Use of an automated fluorescent microsphere method to measure regional blood flow in the fetal lamb[1]

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Abstract: We have developed a method for measuring regional blood flow by means of fluorescent microspheres in all organs and tissues of the fetal lamb, including brain, heart, lung, liver, gut, spleen, kidney, adrenal, brown fat, skin, muscle, bone, and placenta. Five different fluorescent-labeled microspheres were used: blue (B), yellow–green (Y), orange (O), red (R), and crimson (C). An automated, 96-well microplate fluorescent reader (bottom reading) was chosen for the assay because of the rapidity and high throughput that it offers. Tissue samples were digested by 4 M ethanolic KOH. The sedimentation method and dye extraction with Cellosolve acetate, as previously reported by others, were used for the sample processing. The bones were crushed and allowed to directly soak in Cellosolve acetate to extract the dye. The relationship between microsphere number and fluorescent intensity was linear over a broad range of microsphere numbers (80 – 20 000/mL). The coefficients of variation of within-run and between-run precision were 3.39 ± 1.10% and 4.54 ± 1.10%, respectively. Recovery of microspheres from tissues and blood averaged 94.3 ± 2.5% and was not dependent on microsphere number. The spillover of the fluorescent signals into adjacent colors was 4.0 ± 0.1% for O to Y, 8.1 ± 0.4% for O to R, and 9.1 ± 0.5% for R to C, and these values were constant over a wide range in concentrations of the microsphere pairs. No evidence was obtained for quenching of the emission of one fluorophore via photon absorption by another fluorophore. The measurements of regional blood flow obtained with fluorescent microspheres in three chronically instrumented fetal lambs at ~140 days gestation were similar to the flow estimates obtained using radioactive microspheres in four other fetal lambs at the same gestational age. The fluorescent method is thus a viable alternative to the radioactive technique for the measurement of regional blood flow to all fetal organs and tissues, particularly when an automated fluorescent microplate reader is employed to reduce analysis time.

Key words: fluorescent microspheres, organ and tissue blood flow, fluorescent microplate reader, spillover correction.

Résumé : Nous avons développé une méthode utilisant des microsphères fluorescentes pour mesurer le débit sanguin régional dans tous les organes et tissus foetaux de la brebis, notamment le cerveau, le coeur, le poumon, le foie, l'intestin, la rate, le rein, la surrénale, la graisse brune, la peau, le muscle, l'os et le placenta. Les cinq marqueurs fluorescents suivants ont été utilisés : bleu (B), jaune–vert (J), orange (O), rouge (R) et cramoisi (C). Un lecteur fluorescent pour microplaques de 96 puits (lecture plateau inférieur) automatisé a été choisi pour l'essai en raison de sa rapidité et de sa grande efficacité. La digestion des échantillons de tissus a été effectuée en utilisant 4 M de KOH éthanolique. La méthode de sédimentation et l'extraction du colorant par acétate de Cellosolve, telles qu'indiquées antérieurement par d'autres chercheurs, ont été utilisées pour traiter les échantillons. Les os ont été broyés et immergés directement dans l'acétate de Cellosolve pour en extraire le colorant. La relation entre le nombre de microsphères et l'intensité de la fluorescence a été linéaire sur une vaste plage de concentrations de microsphères (80 – 20 000/mL). Le coefficient de variation de la précision à l'intérieur d'une série et entre les séries a été de 3,39 ± 1,10 et 4,54 ± 1,10%, respectivement. La récupération des microsphères des tissus et du sang a été de 94,3 ± 2,5% en moyenne, et a été indépendante du nombre de microsphères. Le débordement des signaux fluorescents sur les couleurs adjacentes a été de 4,0 ± 0,1% pour ce qui est de O vers J, de 8,1 ± 0,4% pour O vers R et de 9,1 ± 0,5% pour R vers C, et ces valeurs ont été constantes sur une vaste plage de concentrations des paires de microsphères. Il n'y a pas eu d'atténuation de l'émission d'un fluorophore via l'absorption photonique par un autre fluorophore. Les mesures du débit sanguin régional obtenues avec des microsphères fluorescentes dans trois foetus chroniquement instrumentés à environ 140 jours de gestation ont été similaires aux estimations de débit obtenues en utilisant des microsphères radioactives dans quatre autres foetus à la même période de gestation. Ainsi, la méthode fluorescente constitue une solution de rechange possible à la technique radioactive pour mesurer le débit sanguin régional dans tous les tissus et organes foetaux, particulièrement lorsqu'un lecteur fluorescent de microplaques automatisé est utilisé pour réduire le temps d'analyse.

Mots clés : microsphères fluorescentes, débit sanguin tissulaire et organique, lecteur fluorescent de microplaques, correction du débordement.
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Introduction

Radioactive microspheres have been used extensively for quantification of systemic, regional, and placental blood flows in fetal lambs (Heymann et al. 1977; Rudolph and Heymann 1967; Rurak et al. 1990*a*). The technique has become a standard method for measuring these blood flows and the distribution of cardiac output in fetal and adult cardiovascular research, and its validity is well documented (Buckberg et al. 1971; Dole et al. 1982; Heymann et al. 1977). However, procedures employing radioactive microspheres have a number of serious disadvantages. These include restriction to specially licensed laboratories, and the short half-lives of some radioisotopes, which requires that they be used soon after manufacture. There is also the inconvenience of prolonged storage of radioactive carcasses and waste before they can be disposed of, and the increasing difficulty in finding disposal sites due to increased regulatory restrictions. Most importantly, there is the potential of hazards to laboratory personnel as well as to the environment due to handling, processing, and disposal of radioactive material.

In response to the practical and regulatory issues involved in the use of radionuclide-labeled microspheres, optical detection techniques involving several new types of microspheres have recently been developed. These include nonradioactive microspheres labeled with various colored dyes (Hakkinen et al. 1995; Hale et al. 1988; Kowallik et al. 1991), and x-ray fluorescence excitation of microspheres loaded with elements of high atomic number (Mori et al. 1992; Morita et al. 1990). In addition, a range of intensely fluorescent labeled latex microspheres specifically for regional blood flow determination are now commercially available (An et al. 1996). Fluorescent microspheres offer higher sensitivity, superior color separation, and greater ease of measurement of regional blood flow compared with colored microspheres (Prinzen and Glenny 1994). Furthermore, effective methods have been developed to extract the microspheres and fluorescent dyes they contain from tissue samples, including tissue digestion and filtration using filtration devices (Glenny et al. 1993). More recently a sedimentation method has been validated for dye extraction that does not require a filtration step (Van Oosterhout et al. 1995). Blood flow estimates have been obtained using fluorescent-labeled microspheres in some but not all tissues from dogs, pigs, and rabbits (heart, skin, kidney, brain, spleen, skeletal muscle, gut, and lung), and compared with measurements obtained using radionuclide-labeled microspheres (Abel et al. 1993; Austin et al. 1993; Chien et al. 1995; Glenny et al. 1993; Van Oosterhout et al. 1995). The correlation between the flow estimates of the two methods was excellent.

In the published studies to date, the fluorescent dyes have been mostly measured with conventional cuvette-type recording spectrofluorometers. Use of automated fluorescence multiwell plate readers would allow for more rapid measurement of samples, and this would be a particular advantage when many or all tissues are studied, as is the case with most fetal sheep research that has employed radioactive microspheres (Cohn et al. 1974; Court et al. 1984; Richardson et al. 1989; Rudolph and Heymann 1967; Rurak et al. 1990*a*). Glenny et al. (1993) have described the use of a 96-well plate reader attached to a conventional fluorimeter via a top–down excitation and emission detection system, and found quenching of the fluorescent signals at higher microsphere concentrations. However, the use of a stand-alone, automated multiwell plate reader does not appear to have been described.

Chronically instrumented fetal lambs are used extensively in prenatal physiological studies. In an effort to eliminate the need to use radioactive microspheres, we have developed a method for measuring regional blood flow by means of fluorescent-labeled microspheres in all individual fetal organs and tissues, including fetal brain, heart, lung, liver, gut, spleen, kidney, adrenal, brown fat, skin, muscle, bone, and placenta. Moreover, we have employed an automated fluorescence microplate reader, which greatly reduces the analysis time. And although we have used the methodology with tissues from fetal sheep, it likely could be utilized with tissues from any large animal species.

Methods

Animal preparation

The study was approved by The University of British Columbia Committee on Animal Care and conformed to the guidelines of the Canadian Council on Animal Care. Seven time-dated pregnant sheep were used and were subjected to surgical preparation at 127–133 days gestation (term ~145 days) using aseptic surgical procedures, as described previously (Kwan et al. 1995). Six of the seven sheep were used in a study of fetal cardiovascular and metabolic functions during spontaneous labor and delivery, which occurred in these animals at a gestational age of 142 ± 2 days (12 ± 2 days following surgery). The flow estimates reported in this paper were obtained 1–2 days prior to the onset of labor. In four of these animals, radioactive microspheres were employed, whereas in the remaining two fluorescent microspheres were used. The other physiologic data obtained from these preparations will be published elsewhere. In the remaining animal, two flow measurements were obtained using fluorescent microspheres, separated by ~7 min, at 141 days gestation (15 days following surgery).

Blood flow measurement

The method for measurement of regional blood flow in the fetus with radioactive microspheres has been described in detail before (Rurak et al. 1990*b*), and was followed for both radioactive and fluorescent microspheres in the current study. Radioactive microspheres (15.5 ± 0.1 μm in diameter), labeled with one of the following gamma-emitting radionuclides: ^{153}Gd, ^{51}Cr, ^{85}Sr, ^{95}Nb, and ^{46}Sc, was obtained from Du Pont - NEN Research Products (Billerica, Mass.). Fluorescent-labeled polystyrene latex microspheres (FluoSpheres, diameter 15.5 ± 0.3 μm) were obtained from Molecular Probes Inc. (Eugene, Oreg.) and were stored in a refrigerator (4°C) away from light when not in use. These microspheres are now available with 10 different color labels (An et al. 1996). In this study, only 5 were used: blue, yellow–green, orange, red, and crimson. The excitation and emission maxima described by the manufacturer for the fluorescent dyes are given in Table 1.

For each experiment, the stock solution containing the radioactive- or fluorescent-labeled microspheres was manually shaken, placed in a sonicating water bath for at least 30 min, and then agitated on a Vortex mixer for 1 min. An aliquot containing approximately 1.2×10^6 microspheres was removed from each of the stock vials just prior to injection. The microspheres were dispersed and mixed by drawing the solution back and forth through a sterile 26-gauge needle into a 1- or 3-mL plastic syringe. Following withdrawal of ~3 mL of fluid from the fetal tarsal vein catheter, they were then injected via this catheter over 30 s, followed by 3 mL of the initially withdrawn fluid and 5 mL of heparinized saline. Reference samples were withdrawn simultaneously from both the carotid and the femoral arterial

Table 1. Excitation and emission wavelength peak and range of fluorescent-labeled microspheres, along with the optimal excitation and emission wavelengths in Cellosolve acetate given by the manufacturer (in parentheses), and the emission and excitation filter combinations used in the CytoFluor™ 2350 instrument.

Color	EX peak (nm)	EX range (nm)	EX filter (nm)	EM peak (nm)	EM range (nm)	EM filter (nm)
Blue	356 (360)	330–385	360/40	417 (420)	380–490	460/40
Yellow–green	497 (490)	435–515	485/20	505 (506)	480–570	530/25
Orange	533 (530)	470–560	530/25	547 (552)	520–600	560/20
Red	568 (565)	495–605	560/20	590 (598)	550–640	590/20
Crimson	610 (600)	500–645	590/20	618 (635)	575–660	645/40

Note: EX, excitation wavelength; EM, emission wavelength. EX and EM filter represent CytoFluor™ 2350 standard filter sets with excitation or emission center wavelength/half bandwidth, respectively.

catheters into heparinized glass syringes attached to a Harvard infusion–withdrawal pump (Harvard Apparatus Co., Millis, Mass.), at a constant rate of 2.06 mL/min from 30 s before microsphere injection to 2 min after the 5-mL saline flush.

Sample processing

After the last microsphere injection, a lethal dose of sodium pentobarbital (Euthanol, MTC Pharmaceuticals, Cambridge, Ont.) was given to the ewe. The fetus was removed from the uterus, towel dried, and then weighed. All fetal organs and tissues were then dissected, cleaned carefully, blotted dry, and weighed. This included pituitary, spinal cord, cerebellum, medulla, pons, midbrain, frontal hemisphere, temporal hemisphere, right and left ventricle, interventricular septum, lung, nuchal muscle, forelimb skin, forelimb muscle (infraspinous), forelimb bone (humerus), diaphragm, omasum, abomasum, rumen, reticulum, spleen, kidney, brown fat, adrenal, small intestine, large intestine, liver, hind limb skin, hindlimb bones (femur, tibia, metatarsal), hindlimb muscle (biceps femoris), and placenta. Each tissue sample was cut into small pieces. The tissues from the animals that received radioactive microspheres were placed in aluminum foil dishes and were carbonized in an oven at 350°C for several days. The carbonized tissues were then packed into counting tubes and counted for 5 min on a Searle gamma-counting system (Searle Analytical, Des Plaines, Ill.).

The tissues obtained from animals that received fluorescent microspheres were processed using the sedimentation and dye extraction methods previously reported by others (Van Oosterhout et al. 1995), with the following modifications. The tissues were placed into preweighed 50-mL polypropylene centrifuge tubes (Elkay, Shrewsbury, Mass.). After being weighed, the tissue samples were further minced finely with scissors inside the test tube. Tissue samples were digested by adding freshly prepared warm 4 M ethanolic KOH with 0.5% Tween 80 (at least 3 mL/g tissue) so that the total volume was 50 mL. The tubes were then left at room temperature for 5 days, protected from the light, and shaken periodically. On the last day of tissue digestion, the tubes with brown fat and skin were placed in a warm water bath at 60°C for ~2 h. After these procedures, all the tissue samples were fully dissolved. They were then centrifuged at $1000 \times g$ in a swinging-bucket rotor at 25°C for 20 min, following which the supernatant was carefully discarded by suction until <1 mL remained above the sediment. The sediment was then resuspended in 50 mL deionized water, which was warmed in a 60°C water bath. The purpose of the 60°C temperature here and with the skin and brown fat digestion was to promote the removal of lipid from the samples. The tubes were centrifuged again, and the supernatant was discarded. Next, the sediment was resuspended in deionized water and centrifuged again, and in some cases, the washing step with deionized water was repeated if the supernatant was not clear. After the final centrifugation, the supernatant was removed carefully by hand using a Pasteur pipette, taking care not to either disturb or dry the pellet (~100 μL of supernatant left). Three millilitres of 2-ethoxyethyl acetate (Cellosolve acetate, Aldrich Chemical Co., Milwaukee, Wisc.) was added to the pellet, and the tubes were stirred using a Vortex mixer and then allowed to stand for at least 4 h to extract the fluorescent dye from the microspheres. They were then stirred again using a Vortex mixer and centrifuged, leaving a clear dye-containing solution from which 200-μL aliquots were pipetted in triplicate into individual wells of a translucent, polypropylene 96-microwell plate (Nunc, Inter Med, well volume 0.3 mL, Canadian Life Technologies, Burlington, Ont.). Fluorescence was then determined. The reference blood samples were processed in the same manner as described above, except for those containing the blue microspheres. With these latter samples, at the final washing step, the supernatant was removed until ~10 mL of deionized water was left. Then nitrogen gas was bubbled through the solution for 10 min, since preliminary work indicated that in the absence of this step, there was interference with the blue fluorescent measurement, as described in the Results section. Further processing of these samples was not different from that of the other samples. After Cellosolve acetate was added, all samples were stored in the dark until ready for reading. All of the fluorescence measurements were made on all of the samples from a single animal on the same day.

Bone tissues required special treatment, since digestion of the bone tissues did not occur with KOH and other methods of bone digestion that were tried were not successful. The bones were crushed with pincers within the test tubes. Six millilitres of Cellosolve acetate was then added, so that the bone tissues were completely immersed in the solvent. Over the next 5 days, the samples were periodically shaken. Thus, the fluorescent dye was extracted from the microspheres without tissue digestion. After centrifugation, 200 μL of the supernatant was transferred from each sample into individual wells of a 96-microwell plate. Fluorescence was then determined and corrected by a factor of 2 to account for the final dilution of the bone samples compared with the other tissues and reference samples.

Fluorescence detection and measurement

The absorption spectra of the five fluorescent microspheres dissolved in Cellosolve acetate were obtained by using an automated spectrophotometer (DU Series, Beckman Instruments, Fullerton, Calif.). The spectral absorbance curves thus established were used to determine the wavelengths at which the fluorescent microspheres have maximum and minimum absorbance. The emission spectra of the microspheres were determined using glass cuvettes with a spectrofluorometer (model RF-540, Shimadzu Corp., Kyoto). Each emission spectrum was obtained by excitation at the optimal wavelength obtained from the absorption spectra.

The fluorescence of the extracted fluorophores in all samples and the solvent blanks was measured with an automated fluorescence multiwell plate reader (CytoFluor™ 2350 fluorescence measurement system, Millipore Corp., Bedford, Mass.). The light source of the instrument is an M32-type halogen bulb (360–700 nm operating range), while the detection system employed a R928 Hamanatsu photomultiplier tube. Both excitation light and emission readings occurred from below the well (i.e., bottom to top orientation). Measurement of multiple fluorescent colors within a sample was accomplished by reading the sample multiple times with paired excitation and emission wavelength filters that were specific for each fluorophore. The filters

were selected from the CytoFluor™ 2350 standard filter set to minimize spillover of fluorescence from one microsphere into the emission filter band of another and are listed in Table 1. However, complete elimination of spillover was not possible with the filter set available and this required estimation of the extent of spillover, as described below. The scan speed of the 2350 Microwell plate reader is 0.25 s/well, or ~24 s/plate. As each plate had to be read at least five times and as all tissues from a single animal required ~10 plates, the total run time for an animal was ~40 min. The computer-acquired fluorescent readings were obtained with the standard CytoFluor™ software and were analyzed using standard computer spreadsheet software.

Standard curve and relationship of fluorescent intensity and microsphere number

To determine the number of fluorescent microspheres in a sample, triplicate 200-μL aliquots of each fluorescent-labeled microsphere were withdrawn and diluted to 10 mL with deionized water containing 0.25% Tween 80. The diluted microsphere specimens were thoroughly suspended, introduced into the counting chamber of a hemocytometer (American Optical, Buffalo, N.Y.), and counted in nine chambers (9 mm^2 in area, and 0.1 mm in depth) under a microscope (Nikon, TMS, Japan) using a ×4 objective with ×10 eyepieces. Each diluted microsphere suspension was counted in triplicate. The total number of microspheres in the solution was computed by the following formula: total microspheres = (number of microspheres counted/0.9 mm^3) × 10^3 × mL suspension. An aliquot of suspension, with known numbers of fluorescent microspheres, was then dissolved in 1 mL of solvent. The solvent remained in contact with the microspheres for at least 4 h to ensure complete extraction of the dye. The fluorescence intensity of the supernatant was then analyzed in the CytoFluor™ 2350 microplate reader. The ratio of fluorescence intensity per number of microspheres (fluorescence units per microsphere number) was then determined and used as the basis of establishing a standard curve for assessing microsphere numbers in tissue samples. The linearity of the CytoFluor™ 2350 instrument was tested by three repeated measurements of the fluorescence intensity of the microspheres in Cellosolve acetate in concentrations ranging from 80 to 20 000/mL. The assay precision was assessed by measuring the within-run and between-run variation of these samples, expressed as coefficient of variation. The stability of the dyes in solvent was evaluated by dissolving fluorescent-labeled microspheres in Cellosolve acetate and reading the fluorescent intensities of each color batch every 2nd day for 2 weeks.

Microsphere recovery

Aliquots (200 μL) with known numbers of fluorescent microspheres labeled with the crimson fluorophore were dissolved in 3 mL Cellosolve acetate. These became the standards of 100% recovery (F_{stand}). Aliquots of the same microspheres were added to the blood and dissected tissue samples obtained from two fetuses in which no microspheres had been injected. The number of microspheres used to spike the blood and tissue samples and in the standards approximated those found in the tissue and blood samples derived from our previous radioactive microsphere studies, and ranged from 400 to 20 000. The blood and tissue samples were processed according to the sedimentation method as described above. Recovery (%) of microspheres from tissue or blood was calculated as $100 \times F_{samp}/F_{stand}$, where F_{samp} was the fluorescence of the tissue and blood samples and F_{stand} was the fluorescence of the standard.

Assessment and correction of spectral overlap and signal quenching

With the relatively large bandpass values of the excitation and emission filters in the CytoFluor™ 2350 instrument (Table 1), it seemed possible that there could be spillover of the emitted light from one fluorophore into the emission band of another label. In addition, the emitted light of one fluorophore can be absorbed by another, if the excitation spectrum of the second label is close to the emission spectrum of the first dye, and this will result in quenching of the first dye's fluorescent signal (Prinzen and Glenny 1994). As an initial check for spillover of a fluorescent signal into the emission spectra of adjacent colors, the fluorescent intensities of each fluorophore dissolved in Cellosolve acetate were measured at each excitation or emission pair listed in Table 1. A spillover matrix representing the signals from the specific fluorescent color in each color band was thus determined, and the results indicated that spillover only occurred for orange into yellow–green, orange into red, red into orange, and red into crimson. Since both spillover and quenching are in part determined by the relative concentrations of the fluorophore pairs, the effect of changes in these concentrations on the fluorescence reading of the affected dye was examined for the following five sets of fluorescent-labeled microsphere pairs: set 1, red effects on crimson; set 2, crimson effects on red; set 3, orange effects on yellow–green; set 4, red effects on orange; and set 5, orange effects on red. For each of these sets we prepared, in triplicate, 25 different combinations of concentration of the two probes. The second color in each set was denoted as A, representing the affected fluorophore, whereas the first one, denoted as B, was the dye causing spillover and (or) quenching. For color A, five microsphere concentrations ranging from 500 to 20 000/mL were employed. For each concentration of A, five increasing concentrations of B (0 – 40 000/mL) were added to different samples (see Fig. 2). To determine the fluorescence intensity of the added microsphere (B) and hence the degree of enhanced fluorescence caused by overlap, the fluorescence intensity for each concentration of A with no added B microspheres was subtracted from the fluorescence readings of the other samples containing that same concentration of A but with added B label. Then the total fluorescence intensity of both colors in each sample was plotted against the fluorescence intensity of the added color (see Fig. 2). The percent spillover of the fluorescence emission signal of the adjacent color was calculated using the following formula: $S = \alpha/\beta \times 100\%$, where α is crossover fluorescence intensity of the specific color in its adjacent window, β is the fluorescence intensity of the specific color in its own window, and S is the spillover expressed as a percentage. To validate these spillover corrections, regression analysis was used to compare the fluorescence intensity of samples with each of the colors alone with samples containing all five labels together, before and after spillover correction. Five concentrations of each microspheres (500 – 20 000) were examined in this analysis. Quenching was also assessed by regression analysis of the fluorescence intensity in the emission band of label A against the microsphere concentration of label B.

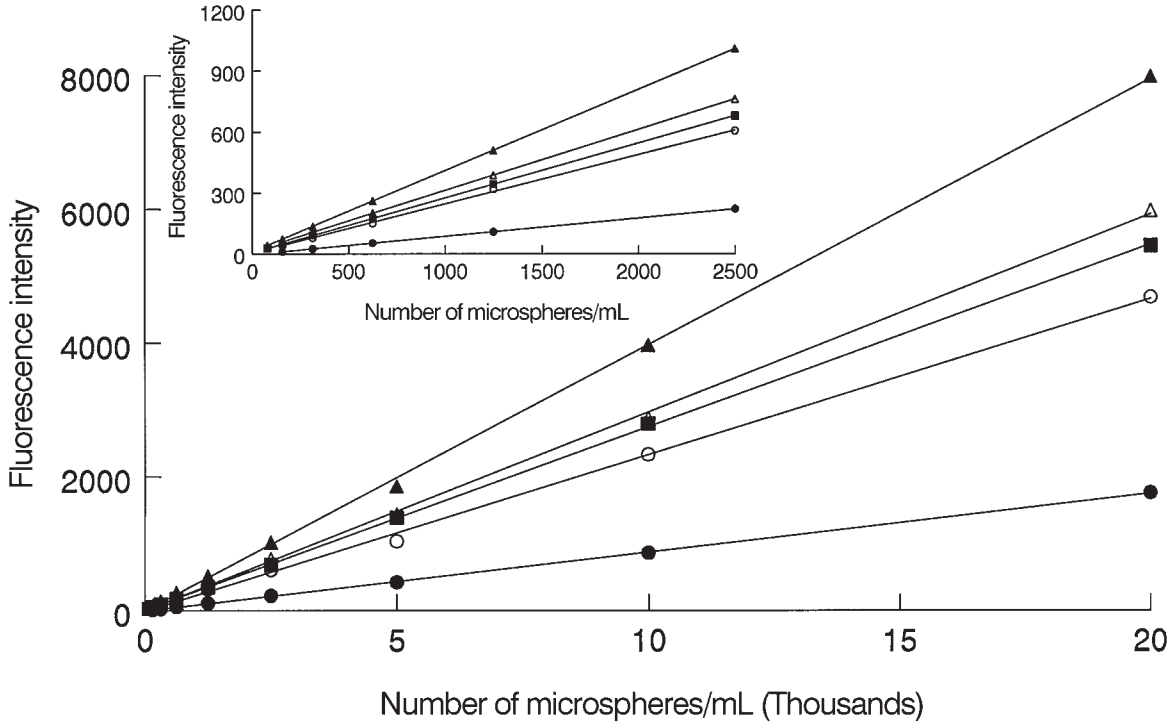
Determination of blood flow

Regional blood flows were computed with the same formula for both radioactive and fluorescent microspheres. The values for fluorescent microspheres were calculated after subtracting fluorescent background, endogenous fluorescence (for blue only), and making corrections for spectral spillover, while for radioactive microspheres, background, decay, and spillover corrections occurred for all isotopes. Organ blood flow was calculated as reference sample withdrawal rate (mL/min) × (I_{organ}/I_{ref}), where I is fluorescent intensity or gamma counts. For the fetal organs and tissues supplied by the ascending aorta, the carotid arterial reference sample was used, whereas for those supplied by the descending aorta, the femoral arterial reference sample was used (Heymann et al. 1977). Lung blood flow was also estimated using the femoral arterial reference sample, since Rosenberg et al. (1984) found this to be an acceptable approach.

Statistical analysis

A commercial software program NCSS (Number Cruncher Statistical System) on an IBM computer system (Copyright © by Dr. Jerry L. Hintze, and NCSS, Kaysville, Utah) was used for the statistical analysis. The regression line was analyzed by the least-squares fitting

Fig. 1. Relationship between the fluorescence intensity and the fluorescent microsphere concentration. Samples were excited and read at the following wavelengths (nm): blue, 360 and 460; yellow–green, 485 and 530; orange, 530 and 560; red, 560 and 590; and crimson, 590 and 645. The sensitivity settings were six for blue (○) and three for yellow–green (●), orange (△), red (■), and crimson (▲).



method. The standard error of the estimate (SEE) was calculated for these regression equations as a measure of random error (Westgard and Hunt 1973). Correlation coefficients were determined by the Pearson method. The differences in blood flow values between the paired organs were analyzed by the paired *t* test. Statistical significance was accepted at $p < 0.05$. The duplicate variability was expressed as the absolute value of the difference of the blood flow values determined by the two fluorescent-labeled microspheres divided by each arithmetic mean × 100. The coefficient of variation (SD/mean) was used to describe the variability of repeated measurements. For comparison of the blood flow estimates obtained in the four animals that received radioactive microspheres (R) with the fluorescent microsphere estimates (F) in the three fetuses, the following formula was used for each tissue and organ: (mean flow_F – mean flow_R)/SD_R. All data are presented as mean values ± SD.

Results

Analytical evaluation

The absorption and emission wavelength peaks and ranges of the five fluorescent labels determined spectrophotometrically are given in Table 1. The optimal absorption and emission wavelengths are in accord with the published data for each fluorescent dye (Glenny et al. 1993). The excitation and emission filter combinations used for each microsphere are also given in Table 1. The CytoFluor™ 2350 instrument has sensitivity settings that enabled us to adjust the voltage on the photomultiplier tube for a scan. Settings range from 1 to 10, with 10 giving the most sensitive readings. For our studies, the gain settings were 6–7 for blue, 3–4 for yellow–green, 3–4 for orange, 3–4 for red, and 2–3 for crimson. Figure 1 shows the fluorescence was linear over a broad range of microsphere numbers examined (80 – 20 000). Crimson appeared to give greatest sensitivity because it has the steepest slope. Correlation coefficients for the five fluorescent labels were 0.999 (all $p < 0.001$), and the SEE ranged from 9.08 to 56.97. The within-run precision, expressed as the coefficient of variation (CV), was evaluated by repeated measurement of eight samples containing low to high concentrations (160 – 20 000/mL) of each fluorescent microsphere label in Cellosolve acetate. The overall CV was 3.39 ± 1.10%. The between-run precision was obtained by analyzing control preparations with 2500 microspheres/mL (dissolved in Cellosolve acetate) of each color in each assay. The overall CV was 4.54 ± 1.10%. The samples assayed for the stability of each color in Cellosolve acetate were stored at 4°C in the dark after each scanning, and run with seven consecutive scans 2 days apart. The fluorescent dyes under these conditions were stable, with no demonstrable loss of fluorescent activity.

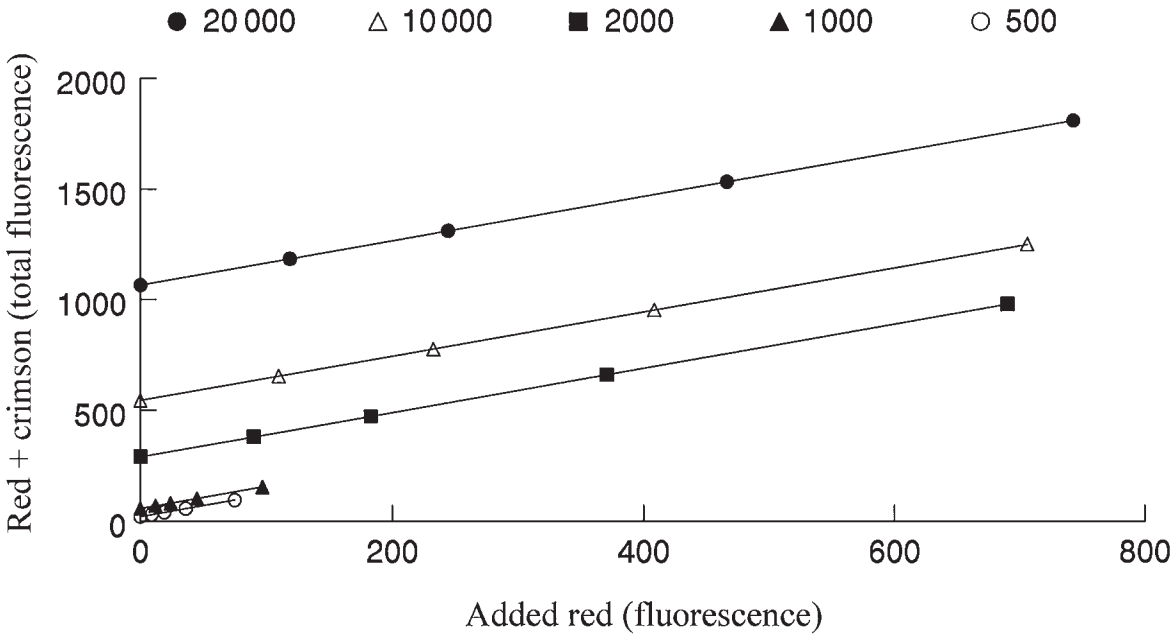
There was no measurable intrinsic fluorescence in control tissues and the blood in the yellow–green, orange, red, and crimson fluorescence settings that were used. However, at the fixed wavelengths of excitation and emission (360 and 460) for the blue color, we observed intrinsic color in the control tissues, with the intensity ranging from 50 to 500 units. This intrinsic blue color was not found in Cellosolve acetate alone. The tissue blue fluorescence obtained for flow determination was thus corrected for those values derived from corresponding blank tissues and organs. In addition, extracted blood reference samples exhibited significant color in the blue band (~2600 units), which interfered with fluorescence reading for the blue dye. This interference was eliminated by bubbling N_2 through the final wash solution. In blood spiked with

Table 2. Recovery of the fluorescent-labeled microspheres added to the blood and tissue samples.

		Fluorescence intensity		
Tissue	Microspheres added	Expected	Observed	Recovery (%)
Brain	20 000	6511	6072	93.26
Heart	20 000	6505	6409	98.53
Lung	2 000	656	640	97.51
Stomach	5 000	1571	1502	95.59
Small intestine	10 000	3122	2959	94.78
Large intestine	8 000	2641	2539	96.13
Liver	400	142	128	90.27
Spleen	10 000	3285	3114	94.80
Kidney	15 000	4788	4633	96.76
Adrenal	20 000	6499	6009	92.46
Brown fat	1 500	501	480	95.76
Skin	1 000	321	295	91.89
Muscle	800	268	242	90.44
Bone	600	205	188	91.73
Blood	1 000	342	324	94.69
				Mean 94.31

Note: Data are average of two experiments.

Fig. 2. Relationship between the total fluorescence intensity of red and crimson fluorophores against the fluorescence intensity of the added color (red) only during serial addition of the red fluorophore to samples containing fixed amounts of the crimson microsphere. The numbers at the top of the illustration give the concentration of crimson microspheres/mL for each curve. The sensitivity setting of the Cytofluor™ instrument was 2.



1500 blue microspheres, the observed fluorescence was then similar to that from the control standards (362 ± 12 vs. 397 ± 13 units).

Recovery

The procedure using tissue digestion, microsphere sedimentation, and dye extraction in the same test tube resulted in minimal loss of microspheres. When fluorescence intensities from the standards were compared with those from the blood and 14 tissue extracts of samples spiked with the crimson-fluorescent microspheres, the recovery was 94.3 ± 2.5% (Table 2). The mean difference from 100% was 5.7 ± 1.0%, and this was significantly different from 0 ($p < 0.001$). However, over the range of microsphere numbers in the spiked samples (400 – 20 000), there was not a significant relationship between the number of microspheres and percent recovery ($r = 0.4332$, $p > 0.1$).

Spectral overlap and signal quenching

As noted in the Methods section, the fluorescence overlap matrix

Table 3. Comparison between radioactive microsphere and fluorescent microsphere method for measurement of fetal upper body organ and tissue blood flows.

Organ and tissue	Radioactive	Fluorescent	SD unit
Brain	271±46	276	0.11
Frontal hemisphere	212±45	260	1.07
Temporal hemisphere	197±54	247	0.93
Midbrain	344±105	397	0.50
Pons	378±120	406	0.23
Medulla	337±86	297	0.47
Pituitary	125±43	116	0.21
Cerebellum	291±49	222	1.41
Spinal cord	127±32	110	0.53
Heart	378±124	222	1.26
Right ventricle	472±229	284	0.82
Left ventricle	358±153	210	0.97
Ventricles	415±190	247	0.88
Septum	348±115	188	1.39
Lungs	79±50	69	0.20
Forelimb skin	20±5	35	3.00
Nuchal muscle	13±5	9	0.80
Forelimb muscle	11±4	8	0.75
Humerus	19±8	24	0.63

Note: Values are means ± SD for fetal organ and tissue blood flows, which are expressed as $mL \cdot min^{-1} \cdot 100\ g^{-1}$. $n = 4$ for radioactive microsphere (R) and 2 for fluorescent microsphere (F) method. SD unit = (mean $flow_F$ – mean $flow_R$)/SD_R. Septum, interventricular septum.

Table 4. Comparison between radioactive microsphere and fluorescent microsphere method for measurement of fetal lower body organ and tissue, systemic and umbilical blood flows, and fetal combined cardiac output.

Organ and tissue	Radioactive	Fluorescent	SD unit
Diaphragm	21±12	16±8	0.42
Stomach	79±42	86±19	0.17
Reticulum	52±30	49±27	0.10
Rumen	35±26	29±16	0.23
Omasum	97±49	107±20	0.20
Abomasum	131±72	160±26	0.40
Small intestine	153±62	101±14	0.84
Large intestine	97±46	71±23	0.57
Liver	3±2	4±1	0.50
Spleen	213±144	160±37	0.37
Kidney	247±66	216±15	0.47
Adrenal	389±144	420±51	0.22
Brown fat	107±63	112±10	0.08
Skin, total	22±10	31±5	0.90
Hindlimb skin	24±6	27±3	0.50
Muscle, total	14±9	9±4	0.56
Hindlimb muscle	17±11	9±4	0.73
Bone, total	22±9	24±3	0.22
Femur	24±11	27±3	0.27
Tibia	26	24±2	
Metatarsal	20	23±3	
Fetal body	320±77	260	0.78
Umbilical	185±47	217	0.68
Cardiac output	503±133	477	0.20

Note: Values are means ± SD for fetal organ and tissue blood flows, which are expressed as $mL \cdot min^{-1} \cdot 100\ g^{-1}$. Fetal body and umbilical blood flows and combined cardiac output are $mL \cdot min^{-1} \cdot kg$ fetal $wt.^{-1}$. $n = 4$ for radioactive microsphere method (R), except for tibia and metatarsal, where $n = 1$; and $n = 3$ for fluorescent microsphere method (F), except for fetal body and umbilical blood flows and combined cardiac output, where $n = 2$. SD unit = (mean $flow_F$ – mean $flow_R$)/SD_R.

data showed that significant spillover into adjacent color windows occurred with orange into yellow–green, orange into red, and red into crimson. There was also a slight spillover of red into orange. There was no spillover of crimson into red; however, there was the possibility of quenching of the red signal by the crimson fluorophore (Prinzen and Glenny 1994). Although the fixed excitation and emission filters employed had a relatively wide bandwidth, the spillover of different fluorescent dyes into windows beyond the immediately adjacent colors was <0.7% in every case. Thus, correction for spillover was only made between adjacent colors as described above.

For the four sets of paired fluorescent-labeled microspheres where spillover was apparent, linear regression was performed to compare the total fluorescence intensity of both colors (A and B) in the samples to the fluorescence intensity of the serially added color (B). The data obtained for one of the four sets (red into crimson) are illustrated in Fig. 2. Irrespective of the amount of A label, the fluorescence was consistently affected by the B label at all concentrations tested. These results suggest that there is a constant degree of fluorescence overlap, depending upon the interfering color concentration, and moreover, that any quenching present did not introduce any nonlinearity into the relationship between fluorescence of B and fluorescence in the A window. For the crimson (B) – red (A) microsphere pair, the slopes of the curves for the relationship between the total fluorescence intensity of both colors (A and B) and the concentration of the serially added color (B) averaged $6.72 \times 10^{-4} \pm 3.53 \times 10^{-4}$ and was not significantly different from 0, indicating the absence of both spillover and quenching effects of crimson on red. The fluorescence overlap values averaged 9.1 ± 0.5% for the red signal in the crimson window (Fig. 2), 4.0 ± 0.1% for the orange signal in the yellow–green, 8.1 ± 0.4% for the orange in the red windows, and 1.0 ± 0.1% for red into orange. No spillover correction was made for the latter microsphere pair. Application of the fluorescence overlap correction factors resulted in a tighter correlation between the expected (x) and measured (y) fluorescence obtained for each microsphere when all five microspheres were present together ($y = 0.993(x + 2.072)$; $r = 0.999$), compared with when the correction factors were not applied ($y = 1.078(x + 66.887)$; $r = 0.995$).

Regional blood flow measurements

The overall CV for the blood flows estimated from the triplicate wells for each tissue was 2.78 ± 1.52%. The ratio of blood flows (mean ± SD) between right and left kidneys and that between paired cerebral hemispheres (frontal and temporal hemisphere) for the three animals that received fluorescent microspheres was 1.03 ± 0.12 and 1.02 ± 0.06, respectively. There was no significant difference in blood flow values between the paired organs ($p > 0.5$), and the duplicate variability was 5.65 ± 2.09%, thus indicating uniformity of distribution of the fluorescent-labeled microspheres.

Tables 3 and 4 give the mean fetal organ and tissue blood

flow estimates obtained from the four fetuses that received radioactive microspheres, and the three animals that received fluorescent microspheres. In the latter group, flow measurements to upper body structures were only available for two of the fetuses, because of failure of the carotid arterial catheter in the other animal. A total of 41 flow estimates were examined in each group, with mean tissue flows ranging over 3–472 mL·min^{-1}·100 g^{-1}. The number of radioactive or fluorescent microspheres in each sample was >400, except for the pituitary and diaphragm, where the numbers were ~100 and ~250, respectively. In general the measurements obtained in the two groups were similar. The difference between the fluorescent and radioactive microsphere estimates divided by the SD of the radioactive estimate was <1 for 36 (88%) of the tissues, between 1 and 2 for 4 (10%), and >2 for only 1 tissue (2%), forelimb skin. For the bones in which flow was measured (humerus, femur, tibia, metatarsal), the percent variation between the fluorescent and radioactive microsphere flow estimates was between –12.5 and 26.0% (mean 9.1 ± 8.1%, $p > 0.1$), and for the humerus, the estimates were within 1 SD. In the one animal in which duplicate fluorescent microsphere flow estimates were obtained with 7 min, the correlation coefficient for the duplicate measurements was 0.997 ($p < 0.001$), and the SEE was 9.55 mL·min^{-1}·100 g^{-1}. The duplicate variability was 10.60 ± 2.16%.

Discussion

Measurement of regional blood flow is important in assessing fetal cardiovascular function. The radioactive microsphere technique is an established method, but is becoming increasingly problematic because of radiation exposure and regulatory restriction. Thus a nonradioactive method is desirable. The current study appears to be the first in which fluorescent-labeled microspheres were employed to measure fetal blood flow in all tissues and organs and also the first in which a 96-well plate fluorescent reader was successfully employed for the fluorescence determination. However, there is a recent report of cerebral blood flow measurement in fetal lambs using colored microspheres (McCrabb and Harding 1995), and a recent abstract on the use of fluorescent-labeled microspheres and a conventional fluorimeter to measure fetal adrenal blood flow (Buchwalder et al. 1996).

Four methods have been employed in adult studies to recover fluorescent microspheres from tissues and reference samples following tissue digestion: vacuum filtration (Glenny et al. 1993), density gradient centrifugation using sucrose (Austin et al. 1993), a sedimentation method (Van Oosterhout et al. 1995), and a method involving addition of Cellosolve to the KOH containing the dissolved tissue, followed by phase separation of the organic and aqueous layers (Abel et al. 1993; Li et al. 1996). However, this latter method has only been applied to myocardial tissue to date, and there was an underrepresentation of fluorescence in the blood reference samples, perhaps due to incomplete extraction of the dye into the organic phase (Li et al. 1996). Van Oosterhout et al. (1995) have calculated that both the time and cost per sample are lowest with the sedimentation method, and as estimation of regional and placental blood flows in fetal lambs generates many samples per animal, we chose this method. However, several modifications were made to the sedimentation method described by Van Oosterhout et al. (1995), largely because of the larger amounts of tissue involved in our study. The main modifications were a larger size (50 vs. 10 mL) and different material (polypropylene vs. glass) for the sample digestion tubes and additional deionized water washes of the digested samples. For most tissues, this procedure was effective in yielding a clear supernatant for fluorescence determination. However for skin and brown adipose tissue, an additional step involving a 2-h incubation at 60°C was required. In addition, bone required direct extraction of the fluorescent dye following crushing of the tissue. The overall estimated recovery of spiked microspheres from all tissues averaged 94.3 ± 2.5% and the percent loss of 5.7 ± 1.0% is significantly different from 0, indicating a small loss of microspheres during the tissue processing procedures. Using a similar approach to estimate microsphere recovery, Van Oosterhout et al. (1995) found a 4% loss, but this was not statistically significant. The apparent lower recovery with our method may be due to the increased number of washing steps. However, as there was no apparent relationship between the number of microspheres added to the tissue and blood samples and microsphere recovery, the loss should not affect the flow calculations and hence was ignored.

Automated microsphere quantification is one of the major issues with the fluorescent microsphere technique (Prinzen and Glenny 1994). The most frequently performed method for determination of fluorescence signals in the tissues is the cuvette method in which the fluorescence of single samples is measured one at a time. However, using this method to measure fluorescence of numerous tissue samples is labor intensive, time consuming, and difficult to automate. A previous attempt to automate spectrometry using a surface-reading 96-microwell plate reader has been reported (Glenny et al. 1993), but this suffered from increased fluorescent quenching with higher microsphere numbers (>2000 per sample), resulting in nonlinearity in the concentration – fluorescence intensity relationship. However, an improved multiwell plate reader with a robotic system to fill the multiwell plates is being investigated (An et al. 1996). Recently, a fully automated system that reads samples via an automated flow cell unit has been developed for measurement of regional lung perfusion. Analysis time of this system is ~3 min/sample, which is much longer than with the microwell plate reader in the current study, although the former method can analyze up to 280 samples unattended (Schimmel et al. 1996).

In the present work, we assessed the applicability of a CytoFluor™ 2350 96-well plate reader, coupled to translucent, polypropylene 96-well plates that are resistant to the solvent used. Measurement can be completed within 40 min for all tissues from one animal. Excellent linearity was observed in the microsphere number – fluorescent intensity relationship, up to at least 20 000 microspheres/mL. This range is considerably wider than that obtained with a 96-well plate fluorescence surface reader (Glenny et al. 1993), probably because quenching effects are more significant with surface-reading instruments (Guilbault 1990). The wide linear range is a particular advantage with organs that receive a high blood flow and hence large microsphere numbers, since serial dilution of the samples can be avoided. The performance characteristics of our method are in accordance with the validated cuvette methods (Glenny et al. 1993; Van Oosterhout et al. 1995). The precision is 3.4 ± 1.1% for within-run CV and 4.5 ± 1.1% for

between-run CV. The potential for well to well crosstalk is small, since the overall CV for the blood flows estimated from the triplicate wells for each tissue was 2.8 ± 1.5%.

A potential disadvantage of our method involves the use of fixed wavelength excitation and emission filters with a 1/2 bandpass of 20–40 nm, compared with the monochromators used in conventional fluorimeters, which can vary excitation and emission wavelengths continuously and with a narrow bandwidth (e.g., 4–6 nm). This could lead to increased problems of spillover of one fluorescent label into the emission window of another label. In addition, there is the potential for one fluorophore to quench the signal of another by absorbing the emitted photons. Evidence for this phenomenon was obtained for the crimson and red fluorescent dyes; in the presence of the former dye, the emitted fluorescence of the red dye was reduced by ~3% at the peak emission wavelength (Prinzen and Glenny 1994). We did observe significant fluorescence overlap for three of the fluorophore pairs: orange into yellow–green and red and red into crimson. However, in no case was there spillover of one label into more than an immediately adjacent label or spillover of more than one label into a single emission band. Hence the matrix correction method that involves solving simultaneous linear equations (Heymann et al. 1977; Schosser et al. 1979) and that is employed to correct for the multiple spectral overlap of radioactive microspheres was not required with our method, at least with the color combination used. Previous estimates of spillover with both radioactive and fluorescent microspheres have utilized a single concentration of each label (Glenny et al. 1993; Heymann et al. 1977; Schosser et al. 1979; Van Oosterhout et al. 1995). However, because it seemed possible that quenching of one fluorophore by another could occur in our measurement system, and because the magnitude of both spillover and quenching are in part determined by the concentration of the interfering dye, we examined the effect of differing concentrations of the fluorophore pairs for which these two problems could occur. In terms of spillover, this occurred with orange into yellow–green, orange into red, and red into crimson. However, the magnitude of the fluorescence overlap signal was relatively small (4–9%) and only slightly larger than the values reported for the same labels in conventional fluorimeters (Glenny et al. 1993). Moreover, the degree of spillover, expressed as a percentage of the fluorescent intensity of the interfering fluorophore in its own emission band, was constant over a wide range of concentrations of both interfering and affected dye. If quenching occurred with these three fluorophore pairs, it was not obvious, and with crimson effects on red, there also was no evidence of quenching of the red emission signal with increasing concentrations of crimson, at least up to the maximum crimson/red ratio examined of 8. This result differs from that of Prinzen and Glenny (1994), who found a slight quenching of the red signal, even with a crimson/red concentration ratio of ~1. This difference may be due to the fact that Prinzen and Glenny examined the entire emission spectrum.

With the exception of the blue emission band, there was no endogenous color in the extracted tissue and samples. However, when reading the blue fluorophore (i.e., ~420–500 nm), endogenous color was present in both the tissues and blood samples, and was particularly large in the latter samples. This phenomenon has also been observed in other studies that employed sedimentation and phase separation methods to recover the fluorophores (Abel et al. 1993; Li et al. 1996; Van Oosterhout et al. 1995), but not with the filtration method (Glenny et al. 1993). Although the blue endogenous color has been suggested to be due to the Cellosolve acetate and Tween 80 (Van Oosterhout et al. 1995), we tested Cellosolve acetate and the final wash supernatant from tissue and blood samples and did not find any color in the blue emission band. We did find that bubbling nitrogen through the final wash of the blood samples completely eliminated the interfering blue color, although the mechanism of this effect is not clear. We did not try nitrogen bubbling with the tissue samples as the intensity of the interference was much less and because to do this for all tissue samples would not be practical. Given these problems with the blue microsphere encountered by us and others, an option would be to omit it and use one of the other available fluorescent labels (e.g., blue–green).

As noted in the Introduction, flow estimates obtained using fluorescent microspheres have been compared with measurements obtained with coadministered radioactive microspheres in several adult studies, and the correlation was excellent (Abel et al. 1993; Austin et al. 1993; Chien et al. 1995; Glenny et al. 1993; Van Oosterhout et al. 1995). Thus we did not feel it necessary to coadminister the two microsphere types in fetal lambs. However, we did compare fluorescent microsphere flow estimates obtained in three fetal lambs with those obtained using radioactive microspheres in four other lambs at the same gestational age. For 88% of the tissues or organs examined, the fluorescent microsphere estimate was within 1 SD of the radioactive measurement, and for all but one of the remaining tissues was within 2 SD. The exception was forelimb skin, where the difference was greater (3 SD). In other studies in which fetal skin blood flow has been measured, the mean values vary considerably, from 19 to 32 $mL \cdot min^{-1} \cdot 100\ g^{-1}$ (see Knight et al. 1996), suggesting that flow to this tissue may fluctuate normally in the fetus. Thus there was good overall agreement between the two methods, particularly given the fact that they were obtained in different animals. The measurements are also similar to the many published estimates of blood flows in the sheep fetus (e.g., Cohn et al. 1974; Court et al. 1984; Richardson et al. 1989; Rurak et al. 1990*a*). The fact that bone blood flow in the two groups was similar indicates that the fluorophore recovery method that we developed for bone (i.e., direct extraction of the dyes by Cellosolve from crushed bone) results in reliable flow estimates. This appears to be the first time that fluorescent microspheres have been used to obtain quantitative flow estimates in bone in either fetal or adult studies.

In conclusion, we have developed a method for measuring regional blood flow by means of fluorescent microspheres in all fetal organs and tissues, including bone, and in the placenta. The method appears to be a viable alternative to the use of radioactive microspheres, thereby eliminating the regulatory, environmental, and health concerns associated with the latter. Moreover, the use of an automated fluorescent microplate reader greatly decreases analysis time compared with the use of a conventional fluorimeter, and this is most valuable when assessing flow in large numbers of samples, as in the case of most studies in fetal lambs.

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