High-resolution imaging reveals a limit in spatial resolution of blood flow measurements by microspheres

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Decking, Ulrich K. M., Vinay M. Pai, Eric Bennett, Joni L. Taylor, Christian D. Fingas, Klaus Zanger, Han Wen, and Robert S. Balaban. High-resolution imaging reveals a limit in spatial resolution of blood flow measurements by microspheres. Am J Physiol Heart Circ Physiol 287: H1132–H1140, 2004. First published April 29, 2004; 10.1152/ajpheart.00119.2004.—Density of 15-μm microspheres after left atrial application is the standard measure of regional perfusion. In the heart, substantial differences in microsphere density are seen at spatial resolutions <5 ml, implying perfusion heterogeneity. Microsphere deposition imaging permits a superior evaluation of the distribution pattern. Therefore, fluorescent microspheres (FMS) were applied, FMS deposition in the canine heart was imaged by epifluorescence microscopy in vitro, and the patterns were observed compared with MR images of iron oxide microspheres (IMS) obtained in vivo and in vitro. FMS deposition in myocardial slices revealed the following: 1) a nonrandom distribution by microspheres are applied into FMS of different color stacked within the same vessel, 2) general FMS clustering, and 3) rather large areas devoid of FMS (n = 3). This pattern was also seen in reconstructed three-dimensional images (<1 nl resolution) of FMS distribution (n = 4). Surprisingly, the deposition pattern of sequentially applied FMS remained virtually identical over 3 days. Augmenting flow by intracoronary adenosine (≥2 μM) enhanced local microsphere density, but did not alter the deposition pattern (n = 3). The nonrandom, temporally stable pattern was quantitatively confirmed by a three-dimensional intermicrosphere distance analysis of sequentially applied FMS. T2-weighted short-axis MR images (2-μl resolution) of IMS revealed similar patterns in vivo and in vitro (n = 6), as seen with FMS. The observed temporally stable microsphere patterns are not consistent with the notion that microsphere deposition is solely governed by blood flow. We propose that at high spatial resolution (<2 μl) structural aspects of the vascular network dominate microsphere distribution, resulting in the organized patterns observed.

myocardial perfusion; microscopy; fluorescence; magnetic resonance; iron oxide particle

Address for reprint requests and other correspondence: U. K. M. Decking, Dept. of Cardiovascular Physiology, Heinrich-Heine-Univ., Universitätsstrasse, Bldg. 22.03, 40225 Düsseldorf, Germany (E-mail: decking@uni-duesseldorf.de).

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tion between microspheres and another molecular flow marker, adenosine, was recently also reported in canine hearts (16). Therefore, microsphere deposition density in principle is a valid measure of local myocardial perfusion.

Because it is based on the measurement of a finite number of microspheres, the precision of the measurement is dependent on the number of microspheres per sample (6). At >400 microspheres in a given sample, the flow determined will be within 10% of the true flow. While the precise measurement of flow in an individual sample therefore requires a relatively high number of microspheres, fewer spheres will be sufficient to estimate the variability of flow within a given organ when several samples are studied (21).

When local myocardial perfusion in individual samples of 0.25 to 1% of the left ventricular mass is studied, a substantial spatial variability of local myocardial blood flow is observed (spatial heterogeneity of perfusion) in several species with the use of either microspheres or molecular markers for flow determinations (e.g., Refs. 3, 4, and 15). In addition, a considerable body of evidence indicates a close correlation of local perfusion and metabolism as well as gene and protein expression (for a review, see Ref. 9). In these studies, the sample size investigated rarely dropped below 0.25 ml, because at a higher spatial scale, the same precision of the flow determination would require a high number of microspheres, possibly interfering with flow regulation and oxygen supply. For the same reason, the spatial variability of local myocardial perfusion in small laboratory animals has not been studied by microspheres, but only by imaging the retention of a molecular flow marker by autoradiography (26).

While recent advances in imaging techniques enable the accurate detection and localization of individual microspheres in the heart at high spatial resolution (5), the validity of the microsphere approach at a high spatial resolution (e.g., 1–50 μl) has not been rigorously tested. Specifically, it is unclear whether the central assumption of the microsphere technique (the deposition density is governed solely by local flow and probability) is valid at all spatial scales. We therefore studied the pattern of microsphere deposition at the highest possible resolution using epifluorescence microscopy, fluorescence imaging coupled to a cryomicrotome, and MRI of MRI-visible microspheres. The results clearly indicate that at a microscopic scale, microsphere deposition is not governed solely by flow and by chance. Therefore, at a high spatial resolution (e.g., <2 μl), microspheres will not be a valid measure of local flow.

METHODS

General. The experiments were conducted in 19 female beagle dogs using procedures and protocols concordant with the “Guiding Principles in the Care and Use of Animals” endorsed by the American Physiological Society. The studies conducted in open-chest dogs at the National Institutes of Health were approved by the Animal Use and Care Committee of the National Heart, Lung, and Blood Institute. The studies in chronically instrumented dogs carried out at the Heinrich-Heine-University Düsseldorf were approved by the local government.

Experimental procedures. In the open-chest studies, anesthesia was achieved by volatile anesthetics (isoflurane, halothane, or enflurane, n = 12) or α-chloralose (n = 5) (100 mg/kg iv loading and 50 mg/kg iv maintenance as required) combined with opioid analgesics (fentanyl). In ventilated beagle dogs (10–12 kg), following the onset of anesthesia, a midline thoracotomy was performed, the pericardium was removed, and the left atrium was cannulated for later application of microspheres. A femoral artery catheter enabled collection of blood from the abdominal aorta as an arterial reference organ. In a subset of experiments, a right branch of the left anterior descending coronary artery was cannulated for regional infusion of adenosine (0.7 mmol/l at 1 ml/min, approximate final concentration 5–10 μmol/l) for 20 min. At the end of the experiment, the animals were euthanized with KCl (10–40 meq rapid iv bolus) and the heart was excised. In one set of experiments, three different-colored fluorescent microspheres (15 μm, 250,000/kg) were given every 20 min, whereas in a second set of experiments, microspheres were applied before, twice during adenosine, and once thereafter.

In a further set of experiments, the deposition pattern of iron oxide microspheres (15 μm) was imaged by magnetic resonance imaging in the open-chest animal in vivo and after cardiac excision in vitro (see below). High-resolution in vivo imaging of iron oxide microspheres required the careful positioning of a surface coil on the myocardial surface of the left ventricular free wall, and the placement of the ventilated, open-chest animal in the MRI scanner. Control images were obtained before the animal was withdrawn from the scanner for application of the iron microspheres (10⁶ microspheres/kg). Afterward, the animal was put back in the identical position it had taken up before.

To test the temporal stability of the myocardial microsphere distribution pattern in the awake dog, and to determine capillary volume and arteriolar density in areas of different basal flow, dogs weighing 20 kg were instrumented after an overnight fast, similar to previously published procedures (17, 20). Left atrial and aortic catheters were placed under general anesthesia for later measurements of flow by microspheres (11). The animals were allowed to recover for 9 days. In one dog, differently colored fluorescent microspheres (15 μm, 250,000/kg) were then applied on days 1–3 via the atrial catheter while blood was simultaneously withdrawn from the aorta (10 ml/min) into a virtual reference organ. For cardiac excision, the animal was anesthetized as previously described (10). In the other dog, radioactive microspheres were applied for local flow determinations in the alert animal. Cardiac excision was followed by glutaraldehyde fixation for electron and light microscopy analysis, as described previously (25).

In all of the other experiments, after cardiac excision the coronary arteries were flushed with a Ca²⁺-free medium composed of (in mmol/l) 280 sucrose, 10 HEPES, 1 EDTA, and 1 EGTA, pH 7.2. Atrial tissue was trimmed away and the heart gently placed in agarose (1%, 42°C), which solidified after cooling.

Fluorescent microspheres: application and imaging. In a first set of experiments in the open-chest dog, yellow-green (Molecular Probes), orange-high, and violet-high (Stason IMT) and crimson (Molecular Probes) microspheres were applied in 20-min intervals. After cardiac excision, transversal (short axis) myocardial slices (~4 mm thickness) were obtained, and microsphere deposition was imaged with the use of a fluorescence stereomicroscope (model MZ FLIII, Leica Microsystems) and a green fluorescent protein filter.

In a second set of experiments, yellow, red, and scarlet microspheres (Molecular Probes) were applied in 40-min intervals, and microsphere deposition was imaged using a commercially available imaging cryomicrotome (Barlow Scientific; Olympia, WA) (5, 14). In brief, the microtome sectioned through the frozen heart at a selected slice thickness of 30–40 μm. Fluorescent images of the newly exposed myocardial surface were digitally obtained with appropriate excitation and emission filters to isolate each of up to four different fluorescent colors. At a 2,000 × 2,000 pixel resolution, the chosen field of view resulted in an individual pixel size of ~30 × 30 μm². As described in detail below (5), software analysis of the acquired images described a list of all microsphere-containing voxels with their x- and y-coordinates according to the respective pixel size and z-coordinates according to the respective slice thickness. Because the pixel size exceeded the size of an individual microsphere, the algorithm em...
ployed would have been unable to identify clustering or clumping of microspheres within a given voxel (which might include in a worst-case scenario up to 8 microspheres, based on the relative sizes of the spheres and the voxels).

In the experiment studying microsphere distribution in the awake animal, yellow microspheres (Molecular Probes) were applied on day 1, red and scarlet microspheres simultaneously on day 2, and green microspheres on day 3. Finally, in the experiments focused on regional elevation of flow by adenosine, red and scarlet microspheres were applied sequentially during adenosine, whereas yellow and green microspheres were applied before and after adenosine. Imaging was carried out as described above.

**Analysis of microsphere deposition pattern.** To describe in a quantitative manner the pattern of microsphere deposition, a program was developed in IDL (Interactive Data Language, version 5.5, Research Systems; Boulder, CO). This program calculated for each individual microsphere containing voxel (identified by x-, y-, and z-coordinates from the image analysis described above) in a defined range of slices or volumes the distance to its nearest neighbor of a different color (intercolor distance) or of the identical color (intracolor distance). The frequencies of all individual next neighbor distances were thereafter displayed as a histogram with the minimal voxel dimension (slice thickness) as bin size.

A random distribution of microspheres in space at a defined density was simulated with the use of the random number generator routine of IDL 5.5. The density of these virtual microspheres was taken from the individual experimental observations. It was thus possible to compare the experimentally observed distance distribution (see above) with a randomly generated microsphere localization pattern of an identical microsphere density.

**Iron oxide microsphere MRI.** In a clinical 1.5-T MR scanner (Sonata, Siemens; Erlangen, Germany), cardiac pacing (120 beats/min) and ventilation (60 breaths/min) were synchronized to avoid movement artifacts. After localizers and a two-dimensional (2-D) flash cine imaging sequence, end-systolic T2-weighted control images were obtained by a 2-D gradient echo GRE sequence with dark blood preparation. The imaging parameters for the sequence were 128 × 128 mm² field of view, 128 × 128 acquisition matrix, 2 mm slice thickness, and 1 k-space line/heart beat. The images were obtained at an echo time of 5, 10, 15, 20, and 25 ms in a single slice. As described above, the animal was withdrawn from the scanner before application of iron oxide microspheres. It was returned to its original position, before a second set of images was obtained.

After cardiac excision, higher-resolution images of iron oxide microsphere deposition patterns were obtained from the heart placed in agar. With the use of a three-dimensional (3-D) version of essentially the same sequence as before, the acquisition matrix was increased to 256 × 256 and the slice thickness reduced to 0.5 mm, resulting in an isotropic voxel size of $0.5 \times 0.5 \times 0.5$ mm³. Repetition time was increased to 1 s. To cover the whole ventricular myocardium in a true 3-D mode, 96 contiguous slices were obtained per heart.

**Chemicals.** Fluorescent microspheres (15 μm) were obtained from Molecular Probes (Eugene, OR) and IMT Stason (Irvine, CA). Iron oxide microspheres (15 μm, ~0.5 ng iron oxide/microsphere) were also obtained from IMT Stason. All other chemicals employed were of analytic grade.

**Statistics.** The arrays describing the intra- and intercolor microsphere distances (see *Analysis of microsphere deposition pattern*) were statistically analyzed with the use of statistical software (version 11.0.1, SPSS, Chicago, IL: www.spss.com), calculating both mean and median. Also, the mode of each distribution of minimal distances binned by the smallest voxel dimension was identified as well as the second mode in case of a bimodal distribution.

**RESULTS**

**Fluorescence microsphere imaging.** When batches of different-colored fluorescent microspheres were applied sequentially, microscopic epifluorescence imaging of myocardial slices revealed a nonrandom pattern of microsphere deposition (Fig. 1). In nine myocardial slices from three dogs, a close proximity among microspheres applied sequentially in 20-min intervals (e.g., red, yellow, and green) was seen in almost every single field of view. The observation of different microspheres aligned apparently within a single vessel (Fig. 1, a–c) contradicted the commonly held assumption that the first microsphere extracted would practically block perfusion in the vessel affected. It also exceeded by far the expected probability of three microspheres arriving sequentially at the same capillary inflow point ($8 \times 10^{-6}$), given the high myocardial capillary density (~3,000 mm⁻²) and low number of microspheres per tissue volume (2.5 mm⁻³) (for details, see DISCUSSION).

To analyze this unexpected microsphere deposition behavior in a more quantitative manner, different-colored microspheres were again applied sequentially in 40-min intervals ($n = 4$ hearts) while systemic hemodynamics remained stable. From the beginning until the end of this 80-min period, aortic end-diastolic pressure (57 ± 12 vs. 58 ± 12 mmHg) and peak systolic pressure (89 ± 27 vs. 81 ± 21 mmHg) as well as heart rate (90 ± 11 vs. 104 ± 12 beats/min) did not change. After cardiac excision, transverse slices ~31 μm thick were removed sequentially from base to apex. Fluorescent images were obtained of the sequentially exposed surfaces for each

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**Fig. 1.** Epifluorescence images of different-colored 15-μm microspheres in myocardial tissue slices. Overview acquired at a ×40 optical magnification and zoomed in examples of close microsphere proximity from different field of views (a–c) acquired at a ×100 optical magnification are shown.
fluorescent color, resulting in a set of 1,200 images per heart and color. Representative images from one heart are presented in Fig. 2. Each panel demonstrates the fluorescence of one microsphere color only and displays several characteristic features. First, the deposition pattern on a qualitative level clearly appears to be nonrandom. Second, clustering of microspheres (highlighted by circles) is readily apparent, which may even take on the form of streak-like patterns. Third, contiguous spaces devoid of microspheres are present that may extend to several millimeters (highlighted by arrows).

Because the microsphere deposition pattern might have been influenced by spatial differences in the capillary and arteriolar density in the beagle heart, a morphometric analysis was performed. The capillary volume fraction in myocardial cross-sections from 12 low- and high-flow samples was 9.44 ± 1.31%, the low coefficient of variation (0.14), indicating a homogenous distribution of capillary density (3,000 mm⁻²), consistent with a previous report from our group (25). In contrast, the arteriolar density in the same samples as estimated by light microscopy (57 ± 34 mm⁻²) displayed a much greater spatial heterogeneity (CV 0.59).

Because the high capillary density, the arteriolar branching pattern (13), and the low number of spheres applied were known, the similarity of the deposition pattern of sequentially applied microspheres was surprising (Fig. 2). In Fig. 2, each panel represents the deposition of one fluorescent batch of microspheres, where the yellow microspheres had been applied first, followed by the red and scarlet microspheres 40 and 80 min later, respectively. It is readily apparent that areas devoid of microspheres after the first application remained frequently devoid of spheres also after the second and third application (see arrows). Also, areas receiving a number of spheres, a cluster, on the first application, frequently also received several spheres on the second or third application, with the local pattern remaining constant (see circles). This stable deposition pattern on subsequent microsphere applications corresponded well with the observation of close proximity of sequentially applied microspheres as seen in Fig. 1. Please note that the microspheres imaged were approximately within 200 μm depth from the myocardial slice surface.

**Quantitative analysis of fluorescence microsphere deposition pattern.** To verify the qualitative impression of close proximity of sequentially applied microspheres and of a non-random deposition pattern, the position (in x-, y-, and z-coordinates) of each MS containing voxel in the heart was determined at a 31 × 30 × 30 μm³ resolution. This was made possible by the many high-resolution images at a small slice thickness, which enabled the application of a software algorithm that identified the voxel position (see METHODS). It was thus possible to calculate the distance of a given reference microsphere to its nearest neighbor of a different or the same color, enabling a thorough quantitative description of the noted microsphere clustering. This was done for all microspheres in all hearts, and the results expressed in frequency histograms as shown in Fig. 3.

When analyzing the distance between, e.g., the yellow and red microspheres in myocardial tissue, i.e., the extracted microspheres from the first and second batch applied, a striking behavior was seen. Consistent with the similarity in the microsphere deposition patterns seen in Fig. 2, the distance between, e.g., an index red MS containing voxel and its nearest yellow neighbor was frequently very small and sometimes the sequentially applied microspheres were even seen in the same tissue voxel. As shown in Fig. 3 for a representative heart, the...
The intercolor distance, i.e., two maxima in the intercolor frequency histogram clearly revealed a bimodal distribution of microspheres below mode 1 was also determined.

Table 1. Bimodal intercolor distance distributions of sequentially applied microspheres

<table>
<thead>
<tr>
<th>Heart 1</th>
<th>Mode 1, µm</th>
<th>Fraction below Mode 1, %</th>
<th>Mode 2, µm</th>
<th>Median, µm</th>
<th>Mean, µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red-Y1</td>
<td>45</td>
<td>3.3</td>
<td>438</td>
<td>452</td>
<td>487</td>
</tr>
<tr>
<td>Sc-Y1</td>
<td>76</td>
<td>7.4</td>
<td>438</td>
<td>497</td>
<td>531</td>
</tr>
<tr>
<td>Heart 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red-Y1</td>
<td>80</td>
<td>16.2</td>
<td>334</td>
<td>242</td>
<td>316</td>
</tr>
<tr>
<td>Sc-Y1</td>
<td>80</td>
<td>14.1</td>
<td>334</td>
<td>295</td>
<td>350</td>
</tr>
<tr>
<td>Heart 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red-Y1</td>
<td>80</td>
<td>11.7</td>
<td>429</td>
<td>323</td>
<td>381</td>
</tr>
<tr>
<td>Sc-Y1</td>
<td>111</td>
<td>19.9</td>
<td>492</td>
<td>350</td>
<td>404</td>
</tr>
<tr>
<td>Heart 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red-Y1</td>
<td>50</td>
<td>7.4</td>
<td>414</td>
<td>390</td>
<td>397</td>
</tr>
</tbody>
</table>

Sc, Scarlet; Yl, yellow. In each heart, the modes were determined based on distance histograms with the bin size given by the smallest voxel dimension (e.g. slice thickness, ~30 µm). The fraction of the total number of microspheres below mode 1 was also determined.

The unexpected deposition pattern was not only seen when studying the distances between sequentially applied microspheres but also within a given microsphere color (batch). For microspheres randomly distributed in space, the distance between an index microsphere and its nearest neighbor could be expected to be close to a Gaussian distribution. In fact, when calculating for eight randomly distributed sets of virtual in silico microspheres the nearest neighbor distances, mean, median, and the mode of the distributions were similar (516 ± 36, 509 ± 35, and 468 ± 58 µm), the skewness was very small (0.26 ± 0.018) and the kurtosis negligible (~0.046 ± 0.039), all very much consistent with a Gaussian normal distribution. In contrast, for eight empirical sets of microsphere containing voxels obtained from four hearts, the distribution was always positively skewed to the left, indicated by a skewness ranging from 0.65 ± 0.01 to 1.185 ± 0.01 (0.95 ± 0.21; P < 0.001 vs. random distribution) and the mode being smaller than median and mean (380 ± 79 vs. 436 ± 24 and 473 ± 20 µm). Because the in silico microspheres were matched to the deposition density of the observed spheres, it is also noteworthy that the randomly distributed microspheres displayed a significantly greater median distance (509 ± 35 vs. 436 ± 24 µm, P < 0.001) than the empirically observed, again reflecting the microsphere clustering.

To test whether the high temporal stability of the microsphere deposition pattern extended from tens of minutes to days, and to study, whether the anesthesia present in open-chest animals might interfere with the local flow and microsphere localization, one animal was chronically instrumented and microspheres were applied starting 9 days after catheter placement. Aortic end-diastolic and peak systolic pressure were 88 and 148 mmHg, respectively, and remained stable during the following 2 days (93 and 161 mmHg on day 3); heart rate varied from 89 to 124 beats/min. As is readily apparent from Fig. 4, again a high fraction of spheres was much closer to its nearest neighbor than expected from two randomly spaced sets of in silico microspheres. It is remarkable that the distance distribution of two batches of microspheres given on day 3 and 1 was almost identical to two batches given simultaneously on day 2 (Fig. 4). Thus the deposition pattern...
Fluorescence MS deposition pattern on local flow enhancement. When local myocardial perfusion was maximally increased due to adenosine infusion into the left anterior descending coronary artery (LAD) via a right LAD branch, a substantially higher fraction of the red spheres applied during adenosine infusion was deposited in the area affected compared with a yellow microsphere injection before adenosine (Fig. 5, compare left and right). The central panel depicts visually the area receiving the enhanced number of red microspheres during adenosine. Surprisingly, areas devoid of microspheres under basal conditions (arrows) received few microspheres during flow elevation, while areas displaying clusters of microspheres before, showed many more microspheres during adenosine (circles).

When analyzing only in the LAD-dependent region, i.e., the tissue receiving enhanced flow (microspheres) during adenosine, the minimal distance between voxels containing microspheres given before (yellow) and during adenosine (red), the frequency distribution was again shifted substantially to the left compared with that of microspheres randomly spaced at deposition densities identical to those observed experimentally in the LAD region. Not surprisingly, when analyzing the minimal distance in the whole slice, containing areas of basal and enhanced microsphere densities, a bimodal distribution was seen (gray lines). And, again, even the less dense population of microspheres demonstrated a minimal distance distribution shifted to the left compared with a random distribution of identical densities.

MR imaging of microsphere deposition. Because the deposition pattern of fluorescent microspheres can only be analyzed post mortem, we explored the possibility of MR visible microspheres for in vivo visualization of microsphere localization. Commercially available 15 μm iron oxide microspheres, containing ~0.5 ng iron oxide each, were applied to the left atrium. Preliminary agarose phantom experiments had indicated that an iron oxide concentration of 2 μg/ml was sufficient to substantially decrease the image intensity in a T2-weighted gradient echo imaging sequence. In consequence, a medium number of microspheres (e.g., 1,000 MS/g, i.e., ~0.5 μg FeO/g) was expected to be too low to significantly alter the imaging contrast in vivo and in vitro. This was confirmed by preliminary canine experiments. Therefore, the amount of microsphere applied was increased by a factor of four compared with fluorescent microspheres. This enabled the visualization of microsphere deposition in a myocardial slice at a voxel size of 2 μl, as demonstrated in Fig. 6. Whereas the top left panel depicts the slice before microsphere application in a T2-weighted flash image, after microsphere application a heterogeneous pattern of microsphere deposition was revealed. Areas apparently devoid of microspheres and streak-like features characterized this pattern, resembling the pattern seen with fluorescent microspheres before. After cardiac excision, in vitro imaging enabled the acquisition at a resolution of 125 nl (0.5 × 0.5 × 0.5 mm3). Two representative slices from 3-D data sets are displayed in the bottom two panels. Again, apparently microsphere-free areas and similar clustering features could be seen. In addition, a higher subendocardial microsphere density was observed.

DISCUSSION

On the basis of a high-resolution imaging approach, the present study demonstrates a spatial limitation of the microsphere method for local flow determinations not visualized before. Contrary to classic assumptions the deposition pattern of microspheres in myocardial tissue was not governed solely by flow and by stochastic laws. Epifluorescence microscopy revealed a nonrandom localization of fluorescent microspheres, supported by fluorescence imaging of whole myocardial slices and further corroborated by a quantitative analysis of individual microsphere localizations. The deposition pattern was characterized by microsphere clusters and voids that were spatially and temporally stable, as seen following sequentially applied microspheres. Local enhancement of perfusion increased the average microsphere density but did only moderately affect the local pattern. Finally, in vivo imaging of the deposition pattern of (iron oxide) microspheres using MRI also demonstrated a substantial heterogeneity of local microsphere deposition, similar to that seen with fluorescent microspheres before.

Nonrandom localization of microsphere deposition. The observed nonrandom localization is surprising, because an approximately random behavior of microspheres was an implicit assumption in all applications of microspheres for local flow measurements. In fact, microsphere deposition had even been formally treated as a mathematical problem that allowed statements regarding precision and distributions predominantly...
This volume element may be tissue volume element of 1 g. The total number of capillaries in apparent vessel (Fig. 1, applied microspheres were frequently observed within one IMS), voxel size 1 \times 1 \times 2 \text{mm}^3. Bottom, representative high-resolution (voxel size 0.5 \times 0.5 \times 0.5 \text{mm}^3) images obtained in vitro after myocardial excision and being embedded in agarose, without and with prior microsphere application (20 ms echo time each).

Already when applying one set of microspheres, the deposition pattern seen, e.g., in Figs. 2 and 6 was nonrandom, characterized by clusters, but also by large, microsphere-free voids (Figs. 2 and 5). This was also indicated by a distance distribution skewed to the left and by a mode being considerably smaller than the mean (see RESULTS). Several factors may contribute to this effect: A nonhomogeneous distribution of microsphere lodging sites, i.e., small (15 \text{m}) arterioles, which is indicated by our data on arteriolar density and consistent with recently published data (see, e.g., Ref. 24), may be one decisive factor. Also, arterio-venous fistulae in the microcirculation may contribute to an inhomogeneous distribution. Finally, spatial heterogeneity of perfusion will also contribute to this effect, although high-resolution measurements of microsphere deposition in canine hearts indicated a rather homogeneous pattern at the microcylinder scale (19).

What could be the reason for this nonrandom distribution? Already when applying one set of microspheres, the deposition pattern seen, e.g., in Figs. 2 and 6 was nonrandom, characterized by clusters, but also by large, microsphere-free voids (Figs. 2 and 5). This was also indicated by a distance distribution skewed to the left and by a mode being considerably smaller than the mean (see RESULTS). Several factors may contribute to this effect: A nonhomogeneous distribution of microsphere lodging sites, i.e., small (15 \text{m}) arterioles, which is indicated by our data on arteriolar density and consistent with recently published data (see, e.g., Ref. 24), may be one decisive factor. Also, arterio-venous fistulae in the microcirculation may contribute to an inhomogeneous distribution. Finally, spatial heterogeneity of perfusion will also contribute to this effect, although high-resolution measurements of microsphere deposition in canine hearts indicated a rather homogeneous pattern at the microcylinder scale (19).

However, given the small number of microspheres and the large number of potential lodging sites (see above), these factors do not explain why, after repeated applications, the microspheres finally lodge in the very same vessels. Because the transport characteristics of rigid 15 \text{m} spheres in the microcirculation will deviate more and more from that of plasma or erythrocytes, we suggest that at branching points of myocardial volume element as defined by the spatial resolution of the imaging cryomicrotome system and the subsequent analysis (Fig. 3).

Fig. 6. Iron oxide microsphere (IMS) deposition pattern as imaged by MRI. Top, \(T_2\)-weighted MR images obtained in vivo before (−IMS) and after application of iron oxide microspheres (+IMS), voxel size 1 \times 1 \times 2 \text{mm}^3. Bottom, representative high-resolution (voxel size 0.5 \times 0.5 \times 0.5 \text{mm}^3) images obtained in vitro after myocardial excision and being embedded in agarose, without and with prior microsphere application (20 ms echo time each).
high flow, ranging from 1 to 0.25% of left ventricular mass (3, 4), and are consistent with in vitro experiments demonstrating a bias of spherical particles at branch points toward the higher flow branch (7). Consequently, for small individual sample sizes, supplied by small arteriolar vessels, microspheres will not represent a valid means for flow determinations.

Spatial distribution and scale of perfusion and microsphere deposition pattern. In contrast, when perfusion of organs and large tissue regions is studied, all available evidence indicates the validity of the microsphere approach (22). The close correlation between plasma flow measured by molecular flow markers and the blood flow determined by microspheres in individual samples as small as 50 mg in rabbits and 250 mg in sheep and dogs (3, 4, 16), clearly supports the validity of local perfusion measurements by microspheres for samples as small as 0.25–1% of the left ventricular (LV) myocardial mass. Dividing the LV myocardium into 4, 8, 16, . . . 256 individual pieces revealed spatial variations of regional flows within the heart (spatial heterogeneity of flow) that could be described by a fractal pattern in the spatial range observed, with increasing flow heterogeneity at higher spatial resolution (2, 27). Individual LV samples displayed not only threefold differences in flow, but also similar differences in local glucose and fatty acid uptake and energy turnover (10) as well as substantial differences in local gene and protein expression (17). This supported the validity of the microsphere flow measurements in this spatial domain, and prompted the question for the smallest unit displaying a rather homogenous perfusion (“microvascular unit”). The results of the present study strongly suggest that well below a spatial scale of 50 μg (μl) microspheres will not be able to answer this question. In addition, while a fit to the observed relation between the sample size and flow heterogeneity appears to open up a seductive possibility to extrapolate the heterogeneity to smaller scales, it is most likely that this will overestimate the spatial heterogeneity present. When the spatial variability of perfusion with a molecular flow marker at a resolution <1 mm² in the rabbit heart is studied, only a very small flow heterogeneity (0.06 conduction velocity) was observed (18), much smaller than expected on the basis of microsphere measurements.

Limitations. For different reasons, the imaging techniques employed did not allow a precise measure of the real microsphere density in the tissue examined. When epifluorescence microscopy is used (see Fig. 1), the distance of an individual microsphere from the slice surface was difficult to estimate and varied between 0 and ~300 μm, influenced by the microsphere color and the optical properties of the tissue. No attempt was made to define a threshold necessary for counting microspheres. When the deposition pattern is analyzed with the use of the cryomicrotome imaging system (see Fig. 2), the resolution obtained in imaging the whole canine myocardial slice at the given pixel size exceeded the size of the individual microsphere. Consequently, two microspheres of the identical color being very close together will have been assigned a common microsphere localization. This does explain why the nearest neighbor of the same color was never observed at the distance range from 0 to 30 μm (data not shown) but could be easily seen when comparing two different colors (see Fig. 3). In fact, at the given resolution microsphere clustering even at a distance from 30–90 μm or even up to 120 μm for one color will have been systematically underestimated because under these circumstances it may not be the nearest neighbor, but rather the second- or third-nearest neighbor that is included in these statistics. Finally, when imaging iron oxide microspheres both in vivo and in vitro (see Fig. 6), both the size of the voxels imaged (2 μl and 125 nl) and the threshold observed in preliminary experiments made it impossible to reliably estimate the number of microspheres per voxel. In addition, because the average microsphere density had to exceed a certain threshold before the image intensity was affected, image areas of normal signal intensity cannot be expected to be devoid of microspheres.

In conclusion, the present study describes in detail the deposition pattern of microspheres. The unexpected findings of sequentially applied microspheres being extracted by one small arteriolar vessel of preferential channeling of microspheres to temporally stable cluster locations and of large microsphere-free voids indicate that at a high spatial resolution, the flow and deposition pattern of microspheres is not only governed by the spatial distribution of blood flow and the low probability of a given particle to end up in one of many precapillary vessels, but will also be influenced by the anatomic features of smaller segments of the arterial vascular tree. This has profound consequences for the spatial scale where microsphere can be a valid means to test the local perfusion. Previous studies supported the validity of microsphere flow determinations down to 200 mg in larger animals (3, 16) and 50 mg in smaller animals (4). The present data indicate that at a spatial scale <2 μg (μl) local flow may not be the dominant force governing microsphere deposition. Clearly, future studies are needed relating local flow to local microsphere deposition at the intermediate scales. To this end, the combination of MRI-based flow measurements (e.g., using Gd) and the application of the iron oxide microspheres introduced in this study may offer a promising avenue.

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