High spatial resolution measurements of organ blood flow in small laboratory animals

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1Division of Pulmonary and Critical Care Medicine and 2Department of Physiology and Biophysics, University of Washington, Seattle 98195; and 3Barlow Scientific, Incorporated, and The Evergreen State College, Olympia, Washington 98505

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Bernard, Susan L., Jon R. Ewen, Clyde H. Barlow, Jeff J. Kelly, Steven A. McKinney, David A. Frazer, and Robb W. Glenny. High spatial resolution measurements of organ blood flow in small laboratory animals. Am J Physiol Heart Circ Physiol 279: H2043–H2052, 2000.—With the use of a newly developed Imaging Cryomicrotome to determine the spatial location of fluorescent microspheres in organs, we validate and report our processing algorithms for measuring regional blood flow in small laboratory animals. Microspheres (15-μm diameter) of four different fluorescent colors and one radioactive label were simultaneously injected into the left ventricle of a pig. The heart and kidneys were dissected, and the numbers of fluorescent and radioactive microspheres were determined in 10 randomly selected pieces. All microsphere counts fell well within the 95% expected confidence limits as determined from the radioactive counts. Fluorescent microspheres (15-μm diameter) of four different colors were also injected into the tail vein of a rat and the left ventricle of a rabbit. After correction for Poisson noise, correlation coefficients between the colors were 0.99 ± 0.02 (means ± SD) for the rabbit heart and 0.99 ± 0.02 for the rat lung. Mathematical dissection algorithms, statistics to analyze the spatial data, and methods to visualize blood flow distributions in small animal organs are presented.

Imaging Cryomicrotome

The Imaging Cryomicrotome (Barlow Scientific, Olympia, WA) is a device that determines the spatial distribution of fluorescent microspheres at the microscopic level. Details of the instrument configuration have been previously reported (13). Briefly, the system rapidly collects microcirculation data from organs containing up to four different colors of fluorescent microspheres. The instrument (Fig. 1) consists of a Kodak Megaplas 4.2 charge-coupled device (CCD) video camera (Eastman Kodak, San Diego, CA), a computer (Dell Computer, Round Rock, TX), a metal halide lamp (HTI 403W/24, Osram Sylvania), an excitation filter-changer wheel, an emission filter-changer wheel, and a cryostatic microtome. Fluorescence images are acquired with the Kodak digital camera (2,000 × 2,000 pixel array) with a 200-mm Nikkor lens (Nikon, Tokyo, Japan) using a macrofocusing baffle. Two motorized filter wheels containing excitation and emission filters are mounted in front of the light source and camera, respectively. The computer, through stepper motors and microsensors, controls the filter positions. A custom-designed microtome is outfitted with a stepper motor to serially section frozen organs. Computer control requires large animals for adequate spatial resolution within the organ. Once dissected, an organ cannot be dissected by an alternative anatomic scheme.

A new system that directly determines the spatial location of every fluorescent microsphere within organs of small laboratory animals has been designed and reported by Kelly et al. (13). We now validate the new system against the radioactive microsphere method, present methods for mathematically dissecting organs, and introduce new methods for characterizing and visualizing spatial distributions of blood flow in small laboratory animals. This new imaging system and processing algorithms provide more spatial information on regional organ perfusion from rats and rabbits than previously available from larger animals such as dogs, sheep, and pigs.

THE MICROSPHERE METHOD developed by Rudolph and Heymann (23) is the current “gold standard” for measuring the distribution of blood flow within organs. Polystyrene spheres, 10–15 μm in diameter and labeled with either radionuclides, color, or fluorescent dyes, are injected into the blood stream and entrapped within the organ of interest. The organ is subsequently dissected into a desired number of pieces in a predetermined manner. The numbers of microspheres in each piece are estimated from either radioactive counts (10), color absorption (14), or emitted fluorescence (7).

The microsphere method is not without problems. It is labor intensive, uses indirect signals to estimate the number of microspheres in each organ sample, and
of the microtome motor, emission filter wheel, and image capture and display is accomplished through a virtual instrument written in LabVIEW 5.1 (National Instruments).

The cryomicrotome serially sections through the frozen organ at a selected slice thickness. Digital images of the tissue surface (en face) are acquired with appropriate excitation and emission filters to isolate each of four different fluorescent colors (Fig. 2). Four-megabyte images, one at each of the four fluorescence excitation/emission wavelengths, are collected in 20–30 s. Each image is processed so that x and y locations of each microsphere are determined. The spatial resolution of the system depends on the size of the organ being processed but is typically 10 μm in the x and y directions and 30 μm in the z direction. Images of organ cross sections produce a three-dimensional binary map defining the spatial location of organ parenchyma. This map determines the organ space locations to be sampled and the three-dimensional space for the statistical sampling (see Fig. 2). Acquired images are analyzed with software written in LabVIEW that applies an intensity threshold to convert microsphere fluorescence into binary images. Each microsphere is represented by a number of clustered pixels. The calculated center of mass of each cluster determines the x- and y-coordinates of each microsphere within each slice, whereas the slice number and thickness determine the z-coordinate. The spatial coordinates and cluster size of each microsphere are written to a text file.

Data reduction is automated through a final analysis program written in the C programming language. A
linked list of all microsphere locations is created, and any microsphere observed in the same x- and y-coordinate across consecutive z slices is reduced to a single observation occurring in the z slice in which the number of pixels representing the microsphere is the largest. The number of consecutive slices in which a given microsphere is observed and the number of pixels representing each microsphere are also used to eliminate artifacts. Artifacts, such as point defects in the camera chip, appear as single pixels in the same x and y location of serial sections and are eliminated in this step.

**METHODS AND VALIDATION STRATEGIES**

To validate the Imaging Cryomicrotome for measuring regional organ blood flow, it is necessary to show both that the fluorescent microspheres are accurately counted and that their spatial locations are faithfully determined. To this end, two different validation strategies are employed. The first counts the number of microspheres in tissues and compares them with predicted numbers. The second determines the spatial distribution of simultaneously injected microspheres of different colors to determine whether the numbers of microspheres in small organ regions are well correlated between the different colors. The second validation strategy uses the premise that differently colored fluorescent microspheres will distribute similarly with blood flow. If a poor correlation between colors is found, the conclusion must be that the Imaging Cryomicrotome cannot accurately determine the spatial locations of the microspheres. The University of Washington Institutional Animal Care and Use Committee approved all animal studies.

**Counting Methods**

A 21-kg pig was chemically restrained with ketamine-xylazine (20 and 2 mg/kg im), anesthetized with thiopental sodium (11 mg/kg), intubated, and mechanically ventilated at a tidal volume and rate sufficient to normalize the arterial partial pressure of CO₂. A left ventricular catheter was placed via the left carotid artery in a retrograde approach and confirmed by pressure tracings. Triple-lumen catheters were placed in each femoral artery for reference blood sampling. Fluorescent microspheres, 15 μm in diameter and of four different colors (green, yellow, red, and scarlet), were simultaneously injected with ¹¹³Sn (New England Nuclear-DuPont, Boston, MA)-labeled microspheres into the left ventricle. The fluorescent microspheres were custom manufactured by Molecular Probes (Eugene, OR) to provide the radioactive counts that were delivered to organs. Withdrawal of the reference blood samples was initiated before microsphere injection and continued for 2 min after the end of the microsphere injection. Placement of the injection catheter in the left ventricle was confirmed by pressure measurement before and after the microsphere injection. The animal was killed with a lethal dose of pentobarbital sodium, and the heart and kidneys were removed.

The heart and kidneys were dissected into 84 pieces. The radioactive counts in each tissue and reference blood sample were determined in a 3 × 3.25 in. sodium well crystal gamma-counter (model 5550, Minaxi gamma-counting system, Packard, Downers Grove, IL). The counts were corrected for background counts and decay.

To estimate the number of fluorescent microspheres in each tissue sample, the quantitative relationship between the number of fluorescent microspheres and radioactive counts was determined from the reference blood samples. The reference blood samples were anticoagulated with citrate-phosphate-dextrose solution and then filtered using Perkin-Elmer centrifugal devices (22). Dyes from the retained fluorescent microspheres were eluted with 2 ml of Cellosolve (Aldrich Chemical, Milwaukee, WI), and the fluorescent signals were determined by an automated fluorescent system (24). This provided a measure of the radioactive counts to the fluorescent signal per milliliter of solvent (counts_rad/ml solvent⁻¹, where counts_rad is radioactive counts, Fl is the fluorescent signal, and ml solvent is the volume of solvent in milliliters). The six reference blood samples provided a mean and standard error of the mean for all four colors.

A calibration standard relating the number of fluorescent microspheres to a given fluorescent signal level was determined for each fluorescent color. The number of fluorescent microspheres in a given volume of the microsphere suspension (microspheres /μl suspension, where microspheres is the number of fluorescent microspheres and μl suspension is the volume of suspension in microliters) was determined in a hemacytometer chamber. This measure was repeated nine times to provide a mean and variance for each color.

To estimate the number of fluorescent microspheres in each tissue sample, the number of fluorescent microspheres in a given volume of the microsphere suspension was determined for each fluorescent color using 30-μm-thick sections. The number of microspheres counted in each sample was compared with the expected numbers of each

\[
\text{Microspheres}_n = \frac{\text{counts}_{\text{rad}} \cdot \text{microspheres}_n}{\left(\frac{\text{Fl}}{\mu l_{\text{suspension}} \cdot \text{ml solvent}}\right) \cdot \text{counts}_{\text{rad}} \cdot \text{ml solvent}}
\]

This relationship was determined for each fluorescent color, and the 95% confidence interval about this value was estimated (19).

Ten heart and kidney samples were selected randomly from the 84 samples. Each sample was surrounded by a clear optimal cutting tissue (OCT) compound (Tissue-Tek, Torrance, CA) and frozen. Individual samples were mounted in the Imaging Cryomicrotome, and the numbers of fluorescent microspheres were determined for each color using 30-μm-thick sections. The number of microspheres counted in each sample was compared with the expected numbers of each.
fluorescent color as estimated from the radioactive counts and Eq. 1.

Spatial Distribution Methods

Fluorescent microspheres, 15 μm in diameter and of four different colors, were injected into the tail vein of a 234-g awake rat and into the left ventricle of a 3-kg anesthetized New Zealand White rabbit via a retrograde catheter from a carotid artery. Between 35,000 and 65,000 microspheres of each color, green, yellow, red, and scarlet, were simultaneously injected into the rat tail vein. Approximately 800,000 of each fluorescent colored microsphere were injected into the rabbit heart. Before injection, all microspheres were sonicated, vortexed, and mixed in the same syringe. The animals were given an anesthetic overdose after the microsphere injection. The rat lungs were excised, reinflated with clear OCT compound, and then frozen. The frozen lungs were placed in a small container, surrounded by cold OCT compound containing carbon black, and then refrozen. The rabbit heart was excised, and the ventricular cavities were filled with OCT compound, frozen, and then processed in the same manner as the rat lungs. The lungs and heart were mounted in the Imaging Cryomicrotome, and spatial locations of every microsphere were determined using 30-μm-thick sections. Three-dimensional binary maps were constructed from tissue images obtained every fifth slice. Each organ was mathematically dissected using the computer algorithms described below.

Mathematical Dissection

The binary map of the organ can be mathematically dissected into multiple regions of preselected volumes. Mathematical dissection allows the organ to be resampled at multiple specified volumes and multiple times. Because organ shapes are not cubical, when dissected along orthogonal planes, many peripheral pieces will be partial cubes. This problem can be avoided by random sampling using spherical volumes of specified sizes. The computer algorithm for random sampling of an organ was written in the C programming language and run on a Sun Ultra 10 workstation (Sun Microsystems, Palo Alto, CA). The program determines a spatial point by choosing x-, y-, and z-coordinates from a pseudorandom number generator (Unix, Berkeley, CA) using the Marsaglia shuffling (17) method to ensure a uniform distribution of numbers. This random spatial point is then located in the binary map of the organ. To be considered an adequate sample, more than 95% of the sampling sphere (defined by its center point and selected radius) must lie within the organ and may not overlap previously sampled regions. This sampling process continues until no other spherical regions can be found within the organ. This approach samples only a fraction of the organ, but in an unbiased manner without sampling overlap. Microsphere locations are stored in a tree structure that allows efficient search algorithms (6) to determine how many microspheres exist within each sampled organ volume.

Microsphere Density Measures of Blood Flow

Traditionally, the numbers of microspheres lodging in organ pieces have estimated the blood flow distribution in organs. Alternatively, microspheres may be thought of as points in space and their spatial distribution characterized by statistical measures of point processes.

The following approach was implemented in a C-written program run on a Sun Ultra 10 workstation. The spatial coordinates of all microspheres are stored in a tree structure that allows efficient determination of distances between microspheres (6). For any given point in the organ, the average distance to the nth nearest microsphere (where n is a pre-determined number of microspheres) is inversely proportional to the regional blood flow at that location. Regions of high flow have relatively more microspheres than low-flow regions. The greater the density of microspheres in a region, the less the average distance to the nth nearest microsphere. The reciprocal of the mean distance to the nth nearest microsphere is therefore proportional to the local blood flow. For example, if the average distance to the five nearest microspheres is 200 μm, then 1/200 is a relative measure of local blood flow.

The organ space can be sampled using various algorithms. One approach systematically selects points on a three-dimensional orthogonal grid. This samples the organ space uniformly without bias. The mean distance to the nearest n microspheres is determined at each sampling point. This measure can then be normalized to the mean of all sampling locations, providing a mean normalized measure of regional blood at each sampling point in an organ.

If a sampling point is located near the edge of an organ, the mean distance to the nth nearest microsphere underestimates the true density of local microspheres because there is less organ volume surrounding the sampling point (5). The distance to the nth nearest microspheres can be corrected for this artifact by normalizing the distance to the volume of tissue explored. This volume is defined by a sphere centered at the chosen sampling point with a radius equal to the distance to the nth nearest microsphere. As an example, for a given sampling point, let the distance to the fifth nearest microsphere be 200 μm. Now, determine the fraction of organ parenchyma that exists within a sphere centered at the sampling point and with a radius of 200 μm. If the sampling point is well within the organ, the entire sphere will contain parenchyma, and the reciprocal of 200 (divided by 1.0) will be a good measure of local blood flow. However, if the sampling point is near the edge of the organ, only a fraction of the sphere, say 0.5 for this example, will contain tissue. In this case, the reciprocal of 200 divided by 0.5 provides a tissue-normalized measure of local blood flow.

The large numbers of sampling points (20,000 or more per organ) make it difficult to visualize their spatial distributions. One approach selects orthogonal planes and represents the density flows as three-dimensional topographic maps. Topographic maps are produced from the spatial data (x, y, z, and flow) using the interpolation and contour mapping functions in MATLAB (Math Works, Natick, MA). Relative flows are represented in units relative to the mean of the entire organ.

Statistics

Counting microspheres. Estimating the number of microspheres that should be present in any given tissue piece requires the use of data with inherent statistical noise. Careful techniques were used to minimize these sources of error, and repeated measures were obtained to accurately estimate the statistical noise. With the use of multiple measurements, means and variances for each component of Eq. 1 were computed, allowing estimation of the number of fluorescent microspheres and calculation of the variance of the number of microspheres present in each tissue sample (19). The sample means and sample variances were used to construct 95% confidence intervals for the estimated number of fluorescent microspheres in each tissue sample. The number of fluores-
cent microspheres counted by the Imaging Cryomicrotome can then be compared with the mean and should, if counted accurately, frequently fall within the 95% confidence interval.

**Correlation coefficients.** The organs were mathematically dissected as described above, and the numbers of microspheres of each different color were determined for each sampled volume. Each organ was randomly dissected 10 times. For each dissection, the correlation coefficient matrix was determined for all four colors and corrected for Poisson noise by the following equation

\[
\hat{r}_{\text{true}} = r_{\text{obs}} \cdot \frac{S_X \cdot S_Y}{\frac{S_X^2}{N - 1} \cdot \bar{X} \cdot \frac{S_Y^2}{N - 1} \cdot \bar{Y}}
\]

(2)

where \( \hat{r}_{\text{true}} \) is the corrected correlation coefficient, \( r_{\text{obs}} \) is the observed correlation coefficient, \( S_X \) and \( S_Y \) are the population standard deviations for the \( x \) and \( y \) distributions, respectively, \( N \) is the number of organ samples, and \( \bar{X} \) and \( \bar{Y} \) represent the means of the \( x \) and \( y \) distributions, respectively (20). The correlation coefficient matrix was determined for each random dissection, and the mean values were reported.

**Relative blood flow frequency distributions.** The organs were mathematically dissected as described above, and the numbers of microspheres of each different color were determined for each sampled volume. Each organ was randomly dissected 10 times. The distribution of microspheres, or blood flow, can be characterized as a coefficient of variation (CV, where CV = SD/mean). The CV was determined for each random dissection, and the mean values were reported.

Microspheres lodge in organ regions in proportion to the amount of blood flow to that region (10). However, because microspheres are discrete particles, there is noise in their distribution. The number of microspheres counted within any given region is therefore an estimate of the true number of microspheres that should be in the region (20). Smaller organ regions have fewer microspheres and hence more counting noise. This noise can be mathematically removed from the observed CV (CV\_obs) with the following formula

\[
\hat{CV}_{\text{true}} = \sqrt{\frac{CV_{\text{obs}}^2 - \left(\frac{N - 1}{N}\right) \cdot \frac{1}{\bar{X}}}{1 - \frac{1}{N \cdot \bar{X}}}}
\]

(3)

where \( N \) is the number of organ volumes in the analysis, \( \hat{CV}_{\text{true}} \) is an estimate of the true CV, and \( \bar{X} \) is the average number of microspheres counted within the sampled regions. 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 \( \bar{X} \) are large, this formula simplifies to

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

(4)

Because the distribution of microspheres is Poisson, the CV with Poisson noise (CV\_noise\_obs) equals \( \bar{X} \cdot \sqrt{\frac{CV_{\text{true}}^2}{N} \cdot \frac{N - 1}{N}} \), where the number of microspheres per volume is considerably less than that required to confidently determine the flow to a region (4), the heterogeneity of perfusion is well determined. We have previously shown that with an average of only four microspheres per piece, the CV is well determined as long as an adequate number of samples is obtained (20).

**RESULTS**

**Counting Microspheres**

In the 10 randomly selected heart and kidney samples, the numbers of microspheres counted ranged from 177 to 2,829 green, 404 to 6,379 yellow, 140 to 2,385 red, and 303 to 4,719 scarlet. All fluorescent microsphere counts fell well within the 95% confidence limits calculated from the radioactive counts in each sample using Eq. 1. Figure 3 shows a plot of the counted versus expected number of yellow fluorescent microspheres in each tissue sample.

**Relative Blood Flow Frequency Distributions**

Between 46,694 and 67,932 microspheres of each color were counted in the rat lungs, and between 14,485 and 20,317 microspheres were counted in the rabbit heart. The rat lungs were mathematically dissected into 431 and 54 regions using randomly selected spherical volumes with radii of 1,000 and 2,000 μm, respectively. A sphere with a radius of 1,000 μm (1 mm) corresponds approximately to the size of a BB or an “O” on this page. The rabbit heart was mathematically dissected into 301 and 21 regions using spheres with radii of 1,000 and 2,000 μm, respectively. When corrected for Poisson noise using Eq. 3, the CV for each color is remarkably similar (Table 1). Figure 4 shows a single distribution of relative flows for yellow microspheres in the rabbit heart and red microspheres in the rat lungs. Note that this distribution is similar to those distributions of larger animals (8).
Spatial Distributions

The rat lungs and rabbit heart were mathematically dissected using randomly selected spherical volumes with radii of 1,000 and 2,000 μm. Figure 5 shows the number of yellow versus green microspheres per sample volume from one mathematical dissection. The correlation coefficients between each color must be corrected for Poisson noise using Eq. 2. Tables 1 and 2 present the corrected correlation matrices for the rat lungs and rabbit heart. Because all four fluorescent colors were injected simultaneously, there should be a high correlation among all four microsphere colors. These excellent correlations show that the spatial locations of the microspheres are accurately determined.

The spatial distribution of blood flow can be explored by plotting the number of microspheres in each sampling sphere in three dimensions. Figure 6 presents the spatial distribution of microspheres counted in the rat lungs and rabbit heart. These images, created using Infini-D (version 4.5, MetaCreations, 1998), can be rotated about any axis to more clearly see the three-dimensional distribution of blood flow using the Quick-Time Movie Player (version 3.0, Apple Computer, 1998).

Microsphere Density Measures of Blood Flow

The local density of microspheres in the organ can also represent the spatial distribution of blood flow. Each sampling point is assigned a flow value. The value is determined from the mean distance to the nth nearest microspheres, normalized by the volume of tissue sampled, and then normalized to the mean value for the organ. Figure 7 shows the flow distributions obtained by sampling the rat lung and rabbit heart with a three-dimensional orthogonal grid and determining the mean distance to the five nearest microspheres at each sampling point. Note that the distributions appear similar to those obtained through random sampling with spherical volumes (Fig. 4).

To visualize the spatial distribution of blood flow using the microsphere density method, contour maps of cross-sectional slices can be constructed. Figure 8

Table 1. Sampling statistics from rat lungs (four colors of microspheres simultaneously injected)

<table>
<thead>
<tr>
<th></th>
<th>Sampling Regions With Radii of 1,000 μm</th>
<th>Sampling Regions With Radii of 2,000 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total microspheres in organ</td>
<td>47,393 46,694 67,932 57,940</td>
<td>47,393 46,694 67,932 57,940</td>
</tr>
<tr>
<td>Regions sampled</td>
<td>431 431 431 431</td>
<td>54 54 54 54</td>
</tr>
<tr>
<td>Mean microspheres/region</td>
<td>28.3 27.4 40.0 34.3</td>
<td>220.7 217.3 316.4 217.3</td>
</tr>
<tr>
<td>Observed CV</td>
<td>0.31 0.30 0.29 0.30</td>
<td>0.21 0.19 0.20 0.19</td>
</tr>
<tr>
<td>Corrected CV</td>
<td>0.25 0.25 0.24 0.25</td>
<td>0.20 0.18 0.19 0.18</td>
</tr>
</tbody>
</table>

Correlation Matrices

<table>
<thead>
<tr>
<th></th>
<th>Green</th>
<th>Yellow</th>
<th>Red</th>
<th>Scarlet</th>
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</thead>
<tbody>
<tr>
<td>Green</td>
<td>0.66</td>
<td>0.69</td>
<td>0.68</td>
<td>0.90</td>
</tr>
<tr>
<td>Yellow</td>
<td>1.0</td>
<td>0.68</td>
<td>0.66</td>
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<tr>
<td>Red</td>
<td>1.0</td>
<td>1.0</td>
<td>0.72</td>
<td>0.99</td>
</tr>
<tr>
<td>Scarlet</td>
<td>1.0</td>
<td>0.99</td>
<td>1.0</td>
<td>0.99</td>
</tr>
</tbody>
</table>

All values are the means of 10 sampling repetitions. CV, coefficient of variation; corrected CV, CV corrected for Poisson noise. Within the correlation matrices, values above diagonal represent observed correlation coefficients, and values below diagonal represent correlation coefficients corrected for Poisson noise.

Table 2. Sampling statistics from the rabbit heart (four colors of microspheres simultaneously injected)

<table>
<thead>
<tr>
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<th>Sampling Regions With Radii of 1,000 μm</th>
<th>Sampling Regions With Radii of 2,000 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total microspheres in organ</td>
<td>14,485 20,317 19,936 17,707</td>
<td>14,485 20,317 19,936 17,707</td>
</tr>
<tr>
<td>Regions sampled</td>
<td>301 301 301 301</td>
<td>21 21 21 21</td>
</tr>
<tr>
<td>Mean microspheres/region</td>
<td>8.1 11.9 11.9 10.4</td>
<td>63.6 91.8 90.4 80.9</td>
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<tr>
<td>Observed CV</td>
<td>0.51 0.47 0.50 0.52</td>
<td>0.28 0.27 0.29 0.32</td>
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<tr>
<td>Corrected CV</td>
<td>0.37 0.37 0.41 0.42</td>
<td>0.25 0.25 0.27 0.30</td>
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</table>

Correlation Matrices

<table>
<thead>
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<th>Green</th>
<th>Yellow</th>
<th>Red</th>
<th>Scarlet</th>
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<tbody>
<tr>
<td>Green</td>
<td>0.56</td>
<td>0.63</td>
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<td>Yellow</td>
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<td>0.63</td>
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<td>Red</td>
<td>1.0</td>
<td>1.0</td>
<td>0.67</td>
<td>1.0</td>
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<tr>
<td>Scarlet</td>
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<td>1.0</td>
<td>1.0</td>
<td>0.92</td>
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All values are the means of 10 sampling repetitions. The corrected CV was corrected for Poisson noise. Within the correlation matrices, values above diagonal represent observed correlation coefficients, and values below diagonal represent correlation coefficients corrected for Poisson noise.
shows contour maps of regional flow in cross-sectional slices from the rat lung and rabbit heart.

DISCUSSION

The important findings of this study are that 1) the Imaging Cryomicrotome accurately counts the numbers of fluorescent microspheres in an organ, 2) the spatial locations of the fluorescent microspheres are faithfully represented, 3) organs can be mathematically dissected repeatedly using different schemes, and 4) statistical measures of microsphere distributions in small laboratory animals are similar to blood flow distributions observed in large animals. This study validates the Imaging Cryomicrotome and our processing algorithms as a method for measuring the regional distribution of blood flow in small laboratory animals.

Until now, fluorescent microsphere studies have adopted radioactive microsphere methods for measuring regional blood flow in organs. This approach physically dissects organs and determines the numbers of microspheres in each organ piece. The methods are labor intensive (21) and do not take advantage of the fact that fluorescent microspheres can be imaged and accurately counted. The new methods presented here have a number of advantages.

The Imaging Cryomicrotome is fully automated. The organ is mounted in the cryomicrotome, the fluorescent colors of interest are selected, and the slice thickness is chosen. If all four colors are used, a rabbit heart can be completely processed in 10 hours without user input. The saved images are processed to produce a text file containing the spatial coordinates of every imaged microsphere. This imaging method directly determines the microsphere numbers and locations compared with indirect measurements using radioactive counting or fluorescent dye extraction. This approach also obviates the need to physically dissect the organ.

The Imaging Cryomicrotome provides the spatial location of every microsphere in an organ on a scale of resolution not previously possible. Blood flow measurements can now be made with very high spatial resolution approaching the capillary level. Depending on the size of the organ being imaged, the instrument is able to determine the spatial location of microspheres with a 10- to 100-μm resolution in the x and y directions and 10- to 100-μm resolution in the z (slice) direction. Most importantly, it offers a method to study organ blood flow distribution in small laboratory animals.

By determining the spatial location of each microsphere in the organ, the resulting data can be mathematically analyzed. With the use of physical dissecting methods, an organ can be dissected by only one scheme. Mathematical dissections allow each organ to be dissected, using multiple schemes, into differently sized and shaped sampling regions. Volumes of varying sizes can be used to obtain fractal dimensions over a broad range of volume (1). Physical dissecting methods also require the organ to be sampled before obtaining the data. Mathematical dissection allows the re-
searcher to explore the data and then resample the organ using another scheme suggested by the first pass through the data.

An important concern with the Imaging Cryomicrotome is the small numbers of microspheres counted in small sampling regions. A commonly held tenet in microsphere methods is that at least 400 microspheres must be found in each piece to be confident in the estimated flow to a given piece (4). However, if one is interested in general measures of flow, such as heterogeneity, correlation between two measures, or directional trends, many fewer microspheres are needed. A recent study by Polissar et al. (20) demonstrated that an average of only four microspheres per piece is needed to accurately measure the CV of perfusion or the correlation between two measurements as long as there are enough pieces in the distribution.

Although too few microspheres exist in our small organ regions to confidently detect small differences in blood flow between two different regions or the same region over time, statistical inferences can still be made with small numbers of microspheres. Whereas a formal statistical analysis of these issues is too detailed for this discussion, an intuitive understanding can be obtained by examining the simultaneous injections performed in the present study. Figure 9 shows the correlation between the numbers of yellow and green microspheres counted in small volumes in a rat lung. As an example, let us use the observation of 20 green microspheres per sample. This observation represents only one measurement from a potential distribution with a true mean for the given sample. Hence, there is some noise in this observation. The correlation plot shows that the number of yellow microspheres in samples with 20 green microspheres is limited to between 5 and 40. Because this distribution contains the Poisson error in both the green and yellow distributions, we can confidently conclude that blood flow is different between two regions if one region has 20 microspheres and the other has either <5 or >40 yellow microspheres. Although these confidence limits are significantly larger than those stated by Buckberg et al. (4) when there are more than 384 microspheres per region, statistical inferences can still be made.

An alternative approach to analyzing regional blood flow patterns is to consider the microspheres as points and apply statistical methods developed to analyze spatial distributions of point processes (3, 5). The average distance to the nth nearest microspheres can be used as an estimate of regional flow. More microspheres will lodge in high-flow regions, and the mean distance between microspheres will therefore be less than in lower-flow regions. The reciprocal of the mean distance between neighboring microspheres can therefore be used as an estimate of local flow. Whereas this approach provides considerably more spatial information, less confidence can be placed in the local values because they are determined from a small number of microspheres. Determining the mean distances be-

Fig. 6. Three-dimensional distribution of sampled spherical volume in the rat lungs (left) and rabbit heart (right). The sampling sphere radii are 1,000 μm in both organs. The spheres are colored according to the number of spheres counted in each sample volume. The images can be rotated about any axis on a computer to best appreciate the three-dimensional distribution of regional blood flow.

Fig. 7. Frequency distribution of microspheres determined from microsphere density method. A quantity of 23,361 and 25,485 points were sampled in the rabbit heart (A) and rat lung (B), respectively. Regional flow is determined by the local density of microspheres at each sampling point. The distributions are similar to larger animals in that they are heterogeneous. Note the similarities in the distributions created by random sampling of the organs with spherical volumes (see Fig. 4).
An important question that is not answered in this study is the following. Do 15-μm-diameter microspheres faithfully represent blood flow distribution at the capillary level? Because of the particulate nature of the microspheres, there is concern that microspheres may not faithfully represent blood flow distribution at the capillary level (15). Bassingthwaighte et al. (2) compared estimates of myocardial blood flow to 54-mg myocardial regions using a molecular microsphere (2-iododesmethylimipramine) and radiolabeled 15-μm-diameter spheres. He found a strong correlation (r = 0.95) between the two estimates. Using lung pieces approximately 1.5 cm³ in volume, Melsom and co-workers (18) demonstrated that 15-μm-diameter microsphere estimates of regional blood flow correlated well (r = 0.99) with estimates from a nonparticulate (molecular) marker. Both of these studies measured blood flow to regions considerably larger than those obtained with the cryomicrotome system. Hence, an answer to the posed question must await development of a molecular blood flow marker that can be imaged at the microscopic level.

**Measures of Heterogeneity**

Our measured CV for the rabbit heart ranged from 25–42%. This is similar to the 20–43% reported by Bassingthwaighte et al. (2). Our measured CV for the rat lung ranged from 19–31%, considerably larger than the 10.2% in larger sample volumes (16). These values are comparable to those reported in other species (9, 11, 25). Perfusion appears to become more variable as smaller sampling volumes are used, suggesting that the “unit of perfusion” (1) is smaller than 1 mm³ in the rat lung. Again, further studies are needed to confirm this observation.

Although we have presented our data in numbers of microspheres or relative flow per sampled region, it is theoretically possible to estimate regional flow in milliliters per minute. A reference blood flow sample (10) would be required with the ability to count the numbers of microspheres in the sample. This can be accomplished by freezing the blood with OCT compound and then using the Imaging Cryomicrotome to count the number of microspheres in the reference sample. Knowing the number of microspheres in the reference sample and the rate at which the sample was obtained allows for the number of microspheres per organ sample to be translated into milliliters per minute.

**Fig. 8.** Two-dimensional reconstructions of local blood flow using the microsphere density method. Regional flow at each point in the organ is determined from the average distance to the 5 nearest microspheres. Regional microsphere densities are corrected for partial volume artifacts and normalized to the mean for the entire organ. A: transverse slice through a rabbit heart. B: transverse slice through a rat lung.

**Fig. 9.** Numbers of microspheres counted in small regions after a simultaneous injection of green and yellow microspheres. Although both estimates of regional flow included Poisson noise, the observed numbers are constrained to a well-defined space. Regions with 20 green microspheres are limited to between 5 and 40 yellow microspheres (solid ellipse). This distribution contains the Poisson error in both the green and yellow distributions. Hence, it can be confidently stated that blood flow is different between 2 regions if 1 region has 20 green microspheres and the other has either <5 or >40 yellow microspheres. If less confidence in the true difference is needed, the limits can be reduced to 9 and 35 (dashed ellipse).
The greatest benefit of this new system is that regional blood flow experiments can now be performed in small laboratory animals, such as rats and mice. To date, we have required larger species to gain sufficient spatial resolution to detect significant changes in blood flow distributions. Utilizing the Imaging Cryomicrotome, we can now obtain more spatial information from animals smaller than those used for traditional microsphere methods. Furthermore, genetically engineered rats and mice provide excellent models of specific diseases. These animal models can now be used to measure changes in organ blood flow in the pathological model and determine how blood flow changes with therapeutic interventions.

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