Viewpoint

Developments in non-radioactive microsphere techniques for blood flow measurement

Frits W Prinzen and Robb W Glenny

Determination of blood flow with radioactive particles that lodge in the microcirculation provides measures of regional perfusion between and within organs. Radioactive particle methods give more detailed information than flow probes and are easier to use than molecular washout tracer methods from both a theoretical and practical perspective. These are probably the main reasons for the success of the radioactive microsphere technique, introduced in 1967 by Rudolph and Heymann.[1] In this first publication on the radioactive microsphere technique, these investigators studied the circulation of lambs in utero.[1] Since that time, microspheres have been used in almost every field of cardiovascular research, and in a wide range of species and preparations varying from isolated organs[2-4] to chronically instrumented animals on treadmills.[5]

Despite a number of potential pitfalls and errors (see below), blood flow measurement with radioactive microspheres is currently regarded as a kind of gold standard of blood flow in experimental cardiovascular research. A new gold standard is needed because the use of radioactivity in general, and to an even greater extent in animal experiments, is becoming increasingly problematic because of restrictive legislation and higher costs of storage and disposal. These factors, along with the desire to reduce radiation load to investigator and technicians, have stimulated the search for non-radioactive microspheres.[6-11] During the last five years considerable progress has been made with such techniques. Although these techniques are still in their infancy, non-radioactive microsphere methods are available for several applications. At present there are three different types of non-radioactive microspheres; coloured, fluorescent, and *x* ray fluorescent.

This paper will serve three purposes: (1) to describe the current state of the art for the available non-radioactive microsphere methods; (2) to compare the measurement errors of the non-radioactive microsphere techniques with those of their radioactive counterparts; and (3) to provide suggestions for future directions of research with non-radioactive microspheres.

Non-radioactive microsphere techniques

Quantification by counting of microspheres

The movement towards non-radioactive microspheres started with a paper by Hale *et al.*[6] These investigators introduced a method for measuring blood flow with coloured microspheres. A similar approach was used by Austin *et al.* employing fluorescent microspheres.[11] Blood and tissue samples were digested with the use of special commercially available solutions[6] or with collagenase and proteinase.[11] The spheres were isolated by centrifugation[6] or gradient centrifugation[11] and then counted in a haemocytometer with the use of a light microscope or a fluorescence activated cell sorter (FACS).[11] Good correlations were reported between blood flow values obtained with simultaneously injected coloured and radioactive microspheres.[6 11] At high myocardial blood flow levels (pharmacologically induced) the coloured microsphere method systematically overestimated blood flow.[6] Fluorescent microspheres were validated for the use in non-ischaemic and ischaemic myocardium.[11] Although coloured microspheres are being used in experiments with myocardial ischaemia,[12] no study was found that validated this application.

The microspheres and specific reagents for the coloured microsphere method are commercially available as E-Z TRAC spheres ®, from E-Z TRAC Inc (West Los Angeles, CA 90025, USA); the fluorescent microspheres are commercially available from Polysciences Inc, Warrington, PA, USA.

Quantification by dye extraction and spectrometry

Coloured microspheres – As an alternative to manually counting coloured microspheres, Kowallik *et al* developed a technique where the microspheres are isolated from tissue and blood by digestion with potassium hydroxide and subsequent microfiltration.[7] The dyes are extracted from the coloured microspheres with a known volume of solvent. Separation of the various colours and their concentration is possible by spectrometry, similar to the gamma spectrometry used with the radioactive microsphere method. In vivo validations were performed by simultaneous injection of radioactive and coloured microspheres in coronary perfusion lines of pig hearts and left atrial injections in dogs (the latter using the reference blood withdrawal technique). Five different colours were compared with five different radioisotopes. Myocardial blood flow was varied by pharmacological intervention as well as obstruction of a coronary artery. These investigators found good correlations ($r = 0.97$-0.98) between myocardial blood flow values obtained with the radioactive and coloured microspheres. After injection in the coronary perfusion line, the slope of the

Department of Physiology, Cardiovascular Research Institute Maastricht, University of Limburg, PO Box 616, 6200 MD Maastricht, The Netherlands: F W Prinzen; Division of Pulmonary and Critical Care Medicine, University of Washington, Seattle, Washington 98195, USA: R W Glenny. Correspondence to Dr Prinzen.

relation was close to unity, indicating a very good match of myocardial blood flow as estimated by the two methods. However, this slope was significantly less than unity in the experiments where the arterial reference method was used. The coloured microsphere technique appeared to cause systematic underestimation of blood flow, especially in the physiological flow range (< 1.1 ml·min^{-1}·g^{-1}), where Kowallik reported a slope of 0.79. These data suggest that some of the microspheres or their dye are lost from tissue samples, that substances in blood or tissue interfere with spectrometry, or that the colour was overestimated in the reference blood sample. A more recent study employing three dyes showed slightly better correlations using myocardial samples as well as samples from various other organs.[13]

The coloured microspheres are commercially available as DYE-TRAC ® spheres from Triton (San Diego, CA, USA).

Fluorescent microspheres – Because of their high sensitivity and specificity, fluorescent dyes are widely used to label biological materials such as proteins and blood cells. Fluorescent microspheres have been used to delineate ischaemic organ regions with the use of light microscopy.[14] Recently dye extraction techniques for fluorescent microspheres have been published.[8, 15] Fresh[8] or formalin fixated tissue[15] was digested with KOH. The microspheres were isolated from the homogenate either by vacuum filtration[8] or by centrifugation[15] and the dye was extracted with (ethoxy)ethylacetate. Pulmonary perfusion was measured by directly extracting the dye from dried lung pieces, thus avoiding the time consuming tissue digestion and subsequent filtration procedure.[8]

Although the whole spectra of the fluorescent dyes overlap to some extent,[15] up to six different dyes could be used without the need for overlap correction, by measuring the emission of each dye only during excitation at its own excitation wavelength.[8] Using this approach good correlations were found between radioactive and fluorescent microspheres for blood flow in heart and kidneys ($r = 0.96$-0.98), and in lungs ($r = 0.99$).[8] In most cases slopes and intercepts were not significantly different from unity and the origin. Preliminary data support the validity of this technique for blood flow measurements in ischaemic myocardium as well as various other organs[16, 17] (see below). By simultaneous injection of two radioisotopes and two fluorescent labels it was shown that the accuracy of the fluorescent microsphere technique was similar to that of the radioactive microsphere technique.[8, 16]

Fluorescent microspheres for dye extraction are commercially available as Fluospheres ® (from Molecular Probes Inc, Eugene, Oregon, USA).

X ray fluorescent microspheres

Another method to detect non-radioactive microspheres is *x* ray fluorescence. Morita *et al*[7] and, more recently, Mori *et al*[10] used a wavelength dispersive *x* ray fluorescence spectrometer to detect microspheres labelled with up to eight different heavy elements. Microspheres are isolated from tissue or blood after digestion as with the other non-radioactive microsphere methods. After centrifugation of the homogenates, the microspheres are trapped from the pellet on filter paper, which is entered into the *x* ray fluorescence spectrometer. In their experiments, Mori and colleagues showed an excellent correlation between regional flows determined by radioactive microspheres and their heavy metal microspheres. The slopes of the comparisons were, however, systematically different from unity. The advantages of this method are the low costs of the microspheres, the possibility of performing measurements without having to digest the tissue,[8] and the potential to use large numbers of different labels, which can be separated without correcting for overlapping spectra.

There are, however, several disadvantages to this method at present. First of all, the measuring system is quite expensive (approximately $200 000). Furthermore, the microspheres used by Mori have a relatively wide diameter distribution (± 1.5-2.0 μm). In addition, approximately 10 times more *x* ray fluorescent microspheres than radioactive microspheres were required in the validation studies.[10]

Therefore, at the current time, the *x* ray fluorescent microsphere technique is not readily accessible to most investigators. However, if the measuring system becomes more sensitive and cheaper, this technique may offer another suitable alternative to radioactive microspheres. Microspheres labelled with heavy metals are commercially available from Sekisui Plastics, Tokyo, Japan.

Sources of error in the microsphere technique

Although considered as a standard for blood flow measurement, it is well known that the microsphere method has its own pitfalls and inaccuracies. Some of these errors cannot be avoided, because they are inherent properties of particles. Most of the errors, established for the radioactive microsphere method also apply to non-radioactive microsphere methods. Several reviews of the sources of error of the microsphere method have been published.[19-21] An overview of potential errors in the microsphere technique is summarised in table I.

To explore the variation in blood flow values obtained with microspheres, two different radioisotopes of 15 μm microspheres were injected simultaneously into the left atrium of four anaesthetised open chest dogs with coronary artery stenosis.[22] An arterial reference withdrawal speed of 20 ml·min^{-1} was used to minimise variations in the measured flows.[18] Similar measurements were performed in various organs of four anaesthetised open chest rabbits. Measurements in both animal models indicate that, if the number of microspheres per sample was above 400, the coefficient of variation was 5% or less (fig 1). With the number of microspheres between 100 and 400, the coefficient of variation was between 6% and 9%, whereas the variation increased considerably if the number of microspheres per sample is below 100 (fig 1). These data show that the number of microspheres in a sample is an important determinant of the variability in the microsphere method, as has been pointed out by several studies.[19, 20, 23-25] This is explained by the fact that microspheres are discrete particles. Consequently, variations in the concentration of spheres occur as a result of statistical variation even with homogeneous blood distribution. This variability approximates the Poisson distribution.[20, 23] Because the variation is due primarily to the random distribution of discrete particles, a similar variation is the minimum to be expected when using non-radioactive microspheres. As demonstrated by the data presented in fig 1 the variation in blood flow measurements increases considerably in samples containing less than 200-400 microspheres. Consequently it is important to suit the number of microspheres to the (expected) level of blood flow and sample size. To this purpose the following equation can be used[26]:

$$T = (CO \times 400)/(Q \times M)$$

where T is the total number of microspheres to be injected, CO is cardiac output (ml·min^{-1}), Q is the approximate flow

Table 1 *Sources of error in blood flow measurements with microspheres.*

Potential error	*Method*					*Solution*
	RA	*CC*	*CE*	*FI*	*XRF*	
Stochastic errors:						
Sphere distribution	●	●	●	●	●	More spheres[19 20 23-25]
Decay distribution	●					More, or more active, spheres[21]
(Gamma) counting error	●					Increase counting time or activity of spheres
Methodological errors:						
Non-uniform mixing	●	●	●	●	●	Proximal injection, mixing chamber, multiport catheter[29 41 42]
Aggregation	●	●	●	●	●	Detergent, dextran, protein, sonication
Additives	●	●	●	●	●	Minimise use of, for example, Tween
Reference sample	●	●	●	●	●	Withdrawal speed, site of sampling[17 20 43]
Circulatory impairment	●	●	●	●	●	Smaller or fewer spheres[41-42 44-46]
Flow biasing	●	●	●	●	●	Smaller spheres[47-53]
Non-entrapment	●	●	●	●	●	Larger spheres[48 50 52]
Diameter variability	●	●	●	●	●	Quantify before use
Loss of microspheres from tissue	●	●	●	●	●	Calibrate between regions[54-56]
Stripping errors	●		●	●	●	Reduce spectral overlap,[54]
						Use of matrix inversion[16 39 52]
Detector: geometry	●					Limit or standardise sample size[54 55]
separation	●					Reduce spectral overlap[54]
saturation	●					Less active spheres, allow decay[54]
Signal quenching				●		Dilute sample, less spheres[5]
Loss of microspheres when isolating		●	●	●	●	Meticulous technique, centrifugation filters, single tube processing
Low signal:noise ratio during dye detection			●		●	Increase number of microspheres to be injected or sample size[7 8]
Inaccurate solvent volumes		●	●	●		Accurate pipettes, larger volumes, robotics
Dye stability			●	●		Check in solvent[7 8]
Background signal			●	●		Choose appropriate colours

RA = radioactive microspheres; CM = counting of microspheres; CE = dye extraction of coloured microspheres; FI = dye extraction of fluorescent microspheres; XRF = *x* ray fluorescent microspheres. (Modified after reference 21.)

(ml·min^{-1}·g^{-1}) in the organ, and M is the sample weight (g). If, after the experiment, the number of microspheres in small samples is too low, data can be pooled among small samples.

When blood flow values at different time intervals are to be compared, it is important to know the physiological variation in blood flow. This was investigated by injecting two different labels separated by 5 min during a stable preparation. Compared to simultaneous injections, separate injections increased the difference between the blood flow values obtained with both labels by approximately 7% at any number of microspheres per sample (fig 1, empty symbols). These data are consistent with those from similar studies comparing the variability of blood flow values after simultaneous and separated injections of microspheres.[27 28]

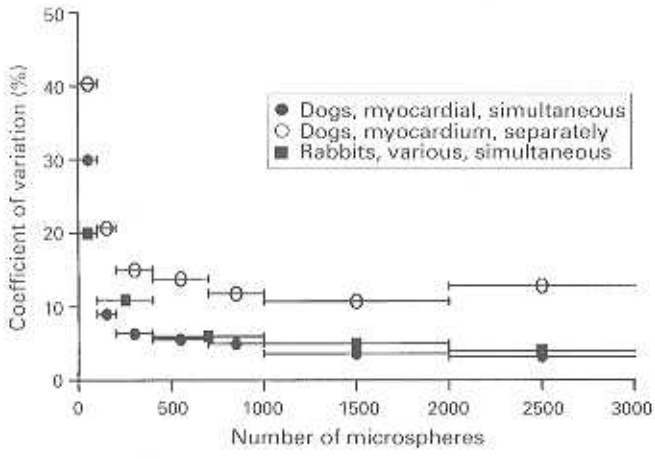

Figure 1 *Variation between blood flow values obtained with different radioisotopes. Data presented are from experiments in anaesthetised dogs with coronary artery stenosis and simultaneous injection of four different labels (n = 2, filled circles) and separate injection of two labels (n = 2, empty circles) in the left atrium and from simultaneous left atrial injection of three labels in four anaesthetised rabbits.[27] Blood flow was determined in the myocardium (all), liver, kidneys and lungs (rabbits only).*

Use of particles to measure blood flow is valid only if the label does not leak from the particles and the particles behave like blood (cells). Checks for these problems have been described for both radioactive[29] and non-radioactive microspheres.[7 8]

Aggregation of the particles is prevented by the use of small amounts of detergent in the injectate (0.05% Tween-80, for example), or by suspending them in a solution containing macromolecules. Dextrans or albumin can be used to coat the microspheres, decreasing the electrostatic attraction between them. All three types of non-radioactive microspheres have less of a tendency to aggregate than the radioactive spheres and they remain more evenly distributed in suspension, due to their relatively low density (1.02-1.05 g·ml^{-1}). Nevertheless, microsphere suspensions should be well vortexed and sonicated before injection.

Because different diameters of microspheres behave differently in the blood stream, a uniform diameter distribution of the microspheres is likely to increase the reproducibility of the blood flow measurements. This has been recognised by the companies delivering the radioactive microspheres, and has resulted in a reduction of the standard deviation of the diameter from 3 μm in the 1970s to <0.5 μm in the 1980s. The latter values also apply to most non-radioactive microspheres available. Currently there is no indication that the difference in density between radioactive microspheres (density 1.3 g·ml^{-1}) and non-radioactive microspheres (density 1.02-1.05 g·ml^{-1}) results in differences in endocardial to epicardial blood flow ratios,[7 15 16] which implies that rheological properties of the microspheres are minimally affected by differences in their density, at least within the range used.

While problems with radioactive decay[21] and geometry of the sample[30] are typical for the radioactive microsphere technique, non-radioactive microsphere techniques share a few possible errors which do not apply to the radioactive technique. Loss of microspheres may occur during the processing of the samples because of the larger number of sample manipulations required. This can be minimised by

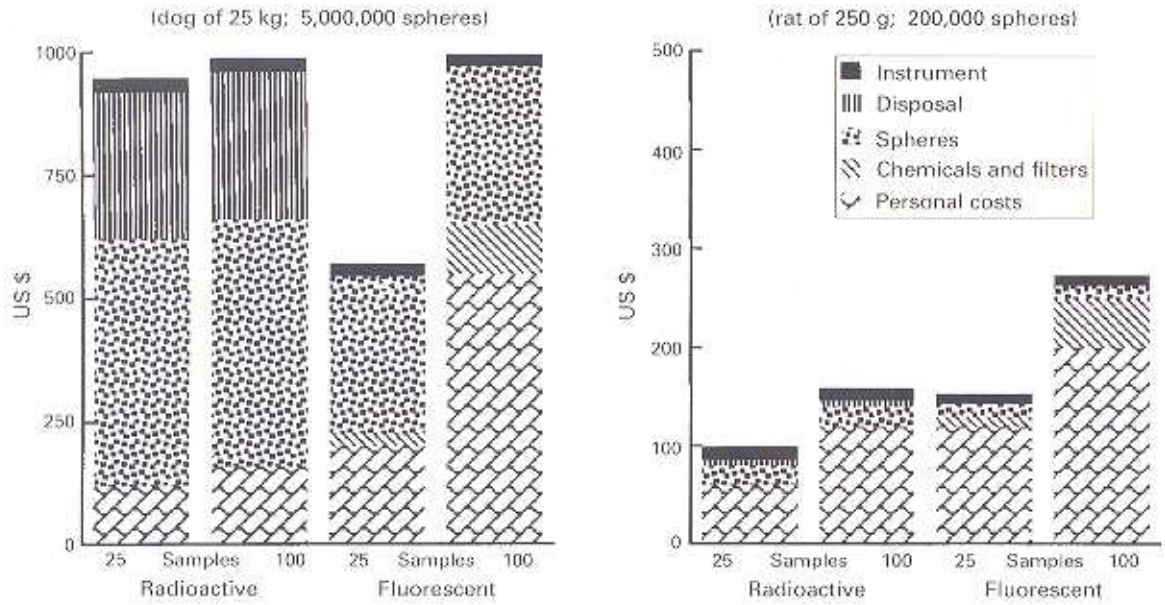

Figure 2 Comparison of the costs of experiments with radioactive and fluorescent microspheres. Presented are the cost for an experiment in a dog of 25 kg, where five million microspheres of four different labels are injected systemically (left panel), and for an experiment in a rat of 250 g, where 200 000 microspheres of four labels are injected systemically. Calculations are based on the following costs: $12.5 per kg for disposal of the radioactive animal, $25 per hour for personnel costs, and $1 per sample for chemicals and filters, whereas the costs of the instrument were divided over 500 experiments (50 experiments in 10 years). Costs of microspheres were estimated to be $250 per 10 million for radioactive microspheres and $160 per 10 million for fluorescent microspheres. Time for microsphere isolation and extraction plus manual spectrometry for the fluorescent microsphere was calculated to be 8 min per sample, based on centrifugal sedimentation (see text).

performing tissue homogenisation, microsphere isolation, and, if applicable, dye extraction, in the same tube (see below). The dyes in coloured or fluorescent microspheres are stable over a temperature range of −20°C to 60°C in saline, blood, and extraction media.[7 8] New procedures will, however, require the stablity of the dyes to be tested. This is especially true for the application of non-radioactive microspheres in chronic experiments. Not only should it be known whether the stability of the dye is the same in the microspheres in the body and in the stored reference blood samples, but it is also important to know to what extent the body destroys or removes these microspheres.

Tissue and blood homogenates, as well as chemicals, may contain substances interfering with the spectra of dyes. Absorbance of white microspheres from Triton interferes with absorbance caused by tissue and blood samples. Tween-80 emits fluorescence in the area of the blue fluorescent spheres from Molecular Probes. These kinds of problems can be solved by increasing the number of microspheres injected, adding a "interfering substance reference" as virtual additional label in the matrix inversion, or just avoiding the use of these labels. Use of chemicals with high purity is also helpful, although it was noted that there were differences in absorbance and fluoresence of chemicals from different suppliers.

Some of these items will be discussed in more detail below ("Future directions of research").

Radioactive or non-radioactive microspheres?

Radioactivity is hazardous. Although the level of radiation to users of radioactive microspheres is usually below that considered dangerous, safety for workers will increase when such work is avoided. More importantly, radioactivity is harmful to the environment. This is probably the strongest argument in favour of decreasing both production and waste of radioactive microspheres. It is, however, important to note that use of non-radioactive microspheres requires chemicals. While most of them (like potassium hydroxide) can be neutralised relatively easily, others (like the extraction media dimethylformamide and ethoxy-ethylacetate) are potentially harmful. Careful handling of the waste from non-radioactive microsphere determinations is therefore almost as important as that from radioactive microsphere determinations. Moreover, the person processing the samples has to obey the safety rules for the chemicals used.

Before comparing the two microsphere techniques, it is important to note that non-radioactive and radioactive spheres can be used together in the same experiment. In this way the number of microsphere injections per experiment can be increased.

The choice of non-radioactive microspheres is mandatory if no license can be obtained to work with radioactive materials and is most attractive when a laboratory has to be adapted to satisfy a legislative code for radioisotope work. Another compelling reason to switch to the use of non-radioactive microspheres may be that some facilities for disposal of radioactive waste may even close. On the other hand, if radioactive microspheres are routinely being used, the decision to use non-radioactive microspheres depends on different factors.

In order to facilitate comparison between various factors, the costs of experiments are estimated when using radioactive and non-radioactive (that is, fluorescent) microspheres. The numbers on personnel costs are meant to quantify the personnel effort required for either method. Estimations are made for varying numbers of samples per experiment and for experiments in small and large animals (fig 2).

Although the measuring instrument requires a considerable investment, the cost per experiment is relatively low if the instruments are in regular use for approximately 10 years. Gamma counters are the most expensive instruments

(approximately $30 000), while fluorimeters and spectrometers can be obtained for $10 000-$25 000. Counting coloured or fluorescent microspheres manually circumvents the need for an expensive instrument, but will increase personnel costs due to the time consuming counting process.

Costs of radioactive waste vary from country to country and also within countries, for example the USA. In Western countries these costs range from $5 to $25 per kg. While users of large animals may have to pay more than $10 000 per year, these costs are considerably lower when small animals are used. As an alternative to the expensive disposal of radioactive carcasses and tissues, some institutes freeze them until radioactivity has decayed (5-10 half lives, which is at least 6 months in the case of the shorter lived isotopes), and then dispose of them as non-radioactive waste. This approach requires an investment of at least $10 000 in a large freezer and space at the laboratory, but is attractive for regular users.

A drawback of all non-radioactive microsphere methods currently described is that tissue digestion, microsphere isolation (filtration), and analysis is time consuming. These disadvantages are multiplied as the number of samples per experiment increases. This indicates that automation of sample processing and microsphere quantification is an important goal for the future (see below). A factor not directly expressed by cost is that the analytical skills required are greater for the non-radioactive microsphere methods.

Costs of microspheres vary depending on the radioisotope and are dependent on the diameter and specific activity. Moreover, considerable discounts can be obtained when ordering large amounts of radioactive and non-radioactive microspheres. Without such discounts the costs per 10 million spheres ranges between $150 and $1000 for radioactive microspheres, and is $22.50 for E-Z TRAC spheres (counting method), $50 for DYE-TRAC spheres (dye extraction), and $160 for fluorescent microspheres from Molecular Probes. In their paper, Kowallik *et al*[7] give indications about the costs of the materials necessary to produce coloured microspheres, and these costs are a fraction of the commercial prices. However, continuous quality control will be required when microspheres are produced in the investigators' laboratory.

It should be noted that in the validation studies published the number of radioactive and fluorescent microspheres was approximately half those of the other types of microspheres.[5-8] Furthermore, the actual costs of radioactive microspheres are higher than mentioned here, because they have a limited lifetime due to isotope decay. This requires accurate estimation of the number of microspheres needed over a certain period of time. When this number is overestimated, the remaining microspheres cannot be used. This is not a concern with non-radioactive microspheres.

Costs which are specific for non-radioactive microspheres are those of chemicals and filters. The chemicals are relatively inexpensive, except for the reagents marketed by E-Z TRAC. The costs of the filters for isolation of the microspheres from the digested tissue is considerable at $0.30-$1.00 each,[7 8] because the diameter of the pores must be precise, allowing as much tissue debris as possible to pass while retaining all of the microspheres. Moreover, the filters need to be resistant to aggressive reagents like potassium hydroxide.

If both blood flow and the content of a chemical substance is to be determined in the same sample, non-radioactive microspheres offer the advantage that instruments used for the chemical analysis are not contaminated by radioactivity.

It should be possible to take advantage of the colour or fluorescent quality of the non-radioactive microspheres. There now exist new possibilities for blood flow measurement, such as determination of the distribution of blood to structures on a microscopic scale. Using methods of quantitative histology[31 32] it should be possible to quantitate perfusion to whole organs as well as intraorgan structures at a level not previously possible. An example is the measurement of blood flow in the capsule, cortex, and medulla of the adrenals. Separation of these tissues by dissection is quite difficult. Jasper *et al*[33] showed that it was possible to measure blood flow by counting the number of fluorescent microspheres under the microscope. Blood flow at three time intervals could be quantified by using three colours and appropriate fluorescence filters in the microscope. Other studies used coloured or fluorescent microspheres to study interarterial microvascular anastomoses in the myocardium of dogs,[34] the direction of flow in the myocardial microcirculation,[35] and the separation of pulmonary and bronchial blood flow in the airways.[36]

Which type of non-radioactive microspheres?

Three different non-radioactive microsphere techniques can now be used with commercially available spheres and instruments: counting microspheres (CM), absorption spectroscopy of extracted dye from coloured microspheres (CE), and fluorescent spectroscopy of extracted dye from fluorescent microspheres (Fl). Each technique has been validated against radioactive microspheres, but are currently used by a limited number of research groups. No study has yet been published comparing the non-radioactive microsphere techniques to each other in vivo. This may not be easy, because each method is so technique dependent that a head to head comparison may be primarily a test of analytical skills. Moreover, some "coloured" spheres also have fluorescent properties and fluorescent dyes have absorbance in the photospectrometer. Therefore ranking of the quality of the three techniques should be done with care.

Specifications in publications and from the different companies, state that the commercially available microspheres (E-Z TRAC® or Polysciences for the CM, DYE-TRAC® for the EC and FluoSpheres® for the Fl method) are all spherical. The diameter uniformity is better for the DYE-TRAC® and FluoSpheres® beads (SD less than $\pm$ 0.3 μm) than for the E-Z TRAC® beats ($=$ 2 μm).[6] This larger variation in the E-Z TRAC® spheres could, at least in part, explain the systematic overestimation of flow in high flow regions in studies by Hale *et al*.[6]

Because there is no indication that the various types of microspheres behave differently in the circulation, the accuracy and precision of these techniques will depend on the analysis of blood and tissue samples. These samples must be digested completely to ensure complete recovery of all microspheres. In the CE and Fl methods these samples are digested in a KOH solution containing a detergent. E-Z TRAC recommends their own ready to use, but quite expensive, reagents for the CM method, but the use of less expensive solutions is possible.[37]

Quantification of microspheres in tissue samples is most different between the CM method and the dye extraction (CE and Fl) methods. Manual counting of the various microsphere colours is probably accurate but quite time consuming. One problem is that only part of the microsphere content of a sample is counted. As mentioned above ("Sources of error"), the number of microspheres in a sample is the main deter-

Table II *Comparison of coloured and fluorescent microsphere techniques.*

	Coloured	*Fluorescent*
Solvent volume	0.1 ml	1-3 ml
Accuracy in vitro (SD)	± 20 spheres	± 2 spheres
Spectral overlap	0-70%	0-18%
(number of labels)	(5)	(7)
Number of labels with overlap < 5%	3	6
Number of microspheres injected	7×10^6	4×10^6
Number of radioactive microspheres injected	3×10^6	4×10^6

minant of the variability in the microsphere method. Hence measurement errors may increase when only a portion of the microspheres is counted. Moreover, pipetting a small volume of microsphere suspension is inaccurate, because microspheres easily aggregate under these circumstances. Errors of 10-20% are not uncommon. Although up to 10 different colours are available, good discrimination of all colours seems questionable. The use of two or three colours per experiment has been validated.[6] A more promising approach is the use of a FACS analyser to count the microspheres.[1]

Quantification of spheres in the CE and Fl method is similar. Once isolated, the microspheres are dissolved in a known volume of solvent and the concentration of each dye measured by spectrometry. There are several differences between the CE and Fl method, which are summarised in table II. First of all, in order to produce an adequate signal, the CE method requires that the dye be dissolved in a small volume of solvent (of the order of 0.1 ml[7]) for spectrometry. Manipulation of such volumes requires considerable analytical skill and the small measuring volume may introduce a significant amount of noise into the measurement.

More important is the quantity of dye per microsphere in relation to the detection level of the spectrometer. Given an absorption of 0.6-1.15 AU per 1000 coloured microspheres (except 4.5 AU per 1000 for yellow) and a noise level of approximately 0.02 AU, it follows that the accuracy of determination of the number of microspheres in a sample is ± SD 20 spheres. The detection of fluorescent microspheres is more sensitive: using a volume of 1 ml of solvent, fluorescence from two microspheres exceeds the noise of the fluorimeter, a sensitivity rivalling the radioactive microsphere method. The difference in sensitivity in vitro indicates that it will be easier to perform reproducible measurements in samples from experiments, but it does not invalidate the coloured microsphere method. The sensitivity of this method (± 20 microspheres) may very well be within the error of the microscope technique due to the number of microspheres per sample (see above, fig 1).

Five different labels are available for the CE method, but there is considerable spectral overlap when using more than three colours (table III). The white and violet dyes have broad spectra and suffer from considerable overlap with adjacent spectra (table IIIA). Moreover, the spectrum of the white dye suffers from relatively high background absorbance in tissue and blood samples. Possibly because of these problems, Kowallik *et al*[7] injected a larger number of microspheres (2-3 times) for the white, violet, and blue microspheres than for the other colours and for the radioactive microspheres, and Wieland *et al* only used the yellow, red, and blue dye.[13] Separation of the contribution from the various dyes may improve if absorbance values at multiple wavelengths are used (or even the entire spectrum) for overlap matrix calculations.[38 39]

Using fluorimetry, the emission of each dye is determined only when it is excited at its own characteristic excitation

Table III *Overlap matrices.*
(A) Overlap matrix of dyes for coloured *microspheres.*

	White	*Yellow*	*Red*	*Violet*	*Blue*
White	**100**	9.1	0.2	0.0	0.0
Yellow	10.4	**100**	0.9	0.0	0.0
Red	4.0	15.6	**100**	6.8	0.0
Violet	11.6	6.0	68.9	**100**	2.0
Blue	19.0	3.0	9.4	15.3	**100**

(B) Overlap matrix of fluorescent *microsphere dyes at excitation and emission wavelengths optimised for maximum signal.*

	Blue	*Blue-green*	*Green*	*Yellow-green*	*Orange*	*Red*	*Crimson*
Blue	**100**	0.0	0.0	0.0	0.0	0.0	0.0
Blue-green	0.2	**100**	18.1	0.0	0.0	0.0	0.0
Green	0.0	8.0	**100**	8.8	0.0	0.0	0.0
Yellow-green	0.0	0.0	1.5	**100**	0.0	0.0	0.0
Orange	0.0	0.0	0.0	0.1	**100**	0.3	0.0
Red	0.0	0.0	0.0	0.0	0.2	**100**	3.3
Crimson	0.0	0.0	0.0	0.0	0.0	2.6	**100**

optimum. As a result overlap between neighbouring spectra is minimal (table II and IIIB). If the green fluorescent microsphere is not used, correction for spectral overlap is not necessary, which is an advantage over the radioactive microsphere method.[29] The spectral overlap between red and crimson dyes is the worst case (if green is excluded). If the amount of one of these dyes is much greater than the other, correction may be necessary. Alternatively, excitation and emission wavelengths different from the optimal wavelengths can be used. While this will decrease the fluorescence signal, it can also decrease the spillover. Since the microspheres provide plenty of fluorescence signal, this is a tempting approach.

A property of fluorimetry is that emitted light from one dye can be absorbed by a dye with an excitation wavelength near the emission wavelength of the first dye. In this case, the amount of fluorescence emission is underestimated by this kind of quenching. In fig 3 spectra of a constant concentration of red with varying concentrations of crimson are depicted. With increasing crimson concentration the

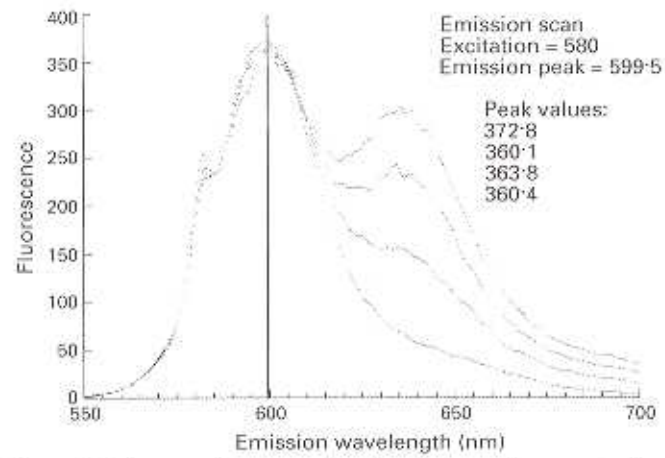

Figure 3 *Spectra of mixtures of red and crimson fluorescent dye. Per ml of solvent there were 2500 red microspheres together with 0, 2000, 4000, or 6000 crimson microspheres. With increasing crimson concentration the reading for red decreases (but by less than 3%) due to absorbance of the emitted red fluorescence by the crimson (see text). Note that the emission at 640 nm, the peak of the crimson emission spectrum, is not read when the sample is excited at 580 nm.*

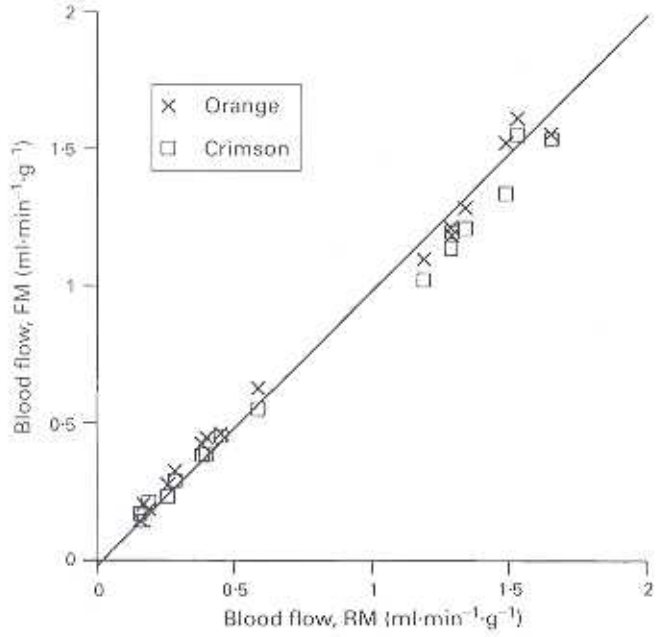

Figure 4 *Regional myocardial blood flow determined with fluorescent microspheres as a function of blood flow determined with radioactive microspheres. Data represent values from endocardial, mesocardial, and epicardial samples from ischaemic and non-ischaemic myocardium of one dog. Two fluorescent dyes and two isotopes were injected simultaneously. The mean of the values from the two isotopes was used as the blood flow on the horizontal axis.*

readings for the emission of the red dye decrease, but by no more than 3%. Therefore, only with very large differences in the intensities between red and crimson does correction for quenching seem to be required. This is supported by the good correlation in blood flow values obtained with fluorescent and radioactive microspheres ([8 16 17]; fig 4) without correction for quenching.

Future directions for research

Because non-radioactive microspheres have only recently been developed for measuring regional organ perfusion, current methodologies are relatively primitive. However, when first introduced by Rudolph and Heymann in 1967, radiolabelled microsphere methods were similarly rudimentary. Given the proper environment, non-radioactive microsphere techniques should evolve in an analogous fashion. Compared to radioactive methods, the greatest problems with non-radioactive microsphere methods are at present practical problems: (1) tissue digestion, (2) microsphere isolation, and (3) automation.

Solid tissue digestion

Until now, digestion of tissue with KOH has been the most common approach. Digestion of some tissues is more difficult than of others. So far, publications have focused on tissues that can be digested relatively easily: lungs, myocardium, and kidneys. Tissues with a high fat content may cause problems with filtration, since fat can occlude the filter pores. This problem can be solved when tissue is digested in 2N ethanolic-KOH rather than 4N aqueous KOH, because fat is more completely digested with 2N ethanolic-KOH.[16] Since the latter solution is more corrosive, the durability of the filters should be checked when in contact with ethanolic-KOH.

Digestion of tissue can be facilitated by mincing the tissue with a tissue homogeniser, such as the Janke & Kunkel Ultraturrax, type 18-10. Fluorescent as well as radioactive microspheres remain intact during this procedure (Mullers-Bouman M-L, and Prinzen FW, unpublished observations). Clearly, investigators will need to tailor the procedures for tissue digestion analysis to the specific organs with which they are working.

Microsphere isolation

Standard isolation of microspheres is performed by microfiltration[7 8] using negative pressure filtration. The advantages of this method are the low costs and the fact that it has been rigorously tested. The disadvantages are that it is labour intensive and that there may be loss of microspheres as the tissue samples are transferred from one vessel to the next. The latter can be circumvented by adding the extraction medium directly on the filter. More time can be saved when using a device designed to perform digestion, filtration, and extraction in the same device.[16]

Currently, techniques are being developed using sedimentation of the microspheres by centrifugation of the homogenate. This can be done when tissue or blood samples are digested in 2N ethanolic-KOH for 48 hours at 60°C. With tissue or blood volumes of less than half the volume of ethanolic-KOH, the density of the microspheres is sufficiently greater than that of the homogenate to spin them to the bottom of the tube (2000 *g* for 15 min). After one washing step with detergent (0.5% Tween-80) and one or two washing steps with distilled water, the dye solvent (in this case 3 ml of 2-ethoxy ethoxyethylacetate) is added to the remaining pellet. After mixing the pellet with the dye solvent and subsequent sedimentation of the debris by a third centrifugation step, the supernatant can be used for fluorimetry.[16] The advantage of this method is that regular glass tubes can be used. These are inexpensive and a large number can be centrifuged simultaneously in a standard laboratory centrifuge. Also, the entire procedure is performed in the same tube, minimising loss of microspheres. An example of the results with ethanolic-KOH digestion in combination with centrifugal sedimentation of the microspheres is presented in fig 4. This figure presents values of myocardial blood flow, as obtained with two different labels of fluorescent microspheres, in samples from endocardial, mesocardial, and epicardial samples from normoxic and ischaemic canine myocardium as a function of blood flow determined with radioactive microspheres. In this particular experiment orange and crimson spheres were injected simultaneously with two radiolabels, while at six other sample times three other radiolabels and three other fluorescent labels were injected. In other experiments, similarly good blood flow estimations were found with the other dyes, even in more fatty organs like brain, liver, and skin,[16] indicating that this microsphere extraction method is useful for many organs.

Automation of microsphere quantification

Although the microsphere isolation procedure is the most time consuming part of the analysis, automated microsphere quantification is desirable. Counting of coloured microspheres can be automated by image analysis software in combination with an automatically moving microscope stage. Such a system is available through E-Z TRAC (approximately $35 000), but a considerably cheaper version has been reported recently.[40] Sample changing is, however, manual.

To automate spectrometry, Glenny *et al* investigated the use of a well plate reader (approximately $5000) connected

to a Perkin Elmer fluorimeter.[8] The increased fluorescent quenching seen with this surface reader resulted in a more curvilinear relationship between the number of microspheres and the fluorescence.[8] Therefore, software for correction of curvilinearity and improved optics are necessary before the currently available well plate reader can replace cuvette methods.

Spectrometry can also be automated by interfacing a liquid handling system with a flow cell in the spectrometer. An autosampling and diluting system, like the Gilson 222 (approximately $10 000), is able to handle volumes as small as 40 µl, and can also return the sample to the original tube rather than wasting the sample.[13] These kinds of instruments may therefore be good alternatives to a well plate reader in the automation of spectrometry.

More fluorescent colours will soon be available and methods to correct for spillover[38, 39] will be needed, as is already the case for the coloured microsphere method. Alternatively, other detection methods, such as a FACS analyser, may be useful in this approach. FACS analysis has the advantage over manual counting of microspheres in that the entire sample is analysed and this could potentially save a considerable amount of time since solid tissue samples do not have to be filtered. Because only particles with a predefined size and colour are counted by FACS analysis, this method may be less sensitive to spectral interference from tissue or blood than the dye extraction methods. A method using an FACS analyser to quantify fluorescent microspheres has recently been published by Austin and coworkers.[11] In their method, an aliquot of each sample was used to estimate the total numbers of microspheres in the tissue samples. At the Experimental Biology '94 meeting a symposium organised by EZ-TRAC presented new studies using an FACS analyser to quantify their fluorescent microspheres. Dr Mazer (Toronto) presented preliminary data indicating excellent quantitation of coloured microspheres when compared to radioactive microspheres in solid tissue. In the discussion following his presentation, Dr Ito (San Diego) mentioned that he has used an alternative approach in which different fluorescent microspheres are labelled with different concentrations of the same dye. These two concepts provide the potential to separate microspheres labelled with combinations of various dyes with varying concentrations, thus effectively increasing the number of labels far beyond that available with radioactive microspheres. This idea is exciting enough to warrant further investigation. Until now, however, validation of the FACS analysis approach is limited and it is certainly too early to compare the practical advantages of this approach, if any, with the spectro(fluori)metric approach. Currently the cost of an FACS analyser is considerably higher than that of a spectrophotometer or fluorimeter.

Thus many procedures in non-radioactive microsphere methods are still in the course of development. It is our experience that relatively small practical problems in these procedures cause considerable problems in validation and delay in the study for which blood flow measurements are meant. By sharing these problems and their solutions among researchers, the development of non-radioactive microsphere methods will be accelerated, benefiting all. The Fluorescent Microsphere Resource Center (FMRC) has grown out the need for a central hub where investigators can share ideas. The principle purpose of the FMRC is to foster the further development of fluorescent, and other non-radioactive, microspheres through dissemination of information and by facilitating communication between scientists. Information can be obtained from a File Transfer Protocol (FTP) server ("fmrc.pulmcc.washington.edu"), through the internet ("glenny@pele.pulmcc.washington.edu") or from an electronic bulletin board via modem.

Summary

Considerable progress is being made in the development of non-radioactive microsphere methods. Validation studies of the three commercially available non-radioactive microspheres are promising. In most experimental conditions the use of non-radioactive microspheres saves money. Avoiding the use of radioactivity facilitates the use of microspheres in chronic animal experiments and when blood flow and chemical measurements are performed in the same sample. Moreover, using histological techniques, distributions of coloured or fluorescent microspheres in subunits of organs could be quantified, opening new scientific possibilities.

Currently, the fluorescent microsphere technique seems to be the most promising non-radioactive microsphere method. Due to the high sensitivity and good spectral separation, the number of microspheres injected can be as small as that used for radioactive microspheres, at least six labels can be used, and the relatively large volume in which fluorescence is measured (~1-3 ml) enables the use of time saving microsphere isolation techniques. Development of these methods and further automation of the quantification process (using either automised spectrometry or FACS analysis) will considerably increase interest in the non-radioactive microsphere techniques. To accelerate these developments, investigators are encouraged to share their experiences.

Key terms: fluorescent microspheres; coloured microspheres; radioactive microspheres; blood flow measurement.

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