

Interactive Medical Technology®

NuFLOW™ FLUORESCENT MICROSPHERE EXTRACTION PROTOCOL FOR REGIONAL BLOOD FLOW MEASUREMENT

NO. 9507.3

PROCEDURAL INSTRUCTIONS

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INTRODUCTION

Interactive Medical Technologies' (IMT) NuFLOW™ Fluorescent Microspheres provides the research scientist with an alternative to the use of radioactive microspheres for the measurement of blood flow in experimental animals. The high equipment and disposal costs normally associated with radioactive microspheres are minimized with fluorescent microspheres. The required laboratory equipment consists of polypropylene centrifuge tubes, a multipurpose centrifuge, a vacuum aspirator, an ultrasound homogenizer and use of a flow cytometer with blue (448nm) or green (543nm) laser excitation. For laboratories requiring high productivity alternatives, IMT offers the NuFLOW™ Flow Cytometer Analysis System, a turnkey package consisting of a desktop blue or green laser flow cytometer and software for fast accurate quantification of fluorescent microspheres.

The fluorescent microspheres prepared by IMT are crosslinked polystyrene-divinylbenzene microspheres, which have been labeled with four separate fluorescent dyes to create ten distinct populations of fluorescent microspheres. The high fluorescent dye content of each individual microsphere allows rapid identification and accurate quantification in a liquid suspension containing varying levels of background cellular debris. The fluorescent microspheres are chemically stable and exhibit no dye leaching in aqueous environments, including strong acid and base solutions. Each color of microsphere is available in three sizes, 10 µm, 15 µm and 25 µm diameter.

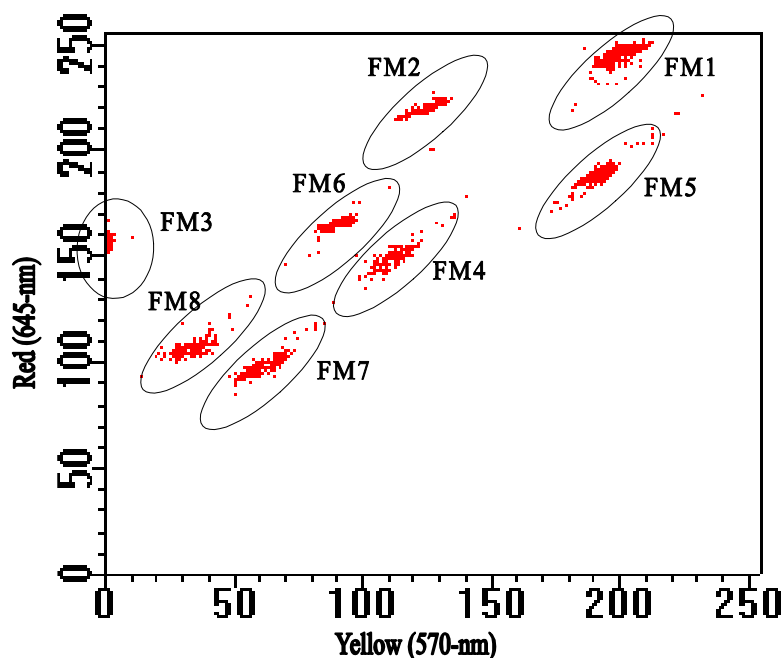
The method of animal preparation and microsphere injection with the NuFLOW™ microspheres are the same as that followed with radioactive microspheres in blood flow studies. To measure regional myocardial blood flow, for example, the microspheres are injected as a bolus into the left atrium of the experimental animal. The microspheres will mix with the arterial blood and flow with it, will distribute to the tissue, and will ultimately lodge in the microvasculature. Following the extraction and quantification of fluorescent microspheres from tissue sections and reference blood samples, blood flow values are calculated according to the following equation:

$$Q_i = (C_i \times Q_r) / C_r$$

where Q_i is the myocardial blood flow per gram (ml/min/g) of sample i , C_i is the microsphere count per gram of tissue in sample i , Q_r is the withdrawal rate of the reference blood sample (ml/min) r , and C_r is the microsphere count in the reference blood sample r .

FIGURE: Yellow/Red Fluorescence Dot Plot.

Each fluorescent microsphere counted by the flow cytometer generates values for yellow and red fluorescence intensity. Each microsphere population is distinguished by elliptical regions-of-interest.



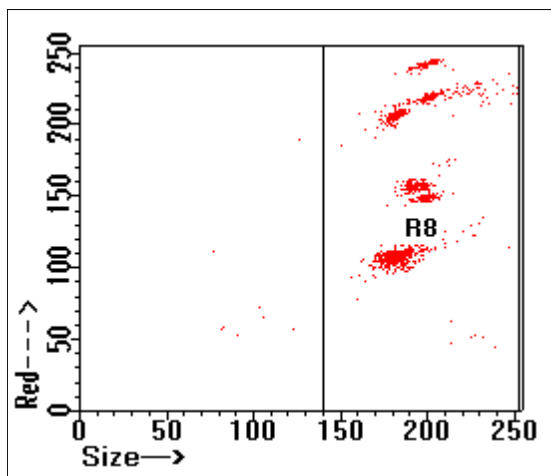
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The total number of microspheres in a sample is determined after completing an extraction protocol that recovers the microspheres from tissue or blood samples for flow cytometric analysis. Following the placing of tissue and blood samples into the appropriate polypropylene centrifuge tube, an alkaline hydrolysis protocol is initiated which digests the tissue and blood samples and ultimately recovers the microspheres in purified form. The first procedural step involves adding equivalent numbers of **Process Control Microspheres (PCM)** of the same size as the experimental microspheres to each sample. In general, one separately adds three colors of **PCM (PC1, PC2, PC3)** at 3,000 microspheres each to all samples and 3 control tubes. The **PCM** microspheres are used as an internal control to calculate the total number of microspheres in the original sample from the actual microspheres counted by flow cytometry. For example, if the actual count data is 1,500 **PCM** microspheres out of the known addition of 3,000 **PCM** microspheres to a sample, then one can assume that 50% (1,500/3,000) of all microspheres in the original sample were also counted. These would include the microspheres used in the regional blood flow measurements. The total number of microspheres in the original sample is then mathematically calculated from the ratio of **PCM** counted in the samples divided by the average number of **PCM** counted in the control tubes. The primary reason that 100% of a sample is not counted is that samples are filtered through a 50um pore strainer to protect the flow cytometer from clogging. About 10%-25% is held up by tissue debris and the filter.

$$\text{Number of Microspheres in Sample} = (\text{Microspheres Counted}) / (\% \text{ of PCM Counted})$$

The variance of the calculated number of **PCM** demonstrates the accuracy of this approach. The minimum expected variance for **PCM** is 4%-5%. This reflects an inherent Poisson distribution variance of 1.8% in attempting to add an average 3,000 microspheres to each sample; some samples will get slightly more or slightly less microspheres than the average 3,000 microspheres. Because calculated **PCM** are dependent on the number of **PCM** microspheres counted, the minimum expected variance is the sum of the variance of all 3 colors of **PCM**.

The completion of the processing procedures will produce approximately 200 µL aqueous solution containing purified fluorescent microspheres from each tissue and blood sample. These samples are then counted by flow cytometer for a period of 4-8 minutes per sample. When the fluorescent microspheres are counted by flow cytometry, each counted microsphere is distinguished by intensity values for Yellow Fluorescence, Red Fluorescence and Side Scattering. The values for Side Scattering indicate the size of the particle counted. Following the analysis of a sample, the values for each particle counted are saved to a computer file corresponding to a standardized Flow Cytometer format. These files are then interpreted by computer software that can discriminate the microsphere populations counted. Oval regions of interest are defined for each microsphere population on **Yellow vs. Red Dot Plots** reflecting the fluorescent signature of each microsphere population. Rectangular regions of interest are defined for 15 µm diameter particle size on **a Side Scatter vs. Red Dot Plot**. The total number of microspheres for each fluorescent population is simultaneously determined as a function of fluorescent signature and particle size (Gated for size and fluorescence).



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Yellow/Red Fluorescence Dot Plot

Particle Size/Red Fluorescence Dot Plot

EQUIPMENT AND PLASTICWARE REQUIRED BUT NOT SUPPLIED

15 and 50 ml Graduated Polypropylene Conical Centrifuge Tubes with Screwcaps (Falcon Blue Max from Becton Dickinson are recommended)

6x50mm and 12x75mm culture tubes for flow cytometry analysis

Centrifuge Racks

Multi-purpose Centrifuge (Min 1700g, 3000rpm on most centrifuges)

Thermolyne Maxi-Mix vortex mixer

Ultrasound Homogenizer (Tapered titanium micro tips of 1/8" and a double step titanium micro tip of 2mm used with a Branson 250 or Cole Parmer 4710 ultrasonic homogenizer)

General Purpose Oven – or Water Bath

Vacuum Aspirator, Flasks with Side Arm, Tubing, and Pasteur Pipette

Repeating 100 µL Pipettor or pipette (Repeating Pipettor recommended)

Wash Bottles

Flow Cytometer with Blue or Green Laser Excitation, Yellow and Red Fluorescence with Side Scattering Detection

Pasteur Pipettes, Dispensers and Filling Bulb, (Supplemental)

12 ml Glass Centrifuge Tubes (Supplemental)

REAGENTS REQUIRED FOR TISSUE AND BLOOD PROCESSING

1. TISSUE/BLOOD DIGEST REAGENT I: (Catalog No. 501-105) 2X Concentrate, 250ml. Before use, dilute bottled reagent two-fold by the addition of 1 volume reagent to 1 volume distilled water. **CAUTION: Strong Alkaline Solution!** Wear gloves and handle with care. Avoid exposure to the skin, eyes and clothing.
2. TISSUE/BLOOD DIGEST REAGENT II: (Catalog No. 501-106) 5X Concentrate, 250ml. Before use, dilute bottled reagent five fold by the addition of 1 volume reagent to 4 volumes distilled water.
3. MICROSPHERE COUNTING REAGENT: (Catalog No. 501-107) 5X Concentrate, 250ml. Before use, dilute bottled reagent five fold by the addition of 1 volume reagent to 4 volume distilled water.
4. BLOOD HEMOLYSIS REAGENT: (Catalog No. 501-108) 5X Concentrate, 250ml. Before use, dilute bottled reagent five fold by the addition of 1 volume reagent to 4 volume distilled water.
5. LIPID EMULSIFIER REAGENT: (Catalog No. 501-109) 1 Liter. Special reagent for processing fatty tissue specimens (e.g., brain, spinal cord). No dilution required.
6. ETHANOL, denatured.
7. PROCESS CONTROL MICROSPHERES (**PCM**): Fluorescent microspheres added to all tissue and reference blood samples. The size of the microspheres should be the same as the ones injected in the animal. The fluorescent color of the microspheres should be different than the fluorescent colors of the microspheres injected in the animal. PCM function as internal controls for microsphere recovery and percentage of sample analyzed. Make 3 process control solutions with approximately 3000 microspheres / 100ul. Dilute concentrated fluorescent microsphere solutions with 0.9% saline solution with 0.5% Tween 80 to achieve 3000 microspheres / 100ul in a beaker with a magnetic stirring bar. Prepare enough solution to add 100ul to all of the tissue, blood and control samples for your study. Verify the concentration by counting 100ul of each fluorescent color three times on the flow cytometer (Note: Count the 100ul with a rinse to analyze 100% of the fluorescent microspheres.)

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8. 50 µm PORE FILTER DEVICE: (Catalog No. 501-110) 1 unit per tissue and blood sample. Used to remove any large particles from final microsphere preparation that could obstruct the flow cytometer counting chamber. Device is reusable following cleaning with soap and water.

Regional Blood Flow Experimental Protocol Recommendations

Regional organ perfusion can be estimated with hematogenously delivered microspheres (Heyman et al., 1997). When appropriately sized microspheres are used, regional blood flow is proportional to the number of microspheres trapped in the region of interest (Bassingthwaighe et al., 1990) a number of excellent review articles describe and validate the use of microspheres for measurement of regional blood flow, but the classic review by Heyman et al. (1977) contains many details for radioactive microsphere use that apply to fluorescent microspheres (Glenny et al., 1999).

Over the last twenty years, new and/or refined methods for measuring regional blood flow have been published. 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 experiment protocol. It is not feasible for this protocol to address the wide variety of methods currently accepted. Outlined below are methods for regional blood flow that have been recommended by our customers and the Fluorescent Microsphere Resource Center (Glenny et al., 1999).

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 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.

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

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).

$[Q_{\text{organ}} / Q_{\text{total}}]$ = fraction of the total cardiac output supplying the 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. Doubling the minimum number (N_{\min}) will make sure that low-flow organ pieces also have adequate number of microspheres (Buckberg et al., 1971; Nose et al., 1985). This 400 microsphere “rule” only applies to measurement 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 estimation of correlation coefficients.

Calculation of Injectate Volume

$$\text{mls of suspension} = N_{\min} / [\text{microspheres concentration}]$$

Preparation of Fluorescent Microspheres for Injection

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Method:

1. Check supernatant solution. It should be clear due to the addition of thimerosal, a bacteriostatic agent. Cloudy fluid may indicate contamination. The fluorescent microsphere suspensions contain a small amount of Tween 80 (0.050% v/v) to prevent microsphere aggregation. If desired, the concentrations of additives can be reduced by centrifuging the suspension, aspirating the supernate and diluting the microspheres with the desired carrier containing a minimum 0.01% (v/v) Tween 80 just prior to injection.
2. Mix by inverting slowly and thoroughly (15 to 30 secs). (Note: Vortexing microspheres can create foam that will cause variations in microsphere concentration.)
3. Place in ultrasonic water bath for at least 2-10 min to disperse the microspheres (Recommended, but not required). Do not cover water bath or sonicate too long because the heat generated can melt the microspheres. For microspheres 15 μ or smaller, sonication time is less (smaller particles are more susceptible to heat).
4. Just prior to injection, invert mix the vial of microspheres again, load and unload the syringe to the desired volume three times. Do not permit the microspheres to settle once they are drawn into the syringe. If injection time is delayed, repeat the loading procedure.
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 mls, then the syringe volume should be 20-30 mls. Glass syringes and containers are preferred; they decrease microsphere loss caused by "static" attraction of the plastic microspheres with the plastic containers or syringes.

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Method for Reference Blood Flow Sampling

There are 3 different anticoagulants that routinely used: Heparin (syringe coated), Citrate Phosphate Dextrose (10 cc per 30 cc syringe) and EDTA*Na₂(6mg/ml of blood).

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 test tubes 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.

Result Calculations

This is most easily accomplished using a computer spreadsheet (available from IMT), but can be processed by hand or with a computer program. The following formula can be used to calculate regional blood flow (ml/min/gram);

Total Microspheres in a Sample

$$\text{Total Spheres}(x)_i = \frac{[\text{Count}(x)_i][\text{PCM}_{\text{avg}}]}{\text{PCM}_i}$$

Where:

Total Spheres(x)_i = the total microspheres of color x in a sample i.

Count(x)_i = the number of color x microspheres counted in sample i.

PCM_{avg} = the average sum of PCM (Process Control Microspheres) added to each sample.

Note: This value is obtained by counting 100% of the three control tubes created at the beginning of the processing protocol. The coefficient of variation between the three samples should be less than 5%.

PCM_i = The sum of PCM counted in sample i.

Regional Blood Flow (ml/min/gram)

$$Q(x)_i = \frac{\text{Total}(x)_i * R_r}{\text{Total}(x)_r * W_i} = \frac{\text{Count}(x)_i * \text{PCM}_r * R_r}{\text{Count}(x)_r * \text{PCM}_i * W_i}$$

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Where:

$Q(x)_i$ = the regional blood flow (ml/min/gram) for color x in sample i.

$Total(x)_i$ = the total microspheres of color x in sample i

$Total(x)_r$ = the total microspheres of color x in sample r

Note: Color x is the color of the microspheres collected in reference blood sample r.

R_r = the reference withdrawal rate for reference blood sample r in (ml/min)

W_i = the weight of tissue sample I in grams

$Count(x)_i$ = the number of color x microspheres counted in tissue sample i.

$Count(x)_r$ = the number of color x microspheres counted in tissue sample r.

Note: The PCM_{avg} is not important in the calculation of regional blood flow (ml/min/gram).

Tissue perfusion in Microspheres per Gram (Tissue samples only)

$$M(x)_i = Total(x)_i / W_i$$

Where:

$M(x)_i$ = Total microspheres in sample i of color x per gram of tissue i.

$Total(x)_i$ = Totals microspheres in sample I of color x

W_i = the weight of tissue sample I in grams

Blood Flow in Microspheres per ml per min (References only)

$$F(x)_r = Total(x)_r / R_r$$

Where:

$F(x)_r$ = the microspheres per ml per min of reference sample r of color x.

$Total(x)_r$ = the total microspheres of color x in reference sample r

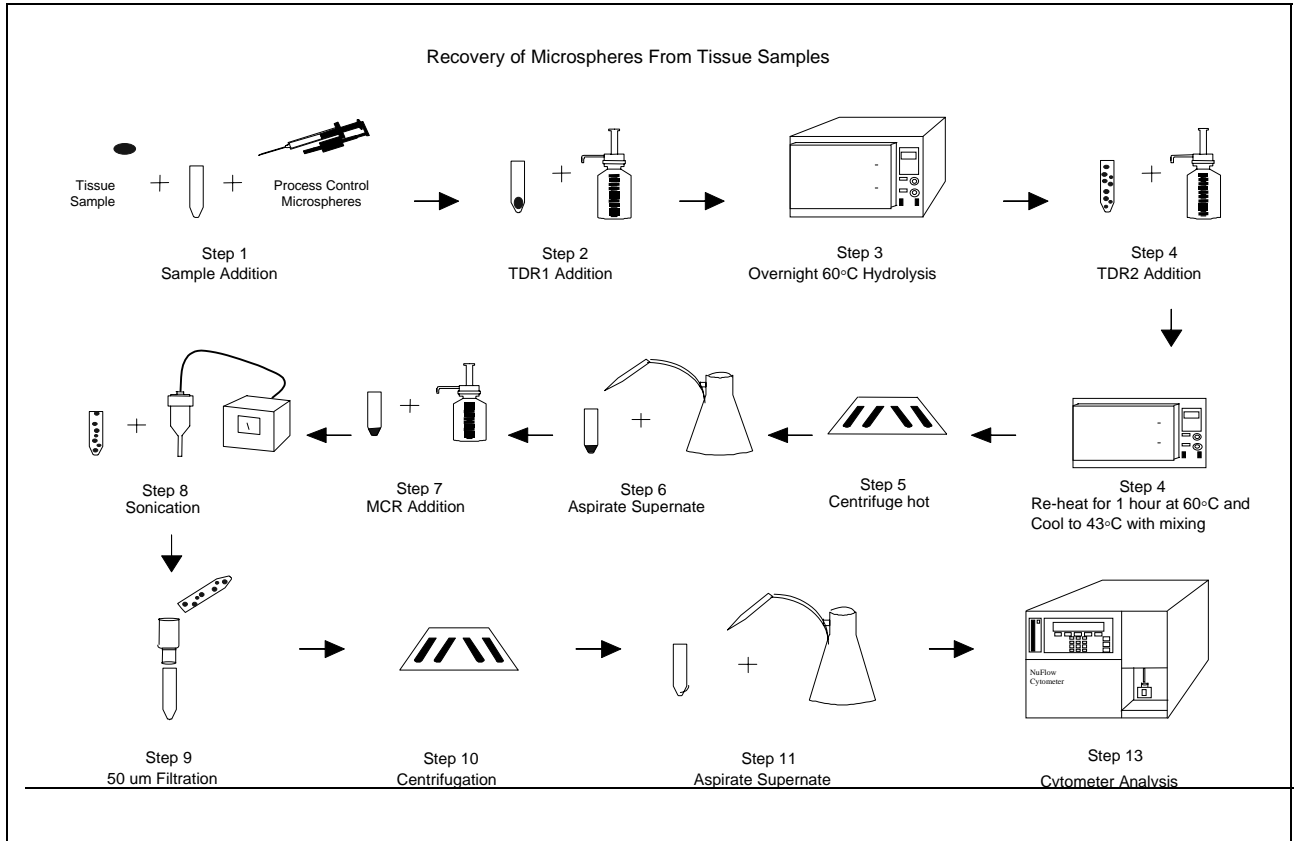
R_r = the reference withdrawal rate for reference blood sample r in (ml/min)

RECOVERY OF MICROSPHERES FROM BLOOD AND TISSUE

GENERAL NOTES

1. Tissue biopsy samples should be refrigerated (less than 1 week) or stored frozen or fixed with formaldehyde until such time as one proceeds with the following extraction procedures. Reference blood samples should be stored refrigerated (Make sure enough anticoagulant is mixed in with the blood).
2. Before mixing by vortex or inversion, all centrifuge tubes should be covered with a screw cap to prevent spillage and loss of microspheres.
3. Unless otherwise noted, all procedures are performed at room temperature.
4. Due to the wide variety of animals and tissue organ types some adjustment may be necessary to dissolve the fatty tissue organs.
5. Unless otherwise noted, all centrifugation steps are at a RCF of 1500 x g with low or no brake (approximately 2,500 – 3,000 RPM for common bench top centrifuges).
6. Unless other wise notes, all 15 ml sample tubes should be sonicated with a 2 mm stepped micro tip and 50 ml tubes with a 1/8" micro tip. Power should be adjusted by starting with a low setting and increase until the pellet begins to break up. Maintain this power setting for the remainder of the samples.
7. Four procedures are used for processing blood and tissue samples depending on the blood and tissue size or volume. The sample processing steps in the first procedure are performed on all samples in an experiment at the same time. The steps in the second procedure are performed in a 15 ml centrifuge tube and are recommended for most organ tissue samples of 1-3 grams. The third procedure is recommended for tissue samples greater than 3 grams or for tissue sample that prove difficult to hydrolyze with the first procedure. The fourth procedure is for blood of different volumes.

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PROCEDURE I. ADDING PROCESS CONTROLS (All Samples)

1. Each whole tissue sample less than or equal to 3 grams is placed in a labeled pre-tarred 15 ml polypropylene centrifuge tube and pushed or briefly centrifuged to the bottom of the tube. Each tissue sample greater than 3 grams and less than 10 grams is placed in a labeled pre-tarred 50ml polypropylene centrifuge tube and pushed or briefly centrifuged to the bottom of the tube. The centrifuge tube is then re-weighed, recording the tissue sample weight.
2. Immediately following reference blood sample collection, the blood sample should be mixed with EDTA*Na₂ (6 mg/ml of blood) in a 15 or 50 ml centrifuge tube to prevent coagulation. Each blood sample is placed in a 15ml or 50ml labeled polypropylene centrifuge tube such that the volume of the blood and the saline rinses is less than half the volume of the test tube (<7.5 ml blood volume in 15ml tube and <25 ml blood volume in 50ml tube).
3. Label 3 additional empty flow cytometer counting tubes as controls for the experiment.
4. Arrange all the blood, tissue and 3 control tubes uncapped and in a random order. (Make sure the tissue and blood are at the bottom of the test tubes.)
5. Mix the **PCM** solution with magnetic stirrer and load a repeating dispenser set to dispense 100ul aliquots. (Don't forget to prime the dispenser by shooting 200ul to 300ul of the **PCM** solution back into the source.)
6. Add 1 shot of the dispenser to every sample tube including the controls. Repeat for the additional process control colors. (Practice this procedure with water, verifying your technique using a scale.)
7. Separate out the control tubes and centrifuge them for 5 minutes, cap or seal and set aside. (Place them in another centrifuge tube for support if necessary. A glass rod of slightly less diameter than the counting tube can be used to remove the counting tube from the centrifuge tube).
8. Begin the recovery of microspheres from tissue and blood sample protocols.

PROCEDURE II. RECOVERY OF MICROSPHERES FROM TISSUE SAMPLES (less than 3 gram)

1. Add 4 ml of diluted **TISSUE/BLOOD DIGEST REAGENT I** (****CAUTION**** Strong Alkaline Solution) to each sample. Place the screw caps on the centrifuge tubes and tighten snugly.
2. **Overnight Alkaline Hydrolysis:** Vortex and place the tubes in a general-purpose oven set to 60°C and allow the tissue to digest by alkaline hydrolysis overnight. Intermittently, remove the centrifuge tubes from the oven, verify that the caps are tightened snugly and vigorously vortex mix for approximately 15-30 seconds (Note: replace the caps that cracked with new ones). The tissue samples in each tube should completely homogenize into suspension, with only small particles of fatty white debris visible.
3. Add diluted **TISSUE/BLOOD DIGEST REAGENT II** to each sample suspension to bring the total liquid volume to the top of the centrifuge tube (14-15 ml). Re-cap and mix by inversion. Visually inspect each tube to verify that suspension is uniformly mixed. Separate out the samples that are not uniformly mixed for future verification of mixing.
4. **Re-heat** the samples for 1 hour in a general-purpose oven set to 60°C. Visually inspect the samples verifying that all tissue and fats are dissolved. If a sample is not dissolved, vortex mix for approximately 15-30 seconds and continue re-heating until it completely dissolves.
5. Vortex mix for approximately 10 to 15 seconds and return to the oven and cool to a 43°C for 1 hour inverting the samples every 15 to 20 minutes to keep the surfactants from separating out.
6. After the oven cools to 43°C vortex mix every sample approximately 15-30 seconds and **Immediately Centrifuge** samples 5 minutes at 1700g with low brake (3000 RPM on most counter top centrifuges). If your centrifuge has temperature control, set the temperature to 20°C. By centrifuging the samples warm, the solvent is less dense and keeps the fats and oils from forming micelles.

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7. Uncap and inspect each sample for floating white fatty or oily material. If floating material is not fluid like, re-heat the sample and transfer it to a larger tube using warm Tissue/Blood Digest Reagent II as a rinse and filling solution go to step 4 in procedure III. All remaining samples can be **aspirated** to a safe level above the pellet (~2ml in a 15ml tube).
8. Each greenish-brown pellet is re-suspended in 10 ml of diluted **MICROSPHERE COUNTING REAGENT** by vortex mixing. If sediment aggregates are visible, they can be disrupted by either (A) brief ultrasound sonication for 15-30 seconds {Recommended}, or (B) returning the centrifuge tubes to the oven for a combination of heating and vortex mixing until all sediment aggregates are dispersed.
9. Centrifuge the microsphere suspension for 15 min., and aspirate the supernate to a level safely above the visible pellet (~2ml).
10. Each tube pellet is re-suspended in 10 ml of diluted **MICROSPHERE COUNTING REAGENT** by vortex mixing. Each microsphere suspension is briefly sonicated for 15-30 seconds to assure disruption of sediment aggregates. A **50 µm Pore Filter Device** is placed on the top of a receiving 15 ml Centrifuge Tube and the suspended microsphere solution is poured through the filter device. The donating centrifuge tube and the filter device are rinsed with **MICROSPHERE COUNTING REAGENT** to complete sample transfer.
11. Centrifuge the microsphere suspension for 15 min., and aspirate the supernate to a level approximate to a safe level above the pellet (~2ml). Centrifuge the sample again for 5 minutes to remove the solution from the test tube walls and aspirate carefully to a solution volume of approximately 200 µL. Briefly sonicate each solution at low power, transfer the sample to an appropriate counting tube with a 200ul pipette set aside for flow cytometer analysis (cap or cover with parafilm if for longer than a day).

PROCEDURE III. RECOVERY OF MICROSPHERES FROM TISSUE SAMPLES (3-10 gram)

1. Add 10 ml of diluted **TISSUE/BLOOD DIGEST REAGENT I** (****CAUTION*** Strong Alkaline Solution) to each sample. Place the screw caps on the centrifuge tubes and tighten snugly.
2. **Overnight Alkaline Hydrolysis:** Vortex and place the tubes in a general-purpose oven set to 60°C and allow the tissue to digest by alkaline hydrolysis overnight. Intermittently, remove the centrifuge tubes from the oven, verify that the caps are tightened snugly and vigorously vortex mix for approximately 15-30 seconds (Note: replace the caps that cracked with new ones). The tissue samples in each tube should completely homogenize into suspension, with only small particles of fatty white debris visible.
3. Add diluted **TISSUE/BLOOD DIGEST REAGENT II** to each sample suspension to bring the total liquid volume to the top of the centrifuge tube (45-50ml). Re-cap and mix by inversion. Visually inspect each tube to verify that suspension is uniformly mixed. Separate out the samples that are not uniformly mixed for future verification of mixing.
4. **Re-heat** the samples for 1 hour in a general-purpose oven set to 60°C. Visually inspect the samples verifying that all tissue and fats are dissolved. If a sample is not dissolved, vortex mix for approximately 15-30 seconds and continue re-heating until it completely dissolves.
5. Vortex mix for approximately 10 to 15 seconds and return to the oven and cool to a 43°C for 1 hour inverting the samples every 15 to 20 minutes to keep the surfactants from separating out.
6. After the oven cools to 43°C vortex mix every sample approximately 15-30 seconds and **Immediately Centrifuge** samples 5 minutes at 1700g with low brake (3000 RPM on most counter top centrifuges). If your centrifuge has temperature control, set the temperature to 20°C. By centrifuging the samples warm, the solvent is less dense and keeps the fats and oils from forming micelles.
7. Uncap and inspect each sample for floating white fatty or oily material. If floating material is not fluid like, re-heat the sample and follow the supplemental procedures for Tissue Samples of the Digestive Track/Skeletal Muscle/Fatty Samples. All remaining samples can be **aspirated** to a safe level above the pellet (~7.5ml in a 50ml tube).

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8. Each greenish-brown pellet is re-suspended in 45 ml of diluted **MICROSPHERE COUNTING REAGENT** by vortex mixing. If sediment aggregates are visible, they can be disrupted by either (A) brief ultrasound sonication for 15-30 seconds {Recommended}, or (B) returning the centrifuge tubes to the oven for a combination of heating at 60°C and vortex mixing until all sediment aggregates are dispersed.
9. Centrifuge the microsphere suspension for 15 min., and aspirate the supernate to a level safely above the visible pellet (~7.5ml).
10. Each microsphere suspension of 7.5 ml is briefly sonicated for 15-30 seconds to assure disruption of sediment aggregates. A **50 µm Pore Filter Device** is placed on the top of a labeled receiving 15 ml Centrifuge Tube and the suspended microsphere solution is poured through the filter device. The donating centrifuge tube and the filter device are rinsed with 3 ml of diluted **MICROSPHERE COUNTING REAGENT** two times to complete sample transfer.
11. Centrifuge the microsphere suspension for 15 min. and aspirate the supernate to a level approximate to a safe level above the pellet (~2ml). Centrifuge the sample again for 5 minutes to remove the solution from the test tube walls and aspirate carefully to a solution volume of approximately 200 µL. Briefly sonicate each solution at low power, transfer the sample to an appropriate counting tube with a 200ul pipette set aside for flow cytometer analysis (cap or cover with parafilm if for longer than a day).

RECOVERY OF MICROSPHERES FROM REFERENCE BLOOD SAMPLES

1. Add diluted **BLOOD HEMOLYSIS REAGENT** to each blood sample to bring the total volume to 50 ml and vigorously vortex for 15 to 30 seconds. Centrifuge the solution for 30 minutes and aspirate the red supernate down to 5-10 ml volume mark leaving the scarcely visible pellet undisturbed.
2. Add 6 ml of diluted **TISSUE/BLOOD DIGEST REAGENT I** (******CAUTION**** Strong Alkaline Solution**) to each sample. Place the screwcaps on the centrifuge tubes, tighten snugly and vigorously vortex for 15 to 30 seconds..
3. **Overnight Alkaline Hydrolysis**: Place the tubes in a general purpose oven and allow the blood samples to digest by alkaline hydrolysis overnight. Intermittently, remove the centrifuge tubes from the oven, verify that the caps are tightened snugly and vigorously vortex mix for approximately 15-30 seconds. The blood samples in each tube should completely homogenize completely into suspension with no visible particulates.
4. Continue with step 3 in procedure II or III depending on the size of the blood sample tube.

COUNTING PROCEDURES**On Becton Dickinson FacScan or similar flow cytometer (“Blow” flow cytometer)**

- 1 Set up the flow cytometer such that all of the microsphere populations are distinguishable and side scatter is centered in the scale (Placing RV-High in the top right corner of the Yellow vs Red Dot Plot). Consult the flow cytometry technician. Following are the setting used for a FacSort:

Detector	Voltage	AmpGain	Mode
SSC	150	1.00	Log
FL1	325	1.00	Log
FL2	231	1.00	Log
FL3	304	1.00	Log

Threshold on FL3 with a channel value of 8.

No compensation.

Flow rate set to Hi (60uL/minute).

Use the analysis to acquisition mode with maximum count of 500,000 and no time limit where the data files are saved sequentially in a folder for analysis later. Note: It is important that every fluorescent particle is counted and saved.

- Quality control of the flow cytometer can be achieved by counting a mixture of the microspheres (about 3000 spheres/sample/color) used in the study repeatedly over time. All count should fall within 10% of the mean with a coefficient of variation of less than 5%. (Spreadsheets available for these calculations)
- 2 Select a counting tube that will allow you to count volumes of about 200ul with as little solution remaining as possible. A 6x50mm tube is recommended for the FacSort. (Note: In order to count with the 6x50mm tube, place it inside of the 12x75mm plastic tube. A glass rod of slightly less diameter than the counting tube can be used to conveniently remove it from the 12x75mm tube.)
- 3 Counting the control tubes.
- 3.1 Start the flow cytometer acquisition with as tube of DI water. (The DI water should have little to no counts)
 - 3.2 Vortex mix the process control tube for 5 to 10 seconds.
 - 3.3 Switch the Control tube with the DI Water and count until there is no more liquid left in the sample. Approximately 5 minutes.
 - 3.4 Remove the control tube and add 70ul of **MCR** solution, vortex mix for 5 to 10 seconds. Continue counting until the rinse solution is completely counted.
 - 3.5 Switch the control tube with the DI water tube.
 - 3.6 Stop the acquisition of data and save the data.
 - 3.7 Record which file corresponds to the control tube in a sample log sheet.
 - 3.8 Repeat for additional control tubes.
- 4 Counting blood and tissue sample tubes
- 4.1 Start the flow cytometer acquisition with as tube of DI water. (The DI water should have little to no counts)
 - 4.2 Vortex mix the blood or tissue sample tube for 5 to 10 seconds.
 - 4.3 Switch the blood or tissue sample tube with the DI water tube.
 - 4.4 Count until there is no more liquid left in the sample. Approximately 5 minutes. (Note: Samples will occasionally clog. If this happens, release the pressure on the counting tube to remove the clog and continue counting. If a sample continues to clog, repeat the pressure release and or remove and vortex the sample, and continue counting without stopping the flow cytometer from acquiring. It is possible to stop the samples acquisition and restart if there is a need to clean the flow cytometer sipper with cleaning wires. The only additional step required if multiple files were used to count the same sample is the addition of the counts for the corresponding colors.
 - 4.5 Stop the acquisition of data and save the data.
 - 4.6 Record which file or files that corresponds to the blood and tissue sample tube in a sample log sheet.
 - 4.7 Repeat for additional blood and tissue sample tubes.

FLOW CYTOMETER DATA FILE ANALYSIS

NuFLOW™ Extraction Protocol 9507.2

With Becton Dickinson's Pclsys Version 1.1 or multiple gates for flow cytometer Software

- 1 Start Pclsys and arrange a yellow vs. red fluorescence Dot Plot, Gat statistics, and spreadsheet windows in a convenient data transfer arrangement. (Procedure can be automated using windows Recorder)
- 2 Open a dot plot of the first file in the experiment with Yellow vs. Red fluorescence as the axis. Add elliptical regions for all the populations noting the fluorescent microsphere color represented by each of the regions. (If more than 7 colors were used than a second set of regions can be used.) Leave one region empty so it can be used for size gating in the next step. Counting small samples of diluted stock microspheres will identify populations.
- 3 Change the axis on the dot plot to side scatter vs. Red Fluorescence. Add a polygon region that will select for size and red fluorescence of the microspheres used. (Use files for the control tubes to draw the region.)
- 4 Change the Gate Specs each fluorescent microsphere to gate for each fluorescent color and size region. (For example gate 1 will read "R1 and R8" where R1 is the region for color and R8 is the region for size.
- 5 Copy the Gate statistics to an excel spreadsheet and paste the data in the appropriate sample locations.
- 6 Open the next data file and arrange the regions to adjust for any population shift. (Shifts can be caused by flow rate changes due to particles clogging the flow cytometer or reduction in fluorescent intensities caused by the tissue sample processing.) The shift is usually very small.
- 7 Copy the gate statistics to the spreadsheet and paste the data in the appropriate sample location. Repeat steps 6 and 7 until there are no more data files.
- 8 Label all of you results in your spreadsheet and calculate regional blood flow using the formulas listed previous. (Spread sheet available with automated features to simplify this procedure.)
- 9 Review the PCM count to verify that the samples were counted correctly. Less than 5% variation in the total microspheres is expected. If greater than 5% variation is found, review the data and adjust regions and gates if necessary. Call for addition assistance in trouble shooting variations.

With single gate flow cytometer software

- 1 Follow procedures 1 thru 3 in above. Because some software only has the ability to gate for one pair of regions at the same time, it is necessary to count each color separately in each data file. Change the gate settings to mach the region and size of the color you are counting and transfer the microsphere count to the excel spreadsheet. Repeat this process for each additional file and then repeat for each color of microspheres.

SUPPLEMENTAL PROCEDURES

Tissue Samples with High Lipid Content (Brain, Spinal Cord)

Following overnight alkaline digestion at 70°C, the addition of **Tissue/Blood Digest II**, centrifugation and aspiration of the supernate, a large quantity of white lipid material will be detected as a pellet. To remove this lipid material, add the following procedure steps.

1. Add **LIPID EMULSIFIER REAGENT (LER)** to each sample to a total 13 ml volume mark (15 ml Centrifuge Tube) or 45 ml (50 ml Centrifuge Tube). Vortex mix very well to dissolve the lipid material in **LER**. Centrifuge for 15 min and aspirate the supernate to a level safely above each visible pellet.
2. Visually inspect the resulting pellet for the continued presence of a white lipid material. If the white sediment is still present, repeat step 1 . If no white sediment is present, continue with **STEP 8 OF PROCEDURE 1 OR 2 DEPENDING ON THE SIZE OF THE TUBE**. Generally, two washes with **LER** are sufficient to remove white lipid from tissue samples.

Tissue Samples of the Digestive Track/Skeletal Muscle/Fatty Samples

- 1 Process all digestive track/fatty samples larger than 1 gram using Processing Procedure III (tissue samples larger than 3 grams).
- 2 If the fats do not dissolve completely at step 4 or 7 then dilute the sample by splitting it into two 50 ml polypropylene tubes (Transferring to a larger centrifuge tube is not recommended due to the possibility of microsphere loss caused by the size of the centrifuge tube).

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- 2.1 Heat the sample in a 60°C oven until as much of the fat is dissolved, remove and vortex thoroughly for 15 to 30 seconds and pour half of the sample into a labeled empty tube. Add TBDRII to both samples to a total 45 ml volume mark, cap, invert and place back in the oven to further dissolve the fats. Repeat this procedure if necessary. After the fats are dissolved, the sample can be processed according to the Processing Procedure III with the addition of the following step.
- 2.2 After step 7 (the first aspiration of the sample), the two sample tubes are combined. Sonicate one of the two samples and pour the 7.5ml of solution with 2 rinses of 3 ml of MCR into the other half of the sample. Continue with step 8 in Processing Procedure III.

Tissue Samples Vital Stains or Formaline treatments (TTC, Evans Blue, Trypan Blue, Buins Yellow and formaldehyde)

No adjustments to the processing protocol are necessary for vital stains or formaline preservative. The only adjustment maybe in the protocols used to stain the tissues. The microspheres fluorescent colors are sensitive to heat and organic solvents. Never use temperatures above 60°C or organic solvents like toluene, chloroform, DMF, acetone, etc.

References

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