Validation of fluorescent microsphere technique for measurement of organ perfusion with simplified sample processing method

abbreviated title: sedimentation technique for fluorescent microspheres

Matthijs F.M. van Oosterhout MD[1]; Henriëtte M.M. Willigers MD[2]; Robert S. Reneman MD PhD[1]; Frits W. Prinzen PhD[1].

Departments of Physiology[1] and Anesthesiology[2], Cardiovascular Research Institute Maastricht, University of Limburg, Maastricht, The Netherlands

Correspondence address:
Frits W. Prinzen, PhD
Department of Physiology
Cardiovascular Research Institute Maastricht
University of Limburg
P.O. Box 616
The Netherlands
telephone: +31-43-881200
telefax: +31-43-671028
E-mail: Frits.Prinzen@FYS.RULimburg.NL

Abstract

A disadvantage of non-radioactive 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 7 anesthetized dogs with coronary artery stenosis up to 6 different FM and 5 different radioactively labeled microspheres (RM) were injected, 2 FM and 2 RM labels being injected simultaneously to enable inter and intra-method comparison. After gamma counting samples of blood, myocardium (n=168) and other organs (n=59) were digested in test tubes using ethanolic KOH 2N (60°C, 48 hour), microspheres were sedimentated by centrifugation and dye was extracted in the same tube followed by fluorescence measurement. Using this processing method recovery of FM was nearly 100%. Good correlations for inter- and intra-method comparisons were found, r was 0.985±0.01 (mean±s.d.), the lower inter-method correlation for blue microspheres (r=0.958) indicate that the use of this label is less desirable. Also RM and FM endo/epicardial blood flow ratios correlated well (r=0.974). Using this one vessel centrifugal sedimentation method and at least 5 fluorescently labeled microspheres, blood flow can be reliably measured in various organs, including ischemic myocardium.

Index Terms

coronary blood flow

myocardial ischemia

radioactive microspheres

tissue digestion

microsphere isolation

organ perfusion

INTRODUCTION

Radioactive microspheres are used to measure regional organ blood flow. This method has been validated in many studies (see for review [14] [8]) 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 radiation exposure to employees, have stimulated the search for non-radioactive microspheres. Recently, fluorescently labeled microspheres have been demonstrated to be a reliable alternative for radioactive microspheres for the determination of blood flow in lung, kidney and normoxic myocardium [7] and normoxic and ischemic myocardium [2]. In these validation studies, organ blood flow was estimated using microspheres with up to 5 fluorescent labels for lung perfusion and 3 fluorescent labels for myocardial and kidney perfusion. In only one study [2] blood flow measurements in ischemic myocardium by means of the fluorescent microsphere method were validated. In a restricted number of ischemic samples (n=11 in four 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 endo/epicardial blood flow ratios by means of fluorescent microspheres. Also, the measurement of blood flow in other organs like liver, brain and skin by means of fluorescent microspheres 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 potassium hydroxide (KOH) [7] [1] 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 involves centrifugal sedimentation rather than microfiltration. The number of spheres is quantified by extracting the dyes in the solvent and subsequently measuring the fluorescence. This method is validated in canine non-ischemic 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 7 mongrel dogs of either sex and unknown age, ranging in weight from 24 to 35 kg. The animals were premedicated with Fentanyl (20 µg/kg body weight i.m.). Anesthesia was induced with sodium thiopental (30 mg/kg body weight iv.) and, after endotracheal intubation, was maintained with nitrous oxide in oxygen (60:40, by volume) 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 controled

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 µm ± 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 ± 0.1 µm, New England Nuclear) were injected into the left atrium. For each determination about $3 \cdot 10^6$ microspheres per fluorescent or radioactive label were injected. In one experiment a double number of blue microspheres ($6 \cdot 10^6$) was injected. Prior to injection, the radioactively and fluorescently labeled microspheres, suspended in 10% dextran with 0.05% 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^{-1} using a Harvard suction pump. Withdrawal of blood started 5 s before injection of the microspheres and was continued for at least one minute after microsphere injection.

Experimental protocol

After the surgical procedure and hemodynamic stabilization the left descending artery (LAD) was narrowed by the occluder (distal LAD pressure 40% of mean aortic pressure), thus producing an area of myocardial hypoperfusion. For inter-method (fluorescent versus radioactive) and intra- method (fluorescent versus fluorescent and radioactive versus 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 4 other fluorescent and up to 3 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 will be discussed in this paper.

The experiments were terminated by administration of an overdose of pentobarbital sodium. From 5 experiments the hearts were excised and stored at -20° or 5°C for further processing. Before dissection, non-muscular structures like fat and vessels at the epicardium, and chordae tendinae, were removed. From each heart approximately 12 transmural pieces were obtained, from the hypoperfused anterior left ventricular (LV) wall (ischemic samples) and from the normally perfused posterior wall (non-ischemic samples). Each transmural sample was divided into three layers of equal thickness, i.e. the subendocardial, mesocardial and subepicardial layers and each sub-sample was weighed (a total of n=168). From 3 experiments samples from skin, kidney, brain, spleen, skeletal muscle, gut and lung (referred to as 'other organs'; in total 59) were collected and weighed. Note that in this set-up "lung" blood flow refers to the microspheres delivered through the bronchial circulation and the pulmonary artery, the latter after shunting through A-V 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). Non-myocardial samples were allowed to autolyze (without KOH) at room temperature in a fumehood for approximately one week. Myocardium, reference blood and, after autolysis, other organs were digested in ethanolic KOH 2N (Sigma) with 0.5% Tween 80. The ratio of tissue or blood to digestion medium

was ≤ 1:3. To enhance digestion the tubes were placed in a 60°C water bath for 48 hours and were manually shaken after 24 hours. Then the tubes with homogenized samples were centrifuged (20 min, 2000 g in a swing out bucket centrifuge) and the supernatant carefully removed with a suction system leaving a volume of less than 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 prior to centrifugation. A last rinsing step was performed using 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 a volume of less than 0.5 ml was left. Finally, 3 ml of an organic solvent: 2-(2-ethoxy) 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 at least 4 hours, vortexed again, and centrifuged (10 min, 2000x g), leaving a clear solvent in which fluorescence was determined. Fluorescence of myocardial tissue and reference blood samples of one experiment was determined at 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. For each dye the fluorescence per microsphere per ml solvent was calculated.

To determine the concentration of microspheres in suspension, an aliquot of microspheres of each dye was counted manually using a fluorescence microscope. The counted microspheres were dissolved in 3 ml of solvent and the fluorescence intensity determined in a cuvette (10 mm path length) or in a flowcell. With these 'standards', a spillover matrix was constructed and the

linearity of the fluorescence signal as a function of microsphere concentration 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 6 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 using the formula :

$$Q_i=(Q_{ref}*Int_i/Int_{ref})$$

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

The ratio of blood flow ($ml \cdot min^{-1} \cdot g^{-1}$) in subendocardial and subepicardial samples was calculated and defined as endo/epi ratio.

For the other (non-myocardial) organ samples the relative ($Qrel_i$) blood flow was calculated using the formula

$$Qrel_i = (Int_i/Int_{mean}) \cdot weight_i^{-1}$$

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 $wheight_i$ 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 ($Q_{FM1or\ 2}$) 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 $Q_{RM1\ or\ 2}$ 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 discussion, section sources of error). For intra- and inter-method 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 to unity and the intercepts compared to 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 ($\bar{Q}_{RM}$ - $Q_{FM1or\ 2}$ for inter- method comparison and Q_{RM1} - Q_{RM2} or Q_{FM1} - Q_{FM2} for intra- method comparison) was plotted against the average of Q_{RM1}, Q_{RM2}, Q_{FM1} and Q_{FM2} (=mean flow: $\bar{Q}$.) For each individual experiment the

mean absolute error ± standard deviation (SD) was calculated and tested for significant deviation from 0. The mean absolute error is a measure for systematic over or under estimation of one of the methods, the inter- or intra- method variability is expressed by the SD of the difference. Significance was tested with an unpaired double tailed t test, $p<0.05$ was considered significant. The inter-method 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 non-ischemic 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 non-ischemic samples in plots (figures 1 and 2) and error calculations were performed for ischemic and non-ischemic 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 non-ischemic 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 2N ethanolic KOH within 48 hour. After a week of autolyzing without KOH all other organ tissues softened and weakened, which facilitated subsequent digestion with ethanolic KOH. When large (approximately 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 hours of digestion. These samples could be completely digested by subdividing the content 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 less than 1% of the total fluorescence. When in another test, orange microspheres were added to the tissue samples as an internal reference prior to digestion and sedimentation, on the 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 near 100%.

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 costs about US$ 0.17

Fluorescence measurements

The variability of repeated measurements (n=10) of fluorescent signals from the same sample as described by the coefficient of variation (c.v.) was 0.3-1.5% and 0.2-1.1% for the cuvettes and flowcell, respectively, when measured on the same day. If the same sample was measured every day for 7 consecutive days, the c.v. was 2.0-9.2%. Using 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 for yellow-green and orange (table 1 shows values as measured in a cuvette). Fluorescence measured in the flowcell 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 higher than 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 ± 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 $ml \cdot min^{-1} g^{-1}$ myocardial tissue. The intra-method correlation is shown in Figure 2 (the same experiment as Figure 1) and in Table 2 the pooled data for all experiments are presented. In one experiment (exp. 5) the data of one of the two simultaneously injected radioactive labeled microspheres (Ru) were discarded because of unacceptable low radioactivity of this isotope [3]. For the pooled data the intra- method correlation for the fluorescent method was slightly lower (r = 0.981±0.01) than for the radioactive method (r = 0.997±0.00). In one experiment (3) the inter- and intra-method correlation for fluorescently labeled microspheres was 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 inter-method correlation (Q_{RM}-Q_{FM}) became 0.988 and the intra-method correlation (Q_{FM}-Q_{FM}) 0.990.

Panels B of Figure 1 and 2 show plots of the absolute error plotted as a function of blood flow for inter- and intra-method 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 under estimation or large random variation in the fluorescent method as compared to the radioactive method. However, blue microspheres led to a slight but significant overestimation of blood flow in experiment 3 and a underestimation of blood flow in experiment 5.

In figure 1A the data points from the ischemic myocardial samples (cross and plus symbols) scatter around the same line as those from the non-ischemic samples (squares and circles). Similarly figure 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 non-ischemic 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 non-ischemic myocardium is presented.

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

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

Also, a good correlation (r = 0.974) was found between the endo/epicardial blood flow ratios as assessed with the fluorescent and radioactive microsphere methods (Figure 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, figure 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 endo/epicardial blood flow ratios equally well.

The inter-method correlation for various non-myocardial tissues is shown in Figure 4 (one experiment) and in Table 2 (pooled data for all experiments are presented). For these organs the inter-method correlation was also good (r = 0.982) while the intercept and the slope of the regression equation were not significantly different from 0 and 1, respectively.

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 6 different dyes in the same experiment.

The accuracy of the blood flow measurements, expressed as the inter-method 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), or negative

pressure filtration in myocardial (r=0.95) and kidney samples (3 fluorescent labels, r=0.96) [7], or using 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. Also, the relative inter-method error for myocardium found in this study (-1.90 ± 11.15 %) was slightly, but not significantly, smaller from that found by Glenny and colleagues (5.10 ± 15.49 %)[7]. The data obtained in the present study also show that the absolute inter-method error for ischemic myocardium (0.00 ± 0.04) is smaller than in non-ischemic 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, liver).

An important practical limitation of the fluorescent or other non-radioactive microspheres is the time and costs involved in processing a sample. For the radioactive microsphere technique this time is relatively short and processing easy. Time and costs 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, while in the vacuum filtration [7] and the sucrose-cushion [2] technique 10 min and at least 9.5 min are involved, respectively. This means a reduction of at least 37% with the simplification described in the present study. Total costs for the sedimentation method amount to $ 0.17, and for the other methods to $ 0.52 and $ 5.77, respectively, which means that a cost reduction of at least 67% can be achieved with the sedimentation method. We therefore conclude, that the sedimentation method

saves time and money compared to negative pressure filtration or counting the microspheres.

***Sources of error*:**

As can be seen in Figure 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 less than 5%. In the present study for each label the number of microspheres injected ($3 \cdot 10^6$ into left atrium) resulted in a mean number of 1580 microspheres, ranging from 10 microspheres in small ischemic samples to 7500 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 the 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 method demonstrates that these possible sources of error are negligible compared to the error related to the number of microspheres per sample. Also the fact that a small, slightly variable amount (less than 0.4 ml) of fluid (mainly water) remained in the test tubes after the 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, since the volume of distribution of the highly lipophylic dyes in the solvent is nearly 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 [13] [16] [6] [10]. There are no differences in diameter between radioactive and fluorescent microspheres, but there is a significant difference in specific gravity (1.3 $g \cdot ml^{-1}$ for radioactive and 1.05 $g \cdot ml^{-1}$ 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 can not conclusively answer this question, comparison of endo/epicardial ratios obtained with fluorescent and radioactive microspheres gives some insight into this aspect. Because of the close similarity between the endo/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 endo/epicardial blood flow ratios.

***Fluorescence Technology*.**

As has be 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 was 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 theoretical less than 1.6%. Spillover between the 6 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-ethoxy) ethyl acetate) has a significant intrinsic background in the blue excitation/emission wavelength range. Also, 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 5 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 to 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 that
5) endo/epicardial blood flow ratios estimated with fluorescent microspheres equals those estimated with radioactive microspheres.

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Legends to Figures

Fig 1. Inter-method 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 ($ml \cdot min^{-1} \cdot sample^{-1}$)

A: Regression plot for inter-method evaluation of the blood flow measured with fluorescent microspheres (Q_{FM}) as a function of the mean value of blood flow determined with Niobium (Nb) and Tin (Sn) labeled microspheres ($\bar{Q}_{RM}$). Blood flow was measured in samples from ischemic (subscript i) and non-ischemic (subscript ni) myocardium with Yellow Green (YG_i and YG_{ni}) and with Crimson (CR_i and CR_{ni}) labeled microspheres, respectively. The regression equations for ischemic and non-ischemic samples together are:

$\bar{Q}_{RM} = 0.05 + 0.98 * Q_{YG}$; SEE = 0.022; R = 0.992 and

$\bar{Q}_{RM} = 0.01 + 0.99 * Q_{CR}$; SEE = 0.031; R = 0.984.

B: Absolute inter-method error (a.e.), as a function of the mean value of blood flow determined with Nb, Sn, YG and CR labeled microspheres ($\bar{Q}$.)

a.e. = $\bar{Q}_{RM} - Q_{YGi}$ = -0.04±0.05 (mean±sd in $ml \cdot min^{-1} \cdot sample^{-1}$) and $\bar{Q}_{RM} - Q_{CRi}$ = -0.00±0.07 for ischemic samples and $\bar{Q}_{RM} - Q_{YGni}$ = -0.02±0.08 and $\bar{Q}_{RM} - Q_{CRni}$ = 0.01±0.12 for non-ischemic samples.

Fig 2: Intra-method comparison

Same experiment and abbreviations as fig 1, flow per myocardial sample ($ml \cdot min^{-1} \cdot sample^{-1}$).

A: Blood flow measured with Nb labeled microspheres ($Q_{Nb(i)}$ or $Q_{Nb(ni)}$) plotted as a function of blood flow measured with Sn labeled microspheres ($Q_{Sn(i)}$ or

$Q_{Sn(ni)}$) for evaluation of the radioactive intra-method variation. Also intra-method evaluation for fluorescent method ($Q_{YG(i)}$ or $Q_{YG(ni)}$ as a function of $Q_{CR(i)}$ or $Q_{CR(ni)}$) is plotted. The regression equations for ischemic and non-ischemic samples together are:

$Q_{NB} = 0.023 + 0.972 * Q_{SN}$, SEE = 0.053, R = 0.995; and

$Q_{CR} = -0.035 + 1.00 * Q_{YG}$, SEE = 0.092, R = 0.986.

B: Absolute intra-method error (a.e.), as a function of the mean value of blood flow determined with Nb, Sn, YG and CR labeled microspheres ($\bar{Q}$.)

a.e. = $Q_{Sn(i)} - Q_{Nb(i)}$ = 0.00±0.05 (mean±sd in ml·min^{-1}·sample^{-1}) and $Q_{YG(i)} - Q_{CR(i)}$ = 0.04±0.08 for ischemic samples and $Q_{Sn(ni)} - Q_{Nb(ni)}$ = 0.01±0.06 and $Q_{YG(ni)} - Q_{CR(ni)}$ = 0.02±0.10 for non-ischemic samples.

Fig 3: Endo/epicardial blood flow ratios determined by simultaneous injection of microspheres labeled with two different fluorescent and two different radioactive labels each per experiment. The plot shows pooled data from all experiments, Line of unity is plotted. The 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.

Fig 4: Inter-method evaluation of regional perfusion measurements for non-myocardial organs as determined by simultaneous injection of fluorescently (Q_{FM}) and radioactively labeled microspheres (Q_{RM}) for one experiment. Relative blood flow (expressed as the relative fluorescence or radioactivity. g^{-1}), is plotted on a logarithmic scale. The drawn line is the line of unity. The 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 ml·min^{-1}·g^{-1} (kidney).

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

Table 2 **Inter- and Intra-method Regression**

Myocardial Samples (n=168)	Slope	Intercept	Correlation
$\bar{Q}_{RM}$ vs. Q_{FM}	0.98±0.03	0.04±0.06	0.985±0.01
Q_{FM} vs. Q_{FM}	0.99±0.04	0.01±0.07	0.981±0.02
Q_{RM} vs. Q_{RM}	0.99±0.02	0.01±0.01	0.997±0.00

Other organs (n=59)	Slope	Intercept	Correlation
Q_{FM} vs. Q_{RM}	0.99±0.05	-0.01±0.01	0.982±0.01

Regression analysis of the flow values in the myocardium (top) and various other organs (bottom) are shown. $\bar{Q}_{RM}$ and Q_{FM}: blood flow determined with radioactive and fluorescent microspheres, respectively. The pooled data from all experiments are presented. values are expressed ± SD.

Table 3 **Absolute Inter- and Intra-method Error**

	Comparison:			
Exp.	$\bar{Q}_{RM}$-Q_{FM1}	$\bar{Q}_{RM}$-Q_{FM2}	Q_{FM1}-Q_{FM2}	Q_{RM1}-Q_{RM2}
	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	

Inter- and intra- method error of myocardial blood flow determination for each experiment separately. Mean difference ± SD (ml/min/sample) is indicated. *:p,0.05 compared with zero. $\bar{Q}_{RM}$: mean flow determined with two radioisotopes, $Q_{FM\ 1\ or\ 2}$ flow determined with one of the two simultaneously injected fluorescent microspheres (fluorescent dyes used: blue (BL), blue-green (BG), yellow-green (YG), orange (OR), red (RD), and crimson (CR); radioisotopes used: Ce, Nb, Sn and Ru.) In experiment 5 only one radioisotope (Ce) was used. Exp. = number of experiment.

Figure 1

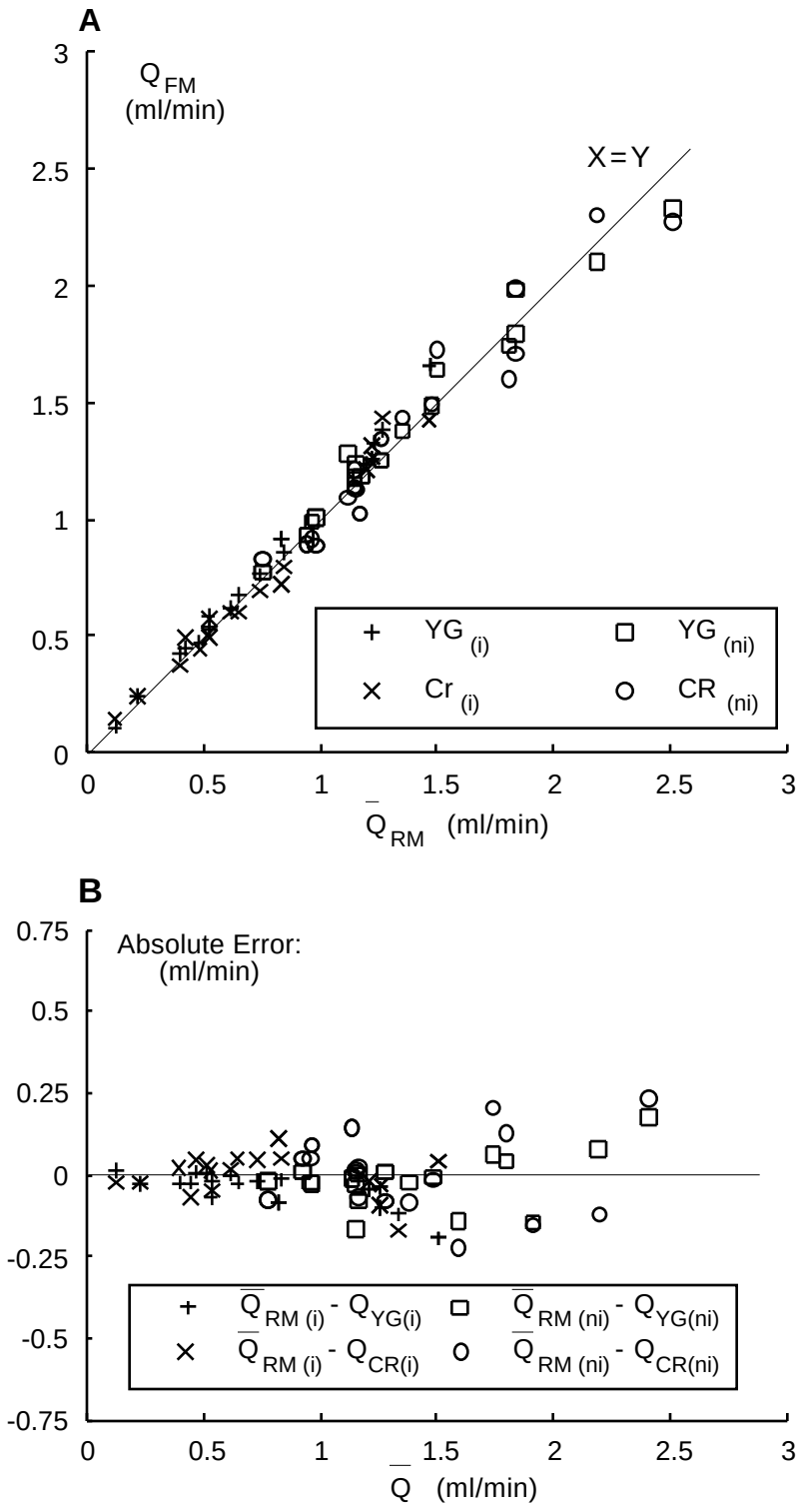

A
Q FM (ml/min)
X=Y
YG (i)
YG (ni)
Cr (i)
CR (ni)
Q RM (ml/min)
B
Absolute Error: (ml/min)
Q RM (i) - Q YG(i)
Q RM (ni) - Q YG(ni)
Q RM (i) - Q CR(i)
Q RM (ni) - Q CR(ni)
Q (ml/min)

Figure 2

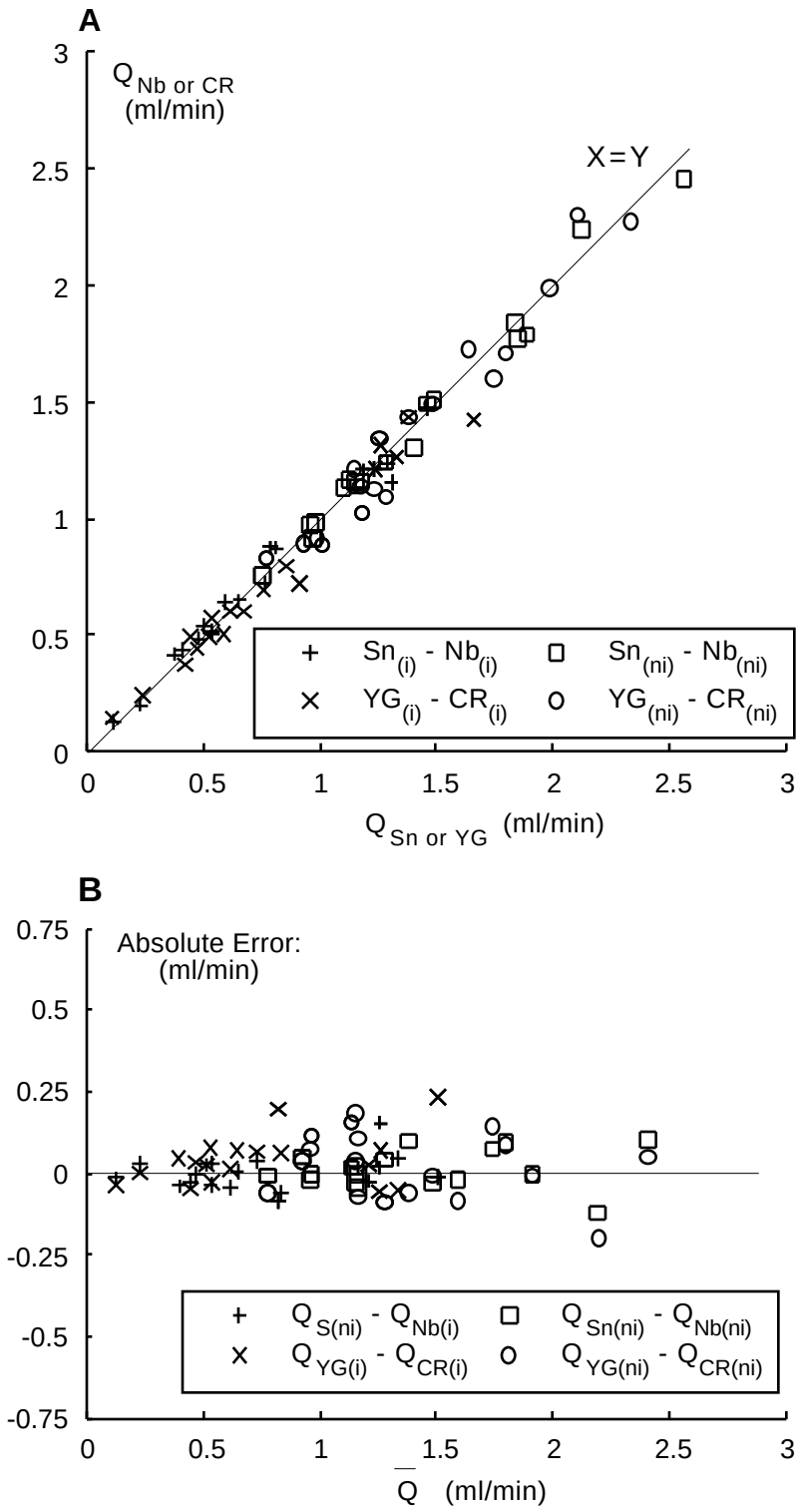

A
Q Nb or CR (ml/min)
X=Y
Sn(i) - Nb(i)
Sn(ni) - Nb(ni)
YG(i) - CR(i)
YG(ni) - CR(ni)
Q Sn or YG (ml/min)
B
Absolute Error: (ml/min)
Q S(ni) - Q Nb(i)
Q Sn(ni) - Q Nb(ni)
Q YG(i) - Q CR(i)
Q YG(ni) - Q CR(ni)
Q (ml/min)

Figure 3

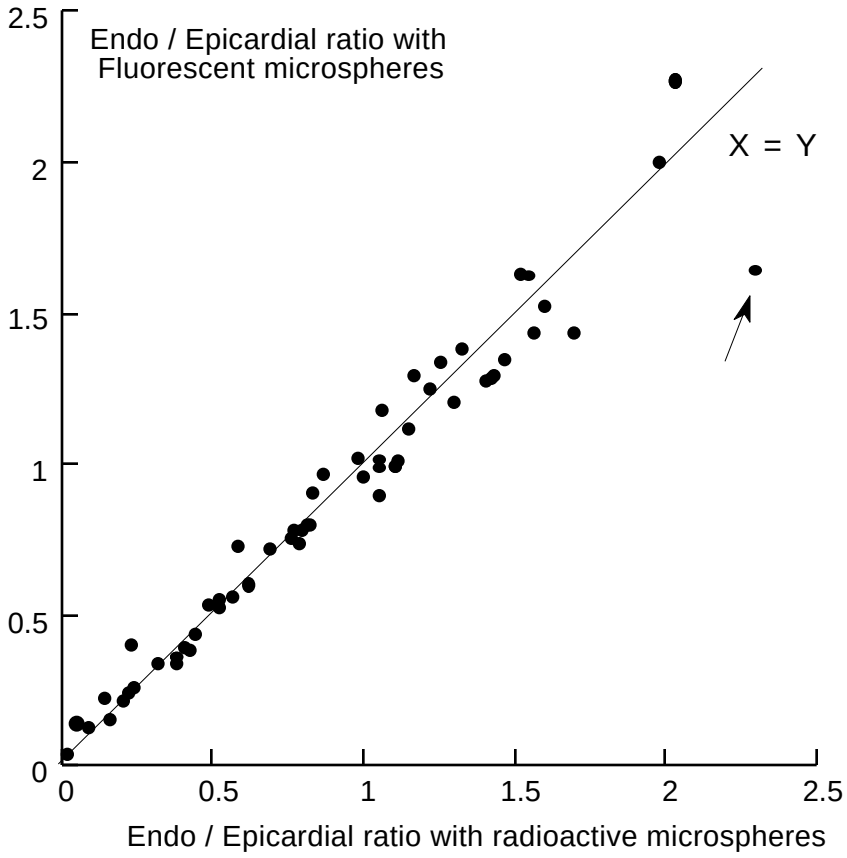
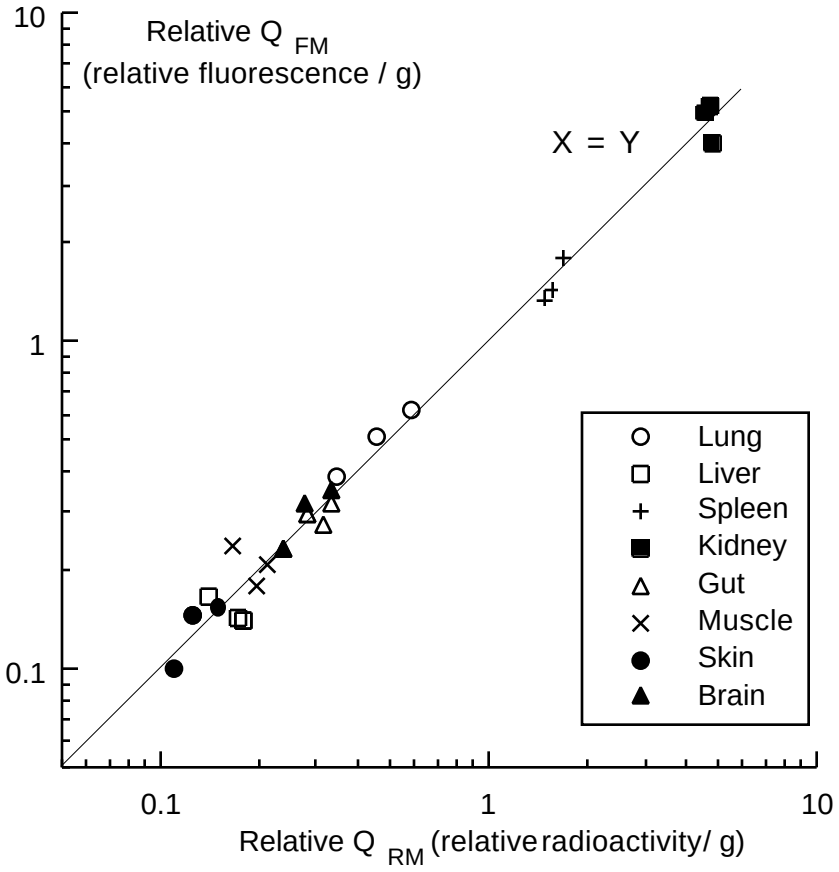

Endo / Epicardial ratio with
Fluorescent microspheres
X = Y
2.5
2
1.5
1
0.5
0
0
0.5
1
1.5
2
2.5
Endo / Epicardial ratio with radioactive microspheres

Figure 4.


10
1
0.1
Relative Q FM
(relative fluorescence / g)
X = Y
Lung
Liver
Spleen
Kidney
Gut
Muscle
Skin
Brain
0.1
1
10
Relative Q RM (relative radioactivity/ g)