special communication

A new sample-processing unit for the fluorescent microsphere method

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Raab, S., E. Thein, A. G. Harris, and K. Messmer. A new sample-processing unit for the fluorescent microsphere method. *Am. J. Physiol.* 276 (*Heart Circ. Physiol.* 45): H1801–H1806, 1999.—The use of fluorescent-labeled microspheres (FM) for measurement of regional blood flow is an attractive alternative to the use of radioactive-labeled microspheres. In the FM method the FM have to be completely recovered from the tissue samples in a time- and labor-intensive process. For this reason, a considerable loss of FM is possible. The aim of this study was to develop a filtration device that allows the tissue sample to remain in a single container throughout the procedure to make the process easier and to avoid the loss of FM. The core of the sample-processing unit (SPU) is a single-tube filtration device with a polyamide wire mesh. The protocol for processing tissue from different organs (heart, kidney, liver, spleen, intestine, muscle, bone, lung, brain) was modified and thus shortened significantly. Furthermore, the SPU allows direct filtration of the blood reference sample without previous digestion. Different experiments showed that the SPU in combination with the new protocol excludes the loss of 15-μm FM. The modifications of the whole procedure render it faster and highly standardized.

filtration; reference blood sample; organ digestion; blood flow measurement; instrumentation

MICROSPHERES (MS) have been used for the measurement of regional blood flow for more than 30 years (5). Until now, radioactive-labeled MS (RM) have been the standard type of MS used. The RM method does, however, have the following disadvantages: *1*) disposal of the radioactive tissue is expensive, *2*) carrying out chronic experiments is difficult because of the short half-lives of the isotopes, and *3*) working with radioactivity poses certain risks to the researcher's health and the environment.

The use of fluorescent MS (FM) eliminates the problems associated with radioactivity (4). However, use of FM entails the complete recovery of the MS from the tissue samples. This is a time-intensive and complex procedure bearing the risk of losing MS.

Two different methods are used to recover FM. The filtration method was introduced by Glenny et al. in 1993 (2). In this procedure the tissue is digested in 1 N KOH for 24 h, and then the digestate is filtered through a polycarbonate (PC) membrane with a pore size of 8 μm. The transfer of the spheres from the digestion container onto the membrane can result in a loss of spheres. Furthermore, the spheres can adhere to the walls of the containers, again resulting in a loss of spheres. A third potential source of loss of MS in this procedure is the instability of the filter membrane against KOH.

In the sedimentation method (3) the organ sample is digested in 1 N KOH for 48 h. The FM are recovered by repeated steps of washing, centrifugation, and aspiration of the liquid. The advantage of this method is that the whole process takes place in a single container. However, the fluorescent dyes are very pH sensitive after they are dissolved from the spheres, so any residual KOH left in the container would result in a loss in fluorescence intensity. Furthermore, FM might be lost when the supernatant liquid is aspirated.

The aim of this study was to design a new method for the recovery of FM that would allow the tissue sample to stay within a single container throughout the procedure, resulting in the complete recovery of the FM. Therefore, a novel filtration device, a sample-processing unit (SPU), was developed. The SPU allows FM recovery by filtration of the digestion fluid, thus avoiding a possible loss of FM by aspiration.

METHODS

SPU Construction

The SPU consists of three units: *1*) a filter unit, *2*) a filter holder, and *3*) a sample tube (Fig. 1). The sample tube can be closed with a cap, as can the entire SPU.

The SPU is made of polypropylene, which is resistant to 4 N KOH and organic solvents such as 2-ethoxyethyl acetate (Cellosolve) and is stable at 60°C.

The core of the SPU is the filter unit, in which digestion, filtration, washing, and dye extraction take place. It is a 20-ml polypropylene tube that is closed at the lower end by a polypropylene grid. Attached to this grid is the filter, which is a polyamide wire mesh. The mesh is a 37-μm-diameter woven monofilament polyamide wire, which is connected in a 2:2 relationship, meaning that two wires in warp direction are interwoven with two wires in weft direction. The mesh opening is 7 μm, which is sufficient to retain 15-μm FM. On-line control of the mesh's quality during production ensures that the mesh opening is constant. Polyamide is inert to 4 N KOH, isopropanol, and the fluorescent dyes used, as given in the product information of the manufacturer (SE-

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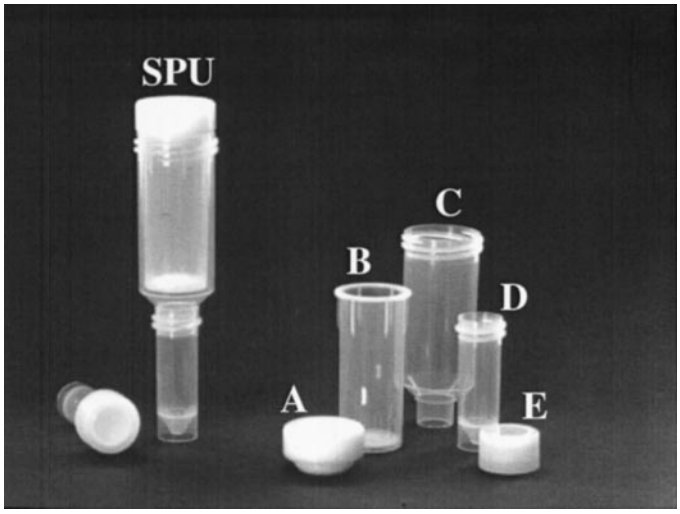

Fig. 1. Sample-processing unit (SPU) and its individual components: SPU consists of filter (*B*), filter holder (*C*), and sample tube (*D*). During digestion of tissue samples, filter is placed into a stainless steel container, which is closed with a Delrin lid (*A*). Sample tube can be closed with a screw-top cap (*E*).

FAR, Rueschlicon, Switzerland). It can be used at 60°C, since its working temperature ranges from −40 to 115°C. Its high tensile strength (41–67 daN/mm^2) allows suction under negative pressure. Its hydrophilic properties (product information, see above) allow easy filtration of aqueous solutions.

The sample tube, a 5-ml polypropylene container, is used to collect the fluorescent dyes that are released from the FM. A polypropylene filter holder can be attached to the upper end of the sample tube. This part of the SPU is used to position the filter over the sample tube when the dyes are dissolved.

For digestion of the tissue samples, the filter unit is placed into a specially designed high-grade steel beaker (Perkin-Elmer, Uberlingen, Germany). During the digestion of the tissue samples, the beaker is closed with a Delrin lid to prevent evaporation. The digestion takes place in a heater rack (Perkin-Elmer), which maintains a constant temperature of 60°C. This aluminum rack is capable of holding 40 beakers.

Required Solutions

The tissue samples are digested using a solution of 4 N aqueous KOH (224.4 g/1,000 ml) and 2% Tween 80 (10 ml/1,000 ml of KOH). Tween is added to the KOH solution to reduce the surface tension. This is necessary to cause the FM to gather in the center of the liquid, thus preventing them from adhering to the walls of the filter unit. The solution is stable for ≤24 h.

To avoid KOH crystallization in the digestion solution during the digestion period, 1.5 ml of 100% isopropanol is added as a layer on top of the aqueous lye in the filter to shield the KOH from the air. The spheres released from the tissue samples are caught between the digestion solution and the isopropanol. Isopropanol also emulsifies fatty tissue in the digestion solution so that fat can be filtered through the hydrophilic filter mesh.

During digestion the dyes are still incorporated in the FM and are, therefore, stable against KOH (2). After they are extracted from the spheres, they may be destroyed by KOH residues. Therefore, the filter unit, the mesh, and the FM are rinsed with a phosphate buffer to remove all KOH residues after digestion. The phosphate buffer consists of 29.9 g of K_2HPO_4 dissolved in 800 ml of distilled water mixed with 5.88 g of KH_2PO_4 dissolved in 200 ml of distilled water. This buffer has the physiological pH of 7.4, which guarantees the stability of the fluorescent dyes.

The dye is extracted from the spheres using Cellosolve (Aldrich, Steinheim, Germany). This solvent is recommended by Molecular Probes (Eugene, OR) for FM.

For the withdrawal of the reference sample, a CPDA-1 stabilizer (26.3 g sodium citrate dihydrate, 3.27 g citric acid monohydrate, 31.9 g glucose monohydrate, 2.51 g sodium dihydrogen phosphate dihydrate, 0.275 g adenine, 1,000 ml distilled water) is used as an anticoagulant. Two milliliters of this solution are added to 10 ml of blood.

Sample-Processing Protocol

Digestion. The digestion procedure is shown in Fig. 2. The SPU is tared and then, after the tissue sample is placed into the filter unit, weighed again to determine the sample weight. The tissue samples that were used in this study weighed 1–4 g. Then the filter unit is placed into the high-grade steel beaker for the digestion of the samples. The filter holder and the sample tube will be needed later for dye extraction.

Digestion solution (15 ml) is added into the filter unit and layered with 1.5 ml of isopropanol.

To avoid the evaporation of isopropanol, the beaker is capped with a Delrin lid. The beaker is then placed into the heater block for 6 h at 60°C to digest the tissue.

Filtration and rinsing. At the end of the digestion period, the FM are confined between the KOH and the isopropanol layer in the center of the liquid (Fig. 3). The filtration and rinsing procedure is shown in Fig. 4. The FM are recovered by filtering the liquid through the wire mesh of the filter. The filter unit is therefore taken out of the beaker and pressed onto a suction mug, which is connected to a vacuum. The digestate is then sucked through the wire mesh in 5–10 s with a maximum negative pressure of 400 hPa. The FM remain on the filter mesh. To remove all residual KOH, the filter unit, the meshwork, and the FM are now rinsed using 20 ml of phosphate buffer while the filter is evacuated. The buffer is sprayed onto the walls of the filter unit in a circular motion to ensure that all FM are rinsed onto the wire mesh. The filter unit is then immersed in a container filled with buffer to clean all KOH residues from the outside. To ensure that no liquid remains in the filter unit, the unit is then placed in a centrifuge tube and centrifuged for 3 min at 4,000

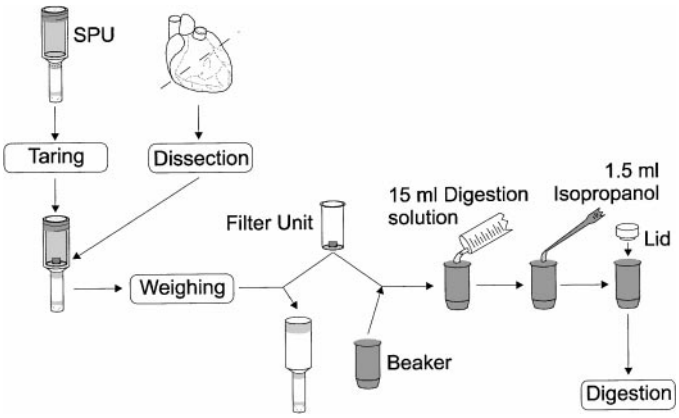

Fig. 2. After SPU is tared and tissue sample is placed into filter unit, sample weight is determined. Filter unit is then placed in high-grade steel beaker, and 15 ml of digestion solution and 1.5 ml of isopropanol are added. Beaker is covered with a Delrin lid and remains in heater rack for 6 h at 60°C.

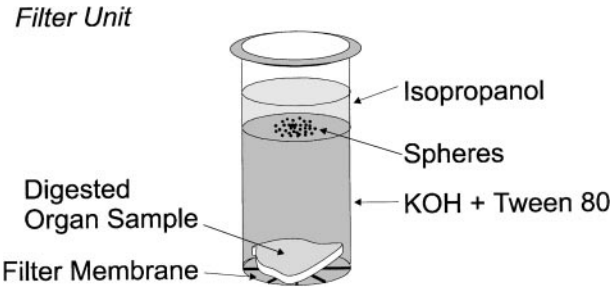

Fig. 3. During digestion, spheres are released from organ sample and float to top of KOH. They are caught in a discontinuous density gradient between KOH and isopropanol layers. Tween prevents adhesion of FM to walls of filter unit and positions them in center of unit.

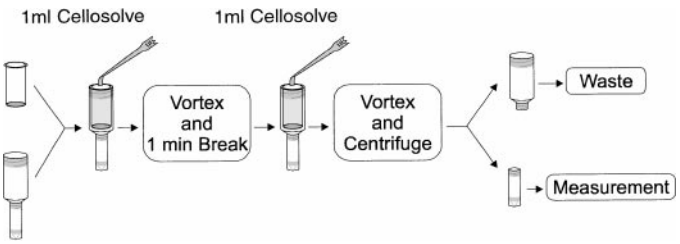

Fig. 5. Filter unit is replaced in filter holder and attached to sample tube. Solvent (1 ml of Cellosolve) is used to dissolve dyes. After soft vortexing for 30 s and a 1-min break, another 1 ml of solvent is added to filter unit to rinse dye through wire mesh. SPU is vortexed again for 30 s and centrifuged (4,000 rpm for 3 min) to ensure that all dye is collected in the sample tube.

rpm. The filter is now dry, all remnants of the digestion solution are removed, and the FM are gathered on the wire mesh.

Dye extraction. The dye is extracted using the organic solvent Cellosolve (Fig. 5). To do so, the filter unit with the recovered FM is replaced into the filter holder, and the sample tube is attached to the base of the filter holder. To release the fluorescent dye from the FM, 1 ml of the solvent is pipetted into the filter unit. To ensure complete dye extraction, the SPU is vortexed softly for 30 s. One milliliter of solvent is added to the filter after a break of 1 min. This break is given to allow enough time for the release of the dyes. Another 1 ml of solvent is added to rinse the extracted dye through the filter's meshwork. The SPU is then vortexed again. To ensure that all the dye is collected in the sample tube, the SPU is centrifuged at 4,000 rpm for 3 min. All the released fluorescent dye is now collected in the sample tube. The filter and filter holder are separated from the sample tube and discarded.

Fluorescence measurement. The fluorescent intensity of the dye is measured using a luminescence spectrometer (model LS50B, Perkin-Elmer) and an autosampler and diluter station using the common autosampler software (models AS90/91 and DS6, respectively, FLWinLab, Perkin-Elmer).

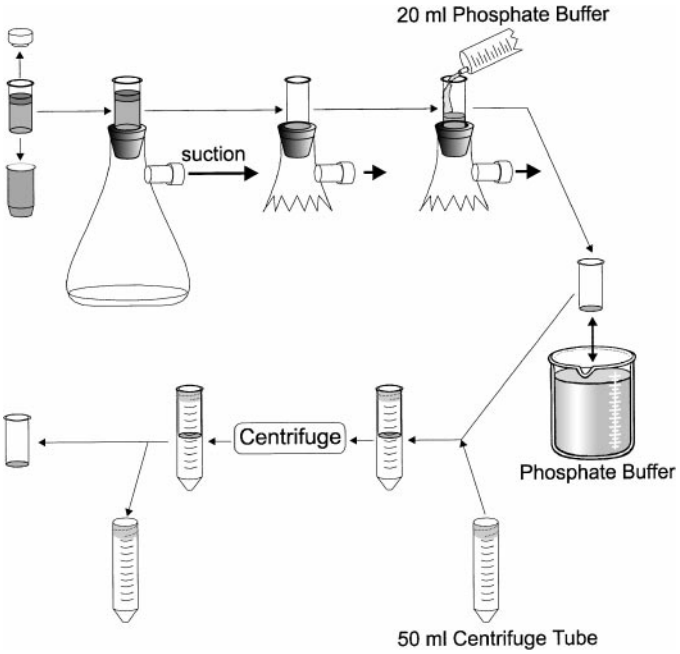

Fig. 4. Filter unit is taken from beaker and brought to filter station. After filtration under low pressure, walls of filter unit are rinsed with 20 ml of phosphate buffer. To wash outside, filter unit is immersed in buffer. Filter unit is then placed in 50-ml centrifuge tube and centrifuged at 4,000 rpm for 3 min to dry filter unit.

Variation of the Sample-Processing Protocol According to Various Organs

The protocol described above is suitable for heart, kidney, liver, spleen, pancreas, intestine, stomach, and muscle tissue. It can be used for Formalin-fixed samples as well as for nonfixed tissues.

If the sample weight is >4 g (maximum 7 g), the digestion liquid should be reduced by 1 ml/g because of the limited volume of the filter unit. In these cases, the normality of the KOH should be increased to 6 N [≤16 N KOH (2) does not harm the FM], and the digestion time should be prolonged to 7 h.

Samples with a high percentage of fat (e.g., brain) require 2.5 ml of isopropanol.

To digest bone tissue, the bone is sawed in half, the marrow is removed, and the sample weight is established. Loss of bone material by sawing does not influence the results, because only the blood flow within the sample and not of the total bone is measured. The marrow can be digested as described above. The crystalline matrix of the bone must be dissolved before the sample can be processed using the SPU. This process takes place in a 50-ml polypropylene test tube, since the polyamide wire mesh of the filter unit is not resistant to acids. Twenty milliliters of 1 N hydrochloric acid are added to the test tube, which is then closed and stored for 5 days. Next the bone is immersed for 5 min in phosphate buffer to neutralize the hydrochloric acid. After this procedure, digestion of the organic matrix takes place in the SPU as described above.

For air-dried lung tissue, the samples are digested in the filter unit for 3 h with the use of 1 N ethanolic KOH, which is not covered with isopropanol. It is particularly important to close the beaker tightly with the Delrin lid to avoid evaporation and crystallization of the KOH.

Processing Reference Blood Samples

The calculation of blood flow with the FM technique requires a reference blood sample. This sample is collected from the aorta by a pump (Harvard Apparatus, S. Natick, MA) with a constant flow while the FM are injected. To prevent clotting of the blood, CPDA-1 anticoagulation stabilizer (see *Required Solutions*) is added to the syringe (2 ml CPDA/10 ml blood). The reference blood sample can be filtered directly through the filter without being digested. To ensure that all the FM contained in the blood sample are recovered, the syringe and the tubing are rinsed with distilled

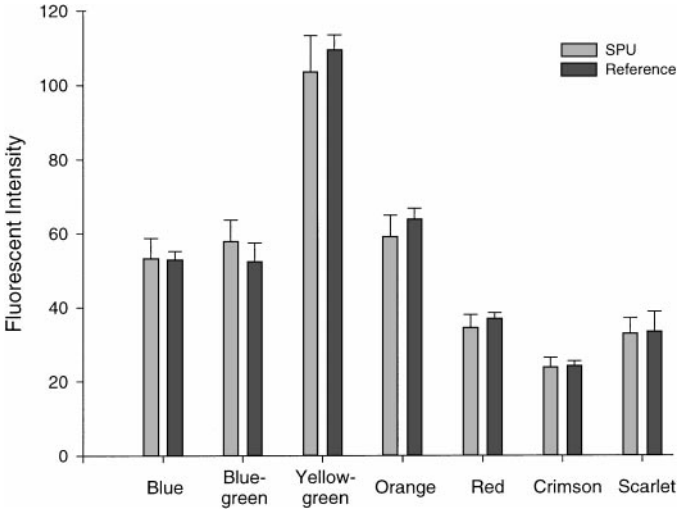

Fig. 6. Fluorescent intensity (means ± SD) of SPU group (n = 100 each), which underwent whole sample processing within SPU, and reference group (n = 20), in which only dye extraction was performed. There is no significant difference among groups ($P < 0.05$ by Student's t-test).

water mixed with 2% Tween 80 (10 ml in 1,000 ml of distilled water), and this solution is again filtered through the same filter unit.

Testing the Reliability of the Filter Unit

Experiment 1. The key to the method is the recovery of all FM. To test whether the polyamide mesh could filter the digested organ and recover 100% of the FM, 2.5×10^3 15-µm-diameter FM (Molecular Probes) of one color were pipetted into 80 filter units. Inasmuch as all the colors (blue, blue-green, yellow-green, orange, red, crimson, and scarlet) behave in the same way, it is not important which color is used. Organ samples were added to the SPU and processed as described above. The filtrate of each filter unit was collected and filtered through PC filters with an 8-µm pore size, according to the method of Glenny et al. (2). The entire filter was investigated systematically using a fluorescence microscope at the different excitation wavelengths of the colors used to determine whether any FM got through the SPU filter unit.

Experiment 2. To test whether FM were adhering to the walls of the filter units, the bottom was removed from the 80 filter units used in *experiment 1*. The tubes of the filter units were cut into six longitudinal strips. These strips were then controlled systematically with a fluorescence microscope at the corresponding FM wavelengths.

Experiment 3. Experiment 3 was designed to clarify whether the fluorescent intensity of the dyes would be influenced in any way during sample processing. Therefore, FM of all colors (2.5×10^3 spheres per color and per filter unit) were pipetted into 100 filter units containing tissue samples (heart). For reference, the same number of spheres was pipetted into 20 glass test tubes and then dried at a constant temperature of 60°C for 48 h. The fluorescent intensities of both groups were measured using the LS50B luminescence spectrometer, and the results were compared using Student's t-test, with $P < 0.05$. In the reference group, there is no loss of FM or fluorescent intensity; therefore, the standard deviation of this group represents the pipetting error.

Experiment 4. To test the reusability of the SPU, 2.5×10^3 FM (orange) were pipetted into the filter units of 25 SPUs. A Formalin-fixed tissue sample (3.4–4.1 g) of porcine livers was added to each filter and processed according to the protocol (digestion in 4 N aqueous KOH for 6 h). The eluate of each filter unit was collected in a glass test tube during the filtration. The liquid was then filtered through a PC membrane with a pore size of 8 µm, according to the method of Glenny et al. (2). The membrane was then controlled for the presence of FM with the help of a fluorescence microscope.

Cellosolve (2 ml) was added to each filter to release the fluorescent dyes, which were then collected in the sample tubes by centrifugation. The intensities of the dyes were measured using the luminescence spectrometer (model LS50B).

The polyamide wire mesh of each filter was investigated for morphological alterations with the help of a microscope (magnification: free changeable zoom from 0.5 to 1.4 × 12.5).

The whole procedure was repeated with each individual filter unit five times.

RESULTS

Experiment 1

Under experimental conditions the filter units recovered 100% of the 15-µm FM, since they were not found on the PC membranes.

Experiment 2

Microscopic examination showed that the polyamide filter had undergone no structural alteration during sample processing. Only a small number of spheres (0–18, 0.0–1.5×10^{-5}%) were found adhering to the walls of the filter units investigated (n = 80). These FM usually adhered at the level of the isopropanol layer. No KOH crystals were encountered. The adherence of the FM to the walls must therefore be attributed to static attraction rather than to the crystallization of KOH.

Experiment 3

Figure 6 shows the results of this experiment: means ± SD for the reference group are 53.3 ± 5.4, 57.9 ± 5.8, 103.5 ± 9.8, 59.0 ± 5.9, 34.4 ± 3.5, 23.8 ± 2.6, and 32.8 ± 4.2 for blue, blue-green, yellow-green, orange, red, crimson, and scarlet, respectively, and for

Table 1. *Reusability of sample-processing unit*

Filter use, n	1	2	3	4	5
Filters failing, n	0	1	1	3	4
Maximum FM in eluate, %	0.0	1.8	2.1	3.5	3.8
Mean intensity, AU	48.3 ± 3.4	47.7 ± 4.1	48.5 ± 2.8	46.4 ± 5.2	46.1 ± 5.4

Repeated use of filter units showed an increasing leakage of filtration meshwork, inasmuch as amount of fluorescent microspheres (FM) found on polycarbonate membranes ranged from 0.0 to 3.8% when filters were used 5 times; 4 filters (16%) failed when used 5 times. Declining mean fluorescent intensities (means ± SE) indicate an increasing loss of FM through filter mesh; AU, arbitrary units.

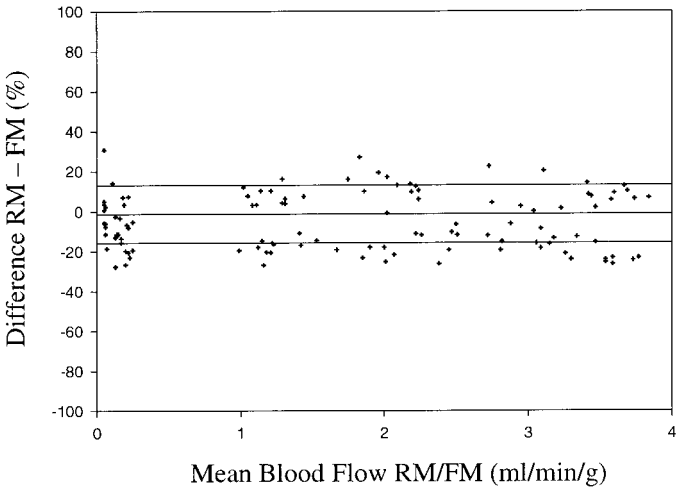

Fig. 7. Comparison of myocardial blood flow values (n = 153) of 1 experiment calculated with data obtained with radioactive (RM) and fluorescent (FM) microspheres under control conditions. After radioactivity of individual tissue samples was determined, samples were processed with SPU. Percent difference between values calculated with FM data and from RM is −1.2 ± 14.3% for this experiment, which indicates that FM and RM measure comparable myocardial blood flow values.

the SPU group, which was processed according to digestion protocol, 53.0 ± 2.2, 50.7 ± 2.8, 109.2 ± 4.2, 63.8 ± 2.9, 36.9 ± 1.7, 24.1 ± 1.8, and 33.8 ± 6.0, respectively. There was no significant difference in fluorescent intensity, indicating that the processing of the samples does not alter the intensity of the dyes. Furthermore, this experiment again indicated that there is no loss of FM during sample processing with the SPU.

Experiment 4

All 25 filter units proved to be tight when used once, inasmuch as no FM were found in the filtrate after filtration through the PC membranes. There was no significant difference in the mean fluorescent intensity between this first group (48.3 ± 3.4) and the control (47.9 ± 3.1). The microscopic investigations of the mesh showed that the polyamide wire was swollen, but there were no signs of lesions. Any further reuses resulted in an increasing loss of FM through the filtration meshwork (Table 1). The meshwork revealed lesions and regions in which it became increasingly translucent.

DISCUSSION

The new SPU and FM recovery protocol presented here represent a significant improvement of the previous methods.

With the help of the SPU[1] we have been able to demonstrate for the first time that the complete recovery of 15-μm FM from tissue and blood samples is possible. Results of one experiment in which RM and FM were injected simultaneously into the left atrium of a pig are presented in Fig. 7. Blood flow values calculated with data obtained with either method show high correlation coefficients (r^2 = 0.89–0.98), low percent difference, and low standard deviation; 99.3% of the values were within the 2-SD range.

For the 15-μm FM there is a very negligible loss of spheres (<1%) due to static adherence to the walls of the filter unit. However, the use of the SPU is more expensive than the commonly used filtration (2) and sedimentation (3) method because of the additional costs of the SPU (Table 2). The filtration and sedimentation methods are more labor and time intensive (Table 3). Most important, the use of the SPU yields more reliable and reproducible results.

The new sample-processing protocol is faster because of a significantly shorter digestion time, and it eliminates the need for aspiration of the digestate and for the transfer between different containers (Table 3). The less time-consuming and highly standardized protocol is particularly advantageous if a large number of samples is to be processed. Because of the modifications of the digestion method presented here, all tissues named in METHODS can be processed with the SPU with a digestion time of 3–6 h, instead of the 24 to 48 h required by the other methods (2, 3). This short period is a result of the use of 4 N KOH. The addition of the isopropanol layer to the top of the digestion solution allows the use of aqueous KOH. In correlation to its normality, 4 N KOH has a density of 1.130 kg/l measured with a densitometer. Inasmuch as the density of the spheres is distinctly lower (1.051–1.056 kg/l measured by a continuous Percoll gradient), the spheres float on top of the digestion solution. If the warm lye (60°C) comes into contact with the air, KOH will crystallize. These crystals might harm the results, because FM may become entrapped in them or the crystals might not be rinsed off the filter units, leading

Table 2. *Cost per sample*

	Cost per Sample
SPU	2.50
KOH/isopropanol	0.10
Buffer	0.30
Organic solvent	0.03
Total cost/sample	2.93

All prices (in US dollars) are based on rate of exchange from German D-Mark of 1:1.65 and might, therefore, vary. SPU, sample-processing unit.

Table 3. *Comparison of methods for FM recovery from tissue samples*

	SPU	Sedimentation	Filtration
Preparation, min	2	2	2
Digestion, h	4–6	48	24
Centrifugation	2×3 min	2×20 min	0
Processing, min	1	1	1

Use of SPU shortens total processing time by a factor of up to 12 because of shorter digestion and centrifugation periods. Data for sedimentation and filtration methods are from Refs. 3 and 2, respectively.

[1] The SPU is available from Perkin-Elmer, Rengoldshauser Str. 11, 88662 Überlingen, Germany.

to the loss of fluorescent intensity when the dyes are released from the FM. Therefore, isopropanol is added to form a layer on top of the KOH to prevent the crystallization of KOH.

The digestion of tissue samples with a very low specific weight, e.g., air-dried lung tissue, can be a problem, since the samples float on top of aqueous KOH. The samples become trapped in the layer of isopropanol on the top of the KOH and are consequently not digested. This problem is solved by using ethanolic KOH. The fact that the density of FM (1.04 kg/l) is distinctly higher than that of 1 N ethanolic KOH (0.88 kg/l) leads to the sedimentation of the FM, so isopropanol is no longer necessary.

Until now, FM were recovered from reference blood samples by digesting the blood in different containers and, finally, pouring the digestate through a filter membrane. This step has the potential for losing FM by spilling drops of the digestate. Because it is now possible to directly filter the reference blood sample, a loss of FM is excluded. The direct filterability of the reference blood sample also saves time, since the sample must not be digested.

The hydrophilic properties of the wire mesh make rinsing and washing the filters with buffer easy, thus solving the problem of a decrease of the fluorescent intensity deriving from nonneutral pH. Even fatty tissues, such as brain, can easily be filtered through the meshwork, because isopropanol dissolves fat, and the physicochemical properties of the meshwork even allow filtration of pure oils.

For extracting the dyes, different solvents can be used. Cellosolve is recommended by Molecular Probes, especially when the color scarlet is used. However, Cellosolve has the disadvantage that it has a low boiling point and, therefore, evaporates rapidly, which can result in an increase in dye concentration. Thus the dyes should be measured as soon as possible after the dissolution of the FM. If the color scarlet is not required, the solvent 2-(2-ethoxy)ethoxyethyl acetate should be used, since it has a higher boiling point and is less hazardous. The excitation and emission wavelengths must be adapted to the solvent used.

A repeated use of the filter units is not recommended, since polyamide is not completely stable in the presence of organic solvents. As microscopic investigations of the filter show, the repeated use of the filters (up to 5 times) causes alterations of the polyamide meshwork. These changes in the filter lead to a loss of FM. For a single use this does not present a problem, since the FM have already been recovered and rinsed before the organic solvent is added to the filter. However, if the same filter is used for a second time or even more times, the altered polyamide wire mesh has mesh openings >7 μm, leading to the loss of FM.

Conclusion

The newly designed SPU results in a significant improvement of the FM method for the measurement of regional organ blood flow. According to the modified filtration protocol, the whole process of FM recovery is performed within a single-tube system, rendering the method faster and more standardized. The complete recovery of 15-μm FM can be guaranteed using the SPU with the novel protocol. With the SPU it is also possible to directly filter nondigested reference blood samples.

Inasmuch as the SPU was designed for an automated use, the FM method, which is still more time and labor intensive than the RM method, can now be completely automated. Thus the FM method has now become a real alternative to the use of RM.

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