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DEVELOPMENT OF THE FLUORESCENT MICROSPHERE TECHNIQUE FOR QUANTIFYING REGIONAL BLOOD FLOW IN SMALL MAMMALS

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SUMMARY

We have demonstrated that the fluorescent microsphere technique can be used in small mammals for accurate determination of regional blood flows. In particular we have shown that 100 % recovery of trapped microspheres is possible, that tissue digestion can be completed in a shorter time than previously reported, and the error-prone filtration method can be replaced with one of sedimentation. The method gave very good agreement among different fluorescent labels ($r^2 > 0{\cdot}99$) and low variability among tissues (mean coefficient of variation = 0·06). Simultaneous injection of radiolabelled and fluorescent microspheres established comparability between these methods ($r^2 = 0{\cdot}96$) for blood flows measured at rest, during vasodilator-induced hypotension, and in muscle hyperaemia during indirect electrical stimulation. Fluorescent microspheres can therefore replace radioactive microspheres for the determination of blood flow with advantages in both safety and cost, without loss of sensitivity.

INTRODUCTION

Microspheres have become the gold standard for measuring regional organ perfusion since their introduction by Rudolf & Heyman (1967), where regional blood flow (RBF) is proportional to the number of microspheres trapped in tissue following intra-ventricular injection. Methods for quantifying the number of microspheres per sample depend on the label used, the most common being the measurement of nuclide decay from radiolabelled microspheres (which we have used in our studies to date). However, the use of radioactive labels is becoming restricted because of the health risks for both the user and for animals in chronic studies, requiring special precautions during their use and subsequent disposal. In addition they have limited shelf lives and are consequently becoming too expensive for routine use, typically costing > £1000 per experimental series for a batch of three different labels used to establish RBF under different conditions. Nevertheless, the microsphere technique is likely to remain the standard against which other techniques are compared, because microspheres can provide simultaneous estimates of cardiac output distribution among different organs, or specific regions within a tissue (Heyman *et al*. 1977).

A number of non-radioactive alternatives have been proposed, including coloured microspheres (Hale *et al*. 1988; Kowallik *et al*. 1991; Hakkinen *et al*. 1995; Degens *et al*. 1996) and X-ray fluorescent microspheres (Morita *et al*. 1990; Mori *et al*. 1992), although they have been shown to have some disadvantages. For example, coloured microspheres may underestimate RBF, since they have limited resolution due to significant spectral overlap among different colours, while data variance is high as a result of the low signal intensity (Hale

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et al. 1988; Kowallik *et al*. 1991; Prinzen & Glenny, 1994; Degens *et al*. 1996). The X-ray fluorescent microspheres technique uses a measurement system that is expensive and not in common use (Prinzen & Glenny, 1994). Based on theoretical considerations and the practical experience of colleagues using them in large animals, fluorescent microspheres appear to be the most viable alternative (Glenny *et al*. 1993; Prinzen & Glenny, 1994; van Oosterhout *et al*. 1995) and provide estimates of RBF for about half the cost of radiolabelled microspheres. They have been used for the determination of blood flow in lung, kidney and myocardium of dog (Austin *et al*. 1990), myocardium, brain, kidney and skeletal muscle of pig (McKiman *et al*. 1994; Hecht *et al*. 1995), and myocardium of rabbit (Chien *et al*. 1995). In addition relative blood flow, but not absolute blood flow, has been estimated in different tissues such as liver, brain, and spleen of dog (van Oosterhout *et al*. 1995), and regional adrenal gland blood flow has been estimated in fetal sheep (Buchwalder *et al*. 1998). So far there are no reports of a systematic application of the fluorescent microsphere (FM) technique in small animals such as the rat, although it has been used to estimate the relative distribution of cardiac output in chick embryos (Mulder *et al*. 1997).

In this study we examined 25 different tissues, representing all major organ systems, in the laboratory rat. For our purposes, examination of RBF was of particular interest in resting and working skeletal muscle in order to quantify differences in inter- and intra-muscle blood flow. As well as validating this technique for use in small mammals, our aim was also to improve the efficiency of existing protocols by reducing digestion and processing time.

METHODS

Animals

Male Wistar rats, body weight 320–420 g, were housed in accordance with the animal welfare regulations of the UK Animals (Scientific Procedures) Act of 1986. Four groups of animals were used: control animals for validation of the methodology ($n = 11$); those subjected to indirect electrical stimulation with injection of either radiolabelled or fluorescent microspheres alone (both $n = 9$); and those co-injected with both labels during stimulation ($n = 9$) or hypotension ($n = 3$).

Surgery

Experiments were performed under sodium pentobarbitone anaesthesia (Sagatal, Rhone Merieux; 6 mg (100 g body weight)$^{-1}$ in 0·9 % saline) given intraperitoneally and supplemented via a jugular vein cannula. The rats were surgically prepared for blood flow determination, as previously described (Hudlická *et al*. 1994). Briefly, both brachial arteries were cannulated with PP50 tubing, the right brachial artery was connected to pressure transducer (type 4, Bell and Howell, Wembley, UK) to monitor blood pressure and heart rate throughout the experiment, and the left brachial artery was used for withdrawing a reference blood sample during microsphere injection. The left ventricle was cannulated (PP50) via the right carotid artery to determine when the cannula tip was in the ventricular lumen by observing changes in the blood pressure waveform, and to subsequently inject microspheres. Existing literature suggests that in order to obtain a reference sample containing microspheres that are adequately mixed, sampling from a major distal artery is desirable. In small animals such as rats or hamsters the probability of interfering with limb blood flow by cannulating the femoral artery is greater than in larger mammals and is therefore generally avoided where possible, particularly when examining blood flow during muscle activity. However, pilot experiments showed similar resting blood flows using brachial or femoral artery cannulation, and our data are consistent with that reported in the literature for these animals. We therefore conclude that any error with our approach is small.

Adjustments in regional blood flow

To examine the influence of contractile activity on relative muscle blood flow nine rats had both feet clamped and m. tibialis anterior (TA) and m. extensor digitorum longus (EDL) of both legs tied together at the distal tendons, and attached to a strain gauge (Dynamometer UF1, 16 oz). Their combined isometric force generation was recorded (Lectromed MT8P, Letchworth Garden City, UK) at optimal muscle

length as g force (g muscle mass)$^{-1}$. Other hindlimb muscles were kept at their *in vivo* position during rest and indirect stimulation (0·3 ms pulse width and supramaximal voltage) which was applied via bipolar silver electrodes to the distal end of the cut common peroneal nerve for 5 min at 4 Hz. Both right and left limbs were stimulated simultaneously to avoid any 'steal' effect. Acute hypotension (defined as a decrease in diastolic pressure to below 50 % of resting) was induced in three rats by administration of sodium nitroprusside (5 mM in saline I.V., approximately 4·5 mg kg^{-1}), with microspheres injected when blood pressure stabilized at approximately 1 min, which recovered to pre-injection levels within a few minutes.

Microspheres and injection

Carbonized microspheres with ^{57}Co, ^{113}Sn, and ^{46}Sc radiolabels had a measured diameter of 15·5 ± 0·1 μm, a density of 1·4 g ml^{-1}, and a specific activity of 11·7 MCi^{-1}, and were shipped at a concentration of 400 000 mg^{-1} (New England Nuclear/DuPont, Boston, MA, USA). Polystyrene microspheres with yellow–green (YG), blue–green (BG), orange (O), and red (R) fluorescent labels were nominally 15·5 μm diameter, with a density of 1·05 g ml$^{-1,}$ and were shipped at 976 000 mg^{-1} (Molecular Probes, Leiden, Netherlands).

Radioactive microspheres (RM) were diluted and suspended in saline from stock vials containing 0·01 % Tween to retard flocculation, fluorescent microspheres (FM) were diluted in Haemocell serum (Gelofusine-plasma substitute, Braun Medical Ltd, Melsungen, Germany) to retard sedimentation. Microspheres were vortexed, sonicated for 3–5 min, quickly vortexed again, then diluted to a final volume of 1 ml. After mixing, 0·9 ml was injected into the left ventricle via the carotid artery cannula; the remaining 0·1 ml was used as a pure microsphere reference and also to quantify the degree of microsphere recovery (see below). For the reference blood flow (BF) sample a precision withdrawal pump was used (Braun Medical Ltd) at a rate of 0·5 ml min^{-1}, and blood was collected into a weakly heparinized (50 i.u.) glass syringe. Pilot experiments established that BF reached a maximum within the first minute of stimulation; injection of microspheres at 4·5 min therefore gave adequate time for functional hyperaemia to develop fully. Microspheres were injected over 15 s and 0·7 ml Haemocell was injected over another 15 s to replace the withdrawn volume of blood and clear the cannula deadspace; withdrawal continued for a further 30 s to ensure full recovery of microspheres. The number of injected microspheres used was determined by the photon abundance of RM labels (for ^{57}Co and ^{113}Sn 0·2–0·4 ml was injected from the original stock, while for ^{46}Sc 0·3–0·5 ml was used, depending on the interval between delivery and use) and quantum efficiencies of FM labels (for YG and O labels 0·2 ml was injected from the original stock, for BG 0·3 ml, and for R 0·4 ml), giving a total injection of $< 0{\cdot}4 \times 10^6$ microspheres. Labels with high quantum efficiency or photon abundance were used for estimations of low blood flows such as those at rest. Similar procedures were performed for either co-injected or separately injected microspheres: two different FM labels simultaneously injected (intra-method comparison) and one radioactive and one fluorescence label (inter-method comparison).

After microsphere injection, experiments were terminated by administration of an overdose of sodium pentobarbitone anaesthesia. Microsphere activity was determined for RM in three energy windows, each corresponding to the major isotope peaks (80–150, 340–440 and 500–2000 keV for ^{57}Co, ^{113}Sn and ^{46}Sc, respectively; Packard Auto Gamma 5650). Tibialis anterior (TA) muscle was divided into two regions of almost equal size by colour: the predominantly glycolytic TA cortex and predominantly oxidative TA core. Following RM counting, FM extraction used the following protocol (Fig. 1).

1. Tissues weighing from 0·006 to 3·0 g were dissected and put directly into 15 ml screw cap polypropylene tubes with a conical base (maximum of 3 g per tube). It proved unnecessary to mince tissue, even though smaller pieces may aid quicker digestion. Five millilitres of 2 M KOH in 99 % (IMS) ethanol with 0·5 % Tween-80 was then added. Tissue digestion was usually completed in 2–4 h using a dry heating block held at 60 °C, with intermittent shaking. Samples were routinely processed using fresh tissues, although storage of frozen tissue (in the dark at −20 °C) introduced no detectable error in BF estimation and tended to aid tissue maceration.

2. After digestion was completed the tubes were centrifuged at 3000 r.p.m. (1500 *g*) for 15 min.

3. The supernatant was carefully aspirated until < 500 μl was left, thereby reducing the possibility of accidental loss of microspheres.

4. After 1 ml dH_2O was added the tubes were quickly vortexed to prevent microsphere flocculation and aid resuspension of remaining pellets, while the subsequent addition of ethanoic Tween (100 % ethanol + 0·5 % Tween-80) allowed complete sedimentation by centrifugation.

5. Nine millilitres of ethanoic Tween-80 was added, and the tubes were vortexed and spun at 1500 *g* for 15 min.

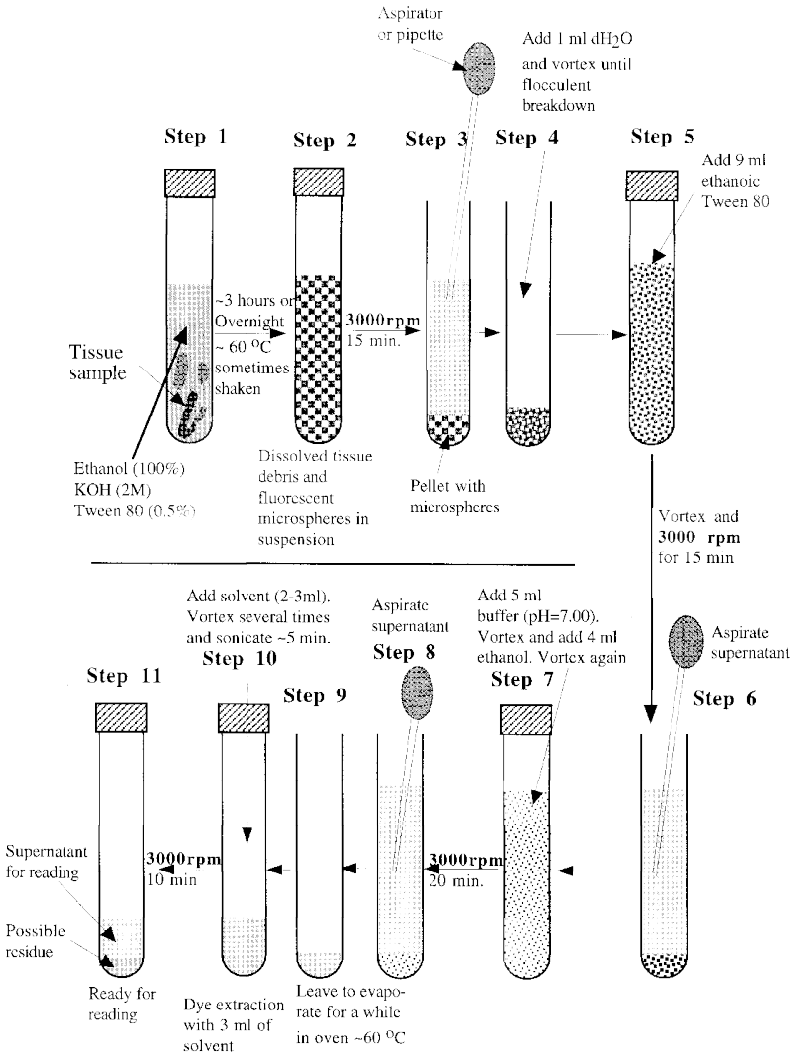

Fig. 1. Schematic diagram of the procedure used for fluorescent microsphere recovery.

6. The supernatant was aspirated as above (Step 3).

7. Five millilitres of 100 mM phosphate buffer (pH 7·00) was added to neutralize the pellet as alkaline solutions quench fluorescence. Using aqueous solutions increased the possibility of microsphere loss by adhesion to the surface of tubes or aggregation, which could readily be seen by eye when tubes were examined under bright light, and hence the buffer was followed by addition of 4 ml absolute ethanol and further vortexing, before spinning (1500 *g*) for 20 min.

8. The supernatant was aspirated, leaving up to 300 μl depending on the amount of tissue residue, and the remaining microspheres and pellet were quickly vortexed to ensure complete resuspension.

9. The tubes were left to evaporate in an oven at 60 °C and then briefly vortexed during this period to disperse more of the flocculent, until around 100 μl fluid remained. This improves solvent extraction of microspheres, which may be less efficient in a completely dry pellet.

10. Two or three millilitres of solvent (di(ethylene glycol) ethyl ether acetate, 98 %; Aldrich Chemical Co., Poole, Dorset, UK) was added according to the expected fluorescence intensity, and vortexed several

times over 3–5 min. After 30 min the tubes were sonicated in a water bath for 5 min to complete dye extraction by the solvent. The tubes were kept as far as possible in the dark after the solvent was added to avoid photo-bleaching of the fluorescent dyes.

11. After sonication, the tubes were ocassionally spun (1500 *g*) once more to sediment any undigested/undissolved material, though this was rarely necessary in our experiments. Readings should be completed within 1 h to avoid loss of signal intensity.

Fluorescence intensity, microsphere number and spillover matrix

Fluorescence intensity was determined with a Perkin-Elmer LS 50B luminescence spectrophotometer with an excitation wavelength range from 200 to 800 nm and emission wavelength range from 200 to 900 nm. Replicate analyses confirmed the nominal precision and accuracy of the spectrofluorimeter, following established analytical test procedures. The absolute value for fluorescence intensity depends on the selection of slit widths, photomultiplier sensitivity, absolute intensity and quantum efficiency (excitation energy/emission energy) of each fluorescent label. In preference to the supplied software package and autosampler, we chose to use the FAC8A program developed by the Fluorescent Microspheres Research Centre (University of Washington, Seattle, WA, USA) which allowed dual scanning of each sample at preset excitation and emission wavelengths and slit widths, and an in-house designed suction-driven sampling device. Fluorescence intensities were measured at their optimal excitation/emission wavelength pairs, using slit widths of 4 nm and an emission filter with a cutoff at 390 nm. The fluorescence of each sample was read in a quartz flow cell of 300 μl capacity in duplicate. Further replication was not required.

To investigate the quantification error (machine replication and possible quenching) five different fluorescent microsphere labels (YG, O, BG, R, and C) at varying concentrations were subjected to successive readings of fluorescence intensity (FI). To minimize dilution inaccuracies we transferred 200 μl aliquots from stock vials of each colour into 9·80 ml of solvent; further dilutions were then performed as required, and the first reading was compared with the mean of 20 subsequent readings. Quantum efficiencies varied among colours, with minimum fluorescence intensities for crimson and maximum intensities for yellow–green. Multiple labels were tested to check the peak separation, and the sensitivity of specific dyes over a microsphere concentration range of 50–10 000 ml^{-1}. Using the paired wavelength analysis when fluorescent dyes are excited at their optimal wavelength, the assessment of the percentage spillover (S) from pure samples of each colour into adjacent colours was calculated as:

$$S = B/A \times 100,$$

where B is spillover intensity of a specific colour in its adjacent spectral peak, and A is the intensity of the same colour measured at its own emission peak. Spillover can be reduced between adjacent colours if necessary by narrowing the slit width of the emission monocromator so that only emitted light within a narrow spectral range is measured, with the compromise of a reduced intensity.

Microsphere stability and recovery

In vitro tests to confirm the stability of the fluorescent labels were carried out at intervals by repeated measurements of fluorescence intensity in solutions of 1000 microspheres ml^{-1} solvent after storage in the light, dark, and/or in the cold. Possible interference by tissue composition was checked by sampling a wide range of tissue types including brain, heart, liver and muscle for their relative fluorescence intensities with different labels.

Several tests were performed to quantify the recovery of microspheres during processing:

1. Supernatants from the reference blood samples and kidney (the tissue with the highest absolute fluorescence intensity) were analysed after (i) completing digestion (Step 3); (ii) absolute ethanol washing (Step 6); and (iii) buffer washing (Step 8).

2. One hundred microlitres of pure microspheres for each label were processed in parallel with tissue samples to provide an absolute reference for microsphere recovery during sample preparation. An additional test was to add the same number of orange microspheres to empty vials and to 16 different tissue samples in which other labels had been used.

3. The effect of tissue on microsphere recovery was tested by adding 100 μl of pure microspheres to *ca.* 1 g of muscle, kidney or liver which did not contain microspheres prior to maceration.

The percentage loss (L) of microspheres was evaluated as:

$$L = \text{supernatant intensity/actual intensity} \times 100.$$

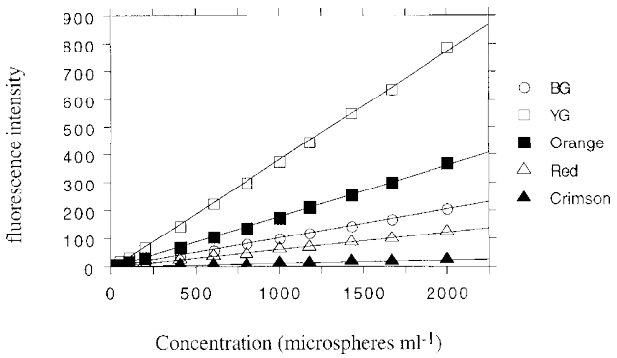

Fig. 2. Linear relationship between the fluorescence intensity (arbitrary units) and microsphere concentration; all regressions extended up to initial concentrations of 12 000 microspheres ml^{-1}

Biocompatibility

The stability of haemodynamic parameters (heart rate, systolic and diastolic blood pressure) was used as an *in vivo* test of the animals tolerance of repeated injections and hence biocompatibility of the microspheres. Possible aortic streaming of microspheres was quantified as the difference between left and right kidney blood flows, and arteriovenous shunting was assessed by the proportion of total injected label collected in the lungs.

Calculation of blood flow

After measuring RM activity, RBF was calculated following correction for background and cross-over; blood flow was also calculated following subsequent measurement of FM intensity for tissue and reference blood samples as follows:

$$Q = (A_t/A_b) \times (s/w) \times 100,$$

where Q is blood flow (in ml min^{-1} $(100\ g)^{-1}$), A_t is individual sample intensity, A_b is reference blood sample intensity, s is reference blood sample withdrawal speed (ml min^{-1}), and w is tissue weight (g). Predictions regarding optimal numbers of microspheres to be injected, based on calculations from Heyman *et al.* (1977) of experimental error, were tested to establish the sensitivity of the method. This is particularly important for our experiments measuring low flow rates (using glycolytic or ischaemic muscle, and for use with small organs or tissue samples).

Statistics

Analyses were performed using least squares linear regression and analysis of variance (ANOVA). The intra-method (co-injection) error was expressed as either absolute or relative value (difference of the blood flow values determined by the two microsphere labels) divided by arithmetic mean of both methods multiplied by 100. Percentage coefficient of variation (c.v.) was calculated as:

$$\text{c.v.} = (\text{s.d.}/\text{mean}) \times 100.$$

In addition to ANOVA, for the inter-method comparison of blood flow estimates obtained by either separately or co-injected radioactive and fluorescent microspheres the following formula was used for each paired tissue:

$$\text{s.d. unit} = \frac{(\text{mean fluorescent BF estimate} - \text{mean radioactive BF estimate})}{\text{standard deviation of radioactive BF estimate}}.$$

Data were also compared using the analysis of Bland & Altman (1986), where the differences between the two methods (RM blood flow − FM blood flow) was plotted against the average of both methods, and the regression line displayed relative to a ± 2 s.d. confidence band. Statistical significance was accepted at $P < 0{\cdot}05$, and all data are expressed as means $\pm$ s.e.m.

Table 1. *Optimal excitation and emission wavelengths for fluorescent microspheres in 98 % di(ethylene glycol) ethyl ether acetate, with slit widths of 4 nm*

Colour	Excitation wavelength (nm)	Emission wavelength (nm)
Blue–green	431 (430)*	466 (465)
Yellow–green	496 (505)	506 (515)
Orange	530 (540)	555 (560)
Red	570 (580)	600 (605)
Crimson	625 (625)	645 (645)

*The nominal values given by the manufacturer are in parantheses.

RESULTS

Fluorescence intensity, microsphere number and spillover matrix

Linearity of the method was excellent, with regression of fluorescence intensity and the number of microspheres giving a linear correlation coefficient $r^2 > 0{\cdot}999$, across a broad range of microsphere concentrations (Fig. 2). There were differences in specific fluorescence intensity among labels, with the highest value for yellow–green and the lowest value for crimson, at the same concentration (Fig. 2). The quantification errors expressed as variability (c.v.) were 0·330, 0·337, 0·537, 1·189 and 1·624 % at intensities of 630·3, 546·4, 203·0, 71·7 and 17·7 fluorescence units, respectively. Similarly, there were no significant differences between the first and subsequent readings within a short time (*ca.* 10 min) at both low and high microsphere concentrations in 22 different tissue samples, and the correlation among duplicate readings was excellent ($r = 1{\cdot}00$).

Optimal excitation and emission wavelengths were determined by dual wavelength scans, and were found to be close to the nominal values supplied by the manufacturer (Table 1). When excited at their optimal wavelength and as narrow a slit width as practicable (4/4 nm), almost no spillover (spectral overlap) occurred among BG, YG, O, and R labels in the eight different concentrations examined (Table 2). Crimson microspheres were not used experimentally due to both a high spillover and low fluorescence intensity; they showed a maximum spillover of 5·29 % into red, 4·94 % into orange, 1·13 % into YG at a concentration of 2857 microspheres ml^{-1}, and 0·61 % into BG at 5000 ml^{-1}. At a concentration more commonly found in tissue ($\leqslant 200$ microspheres) all colours, including crimson, showed essentially no spillover (Table 2), as shown in other studies (van Oosterhout *et al.* 1995). The sensitivity of FMs was at least as great as for the RMs and, with some adjustments in assay conditions, was significantly greater for most tissues. For example, while the minimum number of injected microspheres required for both RM and FM determination of resting BF in tissue of > 100 mg was similar, the lack of significant spillover and essentially zero background meant that reliable estimates of BF could be obtained for much smaller tissue samples, down to *ca.* 10 mg, by the FM method.

Microsphere stability and recovery

Thirty samples were read in duplicate and gave stable readings for around 1 h, with fluorescence intensity (FI) over the next 4 h being lower compared to the first reading by 1–5 %. The loss of FI measured on consecutive days was markedly dependent on the

Table 2. *Spillover matrix* (%) *and absolute intensities of different fluorescent microspheres at different concentrations*

Colour	BG	YG	O	Red	C	No. of microspheres	Fluorescence intensity
BG	100	0·40	0·01	0·00	0·00	10000	1037
YG	0·02	100	0·00	0·00	0·00	10000	3922
Orange	0·05	0·23	100	0·35	0·00	10000	1940
Red	0·09	0·43	0·54	100	0·01	10000	626
Crimson	0·39	1·87	4·32	4·84	100	10000	138
BG	100	0·36	0·00	0·00	0·00	5000	527
YG	0·02	100·0	0·07	0·00	0·00	5000	1960
Orange	0·09	0·21	100	0·36	0·00	5000	970
Red	0·09	0·29	0·53	100	0·00	5000	324
Crimson	0·61	1·71	4·71	5·42	100	5000	67
BG	100	0·16	0·00	0·00	0·00	2857	294·8
YG	0·00	100	0·03	0·00	0·00	2857	1120
Orange	0·00	0·15	100	0·37	0·00	2857	538
Red	0·00	0·18	0·56	100	0·00	2857	178
Crimson	0·00	1·13	4·94	5·29	100	2857	36·3
BG	100	0·16	0·00	0·00	0·00	1666	170
YG	0·00	100	0·01	0·00	0·00	1666	638
Orange	0·00	0·15	100	0·21	0·00	1666	302
Red	0·00	0·24	0·33	100	0·00	1666	102
Crimson	0·00	0·91	3·88	4·15	100	1666	19·8
BG	100	0·33	0·00	0·00	0·00	1000	100
YG	0·00	100	0·00	0·00	0·00	1000	376
Orange	0·00	0·05	100	0·06	0·00	1000	177
Red	0·00	0·07	0·16	100	0·00	1000	59·4
Crimson	0·00	0·00	2·11	1·74	100	1000	11·1
BG	100	0·09	0·00	0·00	0·00	606	58·4
YG	0·00	100	0·00	0·00	0·00	606	226
Orange	0·00	0·00	100	0·00	0·00	606	104
Red	0·00	0·00	0·00	100	0·00	606	34·5
Crimson	0·00	0·00	1·60	0·79	100	606	6·6
BG	100	0·00	0·00	0·00	0·00	202	18·8
YG	0·00	100	0·00	0·00	0·00	202	71·3
Orange	0·00	0·00	100	0·00	0·00	202	33·1
Red	0·00	0·00	0·00	100	0·00	202	10·8
Crimson	0·00	0·00	0·00	0·00	100	202	2·1
BG	100	0·00	0·00	0·00	0·00	50	5·08
YG	0·00	100	0·00	0·00	0·00	50	16·7
Orange	0·00	0·00	100	0·00	0·00	50	7·88
Red	0·00	0·00	0·00	100	0·00	50	2·71
Crimson	0·00	0·00	0·00	0·00	100	50	0·10

BG, blue–green; YG, yellow–green; O, orange; R, red; C, crimson.

fluorescence dye, as reported by others (Glenny *et al*. 1993; van Oosterhout *et al*. 1995). The daily losses of FI over 7 days were 38, 23, 15, 8, 5 % in C, R, BG, YG, and O FMs, respectively. After 14 days the decrease in intensity was minimum (13 %) for YG FMs and maximum (73 %) for C FMs. All samples were held at room temperature in the dark between readings.

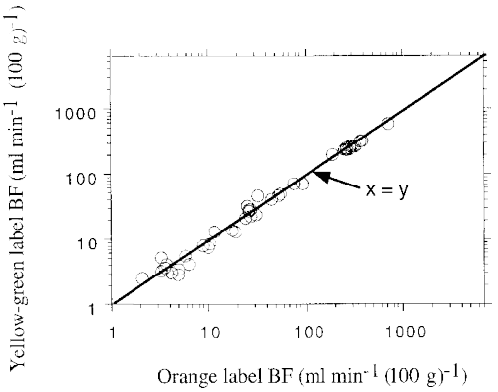

Fig. 3. Intra-method evaluation by simultaneous injection of microspheres with two different fluorescence labels, yellow–green (YG) and orange (O). Results show mean blood flow (BF) estimates in 25 different tissues. Line of identity ($x = y$) is shown. Regression equation is YG = 3·18 + 0·944(O); $r^2 = 0{\cdot}99$.

Leaching of dye, determined by measurement of supernatant fluorescence, was minimal when pure microsphere samples were kept in the dark. Storage of tissue samples in a refrigerator or freezer for up to 10 months had no detectable effect on specific fluorescence or leaching. In our experience fatty tissue posed no problem for tissue digestion, and all tissue used were digested within 4 h without requiring further processing. Aliquots from supernatants at each step of tissue processing were taken ($n = 4$–7) to estimate the percentage loss of microspheres which for pure microspheres, blood and tissue samples up to the buffer washing stage was < 0·1 %. While washing with absolute alcohol gave 100 % recovery, at the aqueous buffer washing stage (Step 7 in Fig. 1) microsphere losses were between 2·9 and 4·1 % in pure YG and R microsphere tubes, respectively. This was higher than at any other stage of processing, and for other samples at the same stage ($P < 0{\cdot}01$). Adding *ca.* 1 g unlabelled tissue to the pure reference samples improved microsphere recovery even further, with, as before, a minimum loss with YG of 0·45 % and a maximum loss with R microspheres of 1·23 %. Spiking tubes with O microspheres at the start of processing revealed a modest (1·9 %) experimental error.

Biocompatibility

Animals were tolerant of repeated injections of fluorescent microspheres, which produced no significant alterations in haemodynamics when compared with radioactive microspheres, and there were no evident changes for up to three injections, from baseline values of heart rate (405 ± 18 beats min^{-1}) or mean blood pressure ($129{\cdot}6 \pm 6{\cdot}9$ mmHg). Any bilateral difference in kidney blood flow was always low (< 5 %; $r^2 = 0{\cdot}9$) indicating adequate mixing *in vivo*, while minimal shunting (< 2 %) likewise indicated that integrity of the cardiovascular system was not compromised by sequential injection of differently labelled microspheres.

Blood flow measurements

When two different types of fluorescent microspheres were simultaneously injected under resting conditions there was a very good agreement ($r^2 > 0{\cdot}99$) between the two estimates of

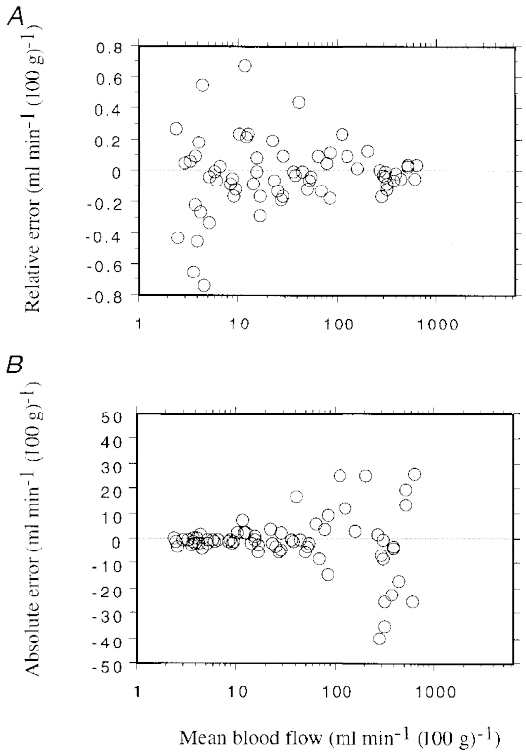

Fig. 4. Intra-method evaluation of regional blood flow in various tissues determined by simultaneous injection of two different fluorescent microspheres (YG and O). *A*, relative intra-method error. *B*, absolute error. Results clearly show highest relative error at low blood flow, and the dependence of absolute error on absolute blood flow, as a function of mean blood flow estimated from the co-injected labels.

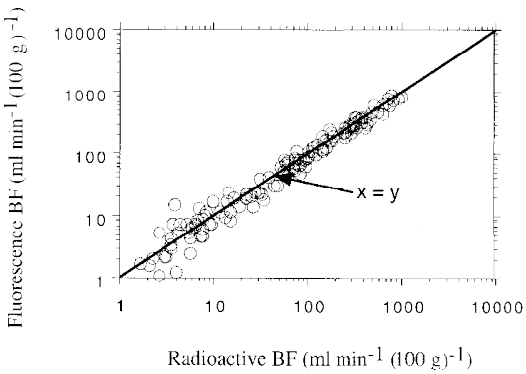

Fig. 5. Inter-method evaluation of regional organ blood flow (BF) by simultaneous injection of fluorescent (BG, YG, O or R) and radioactive (^{57}Co, ^{113}Sn or ^{46}Sc) labelled microspheres in 12 different tissues from 8 different rats. Line of identity ($x = y$) is shown. Regression equation is $y = -0{\cdot}069 + 1{\cdot}022(x) - 3{\cdot}137 \times 10{-}5(x^2)$; $r^2 = 0{\cdot}963$.

Table 3. *Comparison of different regional organ blood flows with simultaneously injected fluorescent and radioactive microspheres*

	Control			Hypotensive		
Tissue	Radioactive	Fluorescent	S.D. unit	Radioactive	Fluorescent	S.D. unit
TA	5·1 ± 0·8	4·8 ± 1·2	0·26	13·6 ± 8·3	11·1 ± 6·6	0·17
EDL	5·1 ± 0·9	3·5 ± 0·9	0·62	13·2 ± 7·8	12·1 ± 6·2	0·08
Soleus	9·9 ± 2·2	6·6 ± 2·1	0·11	36·7 ± 22	31·8 ± 19	0·12
Liver	25·0 ± 3·6	24·5 ± 4·1	0·05	7·2 ± 3·7	6·6 ± 2·9	0·09
Adrenals	587 ± 129	444 ± 83	0·49	205 ± 58	166 ± 43	0·39
Kidney	376 ± 49	364 ± 39	0·11	219 ± 44	211 ± 47	0·10
Left ventricle	150 ± 102	155 ± 87	0·13	382 ± 210	315 ± 178	0·18

Values are means ± S.E.M.; blood flows are expressed as ml min^{-1} (100 g)$^{-1}$. For control, $n = 6$–9; for hypotensive conditions, $n = 3$. TA, tibialis anterior; EDL, extensor digitorum longus. There were no significant differences between radioactive and fluorescent estimates (ANOVA). S.D. unit = (mean flow of fluorescent label − mean flow of radioactive label)/standard deviation of radioactive label.

Table 4. *Comparison of different skeletal muscle blood flows with simultaneously injected fluorescent and radioactive microspheres during rest and indirect electrical stimulation*

	Resting			Stimulated		
Muscles	Radioactive	Fluorescent	S.D. unit	Radioactive	Fluorescent	S.D. unit
TA cortex	5·7 ± 1·0	8·2 ± 0·8	0·96	73·5 ± 15	81·7 ± 11	0·18
TA core	4·4 ± 1·0	6·3 ± 1·5	0·72	135 ± 22	153 ± 33	0·26
Mean TA	5·0 ± 0·8	5·6 ± 1·7	0·11	104 ± 16	110 ± 18	0·12
EDL	5·1 ± 0·9	3·5 ± 0·9	0·68	100 ± 15	93·0 ± 16	0·17
Soleus	9·9 ± 2·2	6·6 ± 2·1	0·62	5·5 ± 1·1	7·8 ± 0·8	0·62

Values are means ± S.E.M.; blood flows are expressed as ml min^{-1} (100 g)$^{-1}$. For resting conditions, $n = 6$–9; for indirect electrical stimulation, $n = 8$–9. There were no significant differences between radioactive and fluorescent estimates (ANOVA). S.D. unit = (mean flow of fluorescent label − mean flow of radioactive label)/standard deviation of radioactive label.

blood flow from lowest to highest flow rates (white adipose tissue to kidney) (Fig. 3), the mean C.V. being 6·03 % for all samples. Relative and absolute errors were also estimated in this intra-method comparison using two different (YG and O) fluorescent microspheres in two rats. As expected, there was a large relative error in low blood flow estimates such as for adipose tissue and different skeletal muscles under resting conditions (Fig. 4*A*), while absolute error was large in high blood flow tissues such as kidney and adrenal gland (Fig. 4*B*).

The inter-method evaluation of regional organ blood flow, using 12 different tissues in eight rats simultaneously injected with radioactive and fluorescent microspheres, showed close agreement between blood flows estimates ($r^2 = 0{\cdot}963$; Fig. 5). The mean blood flows are given in Table 3 under control and hypotensive conditions from different tissues, and in Table 4 for resting and stimulated skeletal muscles. Note that soleus was not stimulated in our experiments, although it is shown in the stimulated column as a reference for non-stimulated skeletal muscle. The RM and FM estimates of blood flows were not significantly different in any tissue under these conditions. The difference between estimates was further tested by calculating the S.D. unit, which for radioactive estimates was < 1 in all tissues (Table 3). When muscle was stimulated there were no significant variations between the two microsphere types, with lower

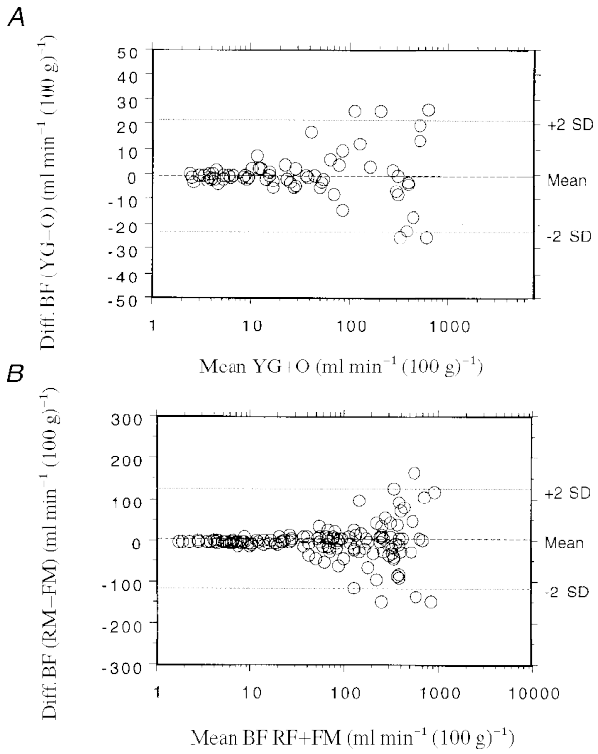

Fig. 6. Comparison of blood flows using the analysis method of Bland & Altman (1986). *A*, intra-method measurement of BF with simultaneously injected fluorescent (YG and O) microspheres, showing the difference in BF plotted against mean BF for 64 different tissue samples. *B*, inter-method measurement of BF with simultaneously injected RMs and FMs at rest, under induced hypotension, and during stimulation of skeletal muscle. Data for 48 tissue samples from 8 rats.

S.D. units of $< 0{\cdot}26$ for all stimulated skeletal muscle and for non-stimulated soleus where S.D. was < 1 (Table 4). For both types of comparison the majority of differences in absolute blood flow lie within the ± 2 S.D. confidence band (Fig. 6).

A wider range of tissues was used in separately injected RM and FM experiments for regional blood flow estimation, from pineal organ to brain. The overall data showed that RM and FM measurements under resting conditions, with few exceptions, were not significantly different (ANOVA), the S.D. unit being < 1 with a mean difference of $2{\cdot}6\,\%$ for 24 tissues (data not shown). In the paired tissue comparison there was some variation between BF measurements in 4 of 31 different tissues (TA cortex, ear, skin, and small intestine), where blood flow estimates were higher with fluorescent microspheres ($P < 0{\cdot}05$). However, the higher blood flow most probably reflects seasonal adjustments in tissue function (see Discussion). Blood flows were also estimated for skeletal muscles with different blood flow capacity, at rest and during functional hyperaemia. There were no significant differences between microsphere types under resting and stimulated conditions (ANOVA) with S.D. unit $\leqslant 1$ for all muscles (Table 5).

Table 5. *Comparison of different muscle blood flows with separately injected fluorescent and radioactive microspheres at rest and during indirect electrical stimulation*

	Resting			Stimulated		
Tissue	Radioactive	Fluorescent	S.D. unit	Radioactive	Fluorescent	S.D. unit
TA cortex	6·0 ± 0·6	8·2 ± 0·9	1·02	115 ± 21	135 ± 24	0·27
TA core	4·9 ± 0·6	4·6 ± 0·6	0·06	190 ± 26	186 ± 24	0·05
TA (mean)	5·5 ± 0·5	6·5 ± 0·6	0·92	152 ± 14	151 ± 22	0·02
EDL	4·5 ± 0·5	6·1 ± 0·7	0·55	138 ± 24	162 ± 30	0·28
EHP	7·2 ± 3·0	7·9 ± 1·5	0·19	158 ± 3	162 ± 30	0·95

Values are means ± S.E.M., blood flows are expressed as ml min^{-1} (100 g)$^{-1}$. For radioactive, $n = 12$; for fluorescent, $n = 6$. There were no significant differences between radioactive and fluorescent estimates (ANOVA). S.D. unit = (mean flow of fluorescent label − mean flow of radioactive label)/standard deviation of radioactive label.

DISCUSSION

Microsphere loss during tissue processing is one of the biggest problems associated with the use of non-radioactive (fluorescent and coloured) microspheres, with both filtration and sedimentation procedures (Hale *et al*. 1988; Kowallik *et al*. 1991; Prinzen & Glenny, 1994; Hakkinen *et al*. 1995; van Oosterhout *et al*. 1995). Although recovery tests were performed in these previous studies, it is not clear whether this varied with stage of processing. In the present study supernatants and pure microsphere samples were analysed from each step of the recovery protocol, which showed that there was essentially 100 % recovery for all blood and tissue samples regardless of mass and blood flow capacity. This is important as estimates of cardiac output (CO) are inversely proportional to the number of spheres in the reference blood sample, which may then lead to erroneously high values of CO if the efficiency of recovery is low, as has been found for coloured microspheres (Degens *et al*. 1996). The only measurable loss was in the pure microsphere tubes (3–5 %), while the presence of tissue during processing reduced microsphere loss to < 1 %. This modest loss occurred only in the aqueous buffer washing stage and has no appreciable effect on calculated RBFs. Nevertheless, complete sedimentation was achieved by the use of ethanoic solutions, which presumably increased the specific gravity difference between microspheres and medium, aided by higher centrifugation speeds than used previously (van Oosterhout *et al*. 1995; Hakkinen *et al*. 1995; Tan *et al*. 1997). Interestingly, while the recovery of pure coloured microspheres was similar to that obtained with FMs, in contrast to the present study the presence of tissue appeared to reduce the efficiency of recovery (Degens *et al*. 1996).

The resolution of the fluorescence method is dependent on the amount of label per microsphere, the quantum efficiency of the fluorescent dye, and the sensitivity of the spectrophotometer (Glenny *et al*. 1993). The latter is determined by light source intensity, transmittance of the filters or monocromators used to select excitation and emission wavelengths, efficiency of photomultiplier tube, and the type of reader used (cuvette or microplate). Using optimal excitation and emission wavelengths there was very good correlation between fluorescence intensity (FI) and the number of microspheres across a wide range of concentrations, FI being maximum in YG FMs and minimum in C FMs at all concentrations. We did not use C in subsequent experiments due to both its low quantum efficiency and significant spillover into adjacent colours at high concentrations. This low FI for

C has been reported previously (van Oosterhout *et al*. 1995), although in another study C showed the maximum intensity among colours used (Glenny *et al*. 1993), presumably reflecting differences in instrument characteristics, and further emphasizing the need for validation trials within each laboratory.

A particular benefit of using the fluorescence method is the possibility of using low numbers of microspheres and volumes of solvent, thereby minimizing the sample dilution required during reading and the potential for vascular blockage (both potential sources of error). Narrow slit widths prevent cross-over among microsphere labels, and may be adjusted according to individual machine sensitivity and specific microsphere FI; e.g. using our high sensitivity fluorimeter YG and O labels have much higher quantum efficiency than C or R microspheres. The resolution was helped by the emission filter cut-off, which by not using blue fluorescence we increased from 350 to 390 nm and therefore minimized cross-over. In other studies where the cut-off had to be left at 350 nm for use with blue microspheres there was a greater variability in blood flow estimations (van Oosterhout *et al*. 1995).

The repeatability of duplicate readings of the same sample was excellent over a wide range of FIs. Although differences in repeat readings were modest, these showed that there was a potential variability according to absolute FI, and parallels the observation that a low number of microspheres increased the error of blood flow estimation in RM measurements (Heyman *et al*. 1977), while relative error increased in tissue with low blood flows. When two different types of FM were simultaneously injected under resting conditions there was a very good relationship between estimated BFs across a wide range of tissue blood flow, the relative error varied inversely with blood flow and absolute error varied directly with blood flow. Likewise, simultaneous injection of radioactive and fluorescent microspheres also gave a good correlation with no significant variations among paired organs under different experimental conditions across the wide range of blood flows induced in our experiment. Importantly, when skeletal muscles were stimulated there were no significant differences between RM and FM estimations and S.D. units showed a tendency toward zero in all muscles.

Fluorescent microspheres produced no significant alteration in blood pressure during or after injection in rats, as found with radioactive microspheres, and hence estimates of RBF were presumably obtained without significant perturbation of haemodynamic status. Indeed, on injecting a much greater number of coloured microspheres in rabbits than was used in the present study, when corrected for differences in body weight, Degens *et al*. (1996) found that $< 10\,\%$ of capillaries were blocked in TA and EDL muscles. Although blood flows were higher with FM than RM measurements in some tissues from rats that were separately injected with either radioactive or fluorescent microspheres, these data can be explained by seasonal differences in temperature, as the FM study was performed in summer and RM experiments in winter, and are most unlikely to reflect different microsphere quality. The resting and stimulated muscle BFs were also estimated with separately injected RM and FM microspheres, again with no significant differences between groups in either condition, with the FM estimates within 1 S.D. of the RM values. Regional differences within TA muscle were evident, with the mainly glycolytic cortex having a consistently lower BF than the mainly oxidative core, evaluated by both FM and RM microspheres (both simultaneously and separately injected) during functional hyperaemia. The measurements were similar to the many published estimates of blood flows using other techniques in the rat for both resting and working muscles, and for other tissues (Goldman & Wurtman, 1964; Jansky & Hart, 1968; Bulow & Madsen, 1976; Foster & Frydman, 1978; Shepherd, 1983; Hudlická, 1985; Kuwahira *et al*. 1993). The pineal organ (weight 7–10 mg) blood flow could only be

estimated using YG microspheres, demonstrating that the quantum efficiency of dye is limiting when low numbers of microspheres are trapped during estimations of small organ and/or low blood flows. The FM technique was also sensitive enough to establish regional blood flow patterns in response to hypothermia in rats and hamsters (Deveci & Egginton, 1998*a*,*b*). Similar differences in inter- and intra-muscle BFs using the FM method have recently been reported in larger animals (Tang *et al*. 1998).

In conclusion, this study has demonstrated that the fluorescent microsphere technique can be used in small mammals for regional blood flow determination in tissues with a wide range of mass. In particular it has shown that full recovery of trapped microspheres is possible, that tissue digestion may be completed in a shorter time than that so far reported, and the error-prone filtration method can be replaced by one of sedimentation since no significant loss of microspheres occurred during routine tissue processing, with the presence of tissue helping to reduce the minor loss of microspheres even further. There was no spillover among the five different colours used which makes any practical difference within the design of this study (an exception was crimson, but then only in higher concentrations than would be used *in vivo*) which, together with essentially no background interference, allows BF estimates to be made in smaller tissue samples than previously possible. Importantly, there were no significant haemodynamic consequences of using FM *in vivo* while measurements of resting, hyporaemic and hyperaemic skeletal muscle blood flows showed excellent agreement between the new FM technique and the established RM methods. Although there are still improvements in technique that may be possible, it is clear that fluorescent microspheres can replace radioactive microspheres to provide similar estimations of regional blood flow, but with advantages in both safety and cost.

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