Am J Physiol Heart Circ Physiol 312: H705–H710, 2017;
First published January 27, 2017; doi:10.1152/ajpheart.00834.2016.

HISTORICAL ARTICLE

The history of the microsphere method for measuring blood flows with special reference to myocardial blood flow: a personal memoir

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Submitted 19 December 2016; accepted in final form 19 January 2017

Hoffman JIE. The history of the microsphere method for measuring blood flows with special reference to myocardial blood flow: a personal memoir. *Am J Physiol Heart Circ Physiol* 312: H705–H710, 2017. First published January 27, 2017; doi:10.1152/ajpheart.00834.2016.—We use many types of equipment and technologies to make our measurements but give little thought to how they developed. Evolution was once described as a series of recoils from blind alleys, and this is exemplified by the gradual development of the microsphere method of measuring blood flows. The microsphere method is one of the most frequently used methods for measuring blood flow to organs and portions of organs. The method can measure myocardial blood flow with reasonable accuracy (within 10%) down to samples weighing >50 mg but probably will not do so for samples weighing 1–10 mg. Microspheres with diameters from 10 to 15 μm provide the best compromise between accurate flow measurement and retention in tissue. Radioactive labels have been almst entirely replaced by fluorescent labels, but colored microspheres and neutron-activated labels are also used.

NEW & NOTEWORTHY The contributions of the various individuals who developed the microsphere method of measuring regional blood flows and how these advances took place are brought to light in this paper.

radioactive microspheres; fluorescent microspheres; neutron activation; colored microspheres

SMALL PARTICLES were used first to study the complex fetal blood pathways when Pohlman (51) injected corn starch granules into the umbilical veins of fetal pigs. Subsequently, Prinzmetal and colleagues (55) injected glass microspheres with diameters of 10–180 μm into the left coronary artery, observed that microspheres from 10- to 80-μm diameter appeared in the right coronary artery and coronary sinus, and concluded that there were interarterial and arteriovenous anastomoses. They applied this method to detecting anastomoses in the liver, spleen, and lungs (54). Measuring was laborious, performed by counting microspheres under a microscope.

Measurement was made easier by radioactive labeling of the microspheres. Initially, glass microspheres were used in which ^{23}Na was transformed to ^{24}Na by neutron bombardment for a week (15, 24, 40), and these were replaced for convenience by radioactive-labeled ceramic microspheres (27, 31, 69). The investigators using glass and ceramic microspheres (20–53 μm in diameter) were concerned about the high specific gravity of the spheres because of difficulty in maintaining the microspheres in suspension and questions about their distribution to regions within organs.

The next development was the introduction of radioactive-labeled macroaggregated albumin microspheres 10–50 μm in diameter that could be used in humans to measure relative blood flow to the lungs and systemic organs (57, 67, 68). The disadvantage of these for physiological studies is that the microspheres break down, move, and metabolize. Nevertheless, these studies formed the basis for several diagnostic methods used today in nuclear medicine.

All of the above methods that helped to advance the use of microspheres could at best determine the proportions of flows to regions, and absolute flows were left to be measured by the simultaneous but inconvenient use of another method; for example, Delaney and Grim (15) used a ^{42}K clearance technique together with collection of venous drainage.

More Recent Developments

Early microsphere methods did not usually measure absolute flows, and no attempt was made to measure flows to small regions within an organ. The big change came in a dental office! Dr. Abraham Rudolph had been interested in measuring fetal blood flows. At that time, these were measured by exteriorizing the animal fetus and implanting catheters for indicator dilution measurements or placing flowmeters around various fetal vessels. It was difficult to make complete measurements of flows to all the organs, and the procedures were usually done on deteriorating preparations. While sitting in the dentist's anteroom, Dr. Rudolph was reading a magazine when he saw an item inserted by Minnesota Mining and Manufacturing (3M Company). They announced that they could supply radioactive plastic microspheres with a variety of labels and sizes. These had originally been produced to inject into the arterial supply to tumors to obtain a high local radiation dose. Unfortunately, many tumors have large arteriovenous anastomoses so that many microspheres would have passed through the tumor and embolized around the body, and the technique was abandoned for human use.

Dr. Rudolph realized that these microspheres might be useful in measuring fetal blood flow. It is possible to separate and quantitate mixtures of microspheres by their spectral gamma emissions in a well scintillation counter; he used a 3-in. thallium-activated sodium iodide crystal with a well of 1.5 in. in diameter. Originally, four isotopic labels were used (^{125}I, ^{51}Cr, ^{85}Sr, and ^{46}Sc (56). Although each radioisotope has a discrete emission energy, scatter and lack of resolution in the scintillation counter produce a broad Gaussian distribution of energy emissions; for example, the full-width half-maximum is

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9.15 keV at 122 keV and 82.75 keV at 662 keV. As a result, the various emission spectra overlap. To estimate the count rates of each isotope, a stripping method was used. A region at the high end of the spectrum with no contribution from lower energy isotopes was used to calculate the total count rate for the isotope with the highest energy. These counts were subtracted from the counts in the adjacent lower energy band to derive the count rate in the next lower energy isotope and so on. There are some inaccuracies in this method because small errors accumulate by the time the lowest energy isotope is reached and because there are small amounts of counts emitted at energies well above their peaks for most isotopes. [An alternative and more accurate method that could be used now with better computer power is multiple regression with matrix inversion (13, 60).]

The "carbonized" microspheres are made of carbon and oxygen, have a specific gravity of 1.3, and so might be expected to behave more like red blood cells. The microspheres are suspended in saline with a detergent (0.01% Tween 80) Some invesigators have used dextran, but this cannot be used in rats, in whom it is an anaphylactic agent. The radioactive label is present throughout the microsphere rather than being on the surface and did not leach out in acute studies, although some leaching has been observed in chronic studies (66). Therefore, several different isotopes can be injected at different physiological states in an intact animal over several hours or days (using implanted vinyl catheters), and at the end of the study, the animal is euthanized and the organs removed for measurement of radioactivity.

Although there is no need to extract the microspheres, the geometry of standard scintillation counters requires the heights of tissue samples to be be the same in each vial, or else a correction factor must be used (44, 58).

In the first publication describing this new method, Rudolph and Heymann (58) used microspheres with an average diameter of 50 μm (SD: 5 μm); absolute flows were measured in the umbilical vein by the antipyrine method, and the flows to the various organs were then calculated from these results. A clever method of deriving absolute flows without needing to use another method was described by Makowski et al. (41). They withdrew blood from an artery into a syringe at a constant known rate. This syringe, the surrogate organ, has a known flow rate, and if the microspheres are distributed in proportion to flow, it is easy to calculate flows as follows:

$$\text{flow}_{\text{organ}} = \text{flow}_{\text{syringe}} \times \frac{\text{counts}_{\text{organ}}}{\text{counts}_{\text{syringe}}}$$

At this time I was an onlooker, with no reason to use microspheres. In 1967, Dr. Raul Domenech, a pathophysiologist and cardiologist from Chile, came to work in my laboratory. He had done experiments with coronary blood flow and knew the difficulties in making accurate measurements. I knew nothing about coronary blood flow and had little interest in it except for curiosity about the subendocardial ischemia often found in severe aortic stenosis. Dr. Domenech went to one of the weekly meetings of the Rudolph group in which they presented some of the early results of this new radioactive microsphere method. He realized that this method could be used to measure not only total coronary blood flow but perhaps even regional flows within the myocardium. Regional flows had previously been measured with diffusible indicators such as rubidium, but the methods were laborious, open to criticism, and could usually be used only once per animal.

In our first experiments on dogs (18), we used 50-μm-diameter microspheres and validated the measurement of coronary blood flow by performing a right heart bypass and collecting the coronary venous drainage. When we calculated flow:g in the inner and outer thirds of the left ventricular free wall, the ratio was ~2.5:1. Soon after obtaining this result, I discussed it with a mathematician, and 3 wk later, I received a manuscript explaining the forces that determined this ratio. By then, however, we had read the relevant literature, which uniformly described an ~1:1 ratio, and realized that despite the mathematical "proof" we had made an error. The probable reason for the overestimation of subendocardial blood flow was found in studies by Phibbs et al. (49, 50), who found that 50-μm-diameter microspheres, unlike 10-μm-diameter microspheres, tended to migrate axially in the artery, so that perhaps they were less likely to make sharp turns into small branch arteries. This finding was demonstrated for the coronary circulation by Sinclair et al. (60). We tested the distribution of microspheres with diameters of 20–23 and 14 μm, and the respective inner:outer flow ratios were 1.4 and 1.3, much closer to the results obtained with diffusible indicators. These findings raised the question about whether even smaller microspheres would be better, but then we ran into the problem that as the microspheres became smaller, a greater proportion of them escaped from the vascular bed (1). For example, up to 26% of microspheres with diameters of 4–12 μm (mainly those <7 μm in diameter) passed through the renal vascular bed in dogs and 3–8% of 9-μm-diameter microspheres escape from the myocardium over 2 h (12). Further studies using microspheres of different diameters confirmed that 10- to 15-μm-diameter microspheres offered the best compromise between correct regional distribution and loss of microspheres through the capillary bed. Not only do these microspheres lodge in small arterioles, but almost all those >12 μm in diameter also remain in the tissue, although some may be found outside the blood vessels (11).

Several studies comparing microspheres and diffusible indicators have shown agreement to within 10% (5, 6, 62, 68), and this was confirmed by the most reliable diffusible indicator, 2-iododesmethyl imipramine (5, 6). [This marker appears to be reliable in rabbits and sheep but not in dogs (6).]

Because it was becoming increasingly expensive to use dogs, we considered using a sheep model. To be sure that the method worked in sheep, we performed a series of right heart bypasses, collected the coronary venous drainage, and compared the microsphere and drainage data. We were upset to find little correlation between them. On investigation, we realized that we had used the same number of microspheres in dogs and sheep, despite the greater size of the sheep. Further analysis based on Poisson statistics showed that to obtain a calculated flow within 10% of "true" flow, there had to be ~400 microspheres/unit of tissue, no matter what the size of the tissue (9). Knowing this and the approximate percentage of cardiac output perfusing the unit, the total number of microspheres needed can be calculated. Subsequently, Nose et al. (48) confirmed this approach, but they and Polissar et al. (52) concluded that under some circumstances, 200 or fewer microspheres per piece might suffice. Richard Austin (deceased) made a more accurate

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estimate of errors; he conceived and carried out the studies as a medical student (3).

When comparing the results from two simultaneously injected sets of microspheres, the resulting correlation coefficient is less than it should be because of Poisson noise. This source of error can be reduced using the following formula:

$$\widehat{r_{\text{true}}} = \frac{r_{\text{obs}} \times s_x \times s_y}{\sqrt{\left[s_x^2 - \left(\frac{N-1}{N}\right)\bar{x}\right] \times \left[s_y^2 - \left(\frac{N-1}{N}\right)\bar{y}\right]}}$$

where the true correlation coefficient ($\widehat{r_{\text{true}}}$) is calculated from the observed correlation coefficient (r_{obs}), the SDs of x (s_x) and y (s_y), the number of pieces of tissue (N), and the means of x and y ($\bar{x}$ and $\bar{y}$) (50).

With a better understanding of the method and its errors, we returned to my original interest in explaining why patients with aortic stenosis often had patchy subendocardial fibrosis. After quantifying this finding in patients with severe aortic stenosis (10), we did experiments based on the findings by Yipintsoi et al. (70) that flows were heterogeneous within any layer of the left ventricular free wall. Utley et al. (64) confirmed these results, and these studies were extended by Austin et al. (2), who found not only heterogeneity of flow in adjacent small (4- to 5-mm cubes, equivalent to ~100 mg) of myocardium but that the heterogeneity of flow during maximal vasodilatation did not match the resting flow patterns. This led Coggins et al. (11) to investigate the coronary flow reserve (ratio of maximal to resting flow per gram) throughout the left ventricle. They found that as perfusion pressure was lowered, coronary flow reserve decreased more in the subendocardium than in the subepicardium, but that even in the subendocardium, some regions lost reserve before others did. This suggests that during conditions that decrease myocardial blood flow, the subendocardium is affected predominantly, but that some regions within the subendocardium become ischemic before others and in persistent or recurrent ischemia become necrotic and fibrotic in a patchy pattern. These findings complemented the studies by Buckberg et al. (8) in which subendocardial ischemia could be predicted from the aortic and left ventricular pressure curves. That study was recently expanded to become applicable to humans (30).

The size of the basic myocardial unit is unknown. The best studies of this subject were those of Bassingthwaighte et al. (33), who used microspheres of different diameters and a molecular flow marker (38, 39) and concluded that flows had a fractal dimension and that the smallest unit might be ~0.5 mm in diameter. The flow values were not random; high-flow regions tended to be associated with other high-flow regions and low-flow regions were associated with other low-flow regions. Microspheres, however, are unreliable for measuring flows in very small pieces of myocardium. There is a tendency for microspheres to exaggerate flows to regions with higher flows, especially in the subendocardium (5, 6, 64) due to particle skimming (62). This mismatch is greater in very small regions because occasionally several microspheres may be found stacked up in the same arteriole (14).

In rabbits, Matsumoto et al. (42) showed that flow variability was manifested by pieces as small as 0.1 mm^2. Stapleton et al. (63) used autoradiography with iododesmethylimipramine in the hamster heart. They observed increasing heterogeneity down to 16 × 16 × 20 μm^3 voxels. This implies that the degree of heterogeneity of flow and flow reserve depends on the sizes of the tissue pieces being examined. The exact sizes of these units and the reasons for the marked heterogeneity of flow have yet to be determined. However, a recent study by Yipintsoi et al. (71) concluded that vascular anatomy determines flow distributions during adenosine vasodilation but that metabolic vasoregulation is the main determinant of heterogeneity in normal physiological states.

It is unlikely that microspheres will solve this problem because although the requirement for 400 microspheres/tissue unit is unlikely to cause physiological changes in 100 mg of tissue (~4.6 mm^3), it will probably cause changes in 0.5 mm^3 of tissue. Numerous studies of different types of microspheres have shown that with the numbers injected no gross functional changes have been observed in cardiac function. This statement, of course, depends on the sophistication of the methods used to test function.

What is the upper limit to the number of microspheres that can be injected without causing physiological disturbance? Hori et al. (30a) injected boluses of 2.5 × 10^6 microspheres of 15 μm in diameter into the canine left anterior descending coronary artery. After the first injection, coronary blood flow decreased for ~30 s and then rose to above its baseline value; there was a slight decrease in fractional shortening. By the eighth injection, flow decreased markedly and remained low, and systolic lengthening occurred. Because the weights of the embolized regions averaged ~32 g, the average microsphere deposition was ~780,000 microspheres/g per injection. [Previously, Monroe et al. (45) had embolized isolated canine hearts with 9.5-μm-diameter polystyrene microspheres with similar findings and also noted ventricular dysfunction, but the numbers of microspheres were not recorded.] In contrast, in our dog experiments, we injected usually ~500,000–1,000,000 microspheres into the left atrium, of which perhaps 25,000–50,000 entered the main coronary arteries for an average deposition of 400-1,100 microspheres/g in the left ventricular free wall. The maximal number of microspheres that can be given without perturbing "normal" function has not been determined.

Glenny et al. (22) showed that in an isolated rat lung, vascular resistance increased <0.8% for every 10,000 microspheres injected and that serial injections demonstrated the same flow distribution. Flaim et al. (20) found no changes in flows, heart rate, or ventricular and aortic pressures with serial injections of microspheres in rats. Kobayashi et al. (35) observed no changes in blood pressure, heart rate, or cardiac output in anesthetized rats after they injected 4 sets of 500,000 microspheres of 15 μm in diameter. The rats averaged 316 g in weight, and their left venticles (weighing ~1 g) would have received ~6,000 microspheres/g. On the other hand, Maxwell et al. (43) observed in anesthetized rats that after the third injection of 100,000 microspheres, coronary flow decreased and coronary vascular resistane increased. These studies did not disclose whether the change were due to the microspheres or diluent.

Flow heterogeneity can be due not only to spatial variability and stochastic variability but also to temporal variability (19, 33). King and Bassingthwaighte (33) and Deussen (16) estimated that spatial variability was the predominant cause of

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Table 1. *Comparison of different types of microspheres*

Type of Label	Advantages	Disadvantages
Radioactive isotope	Measurement made with readily available counters No need to separate microspheres from tissue	Require precautions for safe handling Need to correct for geometry Short half-life isotopes preclude long-term (months) studies Long half-life isotopes cause disposal problems
Colored	Safe to handle No problem with short shelf life	Must dissolve tissue, recover microspheres, and elute dye Measured by spectrometry or fluorimetry
Fluorescent	Safe to handle No problem with short shelf life	Must dissolve tissue, recover microspheres, and elute dye Needs careful control of preparation
Neutron activated	No need to separate microspheres from tissue No problems of short shelf life or disposal No radiation exposure to experimenters	Need to be sent to core laboratory for irradiation and counting

The two radioactive types are the easiest to use and involve minimal manipulation of the tissues. The two dyes require dissolving the tissue, cleaning up the microspheres, and then eluting the dyes for measurement, which must be done with great care. Partial digestion of the tissue and measurement with a cell sorter can be done but is seldom used. All types of microsphere measurements can be automated for batches.

variability and also pointed out that the heterogeneity of flow was fractal and nonrandom.

Later Developments

To use more sets of microspheres per animal, we showed that it was possible to use sets of eight or nine microspheres by adding ^{153}Gd, ^{57}Co, ^{113}Sn, and ^{85}Nb to the other four or replacing ^{125}I and ^{46}Sc with ^{114}In, ^{54}Mn, and ^{65}Zn (4). This allowed for more complex experimental designs and was cost effective. The results were reasonably accurate, but use of the stripping method (58) to separate the contributions of each label added to the error of the method. To try to minimize the counting error, we turned to a high-purity germanium well detector that produced much narrower spectral peaks (32). For example, at 122 keV, the full-width half-maximum was 560 eV for the germanium counter compared with 9.15 keV for the sodium iodide counter. The one disadvantage to this detector is that it has to be kept cooled in liquid nitrogen. Unfortunately, we did not get to use this very sensitive detector. The germanium well was shipped from Europe and arrived without its liquid nitrogen coolant so that it would not function. By the time the new germanium well arrived, the days of radioactive microspheres were fading fast.

Although the radioactive microsphere method, once mastered, is easy to use, the unnecessary use of radioactivity with its associated hazards is frowned upon. Furthermore, some radioactive microspheres have short shelf lives. In addition, disposal of radioactive waste is a problem, especially for the isotopes with long half-lives. We tried to replace radioactive microspheres with nonradioactive microspheres using fluorescent excitation (47) and synchrotron radiation (46), but these methods were not suitable for general use. An alternative was the use of colored microspheres pioneered by Hale et al. (26) and made more practical by Kowallik et al. (36), Hakkinen et al. (25), Hodeige et al. (29), and De Angelis et al. (13); the tissues are dissolved and the dyes are eluted from the freed microspheres and their concentration is then measured by absorption spectrometry or fluorimetry.

For ease of use, currently the best method is that in which the radioactive label has been replaced by fluorescent dyes (21). As many as 13 different sets of fluorescent microspheres can be used (59). This method has been applied to rats (17) and with modifications to mice (37).

Because the dyes do not leach out of the microspheres, they can be used in chronic studies (36). The number of microspheres can be determined by fluorimetry after extraction of the dyes or by flow cytometry. Just as for the radioactive microsphere method, meticulous attention to detail is required to obtain accurate results. For those wishing to use these methods, the publications by Heymann et al. (28), Prinzen and Bassingthwaighte (53), and Glenny et al. (21) are invaluable. Details of how to use the fluorescent microspheres can be found at http://fmrc.pulmcc.washington.edu/documents.shtml.

Detailed information about spatial microsphere distribution has been obtained by microscopy with epifluorescence and examination of sections of myocardium (14) or by a cryomicrotome (7, 61, 65). These studies have confrmed that, in general, microspheres are distributed in proportion to flow unless regions become very small. Fluorescent microspheres have been used together with a fluorescent polymer cast and an imaging cryomicrotome to define the anatomy of the vessels (23, 65). The microspheres were used to standardize measurements rather than to measure flow and, unlike an earlier study (14), also showed that the microspheres did not form clusters in any single vessel and were distributed randomly.

Because of the advantages of using radioactivity, neutron activation has been resuscitated (34, 56). Reinhart et al. (54) simultaneously used eight stable isotopes made radioactive by neutron activation; all had half-lives of 1.7–4 days except for europium, with a half-life of 8.59 yr. (More isotopes are available.) These microspheres are not radioactive during the experiment but are sent to a core laboratory where they are made radioactive by neutron bombardment.

A comparison of the advanages and disadvantages of different types of micropsheres is shown in Table 1.

ACKNOWLEDGMENTS

I am grateful to Dr. Rudolph and Dr. Heymann, who introduced the method and helped us in our investigations; to the pre- and postdoctoral Fellows, who contributed ideas and skills to the experiments; to Bruce Payne, whose technical ability helped us to overcome numerous problems; and to Dr James Bassingthwaighte, who not only drew my attention to some important publications but has provided advice for many years.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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AUTHOR CONTRIBUTIONS

J.I.E.H. conceived and designed research; J.I.E.H. performed experiments; J.I.E.H. analyzed data; J.I.E.H. interpreted results of experiments; J.I.E.H. drafted manuscript; J.I.E.H. edited and revised manuscript; J.I.E.H. approved final version of manuscript.

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