5 Microsphere Measurement of Regional Organ Perfusion

Regional organ perfusion can be estimated with hematogenously delivered microspheres (Heyman et al., 1977). When appropriately sized microspheres are used, regional blood flow is proportional to the number of microspheres trapped in the region of interest (Bassingthwaighte et al., 1990). A number of excellent review articles describe and validate the use of microspheres for measurement of regional organ perfusion, but the classic review by Heyman *et al*. (1977) contains many details for radioactive microsphere use that apply to fluorescent microspheres.

Over the last twenty years, new and/or refined methods for measuring regional organ perfusion have been published. Many of these publications are listed in our reference section. A careful search of the literature should be done prior to starting a study to determine the most appropriate method for measuring regional organ perfusion for any given experimental protocol. It is not feasible for this manual to address the wide variety of methods currently accepted. We have outlined below, methods for measuring regional organ perfusion that we have utilized in our laboratory.

Preparation of Fluorescent Microspheres for Injection

Calculation of Microspheres for Injection

A minimum of 400 microspheres are needed per tissue piece to be 95% confident that the flow measurement is within 10% of the true value (Buckberg et al., 1971). If measurement of regional blood flow (ml/min) is the primary objective of a study, the number of microspheres injected must be calculated to assure a sufficient number reach the organ of interest.

The following equation estimates the minimum total number of microspheres needed per injection to accurately measure blood flow.

$$N_{min} = 400(n)/[Q_{organ}/Q_{total}] \quad (1)$$

where:

N_{min} = minimum number of microspheres needed for injection

n = total number of organ pieces

Example: in a 200-gram heart, if you plan to evaluate ten, 1-gram pieces, the total number of pieces per organ should be 200 (even though you plan to evaluate only 5% of the organ).

$[Q_{organ}/Q_{total}]$= fraction of total cardiac output supplying organ of interest

With this calculation, 400 microspheres are provided for each organ piece with an average blood flow. A piece with twice the average flow will have 800 microspheres and a piece with 0.5 times the average

flow will have 200 microspheres. We usually double the minimum number (N_{min}) to make sure that low-flow organ pieces also have an adequate number of microspheres (Buckberg et al., 1971; Nose et al., 1985). This 400 microsphere "rule" only applies to measurements of perfusion to a single region or organ piece. A recent paper by Polissar et al. (1999), reports that fewer microspheres are required for accurate measurements of heterogeneity and correlation. Polissar suggests a minimum of 15,000 microspheres, total, for all pieces combined for accurate measurements of heterogeneity and 25,000 microspheres, total, for accurate estimates of correlation coefficients.

Estimating the Number of Microspheres per ml

The following equation (based on percent solids) estimates the number of microspheres per ml of microsphere suspension:

$$N(\text{microspheres/ml}) = (6.03\text{x}10^{10})(\%\text{solids})/(3.3144d^3) \quad (2)$$

where:

d = diameter of microspheres in microns (μ)
% solids supplied by manufacturer (use %, 2% = 2, not 0.02)

Calculation of Injectate Volume

$$\text{mls of suspension} = N_{\text{min}}/N(\text{microspheres/ml}) \quad (3)$$

Preparation of FMS for Injection

Note: The dye "load" varies for each lot of fluorescent microspheres; therefore, signal intensities should be checked for each new lot (Section 4). Also, the number of microspheres per ml can be checked with a hemocytometer. Do not mix fluorescent microspheres from different lots.

Method:

1. Remove from refrigerator and check supernatant solution. It should be clear due to the addition of thimerosal, a bacteriostatic agent. Cloudy fluid may indicate contamination.

2. Vortex (vigorous agitation) thoroughly (5 - 15 secs).

3. Place in ultrasonic water bath for at least 2-10 min to disperses the microspheres. Do not cover water bath or sonicate too long because the heat generated can melt the microspheres. For microspheres 15 μm or smaller, sonication time is less (smaller particles are more susceptible to heat).

4. Just prior to injection, vortex the vial of microspheres again and withdraw the desired volume based on the calculated number (see Calculation of Microspheres for Injection, previous page). Do not permit the microspheres to settle once they are drawn into the syringe. If injection time is delayed, vortex them thoroughly again.

5. Injection time varies for each experimental design and must be determined prior to injection. Most left-heart injections are done over short periods of time (5-15 seconds) while the reference withdrawal pump is withdrawing the sample. A left-heart injection should not appreciably change the animal's stroke volume. Slow and steady injections allow for uniform mixing of microspheres, whereas bolus injections often result in streaming (not desirable).

   Following injection, a visible rim of microspheres will remain in the syringe if plastic syringes are used. This can be expected and is of little concern since the remaining microspheres represent only a small fraction of the total used.

6. After injection, flush the dead space of the catheter thoroughly (at least three times the volume of the catheter) and change the stopcock (microspheres get caught in the stopcocks; discard along with the used syringes after each injection to avoid contamination of subsequent injections).

Reference Blood Flow Sampling

A reference blood flow sample allows calculation of regional flow in ml/min. It is essential that the reference blood withdrawal catheter be accurately positioned so that a representative sample of well-mixed microspheres and blood can be obtained. If pulmonary perfusion is being measured, the reference blood sample should be obtained from a pulmonary artery. If systemic organ flows are measured, reference blood samples can be obtained from the descending aorta. The blood samples should be obtained as close to the organ of interest as possible without interfering with blood flow.

The site of microsphere injection is very important. For systemic blood flow measurements, left atrial injection of microspheres are best. If a left atrial catheter is not possible, then a left ventricular catheter is adequate. Left atrial injections allow for two-chamber mixing and more uniform distribution of the microspheres in the blood. Left ventricular injections allow one chamber mixing, shown to be sufficient in most species.

The reference withdrawal pump must be accurately calibrated so that reference blood is withdrawn at a uniform rate. If problems exist in the rate of withdrawal, the reference sample is invalid. Whenever possible, two reference blood samples should be withdrawn simultaneously, in case one catheter clots or one sample is lost.

Withdrawal syringes must be large enough to hold the volume of blood in the reference sample, heparin, and dead space volume. Example: if withdrawal rate is 5 ml/min and withdrawal time is 2 min after completion of a 1-min injection, heparin volume is 1 ml and dead space is 3 ml, then the syringe volume should be 20-30 ml. Glass syringes and containers are preferred; they decrease microsphere loss caused by "static" attraction of the plastic microspheres with the plastic containers or syringes.

Method for Reference Blood Flow Sampling

There are 2 different anticoagulants that we routinely use: Heparin (syringe coated) and Citrate Phosphate Dextrose (10 cc per 30 cc syringe).

1. Using whole blood, calibrate the reference withdrawal pump at the predetermined withdrawal rate, including the catheters, extension tubing and matched anticoagulated glass syringes that will be used for the reference withdrawal. Have new stopcocks and flush syringes available.

2. Connect the matched glass anticoagulated syringes in the withdrawal pump to the catheters and the extension tubing so that everything is set up for withdrawing the reference sample. Do not turn the stopcock on the catheters until you are ready for injection (the blood will flux into the catheter dead space and may clot).

3. Once the microspheres have been drawn into the injection syringes, start the withdrawal pump and make sure blood is flowing freely into the extension tubing.

4. Inject the microspheres over the designated time period (sec or min) followed by a flush of warmed saline three times the volume of the catheter dead space.

5. A timer is set for 2 min after completion of injection for the reference blood withdrawal. At the end of the withdrawal, the pump is turned off, the stopcocks are opened and the blood remaining in the extension tubing is drawn into the syringe.

6. Transfer blood into labeled vials for further processing (see Digestion of Blood and Tissue, next page). Rinse syringes and extension lines with 2% Tween-80® (using approximately twice the volume of the blood) and add this rinse to the blood samples.

7. Flush the catheters again and change the stopcocks.

Calculation of Regional Perfusion

Regional perfusion to the target organ can be calculated by one of two methods.

Flow to Each Piece Relative to the Mean

This is most easily accomplished using a computer spreadsheet, but can be processed by hand or with a computer program. If the fluorescence of each organ piece is denoted by fl_i, where i is the sample number and n is the number of pieces, then flow to piece i relative to the mean, Q_{mi}, is given by the equation:

$$Q_{mi} = fl_i/(\Sigma fl_i/n) \qquad (5)$$

The denominator in equation 6 consists of the mean fluorescence for all of the pieces. Q_{mi} is a dimensionless number.

Flow to Each Piece in ml/min

This is most easily accomplished using a computer spreadsheet, but can be processed by hand or with a computer program. A reference blood flow sample must be obtained at the time of microsphere injection (see section on Reference Blood Flow Sampling). If the fluorescence of each organ piece is denoted by fl_i where i is the sample number, fl_{ref} is the fluorescence of the reference blood flow sample, and R is the withdrawal rate of the reference blood flow sample in ml/min, then flow to piece i , Q_i, is given by the equation:

$$Q_i(\text{ml/min}) = (fl_i/fl_{ref})\cdot\text{R(ml/min)} \qquad (6)$$

Recovering Microspheres from Samples for Quantification

With the exception of lung tissue, microspheres must be physically separated from the tissue or blood in order to quantify the number of microspheres in each sample. Because lung tissue is porous, direct extraction of the microspheres from air-dried tissue (dried at total lung capacity) is possible.

The most practical methods to recover the microspheres from digested tissues are:

1) Negative Pressure Filtration
2) Sedimentation
3) Perkin Elmer polyamide woven filtration devices

Blood samples anticoagulated with Citrate Phosphate Dextrose can be directly filtered with the Perkin Elmer polyamide woven filtration devices.

How samples are digested depends on the method used for microsphere recovery. As a rule, potassium hydroxide (KOH) digests kidney and heart, but does not completely digest fat (brain), fascia, cartilage, etc. Ethanolic KOH, a very powerful digestion solution, successfully digests most tissue, including brain and fascia.

Note: Both solutions recommended for tissue digestion are extremely caustic; adequate precaution should be used when handling these solutions.

Blood and Tissue Digestion Followed by Negative Pressure Filtration

Tissue and **heparinized** blood samples containing microspheres must be digested to recover the microspheres. Use of glass vials with caps for tissue digestion decrease the likelihood of spillage or microsphere loss. If negative pressure filtration method is used, the volumes and concentrations of solutions are not critical. However, when using the sedimentation method, the concentration of solutions is very important (see note under sedimentation regarding specific gravity).

Digestion of Heparinized Blood for Negative Pressure Filtration

1. Add 7 ml of 89.2% KOH (16 N, 89 g in final volume of 100 ml H_2O) to each 30 ml of diluted blood (see step 6 on previous page) (i.e., ~10 ml of blood plus 20 ml 2% Tween-80® rinse solution) and digest overnight.

2. Filter blood (filter pore size must be smaller than the microspheres; e.g., 10 µm for 15-µm microspheres).

   Note: Digested blood samples can be stored (tightly capped) at room temperature and filtered at a later date.

Digestion of Solid Tissue for Negative Pressure Filtration

1. For each 1-2 g of solid tissue (heart, kidney, etc.), add 5-10 ml of 22.4% KOH (4 N, 22.4 g in final volume of 100 ml H_2O). Freshly made KOH solution, warm due to the exothermic reaction, aids in tissue digestion.

2. Digestion time varies depending on the tissue; usually 24 hours is sufficient.

Digested tissue samples should not stand unfiltered for long periods of time since fat in them may solidify.

After samples have been digested with KOH, the microspheres are physically separated by negative pressure filtration. This method is inexpensive and has been rigorously tested; however, it is labor-intensive and may cause microsphere loss when the tissue sample is transferred from one vessel to another or if the filter fails.

Assemble filtration system as shown in Figure 5-1. A new (or sometimes multiple) filter is used for each sample. We have had success with the Poretics filtration device using Poretics polycarbonate filters. Although the manufacturer states that these filters are not compatible with alkaline solutions (KOH), we have found that they work well when allowed to remain in contact with the KOH for no longer than 3 min.

1. Assemble filtration apparatus and connect to suction (make sure it does not exceed maximum value allowed for the membrane). Place filter between graduated cylinder and filter screen. Clamp graduated cylinder to filter holder.

2. Make buffer rinse solution as follows:

   - Dissolve 5.88 g potassium phosphate (monobasic) KH_2PO_4 in 200 ml H_2O and 22.9 g.
   - Dissolve K_2HPO_4 potassium phosphate (dibasic) in 800 ml H_2O.
   - Combine the two solutions.

3. Pour digested sample into the graduated cylinder and allow suction to filter sample. Exposure time of the filter to KOH should be minimized to assure that filters remain competent. Rinse

sample tube into filtration device with 2% Tween-80® solution. Rinse graduated cylinder two to three times (approximately 20 ml).

4. Do a final rinse with the 5-10 ml of the buffer rinse solution made in step 2.

5. Let vacuum air-dry the filter.

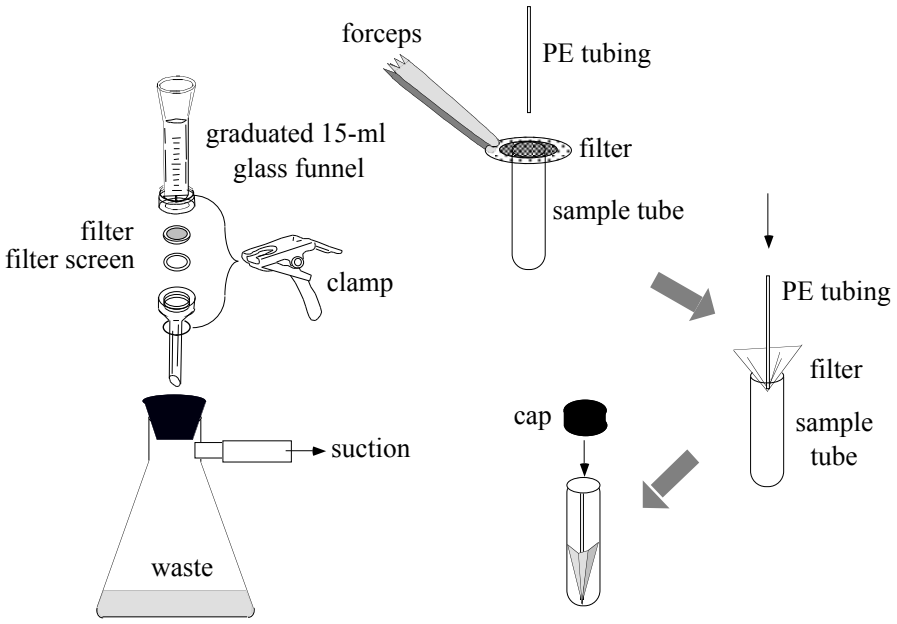


Figure 5-1

Schematic diagram for filtering apparatus using polycarbonate filters.

6. Remove filter with forceps. Handle only filter rims that are free of microspheres. Carefully place filter in sample tube with assistance from small plunger (we use PE 50 tubing [see Figure 5-1] cut into appropriate lengths). Leave the plunger in the polypropylene container, as it may contain microspheres (PE tubing will not affect the results).

   Note: If the filter becomes clogged with microspheres or tissue debris, the filtration process may slow or stop, due to filter failure caused by increased contact time with KOH. In this event, you should use smaller volumes of digested sample and multiple filters placed in one sample tube and the combined fluorescence measured.

Polyamide Woven Filtration Devices

Perkin-Elmer offers filtering devices that have been specifically made to isolate fluorescent microspheres from CPD anti-coagulated blood or digested tissues. The major advantage of this system is that each tissue sample is digested, filtered, and the fluorescent dyes extracted in a single container, obviating the need to transfer materials from one vessel to another and theoretically decreasing microsphere loss. The devices are polypropylene and consist of three stages (Figure 5-2). The first stage has a polyamide membrane integrated into the bottom. Each tissue sample is placed in this first stage and digested with KOH. CPD anticoagulated blood can be directly filtered. The digested material is filtered by suction and dried by centrifugation. The filtered sample and container are then placed within the second stage and the third stage attached to the bottom of the second stage. The organic solvent is added to the first stage and then transferred to the last stage by centrifugation. The last stage then contains the organic solvent with the extracted dyes.

Figure 5-2

Ethanolic KOH, Tissue and Blood Digestion, and Sedimentation

To use the sedimentation method for microsphere recovery, the sample must be digested in ethanolic KOH. Sedimentation of the microspheres is possible if the **specific gravity** of the solution is **less** than that of microspheres (van Oosterhout et al., 1994). Tissue digested with ethanolic KOH (as opposed to KOH in water), has a specific gravity that is much less than the microspheres. The following procedure has been successfully used for brain tissue (Powers et al., 1999).

Solutions required:

A. 2.3 M ethanolic KOH with 0.5 % Tween 80.
B. Internal standard (a non-study color)
C. 1% Triton X-100
D. Distilled Water Phosphate buffer
E. 0.25% Tween-80
F. 2-Ethoxyethyl acetate

Recipes

A. 2.3 M ethanolic KOH with 0.5 % Tween 80.
   3 g KOH and 0.5 g Tween 80 are put into a glass beaker. 100 ml ethanol is added to the 100 ml line. The solution is stirred until a clear solution is obtained (approx. 20 min.).
B. Internal standard (a non-study color). **This is optional:**
   Use a non study color
   1. Vortex and sonicate microspheres (2% solids) in vial for 30 seconds.
   2. Vortex again and immediately withdraw 1 ml of solution with a sterile syringe.

3. Add microspheres to 100 ml of solution E (0.25% Tween-80)
4. Add stir bar and keep solution stirring with a stir bar while in use.

C 1% Triton X-100.
10 g Triton X-100 (Sigma no. X-100) is brought to 1 liter with distilled water and stirred until in solution.

D. Distilled Water Phosphate buffer (solution for rinsing)
5.88 g KH_2PO_4 in 200 ml distilled water (monobasic)
22.9 g K_2HPO_4 in 800 ml distilled water (dibasic)
Combine the two solutions.
Add 28.6 ml of the combined solution to 1000 ml distilled H_2O

E. 0.25% Tween-80
2.5 g Tween 80 is put into a beaker and the volume is brought up to 1 liter with distilled water. Stir with a stir bar until in solution.

F. 2-Ethoxyethyl acetate
Solvent used to dissolve microspheres and release dye (final solution for fluorimetry).

Tubes

Polypropylene 15 ml centrifuge tubes with tightly fitting caps. Do not use polystyrene as this is what the microspheres are mode of and the 2-ethoxyethyl acetate will dissolve the tube. The tubes must be able to take 2000 x G for 20 min., 3 times. A tolerance of 6000 x G is therefore recommended.

Taking Samples

1. Weigh 15 ml polypropylene tubes with caps.
2. Put tissue into tubes, pushing them to below the 7 ml line.
3. Store tissues for 2 weeks at room temp (in a fume hood) for autolysis to occur. If the samples have been stored cold, let them stand out for at least 2 days prior to processing.

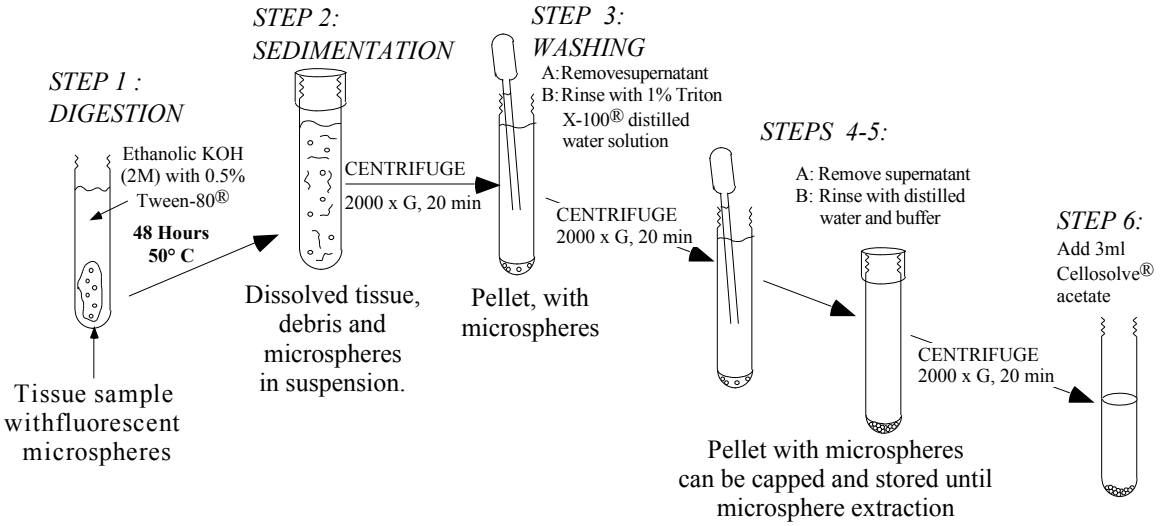


Figure 5-3:

Schematic diagram for sedimentation microsphere recovery.

Tissue Processing

Day 1:

1. Weigh tubes and record tissue weight.
2. Add exactly 200 µl internal standard (if internal standards are being used)
3. Add approximately 8 ml ethanolic KOH. (Step 1)
4. Cap tightly and vortex for 20 sec.
5. Put tubes into 50 degree centigrade water bath with shaker.
6. Vortex at 24 hours for 20 sec per tube.

Day 3

1. Vortex at 48 hours or until particles are re-suspended (about 30 seconds). (Step 2). After vortexing, all samples, vortex again for 5 sec.
2. Centrifuge 20 min at 2000 x G.
3. Take off all but 1.5 ml supernatant using a Pasteur pipette attached to suction with a trap, and discard sup.
4. Add approximately 8 ml 1% Triton X-100 and vortex for 30 second. (Step 3)
5. Centrifuge 20 min., 2000 x G.
6. Take off all but 0.5-1 ml supernatant and discard. (Step 4)
7. Add 7 ml distilled water phosphate buffer to each tube. .(Step 5)
8. Vortex for 30 seconds until all particles are suspended.
9. Centrifuge 20', 2000 x G
10. Take off all but 1.0 ml sup and discard, using the suction with trap.
11. Remove all but 150 microliters of sup carefully by hand using a Pasteur pipette. (Step 6) More than 150 µl water when mixed with 2-Ethoxyethyl acetate creates two phases.
12. Add 3 ml 2-Ethoxyethyl acetate and vortex until particles are well suspended. Let samples sit in the dark and away from heat for 5 days.
13. Let samples sit in the dark and away from heat for 5 days.

Day 5

1. Vortex gently until pellet breaks up (approximately 20 sec).

Day 8

1. Vortex vigorously until pellet breaks up (approximately 30 seconds)
2. Centrifuge 20', 2000 x G
3. Supernatant is then used for fluorimetry-read all blood and tissue samples on the same day.

WARNING: Tissue digested with ethanolic KOH cannot be filtered through Poretics polycarbonate filters because the filters will not withstand the ethanolic KOH. The ethanol available in Europe contains additives to discourage human consumption. These additives turn the ethanol brown in the presence of KOH, which renders the ethanol unsuitable for use in this technique.

Additional notes:

1. do not mix caps up, it may render your weights in accurate.
2. Spilled ethanolic KOH or Cellosolve can remove writing on tubes
3. Accurate volumes of 2-Ethoxyethyl acetate are required for accurate flow measurements.

Quantification of Fluorescent Microspheres

Internal Standard Test for Complete Microsphere Recovery

The method introduced by Chien et al. (1995) is used to check and correct for any microsphere loss during the filtration or sedimentation process. A known amount of microspheres of a color not used in your experiment is added to each sample vial of tissue prior to digestion. This microsphere acts as an internal standard. After the filtration or sedimentation is complete, all samples should have comparable signals. If large discrepancies in the signal between samples exist, your technique should be evaluated to determine the source of loss. If discrepancies are small, the microsphere signals can be corrected by the proportion of loss to yield a corrected set of signals for each sample. The correction formula is:

$$I_{true} = I_{obs} \cdot \left(\frac{I_{st,true}}{I_{st,obs}} \right) \quad (4)$$

Where I_{true} is the true fluorescence signal, I_{obs} is the observed (measured) fluorescence signal, $I_{st,true}$ is the true florescence standard signal and $I_{st,obs}$ is the measured fluorescence standard signal. This method assumes that the relative number of lost microspheres is similar for the internal standard and all other colors.

Fluorescent Dye Extraction

Organic Solvent for Fluorescent Dye Extraction

The following organic solvent has been tested and used extensively to dissolve the microspheres and release the encapsulated dyes:

Solvent	Boiling Point	Hazard
2-ethoxyethyl acetate (Cellosolve® acetate)	156º C	teratogen

The Material Safety Data Sheets (MSDS) should be obtained for any solvent being considered to determine risks and proper precautions. Your chemical vendor will supply this data on request.

Do not confuse the fluorescent microsphere solvent, 2-ethoxyethyl acetate (Cellosolve® acetate) with ethyl Cellosolve, which will not dissolve the microspheres.

All potential solvents have not been tested, and other solvents may give satisfactory results. It is advantageous to use a solvent with low volatility to minimize evaporation and potential safety hazards from inhaling the vapors. Dimethyl sulfoxide and dimethyl formamide are not suitable solvents as they render the fluorescent dyes unstable.

Note: Fluorescence degrades if exposed to light for any length of time.

The maximum excitation and emission wavelengths for some fluorescent dyes are solvent-dependent (see Table 2-2). Therefore, the choice of solvent may depend on the fluorescent colors chosen for the study.

Different lots of solvent may contain impurities that can degrade some of the fluorescent colors over time. We suggest making a single test solution containing solvent and all the fluorescent colors to be used. This solution should be read daily for as long as you normally allow your samples to remain in the solvent before reading them in the fluorimeter (e.g., if samples are to remain in solvent for 72 hours, read the test solution every day for 3 days). Repeat this with each new solvent lot. If degradation occurs in specific colors, avoid these colors or use a new solvent lot. We have observed that the stability of fluorescent colors vary between different lots of Cellosolve® acetate.

Direct Extraction of Air-Dried Lung Tissue

Microspheres can be directly extracted from air-dried lung tissue without prior tissue digestion by adding organic solvent directly to the tissue and allowing at least 2-7 days for complete extraction of dyes from the microspheres. The time for complete extraction varies with species and lung injury.

Extraction of Microspheres Following Sedimentation or Filtration Recovery Techniques

Once the microspheres are separated from the samples by sedimentation or filtration, the fluorescent dye is extracted from the microspheres by adding an accurately measured volume of organic solvent to the microspheres. The solvent dissolves the microspheres and releases the fluorescent dye into the organic solvent. The fluorescent signal in this solution is proportional to the number microspheres present in the sample; therefore, the accuracy of the volume of solvent is critical.

We routinely add 2 ml of 2-ethoxyethyl acetate to each sample with an Eppendorf repeater pipette with an accuracy of ±0.3-2.5% and precision of <0.1-1.0%. Once the organic solvent has been added to the sample, it is then vortexed and allowed to sit for ~1 hour before the fluorescence is read. If the fluorescent signal is high, dilute the sample (see Section 3 on linearity).

Fluorescence Measurement

Depending on the fluorimeter used, fluorescence can be measured using matched glass cuvettes, a 96-wellplate reader, or an automated flow cell. We routinely measure fluorescence with matched glass cuvettes. The excitation and emission wavelengths recommended in this manual (Table 2-1) are optimal in our Perkin-Elmer LS-50B using red-sensitive photomultiplier tubes.

Machine Settings

The excitation and emission monochromators and slit widths are most important since they greatest effect on resolution and signal to noise. These settings can be specified for each color for a given analysis and should selected based on all the colors used and the expected intensity range. Count time or

scan speed is also important, since fluorescent intensity is actually an average emission over the specified time period. In general, count times of 1 second and scan speeds of 240 - 480 nm per minutes allow sufficient counts within a reasonable total analysis time. PMT gain is automatically adjusted according to the slit width. All samples from a given experiment must be processed with identical parameter settings.

Increasing the emission slit widths increases the measured fluorescent signal and therefore, improves signal to noise. Increasing the excitation slit width in the Perkin Elmer LS-50 improves resolution and signal to noise. Slit widths should be customized to the colors used in a given experiment. When using multiple colors, we recommend excitation and emission slit widths of 4–8 nm, for optimal resolution and signal to noise. For less than 3 or 4 colors, which are separated by 20–40 nm, slit widths can be increased to 10-15 nm to improve the signal-to-noise ratio.

Excitation wavelengths should be set to optimal wavelengths for fluorescent dyes used in a given experiment to achieve the best emission signals. Selecting excitation and emission wavelengths that are not at the respective peak wavelengths can optimize separation of adjacent colors. Protocols requiring dyes that spillover into adjacent colors need to be mathematically corrected for spillover (see Section 4).

Sample Dilution

The concentration of dyes, from tissue samples, in the final solvent solution is important for two reasons. Intensity is linearly proportional to concentration only for sufficiently dilute solutions. At high dye concentration intensity is reduced or "quenched" relative to increasing concentration. This quenching effect is more pronounced with increasing numbers of colors used, although the true linear range remains essentially unchanged. We recommend standard curves be made for every color to be used and for the maximum colors to be used in a single experiment. Injecting only a sufficient number of p necessary for accurate counting statistics is the first means to minimize quenching. During the final sample processing, increasing the amount of solvent volume used per sample is the best way to minimize quenching. Additionally, intensity values are reported using an arbitrary scale with a maximum value of 1000, based on the count range of the PMT. If a broad range of intensities are expected the slit widths can be decreased to avoid maxing out the PMT. If you plan to markedly change blood flow to an organ of interest and blood flow increases dramatically, serial dilutions may be necessary to accurately measure fluorescence intensities. To prevent serial dilutions, the number of microspheres injected can be reduced to optimize the intensities under varying flow conditions.

Samples must be diluted accurately by careful pipetting or by weight to minimize introduced errors that will be amplified by serial dilution. Dilution of samples with solvent should continue until the fluorescence reaches an appropriate range. At each step, the dilution factor must be calculated. The true fluorescence signal is then calculated by multiplying the final fluorescence reading by the dilution factor. This must be done accurately because, when many dilutions are required, any error in measurement will be magnified by the multiplication factor. When a final dilution is reached and the fluorescence measured, a further dilution should be performed and the fluorescence measured to assure that quenching has not occurred.

Cuvettes

Because of the solvent used to extract the fluorescent dyes from the microspheres, only glass or quartz cuvettes can be used. The additional expense of quartz cuvettes is not necessary, as the wavelengths of the fluorescent colors are in the 350 to 800 nm range.

If more than one cuvette is used to make multiple fluorescent measurements, it is imperative to use matched cuvettes. A statement by the cuvette manufacturer that two cuvettes are "matched" is not a guarantee that they are. Cuvettes should be tested prior to reading a series of samples by measuring a fluorescent solution in each cuvette to ascertain whether or not they produce similar fluorescence signals (within 5% variation). We routinely test our matched cuvettes when they are new, and also with our fluorescent controls prior to and during each run. This acts as a quality control and is recorded in a separate control program (see Section 3).

Meticulous cleanliness is mandatory when using optical instrumentation. Any scattering of light will alter the fluorescence signal. Use powder-free gloves whenever handling cuvettes. Cuvettes should be washed with methanol between each sample (we use a commercially available cuvette washer). We also read a Cellosolve blank every 50 samples to check for cleanliness and background noise.

PLEASE NOTE THAT WE ACCEPT NO RESPONSIBILITY FOR THE USE OF OR THE SUCCESS OF THESE SUGGESTIONS. THE IMPLEMENTATION IS ENTIRELY AT THE DISCRETION OF THE USER.

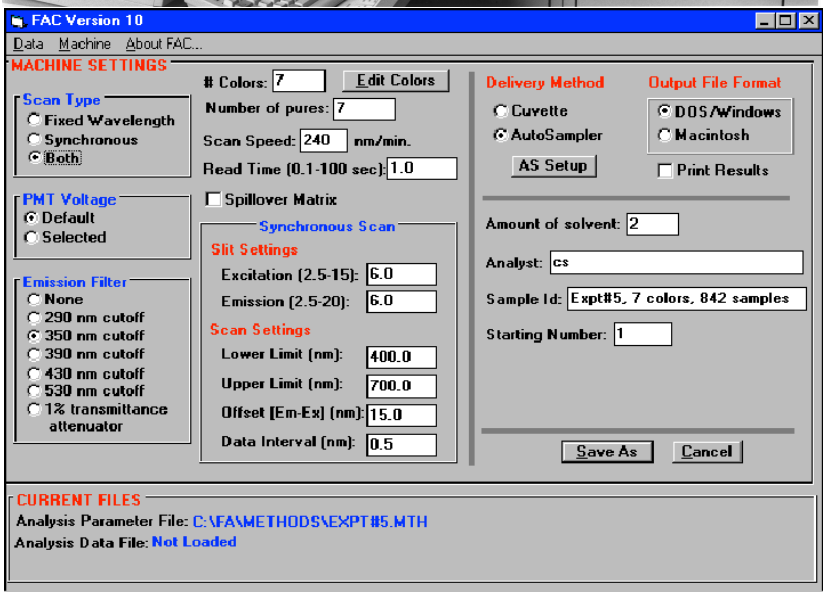
Software/Data Management

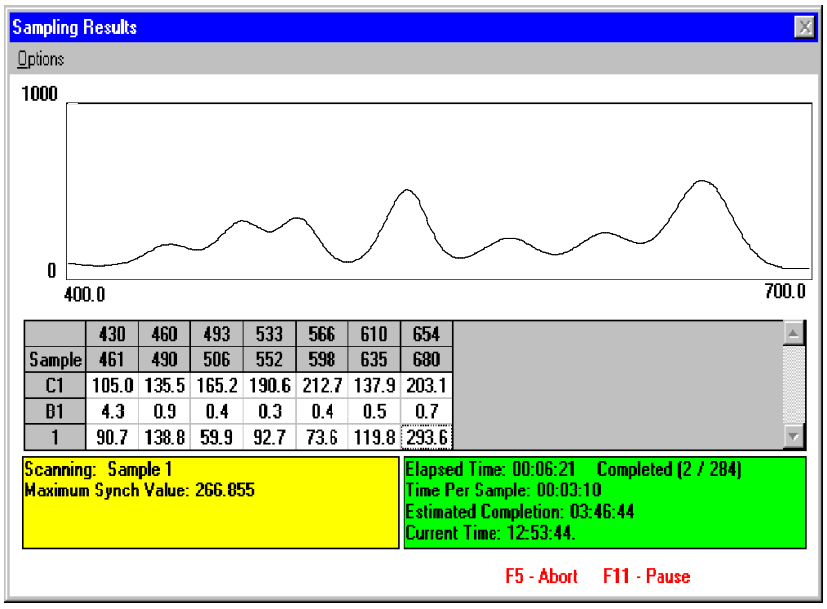
Because only the peak emission value is required for quantification, it is not necessary to obtain full spectral curves, as shown in Figure 2-3. Some instruments (such as the Perkin-Elmer LS-50) can be programmed to output peak intensity readings at specified wavelengths, thus shortening the analysis time.

WINFAC, a public domain program, is available to drive the Perkin Elmer LS-50 and LS-50B to measure peak intensity readings at specified wavelengths. This program calculates either fluorescent intensities or number of microspheres per sample and produces text files that are easily imported into spreadsheet programs. WINFAC is available through the Fluorescent Microsphere Resource Center.

Wellplate Reader

The Perkin Elmer LS-50B can accommodate a 96-wellplate reader. The wellplates used for this accessory must be opaque and resistant to the solvents used Dan Rurak's lab in Vancouver has successfully adapted a 96-well plate reader to measure regional blood flow in all organs (Tan, W. P., K, 1997).





	430	460	493	533	566	610	654
Sample	461	490	506	552	598	635	680
C1	105.0	135.5	165.2	190.6	212.7	137.9	203.1
B1	4.3	0.9	0.4	0.3	0.4	0.5	0.7
1	90.7	138.8	59.9	92.7	73.6	119.8	293.6

Figure 5-4. Automated fluorescent sampler.
Top: Computer and hardware configurations integrate a Perkin-Elmer LS-50B, AS-91 autosampler, and a dilutor station through a computer running Windows 95.
Middle: User definable options for acquiring fluorescent data.
Bottom: Data window as samples are read showing synchronous scans and fixed wavelength intensities.

Automated Flow Cell

Working in conjunction with Perkin Elmer, we have developed a fully automated system (Figure 5-4) that reads fluorescent samples (Schimmel, et al., 2000). Dave Frazer and Carmel Schimmel developed the software to drive the automated fluorescent sampler. They integrated a Perkin-Elmer LS-50B, AS-91 autosampler, and a dilutor station through a computer running Windows 95. The autosampler allows 270 samples to be read without technician intervention. This system has greatly reduced the analysis error and labor required for the fluorescent microsphere method (Schimmel, et al., 2000). The software package is available without charge via the Fluorescent Microsphere Resource Center web page (http://fmrc.pulmcc.washington.edu/).

Other Methods for Quantitating Fluorescent Microspheres

Three additional methods for measuring regional organ perfusion using fluorescent microspheres without extracting the dye are currently being used.

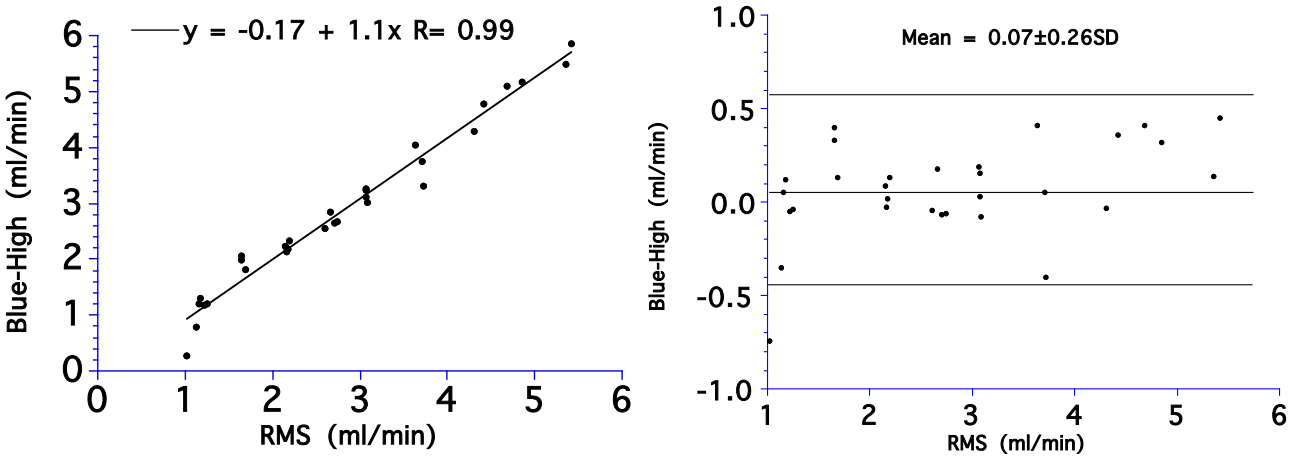
Direct Counting of Microspheres

Direct counting of serial dilutions in the digested sample is less accurate, labor-intensive, and requires a fluorescent microscope and a counting chamber (hemocytometer).

Flow Cytometer

Flow cytometry has been used for counting fluorescent microspheres (Austin et al., 1989). A laser detects individual fluorescent particles and can differentiate particle size as well as different dye loads. Sample processing is available commercially by Interactive Medical Technologies (IMT), where both 10- and 15-µm microspheres (NuFlow™) can be purchased. Ten markers are available; three are used as internal controls, leaving seven colors to act as blood flow markers. The tissue processing requires digestion, sonication, centrifugation, filtration, and a second centrifugation. The samples are then read by a flow cytometer. IMT will process tissues and return the results in a formatted spreadsheet. The FMRC compared the NuFLOW™ Microspheres and Investigator Partner Service (IPS) for measuring regional organ blood flow and found it compares well with radioactive microspheres.

Methods: Seven different colored NuFLOW™ microspheres 15 µm in diameter were obtained from Triton Technologies (San Diego, CA) in a kit costing *$255.* The specific colors used were red-high, violet-high, blue-high, red-med, orange-high, violet-med, and red-low. Each 2.1 ml bottle contained 50 million spheres in a saline solution, 0.01% Tween 80 and 0.01% Thimerosal as a bacteriostat. 1.25 million microspheres of each color were mixed with 1.3 million Ruthenium labeled microspheres and simultaneously injected into the left ventricle of an anesthetized and mechanically ventilated pig (15 kg). Two simultaneous reference blood samples were obtained from right and left femoral arterial lines. The animal was killed by anesthetic overdose and the heart and kidneys removed, diced into approximately 1 gram pieces and the radioactivity determined in a scintillation counter. The radioactive count in each sample was corrected for background and decay. Blood flow to each piece (ml/min) was determined in duplicate from the two arterial reference blood samples. The organ and blood samples were frozen and stored until the radioactivity decayed to a safe level for shipment (3 months).



Thirty samples were selected on the basis of their initial radioactive counts to provide a wide range of flow values. Each sample was placed in an individual tube and sent to the IPS laboratory along with the reference blood samples ($12.50/sample). An electronic spreadsheet with the estimated blood flow for each sample (ml/min) was returned by email 2 weeks later for a fee of $60.

Results: Blood flows to each sample estimated from the NuFLOW™ Microspheres and Investigator Partner Service were compared to blood flows determined by the radioactive microsphere method. One correlation plot and its companion Bland-Altman plot are presented below. The table summarizes all plots for all colors tested. Left and right arterial samples produced similar results. Only the results from the left reference sample are presented.

Figure 5-4 correlation plots and its companion Bland-Altman plot comparing radioactive microspheres (RMS) and fluorescent microspheres counted by flow cytometry.

	Correlation Plots			Bland-Altman Plots	
Color	**Slope**	**Intercept**	**R**	**mean**	**SD**
Red-High	1.00	-0.10	0.98	-0.04	0.24
Violet-High	.87?	0.21	0.97	-0.15	0.31
Blue-High	1.10	-0.17	0.99	0.07	0.26
Red-Med	.99	-0.01	0.99	-0.03	0.19
Orange-High	1.00	-0.17	0.98	-0.12	0.24
Violet-Med	.96	-0.04	0.98	-0.14	0.29
Red-Low	1.00	-0.06	0.99	-0.07	0.19

Conclusions: The NuFLOW™ Microspheres and Investigator Partner™ Service provide an accurate determination of regional myocardial and renal blood flow. We cannot comment on other tissues because of potential problems with digesting lipid rich tissues such as brain and gut. The primary drawback is the cost ($390 for 30 samples, 2 reference blood samples, a report, and shipping). Although the entire process has not been validated in the peer review literature, validation studies are reportedly in progress [source: Triton Technologies].

Cryomicrotome/Fluorescent Imaging System

The Regional Flow Imaging System (Barlow Scientific, Inc. Olympia WA) is a fluorescence cryomicrotome that determines the spatial distribution of organ blood flow at the microscopic level. Briefly, the system rapidly collects microcirculation data from organs containing up to four different colors of fluorescent microspheres. The instrument (Figure 5-4) consists of a charge-coupled device (CCD) video camera, a computer (Dell Computer Inc., Round Rock, Texas), metal halide lamp (HTI 403W/24, Osram, Sylvania), excitation filter-changer wheel, emission filter-changer wheel, and a cryostatic-microtome. Fluorescence images are acquired using a Kodak digital camera (2040 x 2040 pixel array) with a 200 mm zoom Nikkor lens (Nikon) with a macro-focusing baffle. Two motorized filter wheels containing excitation and emission filters are mounted in front of the light source and camera, respectively. The computer through photomicrosensors controls the filter positions. A custom designed microtome is outfitted with an asynchronous stepper motor to serial section frozen organs. Computer control of the microtome motor, emission filter wheel, and image capture and display is accomplished through a virtual instrument written in LabView 5.0.1 (National Instruments Inc.).

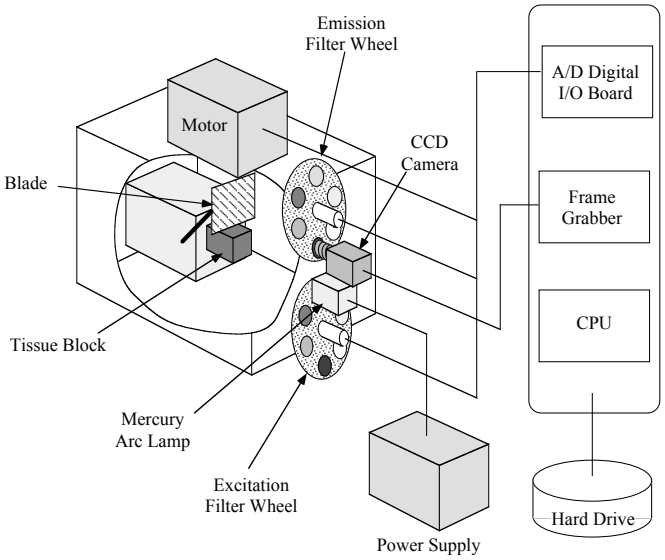


Figure 5-4

Schematic of imaging cryomicrotome.

The imaging cryomicrotome serially sections through the frozen organ at a selected slice thickness. Digital images of the tissue surface (en face) are acquired with appropriate excitation and emission filters to isolate each of four different fluorescent colors (Figure 5-5). Four 1-4 MB images at each of four fluorescence excitation/emission wavelengths are collected. Each image is processed so that X, Y, and Z (slice) locations of each microsphere are determined and saved in a text file. The spatial resolution of the system depends on the size of the organ being processed, but is typically 15 μm in the X and Y directions and 30 μm in the Z direction. Images of organ cross-sections produce a three-dimensional binary map defining the spatial location of organ parenchyma. This map determines the organ space locations to be sampled and the three-dimensional space for the statistical sampling (see below)

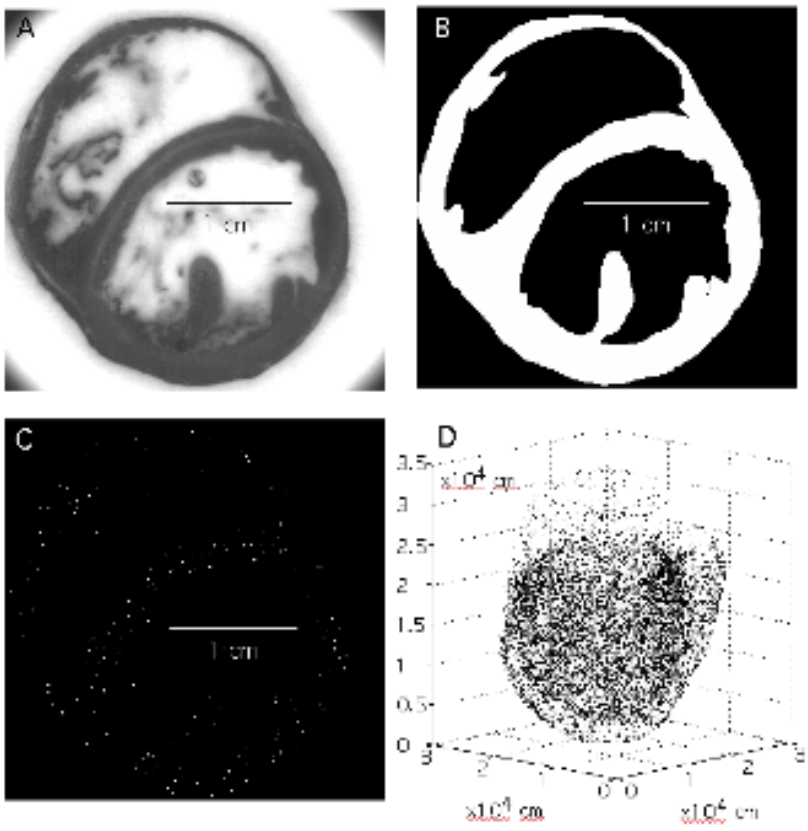


Figure 5-5. Processing of fluorescent images. A. 2000 x 2000 pixel outline image of rabbit heart en face. B. Bitmap image (black = 0 and white = 1) defining the spatial location of heart tissue. C. Fluorescent image obtained with a specific excitation/emission filter pair to determine individual microsphere location. Each point represents a microsphere located at x, y, and z (slice) location. D. Three-dimensional distribution of 26083 yellow microspheres.

Acquired images are analyzed with an analysis program also written in LabView, which applies an intensity threshold to convert areas of microsphere fluorescence into binary image blobs. The center of mass of each blob is calculated to fine the X and Y positions of each microsphere, with the Z coordinate being the slice number. The spatial coordinates and blob size is written to a text file.

Data reduction is automated through a final analysis program. A linked list of all microsphere locations (X, Y, and Z coordinates) is created. Any microsphere observed in the same X and Y coordinate across consecutive Z slices is reduced to a single observation occurring in the Z slice in which the microsphere is the largest. The number of consecutive slices in which a given microsphere is observed and the number of pixels representing each microsphere are also used to eliminate artifacts.

The imaging cryomicrotome provides the spatial location of every microsphere in an organ. It allows blood flow measurements to made be at a scale of resolution not previously possible. Microsphere locations are determined in a fully automated system. If all four colors are used, a rabbit heart can be completely processed in 20 hours without user input. The saved images are processed to produce a text file containing the spatial coordinates of every imaged microsphere. This approach obviates the need to physically dissect the organ and to separate the microspheres and tissue prior to eluting the fluorescent signals. Most importantly, it offers a method to study organ blood flow distribution at a very high resolution in small laboratory animals.

References

Austin, R. E., Jr., W. W. Hauck, G. S. Aldea, A. E. Flynn, D. L. Coggins and J. I. Hoffman. Quantitating error in blood flow measurements with radioactive microspheres. *Am J Physiol*. 257:H280-8, 1989.

Bassingthwaighte, J. B., M. A. Malone, T. C. Moffett, R. B. King, I. S. Chan, J. M. Link and K. A. Krohn. Molecular and particulate depositions for regional myocardial flows in sheep. *Circ Res*. 66:1328-44, 1990.

Buckberg, G. D., J. C. Luck, D. B. Payne, J. I. E. Hoffman, J. P. Archie and D. E. Fixler. Some sources of error in measuring regional blood flow with radioactive microspheres. *J. Appl. Physiol.* 31:598-604, 1971.

Chien, G. L., C. G. Anselone, R. F. Davis and D. M. Van-Winkle. Fluorescent vs. radioactive microsphere measurement of regional myocardial blood flow. *Cardiovasc Res*. 30:405-12, 1995.

Heyman, M. A., B. D. Payne, J. I. Hoffman and A. M. Rudolf. Blood flow measurements with radionuclide-labeled particles. *Prog. Cardiovasc. Dis.* 20:55-79, 1977.

Polissar, N. L., D. Stanford and R. W. Glenny. The 400 microsphere per piece "rule" does not apply to all blood flow studies. Am. J. Physiol. in press.

Powers, K. M., C. Schimmel, R. W. Glenny and C. M. Bernards. Cerebral blood flow determinations using fluorescent microspheres: variations on the sedimentation method validated. *Journal of Neuroscience Methods*. 87:159-165, 1999.

Schimmel, C, D. Frazer, S.R. Huckins and R.W. Glenny. Validation of Automated Spectrofluorimetry for Measurement of Regional Organ Perfusion Using Fluorescent Microspheres. *Comput. Methods Programs Biomed.* 62:115-25, 2000.

Tan, W. P., K. W. Riggs, R. L. Thies and D. W. Rurak. Use of an automated fluorescent microsphere method to measure regional blood flow in the fetal lamb. *Canadian Journal Of Physiology And Pharmacology*. 75:959-968, 1997.

van Oosterhout, M. F. M. and F. W. Prinzen. The fluorescent microsphere method for determination of organ blood flow. *FASEB J.* 8:A854, 1994.