}^2 \) and combine them into a new estimator for CV<sub>adj</sub>. Define

\[ S_{adj}^2 = S^2 - \left( \frac{N - 1}{N} \right) \cdot \overline{X} \]

\[ \overline{X}_{adj}^2 = \overline{X}^2 - \left( \frac{\overline{X}}{N} \right) \]

Now we form an adjusted estimate of CV<sub>adj</sub>

\[ CV_{adj}^2 = \frac{S_{adj}^2}{\overline{X}_{adj}^2} \]

\[ E(CV_{adj}^2) = E \left( \frac{S_{adj}^2}{\overline{X}_{adj}^2} \right) = \frac{E(S_{adj}^2)}{E(\overline{X}_{adj}^2)} = \frac{\sigma^2}{\Lambda^2} \]
So we use
\[
C V_{\text{true}} = \sqrt{\frac{S^2 - \frac{(N - 1)}{N} \cdot \bar{X}}{\bar{X}^2 - \frac{\bar{X}}{N}}} = \sqrt{\frac{CV_{\text{obs}}^2 - \frac{(N - 1)}{N} \cdot \frac{1}{\bar{X}}}{1 - \frac{1}{N} \cdot \frac{1}{\bar{X}}}}
\]
as our estimate. Note that if \(N\) is large, this simplifies to the familiar formula
\[
CV_{\text{true}} = \sqrt{CV_{\text{obs}}^2 - \frac{1}{\bar{X}}}
\]

**APPENDIX 2**

**Derivation of Adjusted Correlation Coefficient**

We have \(N\) pieces with two different measurements of flow, based on the number of microspheres in each piece. We are interested in the correlation between flows in the absence of Poisson noise. An estimate of this true correlation coefficient can be derived:

Let \(X\) and \(Y\) be two distributions of microspheres per piece. \(X_i\) and \(Y_i\) represent the number of microspheres in the \(i\)th piece for \(i = 1, \ldots, N\) pieces.

For the model
\[
X_i = \mu_x + \Delta_x + \delta_x + \epsilon_x
\]
\[
Y_i = \mu_y + \Delta_y + \delta_y + \epsilon_y
\]
where \(\mu_x\) is the expected mean number of microspheres for all pieces from the \(X\) injection, and \(\mu_y\) is the expected mean number of microspheres for all pieces from the \(Y\) injection.

The deviation of piece \(i\) from \(\mu\) can be broken into three components, \(\Delta, \delta,\) and \(\epsilon,\) defined as follows. \(\Delta_x\) is the expected common deviation of piece \(i\) from \(\mu_x\) in common with \(\Delta_y,\) except for a scale factor depending on the number of microspheres injected. \(\Delta_y\) is the expected common deviation of piece \(i\) from \(\mu_y,\) in common with \(\Delta_x,\) except for a scale factor depending on the number of microspheres injected. \(\delta_x\) is the unique deviation of piece \(i\) from \(\mu_x,\) not in common with the deviation from the \(Y\) injection. \(\delta_y\) is the unique deviation of piece \(i\) from \(\mu_y,\) not in common with the deviation from the \(X\) injection. \(\epsilon_x\) is the Poisson noise for piece \(i,\) \(X\) injection. \(\epsilon_y\) is Poisson noise for piece \(i,\) \(Y\) injection. \(X_i\) is \(\sim\) Poisson\((\mu_x - \Delta_x - \delta_x),\) \(Y_i\) is \(\sim\) Poisson\((\mu_y - \Delta_y - \epsilon_y,\)

\[E(\Delta_x) = E(\Delta_y) = E(\delta_x) = E(\delta_y) = E(\epsilon_x) = E(\epsilon_y) = 0\]

We note that if \(\alpha\) is the ratio of total microspheres injected (\(X\) spheres/\(Y\) spheres), then
\[
\mu_y = \alpha \mu_x
\]
and
\[
\Delta_y = \alpha \Delta_x
\]

Variances are
\[
\text{Var}(X_i) = \sigma_{\alpha x}^2 + \sigma_{\mu x}^2 + \sigma_{\delta x}^2 = \sigma_x^2
\]
where \(\sigma_x^2 = \text{Var}(\Delta_x),\) the variance of the common part; \(\sigma_{\mu x}^2 = \text{Var}(\delta_x),\) the variance of the unique part; and \(\sigma_{\delta x}^2 = \text{Var}(\epsilon_x),\) the variance of the Poisson noise.

A similar set of definitions holds for \(\sigma_{\alpha y}^2, \sigma_{\mu y}^2,\) and \(\sigma_{\delta y}^2.\) Note that, by definition of the Poisson distribution, \(\sigma_{\mu x}^2 = \mu_x\) and \(\sigma_{\delta y}^2 = \mu_y.\)

The population correlation coefficient is calculated as
\[
r_{\text{obs}} = \frac{\frac{1}{N} \sum_{i=1}^{N} (X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\left[ \frac{1}{N} \sum_{i=1}^{N} (X_i - \bar{X})^2 \right] \sum_{i=1}^{N} (Y_i - \bar{Y})^2}}
\]

Let \(S_x^2\) denote the population variance of \(X.\) The expected value of the population variance is
\[
E(S_x^2) = E \left[ \frac{1}{N} \sum_{i=1}^{N} (X_i - \bar{X})^2 \right] = \frac{N - 1}{N} \cdot (\sigma_{\alpha x}^2 + \sigma_{\mu x}^2 + \sigma_{\delta x}^2)
\]
So the expected value of \(r_{\text{obs}}\) is approximately
\[
E[r_{\text{obs}}] = \frac{E[S_x^2] \cdot E[S_y^2]}{E[S_x^2] \cdot E[S_y^2]}
\]
\[
= \frac{\sigma_{\alpha x}^2}{\sqrt{(\sigma_{\alpha x}^2 + \sigma_{\mu x}^2 + \sigma_{\delta x}^2) \cdot (\sigma_{\alpha y}^2 + \sigma_{\mu y}^2 + \sigma_{\delta y}^2)}}
\]
We want
\[
r_{\text{true}} = \frac{\alpha \sigma_{\alpha x}^2}{\sqrt{(\sigma_{\alpha x}^2 + \sigma_{\mu x}^2 + \sigma_{\delta x}^2) \cdot (\sigma_{\alpha y}^2 + \sigma_{\mu y}^2 + \sigma_{\delta y}^2)}}
\]
We approximate this by
\[
\hat{r}_{\text{true}} = \frac{r_{\text{obs}} \cdot S_x \cdot S_y}{\sqrt{S_x^2 - \frac{(N - 1)}{N} \cdot \bar{X} \cdot S_y^2 - \frac{(N - 1)}{N} \cdot \bar{Y}}}
\]
Our adjusted estimate is approximately unbiased, because
\[
E[\hat{r}_{\text{true}}] = \frac{E[r_{\text{obs}}] \cdot E[S_x^2] \cdot E[S_y^2]}{E[S_x^2] \cdot E[S_y^2]}
\]
\[
= \alpha \sigma_{\alpha x}^2
\]
\[
= \frac{\alpha \sigma_{\alpha x}^2}{\sqrt{(\sigma_{\alpha x}^2 + \sigma_{\mu x}^2 + \sigma_{\delta x}^2) \cdot (\sigma_{\alpha y}^2 + \sigma_{\mu y}^2 + \sigma_{\delta y}^2)}}
\]
\[
= r_{\text{true}}
\]
To allow negative correlations in this model, we define \(Y_i\) in a slightly different way
\[
Y_i = \mu_y - \Delta_y + \delta_y + \epsilon_y
\]
which implies that \(Y_i\) is \(\sim\) Poisson\((\mu_y - \Delta_y + \epsilon_y).\)

The derivation of the adjusted correlation coefficient leads to Eq. 5 with or without this change, so our adjusted correlation coefficient can be used regardless of whether the correlation is positive or negative.
APPENDIX 3
Method for Generating Simulated Organs

The fractal model of van Beek (11) was used to generate organ blood flow distributions. In this model, regional blood flows are determined by repetitively subdividing an organ into pieces. After k subdivisions there are 2^k organ pieces. At each subdivision, blood flow to a piece is asymmetrically portioned into its two daughter pieces. A fraction of blood (γ) is portioned to one piece and the remaining flow (1 − γ) is portioned to the other piece. At each subdivision, γ is randomly determined from a normal distribution with mean of 0.5 and a standard deviation of σ. The value of γ is constrained to lie between zero and unity. If σ = 0, blood flow will be uniformly distributed to all organ pieces. As σ deviates from 0, blood flow becomes more heterogeneous. At the smallest piece size, the fractional amount of blood flow, fi, to each piece i is determined.

The expected numbers of microspheres depositing in these simulated organs are determined by multiplying the number of microspheres injected into the organ, M, by the fractional flow to each organ piece, fi. Each quantity of the distribution Xtrue = M · fi is rounded to the nearest integer and designated as the “true” blood flow to each organ piece. The true coefficient of variation, CVtrue, is determined for each organ. These true CV values serve as a comparison for the noise-correlated CV values generated with simulated data.

We also simulated observed and corrected correlations. Starting with Xtrue, the expected number of microspheres deposited in the same piece at another time (e.g., under another experimental condition) is simulated by creating a second distribution, Ytrue, with a specified correlation to Xtrue. We consider nonnegative (zero or positive) correlations and negative correlations separately. For nonnegative correlations, the specified correlation r, the second distribution is generated with the following equation

\[ \text{Y}_{\text{true},i} = K_1 \text{X}_{\text{true},i} + K_2 \text{X}_{\text{true},i} + K_3 \text{e}_i \]  

where e_i is a random normal number from a distribution with a mean of zero and a variance of 1.0. The constants K_1, K_2, and K_3 are chosen by the user to yield the desired correlation between Xtrue and Ytrue while keeping the total flow of Y equal to the total flow of X. We also attempt to keep the true CV of X close to the true CV of Y. By chance, negative values of Y may occur; these numbers are discarded and a new e_i is redrawn. Because of the random generation of Ytrue, the actual correlation between Xtrue and Ytrue may differ slightly from the desired correlation r. The true correlation, r_{true}, is determined from the generated Xtrue and Ytrue, rather than from the target value, r. If needed, the process is repeated until r_{true} is sufficiently close to the desired r, and the final set of values Xtrue and Ytrue is designated as the true organ observed under two conditions.

When the desired r is negative, we use the method described above to generate Ytrue using the absolute value of r as the target correlation. The maximum and minimum values of Ytrue are then determined. Each Yi is replaced by the value of \(\text{max} + \text{min} - Y_i\), and then this new Ytrue is normalized so that the total flow of Y is equal to the total flow of X. As with the nonnegative correlations, the process is repeated until r_{true} is sufficiently close to the desired negative r, yielding a final set of Xtrue and Ytrue, which comprise a true organ observed under two conditions.

Observed microsphere distributions, \(X_{\text{obs},i}\), are generated by adding Poisson noise to the true number of microspheres in each organ piece. Poisson noise is estimated by choosing a random number (m) from a uniform distribution between 0 and 1 and then determining the largest value of k, such that

\[ \sum_{j=0}^k \left( \frac{X_{\text{true},i}^j}{j!} \right) e^{-X_{\text{true},i}} \leq m \]  

where Xtrue is the true number of microspheres in piece i. Then Xobs is set equal to k. A normal distribution with a mean of Xtrue and a standard deviation of \(\sqrt{X_{\text{true},i}}\) is used to estimate Xobs for Xtrue > 100. For the paired simulated true flow distribution, Ytrue, the observed microsphere distribution, Yobs, is generated in the same manner as the Xobs, using Eq. 9 or the normal distribution.

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