On the validity of blood flow measurement using colored microspheres

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Hodeige, D., M. De Pauw, W. Eechaute, J. Weyne, and G. R. Heyndrickx. On the validity of blood flow measurement using colored microspheres. *Am. J. Physiol.* 276 (*Heart Circ. Physiol.* 45): H1150–H1158, 1999.—The aim of this study was *1*) to investigate the validity of repeated estimations of blood flow using colored microspheres (CMS) and *2*) to develop and validate a method that permits four consecutive estimations in the same animal using nonradiolabeled microspheres (NRMS). Several mixtures of different types of microspheres were injected in dogs, with each mixture containing the radiolabeled microspheres (RMS; labeled with ^{113}Sn) with either three CMS, four CMS, or three CMS and one type of fluorescent (crimson labeled) microsphere (FMS). The blood flows estimated with the use of any of the injected microspheres were compared with those measured using the RMS as the "gold standard." The results were analyzed by *1*) regression analysis, *2*) variance analysis (ANOVA I), and *3*) estimation of the limits of agreement between RMS and NRMS flow rates. The results indicate that simultaneous estimations of blood flow obtained with the use of more than three CMS lack accuracy and reliability. A combination of three types of CMS with crimson-labeled FMS, however, offers the possibility to estimate consecutively four different flow rates in the same animal in an accurate way and with relatively high precision.

radioactive microspheres; fluorimetry; absorptiometry; gamma spectrometry

COLORED (CMS) as well as fluorescent microspheres (FMS) are used for measuring regional blood distribution in animals (7, 9, 13). The method in which either CMS or FMS are used has been validated (7, 8, 11) indirectly by comparing the results with those obtained via the method in which radioactive microspheres (RMS) are used, which are regarded as a "reference standard" for the measurement of the blood flow in experimental research.

Frequently, repeated blood flow measurements in the same animal (same tissue or organ) must be performed (1, 16). For this purpose, microspheres labeled with different color or fluorescent components are injected, and, thereafter, the components are measured simultaneously. The determination of the amounts of the different components may be disturbed by interference between the different absorption or fluorescent spectra or by (negative or positive) background effects due to incompletely digested substances from blood or tissue.

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For several years, we have conducted experiments in dogs in which regional blood flow measurements were performed. In these studies (3, 10), microspheres labeled with ^{113}Sn, ^{60}Co, ^{86}Rb, or ^{85}Sr were used for repeated (usually four) estimations of the blood flow in the same dog under different experimental conditions. The disadvantages (manipulation, radioactive waste, costs) inherent in the use of RMS have incited us to replace them, when possible, with nonradiolabeled microspheres (NRMS). The need for the use of CMS was obvious, because no apparatus for simultaneous fluorimetric measurement of the different fluorescent components currently used was available in our laboratory. Preliminary experiments, however, showed that the required accuracy and reliability were not attained in a number of experiments in which more than three CMS were injected. Because our experimental protocol necessitates four repeated estimations in the same animal, we undertook an analytic study with respect to the validation of a method for obtaining four repeated blood flow estimations using a combination of different CMS with one FMS. This was performed by comparing the results of in vitro as well as in vivo experiments.

MATERIALS AND METHODS

Materials

CMS [diameter (mean ± SD): 15.5 ± 0.2 μm], solubilized in saline + 0.1% Tween 80 + thimerosal and containing 60 million spheres/20 ml suspension, were purchased from Triton Technology. They were labeled with one of the following color components: white (Blancophor) with maximum absorbance at 370 nm; yellow (Resolin Brillantgelb) with maximum absorbance at 780 nm; red (Rouge Terasil E-BST) with maximum absorbance at 530 nm; blue (Resolin Brillantblau) with maximum absorbance at 672 nm; or violet (Resolin Rotviolet) with maximum absorbance at 740 nm.

The pure white, yellow, blue, and violet components were from Bayer (Leverkusen, Germany), and the pure red component was from Ciba-Geigy (Zurich, Switzerland). Microspheres labeled with the crimson fluorescent component from Molecular Probes (Eugene, OR) were also used in a series of experiments. The RMS labeled with ^{113}Sn [diameter (mean ± SD): 15 ± 0.5 μm; solubilized in saline + 10% dextran + 0.01% Tween 80; specific activity = 12.5 mCi/g] were from NEN (Boston, MA). The following products or reagents were used for the isolation of the microspheres from tissue or blood: 4 M KOH solution, Tween 80, and *N*,*N*-dimethylformamide (DMF) from Aldrich (Milwaukee, WI); and a 22-mm drain disk polyethylene (PE) support filter (SN 1800614) from Nucleopore (Costar, Bodenheim, Germany).

Methods

Digestion procedure for tissue and blood. Seven milliliters of KOH solution (4 M for tissue; 12 M for blood) containing 2% Tween 80 (freshly prepared) were added to a tube (with glass stop) containing either 500–600 mg of tissue or 2 ml of blood; the tube was shaken continuously in a water bath at 70°C for 24 h. After digestion, the hot liquid in the tube was filtered, under light vacuum, through a 25-mm PE membrane (8.0 μm) laying on a PE drain disk filter (22-mm diameter), with both membrane and drain disk supported by a metallic grate of 25 mm in diameter and 0.5 mm in thickness; the filter, membrane, and grate were clamped between two round clamps of stainless steel, and the underclamp was connected to a vacuum pump. The tube and membrane filter were washed three times with 5 ml of 4 M KOH solution containing 2% Tween. This filtration system has been shown to give a 100% recovery of any of the microspheres used.

After filtration, the 25-mm membrane (8.0 μm) containing the microspheres was taken with a tweezer, transferred onto the bottom of a glass tube, and allowed to dry at room temperature (overnight); 0.3 ml DMF was then added, and the tube was vortexed for 10 s. After centrifugation, the supernatant DMF extract was transferred to a small tube (0.5 ml); the tube was closed until measurement was performed by absorptiometry and fluorimetry.

Gamma spectrometry. Before the tissue or blood was digested with KOH solution, the tube containing the tissue or blood was put into a plastic tube (inner diameter: 2 cm; height: 20 cm) with a round bottom, and gamma emission was counted (counts/min) for at least 5 min in a Berthold gamma spectrometer with settings corresponding to the total energy of the ^{113}Sn radioisotope. All measurements of radioactivity were performed under the same conditions. Count ranges of tissues were >2,000 counts/min and were corrected for the background count range (40 counts/min).

Absorption spectrophotometry. The absorbance (A) from 325 to 750 nm of the DMF extract was measured with a Beckman (UV/VIS 7000) diode array spectrophotometer (wavelength accuracy: 1.25 nm; wavelength repeatability: 0.05 nm; spectral bandwidth: 2 nm; photometric accuracy: 0.005 absorption units). The software program of the spectrophotometer makes it possible to calculate the concentration (in μg/ml) of each color component in the DMF extract by comparing the measured absorbance with that of an adequate standard curve constructed using the instrument for either one color component or a mixture of three or four color components; the standard curves were calculated by Fourier analysis on the basis of 12 different concentrations of one color component or 12 different mixtures of color components. The number of microspheres per milliliter of DMF extract was calculated by multiplying the concentration of the color component of the extract by the number of microspheres per microgram of color component, which was determined experimentally by measuring the amount (in μg) of color component per milliliter of microsphere suspension for which the number of microspheres per milliliter of suspension was known.

Fluorimetry. Fluorimetry of the DMF extract was performed immediately after absorptiometry using a Farrand K2 spectrofluorometer (bandwidth: 3 nm). DMF extracts (200 μl) were diluted to 1 ml with 2-ethoxyethyl acetate (Aldrich), and the relative fluorescence at 640 nm was measured using excitation light of 605 nm. The number of microspheres in the DMF extract (0.3 ml) was calculated from a standard curve plotting the relative fluorescence versus the number of microspheres; the number of microspheres was determined experimentally from a DMF extract of the original crimson-labeled microsphere suspension for which the number of microspheres per milliliter of suspension was known. The fluorescence standard curve was linear up to 5,000 microspheres, and the sensitivity was ≤30 microspheres.

Calculation of blood flow. Tissue blood flow (F; expressed in $ml \cdot g^{-1} \cdot min^{-1}$) was calculated from the reference blood flow (7.64 ml/min) and the number of microspheres in tissue and reference blood according to

$$F = 7.64 \text{ ml/min} \times \frac{\text{No. of microspheres/g tissue}}{\text{No. of microspheres in reference blood}}$$

Experimental protocol

In vivo experiments. In this series of experiments blood flow rates in different organs (heart, lung, kidney) were simultaneously measured using different types of CMS and then compared with measurements obtained using RMS, which are accepted as the gold standard. A total of 10 mongrel dogs (weighing between 18 and 24 kg) were anesthetized with pentobarbital sodium (Nembutal; 30 mg/kg iv). A left thoracotomy was performed in the fourth intercostal space. The pericardium was opened and the heart exposed. Catheters were inserted into the descending thoracic aorta and the left atrial appendage. The left circumflex coronary artery was carefully dissected and instrumented with a snare occluder. Simultaneous injections of 3 or 4 NRMS (CMS or CMS + FMS) together with RMS were performed in basal conditions (3 dogs), during maximal coronary hyperemia with dipyridamole (1.12 mg/kg iv; 4 dogs), and during left circumflex coronary artery occlusion (3 dogs). NRMS and RMS were sonicated and vortexed before and after mixing, and the mixture of microspheres was injected into the left atrial appendage. Simultaneously, a blood sample was withdrawn from the descending thoracic aorta at a speed of 7.64 ml/min for 150 s. At the end of the experiment, the dogs were killed with a lethal dose of pentobarbital sodium (50 mg/kg). Tissue samples were taken from the left ventricular free wall, the lungs, and the kidneys. The samples were weighed, placed in tubes, and counted for gamma emission (see METHODS). Thereafter, the samples were processed for digestion, followed by absorptiometry and/or fluorimetry.

Three groups of dogs were distinguished according to the composition of the injected mixture of NRMS with RMS. *Group I* (6 dogs) comprised white, yellow, and red CMS plus RMS; *group II* (2 dogs) comprised white, yellow, red, and blue CMS plus RMS; and *group III* (2 dogs) comprised white, yellow, and red CMS plus crimson FMS and RMS.

The mixture for *group I* contained the following numbers of microspheres: 2×10^6 RMS, 6×10^6 white, 4.5×10^6 yellow, and 5×10^6 red. In experiments with *groups II* and *III*, the number of microspheres differed by dog and by type of microsphere as follows: RMS, 2.0×10^6 (all dogs); white, 6.0×10^6 (*dogs G*, *H*, *K*, and *L*), 4.5×10^6 (*dogs I* and *J*), or 5.0×10^6 (*dogs M* and *N*); yellow, 4.5×10^6 (*dogs G*, *H*, *K*, and *L*), 5.5×10^6 (*dogs I* and *J*), or 4.8×10^6 (*dogs M* and *N*); red, 5.0×10^6 (*dogs G*, *H*, *K*, and *L*), 5.6×10^6 (*dogs I* and *J*), or 5.8×10^6 (*dogs M* and *N*); blue, 5.0×10^6 (*dogs G* and *H*), 4.0×10^6 (*dogs I* and *J*); and FMS, 2.0×10^6 (*dogs K* and *L*) or 3.0×10^6 (*dogs M* and *N*).

In vitro experiments. This series of experiments was performed to examine the accuracy and precision of the analytic technique (isolation procedure and measurement of color or fluorescent components). First, each type of microsphere was subjected to the isolation procedure, either individually or as a mixture of different microspheres (either white, yellow, and red CMS + crimson FMS or white, yellow, red, and blue

CMS), and extracted with 0.3 ml DMF; the recovery value for each type of microsphere was calculated by comparing the absorbance or fluorescence of the extract with those of the same number of microspheres directly extracted with 0.3 ml DMF and measured either individually or as a mixture.

Second, to examine whether absorptiometry or fluorimetry is disturbed by interfering substances from tissue, different samples of noncontaminated fresh heart tissue (n = 10, ranging from 1,155 to 1,235 mg) were dissolved, extracted with 0.6 ml DMF, and divided in two parts. Each part was added to a reference mixture of either white, yellow, and red CMS and crimson FMS or white, yellow, red, and blue CMS; their absorbance and fluorescence values were compared with those of the pure reference mixture.

Third, to obtain an idea about the precision of a simultaneous measurement of the flow rate in a dog by using microspheres labeled with white, yellow, red, and crimson components, duplicate samples (N = 30 samples) were prepared containing different amounts (from 578 to 612 mg) of fresh heart tissue and a mixture of a variable number of (white, yellow, red, and crimson) microspheres corresponding with flow rates between 0.85 and 4.65 $ml \cdot g^{-1} \cdot min^{-1}$. The series of duplicate samples was digested and extracted as described in *Digestion procedure for tissue and blood*. The flow rates were calculated using the blood reference standard of *dog I*.

All experimental procedures were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Statistical Analysis

To establish whether flow rates estimated using CMS or FMS agreed with those measured using RMS, the results were analyzed with the use of three different types of statistical analysis: regression analysis, variance analysis, and estimation of the precision of the limits of agreement.

Regression analysis. The curve that best fits the points when the CMS or FMS flow rates (y-axis) of the tissues of all dogs of the in vivo experiments (*groups I–III*) are plotted versus the RMS flow rates (x-axis) was calculated by linear regression analysis. The strength of the relation between both variables was expressed by the correlation coefficient (R). The 95% confidence interval (CI) of each regression curve was calculated, and the slope of the regression curve, or regression coefficient (b), was compared with unity (see Table 1).

Variance analysis. The flow rates of all analyzed tissues of each dog (*groups I–III*) measured using the same type of microsphere were considered as a set of data. Differences between the means of the different sets (different types of microspheres) were analyzed by the method of repeated measures of variance (ANOVA I, with Dunnett's multiple-comparison test), with the flow rates of each tissue item measured using the different types of microspheres being linked.

Estimation of precision of agreement. The precision of the agreement between the RMS flow rates (as reference) and those measured using each of the CMS (i.e., white, yellow, red, and blue) or the FMS was calculated according to the method of Bland and Altman (2). Therefore, the RMS flow rates of each type (*group I*, *II*, or *III*) of the in vivo experiments were divided into classes (see Tables 5–7) and the following parameters calculated: *1*) the differences (D) between RMS and either CMS (white, yellow, red, or blue) or FMS flow rates; *2*) the mean difference ($\overline{D}$) and standard deviation (s) of each flow rate class; *3*) the limits of agreement ($\overline{D} - 2s$ and $\overline{D} + 2s$); *4*) the 95% CI extreme lower (LL) and upper precision limits (UL) of $\overline{D} - 2s$ and $\overline{D} + 2s$, respectively; and *5*) the $\overline{D}$ values as well as the LL and the UL values of each class expressed as percentages of the RMS flow rate mean ($\overline{F}$) of the class (i.e., $100\overline{D}/\overline{F}$, $100LL/\overline{F}$, and $100UL/\overline{F}$, respectively). With respect to the in vitro experiments, an estimate of the standard deviation was calculated from the differences (d) between the two results of the duplicates using the formula $s = \sqrt{\Sigma d^2/2N}$, and the CI was calculated using the formula $CI = 2.042s/\sqrt{N}$ ($\alpha = 0.05$).

Table 1. *Overlap matrix of colored components*

	White	Yellow	Red	Violet	Blue
White	100.0	8.7	0.0	0.0	0.0
Yellow	11.2	100.0	1.1	0.0	0.0
Red	4.4	16.2	100.0	6.1	0.0
Violet	12.1	6.4	70.7	100.0	0.0
Blue	20.2	2.8	8.6	14.1	100.0

Data represent spectral overlap (%) calculated from absorption measurements of equal concentrations of colored microspheres in *N*,*N*-dimethylformamide.

RESULTS

Reciprocal Interference of Color and Fluorescent Components

The reciprocal interference of the absorption spectra of the color components was derived from measurements of the absorption spectra of equal concentrations of the components in DMF. The results (Table 1) show *1*) no spectral overlap of white or yellow with red, violet, or blue component; *2*) an important overlap of red with yellow component; *3*) a very high overlap of violet with red component; *4*) an important overlap of blue with white component; and *5*) a yet higher overlap of a combination of blue and red or of blue and yellow with the white component.

Fluorescence measurements have shown that the relative fluorescence (at 640 nm, using excitation light of 605 nm) of amounts of crimson component, corresponding with 400 crimson-labeled microspheres, is not disturbed by comparable amounts of the color components used.

In Vivo Experiments

Regression analysis. Table 2 summarizes the parameters of the regression curves between CMS (or FMS) flow rates (y-axis) and RMS flow rates (x-axis) measured in all analyzed tissues of each group (*groups I–III*) in the in vivo experiments. When white, yellow, and red color components with or without the crimson fluorescent component (*groups I* and *III*) are estimated simultaneously, a strong linear relationship is seen between CMS or FMS flow rates and RMS flow rates. Table 2 shows that *1*) the correlation coefficients R, being >0.980 (a somewhat lower $R = 0.974$ was found for the curve for red CMS), express a high correlation between any of the CMS or FMS flow rates and the RMS flow rates, and *2*) the regression coefficients b (between 0.960 and 0.982; a somewhat lower value was

Table 2. *Regression analysis between flow rates measured by RMS and CMS or FMS*

Experiment Group	Type of Microsphere	Regression Curve	95% CI for *b* LL	95% CI for *b* UL	*R*
I	White	$y = 0.023 + 0.976x$	0.958	0.992	0.990†
	Yellow	$y = 0.012 + 0.979x$	0.960	0.994	0.991†
	Red	$y = 0.016 + 0.960x$	0.951	0.985	0.992†
II	White	$y = 0.022 + 1.022x$	0.970	1.089	0.976†
	Yellow	$y = 0.201 + 0.798x$*	0.754	0.818*	0.942†
	Red	$y = 0.251 + 0.652x$*	0.592	0.704*	0.870†
	Blue	$y = 0.171 + 0.837x$*	0.796	0.869*	0.954†
III	White	$y = 0.023 + 0.968x$	0.932	1.016	0.990†
	Yellow	$y = 0.040 + 0.982x$	0.966	1.018	0.985†
	Red	$y = 0.092 + 0.931x$	0.941	0.982	0.974†
	Crimson	$y = 0.045 + 0.972x$	0.942	0.995	0.982†

Regression curves (represented as $y = a + bx$) are between flow rates measured using colored (CMS) or fluorescent microspheres (FMS; y) and those measured by ^{113}Sn radiolabeled microspheres (RMS; x), where b is regression coefficient. LL and UL represent lower and upper limits, respectively, of 95% confidence interval (CI) of b. R, Pearson correlation coefficient. * Significantly different ($P < 0.05$) from $b = 1$ or $a = 0$. † Significantly different ($P < 0.01$) from zero.

found for the curve for red CMS of *group III*) are close to 1 and have narrow 95% CI that include 1, indicating that, in the mean, the change of y equals that of x.

When the four color components (white, yellow, red, and blue; *group II*) are measured simultaneously, the agreement between CMS and RMS flow rates is less pronounced. Indeed, some R values (i.e., $R = 0.942$ for the curve for yellow CMS and especially $R = 0.870$ for the curve for red CMS), although highly significant ($P < 0.01$), indicate that the association of the points to the regression curve is less perfect than for a simultaneous measurement of the white, yellow, and red color components. Moreover, the b values, especially those for the curve for red CMS, are significantly ($P < 0.05$) lower than 1, and their respective 95% CI limits do not include unity.

Variance analysis. The flow rates measured by a specific type of microsphere in the tissues of a given dog were considered as a set of data (see MATERIALS AND METHODS). The mean values and standard deviations of these sets of flow rates are represented in Tables 3 and 4 for each dog and each group (*I–III*) of the in vivo experiments. Statistical analysis performed to assess a possible difference between the means of these sets, using ANOVA I with repeated measures of variance, indicates first that when dogs were treated with three (white, yellow, and red) CMS (*group I*), only small or nonsignificant differences were seen between the mean flow rates measured by CMS and RMS (Table 3). Indeed, the mean flow rates of *dogs A–F* as measured by white CMS do not differ significantly ($P > 0.05$) from the RMS values. The same holds for the mean flow rates measured by yellow CMS, with the exception of *dog A*, because its mean flow rate was 9% lower ($P < 0.01$) than the mean RMS flow rate. The flow rate means for red CMS are also in good agreement with the RMS values, with the exception of *dogs A* and *F*; the flow rate means for red CMS for these dogs are ~10% (*dog A*) or 9% (*dog F*) lower ($P < 0.05$) than the RMS flow rate mean.

Second, when dogs received four different CMS labeled with white, yellow, red, and blue component, respectively (*group II*), larger differences between the flow rate means measured by CMS and RMS were observed (Table 4). Indeed, compared with the RMS flow rate means, lower ($P < 0.05$) flow rate means for red (from 15 to 33%), blue (from 8 to 12%), and yellow CMS (from 5 to 12%) were seen for *dogs G–J*, whereas the flow rate means for white CMS were ~9% higher ($P < 0.05$). Table 4 also shows the flow rate means of *dogs K–N*, which were treated with RMS, white, yellow, and red CMS, and crimson FMS (*group III*). No significant differences ($P > 0.05$) were observed between the RMS flow rate means and those measured by any of the CMS or the FMS.

Estimation of precision of agreement. The degree of the precision of the agreement between RMS flow rates on the one hand and those measured by each of the CMS or the FMS on the other hand was calculated for the in vivo experiments (see *Statistical Analysis*).

With regard to the experiments with three CMS (*group I*), the results (Table 5) show that *1*) the mean differences ($\bar{D}$), as such or expressed as percentages of the mean RMS flow rates of the classes ($100\bar{D}/\bar{F}$), are almost all positive and low, indicating that somewhat lower flow rates were measured with white (<2.8%),

Table 3. *Blood flow rates in tissues from dogs measured using RMS and CMS*

Dog	Blood Flow Rates, ml·g^{-1}·min^{-1} RMS	White MS	Yellow MS	Red MS
A				
M	5.01	4.85	4.58†	4.49†
s	1.621	1.629	1.526	1.447
n	41	41	41	41
B				
M	1.33	1.27	1.30	1.30
s	0.903	0.912	0.878	0.964
n	42	42	42	42
C				
M	0.66	0.65	0.66	0.66
s	0.500	0.518	0.523	0.504
n	46	46	46	46
D				
M	1.83	1.78	1.79	1.79
s	0.175	0.170	0.176	0.177
n	46	46	46	46
E				
M	1.18	1.21	1.23	1.20
s	0.247	0.250	0.251	0.241
n	32	32	32	32
F				
M	3.91	3.90	3.86	3.60†
s	0.243	0.236	0.232	0.215
n	42	42	42	42

Data are mean flow rates (M) and standard deviations (s) measured in tissues (heart, lung, kidney) from dogs injected with a mixture of RMS and white-, yellow-, and red-labeled microspheres (MS) for in vivo experiments in dogs of *group I*; n = no. of analyzed tissues per dog. † Highly significant differences ($P < 0.01$) between RMS and CMS mean flow rates.

Table 4. *Blood flow rates measured using RMS, CMS, and/or FMS*

Dogs	Blood Flow Rates, ml·g^{-1}·min^{-1}					
	RMS	White MS	Yellow MS	Red MS	Blue MS	Crimson MS
G						
M	2.28	2.45*	2.02†	1.53†	2.05†	
s	0.469	0.484	0.430	0.368	0.394	
n	38	38	38	38	38	
H						
M	1.49	1.64*	1.40*	1.27†	1.32†	
s	0.603	0.674	0.545	0.446	0.551	
n	63	63	63	63	63	
I						
M	1.25	1.39*	1.10*	1.04†	1.17*	
s	0.697	0.740	0.731	0.724	0.732	
n	42	42	42	42	42	
J						
M	1.91	2.12†	1.78*	1.70†	1.54†	
s	0.612	0.601	0.493	0.594	0.562	
n	48	48	48	48	48	
K						
M	1.69	1.74	1.77	1.76		1.71
s	1.369	1.455	1.419	1.382		1.343
n	49	49	49	49		49
L						
M	1.95	1.96	1.94	1.86		1.91
s	0.801	0.802	0.811	0.773		0.798
n	62	62	62	62		62
M						
M	0.82	0.78	0.84	0.80		0.79
s	1.231	1.208	1.182	1.151		1.224
n	48	48	48	48		48
N						
M	1.54	1.62	1.50	1.59		1.50
s	1.111	1.182	1.160	1.124		1.172
n	40	40	40	40		40

Data are mean flow rates (M) and standard deviations (s) measured in tissues (heart, lung, kidney) from dogs injected with a mixture of RMS and either 4 CMS (white, yellow, red, and blue) or 3 CMS (white, yellow, and red) and 1 FMS (crimson) for in vivo experiments in dogs of *groups II* and *III; n* = no. of analyzed tissues per dog. *Significant ($P<0.05$) and †highly significant ($P<0.01$) differences between RMS and CMS mean flow rates.

yellow (<5.4%), and red CMS (<8.3%) than with RMS, and *2*) the values of 100LL/$\overline{F}$ and 100UL/$\overline{F}$ vary from −22.4 to +23.4 for white CMS, from −22.0 to +23.8 for yellow CMS, and from −20.0 to +23.7 for red CMS. Thus, when the blood flow in a dog is measured simultaneously using white, yellow, and red CMS, the mean percentage error of any of the CMS flow rates with respect to the RMS value is low. The 100LL/$\overline{F}$ and 100UL/$\overline{F}$ values, however, indicate that these mean percentage errors may vary between about −22 and +22%.

Comparable results were obtained for repeated blood flow measurements using white, yellow, and red CMS in combination with crimson FMS (*group III*; Table 6). The 100$\overline{D}$/$\overline{F}$ values are also low and negative (for white and yellow CMS) or positive (for red CMS and crimson FMS), indicating that, in the mean, with white and yellow CMS a somewhat higher blood flow, and with red CMS and FMS a somewhat lower blood flow, than with RMS may be anticipated. The ranges of these relative percentage errors, as expressed by the 100LL/$\overline{F}$ and 100UL/$\overline{F}$ values, are comparable with those seen for the dogs of *group I*.

Table 5. *Statistical parameters for differences between flow rates in group I*

Type of Microsphere	Class Flow Rate, ml·g^{-1}·min^{-1}			Statistical Parameters			
	Limits	$\overline{F}$	*n*	$\overline{D}$	100$\overline{D}$/$\overline{F}$	100LL/$\overline{F}$	100UL/$\overline{F}$
White	0.5<F<1.5	1.24	135	0.035	+2.8	−11.4	+21.9
	1.5<F<3.0	2.10	31	0.011	+0.5	−22.4	+23.4
	3.0<F<5.0	4.27	47	0.027	+0.6	−17.5	+18.8
	5.0<F<6.8	5.79	36	0.106	+1.8	−16.8	+20.4
	0.5<F<6.8			0.041	+1.5	−17.0	+21.1
Yellow	0.5<F<1.5	1.24	135	0.012	+0.9	−19.4	+21.3
	1.5<F<3.0	2.10	31	0.012	+0.5	−22.0	+23.2
	3.0<F<5.0	4.27	47	0.194	+4.5	−13.3	+22.3
	5.0 F>6.8	5.79	36	0.312	+5.3	−13.1	+23.8
	0.5<F<6.8			0.090	+3.4	−16.1	+22.3
Red	0.5<F<1.5	1.24	135	−0.008	−0.6	−20.0	+18.8
	1.5<F<3.0	2.10	31	0.066	+3.1	−17.4	+23.7
	3.0<F<5.0	4.27	47	0.257	+6.0	−15.4	+23.6
	5.0 F>6.8	5.79	36	0.485	+8.3	−16.8	+23.5
	0.5<F<6.8			0.122	+4.7	−17.4	+22.4

Data represent mean differences ($\overline{D}$) between flow rates (F) measured using RMS and those measured using 3 CMS (white, yellow, and red) for in vivo experiments in *group I* and divided into classes; *n* = no. of differences per class. $\overline{F}$, mean flow rate per class. LL and UL, lower and upper precision limits, respectively. Statistical parameters include $\overline{D}$ values (100$\overline{D}$/$\overline{F}$) with LL (100LL/$\overline{F}$) and UL (100UL/$\overline{F}$) expressed as percentages of $\overline{F}$ of each class.

When dogs were injected with a mixture of white, yellow, red, and blue CMS (*group II*), considerably higher $\overline{D}$ and 100$\overline{D}$/$\overline{F}$ values were found (Table 7) than for the dogs of *group I* or *III*; the values are negative for white CMS and positive for yellow, red, and blue CMS, indicating that higher flow rates are obtained with white CMS (mean: −8.7%) and lower flow rates are obtained with yellow (mean: 7.7%), red (mean: 19.3%), and blue CMS (mean: 9.6%) than with RMS. Moreover, the 100$\overline{D}$/$\overline{F}$ values vary within a considerably larger range as indicated by the extreme 100LL/$\overline{F}$ and 100UL/$\overline{F}$ limits, with the latter values lying between −81.2

Table 6. *Statistical parameters for differences between flow rates in group III*

Type MS	Class Flow Rate, ml·g^{-1}·min^{-1}			Statistical Parameters			
	Limits	$\overline{F}$	*n*	$\overline{D}$	100$\overline{D}$/$\overline{F}$	100LL/$\overline{F}$	100UL/$\overline{F}$
White	0.5<F<1.5	0.96	125	−0.029	−3.0	−23.2	+17.2
	1.5<F<3.8	2.22	74	−0.091	−4.1	−24.2	+16.1
	0.5<F<3.8			−0.052	−3.6	−24.0	+16.9
Yellow	0.5<F<1.5	0.96	125	−0.060	−6.2	−27.6	+15.2
	1.5<F<3.8	2.22	74	−0.089	−4.0	−22.8	+14.9
	0.5<F<3.8			−0.070	−4.9	−23.9	+14.3
Red	0.5<F<1.5	0.96	125	−0.042	−4.4	−24.8	+16.0
	1.5<F<3.8	2.22	74	+0.084	+3.8	−14.7	+22.3
	0.5<F<3.8			+0.020	−1.4	−21.2	+18.4
Crimson	0.5<F<1.5	0.96	125	−0.028	−2.9	−22.9	+17.1
	1.5<F<3.8	2.22	74	+0.064	+2.9	−15.8	+21.7
	0.5<F<3.8			+0.006	+0.4	−19.6	+20.4

Data represent mean differences between flow rates measured using RMS and those measured using 3 CMS (white, yellow, and red) and 1 FMS (crimson) for in vivo experiments in *group III* and divided into classes; *n* = no. of differences per class.

Table 7. *Statistical parameters for differences between flow rates in group II*

Type of Microsphere	Class Flow Rate, ml·g^{-1}·min^{-1} Limits	$\bar{F}$	*n*	Statistical Parameters $\bar{D}$	100$\bar{D}/\bar{F}$	100LL/$\bar{F}$	100UL/$\bar{F}$
White	0.5<F<1.5	0.90	86	−0.121	−13.3	−81.2	+54.5
	1.5<F<3.6	2.38	105	−0.134	−5.6	−40.2	+29.2
	0.5<F<3.6			−0.129	−7.5	−56.3	+41.2
Yellow	0.5<F<1.5	0.90	86	0.049	+5.4	−29.8	+40.8
	1.5<F<3.6	2.38	105	0.198	+8.3	−25.1	+41.6
	0.5<F<3.6			0.131	+7.7	−26.0	+41.1
Red	0.5<F<1.5	0.90	86	0.178	+19.8	−22.8	+62.4
	1.5<F<3.6	2.38	105	0.452	+19.0	−19.4	+57.2
	0.5<F<3.6			0.329	+19.3	−20.7	+59.4
Blue	0.5<F<1.5	0.90	86	0.104	+11.5	−28.1	+51.1
	1.5<F<3.6	2.38	105	0.212	+8.9	−40.4	+58.4
	0.5<F<3.6			0.164	+9.6	−34.4	+53.6

Data represent mean differences between flow rates measured using RMS and those measured using 4 CMS (white, yellow, red, and blue) for in vivo experiments in *group II* and divided into classes; *n* = no. of differences per class.

and +54.5 for white CMS, between −29.8 and 41.6 for yellow CMS, between −22.8 and 62.4 for red CMS, and between −40.4 and +58.4 for blue CMS, respectively. The higher range of the differences between RMS and CMS flow rates in the experiments of *group II* compared with those of *group III* is also illustrated in Figs. 1 and 2.

Finally, attempts to perform four repeated estimations in the same dog using white, yellow, red, and violet (instead of blue) CMS resulted in a considerably lower accuracy and reliability of the calculated flow rates (data not discussed in detail here).

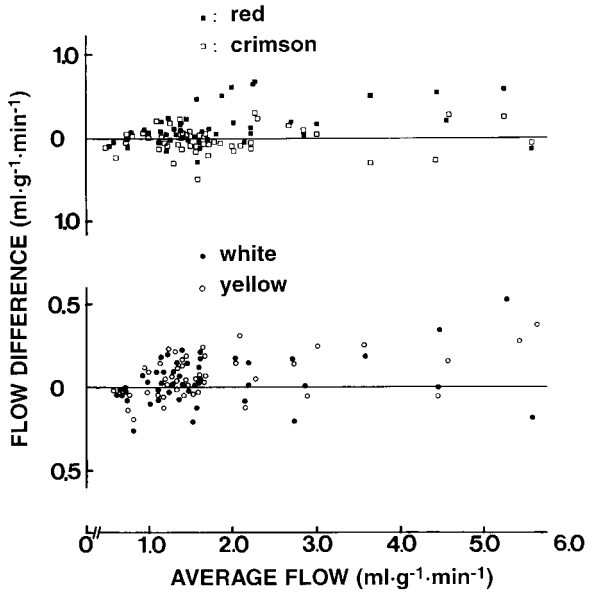

Fig. 1. Blood flow estimation using radiolabeled (RMS), white, yellow, and red colored microspheres (CMS), and crimson fluorescent microspheres (*group III*). Absolute differences shown are between blood flows estimated using RMS and nonradiolabeled microspheres (NRMS) and corresponding mean blood flows estimated for each labeled microsphere.

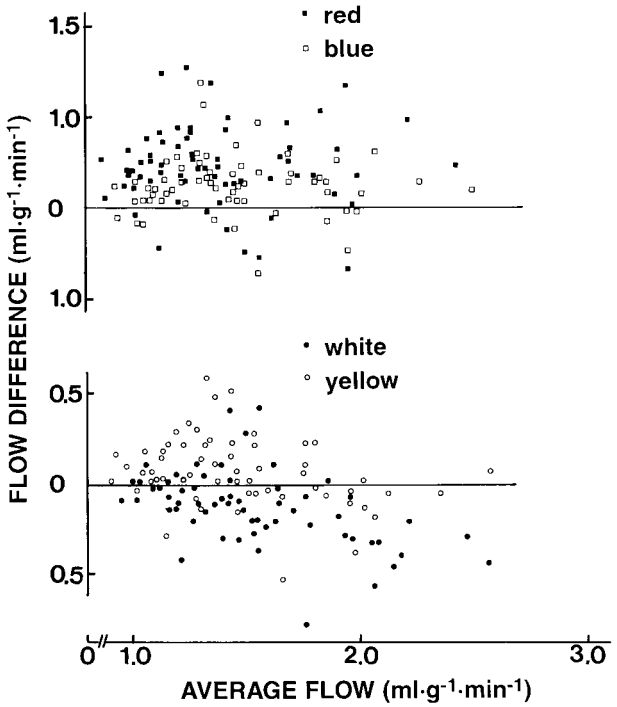

Fig. 2. Blood flow estimation using RMS and white, yellow, red, and blue CMS (*group II*). Absolute differences shown are between blood flows estimated by RMS and NMRS and corresponding mean blood flows estimated for each labeled microsphere.

In Vitro Experiments

The purposes of the in vitro experiments were *1*) to obtain an idea about the accuracy and precision of the analytic procedure in the absence of tissue and *2*) to examine whether the absorptiometry or fluorimetry was disturbed due to components from incompletely digested tissue. The results with respect to the accuracy and precision (Table 8) show that microspheres, when treated separately through the analytic procedure, were almost completely recovered (~98.3–99.3%) with a good precision, with the coefficients of variation (CV) lying between 1.6 and 2.4%.

When a mixture of white, yellow, and red CMS and crimson FMS was treated in the same way, comparable recoveries (between 97.8 and 98%) and CV values (between 1.9 and 2.9%) were found. For a mixture of white, yellow, red, and blue CMS, somewhat lower recovery (between 95.8 and 97.1%) with higher CV values (between 3.6 and 4.7%) were found.

The results of the experiments concerning the suitability of the digestion procedure (Table 8, labeling component data) show that *1*) the absorbance or fluorescence of white, yellow, red, and crimson labeling components, when measured as a mixture, is recovered almost completely (97.6–100.6%) and with high precision (CV: between 1.1 and 2.4%) in the presence of tissue extract; and *2*) somewhat lower recoveries (92.1–96.2%) with lower precision (CV: between 3.8 and 5.7%) are found when the four color components (white, yellow, red, and blue) were measured in the same conditions.

Table 8. *Recovery of microspheres or labeling components*

	Recovery, %				
	Mode of microsphere addition			Labeling components	
	Individual	3 CMS + 1 FMS	4 CMS	3 Colors + 1 fluorescent	4 Colors
White	98.4 (1.9)	98.3 (2.6)	96.9 (4.2)	98.9 (2.1)	96.2 (3.8)
Yellow	99.1 (2.2)	98.2 (2.1)	96.4 (4.7)	97.6 (1.1)	94.2 (4.1)
Red	98.4 (2.1)	97.8 (2.9)	95.8 (3.8)	100.6 (2.4)	92.1 (5.7)
Blue	98.4 (1.9)		97.1 (3.6)		95.4 (3.9)
Crimson	99.3 (1.6)	98.7 (1.9)		98.9 (1.4)	

Data represent percentages of mean recovery of microspheres or color or fluorescent components of microspheres added to tissue or tissue extract (in vitro experiments); nos. in parentheses are coefficients of variation. Microspheres were added individually or as mixtures of either 3 CMS plus 1 FMS or 4 CMS. Labeling components were added to tissue extract as mixtures of either 3 color components plus 1 fluorescent component or 4 color components.

Finally, from the series ($N = 30$) of duplicate determinations of white, yellow, and red CMS and crimson FMS added to heart tissues (see MATERIALS AND METHODS), the mean flow rates, standard deviations of the duplicates, and confidence intervals ($CI = t \cdot s/\sqrt{N = 1}$, using t statistic with $\alpha = 0.05$) for one determination were calculated for each type of microsphere. The values ($M \pm s$) were as follows: 1.29 ± 0.0911 for white CMS, 3.41 ± 0.264 for yellow CMS, 1.45 ± 0.101 for red CMS, and 1.87 ± 0.088 for crimson FMS. Thus, when blood flow is estimated repeatedly in a dog with the use of these four types of microspheres, there is a 95% chance that any true flow rate X (in $ml \cdot g^{-1} \cdot min^{-1}$) will lie between $X \pm t \cdot s/\sqrt{N = 1}$, i.e., $X \pm 0.186$ for white CMS, $X \pm 0.538$ for yellow CSM, $X \pm 0.204$ for red CMS, and $X \pm 0.176$ for crimson FMS. When the confidence intervals are expressed as percentages of the flow rate means of the series of duplicate determinations, the following percentage errors for one determination were found: 14.4% for white CMS, 12.7% for yellow CMS, 14.0% for red CMS, and 9.4% for crimson FMS.

DISCUSSION

It was the aim of this study to examine the possibility of using nonradioactive (colored and/or fluorescent) microspheres for repeated estimations of blood flow in the same animal. This requires an accurate and reliable determination of several color (and/or fluorescent) components in the same tissue. The validation of the method using NRMS was approached in two ways: first, in a comparative way, i.e., by comparing the results found using NRMS with those obtained using RMS as a "gold standard," and second, in an analytic way by checking the criteria of accuracy and precision of the first method. Because blood flow to any organ may vary with time, it was considered essential to inject the different microsphere types at the same time to assess possible differences between the flow rates. To ensure that the analyzed tissues should contain at least 200 microspheres of each type, between 3×10^6 and 6×10^6 microspheres (according to type) were injected. In this way, an adequate spectrophotometric or fluorimetric estimation was made possible (8) and the error due to statistical variation (4–6, 8, 12, 14, 15) was reduced to a minimum. Because several types of microspheres were injected simultaneously, the total number of injected microspheres was rather high but was lower than the number needed to result in permanent hemodynamic alterations (1). Moreover, it is justifiable to assume that the different microsphere types are distributed to the organs in proportion to their concentration of the injected mixture so that the flow rates measured by them should be equal for each tissue.

The simultaneous injection of different types of microspheres implies that, in case of a complete agreement between the estimated flow rates, the proportion between the number of different microspheres in any analyzed tissue should be the same as in the injected mixture. However, the proportion between the numbers of different microspheres is not a determining factor for concluding whether there is agreement or disagreement between flow rates. Nevertheless, we found it suitable to compare the flow rates in experiments for *groups II* and *III* (four NRMS) on the basis of two different mixtures (see MATERIALS AND METHODS).

The results of the comparative study (in vivo experiments) were analyzed statistically in three ways to conclude whether there was agreement between RMS and CMS or FMS flow rates. Regression analysis shows a very high correlation between these flow rates and each type (*groups I–III*) of experiment. Correlation coefficients, however, although highly significant ($P < 0.01$), only express an extent of association between pairs of x,y variables and do not permit us to conclude whether there is equality between a pair of x,y variables. The estimated derivative of a regression curve or regression coefficient (b), expressing the change of y with x, provides more information about the agreement between two variables having the same dimension; b will indeed be 1 when the two variables of each x,y pair are equal.

The 95% CI of the curves of the experiments with three CMS (*group I*) or with three CMS and one FMS (*group III*) include or nearly include 1, indicating that the flow rates measured by any of them do not (or nearly not) differ from those measured by RMS. On the contrary, the b coefficients of the curves corresponding to the experiments with four CMS (*group II*) are considerably <1, except for the curve for white CSM, and their 95% CI do not include 1. Thus regression analysis reveals a lack of agreement between the blood flow rates measured by yellow, red, and blue CMS and those measured by RMS when repeated blood flow measurements in the same animal are performed using white, yellow, red, and blue CMS.

The method of repeated measurements of analysis of variance, with the number of analyzed tissues taken as replicates of blood flow estimation in different experimental conditions (different types of microspheres), leads to an analogous conclusion. In the experiments in which three types (white, yellow, and red) of CMS were used with or without FMS, the differences between the

mean flow rates measured by RMS and NRMS are either small or not significant, respectively. In the experiments in which four (white, yellow, red, and blue) CMS were used, however, very important and statistically significant differences are seen between RMS and CMS flow rates.

When the differences between the flow rates measured by RMS and CMS or FMS were analyzed in a more direct way, as proposed by Bland and Altman (2), considerably less agreement was found when blue CMS, in addition to the three other CMS, were measured. More importantly, larger 95% CI precision limits of the differences were found under these conditions, indicating a high degree of unreliability of the CMS flow rates. On the contrary, the agreement between RMS and CMS or FMS flow rates and the reliability were much better when blue CMS were omitted or replaced by FMS.

The lack of reliability of blood flow estimations for which a combination of white, yellow, red, and blue CMS were used (*group II*) is not likely due to technical shortcomings: tissues of all experiments were analyzed by the same person in the same way and according to a rigorous analytic procedure. The in vitro experiments (see Table 8), however, indicated that the addition of a mixture of colored components to tissue extract immediately before spectrophotometry yielded lower mean recovery values and a larger range of individual recovery values when the mixture contained blue component. A still lower precision may thus be expected for the blood flow estimations of in vivo *group II*, because each estimated value incorporates the sum of errors of the different steps of the analytic procedure. Moreover, the lack of reliability of estimates for *group II* may also be due to the fact that the blue absorption spectrum, having a maximum peak at 672 nm, shows a slight, yet increasing, absorption in the wavelength zone from 500 to 325 nm, i.e., in the region of the absorption peaks of white, yellow, and red components. Indeed, this overlapping zone increases the risks for *1*) interference between the absorption spectra of the different color components and *2*) disturbances from aspecific components of digested tissue, both negatively affecting the precision of the flow estimations.

In conclusion, the results of our study demonstrate that *1*) three repeated blood flow measurements in the same dog with the use of three CMS (white, yellow, and red) can be performed in an accurate way and with the necessary precision; *2*) a quadruple estimation of the blood flow in the same dog with the use of four CMS (white, yellow, red, and blue) leads to unreliable results that are difficult to interpret, especially in those studies in which changes (increases or decreases) of $<25\%$ of the blood flow are to be measured; and *3*) four repeated estimations of the blood flow in the same animal with a high degree of precision and accuracy are made possible only with the use of the three CMS labeled with white, yellow, and red components, respectively, in combination with FMS labeled with the crimson component.

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