

Fluorescent microspheres to measure organ perfusion: validation of a simplified sample processing technique

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Fluorescent microspheres to measure organ perfusion: validation of a simplified sample processing technique. *Am. J. Physiol.* 269 (Heart Circ. Physiol. 38): H725–H733, 1995.—A disadvantage of nonradioactive microsphere techniques is that the processing of samples is time-consuming and complex. We developed and validated a simplified processing method for the fluorescent microsphere (FM) technique. In seven anesthetized dogs with coronary artery stenosis up to six different FM and five different radioactively labeled microspheres (RM) were injected. Two FM and two RM labels were injected simultaneously to enable inter- and intramethod comparison. After gamma-counting samples of blood, myocardium ($n = 168$), and other organs ($n = 59$) were digested in test tubes with 2 N ethanolic KOH (60°C, 48 h), microspheres were sedimentated by centrifugation, dye was extracted in the same tube, and fluorescence was measured. With this processing method, recovery of FM was $\sim 100\%$. Good correlations for inter- and intramethod comparisons were found [$r = 0.985 \pm 0.01$ (mean \pm SD)]. The lower intermethod correlation for blue microspheres ($r = 0.958$) indicates that the use of this label is less desirable. RM and FM endocardial-to-epicardial blood flow ratios correlated well ($r = 0.974$). With this one-vessel centrifugal sedimentation method and at least five fluorescently labeled microspheres, blood flow can be reliably measured in various organs, including ischemic myocardium.

coronary blood flow; myocardial ischemia; radioactive microspheres; tissue digestion; microsphere isolation

RADIOACTIVE MICROSPHERES are used to measure regional organ blood flow. This method has been validated in many studies (for reviews see Refs. 8 and 14) and is currently regarded as a “gold standard” for (regional) blood flow measurements in experimental cardiovascular research. The use of radioactivity is becoming increasingly problematic due to restrictive legislation and higher costs of storage and disposal. These factors, along with the desire to reduce employee radiation exposure, have stimulated the development of nonradioactive microsphere techniques. Recently, fluorescently labeled microspheres have been demonstrated to be a reliable alternative to radioactive microspheres for the determination of blood flow in lung, kidney, normoxic myocardium (7), and normoxic and ischemic myocardium (2). In these validation studies, organ blood flow was estimated using

microspheres with up to five fluorescent labels for lung perfusion and three fluorescent labels for myocardial and kidney perfusion. In only one study (2) blood flow measurement with fluorescent microspheres in ischemic myocardium has been validated. In a restricted number of ischemic samples ($n = 11$ in 4 dogs) it was shown that the results correlated well with those obtained with radioactive microspheres. However, no data were reported on the validity of the assessment of endocardial-to-epicardial blood flow ratios by means of fluorescent microspheres. Blood flow measurement with fluorescent microspheres in other organs, such as the liver, brain, and skin, has not been validated.

Before quantifying the number of microspheres in a sample, a microsphere isolation and purification procedure is required. For this purpose, digestion of solid tissues and blood with KOH (1, 7) or proteases (2) has been used. Subsequently, the homogenate is isolated by negative pressure filtration (7) or the use of sucrose gradients (2). As has been suggested before (7), the disadvantage of these procedures is that sample processing does not take place in a single vial, which could result in loss of microspheres. Moreover, the procedures are relatively expensive (filters, proteinases) and time consuming. When fatty tissues (e.g., brain and liver) are processed, filters are easily occluded.

This paper describes and validates a simplified processing technique in which tissue digestion, microsphere isolation, and dye extraction are performed in one vessel and in which centrifugal sedimentation rather than microfiltration is used. The number of spheres is quantified by extracting the dyes in the solvent and subsequently measuring the fluorescence. This method is validated in canine nonischemic and ischemic myocardium as well as in various other organs, using microspheres labeled with up to six different fluorescent dyes.

METHODS

Animal preparation. The experiments were performed on seven mongrel dogs of either sex and unknown age, ranging in weight from 24 to 35 kg. The animals were premedicated with fentanyl (20 $\mu\text{g/kg}$ body wt im). Anesthesia was induced with thiopental sodium (30 mg/kg body wt iv) and, after endotracheal intubation, was maintained with nitrous oxide in oxygen (60:40 by vol) and halothane 0.5–1% using a volume-controlled respirator (Servo 900, Siemens). The preparation

has been described in detail before (12). In short, the heart was exposed through a left anterior thoracotomy. An inflatable occluder cuff was placed around the left anterior descending coronary artery (LAD) to induce a pressure-controlled stenosis (11). Saline-filled catheters were placed in the left atrium for microsphere injection and in the femoral artery for taking reference blood samples.

Microsphere injection. In each experiment microspheres with blue, blue-green, yellow-green, orange, red, and crimson fluorescent labels (polystyrene, $15.5 \mu\text{m} \pm 2\%$; Molecular Probes, Eugene, OR) and microspheres with a maximum of five different isotopes (^{141}Ce , ^{113}Sn , ^{103}Ru , ^{95}Nb , and ^{46}Sc ; $15.5 \pm 0.1 \mu\text{m}$; New England Nuclear, Boston, MA) were injected into the left atrium. For each determination $\sim 3 \times 10^6$ microspheres per fluorescent or radioactive label were injected. In one experiment a double number of blue microspheres (6×10^6) was injected. Before injection, the radioactively and fluorescently labeled microspheres, suspended in 10% dextran with 0.05% polyoxyethylenesorbitan monooleate (Tween 80), were mixed, sonicated, and vortexed. To allow calculation of absolute blood flow rates, a reference blood sample was taken from the femoral artery at a rate of 20.7 ml/min with a Harvard suction pump. Withdrawal of blood started 5 s before injection of the microspheres and was continued for ≥ 1 min after microsphere injection.

Experimental protocol. After the surgical procedure and hemodynamic stabilization, the LAD was narrowed by the occluder (distal LAD pressure = 40% of mean aortic pressure), producing an area of myocardial hypoperfusion. For intermethod (fluorescent vs. radioactive) and intramethod (fluorescent vs. fluorescent and radioactive vs. radioactive) evaluation, microspheres labeled with two different fluorescent dyes and two different radioactive isotopes were mixed in one syringe and injected simultaneously during stabilized LAD stenosis. Up to four other fluorescent and up to three other radioactive labels were injected at other time intervals, either during coronary stenosis or after deflation of the cuff. Only the results of the simultaneously injected microsphere pairs are discussed.

The experiments were terminated by administration of an overdose of pentobarbital sodium. From five experiments the hearts were excised and stored at -20° or 5°C for further processing. Before dissection, nonmuscular structures like fat and vessels at the epicardium as well as chordae tendineae were removed. From each heart ~ 12 transmural pieces were obtained from the hypoperfused anterior left ventricular wall (ischemic samples) and from the normally perfused posterior wall (nonischemic samples). Each transmural sample was divided into three layers of equal thickness, i.e., the subendocardial, mesocardial, and subepicardial layers, and each subsample was weighed (a total of $n = 168$). From three experiments, samples from skin, kidney, brain, spleen, skeletal muscle, gut, and lung (referred to as "other organs"; in total $n = 59$) were collected and weighed. Note that in this protocol "lung blood flow" refers to the microspheres delivered through the bronchial circulation and the pulmonary artery, the latter after shunting through arteriovenous anastomoses. The radioactivity of the tissue and arterial blood samples was determined in a gamma counter (LKB 1282, Compugamma, Wallac).

Tissue and blood digestion. All tissue samples and reference blood samples were transferred to regular 10 ml glass screw-cap test tubes (caps with Teflon inlay because of the aggressive reactants used). Nonmyocardial samples were allowed to autolyze (without KOH) at room temperature in a fume hood for ~ 1 wk. Myocardium, reference blood, and, after autolysis, other organs were digested in 2 N ethanolic KOH (Sigma, St. Louis, MO) with 0.5% Tween 80. The ratio of tissue or blood to digestion medium was $\leq 1:3$. To enhance digestion, the tubes

were placed in a 60°C water bath for 48 h and manually shaken after 24 h. Then the tubes with homogenized samples were centrifuged (20 min, 2,000 g in a swing-out bucket centrifuge), and the supernatant was carefully removed with a suction system, leaving < 1 ml. The difference in specific gravity between digestion medium (0.893 g/cm^3) and fluorescent microspheres (1.055 g/cm^3) allowed the formation of a pellet after centrifugal sedimentation. This pellet, containing microspheres and some debris, was rinsed with 9 ml 0.25% Tween 80 in demineralized water (60°C), vortexed, and centrifuged again at the same force and duration. The pellet was completely resuspended before centrifugation. A last rinsing step was performed with demineralized water without Tween 80. After centrifugation, the supernatant was carefully removed while preventing disturbance of the pellet. To avoid clumping, the pellet was not dried, but < 0.5 ml was left. Finally, 3 ml of an organic solvent, 2-(2-ethoxyethoxy)ethyl acetate (Aldrich Chemical, Milwaukee, WI), was added to the pellet to extract the fluorescent dye from the microspheres. Tubes were vortexed and allowed to stand for ≥ 4 h, vortexed again, and centrifuged (10 min, 2,000 g), leaving a clear solvent in which fluorescence was determined. Fluorescence of myocardial tissue and reference blood samples of one experiment was determined the same day, whereas fluorescence of the other organs was determined later.

Microsphere recovery. To confirm that no microspheres were lost during the procedure of digestion and subsequent sedimentation with supernatant removal, two tests were performed. In the first test, the supernatant of all rinsing steps from the samples of one experiment was collected and filtered (filter device, Molecular Probes). Fluorescence of any retained microspheres was determined by soaking the filters in solvent (3 ml) and determining the fluorescence. In the second test, 200 μl of a suspension containing orange microspheres was added to empty vials and, as an internal reference, to vials containing myocardial samples from an experiment in which dyes other than orange had been used. The myocardial samples were processed according to the sedimentation method. Orange fluorescence from the microspheres alone (without further processing) and those combined with tissue samples (after sample processing) were compared.

Determination of fluorescence. Fluorescence was determined with a Perkin-Elmer LS-50B luminescence spectrophotometer with an excitation wavelength range from 200 to 800 nm and an emission wavelength range from 200 to 900 nm. The machine was equipped with a pulsed xenon light source, variable excitation and emission monochrometers with variable slit widths, a red-sensitive photo multiplier tube (wavelength range 200–900 nm), and a cuvette reader or a flow cell (L215–1247, Perkin-Elmer). All fluorescence measurements were made with excitation and emission slit widths of 6 nm and an emission filter blocking all light below 350 nm. The optimal excitation and emission wavelengths for each of the six fluorescent dyes were determined.

To determine the concentration of microspheres in suspension, an aliquot of microspheres of each dye was counted manually with a fluorescence microscope. The counted microspheres were dissolved in 3 ml of solvent, and the fluorescence intensity was determined in a cuvette (10 mm path length) or in a flow cell. For each dye the fluorescence per microsphere per milliliter solvent was calculated. With these "standards," a spillover matrix was constructed, and the linearity of the fluorescence signal as a function of microsphere concentration was determined.

In serial measurements the FAC4A software package (developed by Perkin-Elmer and the Fluorescent Microsphere Resource Center, University of Washington, Seattle) allowed

automatic scanning of each sample for the six specific excitation and emission wavelength pairs.

Blood flow calculation. Blood flow values for radioactive and fluorescent microspheres were calculated after subtracting radioactive and fluorescent background from raw data, respectively, making corrections for spectral spillover by means of the matrix inversion method (15). For the fluorescent data, blood flow values with and without correction for spillover were compared. Because no significant difference in blood flow values derived from fluorescent microspheres with and without correction could be detected, the uncorrected data were used.

For myocardial samples, blood flow was calculated with the formula

$$\dot{Q}_i = (\dot{Q}_{\text{ref}} \cdot \text{Int}_i / \text{Int}_{\text{ref}})$$

where \dot{Q}_i and \dot{Q}_{ref} are the flow in sample i and the reference withdrawal speed, respectively, and Int_i and Int_{ref} are the radioactivity or fluorescence intensity in sample i and in the reference blood sample, respectively. Myocardial blood flow values are expressed in $\text{ml} \cdot \text{min}^{-1} \cdot \text{sample}^{-1}$, or normalized per unit of weight and expressed in $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$.

The ratio of blood flow ($\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$) in subendocardial and subepicardial samples was calculated and defined as the endocardial-to-epicardial ratio.

For the other (nonmyocardial) organ samples the relative blood flow (\dot{Q}_{rel_i}) was calculated using the formula

$$\dot{Q}_{\text{rel}_i} = (\text{Int}_i / \text{Int}_{\text{mean}}) \cdot \text{weight}_i^{-1}$$

where Int_i is fluorescence or radioactivity in sample i , Int_{mean} is mean fluorescence or radioactivity of all organ samples from each experiment, and weight_i is the weight of sample i . For these organs, relative blood flow was used rather than absolute blood flow because the fluorescence of the other organs was not measured on the same day as that of the arterial reference samples. As will be shown, repeatability of fluorescence measurements is less when tissue and reference samples are measured on different days rather than on the same day (see DISCUSSION). Absolute blood flow in the other organs, however, could be derived from the radioactive microspheres.

Statistics. Blood flows determined with each of the two simultaneously injected fluorescently labeled microspheres ($\dot{Q}_{\text{FM}_{1\text{or}2}}$) were compared with the mean of the blood flow values obtained from the two simultaneously injected radioactively labeled microspheres (\bar{Q}_{RM}). \bar{Q}_{RM} was used rather than $\dot{Q}_{\text{RM}_{1\text{or}2}}$ from individual isotopes to obtain the most precise blood flow standard. Because the error made with microspheres is related to the number of microspheres per sample, the use of data from two labels will increase accuracy, especially in ischemic samples (see also *Sources of error* in DISCUSSION). For intra- and intermethod comparison, linear regression analysis was performed. Regression coefficients (r) and standard error of estimate (SEE) were calculated for the best-fitting lines. The slopes of the lines were compared with unity, and the intercepts were compared with the origin. Data were also compared using the analysis of Bland and Altman (4), where the difference (which is the absolute error) between the two methods ($\bar{Q}_{\text{RM}} - \dot{Q}_{\text{FM}_{1\text{or}2}}$ for intermethod comparison and $\dot{Q}_{\text{RM}_1} - \dot{Q}_{\text{RM}_2}$ or $\dot{Q}_{\text{FM}_1} - \dot{Q}_{\text{FM}_2}$ for intramethod comparison) was plotted against the average of \dot{Q}_{RM_1} , \dot{Q}_{RM_2} , \dot{Q}_{FM_1} , and \dot{Q}_{FM_2} (mean flow: \bar{Q}). For each individual experiment the mean absolute error \pm standard deviation (SD) was calculated and tested for significant deviation from zero. The mean absolute error is a measure for systematic over- or underestimation of one of the methods; the

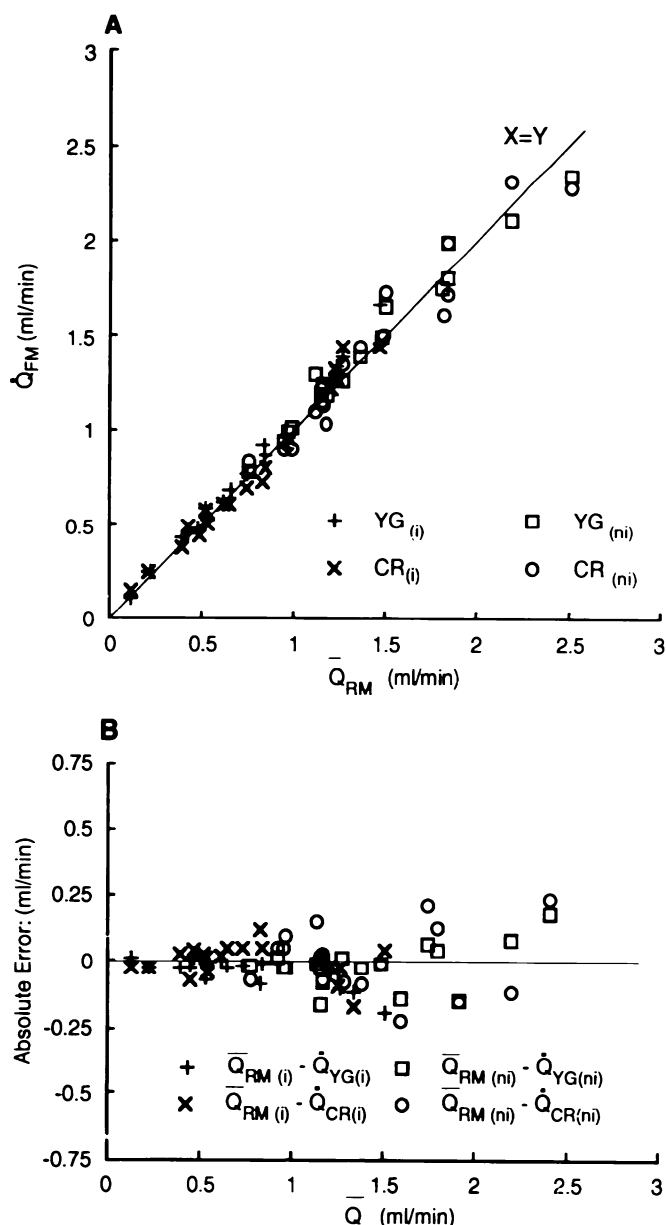


Fig. 1. Intermethod evaluation. Regional myocardial perfusion as determined by simultaneous injection of microspheres labeled with two different fluorescent and two different radioactive labels each in one experiment. Blood flow per myocardial sample (in $\text{ml} \cdot \text{min}^{-1} \cdot \text{sample}^{-1}$). A: regression plot for intermethod evaluation of the blood flow measured with fluorescent microspheres (\dot{Q}_{FM}) as a function of the mean value of blood flow determined with niobium (^{95}Nb)- and tin (^{113}Sn)-labeled microspheres (\bar{Q}_{RM}). Blood flow was measured in samples from ischemic (subscript i) and nonischemic (subscript ni) myocardium with yellow-green- [$\text{YG}_{(i)}$ and $\text{YG}_{(ni)}$] and with crimson [$\text{CR}_{(i)}$ and $\text{CR}_{(ni)}$]-labeled microspheres, respectively. Regression equations for ischemic and nonischemic samples together are $\bar{Q}_{\text{RM}} = 0.05 + 0.98\dot{Q}_{\text{YG}}$; standard error of estimate (SEE) = 0.022; $r = 0.992$; and $\bar{Q}_{\text{RM}} = 0.01 + 0.99\dot{Q}_{\text{CR}}$; SEE = 0.031; $r = 0.984$. B: absolute intermethod error as a function of mean value of blood flow determined with ^{95}Nb -, ^{113}Sn -, YG-, and CR-labeled microspheres (\bar{Q}). Absolute intermethod error = $\bar{Q}_{\text{RM}} - \dot{Q}_{\text{YG}_{(i)}} = -0.04 \pm 0.05$ (mean \pm SD, in $\text{ml} \cdot \text{min}^{-1} \cdot \text{sample}^{-1}$). $\bar{Q}_{\text{RM}} - \dot{Q}_{\text{CR}_{(i)}} = -0.00 \pm 0.07$ for ischemic samples, and $\bar{Q}_{\text{RM}} - \dot{Q}_{\text{YG}_{(ni)}} = -0.02 \pm 0.08$ and $\bar{Q}_{\text{RM}} - \dot{Q}_{\text{CR}_{(ni)}} = 0.01 \pm 0.12$ for nonischemic samples.

inter- or intramethod variability is expressed by the SD of the difference. Significance was tested with an unpaired double-tailed *t*-test, and *P* < 0.05 was considered significant. The intermethod error was also calculated by means of the mean absolute error ($Err_{abs} = \bar{Q}_{RM} - \bar{Q}_{FM}$) and the mean relative error [$Err_{rel} = 200(\bar{Q}_{RM} - \bar{Q}_{FM})/(\bar{Q}_{RM} + \bar{Q}_{FM})$] in both ischemic and nonischemic areas. For most error calculations and regression analysis blood flow per sample was used. To evaluate the effect of flow rate on the accuracy of blood flow measurements, different symbols were used for ischemic and nonischemic samples in plots (Figs. 1 and 2) and error calculations were performed for ischemic and nonischemic samples separately. To give an impression of the degree of ischemia, blood flow (\bar{Q}_{RM} and \bar{Q}_{FM}) in the subepicardial, mesocardial, and subendocardial layers in ischemic and nonischemic myocardium was normalized per gram tissue and expressed in $ml \cdot min^{-1} \cdot g^{-1}$. The variability of repeated fluorescent signals was quantified by the coefficient of variation (*C_v*).

RESULTS

Digestion of tissue. Myocardium, weighing 0.4–3 g, and reference blood readily digested in 7 ml 2 N ethanolic KOH within 48 h. After 1 wk of autolyzing without KOH all other organ tissues softened and weakened, which facilitated subsequent digestion with ethanolic KOH. When large (~3 g) tissue samples of fatty tissue (intestine, liver, or skin) were digested, a fatty debris occasionally floated on top of the ethanolic KOH after 48 h of digestion. These samples could be completely digested by subdividing the contents of one tube over four tubes and repeating the incubation in ethanolic KOH at 60°C.

Microsphere recovery. Filtration of the supernatant from 36 myocardial samples was found to contain <1% of the total fluorescence. When, in another test, orange microspheres were added to the tissue samples as an internal reference before digestion and sedimentation, average fluorescence was 4% lower than the fluorescence of the same number of microspheres directly extracted (15.5 ± 0.9 and 16.1 ± 0.7 , respectively). This difference, however, was not statistically significant. Both tests showed that there was no significant loss of microspheres with the supernatant, and the recovery of microspheres was ~100%.

Table 1. Spillover matrix of fluorescent colors

Color	Blue	Blue-Green	Yellow-Green	Orange	Red	Crimson
Blue	100.0	0.0	0.0	0.0	0.0	0.0
Blue-green	0.8	100.0	0.1	0.0	0.0	0.0
Yellow-green	0.0	0.0	100.0	0.1	0.0	0.0
Orange	0.0	0.0	0.0	100.0	0.4	0.0
Red	0.0	0.0	0.0	0.5	100.0	0.5
Crimson	0.0	0.0	0.0	0.0	3.2	100.0
Intensity	10.26	10.49	31.57	14.12	8.52	4.39
Excitation, nm	358	431	496	530	570	614
Emission, nm	420	466	506	555	600	639

For construction of spillover table, fluorescence of pure samples was measured in a cuvette or in a flow cell (no difference in spillover, fluorescence of flow cell 80% compared to fluorescence measured in cuvette), using excitation and emission wavelength pairs indicated. Spillover is given in percentages. Fluorescence of 100 microspheres dissolved in 1 ml solvent measured in a cuvette is presented.

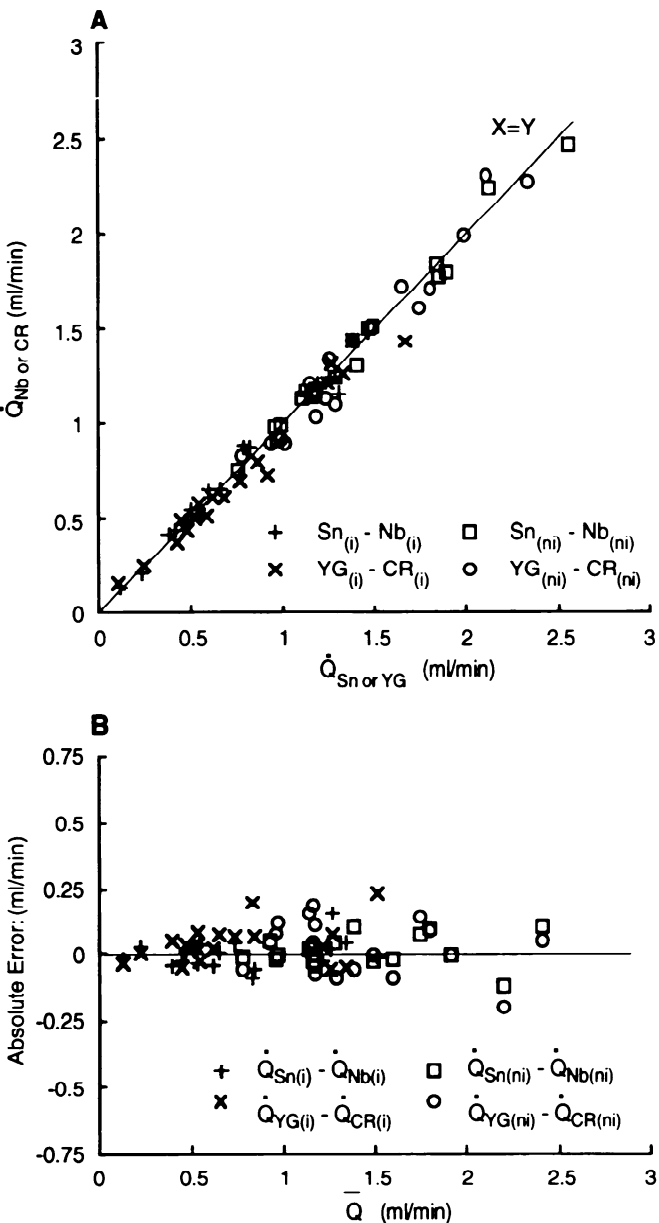


Fig. 2. Intramethod comparison. Same experiment and abbreviations as Fig. 1; blood flow given per myocardial sample ($ml \cdot min^{-1} \cdot sample^{-1}$). A: blood flow measured with ^{95}Nb -labeled microspheres [$\dot{Q}_{Nb(i)}$ or $\dot{Q}_{Nb(ni)}$] plotted as a function of blood flow measured with ^{113}Sn -labeled microspheres [$\dot{Q}_{Sn(i)}$ or $\dot{Q}_{Sn(ni)}$] for evaluation of the radioactive intramethod variation. Intramethod evaluation for fluorescent method shown as well; $\dot{Q}_{YG(i)}$ or $\dot{Q}_{YG(ni)}$ is plotted as a function of $\dot{Q}_{CR(i)}$ or $\dot{Q}_{CR(ni)}$. The regression equations for ischemic and nonischemic samples together are $\dot{Q}_{Nb} = 0.023 + 0.972\dot{Q}_{Sn}$; SEE = 0.053; *r* = 0.995; and $\dot{Q}_{CR} = -0.035 + 1.00\dot{Q}_{YG}$; SEE = 0.092; *r* = 0.986. B: absolute intramethod error as a function of the mean value of blood flow determined with ^{95}Nb -, ^{113}Sn -, YG-, and CR-labeled microspheres (\bar{Q}). Absolute intramethod error = $\dot{Q}_{Sn(i)} - \dot{Q}_{Nb(i)} = 0.00 \pm 0.05$ (mean \pm SD, in $ml \cdot min^{-1} \cdot sample^{-1}$), and $\dot{Q}_{YG(i)} - \dot{Q}_{CR(i)} = 0.04 \pm 0.08$ for ischemic samples and $\dot{Q}_{Sn(ni)} - \dot{Q}_{Nb(ni)} = 0.01 \pm 0.06$ and $\dot{Q}_{YG(ni)} - \dot{Q}_{CR(ni)} = 0.02 \pm 0.10$ for nonischemic samples.

Duration and costs of analysis. When processing a series of 50 samples, the average time for processing one sample with the sedimentation method required 6 min and cost ~\$0.17 (US).

Table 2. *Inter- and intramethod regression*

Sample Type	Slope	Intercept	Correlation
Myocardial			
\bar{Q}_{RM} vs. \bar{Q}_{FM}	0.98 ± 0.03	0.04 ± 0.06	0.985 ± 0.01
\bar{Q}_{FM} vs. \bar{Q}_{FM}	0.99 ± 0.04	0.01 ± 0.07	0.981 ± 0.02
\bar{Q}_{RM} vs. \bar{Q}_{RM}	0.99 ± 0.02	0.01 ± 0.01	0.997 ± 0.00
Other organs			
\bar{Q}_{FM} vs. \bar{Q}_{RM}	0.99 ± 0.05	-0.01 ± 0.01	0.982 ± 0.01

Values are means \pm SD. Regression analysis of flow values in myocardium ($n = 168$ samples) and various other organs ($n = 59$ samples) are shown. \bar{Q}_{RM} and \bar{Q}_{FM} , blood flow determined with radioactive and fluorescent microspheres, respectively. Pooled data from all experiments are presented.

Table 3. *Absolute inter- and intramethod error*

Experiment	$\bar{Q}_{RM} - \bar{Q}_{FM}$	$\bar{Q}_{RM} - \bar{Q}_{FM_2}$	$\bar{Q}_{FM_1} - \bar{Q}_{FM_2}$	$\bar{Q}_{RM_1} - \bar{Q}_{RM_2}$
	OR	CR	OR - CR	Sn - Nb
1	-0.03 ± 0.09	-0.04 ± 0.07	0.01 ± 0.06	0.01 ± 0.02
	YG	CR	YG - CR	Ce - Nb
2	0.01 ± 0.16	0.04 ± 0.18	0.03 ± 0.16	0.00 ± 0.04
	BL*	RD	BL - RD*	Sn - Ru
3	-0.16 ± 0.11	0.00 ± 0.05	-0.16 ± 0.12	0.01 ± 0.04
	YG*	CR	YG - CR*	Sn - Nb
4	-0.03 ± 0.07	0.00 ± 0.10	0.03 ± 0.02	0.00 ± 0.06
	Ce - BL*	Ce - BG*	BL - BG*	
5	0.11 ± 0.20	0.05 ± 0.15	-0.06 ± 0.13	

Values are mean differences \pm SD ($\text{ml} \cdot \text{min}^{-1} \cdot \text{sample}^{-1}$). Inter- and intramethod error of myocardial blood flow determined for each experiment separately. \bar{Q}_{RM} , mean flow determined with two radioisotopes; $\bar{Q}_{FM_{1 \text{ or } 2}}$, flow determined with one of two simultaneously injected fluorescent microspheres. Fluorescent dyes used were blue (BL), blue-green (BG), yellow-green (YG), orange (OR), red (RD), and crimson (CR). Radioisotopes used were ^{141}Ce , ^{95}Nb , ^{113}Sn , and ^{103}Ru . In experiment 5 only one radioisotope (^{141}Ce) was used. * $P < 0.05$ vs. zero.

Fluorescence measurements. The variability of repeated measurements ($n = 10$) of fluorescent signals from the same sample, as described by C_v , was 0.3–1.5 and 0.2–1.1% for the cuvettes and flow cell, respectively, when measured on the same day. If the same sample was measured every day for seven consecutive days, the C_v

was 2.0–9.2%. With the use of optimal excitation and emission wavelengths for each dye, the spillover of different fluorescent dyes was small and only substantially larger than zero for crimson to red: 3.2% (Table 1). There was a difference in fluorescence per microsphere between colors. The lowest values were found for red and crimson, and the highest values were found for yellow-green and orange (Table 1 shows values as measured in a cuvette). Fluorescence measured in the flow cell was 80% of that measured in a cuvette. Fluorescence intensity ranged from 7 to 400 for all organ and reference blood samples. When fluorescence was > 200 , samples were diluted to be sure that the measurements were made in the fluorescence range where the relation between microsphere concentration and fluorescence signal was linear.

Myocardial perfusion studies. Blood flow values obtained with fluorescent microspheres correlated very well with those obtained with radioactive microspheres. Figure 1 shows the data for one typical experiment, and in Table 2 the pooled data for all experiments are presented ($r = 0.985 \pm 0.02$, slope and intercept not significantly different from 1 and 0 respectively). In these experiments, blood flow varied from 0.1 to 2.5 $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ myocardial tissue. The intramethod correlation is shown in Fig. 2 (the same experiment as Fig. 1), and in Table 2 the pooled data for all experiments are presented. In one experiment (expt. 5), the data of one of the two simultaneously injected radioactive labeled microspheres (^{103}Ru) were discarded because of unacceptably low radioactivity of this isotope (3). For the pooled data, the intramethod correlation for the fluorescent method was slightly lower ($r = 0.981 \pm 0.01$) than for the radioactive method ($r = 0.997 \pm 0.00$). In one experiment (expt. 3), the inter- and intramethod correlations for fluorescently labeled microspheres were 0.958 and 0.946, respectively, due to the use of blue microspheres. If the data obtained with the blue microspheres in experiment 3 are discarded, then the pooled intermethod correlation ($\bar{Q}_{RM} - \bar{Q}_{FM}$) became 0.988, and the intramethod correlation ($\bar{Q}_{FM_1} - \bar{Q}_{FM_2}$) became 0.990.

Table 4. *Blood flow and intermethod error in nonischemic and ischemic myocardium*

	Nonischemic				Ischemic			
	$\bar{Q}, \text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$				$\bar{Q}, \text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$			
	Epi	Meso	Endo	Endo Epi	Epi	Meso	Endo	Endo Epi
RM	1.12 ± 0.35	1.30 ± 0.22	1.48 ± 0.21	1.38 ± 0.31	0.90 ± 0.60	0.56 ± 0.50	0.38 ± 0.32	0.44 ± 0.26
FM	1.13 ± 0.30	1.35 ± 0.18	1.42 ± 0.17	1.36 ± 0.31	0.89 ± 0.55	0.58 ± 0.45	0.41 ± 0.30	0.44 ± 0.22
Err _{abs.} , $\text{ml} \cdot \text{min}^{-1} \cdot \text{sample}^{-1}$	Offset		Variation		Offset		Variation	
	0.02 ± 0.06		0.15 ± 0.07		0.00 ± 0.04		0.07 ± 0.04	
	0.32 ± 2.58		8.25 ± 3.51		-4.46 ± 10.31		16.16 ± 15.57	

Values are means \pm SD of pooled data from all myocardial samples (except flow values obtained from blue microspheres in experiment 3). \bar{Q} , mean blood flow assessed with microspheres labeled with two radioactive labels (RM) or with two fluorescent labels (FM), in epicardial (Epi), mesocardial (Meso), and endocardial (Endo) layers of nonischemic ($n = 90$ samples) and ischemic ($n = 78$ samples) myocardium. Blood flow values in $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ \pm SD. Endo Epi = the ratio of endocardial and epicardial blood flow. Systemic differences and random errors between blood flow assessed with RM and FM are represented by offset and variation, respectively. Err_{abs.}, absolute intermethod error; Err_{rel.}, relative intermethod error.

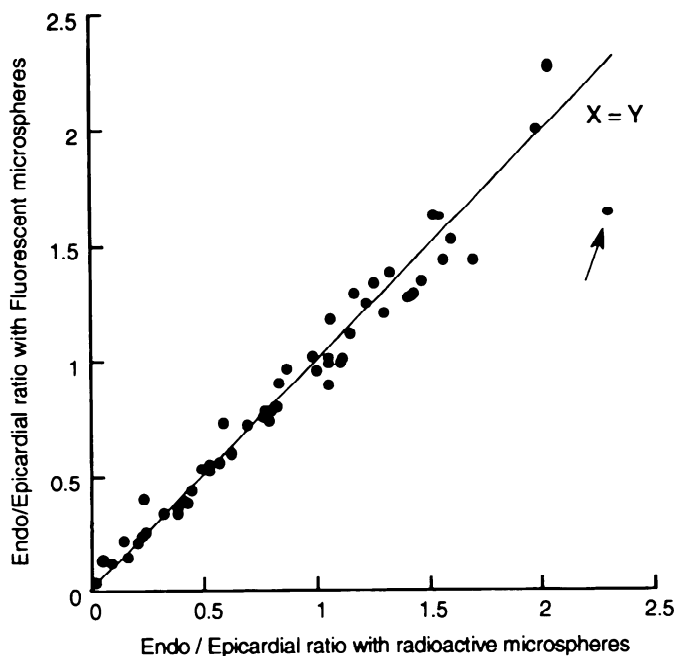


Fig. 3. Endocardial (Endo) to-epicardial blood flow ratios determined by simultaneous injection of microspheres labeled with two different fluorescent and two different radioactive labels each per experiment. Plot shows pooled data from all experiments; line of unity is plotted. Regression equation for all data points is $y = 0.91x + 0.07$; $r = 0.974$. Discarding one stray point (arrow), the equation changes to $y = 0.96x + 0.03$; $r = 0.985$; slope and intercept are not statistically different from identity and origin, respectively.

Figures 1B and 2B show plots of the absolute error plotted as a function of blood flow for inter- and intramethod comparison, respectively. Table 3 summarizes the values of the mean absolute error for the individual experiments. The pooled data as well as the data for individual experiments show that for most experiments there was no systematic over- or underestimation or large random variation in the fluorescent method compared with the radioactive method. However, blue microspheres led to a slight but significant overestimation of blood flow in *experiment 3* and an underestimation of blood flow in *experiment 5*.

In Fig. 1A the data points from the ischemic myocardial samples scatter around the same line as those from the nonischemic samples. Similarly, Fig. 1B shows that the absolute error between the flow values determined with fluorescently and radioactively labeled microspheres at a given flow per sample is similar for ischemic and nonischemic myocardial samples. This indicates that the error between the radioactive and fluorescent methods is independent of flow rate. This aspect is further elaborated in Table 4, in which the statistical analysis of the pooled blood flow data for ischemic and nonischemic myocardium is presented.

Mean blood flow in all layers was 0.62 in ischemic myocardium and $1.3 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ in nonischemic myocardium. There was no significant difference in the blood flow values assessed with fluorescently or radioactively labeled microspheres.

The absolute intermethod errors are presented. There was no significant offset (mean differences, 0.00 and $0.02 \text{ ml} \cdot \text{min}^{-1} \cdot \text{sample}^{-1}$) and only a slight variation (SD of the mean difference, 0.07 and $0.15 \text{ ml} \cdot \text{min}^{-1} \cdot \text{sample}^{-1}$) between the two methods for ischemic and nonischemic myocardium, respectively. For ischemic and nonischemic myocardial samples together the relative intermethod error was $-1.90 \pm 11.15\%$.

A good correlation ($r = 0.974$) was found between the endocardial-to-epicardial blood flow ratios as assessed with the fluorescent and radioactive microsphere methods as well (Fig. 3). The slope (0.91) and intercept (0.07) of the regression equation were statistically different from identity and origin, respectively. However, the regression equation was strongly influenced by one (unexplained) stray value (arrow, Fig. 3). If this point was omitted from the calculation of the regression line, regression improved to $r = 0.985$, and the slope (0.96) and the intercept (0.034) were not statistically different from identity and origin, respectively. It was therefore concluded that fluorescent and radioactive microspheres estimated endocardial-to-epicardial blood flow ratios equally well.

Other organ perfusion studies. The intermethod correlation for various nonmyocardial tissues is shown in Fig. 4 (one experiment) and in Table 2 (pooled data for all experiments). For these organs the intermethod correlation was also good ($r = 0.982$), whereas the intercept and the slope of the regression equation were not significantly different from 0 and 1, respectively.

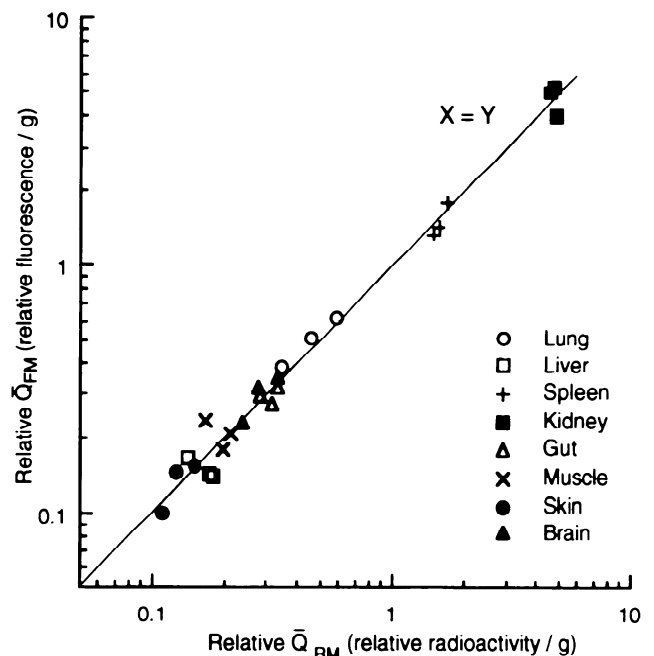


Fig. 4. Intermethod evaluation of regional perfusion measurements for nonmyocardial organs as determined by simultaneous injection of fluorescently (\bar{Q}_{FM}) and radioactively labeled microspheres (\bar{Q}_{RM}) for one experiment. Relative blood flow (expressed as the relative fluorescence or radioactivity g) is plotted on a logarithmic scale. Drawn line is line of unity. Regression equation for all organs together in this experiment is $y = 0.995x + 0.004$, and $r = 0.991$. Absolute blood flow (derived from the radioactive microspheres only) in these organs ranged from 0.14 (skin) to $6.11 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ (kidney).

DISCUSSION

A new technique for sample digestion and isolation of fluorescent microspheres is described and validated. In the present study this time- and money-saving, one-vessel, sedimentation technique was suitable for myocardium as well as a variety of other organs. With the use of subsequent dye extraction and fluorimetry, reliable blood flow estimates can be obtained with up to six different dyes in the same experiment.

The accuracy of the blood flow measurements, expressed as the intermethod correlation, reported here ($r = 0.985$) is comparable to that found by others using direct extraction from lung tissue (5 fluorescent labels, $r = 0.99$), negative pressure filtration in myocardial ($r = 0.95$) and kidney samples (3 fluorescent labels, $r = 0.96$) (7), or protease tissue digestion and purification through sucrose gradients in normal and ischemic myocardium (3 fluorescent labels, $r = 0.99$) (2). Slopes and intercepts from the regression equations were not statistically different from 1 and 0, respectively, for all methods. The relative intermethod error for myocardium found in this study ($-1.90 \pm 11.15\%$) was also slightly, but not significantly, smaller than that found by Glenn and colleagues ($5.10 \pm 15.49\%$) (7). The data obtained in the present study show that the absolute intermethod error for ischemic myocardium (0.00 ± 0.04) is smaller than in nonischemic myocardium (0.02 ± 0.15). The accuracy of the fluorescent microsphere method in ischemic myocardium and in various other organs was excellent, indicating that the sample processing technique is reliable in organs with low perfusion and in organs that are normally hard to digest due to a high fat content (e.g., brain, skin, and liver).

An important practical limitation of fluorescent or other nonradioactive microspheres is the time and cost involved in processing a sample. For the radioactive microsphere technique this time is relatively short, and the processing is easy. Time and cost involved in the processing of solid tissue samples varies for the different methods used (Table 5). With the sedimentation method, processing of a sample takes 6 min, whereas in the vacuum filtration (7) and the sucrose-cushion (2) techniques 10 min and ≥ 9.5 min are involved, respectively. This means a reduction of $\geq 37\%$ with the simplification described in the present study. Total cost for the sedimentation method amounts to \$0.17, and for the other methods to \$0.52 and \$5.77, respectively, which means that a cost reduction of $\geq 67\%$ can be achieved with the sedimentation method. We therefore conclude that the sedimentation method saves time and money compared with negative pressure filtration or counting of microspheres.

Sources of error. As can be seen in Fig. 2, the radioactive microsphere method has small inherent errors, which have been well documented (3, 5, 9). The error is mainly determined by statistical variation in the distribution of the microspheres and is dependent on the number of microspheres in each tissue and reference sample. A minimum number of 400 microspheres per sample should guarantee a relative error of $< 5\%$. In the

present study, for each label the number of microspheres injected (3×10^6 into left atrium) resulted in a mean of 1,580 microspheres, ranging from 10 microspheres in small ischemic samples to 7,500 microspheres in normoxic samples.

The results of the present study demonstrate that the flow values obtained with the fluorescent microsphere technique correlate quite well with those obtained with the radioactive microsphere method and that the error made with the fluorescent microsphere technique is only slightly larger than the error made with radioactive microspheres. This indicates that possible additional sources of error of the fluorescent microsphere technique are small. Such possible errors are incomplete isolation of microspheres from the samples and loss of microspheres or dye from microspheres during sample processing. Incomplete isolation of microspheres from tissue or blood would have resulted in underestimation and overestimation of organ blood flow, respectively, or in a greater variability in the flow values. The same holds for loss of microspheres or loss of dye during sample processing. Absence of loss of microspheres during sample processing was also demonstrated by the very low number of microspheres in the supernatant and the complete recovery of microspheres when these spheres were added as an internal reference. The absence of large systematic bias or large variability between the radioactive and fluorescent microsphere methods demonstrates that these possible sources of error are negligible compared with the error related to the number of microspheres per sample. In addition, the fact that a small, slightly variable amount (< 0.4 ml) of fluid (mainly water) remained in the test tubes after the

Table 5. Time and costs of sedimentation, filtration, and sucrose-cushion methods

Processing Step	Sedimentation (A)		Filtration (B)		Sucrose-Cushion (C)	
	Time, min	Cost, \$	Time, min	Cost, \$	Time, min	Cost, \$
Isolation	2.9	0.05	6.9	0.45	> 3	0.05
Other	2.0	0.01	2.0	0.01	2.0	5.72
Fluorescence	1.1	0.11	1.1	0.06	**4.5 *15	
Total	6.0	0.17	10	0.52	9.5 20	5.77

Estimation of time and costs (in U.S. dollars) to process a sample (calculated as 1/50 of the time or cost for a series of 50 samples) by the sedimentation technique (this paper), the filtration technique [Glenn et al. (7)], and the sucrose-cushion technique [Austin et al. (2)]. *Isolation*, sedimentation in centrifuge (A, C) or vacuum filtration (B); *Other*, numbering tubes, weighing samples, and preparing chemicals; *Fluorescence*, determination of fluorescence by spectrophotometry (A, B), by manual counting with fluorescence microscopy (*), or by fluorescence-activated cell sorter (**, C). Costs were related to cost of tubes (5 times reusable) (A, B, C) and filters (B) for *isolation* and to chemicals [KOH (A, B) or proteinase collagenase (Sigma) (C) for *other* and 2-(2-ethoxyethoxy)ethyl acetate (Aldrich) (A, B) for *fluorescence*]. Costs of laboratory personal or apparatus were not included. Time was not always subdivided into categories by authors; in B a total time and time for *fluorescence* was given, in C only an estimation of time for *fluorescence* was given. We estimated that time for *other* would not differ for different methods (2 min) and *isolation* in (C) would take at least 3 min.

final removal of supernatant does not seem to be a source of error. The presence of this small amount of supernatant together with a relatively large amount (3 ml) of solvent induces only a small error, if any, because the volume of distribution of the highly lipophilic dyes in the solvent is ~100%.

Effects of microsphere density. Rheological properties of microspheres can influence blood flow estimation. The diameter of microspheres has a significant influence on intraventricular and intrarenal blood flow distribution (6, 10, 13, 16). There are no differences in diameter between radioactive and fluorescent microspheres, but there is a significant difference in specific gravity (1.3 g/ml for radioactive and 1.05 g/ml for fluorescent microspheres). To the best of our knowledge no studies are available on the effect of the specific gravity of microspheres on the blood flow values obtained. Although the design of our study cannot conclusively answer this question, comparison of endocardial-to-epicardial blood flow ratios obtained with fluorescent and radioactive microspheres gives some insight into this aspect. Because of the close similarity between the endocardial-to-epicardial ratios as determined with fluorescent and radioactive microspheres found in this study (Fig. 3), it may be concluded that the rheological properties of both types of microspheres are not different, despite the difference in specific gravity. If more streaming had occurred with the heavier radioactive microspheres under normoxic circumstances, these microspheres would have been preferentially deposited in the endocardial layers, resulting in significantly higher endocardial-to-epicardial blood flow ratios.

Fluorescence technology. As has been shown before (7), spectrofluorometry provides a sensitive, highly repeatable measure that is linear with respect to the number of fluorescently labeled microspheres per sample. Repeatability of fluorescence measurements was highest when analyzing samples the same day ($C_v = 0.2$ – 1.1%). The repeatability decreased when the samples were analyzed on different days ($C_v = 2$ – 9%), which has been observed before (7). Therefore, in the present study absolute blood flow calculations were only performed if fluorescence in tissue and the arterial reference sample were determined on the same day.

An advantage of fluorescent dyes over radioisotopes is the minimal spectral spillover, being largest for crimson to red (3.2%). The actual overestimation of fluorescence of a dye because of spillover from another fluorescent dye is dependent on 1) the percentage of spillover and 2) the difference in fluorescence between dyes. For example, the overestimation of red (because of a spillover from crimson to red of 3.2%) would be 7 and 35% when the ratio of crimson to red fluorescence equals 2:1 and 10:1, respectively. In this study, in all samples the fluorescence of crimson was less (20–50%) than the fluorescence of red, so the actual overestimation of red due to spillover from crimson was theoretically <1.6%. Spillover between the six different dyes used in this study did not affect the accuracy of the blood flow values obtained. This was demonstrated by the fact that blood flow values derived from fluorescence data corrected for

spillover did not differ significantly from those not corrected for spillover.

It was observed that blue fluorescent microspheres cause a greater variability in the blood flow estimation than other dyes (Table 3). This could be explained by the varying background fluorescence of blue. The solvent used in this study [2-(2-ethoxyethoxy)ethyl acetate] has a significant intrinsic background in the blue excitation/emission wavelength range. Moreover, Tween 80 dissolves in the solvent and has a significant background fluorescence in the blue wavelength range. To circumvent this problem, the final concentrations of Tween 80 were minimized, using water without Tween for the final washing step in the microsphere isolation procedure. Although the mean background fluorescence value was subtracted from the value of each sample, variation of background in individual samples may have contributed to a larger variability in blood flow estimation for the blue microspheres.

Therefore the present study shows that five fluorescent dyes can be used in the same experiment (the use of the blue label is less desirable) without the need for spectral spillover correction. When such correction is applied, at least one other fluorescent dye (green) can be used (7).

In conclusion, this study shows that 1) with autolysis and ethanolic KOH digestion, samples from blood and all kinds of organs can be easily digested, 2) all microspheres can be recovered from the samples by subsequent centrifugal sedimentation, 3) this easy method saves time and money compared with classical negative pressure filtration, 4) blood flow can be estimated, using up to six different fluorescent labels in the same experiment, with an accuracy similar to that of the radioactive microsphere technique in normally and hypoperfused myocardium and in various other organs, and 5) endocardial-to-epicardial blood flow ratios estimated with fluorescent microspheres equal those estimated with radioactive microspheres.

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