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Laboratory Investigation

Measurement of Regional Myocardial Blood Flow With Multiple Colored Microspheres*

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Background. **The use of radioactive microspheres (RM) for the measurement of regional myocardial blood flow (RMBF) is limited and inaccessible to many investigators due to radiation safety concerns and radioactive waste disposal problems. Therefore, a new method for the measurement of RMBF using colored microspheres (CM) was developed.**

Methods and Results. **Polystyrene spheres (diameter, 15±0.1 [SD] μm; density, 1.09 g/ml) were dyed with one of five colors. With the injection of CM into the left atrium or into a coronary perfusion line, RMBF and its distribution can be determined. CM are extracted from the myocardium and blood by digestion with potassium hydroxide and subsequent microfiltration. The dyes are then recovered from the CM within a defined volume of a solvent, and their concentrations are determined by spectrophotometry. The separation of composite absorbance spectra by spectrophotometry with the CM technique was as good as the separation of energy spectra by a γ-counter using the RM technique. Leaching of dye from the CM was less than 0.1% during a 2-month period in vitro. Significant leaching of dye from the microspheres also did not occur during 8 hours in the blood and myocardium of four anesthetized dogs in vivo. For further validation of this method, pairs of CM and RM (15.5±0.1 [SD] μm) were simultaneously injected under five different RMBF conditions (range, 0–10 ml/[min·g]) into the left anterior descending coronary artery of four anesthetized pigs, with coronary inflow as a flow reference, or into the left atrium of four anesthetized dogs using aortic blood withdrawal as a reference. The relation between RMBF determined by CM and RM was $CM=0.01+1.00 \cdot RM$ ($r=0.98$, $n=1,080$ data points) in the pigs, and $CM=-0.19+0.92 \cdot RM$ ($r=0.97$, $n=1,813$ data points) in the dogs.**

Conclusions. **Measurement of RMBF with CM yields values very similar to those of RM. Their use is less expensive and avoids all the disadvantages related to radioactivity, thus offering an alternative method for as many as five RMBF measurements in a single experiment. (*Circulation* 1991;83:974–982)**

In 1967, plastic, radioactively labeled microspheres (RM) were introduced for the measurement of regional perfusion.[1] One year later, Makowski et al[2] introduced the reference blood withdrawal technique for the quantification of regional blood flow. In 1969, Domenech et al[3] first validated the use of RM for the measurement of regional myocardial blood flow (RMBF). Thereafter, this method has become the standard technique for the measurement of RMBF in various experimental settings. However, because of the precautionary measures needed to minimize radiation exposure, use of RM is restricted to specially licensed laboratories. Storage of microspheres, as well as disposal of radioactive waste, is expensive and an environmental hazard.

To avoid some of these limitations inherent in the RM method, Hale et al[4] proposed a method for measuring RMBF with nonradioactive, colored microspheres (CM). According to this technique, samples are digested by a combination of enzymatic and chemical methods. Aliquots of the microspheres trapped within a given sample are then counted in a hemacytometer by an investigator using light microscopy. There are, however, significant limitations to

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this technique: 1) RMBF is extrapolated from only a small aliquot of the microspheres actually trapped within the sample, thereby entailing a substantial statistical error in RMBF calculations. 2) The use of only three different colors was validated in only a small number of samples, whereas it is clearly desirable to be able to make more than three measurements of RMBF in many experimental protocols. 3) There was a considerable variation in the diameter of the CM used in their study, as admitted by Hale et al.[4] 4) This method requires substantial time for the tedious counting of individual microspheres. 5) In preliminary experiments, we found it almost impossible to visually distinguish the nine commercially available microsphere colors in the reddish background of digested myocardium.

Recently, an alternative, nonradioactive method for measuring RMBF was developed by Morita et al[5] using x-ray fluorescence excitation of microspheres loaded with elements of high atomic number. So far, only two different labels were validated by comparison with RM after intracoronary injection in two dogs. This method could be hampered by leaching of the label from the microspheres over years. Another disadvantage is the need for sophisticated and relatively expensive equipment for x-ray excitation and fluorescence detection that is not commercially available.

To circumvent these limitations, we have developed a new method for measuring RMBF, featuring easy tissue processing and quantitative, automated counting of all CM within an individual sample. In nearly 3,000 individual myocardial samples, we validated this method by comparison with RM over a range of RMBF from 0 to 10 ml/(min · g).

Methods

Preparation of Colored Microspheres

Polystyrene microspheres (15±0.1 μm, mean±SD) (Dynospheres, Dyno Particles A · S, Lillestrøm, Norway) were purchased as a 10% aqueous suspension from Paesel GmbH & Co., Frankfurt, FRG. Five hundred microliters (approximately 28×10^6 microspheres) of the colorless microsphere suspension was put into a closed, nonairtight test tube. For purification, the microspheres were washed twice with 70% ethanol and vacuum dried. The microspheres were then dyed with one of five different colors: white (Blankophor MAR), yellow (Resolin–Brillant–Gelb 10GN 200%), violet (Resolin–Rot–Violett FBL 200%), and blue (Resolin–Brillant–Blau BGLN 200%) obtained from Bayer AG, Leverkusen, FRG; and red (Terasil Rot E-BST) obtained from CIBA–GEIGY AG, Wehr, FRG. For purification before use, the yellow and blue dyes were recrystallized in dichloromethane. For dyeing of red or violet microspheres, 400 mg dye, 2 ml double-distilled water, and 1 ml 1% aqueous emulsion of Levegal PTN (Bayer AG) were combined in the test tube containing the dried microspheres from 500 μl of the original suspension. For white and blue microspheres, 8 ml solvent–mixture of toluene–dimethylformamide (DMF) (85%/15% vol/vol), saturated with the dye at 80°C (white dye) or 100°C (blue dye), was added to the dried microspheres. For yellow microspheres, 100 mg dye and 1 ml 1,2,4-trichlorobenzene were added to the dried microspheres. These mixtures were then heated in a boiling water bath for 5 (yellow dye) or 90 (white, red, and violet dye) minutes or in a paraffin bath at 125°C for 90 minutes (blue dye). The white, yellow, and blue microspheres were recovered by filtration through polyester microfilters (pore size, 8 μm; diameter, 47 mm; Nuclepore, Tübingen, FRG). Any remaining free dye between the filtered microspheres (white, yellow, and blue) or the solution containing the red and violet microspheres was washed out with absolute ethanol. For that purpose, the microspheres were resuspended in absolute ethanol, ultrasonicated, centrifuged (3 minutes, 2,500*g*), and the supernatant was drawn off. The purified CM were vacuum dried, resuspended in isodense solution of glycerol/Tween 80/double-distilled water (25%/0.5%/79.5% vol), ultrasonicated, and divided into appropriate portions, that is, four portions containing approximately 7×10^6 CM each, for later use. CM were washed twice with 70% ethanol and vacuum dried before their final suspension in physiological saline solution for injection.

A photomicrograph of a mixture of CM of different colors is shown in Figure 1. The amount of the previously mentioned dyeing reagents can be extrapolated up to the production of 100×10^6 CM. A portion of dried microspheres of each color has been stored for more than 12 months without any effect on their usability.

To test the stability of dye attachment to the microspheres in vitro, we suspended 5×10^5 CM of each color in 2 ml saline and stored each for 2 months. When we analyzed the dye content in the supernatant after microfiltration of four 2-ml samples for each color, less than 0.1% of dye was recovered. To test the homogeneity of staining, we determined the difference in dye recovered from six aliquots containing 400–1,000 CM of each color and found the difference to be less than 1.5%.

The density of our CM is 1.09 g/ml as measured by density gradient centrifugation. To test for potential alterations in the size distribution by the chemical, mechanical, and thermic dyeing procedures, 2,000 microspheres dyed with different colors were examined by scanning electron microscopy. The small deviation from the average size of 15 μm, which was indicated by the manufacturer (15±0.1 μm, mean±SD), was confirmed. There were no microspheres smaller than 14 μm, and only eight of 2,000 were as large as 17 μm. In 100 RM (Du Pont NEN Products, Dreieich, FRG), which were used for comparison, the small deviation from the average size indicated by the manufacturer (15.5±0.1 μm, mean±SD) was also confirmed.

Quantification of Colored Microspheres

CM were quantified by their dye content. The dye was recovered from the microspheres by adding 100 μl

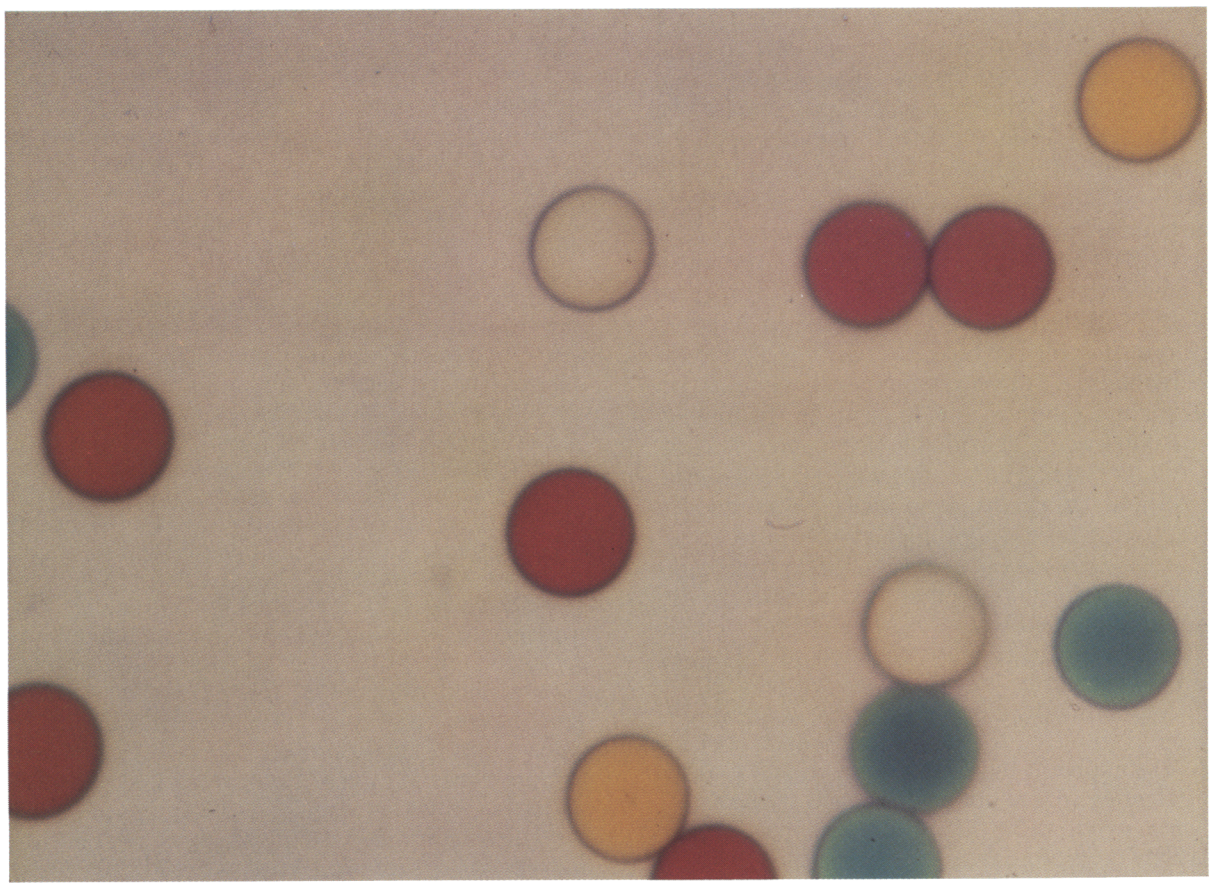

FIGURE 1. *Photomicrograph of a mixture of colored microspheres with five different colors at an original magnification of ×1,100.*

DMF as a solvent with a Hamilton syringe (Microlab P, Hamilton, Bonaduz, CH). To ensure complete recovery of the dye, we placed CM and DMF together into a conical centrifuge tube and vortex mixed the tube for 30 seconds. The tube was then centrifuged (5 minutes, 2,000*g*), and the dye solution was transferred into 0.3-ml glass tubes (JWGE 19165 Millipore, Eschborn, FRG). Last, the transferred dye solution was separated from remaining particles and microspheres by centrifugation (3 minutes, 2,000*g*) to minimize scatter in the subsequent spectrophotometry.

The completeness of dye removal from the microspheres was tested by adding increasing amounts of the DMF solvent from 50–800 μl in eight samples of dried CM and by exposing the CM to DMF for increasing durations from 20 seconds to 1 hour. Confirming the completeness of dye removal from the microspheres by 100 μl DMF for a few minutes (30 seconds of vortex agitation plus 3 minutes of centrifugation), no increase in dye removal occurred with increasing amounts of and exposure to DMF.

The photometric absorption of each dye solution was determined by a Diode-array UV/Visible Spectrophotometer (model 8452A, Hewlett–Packard Co., Palo Alto, Calif.) (wave length range, 190–820 nm with 2-nm optical band width). In a manner similar to the overlap correction in counting the RM, the composite spectrum (Figure 2A) of each dye solution was resolved into the spectra of the single constituents (Figure 2B) by a matrix inversion technique.[6] The absorption spectrum of each dye was measured separately and was used as a reference for the matrix inversion, determining the contribution of each color to the measured composite spectra at five fixed wavelengths: 370, 448, 530, 594, and 672 nm (arrows in Figure 2). Within 10 seconds, 100 single measurements of 0.1-second duration each were performed and averaged so that the standard deviation of each absorbance value was less than 0.1% of the mean. The amount of dye or the number of CM in a given sample was adjusted to achieve absorbance values of no more than 1.3 AU (absorbance unit, 1 AU = −lg [10% light transmission/100%]) and, thus, to ensure the linearity between absorbance and dye concentration according to the Lambert–Beer law. Samples with absorbances higher than 1.3 AU were diluted and analyzed again. The lower limit of detection that could be reliably distinguished from background noise was 0.05 AU.

To test the error of separation by spectrophotometry and matrix inversion resolution of composite spectra, we used an overdetermined set of linear equations as previously applied by Baer et al[7] to RM. Therefore, the absorbance of each color in the dye

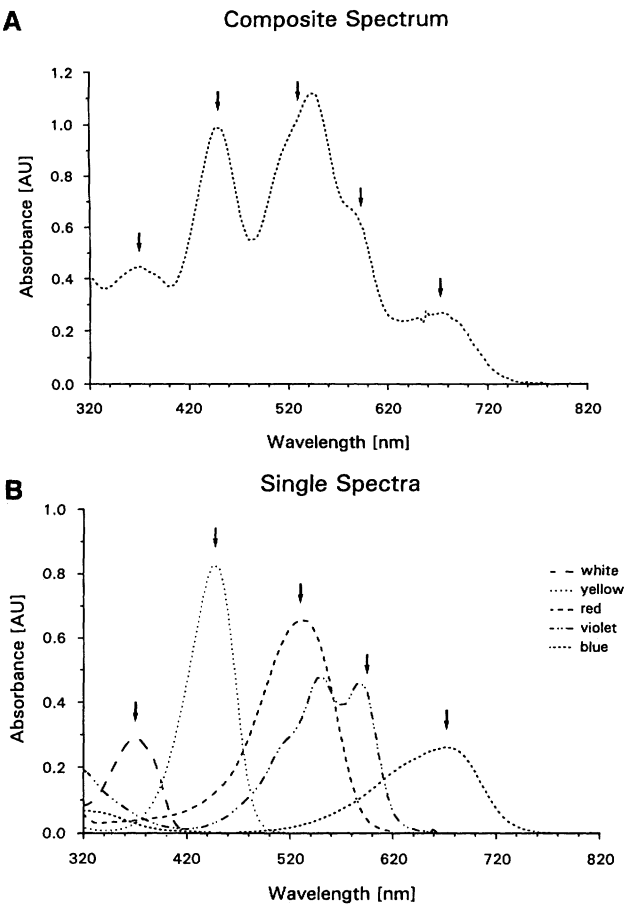

FIGURE 2. Panel A: *Composite spectrum of five different colors of the dye solution recovered from microspheres of an individual sample.* Panel B: *Single spectra of the five different colors contributing to the composite spectrum in panel A.*

solution was determined at three different wavelengths: at the normally used wavelength as described above and at 10 nm above and below this wavelength. A prerequisite for this approach is an equal variance of absorbance or a normalization with respect to the variance. Therefore, the square of the difference between the measured and the calculated absorbance values was weighted with the variance of 100 consecutive measurements. It must be realized that the variance of consecutive measurements of the same amount of absorbance by the CM technique is a variance different from that of increasing durations of counting radioactivity by the RM technique. Nevertheless, the goodness of fit or so-called χ^2 was determined in the same way as described by Baer et al[7] for RM.

To test potential interactions of different colors within a composite spectrum, six increasing concentrations of a fifth dye were added to a given four-color set. A linear regression analysis was performed between increasing absorbance of the added fifth color and the absorbance of the remaining four colors, using mean values of sixfold determinations for each added color.

To test the completeness of recovery of each dye by the matrix inversion resolution of a composite spectrum, the same set of data was used. The increase in total absorbance after addition of a fifth dye to a given four-color set was measured. After calibration of total absorbance within the spectral range with the absorbance value at the predetermined wavelength for each color separately, the measured increase in total absorbance after addition of a fifth color could be compared with the absorbance value attributed to this fifth color by matrix inversion resolution of the composite spectrum.

In Vivo Validation of Colored Microspheres

Animals used in this study were handled in accordance with the guidelines of the animal welfare regulations of the American Physiological Society, and the experimental protocol was approved by the Bioethical Committee of the District of Düsseldorf, FRG.

Protocol 1: Stability of dye attachment in vivo. Four beagle dogs (13–18 kg body weight) were anesthetized with thiamylal sodium (15 mg/kg) administered through a brachial vein. An endotracheal tube was positioned and connected to a respirator equipped with an enflurane vaporizer (Spiromat 650, Dräger, Essen, FRG). Anesthesia was maintained with enflurane (0.5–1.5%) with a mixture of nitrous oxide and oxygen, and ventilation was adjusted to keep P_{CO_2} between 35 and 40 mm Hg, P_{O_2} greater than 100 mm Hg, and pH within the normal range. One common carotid artery was cannulated with a large polyethylene catheter that served as the blood supply for an extracorporeal circuit. The other carotid artery was cannulated with a small polyethylene catheter for pressure measurement (type 4-327-I, Bell & Howell, Pasadena, Calif.) and blood sampling. An internal jugular vein was cannulated to return blood from the extracorporeal circuit before coronary cannulation, and a second jugular vein was cannulated for saline infusion.

A left lateral thoracotomy was performed in the fourth intercostal space, and the pericardium was opened and sutured to cradle the heart. A catheter-tipped manometer (type 501-869/2, Braun–Melsungen AG, FRG) was placed in the left ventricle through the right femoral artery for measurement of left ventricular pressure. The first derivative of left ventricular pressure (dP/dt) was calculated from digitized left ventricular pressure data using CORDAT software[8] on an AT-type computer.

Ultrasonic crystals were implanted in the anterior wall within the perfusion area of the left anterior descending coronary artery (LAD) to measure midwall segment shortening according to standard techniques[9] (System 6, Triton Technology, San Diego, Calif.). A second set of ultrasonic crystals was implanted in the posterolateral wall within the perfusion area of the left circumflex coronary artery. The proximal LAD was dissected free from surrounding tissue for a distance of approximately 1 cm. After the dogs were heparinized (20,000-IU bolus, 10,000 IU hourly), the LAD was ligated and rapidly cannulated.

Perfusion pressure was measured through a distal sidearm of the cannula. Blood was supplied by an extracorporeal circuit with two side ports: one for microsphere injection and one for regional drug infusion. The extracorporeal circuit included an occlusive pump (Masterflex, Cole–Parmer Instrument Co., Chicago), which was servocontrolled to regulate coronary pressure, and a 50-ml windkessel. The microsphere injection port was just distal to the pump and windkessel in the extracorporeal circuit, and a mixing chamber with a vortex mixer was positioned just distal to the injection port. Thorough mixing of microspheres with blood by this perfusion system has been documented previously.[10] Arterial and left ventricular pressures, coronary arterial pressure, coronary blood flow to the LAD, and segment length of the anterior and the posterolateral walls were recorded on an eight-channel recorder (model MK 200 A, Statham–Gould, Oxnard, Calif.) and on-line converted to digital data for subsequent analysis using the CORDAT software.

To test for a potential leaching of the dyes from the microspheres in blood or tissue, we compared CM dissolved in saline and immediately processed with CM injected into and kept in blood or myocardium. Previously portioned and dried CM were resuspended in 9 ml saline containing 0.02% Tween 80 and ultrasonicated for 1 minute. Before their injection, CM were thoroughly dispersed by vortex mixing, and 1.5 ml of this suspension was drawn up with an automatic Hamilton syringe. As indicated by the manufacturer, this syringe can reproducibly draw up the same volume of a fluid with an error of less than 0.3%. Identical (within this range of accuracy) amounts of CM were then injected: 1) through a saline-filled copy of the perfusion system from the microsphere injection port to the cannula tip, and the perfusion system was flushed with 50 ml saline, 2) through another copy of the perfusion system filled with freshly withdrawn heparinized blood, and the perfusion system was flushed with 50 ml blood, and 3) into the above-described coronary perfusion line of four anesthetized dogs. The number of injected CM varied among 75,000 (red), 330,000 (white), 400,000 (violet), 500,000 (yellow), and 550,000 (blue) to account for their different absorbance characteristics. The saline and blood perfusates were collected in Teflon-sealed 16-ml screw-cap glass tubes (Schott, Mainz, FRG) in 3.5-ml portions. The saline samples were immediately processed and served as the 100% reference. The blood samples were stored for 8 hours before further processing. The four dogs were kept in stable hemodynamic condition for 8 hours. Before euthanization by potassium chloride injection, the LAD-perfused myocardium was delineated by methylene blue injection into the perfusion line. The entire delineated area was then removed and further processed.

Protocol 2: Comparison of CM to RM after intracoronary injection. Four swine (25–35 kg body weight) were initially sedated with ketamine hydrochloride (30 mg/kg i.m.) and then anesthetized with thiamylal sodium (250–500 mg) administered through an ear vein. A tracheostomy was performed through a midline cervical incision, and an endotracheal tube was positioned and connected to a respirator equipped with an enflurane vaporizer. Anesthesia was maintained as described above. The further instrumentation was also identical to that described above for the four beagle dogs, except that transmural wall thickening was measured instead of midwall segment shortening.

Protocol 3: Comparison of CM to RM after left atrial injection. Four beagle dogs (15–18 kg body weight) were anesthetized and instrumented as described above. However, in these four dogs, the LAD was not cannulated for perfusion, and coronary arterial inflow was measured with an electromagnetic flow probe (Statham–Gould). Microspheres were injected through a Teflon catheter into the left atrium. A second Teflon catheter was inserted into the descending aorta for withdrawal of reference blood samples.

RMBF distribution in protocols 2 and 3 was measured using 15.5 ± 0.1-μm RM labeled with one of the following radionuclides: cerium-141, chromium-51, ruthenium-103, niobium-95, or scandium-46 simultaneously with one of the CM. For each intracoronary injection in the four pigs, approximately 130,000 RM were injected. The number of CM injected varied among 80,000 (red), 300,000 (yellow), 400,000 (violet), and 500,000 (white and blue) to account for the different absorbance characteristics of the CM. On the day of the experiment, the previously portioned and dried CM were resuspended in 3 ml saline (containing 0.02% Tween 80) for intracoronary injection and 6 ml saline (containing 0.02% Tween 80) for intra-atrial injection and were ultrasonicated for 1 minute. Before injection, RM suspended in saline were thoroughly dispersed by vortex mixing and were then added to the vial containing the CM, and the mixture was vortex agitated again for an additional minute. Each microsphere injection was followed by a flush of saline solution. For each measurement in the four dogs, approximately 3×10^6 RM and 1.8×10^6 (red) to 7×10^6 (white, yellow, violet, and blue) CM were injected. After occlusion of the LAD, more colored spheres (20×10^6) were injected to improve the precision of RMBF measurements in this low-flow situation. Stable steady-state conditions of systemic hemodynamics, regional myocardial function, and coronary blood flow before and during microsphere infusion and the withdrawal period were ensured under each condition in all experiments. In the dog studies with left atrial injection, reference arterial blood samples were collected through the aortic catheter starting 10 seconds before injection of the microspheres and continuing for 100 seconds at a rate of 6.3 ml/min.

Five different myocardial blood flow conditions were produced for the pigs and dogs. During steady-state conditions, one pair of colored and randomly matched RM was simultaneously injected.

In the four pigs, the first RMBF measurement was obtained under baseline conditions. LAD blood flow

was then increased in three steps by intracoronary adenosine infusion (100–400 μg/min) at a constant coronary perfusion pressure, and measurements were again obtained at stable steady states of elevated flow. Last, coronary perfusion pressure was reduced to produce regional myocardial akinesia, and a last measurement was obtained during steady-state ischemia.

In the four dogs, after control measurements, coronary blood flow was increased in three steps by intravenous infusion of dipyridamole (0.1–0.3 mg/kg) and epinephrine (0.5–3 μg/[kg · min]), and measurements were obtained at the respective steady states. A final measurement was begun after 3 minutes of complete occlusion of the LAD during steady-state regional myocardial dysfunction.

After completion of the study, animals were euthanized. The hearts were removed and carefully dissected free from epicardial fat. In the four dogs of protocol 1 and in the four pigs of protocol 2, the LAD-perfused myocardium was delineated by methylene blue injection into the perfusion line before euthanization. The stained myocardium was cut into samples of 0.3–1.8 g (mean, 1.1 g), and these were placed into Teflon-sealed 16-ml screw-cap glass tubes (Schott). The mean number of samples per animal was 54 in the four pigs of protocol 2 and 91 in the four dogs of protocol 3. The left ventricular samples were divided into transmural thirds. In the four dogs of protocol 3, both ventricles and atria were processed in the same way. After counting gamma radioactivity in a 3-in. NaI (Tl) well detector (model BF5300, Berthold, Wildbad, FRG) for 1 minute with a multichannel pulse-height analyzer, the samples were processed for CM quantification.

Seven milliliters of a 4 M KOH solution containing 2% Tween 80 was added to each sample for digestion of the tissue. The glass tubes were closed, placed in a water bath shaker for 4 hours at 72°C, and hand mixed every hour. A high-grade steel vacuum filtration chamber was fitted to a polyester filter (pore size, 8 μm; diameter, 25 mm; Nuclepore). The digested tissue solution was mixed with a Teflon-coated magnetic stirring bar and then placed on the filter with a burette, while the filter was rinsed with 2% Tween 80 solution. To avoid a loss of microspheres, the test tube and burette were rinsed twice with 2% Tween 80 solution, which was then filtered, as was the tissue solution.

The reference blood samples were processed in the same manner as the tissue samples. Each blood sample and 2% Tween 80 washing fluid (7 ml twice) of the blood withdrawal syringe were divided into 3.5-ml portions for the counting of RM. For subsequent digestion, 1.1 ml 16 M KOH and 0.5 ml 20% Tween 80 solution were added. Thus, the KOH and Tween 80 concentrations were the same as in the tissue samples. The further preparation of the blood samples was not different from that of the tissue samples.

For use in the calculation of RMBF in the pigs (protocol 2), the radioactivity and dye content of the entire perfusion area were determined. Hence, in the four pigs, RMBF, corrected for wet weight, was calculated with the equation: (X per sample divided by blood flow to sample) = (total tissue X divided by coronary inflow), where X is the number of counts per minute for the RM or the specific absorbance for the CM.[10] The total tissue radioactivity content was determined by totaling all counts from the perfusion bed, whereas the amount of dye injected was determined by totaling the absorbance of all samples. Coronary inflow was set by the extracorporeal coronary perfusion circuit. In the dogs (protocol 3), RMBF, corrected for wet weight of the tissue, was calculated with standard techniques[2]: (X per sample divided by blood flow to sample) = (X in arterial withdrawal divided by reference withdrawal rate).

Comparisons With Other Microsphere RMBF Measurement Techniques

Time requirements. For all RMBF techniques, cutting and weighing of tissue samples is necessary. With our technique, the further processing of an individual tissue or blood sample requires approximately 8–10 minutes and an additional 3 minutes for the spectrophotometric measurement. For all samples together, an additional 4 hours are necessary for the digestion procedure. With the technique proposed by Hale et al,[4] processing of an individual sample requires 45 minutes. However, the time for microscopic counting of an aliquot of microspheres in a hemacytometer was not mentioned. With the technique proposed by Morita et al,[5] tissue samples have to be further cut to achieve a homogeneous distribution within the irradiated volume of the vial, and blood samples have to be further processed and suspended in gelatin for a homogeneous distribution of microspheres in the vial. The measurement, as such, requires an additional 10 minutes per sample.

Costs. The costs for the measurement of RMBF under five different flow conditions in a 30-kg dog, including preparation and dyeing of our CM as well as tissue and blood processing of 100 samples, amount to approximately $150. No special and expensive disposal of carcasses is necessary. Shelf life of our CM is not limited as is that of RM, which individually are subject to different half-life characteristics. Such differential decay times complicate inventory keeping and cause additional costs. Measurement of only four flow situations and processing of only 10 tissue samples and four blood samples cost $350 with the method proposed by Hale et al.[4] With RM from Du Pont NEN Products, five flow measurements also cost approximately $350. Information about the costs of microspheres by the technique of Morita et al[5] is not available.

Equipment. Laboratory equipment for microsphere dyeing and sample processing (centrifuge, ultrasonicator, vortex mixer, magnetic stirrer, shaking water bath, and glassware) costs approximately $15,000. However, most of this equipment is already possessed by most laboratories. The cost of the counting unit is approximately $15,000 for the spectrophotom-

TABLE 1. Interactions for Five-Color Set

	Average slope				
Color added	White	Yellow	Red	Violet	Blue
White		−0.41	−0.05	−0.05	−0.18
Yellow	−2.7		−0.99	−0.22	−0.76
Red	−2.3	−0.93		−0.18	−0.42
Violet	−1.2	−0.93	−0.59		−0.16
Blue	−0.16	−0.72	−0.71	−0.59	

Values represent 100 times the slope of the regression equation. Average slope calculated by (absorbance other color/absorbance added color)×100.

eter and approximately $40,000 for a sodium iodide gamma counter including an autosampler. For the technique proposed by Hale et al,[4] a light microscope and a hemacytometer are required, again in addition to standard laboratory equipment (centrifuge, ultrasonicator, vortex mixer, water bath, and glassware). The equipment for x-ray fluorescence excitation and detection according to the technique of Morita et al[5] is estimated to cost approximately $120,000.

Data Analysis

The regression analyses were based on a least-squares fitting method using the commercial software program SYSTAT on an IBM AT. To test the hypothesis that measurement of RMBF would be identical with RM and CM, slope and intercept were compared to the line of identity (intercept=0, slope=1) by two-tailed *F* tests.

Results

In Vitro Studies

The interaction of single colors within a composite spectrum was negligible, as evidenced by minimal changes in the absorbance of a given four-color set by adding increasing concentrations of a fifth color (Table 1).

The recovery of dye by matrix inversion resolution of composite spectra was almost complete because slopes of regression lines between the real and the calculated absorbance values were within 5% of the line of identity (Table 2).

The χ^2 value as an indicator of the goodness of separation of colors by spectrophotometry and matrix inversion was between 50 and 200, and the variation coefficient determined for each calculated absorbance value by use of the respective χ^2 was less than 1% for each color.

TABLE 2. Recoveries for Five-Color Set

Color added	Slope	Variation coefficient (%)
White	0.957	3.636
Yellow	1.028	3.346
Red	1.021	1.525
Violet	1.009	1.287
Blue	0.984	1.513

All correlation coefficients were greater than 0.998.

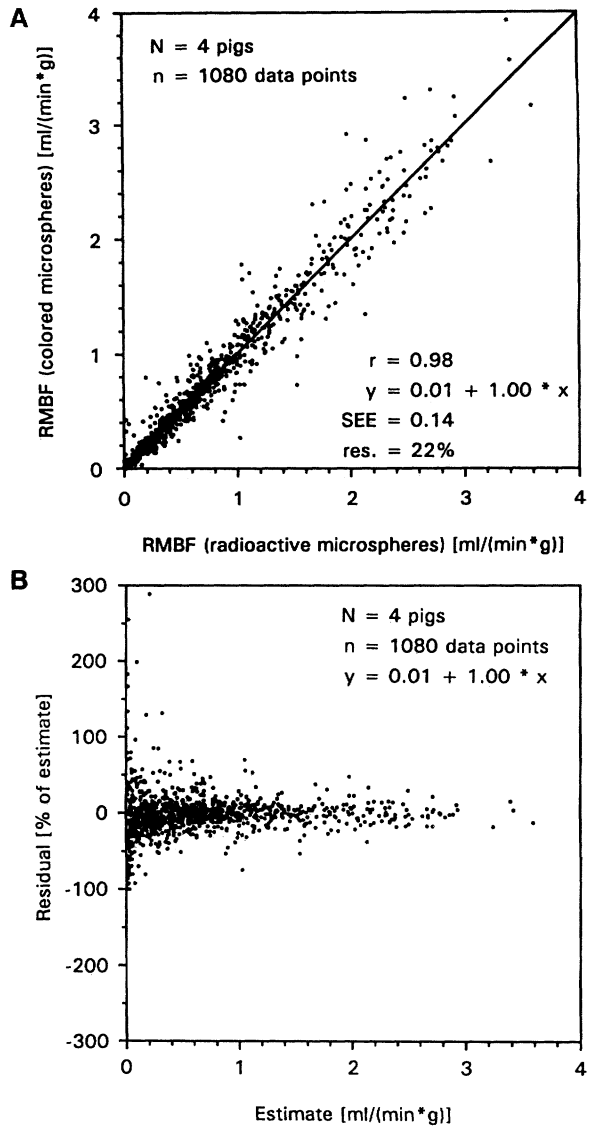

FIGURE 3. Panel A: *Regression plot of all regional myocardial blood flow (RMBF) values (n=1,080) measured by radioactive and colored microspheres during systematic flow variations in four pigs by intracoronary microsphere injection. Each point represents one tissue sample. SEE, standard error of the estimate; res., mean percentage of the residuals.* Panel B: *Plot of percentage residuals versus estimated flow for all data points of panel A.*

In Vivo Studies

A significant leaching of dye from microspheres either injected into blood ex vivo or trapped in myocardium in vivo during an 8-hour period did not occur for any color compared with the immediately processed microspheres exposed to saline only (Table 3).

In the pigs, in 1,080 samples, RMBF measured by intracoronary injection of RM and CM was almost identical. The linear regression was not different from identity (Figure 3). Subdividing the regression

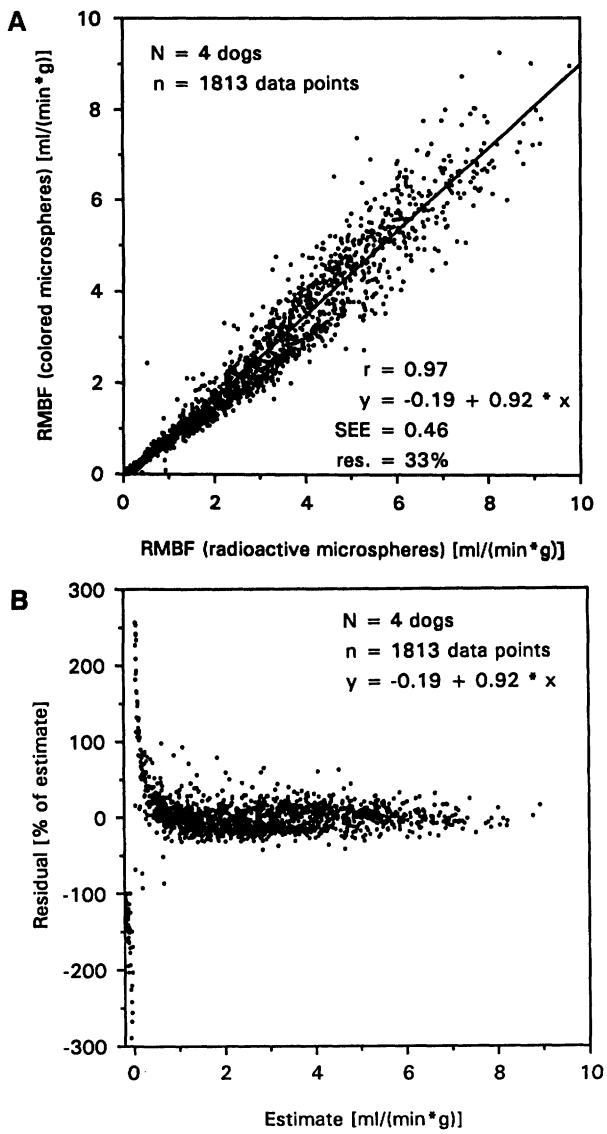

FIGURE 4. Panel A: *Regression plot of all regional myocardial blood flow (RMBF) values (n=1,813) measured by radioactive and colored microspheres during systematic flow variations in four dogs by left atrial microsphere injection. Each point represents one tissue sample. SEE, standard error of the estimate; res., mean percentage of the residuals.* Panel B: *Plot of percentage residuals versus estimated flow for all data points of panel A.*

TABLE 3. Stability of Dye Attachment In Vivo

	Recovered dye (%)		
Color	Saline	Blood	Myocardium
White	100	100.6±0.5	99.6±0.8
Yellow	100	99.2±1.1	99.2±0.8
Red	100	100.5±0.7	99.5±1.4
Violet	100	98.8±1.3	100.5±1.8
Blue	100	99.3±1.3	98.5±2.7

Values are mean±SD from four dog experiments.
Dye recovered after saline perfusion was set 100%.

TABLE 4. Regression Data for Individual Animals

Animal	Slope	Intercept	*r*	SEE
Pig 1	0.99	0.00	0.99	0.08
Pig 2	0.98	0.01	0.98	0.11
Pig 3	0.96	0.05	0.98	0.17
Pig 4	1.07	−0.02	0.98	0.15
Dog 1	0.81	−0.01	0.97	0.24
Dog 2	0.86	−0.17	0.99	0.31
Dog 3	0.90	−0.24	0.96	0.46
Dog 4	1.03	−0.22	0.98	0.43

SEE, standard error of the estimate.

into low-to-normal (<1.1 ml/[min · g]) and normal-to-high flows (≥1.1 ml/[min · g]) revealed no differences in either slopes or intercepts.

In the dogs, in 1,813 samples, RMBF measured by intra-atrial injection of RM and CM correlated closely. The correlations for individual animals are presented in Table 4. RMBF determined with CM was systematically somewhat lower, as indicated by the slope of the regression line (Figure 4). Subdividing the regression into low-to-normal (<1.1 ml/[min · g]) and normal-to-high flows (≥1.1 ml/[min · g]) revealed a lower slope for lower flows (0.78) and a higher slope for higher flows (0.94).

Discussion

This study demonstrates that RMBF can be accurately measured with a cost-effective, nonradioactive technique that currently allows up to five measurements in a single experimental preparation.

In the in vitro studies, our CM technique proved to be as accurate as the established RM technique. The size distribution of our CM is highly uniform, whereas the size of the CM used by Hale et al[4] varied in diameter from 3.3 to 29.2 μm. The density of our CM is 1.09 g/ml, thus closely approximating the density of erythrocytes. The density of the CM used by Hale et al[4] is somewhat lower (1.05 g/ml), whereas the density of RM is 1.3 g/ml, and the density of the non-RM proposed by Morita et al[5] is even greater than 2 g/ml. A significant leaching of the label from the microspheres did not occur with our CM. The separation characteristics by use of spectrophotometry and matrix inversion resolution of composite spectra revealed almost complete recovery of each dye and only minimal interaction between different dyes of a composite spectrum (Tables 1 and 2). Also, the variation coefficients calculated from χ^2 values by use of an overdetermined set of linear equations as previously applied by Baer et al[7] to RM were less than 1%.

In the in vivo studies, no significant leaching of the dyes from the microspheres occurred during an 8-hour period in blood or in myocardium. The small deviation from the saline reference (Table 3) is well within the accuracy of pipetting multiple, identical aliquots or the shunting of microspheres from myocardium.[11,12]

During the in vivo comparison of CM and RM, higher numbers of CM than of RM were used for measuring RMBF. However, left atrial injection of 13×10^6 microspheres[13] or serial injections of up to 48×10^6 microspheres[7] have been reported to be without significant effects on systemic hemodynamics. Thus, the injection of this many CM obviously did not result in a critical embolization of the coronary vasculature in our experiments. That would have been indicated by a deterioration of regional myocardial function, which correlates closely with myocardial perfusion under ischemic conditions.[14]

After intracoronary administration, there was no difference in RMBF measured by CM or RM. However, after intra-atrial injection, RMBF calculated from RM was slightly higher than that determined with CM. Whether this difference between RMBF measurements by CM and RM reflects an additional statistical error by the additional analysis of reference blood samples or represents a systematic error introduced by the reference blood withdrawal as such cannot be distinguished. Fewer RM than CM being sampled in the reference blood withdrawal relative to the myocardium would result in higher RMBF calculated from RM than from CM. However, a preferential streaming of RM with the direction of higher flow, for example, aorta versus the withdrawal syringe, has been documented for microspheres of larger diameter[15,16] but not for microspheres of equal diameter and different density.[17] In any event, the density of our CM is closer to that of erythrocytes, and the difference between RMBF measurements by CM and RM is small.

In conclusion, measurement of RMBF with our new technique with CM yields values very similar to those yielded by RM. Our technique avoids all problems related to radioactivity and is cost effective. To avoid radioactivity, more time for dyeing of microspheres and for tissue and blood sample processing is required, that is, about 8 hours for preparing a batch of 100×10^6 CM and about 20 hours for processing of 100 samples. Potentially, this time can be reduced by the future use of an autosampler in conjunction with the spectrophotometer. An extension for use of more colors is also conceivable. This technique enables laboratories without access to RM to measure RMBF and enables others to perform more sequential measurements.

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