Visualizing Regional Myocardial Blood Flow in the Mouse

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Rationale: The spatial distribution of blood flow in the hearts of genetically modified mice is a phenotype of interest because derangements in blood flow may precede detectable changes in organ function. However, quantifying the regional distribution of blood flow within organs of mice is challenging because of the small organ volume and the high resolution required to observe spatial differences in flow. Traditional microsphere methods in which the numbers of microspheres per region are indirectly estimated from radioactive counts or extracted fluorescence have been limited to larger organs for 2 reasons; to ensure statistical confidence in the measured flow per region and to be able to physically dissect the organ to acquire spatial information.

Objective: To develop methods to quantify and statistically compare the spatial distribution of blood flow within organs of mice.

Methods and Results: We developed and validated statistical methods to compare blood flow between regions and with the same regions over time using 15-µm fluorescent microspheres. We then tested this approach by injecting fluorescent microspheres into isolated perfused mice hearts, determining the spatial location of every microsphere in the hearts, and then visualizing regional flow patterns. We demonstrated application of these statistical and visualizing methods in a coronary artery ligation model in mice.

Conclusions: These new methods provide tools to investigate the spatial and temporal changes in blood flow within organs of mice at a much higher spatial resolution than currently available by other methods. (Circ Res. 2013;112:e88-e97.)

Key Words: 3-D imaging ■ blood flow ■ fluorescent microspheres ■ Poisson distribution

Integrative physiology explores the influence of genes, proteins, and molecular pathways on physiology at the level of the whole organ or animal. Physiology of genetically altered mice is of special interest in studying the connection between cellular-level processes and the whole animal. The distribution of blood flow to and within organs is a phenotype of interest because derangements in blood flow may precede detectable changes in organ function. Although a number of studies have explored the distribution of blood flow to different organs in genetically altered mice, none have explored the spatial distribution of blood flow within organs of these mice.

Using fluorescent microspheres and an in situ imaging approach, we present a new method of visualizing regional flow patterns within mouse hearts that typically have tissue volumes of ≈100 to 200 µL. Because of the fewer numbers of microspheres that can be used in these hearts, we develop and validate statistical methods to compare blood flow between regions and with the same regions over time. We further demonstrate application of these statistical and imaging methods in a coronary artery ligation model in mice and through determination of endocardial to epicardial perfusion ratios.

These new methods provide tools to investigate the spatial and temporal changes in blood flow within organs of mice at a much higher spatial resolution than currently available by any other method. They allow investigators to phenotype genetically altered mice with respect to blood flow distribution and to explore novel interventions to alter regional organ blood flow in these animals.

Methods

Heart Preparation

All procedures were approved by the Animal Care and Use Committee of Vanderbilt University and conformed to the Guide for the Care and

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Use of Laboratory Animals published by the US National Institutes of Health. Hearts were harvested after mice were deeply anesthetized with 3% to 5% isoflurane in 100% oxygen. The aortas were cannulated and hearts perfused in retrograde mode with filtered Tyrode solution containing (in mmol/L) NaCl 139, KCl 4, NaHCO3 14, NaH2PO4 1.2, MgCl2, Na2HPO4 1.5, glucose 10, and 8-propanolol 0.0002. The pH was adjusted to 7.4 using carbogen gas (95% O2/5% CO2). The hearts were perfused with a constant pressure of 70 mmHg at 37°C for up to 30 minutes. Hearts were allowed to beat spontaneously, and rate and rhythm were monitored via ECG recording. After a stable baseline was attained, typically between 3 and 8 minutes, the coronary flow was assessed in 2 different ways (in retrograde perfused hearts, the total flow through equals the coronary flow): (1) Hearts were lifted out of the bath, and the flow through was collected for 1 minute; and (2) for continuous flow measurements, we used an in-line flow probe (1PXN, AD Instruments, Colorado Springs, CO) connected to a flowmeter (Transonic Systems TS410).

Microsphere Preparation and Injection

Six microliters of stock green and yellow 15-µm microspheres (FluoSpheres F21010 & F2101, Invitrogen, NY) were vigorously vortexed and diluted with Tyrode buffer to a final volume of 500 µL. It was determined in pilot experiments that ≈50% of the injected microspheres reach the heart (trapping/loss of 50%). Further studies patterned after a Langendorff-perfused rat heart study were conducted to determine the relationship between number of injected microspheres and coronary flow in a mouse heart. Methods and results of these studies are provided in the online-only Data Supplement. In the final studies, ≈5000 green or yellow 15-µm fluorescent microspheres were injected over 30 seconds into the perfusion line placed just over a small heating coil column directly above the heart.

Four different patterns of microsphere color injections were performed in a number of different hearts. In 2 hearts, a mixture of yellow and green microspheres was injected (simultaneous injection). In 4 hearts, 1 microsphere color was injected, followed by a second injection of a different color 10 to 15 minutes after the first (serial injection). In 3 hearts, after the injection of 1 color, a large branch of the left anterior descending artery was tied with 7-0 braided silk, and second color was injected after the hearts re-equilibrated. In 5 additional hearts, a single color of microspheres was injected to estimate endocardial to epicardial blood flow ratios. The order of microsphere colors varied across experiments. After the final microsphere injection, optimal cutting temperature medium (Sakura Finetek, Torrance, CA) was injected into both ventricles, and the hearts were frozen at –80°C.

Microsphere Imaging

The Imaging Cryomicrotome (Barlow Scientific, Inc, Olympia, WA) determines the spatial distribution of fluorescent microspheres at the microscopic level. Details of the instrument configuration have been previously reported, but components of the instrument have been upgraded. The instrument now consists of a Redlake Megapixel II digital camera (Redlake MAS Tucson, AZ), a computer (Dell Computer Corp, Round Rock, TX), a metal halide lamp (DayMax 400 W, ILC Technology, Inc, Sunnyvale, CA), an excitation filter-changer wheel, an emission filter-changer wheel, and a cryostatic-microtome. Fluorescence images are acquired with the Redlake digital camera (2184×1472 pixel array) with an AF Micro-Nikkor 200 mm f/4D IF-ED lens (Nikon, Corp, Tokyo, Japan). The computer controls 2 motorized wheels containing excitation and emission filters through stepper motors and microsensors. A custom-designed microtome serially sections frozen organs. Computer control of the microtome motor, filter wheels, image capture, and display is accomplished through a program written in LabVIEW (8.2, National Instruments Inc, Austin, TX).

The cryomicrotome sections the frozen hearts with a slice thickness of 14.3 µm. Digital images of the remaining tissue surface are acquired with appropriate excitation and emission filters to isolate each fluorescent color. Images at each fluorescence excitation/emission wavelength pair are collected and processed so that x, y, and z (slice) locations of each microsphere can be determined. The spatial resolution of the system depends on the magnification used to image the tissue and was 13.8 µm in the x and y directions for this study. Image processing is completed in Fiji (http://fiji.sc), an open-source bundle of ImageJ, and a variety of plug-ins. A trainable segmentation algorithm, a standard plug-in provided with Fiji, is applied to images of tissue cross sections to produce a 3-dimensional (3D) binary map defining the spatial locations of the myocardium. Ventricular cavities are identified in the images and designated as nontissue. This map determines the 3D organ space to be sampled. The spatial locations of each microsphere as a 3D point are determined through previously described imaging methods.

Random Sampling Methods to Visualize Flow in Spherical Regions

Random sampling of the organ is performed by choosing x, y, and z coordinates from a pseudorandom number generator. Marsaglia shuffling is used to ensure a uniform distribution of numbers. Each random spatial point is located in the binary map of the heart. If >95% of the sampling sphere (defined by its center point and selected radius) lies within heart tissue, the spherical region is considered adequate to sample. If not, another point is randomly chosen, and the volume around this point is examined. Sampled volumes are allowed to overlap slightly with center points no closer than 75% of the diameter of a sphere. We noted an approximate oversampling of 1% of the entire sampled volume. This sampling process continues until no other spherical regions can be found within the organ. With this approach, only a fraction of the organ is sampled, but it is done in an unbiased manner with limited sampling overlap. This allows statistical comparison of flow between different spheres and by using different microsphere colors also within the same region over time.

Rolling Ball Average to Characterize Regional Blood Flow

The random sampling method provides limited 3D information because the number of samples is constrained by restricting the overlap. A way to refine the 3D flow information is to assess the number of microspheres within a given distance from each voxel in the myocardium. This can be envisioned as rolling a ball through the x, y, and z grid, counting the microspheres existing inside the spherical surface, and assigning this value to the voxel at the center of the ball. Grid points near the myocardial boundaries that also sample empty space, either outside the heart or inside the ventricles, have their raw number of microspheres scaled by the fraction of tissue within the sphere. Thus, a sampling volume with 75% of its voxels counted as myocardium and a raw microsphere count of 10 would obtain a value of 100/0.75=13.3. With the use of this rolling ball method, the entire myocardium can be sampled, and microsphere numbers can be assigned to each voxel in the heart.

Partitioning Hearts Into Endocardial and Epicardial Regions

The hearts can be virtually dissected into epicardium and endocardium using an automated method in MATLAB (R2011b, The MathWorks Inc, Natick, MA). The MATLAB script reads a substack of our binary map images, limited to those in which the left ventricle is patent, and determines the internal and external boundaries of the heart tissue. The pixels along the boundaries are used as seed points for forming a Voronoi diagram that is used to approximate the medial axis of the tissue. The endocardium is defined as all heart tissue inside the Voronoi polygons associated with the ventricular cavity seed points. The remaining tissue is designated as epicardium. Once the heart mask is split between endocardium and epicardium, the x,y,z coordinates of the microspheres are used to apportion the microspheres between the 2 compartments.
Displaying Results

Results from the sampling and rolling ball methods were imported into Paraview (Kitware Inc, Clifton Park, NY), an open-source data visualization application (http://www.paraview.org/). Paraview facilitates interactive data exploration in 3 dimensions. Because spatial patterns are more obvious when interacting with 3D data, movies of spinning 3D plots are provided in the online-only Data Supplement.

Results

Statistical Framework

Estimating Confidence Intervals

Because microspheres are individual particles, their delivery to any given organ region has a strong stochastic component. These discrete events occur at rates described by the Poisson distribution. If a microsphere experiment could be repeated many times, then the mean number of microspheres, \( \mu \), found in a given organ region would provide the best estimate of the true blood flow (and numbers of microspheres) to that region. The Poisson distribution is a discrete function that is defined for only positive integer values and is described completely by its mean, \( \mu \):

\[
P(x = k) = \frac{\mu^k e^{-\mu}}{k!}
\]

where \( P(x=k) \) is the probability of a value \( x \) being equal to the integer \( k \). The Poisson distribution is symmetrical about its mean for \( k \geq 10 \) but becomes increasingly right-skewed as \( k \) approaches zero. The cumulative distribution function is given by

\[
CDF = e^{-\mu} \sum_{i=0}^{k} \frac{\mu^i}{i!}
\]

Buckberg et al. were interested in the accuracy of the microsphere method and used the Poisson distribution to calculate the numbers of microspheres needed in an organ region to be confident in the accuracy of the observation. They determined that 384 microspheres need to be entrapped in an organ region to be certain at the 2.5% level (46.3 rounded to nearest integer). In this example, 33 microspheres are counted in an organ region. There is a <2.5% chance that the observed number, 33, could have arisen and to have a certain CI that this range includes the true distribution. Using a 95% CI, the error rate, \( \alpha \), is 5%. The lower and upper 95% CI limits for the observed mean are the values, \( \mu_L \) and \( \mu_U \), for which \( x=33 \) is just significant at the 2.5% level (\( \alpha/2 \) using a 2-tailed test). In other terms, determine the mean of the distribution that has 33 just barely in the upper 95% CI tail and the mean of the distribution that has 33 just barely in the lower 95% CI tail (Figure 1).

These 2 means define the 95% CIs for the observed number of microspheres in the given organ region and can be obtained as solutions to the following equations:

\[
\sum_{k=33}^{\infty} \frac{e^{-\mu} \mu^k}{k!} < 0.025
\]

and

\[
\sum_{k=0}^{32} \frac{e^{-\mu} \mu^k}{k!} < 0.025
\]

For this example, \( \mu_L = 22.7 \) and \( \mu_U = 46.3 \). These values can be estimated from closed equations or look-up tables. Broader CIs, for example, 99th percentile and \( \alpha=1\% \), could be determined by comparing the cumulative functions in Equations 3 and 4 to 0.005 rather than 0.025.

Using CIs, it is possible to compare 2 different observations, either between 2 regions or with the same region after a second microsphere injection. If the observed number of microspheres in another region is observed to be, say, 12, then \( \mu_L \) and \( \mu_U \) for that region would be 6.21 and 20.97. Despite the relatively few numbers of microspheres in each region, 12 and 33, it is possible to infer that they are statistically different with >95% certainty because their CIs do not overlap.

Although Buckberg et al. were more concerned with accuracy in measuring flow to each region, the approach presented here focuses on determining whether a difference exists

![Figure 1. Poisson distributions for \( \mu_L \) and \( \mu_U \) determined in the example from the text. In this example, 33 microspheres are counted in an organ region. There is a <2.5% chance that the observed number, 33, could have come from a Poisson distribution with a true mean <23 (22.7 rounded to nearest integer). There is also a <2.5% chance that the observed number, 33, could have come from a Poisson distribution with a mean >46 (46.3 rounded to nearest integer).](http://circres.ahajournals.org/doi/pdf/10.1161/01.RES.000.000.000.000)
between 2 measures. However, because of the small numbers of microspheres in sampled regions from small organs, confidence in the magnitude of the differences between any 2 measures is lessened. The magnitude of the difference between 2 numbers can be characterized by the ratio of the 2 numbers. Barker and Caldwell have reviewed different methods to estimate the CIs on ratios of counts derived from a Poisson distribution when the events are rare. A log-linear approach provides CIs on the ratio $x_1/x_2$, where $x_1$ and $x_2$ are 2 counts from a Poisson distribution. The lower and upper CIs on the ratio $x_1/x_2$ can be estimated from the following formulas:

\[
\text{lower 95% CI} = \frac{x_1}{x_2} e^{-1.96\left(\frac{1}{x_1} + \frac{1}{x_2}\right)}
\]

and

\[
\text{upper 95% CI} = \frac{x_1}{x_2} e^{1.96\left(\frac{1}{x_1} + \frac{1}{x_2}\right)}
\]

Using the example above with $x_1=33$ and $x_2=12$, the observed ratio is 2.75 but the 95% CI around this ratio is rather broad at 1.42 to 5.32. Note that the CI does not include 1.0, confirming that 33 and 12 are significantly different.

The CI of a ratio between 2 microsphere counts is greatly dependent on the numbers of microspheres, and CIs are necessarily broader with fewer numbers of microspheres. For example, if there is a 20% increase in the flow to a region between time 1 and time 2, the observed ratio of 1.2 has a range of CIs depending on the numbers of microspheres counted at each time point. Figure 2 shows the relationship between the 95% CIs and the observed microsphere counts for an observed ratio of 1.2. A corollary of the broad CIs with small microsphere numbers is the increased likelihood of not declaring a difference when in fact a difference exists. This is typically called a type II error and is characterized by $\beta$, the probability of making this error. The $\beta$ for varying ratios of $x_1/x_2$ as a function of microspheres counted is further explored with numeric simulations below.

**Numeric Simulations**

To validate the theoretical approach outlined above, numeric simulations of organ blood flow were performed. Blood flow to organ regions was simulated with an asymmetrical branching model of Van Beek et al. Their model III was implemented to create a log-normal distribution of flows to 128 regions, similar to that observed in hearts. The regional organ blood flow created with 1 pass of this model was designated as the true relative flow to each organ region, $i(R_{true})$. Repeated microsphere studies were simulated by initially designating the total number of microspheres injected, $M$ (ranging from 125 to 500,000), for each realization. The true numbers of microspheres that would go to each organ region, $N_{true}$ (without Poisson noise), was determined by multiplying the true relative flow to each region by the average number of microspheres injected per region, $N_{true} = R_{true} \cdot M/128$. Repeated realizations of microspheres lodging in each region were simulated by using these true microsphere numbers to generate random numbers from a Poisson distribution with the mean to each region, $\mu_i$, equal to the true number of microspheres to each region, $N_{true}$ (Online Tables II and III). Random numbers from Poisson distributions with specified means were generated from the Knuth algorithm. In this way, repeated simulations could be performed in which the total numbers of microspheres lodging in the organ could be varied. The validity of the model was tested by running 100 simulations with a fixed number of total microspheres and demonstrating that the numbers of microspheres going to each organ region varied as a Poisson distribution with a mean similar to the set or true numbers of microspheres.

In these simulations, the true blood flow to each region remained fixed across realizations, but the numbers of microspheres lodging in each region varied because of Poisson noise. Ninety realizations were simulated for each of 9 different microsphere injections (Online Tables II and III for 2 examples). In simulations using the fewest numbers of total microspheres, the numbers of microspheres per region ranged from 1 to 50 with a mean of 9.8. In simulations using the greatest numbers of total microspheres, the numbers of microspheres per region ranged from 50 to 1281 with a mean of 390.6. Using the statistical approach developed above, CIs and statistical comparisons were made between the same regions at each of the different realizations.

**False Discovery Rate**

CIs were calculated using a normal approximation proposed by Molenaar. Because a 95% CI was chosen, the numbers of microspheres to a given region should be declared as being different between realizations 5% of the time, although the true blood flow remained the same. A term borrowed from high-throughput genetic analyses calls this the false discovery rate (FDR). Through repetition, 143,360 comparisons were made at each level of injected microspheres (Figure 3). When the mean number of microspheres per region was >100, the FDR was equal to the expected rate of 5%. When fewer microspheres lodged per region, the FDR was significantly less than expected, likely because of the skewed shape of the Poisson distribution or a bias in the methods of Molenaar used to calculate the CIs at smaller mean values.
and $x_2$. Ten thousand realizations were run at each $x_1$ and $x_2$ pair difference of 20%, 50%, and 200% in blood flow between $x_1$ were simulated as being 1.2, 1.5, and 2.0, representing a true ratio of $x_1/x_2$. The simulations were performed to see whether the error rate varied with a range of mean numbers of microspheres per region. When the mean number of microspheres per region is >100, the error rate approaches the theoretical value of 5%. Below 100 microspheres per region, the confidence intervals were more conservative.

**Figure 3.** Using a confidence interval of 95% around each of 2 observations from a Poisson distribution with the same mean, one would expect a 5% chance that the confidence intervals would not overlap. Because the 2 observations were generated from the same Poisson distribution, nonoverlapping confidence intervals represent an error. Numeric simulations were performed to see whether the error rate varied with a range of mean numbers of microspheres per region. When the mean number of microspheres per region is >100, the error rate approaches the theoretical value of 5%. Below 100 microspheres per region, the confidence intervals were more conservative.

**Power ($\beta$ Error)**

The probability of declaring microsphere counts in 2 regions (or the same region at 2 different times) as not different, when in reality they are, is called $\beta$. The power to identify statistical differences is defined as $1-\beta$. To determine the relationship between $\beta$ and the numbers of microspheres in each region, we simulated microspheres lodging in 2 different regions with known true blood flow to each region. Known ratios of $x_1/x_2$ were simulated as being 1.2, 1.5, and 2.0, representing a true difference of 20%, 50%, and 200% in blood flow between $x_1$ and $x_2$. Ten thousand realizations were run at each $x_1$ and $x_2$ pair for a given ratio by generating random numbers from a Poisson distribution with means equal to the true number of microspheres to each region; 95% and 80% CIs were calculated for each observation as described above. A type II error occurred if the 2 observations were determined to be not significantly different. The probability of a type II error ($\beta$) was plotted as a function of the mean number of microspheres in the 2 regions for the true ratios of 1.2, 1.5, and 2.0 (Online Figure I). As expected, $\beta$ increases with decreasing numbers of microspheres counted, and $\beta$ decreases as the true difference between the 2 regions increases (increasing ratio of $x_1/x_2$). The simulations also demonstrate that the more stringent the requirement to declare a difference is (wider CIs and smaller $\beta$), the less power (1-$\beta$) there is to identify small but true differences.

**Scaling Microsphere Numbers**

In experiments using animals, the number of microspheres injected at different time points cannot be precisely controlled. When comparing the numbers of microspheres lodging in the same region at different time points (2 separate microsphere injections), either the same numbers of microspheres need to be injected into the whole organ at each time point or the resulting observations need to be scaled so that they are comparable. If different numbers of microspheres are injected, the injection with the fewer numbers of microspheres can be scaled up or the injection with the greatest numbers of microspheres can be scaled down. When scaling up, any counting noise introduced by the stochastic nature of the microsphere lodging will be magnified, and consequently, the error rate will increase. A more conservative approach is to scale the injection with the greater number of microspheres down to be comparable to the injection with fewer total microspheres. We ran numeric simulations with different numbers of microspheres in sequential microsphere injections and confirmed this result (data not shown).

**Mouse Heart Experiments**

All hearts were randomly sampled using spheres with radii of 250, 500, 750, and 1000 µm, corresponding to sampling volumes of 0.065, 0.53, 1.8, and 4.2 µL, respectively. The numbers of microspheres counted and comparison statistics for 1 heart that had a simultaneous injection of yellow and green microspheres are presented in Online Table I. At the smallest sampling volume, many more sampling regions were obtained but with very few microspheres per region. Despite the very low numbers of microspheres counted in each region (means of 0.88 and 1.27 for the 2 colors), measures of perfusion heterogeneity (coefficient of variation) were very similar, and the correlation between the 2 colors was perfect when corrected for Poisson noise. Using the statistical methods developed above, none of the sampled regions were found to be statistically different, resulting in an FDR of 0%. Similar findings were observed for the different sampling volumes, and as expected, measures of heterogeneity decreased with increasing sampling volumes.

Regional perfusion can be expressed as flows relative to the mean flow in the heart. Using a sampling volume of 1.8 µL, a histogram for 1 color of microspheres in 1 heart is shown in Figure 4. The numbers of microspheres per region ranged from 8 to 42 with a mean of 21.2. Despite these relatively low numbers of microspheres per region, low-flow regions could be identified as being statistically different from high-flow regions. Three-dimensional visualizations of microsphere locations and sample volumes, color-coded for the relative blood flow per region, were created in Paraview (Online Movie I).

Data from hearts randomly sampled using spheres with a volume of 1.8 µL are presented in the Table. These data show that the microsphere numbers in the hearts receiving the simultaneous injection were all perfectly correlated (when corrected for Poisson noise) and had an FDR of 0%. The microsphere numbers in the hearts receiving the serial injections were less well correlated but still had relatively high correlations of 0.69, 0.94, 0.95, and 0.99. Assuming the different colored microspheres were well mixed and distributed similarly with blood flow, we would not expect any differences between their observed distributions. We therefore classify any observed difference as a false discovery. The FDR (see Discussion) for the hearts with serial microsphere injections therefore ranged from 0% to 3.1%, below the expected FDR of 5%. A number of regions were identified as having significant changes in blood flow in the hearts in which a coronary artery was ligated (Table). As expected, the correlation coefficient between the 2...
injections was much lower because of the significant change in blood flow to regions within the distribution of the occluded coronary artery. Three-dimensional plots showing where blood flow changed after coronary artery ligation are shown in Figure 5. Despite the relatively few numbers of microspheres in the sampled regions, it was possible to identify regions that had statistically significant changes in microsphere numbers. These plots clearly demonstrate the spatial relationship among the regions with flow changes. The magnitude of the change in flow in these regions is less certain because of the small numbers of microspheres counted in each region (see Power (β Error) section under Numeric Simulations above). The ratios of microspheres counted before and after coronary artery ligation in regions of significant change are presented in the online only Data Supplement (Online Figure IV).

Regional blood flow can be visualized on a finer scale with a flow value assigned to each voxel in the heart using the rolling ball method described above. The smaller the rolling ball is, the greater the observed heterogeneity in perfusion. Three orthogonal planes from 1 heart using a rolling ball size of 1.8 µL (750 µm radius) are presented in Figure 6. A movie running through a stack of transverse sections depicting regional blood flow can be viewed in the online Data Supplement (Online

<table>
<thead>
<tr>
<th>Heart (Color Order)</th>
<th>Regions, n</th>
<th>Average Green Microspheres per Region, n</th>
<th>Average Yellow Microspheres per Region, n</th>
<th>Regions With Statistical Difference, n</th>
<th>CV, Green</th>
<th>CV, Yellow</th>
<th>Correlation Coefficient, r</th>
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<tr>
<td>Simultaneous</td>
<td>39</td>
<td>20.5</td>
<td>25.8</td>
<td>0</td>
<td>0.70</td>
<td>0.68</td>
<td>1.00</td>
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<tr>
<td>Simultaneous</td>
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<td>21.3</td>
<td>31.6</td>
<td>0</td>
<td>0.29</td>
<td>0.27</td>
<td>1.00</td>
</tr>
<tr>
<td>Serial (G, Y)</td>
<td>55</td>
<td>12.0</td>
<td>17.0</td>
<td>0</td>
<td>0.47</td>
<td>0.40</td>
<td>0.94</td>
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<tr>
<td>Serial (Y, G)</td>
<td>55</td>
<td>18.0</td>
<td>17.1</td>
<td>0</td>
<td>0.49</td>
<td>0.40</td>
<td>0.69</td>
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<td>Serial (Y, G)</td>
<td>41</td>
<td>22.5</td>
<td>21.9</td>
<td>0</td>
<td>0.42</td>
<td>0.36</td>
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<tr>
<td>Serial (Y, G)</td>
<td>32</td>
<td>21.0</td>
<td>32.1</td>
<td>1</td>
<td>0.41</td>
<td>0.38</td>
<td>0.95</td>
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<tr>
<td>Coronary ligation (G, Y)</td>
<td>49</td>
<td>20.3</td>
<td>17.5</td>
<td>14</td>
<td>0.52</td>
<td>0.72</td>
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<tr>
<td>Coronary ligation (Y, G)</td>
<td>40</td>
<td>13.5</td>
<td>37.3</td>
<td>12</td>
<td>0.87</td>
<td>0.43</td>
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<tr>
<td>Coronary ligation (G, Y)</td>
<td>32</td>
<td>12.8</td>
<td>25.4</td>
<td>5</td>
<td>0.64</td>
<td>0.56</td>
<td>0.46</td>
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</tbody>
</table>

CV indicates coefficient of variation; G, green; and Y, yellow.
Movie II). The CIs for Poisson distributions can be used to determine which regions have significant differences in microsphere numbers between the interventions. Figure 7 compares the microsphere flow values and the resulting difference map for a single cross section of 1 heart with a coronary ligation.

A movie showing the spatial grouping of significantly different flows can be viewed in the online-only Data Supplement (Online Movie III).

To demonstrate other schemes for virtually dissecting the heart, the hearts from the serial (n=6) and simultaneous (n=5) injection mice were virtually dissected into endocardial and epicardial regions using a Voronoi algorithm. Only the first injection from the serial injection series was used. The numbers of microspheres per 1µL of heart tissue were determined in each region. The mean±SEM ratio of endocardial to epicardial microspheres is 1.33±0.11 (n=11), consistent with observations in dogs and humans.

The serially and simultaneously injected hearts were also virtually dissected into endocardial and epicardial regions, and the numbers of microspheres per 1µL of heart tissue were determined in each region. CIs around the endocardial/epicardial ratios can be constructed with Equations 5 and 6 and then used to determine whether the endocardial/epicardial ratio is different between the 2 colors. None of endocardial/epicardial ratios are statistically different between the simultaneous or serial injections, as expected.

Discussion
Our study demonstrates that regional blood flow can be visualized in mice hearts and that, despite the few numbers of microspheres per region, it is possible to identify differences in flow between regions at 1 time point or within the same regions over time. We provide the statistical framework to create confidence levels in identifying these differences. These new approaches open novel avenues of investigation with regard to organ blood flow in genetically altered mice.

We have previously used the Imaging Cryomicrotome to study blood flow distributions in relatively larger organs such as rat lungs and armadillo hearts and skeletal muscle. Compared with the present study, these previous studies used more microspheres, and blood flow was characterized by coefficients of variation and fractals that require fewer microspheres than needed for regional flow measurements. The present study uses far fewer microspheres because of the small heart sizes and requires greater optical magnification to visualize the hearts. Despite these limitations, we show that it is possible to generate 3D images of regional myocardial blood flow.

The microsphere method introduced by Rudolph and Heymann has become the gold standard for measuring regional organ blood flow. A commonly held tenet is that at least 400 microspheres must lodge in each region of interest for there to be confidence in the measurements. This guideline is derived from the work by Buckberg et al, who concluded that to have 95% CI that the observed flow to a region is within 10% of the true flow, at least 384 microspheres must lodge within the organ region. Many investigators have interpreted the Buckberg et al guideline to mean that the microsphere methods cannot be used if organ samples have <400 microspheres per region of interest. However, if investigators do not need to know the absolute flow (eg, mL/min) to regions but rather are interested in identifying statistical differences in blood flow between regions or after interventions, many fewer microspheres can be counted. If larger organ regions are used such as partitioning the heart into endocardial and epicardial regions, absolute
flows can be estimated with confidence because many more microspheres will be counted in each region. When regional blood flows over the entire heart are compared, multiple statistical comparisons are made, and differences will be found that do not really exist (type I error). It is possible to use corrections such as the Bonferroni adjustment for multiple comparisons, but they commonly result in very conservative statistical inferences and a decreased ability to identify true differences. An alternative approach that has become popular with high-throughput science is to report the expected FDR. The FDR is the fraction of false-positive findings or the error rate, \( \alpha \), in our statistical comparisons. Because we chose to use 95% CIs, we would expect multiple statistical comparisons to have an FDR of 5%. However, numeric simulations demonstrated that the FDR decreases with decreasing numbers of microspheres per region below a mean microsphere count of 100. In the experiments in which 2 colors of microspheres were injected simultaneously, we sampled the hearts at different sampling volumes. At the smallest sampling volumes, nearly 1000 regions were identified, but the mean number of microspheres per region was \( \sim 1 \). Our numeric simulations indicate that the FDR for an average of only 1 microsphere per region was \( <1\% \), and we did not expect to observe any statistical difference between the simultaneously injected microspheres. At larger sampling volume, the mean number of microspheres increased, but the numbers of comparisons decreased. For all of the combinations of region volumes and counted microspheres, we did not see any statistical differences, which is consistent with our numeric simulations (Figure 2).

Although the statistical approach presented here identifies differences among organ regions with fewer microspheres than traditionally used, it comes at the expense of being less sensitive to smaller differences in flow. This tradeoff is inherent to any statistical inference. The more stringent the requirement to declare a difference (wider CIs and smaller \( \alpha \)) is, the less power \( (1-\beta) \) there will be to identify small but true differences. The simulations to determine the power of the present statistical approach demonstrate that it is difficult to identify differences of 20% even when there are 400 microspheres per region. The ability to identify larger changes, say 50% or 100%, is increased and requires many fewer microspheres. It is possible to increase the power of these methods by accepting less confidence in declaring difference between the counts in 2 regions (eg, 90% CIs or \( \alpha=0.1 \)). Unfortunately, there is no way to circumvent this compromise in a single experiment. This problem can be ameliorated through repeat experiments, in which an increased number of observations can provide greater statistical power and the ability to identify a weaker signal through the background noise.

The statistical methods used to compare changes within a region over time require that the same numbers of microspheres are injected and counted for the 2 different microsphere colors. Because this requirement cannot be practically achieved, the numbers of microspheres in each region must be proportionally scaled so as to be the same between injections. Our numeric simulations confirmed that it is best to scale the color with the larger number of counted microspheres down to the numbers of the other color. Statistical comparisons are then performed on the scaled numbers of microspheres per region.

In the rolling ball method, used to visualize flow across the myocardium, the radius of the ball is arbitrary. Smaller ball sizes will reveal more heterogeneity of perfusion but will also introduce more noise into the local flow values because of lower microsphere numbers counted within the ball. Although relative flow values are assigned to each voxel within the heart tissue, the voxels are much smaller than the radius of the rolling ball, and neighboring voxel values influence the flow values within any voxel. Because statistical comparisons assume independence between compared values, it is only appropriate to compare flow values with voxels that are separated in space by a distance greater than the radius of the rolling ball.

Limited numbers of microspheres can be administered to a heart because of hemodynamic and ischemic changes caused by occlusion of the microcirculation. We conducted studies similar to those of Zuurbier et al\(^{10}\) in which microspheres were serially injected into an ex vivo perfused mouse heart. In adenosine-treated hearts, in which the coronary system is fully dilated, vascular resistance increases with each microsphere injection. In hearts that are pretreated with adenosine, microsphere injections with Tween initially cause vasodilation. These studies confirm that microspheres affect the vascular bed. When corrected for Poisson noise, the correlation coefficient \( (r) \) between simultaneously injected microspheres was perfect \( (r=1.0) \), whereas the correlation between serially injected microspheres was in 3 hearts still very high \( (0.94,0.95,0.99) \) but in 1 heart was less \( (0.69) \). This may reflect a natural fluctuation in blood flow, as has been suggested before.\(^{32}\) However, it cannot be ruled out that the initial microsphere injection affected the distribution of blood flow and microspheres in the second injection in this heart to a greater degree than in the other hearts.

One significant advantage of an imaging approach is that an organ can be repeatedly virtually dissected using a number of different schemes. Traditional methods using microspheres to measure regional blood flow require that an organ be physically dissected into pieces along predetermined patterns and the numbers of microspheres counted in each piece. Using imaging methods, the organ parenchyma and spatial locations of each microsphere are stored in a digital format and can therefore be repeatedly virtually dissected in any number of spatial patterns to explore different blood flow distributions. If desired, mouse hearts can be virtually dissected using the standardized myocardial segmentation and nomenclature for tomographic imaging of the heart.\(^{33}\) In addition, spatial trends can be explored using various coordinate systems such as blood flow as a function of distance from epicardial surface. An additional advantage of virtual dissection is that the volumes of sampling regions can be selected after the microspheres are counted to attain a targeted mean number of microspheres per region to improve statistical confidence in the observed numbers. The inherent tradeoff with better counting statistics from larger sampling regions is the decrease in the spatial resolution of the observations.

We chose to present our flow data as relative to the mean flow within each heart so that we could present flow across hearts and over time in the same heart on similar scales.
Absolute flow in milliliters per minute can be determined for each organ region by multiplying the number of microspheres counted in any region by the total blood flow to the organ and dividing this by the number of sampled regions. We have added an example to the online-only Data Supplement materials of a heart in which absolute flows are visualized.

Fluorescent microsphere methods provide a means for assessing regional organ perfusion in mice using equipment available at most research institutions. At present, dedicated small-animal magnetic resonance images can provide estimates of regional organ blood flow in mice. But such instruments may not be readily available to many researchers, and high resolution requires long imaging times. Although our study was performed in an ex vivo heart preparation, the microsphere methods can be easily applied to living mice, other organs, and physiological conditions. We used an Imaging Cryomicrotome, but a number of different approaches, including paraffin-embedded histo logical methods, can be used to count and determine the locations of fluorescent microspheres within organs. Investigators willing and able to dissect mice organs into multiple regions and then use established methods to count the microspheres can now be confident in using samples with <400 microspheres per piece. The statistical methods developed within this study are applicable to any method that counts discrete numbers of microspheres within organs.

Although these methods have valuable applications for assessing regional organ perfusion in genetically modified mice, several limitations should be acknowledged. The microsphere method requires that animals be euthanized. The number of time points that can be obtained is limited by the number of different colors of microspheres that can be injected and counted. Although repeated fluorescent microsphere studies have been conducted over months in larger animals, the feasibility of this approach in mice has not been investigated.

This study demonstrates that blood flow distributions can be explored within the mouse myocardium. Statistical methods can be used to identify regions that have different flows in space or over time. Although multiple colors of microspheres can be visualized, the numbers of microspheres within any 1 injection would have to be decreased, thereby decreasing the spatial resolution of the methods. The approach presented here is not dependent on the Imaging Cryomicrotome and could be adapted to serial histological sections.

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Disclosures
None.

References


What is Known?

- The gold standard method for measuring blood flow within organs uses the deposition of microspheres to quantify local perfusion.
- Microsphere methods are well established and have been thoroughly developed for use in large laboratory animals.

What New Information Does This Article Contribute?

- Methods to use microspheres for measuring regional blood flow in mouse hearts.
- Statistical foundation to compare blood flow estimates between regions or over time.

Measuring the distribution of blood flow within organs of large experimental animals has provided important insights into physiology and pathophysiology at the whole-animal and organ level. As physiological studies have moved into smaller animals such as mice, blood flow measurements within organs become challenging because of the small size of the organs. Hence, traditional methods using microspheres to measure regional organ blood flow need to be adapted to these smaller animals. We therefore developed methods to virtually dissect hearts from mice and visually count the numbers of fluorescent microspheres within the partitioned regions. We also developed the statistical methods needed to compare blood flow changes over time or differences between regions when the numbers of microspheres within the regions are relatively few. These new methods allow investigators to conduct blood flow experiments within mice at a much higher spatial resolution than previously possible.
Visualizing Regional Myocardial Blood Flow in the Mouse
Melissa A. Krueger, Sabine S. Huke and Robb W. Glenny

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doi: 10.1161/CIRCRESAHA.113.301162

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Supplement Material

Power (β error)
The probability of declaring microsphere counts in two regions (or the same region at two different times) as not different when in reality they are, is termed β. The power to identify statistical differences is defined as 1-β. Simulations were performed to determine the relationship between β and the numbers of microspheres in each of two different regions with known true numbers of microspheres, x₁ and x₂, to each region, respectively.

Figure I. β is the probability of declaring microsphere counts in two regions as not different when in reality they are different. The power to identify a true difference between two regions is 1-β. β is dependent on the chosen significance level and the numbers of microspheres in the two regions. Known ratios of x₁/x₂ were simulated as being 1.2, 1.5 and 2.0, representing a true difference of 20%, 50% and 200% in blood flow between x₁ and x₂. The plot on the left shows curves for α = 0.05 or a CI of 95%. The plot on the right shows curves for α = 0.20 or a CI of 80%. Four curves are presented for true differences of 20%, 30%, 50% and 100%. In the left-hand plot, the curve of x₁/x₂ = 1.5 demonstrates that there is a 50% chance of identifying a 50% difference in the numbers of microspheres between two regions that have an average of 100 microspheres in each region (dashed line). Note in the left hand plot where α = 0.05, that even in regions with 400 microspheres (solid line), the power to identify a difference of 20% is only 40% (1-β).

Table I Data from one heart with simultaneous injection at different sampling volumes

<table>
<thead>
<tr>
<th>Sampling Volume (µL)</th>
<th>Number of Regions</th>
<th>Mean Number of Green Microspheres per Region</th>
<th>Mean Number of Yellow Microspheres per Region</th>
<th>Number of Regions with Statistical Difference</th>
<th>CV Yellow</th>
<th>CV Green</th>
<th>Correlation Coefficient (r)</th>
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</thead>
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<tr>
<td>0.065</td>
<td>878</td>
<td>0.88</td>
<td>1.27</td>
<td>0</td>
<td>0.56</td>
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<td>0.53</td>
<td>124</td>
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<td>0</td>
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<td>0.32</td>
<td>1.0</td>
</tr>
<tr>
<td>1.8</td>
<td>40</td>
<td>21.28</td>
<td>31.55</td>
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<td>4.2</td>
<td>17</td>
<td>44.94</td>
<td>71.18</td>
<td>0</td>
<td>0.24</td>
<td>0.23</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Pilot microsphere studies in Langendorff-perfused mouse heart

We measured the effect of microsphere injection on flow by serial injections of 15 µm diameter microspheres into the perfusate line of the isolated mouse hearts. Hearts were paced at 8 Hz via atrial stimulation to prevent a change in flow due to changes in heart rate. The estimated number of microspheres per aliquot may not be the actual number of microspheres that reach the heart for various reasons such as the number of microspheres in each aliquot varies due to the particulate nature of microspheres, the mixing of microspheres may not always be complete and there is trapping of microspheres in the syringe and perfusion line. Therefore, the hearts were imaged in the cryomicrotome to count the number of lodged microspheres. The number of microspheres injected with each aliquot was then determined as the counted microspheres divided by number of aliquots injected. The left side of figure II shows that flow initially increase and then decreases in each of the four separate experiments. The initial increase in flow has been previously reported to be an effect of the anti-aggregation agent Tween in the microsphere storage buffer.\(^1\)

To prevent the masking effect of Tween, we used 1.2 µM adenosine to fully vasodilate the coronary circulation. We then repeated the microsphere injections and counting of the lodged microspheres. We saw an immediate and linear decrease in flow (figure II right). These results are similar to those in Zuurbier's rat study. The typical perfusion time for the experiment was around 30 minutes and we did not observe any changes in QRS duration (data not shown).

\(n = 4\)

\(n = 4\)

**Figure II.** Total flow through isolated mouse heart preparation as a function of increasing numbers of injected 15 µm diameter microspheres. Driving pressure is constant so flow decreases as vascular resistance increases. Left: Blood flow initially increases due to vasodilation and then decreases with additional microsphere injections. Right: Vasculature is pre-dilated with adenosine and vascular resistance increases in linear fashion.
False Discovery Rates

Tables II and III provide examples of the numerical simulations that were performed to identify the false discovery rate for differences in microsphere numbers counted in organ regions. See manuscript for details.

**Table II.** Numerical simulations of microsphere experiments for 128 organ regions repeated 90 times in which 1000 microspheres were injected into the organ.

<table>
<thead>
<tr>
<th>Region Number, (i)</th>
<th>True Relative Flow to Region, (i)</th>
<th>True number of microspheres lodging in Region, (i)</th>
<th>Realization Number</th>
<th>Mean numbers of microspheres to Region (i) over all realizations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>1.39</td>
<td>11</td>
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<td>2</td>
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<td>8</td>
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<td>1.16</td>
<td>9</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
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<td>6</td>
<td>7</td>
<td>3</td>
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<td>5</td>
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<td>6</td>
</tr>
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<td>6</td>
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<td>2.05</td>
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<td>22</td>
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<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>0.94</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

**Table III.** Numerical simulations of microsphere experiments for 128 organ regions repeated 90 times in which 20000 microspheres were injected into the organ.

<table>
<thead>
<tr>
<th>Region Number, (i)</th>
<th>True Relative Flow to Region, (i)</th>
<th>True number of microspheres lodging in Region, (i)</th>
<th>Realization Number</th>
<th>Mean numbers of microspheres to Region (i) over all realizations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
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</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>1.06</td>
<td>165</td>
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</tr>
<tr>
<td>3</td>
<td>1.16</td>
<td>182</td>
<td>153</td>
<td>183</td>
</tr>
<tr>
<td>4</td>
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<td>127</td>
<td>121</td>
<td>134</td>
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<tr>
<td>5</td>
<td>0.67</td>
<td>104</td>
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<td>116</td>
</tr>
<tr>
<td>6</td>
<td>0.61</td>
<td>96</td>
<td>84</td>
<td>92</td>
</tr>
<tr>
<td>7</td>
<td>1.70</td>
<td>238</td>
<td>247</td>
<td>272</td>
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<tr>
<td>8</td>
<td>2.05</td>
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<td>327</td>
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<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>0.94</td>
<td>147</td>
<td>132</td>
<td>147</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>20000</td>
<td>20000</td>
<td>20000</td>
</tr>
</tbody>
</table>
The magnitude of the difference in flow between two regions (or the same region over time) has less certainty due to the small numbers of microspheres counted in each region (see Power section under Numerical Simulations in manuscript). The ratios of microspheres counted before and after coronary artery ligation in regions of significant change are presented in figure III.

**Figure III.** Plot of change in flow as a ratio of the microsphere counted in each region before and after coronary artery ligation. Only the regions identified as having significant changes in flow are presented from 3 different hearts. A point on the vertical axis above 1.0 (dotted line) indicates an increase in blood flow and a point below this line indicates a decrease in flow. The confidence intervals around the ratios are shown for each region. Note that none of the confidence intervals cross the dashed line equal to 1.0. Also note the log scale on the vertical axis to accommodate the wide range of values. Ratios of 0 have been converted to 0.01 to allow them to be plotted on a log scale.

**Visualizing Absolute Flows**

Absolute flows in mL/min can be determined to any organ region using the following equation:

\[
Q_t \text{mL/min} = \frac{N_t \cdot Q_r \text{mL/min}N}{N}
\]
where \( Q_i \) is the absolute flow to region \( i \), \( N_i \) is the number of microspheres counted in region \( i \), \( Q_T \) is the total blood flow to the organ and \( N \) is the total number of microspheres in the organ. Figure IV presents the spatial distribution of absolute flows in a heart before and after coronary artery ligation. This is the same heart that is presented in figure 5 of the manuscript.

**Figure IV.** Visualizing absolute flow. Left panel shows regional blood flows in ml/min for 49 regions in a mouse heart at baseline prior to coronary artery occlusion. The regions have a volume of 1.8 \( \mu \)L. The graph on the right plots absolute flows to the 49 regions before and after coronary artery occlusion. Absolute flows were calculated accounting for a decrease in total flow after coronary occlusion. The dashed line depicts the line of identity. Points below the line lost flow with occlusion and the points above the line gained flow due to redistribution away from the ischemic region. As expected, absolute flow is very low in the ischemic region, but there is also some flow redistribution in the non-ischemic region and the conversion into absolute flows reveals that in some of those regions the absolute flow increases slightly.

Because the hearts are virtually dissected, the sampled regions cannot be weighed to provide flow in units of mL/min/g. Using a density of 1.053 g/mL it is possible to estimate the mass for the 1.8 \( \mu \)L volumes as 0.0019 g. Using this estimate of mass, the flows axes in figure IV would range from 0 to 26 mL/min/g.

Movie I: Three-dimensional visualizations of microsphere locations and sample volumes, color-coded for the relative blood flow per region, were created in Paraview.

Movie II: Movie running through a stack of transverse sections depicting regional blood flow.

Movie III: A movie showing the spatial grouping of significantly different flows.
References