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Use of fluorescent micropsheres to measure coronary flow reserve in rat animal model

Riddhi Harsh Shah

New Jersey Institute of Technology

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ABSTRACT

USE OF FLUORESCENT MICROSPHERES TO MEASURE CORONARY FLOW RESERVE IN RAT ANIMAL MODEL

by

Riddhi Harsh Shah

Heart attacks result from reduced or blocked blood flow through major coronary arteries, resulting in permanent damage to heart muscle. Coronary blood flow (CBF) is thus important to measure in experimental animal models of heart disease. A standard method to measure CBF uses tracer microspheres (Ø = 15 μm) injected into the left ventricle that flow through coronary arteries but cannot pass through capillaries and so become trapped in heart muscle. Previously, radioactive or colored microspheres have quantified the number of tracers trapped in the muscle. Fluorescent microspheres offer a more recent and more sensitive measurement mode. However, fluorescent microspheres have not often been used to measure CBF in small animals (rats, mice) that are now the most common animal models used in heart research. This thesis aimed to develop the techniques for use of fluorescent microspheres to measure CBF in rat animal models used by the cell biology laboratories at UMDNJ-Newark. Two non-overlapping fluorescent wavelengths were chosen (yellow-green; red). Using a spectrophotometer, fluorescence intensity was calibrated for known numbers of microspheres (set via controlled dilution). CBF in two rats was measured at rest and during maximal vasodilation (adenosine) using procedures for colored microspheres. After euthanasia, hearts were removed, and blood samples and left ventricular tissue were processed using a sedimentation method for full recovery of fluorescent microspheres, which were scanned through the spectrophotometer

to count fluorescence intensity. Using the predetermined calibration curve, the number of microspheres in each sample was determined; from this CBF was calculated. CBF averaged 5.9 ml/min/g at rest, which was within the normal range for rats quoted in recent literature. With maximal vasodilation, CBF increased to an average of 12.9 ml/min/g, which indicated a coronary flow reserve that was 2.2 times the resting level. The same value for coronary flow velocity reserve (2.2) was measured in 6 rats using Doppler echocardiography. The consistency of these results suggests that the procedures developed for fluorescent microspheres lead to repeatable and reliable measurement of coronary blood flow in rats.

USE OF FLUORESCENT MICROPSHERES TO MEASURE CORONARY FLOW RESERVE IN RAT ANIMAL MODEL

by
Riddhi Harsh Shah

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APPROVAL PAGE

USE OF FLUORESCENT MICROSPHERES TO MEASURE CORONARY FLOW RESERVE IN MICE MODEL

Riddhi Harsh Shah

Dr. William Hunter, Thesis Advisor — Date
Professor of Biomedical Engineering, NJIT

Dr. Van Buskirk, Thesis Advisor — Date
Distinguished Professor and Chair of Biomedical Engineering, NJIT

Dr. Raquel Perez Castillejos, Committee member — Date
Assistant Professor, NJIT

BIOGRAPHICAL SKETCH

Author: Riddhi Harsh Shah

Degree: Master of Science

Date: August 2011

Undergraduate and Graduate Education:

- Master of Science in Biomedical Engineering,
  New Jersey Institute of Technology, Newark, NJ, 2011

- Bachelor of Science in Biomedical Engineering,
  Mumbai University, Mumbai, India, 2009

Major: Biomedical Engineering

Om NamahShivay!!!

I would like to dedicate this thesis to my parents and to all my friends. There is no doubt in my mind that without their continuous support, blessings and encouragement I could not have achieved this.

My strong belief and conviction in Almighty GOD and HIS grace has made my dreams come true.

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CHAPTER 1

INTRODUCTION

1.1 Heart Attack and Coronary Blood Flow

Heart disease and related conditions affect 12 million Americans and cost $274 billion a year [5]. It is the leading cause of death in United States [5]. Unfortunately, many people do not realize any potential problems with their heart until they have a heart attack. The treatments are generally expensive. The patient has to undergo coronary bypass or coronary angioplasty surgery. Today's lifestyle, eating habits and drinking habits are the major cause of heart attacks and stroke [5]. A heart attack occurs when blood vessels that supply blood to the heart are blocked, preventing enough oxygen from getting to the heart, resulting in death or permanent damage of heart muscle.

In atherosclerosis, plaque builds up in the walls of coronary arteries. This plaque is made up of cholesterol and other cells. A heart attack can occur as a result of the following:

1) The slow buildup of plaque may almost block one of the coronary arteries. A heart attack may occur if not enough oxygen-containing blood can flow through this blockage. This is more likely to happen during exercise.

2) The plaque itself develops cracks (fissures) or tears. Blood platelets stick to these tears and form a blood clot (thrombus). A heart attack can occur if this blood clot completely blocks the passage of oxygen-rich blood to the heart. This is the most common cause of heart attack.

3) Sudden, significant emotional or physical stress, including an illness, can trigger a heart attack, too.

Slow build up of plaque and blockage of coronary arteries is the cause of heart attacks. Researchers say that there are two types of plaque: soft plaque, also known as

vulnerable plaque, and hard plaque, also known as healed plaque. Soft plaque poses more risk to cause a heart attack. Soft plaque produces a small bump beneath the inner lining of an artery, the endothelium. If the plaque cracks open or is torn up, it punctures a hole in endothelium and thus exposes the contents of plaque to the cells in the blood stream. A blood clot then develops at the site of this "injury" as part of what in other circumstances would be a healing process. This clot (along with a possible spasm of the affected artery) is what most commonly causes obstruction of blood flow, resulting in a heart attack. The amount of damage to the heart muscle depends on the size of the area supplied by the blocked artery and the time between injury and treatment [18].

The coronary arteries carry blood to the heart muscle. Because the heart muscle is continuously working (as opposed to other muscles of the body, which are often at rest), it has a very high requirement for oxygenated blood. The coronary arteries are vitally important for supplying that blood, and allowing the heart to work normally. Because a blockage in any of the coronary arteries produces a heart attack or myocardial infarction, special care must be taken to monitor coronary arteries and measure coronary blood flow.

1.2 Significance of Coronary Blood Flow

Coronary arteries are the major arteries that carry oxygenated blood to the heart muscle. Two major coronary arteries arise from the aorta- the right coronary artery (RCA) and the left main coronary artery (LM). The left main artery quickly branches into two large arteries – the left anterior descending artery (LAD) and the circumflex artery. All of these coronary arteries lie on the surface of the heart and distribute oxygenated blood to different regions of the heart muscle. In case of normal, non-diseased vessels, they have

low vascular resistance relative to their more distal and smaller branches that comprise the microvascular network [17].

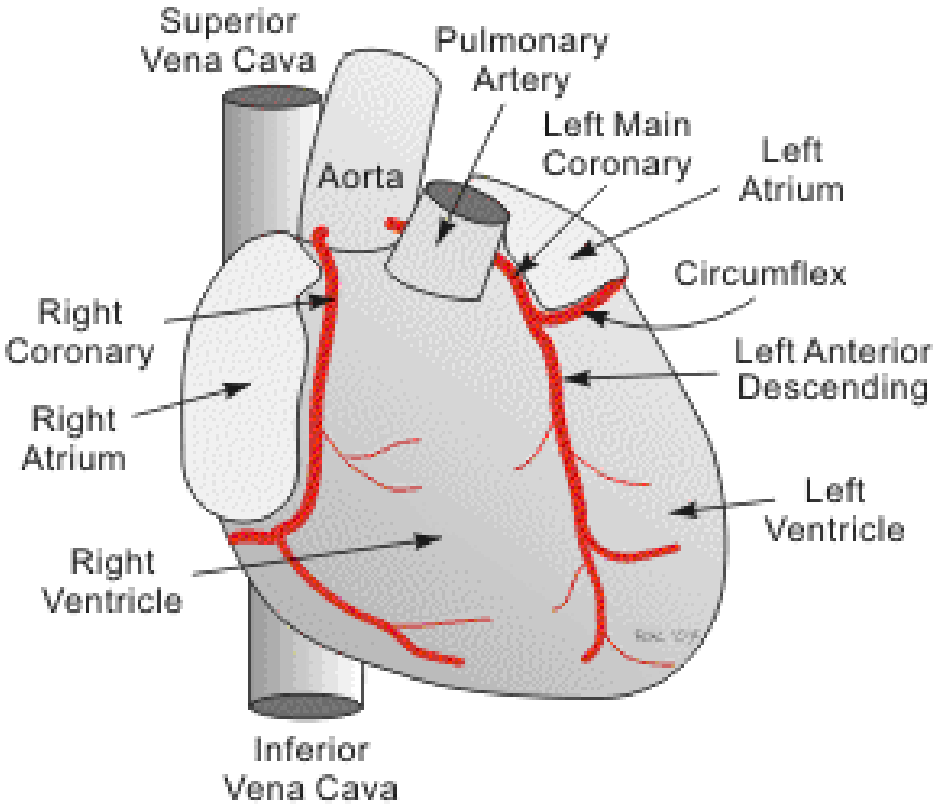

Figure 1.1 Coronary circulation in the heart [7]
(Source: http://www.cvphysiology.com/Blood%20Flow/BF001.htm)

The important features of coronary blood flow:

1) Flow is tightly coupled to oxygen demand. In non-diseased coronary vessels, whenever cardiac activity and oxygen consumption increases, there is an increase in coronary blood flow that is nearly proportional to the increase in oxygen consumption.

2) Good autoregulation between 60 and 200 mmHg perfusion pressure helps to maintain normal coronary blood flow whenever coronary perfusion pressure changes due to changes in aortic pressure.

3) Adenosine serves as a metabolic coupler between oxygen consumption and coronary blood flow.

4) In the presence of coronary artery disease, coronary blood flow may be reduced. This will increase oxygen extraction from the coronary blood and decrease the venous oxygen content [7].

Since oxygenated blood supplied by coronary arteries is an essential factor for heart muscle to function normally, it is of prime importance to check for the ability of coronary arteries to deliver the required blood flow.

1.3 Use of Laboratory Animals in Biomedical Research

An animal model represents some, most, or all aspects of a normal or abnormal condition in another animal or human being. Abnormal model conditions may be inherited, spontaneous, or experimentally-induced. Because of a large number of people suffering from heart disease, cerebral strokes, and many other such diseases, tests are being performed on animals. Also, death rates are declining because of advances in diagnosis, treatment and prevention made through animal research. Between 17 million and 22 million animals have been estimated annually to be used in biomedical and behavioral research, education and testing. About 85% of these are rats and mice, and less than 2% are cats, dogs and non-human primates. In the past, most cardiovascular research was

performed using canine animal models. For example, the use of dogs as animal models made open-heart surgery through the use of a heart lung machine possible today in human beings. Surgery to replace heart valves and large arteries has also been made possible only after experimenting on dogs. Although the use of dogs has proved quite effective in cardiovascular research, in the last two decades, the use of mice and rats has increased to a greater extent, so that now they are the most common animal models [30].

The commercial availability of rats and mice, plus their small size, high reproductive rate, and minimal costs of purchase and maintenance, have made them the most studied and perhaps best understood laboratory animal species. In addition, they are understood and characterized anatomically, physiologically and genetically. Several stocks of rats and mice have withstood the process of inbreeding, allowing the commercial production of a large variety of inbred strains and providing the researcher with thousands of genetically similar individuals. A large number of mutant strains and stocks, with naturally occurring anatomical, physiological, or biochemical diseases, have been developed as animal models for similar conditions in humans and other animals. They are amenable to germ-free and pathogen-free production techniques, thereby greatly reducing attendant, unwanted disease as a variable. Large databases on rodents are available as a result of years of selective breeding designed to meet specific research requirements for models of human disease [11].

Mice are used for a broad range of research. Their relatively short life span makes them useful in aging research. Mice are the primary mammal used in genetics research because of their high reproductive potential and short generation time. They are also used

widely in drug testing and cardiovascular research because they respond favorably and are economical to use in large numbers [12].

Transgenic studies in mice have introduced new and valuable strains and mutations to biomedical research. Transgenics are produced when foreign DNA is integrated into animal cells by experimental means. Such foreign DNA can mimic the changes in DNA that cause inherited disease in humans. In addition, changes in DNA produce altered proteins whose function differs from normal, which offers basic scientists a method to study the function of these proteins. Mice are ideal for transgenic use due to their pronuclei, which are suitable for manipulation [12].

1.4 Techniques Used Previously to Determine Blood Flow in Rats and Mice

Radioactive microspheres had originally become the gold standard for measuring regional organ perfusion since the technique was introduced in 1967, by Rudolph and Heymann for examining regional blood flow in sheep fetuses in utero [35]. This technique became an essential tool in cardiovascular research by enabling measurements of regional blood flow in any organ. Regional blood flow is proportional to the number of microspheres trapped in that region of tissue following injection of the microspheres upstream – usually in the left ventricular chamber [35]. Methods for quantifying the number of microspheres per sample depends on the label used to track the microspheres, the most common being the measurement of nuclear isotope decay from radiolabeled microspheres. However the use of radiolabeled microspheres was becoming restricted because of the health risks for both the user and the animal in chronic studies, requiring

special precautions during the experiments and subsequently during disposal of the animals and tissue samples.

A number of non-radioactive techniques have been proposed, including the use of colored microspheres [8][16][23][38] and X-ray fluorescent microspheres [26-27]. But they too were not compatible in many different aspects. Colored microspheres underestimated regional blood flow since they had limited resolution due to significant spectral overlap among different colors, while data variance was high as a result of low signal intensity [8][19][27]. The measurement system used for X-ray fluorescent microspheres was expensive and available rarely and not in common use [32].

Consequently, techniques using optically fluorescent microspheres were developed to measure regional organ blood flow. These techniques have been validated against traditional radioactive methods, and they provide estimation of regional blood flow for about half the cost of radiolabeled microspheres [31-32][38]. Optically fluorescent methodologies are currently being used world-wide in cardiovascular research.

1.5 Use of Fluorescent Microspheres to Determine Coronary Blood Flow in Animals

Microsphere methods provide information on regional perfusion between and within organs that is more detailed than that available from blood-flow probes, which can only be placed around one or two large arteries. Recently, fluorescent microspheres have become more commonly used in experiments as they offer numerous advantages as compared to radioactive and colored microspheres:

a) Fluorescent microspheres are cost effective as compared to radioactive microspheres

b) Fluorescent microspheres eliminate the hazard and disposal problems caused as in use of radioactive microspheres

c) Blood flow measurements in kidney, lung, pancreas, adrenal glands and teeth are easily feasible using fluorescent microspheres

d) Fluorescent microspheres offer a shelf life of at least one year, so they can be retained in the tissue organ which is not possible in case of radioactive microspheres that have short lives and their retention is harmful to the tissue organ.

e) Lastly, fluorescent microspheres offer higher sensitivity, superior color separation and greater ease of measurement as compared to colored and radioactive microspheres [2][22].

Fluorescent microspheres have been used for the determination of blood flow in lung, kidney and myocardium of dog [1][13]. They have been used for the determination of blood flow in myocardium, brain, kidney and skeletal muscle of pig [13][19][25] and myocardium of rabbit [2][6]. In addition to absolute blood flow, fluorescent microspheres have been used for determination of relative blood flow in different tissues such as liver, brain, spleen of dog [38] and regional adrenal gland blood flow in fetal sheep [4]. Fluorescent microspheres have even been used to estimate the cardiac output in chick embryos [29]. Recently, fluorescent microspheres have been used for determination of regional and systemic hemodynamics in rats. Given the validation of fluorescent microspheres in rats, fluorescent microspheres have even been attempted for assessment of regional and systemic hemodynamics in genetically modified mice [34].

Fluorescent microspheres have been used for a wide range of applications including blood flow determination, tracing, in vivo imaging, calibration of images and

flow cytometry. As the fluorescent dye is incorporated throughout the bead and not just on the surface, they are relatively immune to photobleaching and other environmental factors. Fluorescent microspheres are available in many different colors: Red, Orange, Crimson, Blue, Yellow-green, Green, Blue-green, Scarlet. Each color exhibits a unique pair of optical excitation and emission wavelengths. This allows researchers to study the effects of multiple physiological variables in the course of a single experiment. The exact excitation and emission spectra depend on the solvent used to extract the fluorescent dyes. The principal advantage of fluorescence over radioactivity and absorption spectroscopy is the ability to separate compounds on the basis of either their excitation or emission spectra, as opposed to a single spectrum, as in colored microspheres.

Fluorescent microspheres are now an emerging technique used in rodents for the measurement of regional tissue blood flow. In a few labs, they have been tested on myocardial infarction models and pressure overload hypertrophy models in rodents. 15-micron diameter fluorescent microspheres have also been used to measure cerebral blood flow in rats [9]. Measurement of bone blood supply in mice has been recently determined by using fluorescent microspheres [36]. However, fluorescent microspheres have not yet been used in rodent animal models by the cardiovascular research laboratories at the New Jersey Medical School, a division of the University of Medicine and Dentistry of New Jersey (UMDNJ). The work in this thesis lays the groundwork that will enable the use of fluorescent microspheres to determine coronary blood flow in rodent animal models in the cardiovascular laboratories at UMDNJ.

1.6 Calculation of Myocardial Blood Flow by use of Fluorescent Microspheres

Fluorescent microspheres can be used for the measurement of regional blood flow without any concern of spillover of emitted fluorescence. Fluorescent microspheres are chemically stable and exhibit no dye leaching in aqueous environments, including strong acid and base solutions. 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 [26].

To measure myocardial blood flow, microspheres are injected into the left ventricle of an experimental animal. The microspheres mix uniformly with the arterial blood and flow with it. Some microspheres flow into the blood going through the ascending aorta and on to all other parts of the body, and some microspheres are distributed into the blood flowing through the coronary arteries. In the heart tissue fed by the coronary arteries – as well as in all other body tissues – the microspheres become trapped in the microvasculature because their diameter is too large to allow them to pass through capillaries.

To be able to relate the amount of blood flow in a specific tissue to the number of microspheres trapped in that tissue, a reference sample of blood flow must be obtained at the same time that the microspheres are injected and flowing throughout the entire systemic arterial circulation. This reference blood flow is usually obtained via a catheter placed through the femoral artery into the abdominal aorta of the animal. The magnitude of the reference blood flow is controlled and accurately determined by using a high-precision syringe pump to withdraw a precisely known blood flow through the aortic catheter.

After mixing well with the blood in the left ventricle, the concentration of the injected microspheres is the same in all the arterial blood flowing to all tissues in the body. Thus, the number of microspheres trapped in a particular tissue will be proportional to the magnitude of the blood flow going to that tissue. This proportion also holds for the blood flow going into the reference blood-flow sample. Consequently, the following equation is valid:

$$\frac{\text{Blood flow to heart}}{\text{Reference blood flow}} = \frac{\text{Number of microspheres in heart sample}}{\text{Number of microspheres in blood sample}} \quad (1.1)$$

Following extraction and quantification of the number of fluorescent microspheres from both the heart tissue and the reference blood sample, all of the values in the formula are known except for the coronary blood flow. Hence, this formula enables the calculation of the coronary blood flow.

1.7 Use of Doppler Echocardiography to Determine Coronary Blood Flow

Another technique to measure coronary blood flow is needed to test the validity of the measurements by fluorescent microspheres that are planned for the research in this thesis, the velocity of blood flow can also be measured by Doppler echocardiography [20], and data from this second technique will be used in this thesis.

An echocardiogram is a sonogram of the heart. This method uses standard ultrasound techniques to image two-dimensional slices of the heart. In addition to creating two-dimensional pictures of the heart or other structures in the cardiovascular system, an echocardiogram can also measure the velocity of blood at any arbitrary point using the Doppler shift measured in the returning ultrasound echo. Such Doppler echocardiography is often used to assess cardiac valve function and to look for any

abnormal fluid pathways communicating between the left and right sides of the heart. It is also used to measure cardiac output by measuring the velocity of blood flow in the aorta. The Doppler effect (or Doppler shift) is the change in frequency of a sound wave when the source of the wave is moving with respect to the observer. Doppler echocardiography is a procedure which uses ultrasound technology to examine the velocities of motion of blood within and around the heart and also velocities of the heart tissue itself [37]. Doppler measurements of coronary blood velocity have been made in human subjects [20]. In addition to measurements in humans, transthoracic Doppler echocardiography has also proved to be reliable in measurement of coronary blood flow and coronary flow reserve in rat and mouse animal models [14-15].

During our comparison study, pulsed-wave Doppler echocardiography will be used to determine coronary blood flow in rats. Pulsed wave (PW) Doppler systems use a transducer that alternates transmission and reception of ultrasound in a way similar to an M-mode ultrasound transducer [37]. One main advantage of pulsed Doppler is its ability to provide Doppler shift data selectively from a small segment along the ultrasound beam, referred to as the "sample volume". The location of the sample volume is operator controlled. An ultrasound pulse is transmitted into the tissues and travels for a given time (time X) until it is reflected back by a moving red cell. It then returns to the transducer over the same time interval but at a shifted frequency. The total transit time to and from the area is 2X. Since the speed of ultrasound in the tissues is constant, there is a simple relationship between roundtrip travel time and the location of the sample volume relative to the transducer face (i.e., distance to sample volume equals ultrasound speed divided by

round trip travel time). This process is alternately repeated through many transmit-receive cycles each second [10].

This range gating is therefore dependent on a timing mechanism that only samples the returning Doppler shift data from a given region. It is calibrated so that as the operator chooses a particular location for the sample volume, the range gate circuit will permit only Doppler shift data from inside that area to be displayed as output. All other returning ultrasound information is essentially "ignored" [10]. Hence, the blood velocity can be determined exclusively from one small region – in our case from one of the coronary arteries on the heart.

1.8 Specific Aims of this Thesis

This research study had four primary aims:

1) To develop techniques for reliably measuring the number of fluorescent microspheres in a sample by using a commonly available laboratory fluorescent spectrophotometer.

2) To develop techniques for reliably recovering 100% of microspheres in a tissue or blood sample by using the sedimentation method

3) To test the ability of fluorescent microspheres to reliably measure coronary blood flow in rats by comparing results of the coronary blood flow reserve obtained using fluorescent microspheres with flow reserve measurements from Doppler echocardiography.

4) To test the repeatability of fluorescent microsphere measurements of coronary blood flow in rats.

CHAPTER 2

DETERMINATION OF CALIBRATION CURVES FOR FLUORESCENT MICROSPHERES

2.1 Principles of Fluorescence Spectrophotometry

Fluorescence spectroscopy is a type of electromagnetic spectroscopy which analyzes fluorescence from a sample. Spectrophotometers use the principle of fluorescence spectroscopy. Spectrophotometers use the diffraction grating monochromators to isolate the incident light and fluorescent light to narrow ranges of wavelengths. They use the following scheme: The light (often from a broadband excitation source) passes through a filter or monochromator, and strikes the sample. A proportion of the incident light is absorbed by the sample, and some of the molecules in the sample fluoresce. The fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second filter or monochromator and reaches a detector, which is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector.

Various light sources may be used as excitation sources, including lasers, photodiodes, mercury-vapor lamps, and halogen arc lamps. A laser only emits light within a very narrow wavelength interval, typically less than 0.01 nm, which makes an excitation monochromator or filter unnecessary. The disadvantage of this method is that the wavelength of a laser cannot be varied easily. A mercury vapor lamp is a line lamp, meaning it emits light only at several specific wavelengths. By contrast, a halogen arc lamp has a continuous emission spectrum with nearly constant intensity in the range from 300-800 nm

Filters and/or monochromators are often used in spectrophotometers. The most common type of monochromator utilizes a diffraction grating; that is, collimated light illuminates a grating and exits with a different angle depending on the wavelength. By selecting the monochromator, light with an adjustable central wavelength and with an adjustable bandwidth can be used to excite the fluorescence in the sample and to limit the range of emitted light that will be detected.

2.2 Biotek Instruments FL 500 Microplate Reader

The specific spectrophotometer used in this research was contained within a microplate reader. They are widely used in research, drug discovery, validation, quality control and manufacturing processes in the pharmaceutical and biotechnological industry and academic research. The most common microplate format used in academic research laboratories or clinical diagnostic laboratories has 96-wells (arranged in an 8 by 12 matrix) with a typical reaction volume between 100 and 200 μL per well.

Figure 2.1 Microplate reader used for fluorescence spectrophotometry [3].
(Source: Manual for Bio-Tek Instruments FL500 Microplate reader, accessed on April,2011)

Figure 2.1 shows an example of the Bio-Tek Instruments FL500 Microplate Reader that was used to calculate fluorescence intensity from samples containing microspheres. It works on the following principle: a halogen lamp provides broadband illumination, which is then restricted to a narrower bandwidth by passing through an optical filter (the excitation filter). As a result of this optical excitation, the sample emits light (it fluoresces) and a second optical system (emission system) collects the emitted light over a different narrow band of wavelengths using a second optical filter (the emission filter). This system collects and filters fluorescent light that was emitted at 90° to the incident excitation. A photo-multiplier tube (PMT) quantifies the intensity of the resulting fluorescent light.

The fluorescence filters are arranged in filter wheels: four excitation filters and four emission filters were provided. The selection of filters available in the particular instrument used in this research is shown in Figure 2.2. Each filter's central wavelength and bandwidth (in nm) are specified. Spectral representations are shown on the next page.

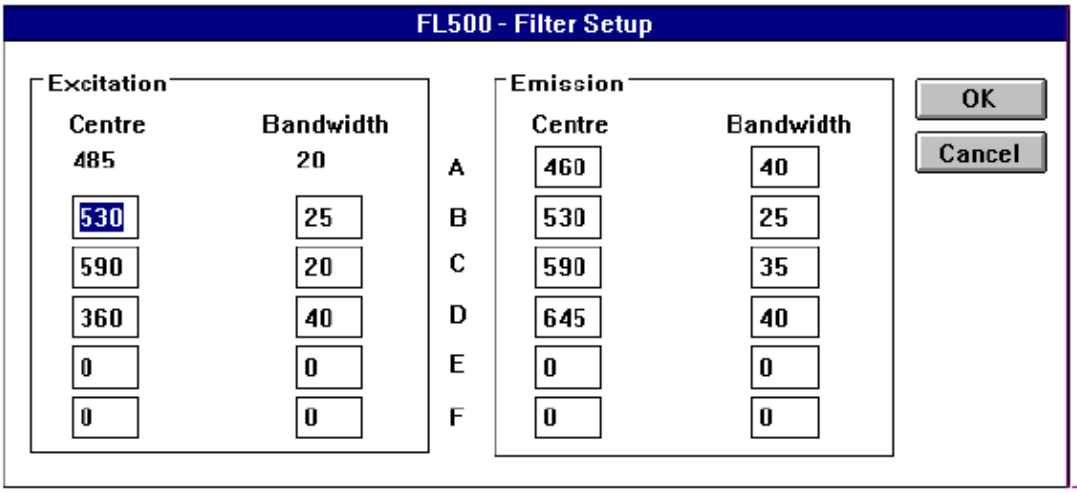

Figure 2.2 Selection of excitation and emission filters in BioTek FL500 [3].
(Source: Manual for using Bio-Tek Instruments FL500 Microplate reader, accessed on April, 2011)

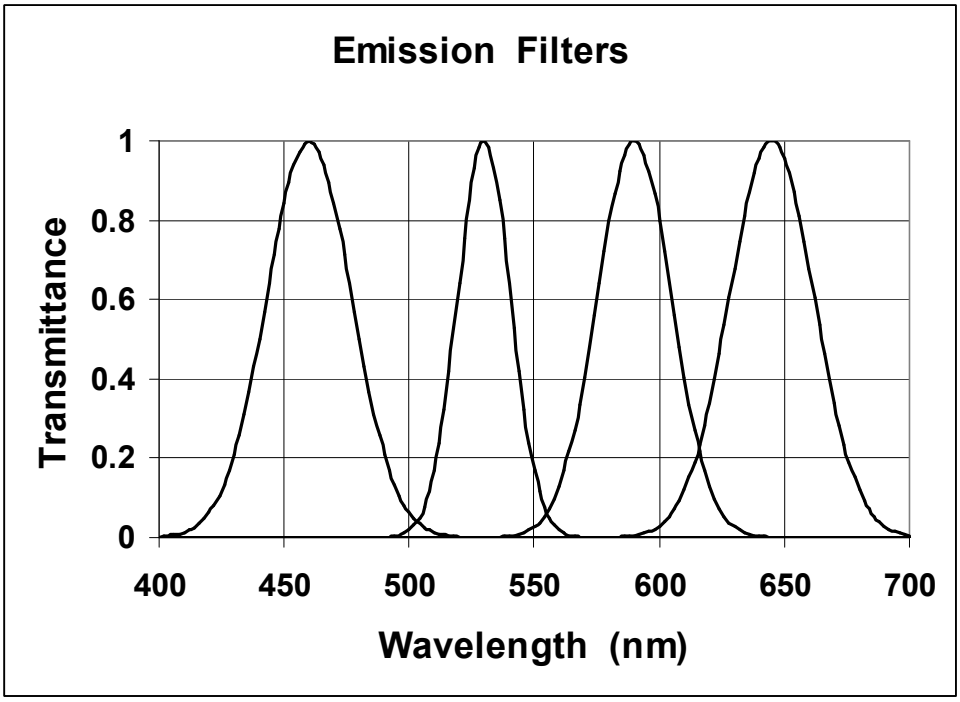

Figure 2.3 Spectra of emission filters available in the Bio Tek FL500.

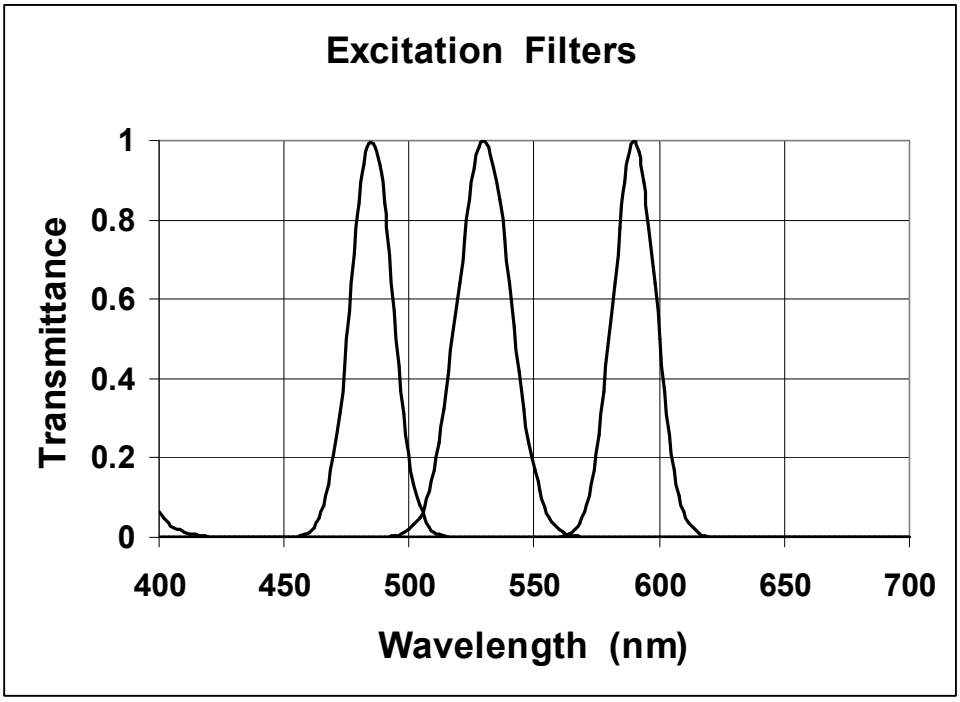

Figure 2.4 Spectra of excitation filters available in the Bio Tek FL500.

2.3 Fluorescence Spectra of Commercial Microbeads

As mentioned in Chapter 1, two suppliers (Molecular Probes and Triton Technology) provide microbeads that contain a range of fluorescent dyes. The set of emission wavelengths for microbeads available from the supplier chosen for this research (Triton Technology) are shown in Figure 2.5. This figure was generated by fitting each emission spectrum to a skew-normal distribution. Only by having such a variety of emission spectra – and limited bandwidth detectors – can multiple measurements of coronary blood flow be performed in the same experimental animal, since all injected beads become lodged together in the same tissue sample and could fluorescence.

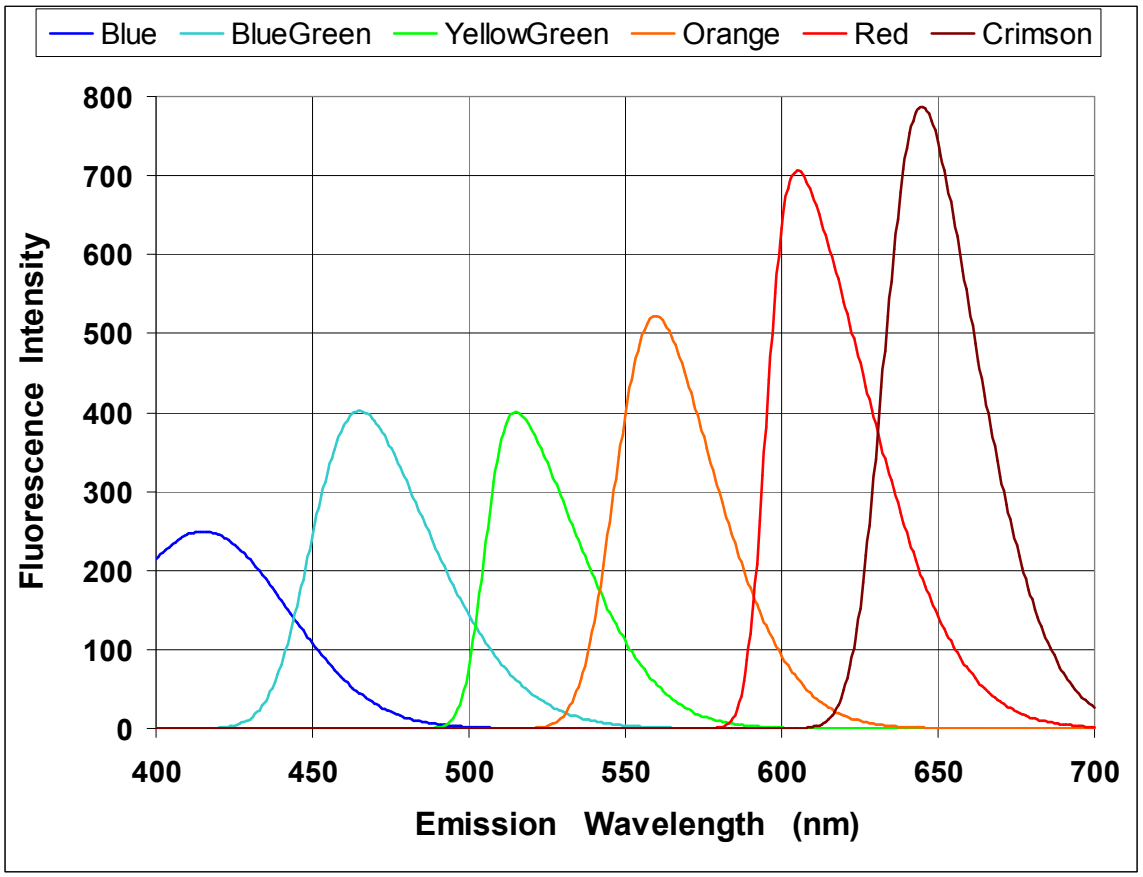

Figure 2.5 Spectra of emitted fluorescence from a suite of commercial microbeads. Data for the peak frequency and bandwidth is obtained from [21].

Additional separation between the fluorescent signals from multiple varieties of beads embedded in a tissue sample can be obtained by taking advantage of the different excitation spectra for the fluorescent dyes in the beads. Fluorescence is excited by photons of higher energy than the photons that are released during fluorescence. By Planck's law, these higher energy photons have higher frequency and thus shorter wavelength. Some photon energies are better able to excite the fluorescent response of the dye, and this "efficiency" of excitation is expressed as the excitation spectrum for the fluorescent response. For example, the excitation spectrum and the emission spectrum for yellow-green fluorescent microbeads is shown in Figure 2.6 The excitation spectrum –in blue – peaks at a lower wavelength (higher frequency and energy); in this case, the difference is 10 nm. For the greatest excitation of fluorescence, the excitation should

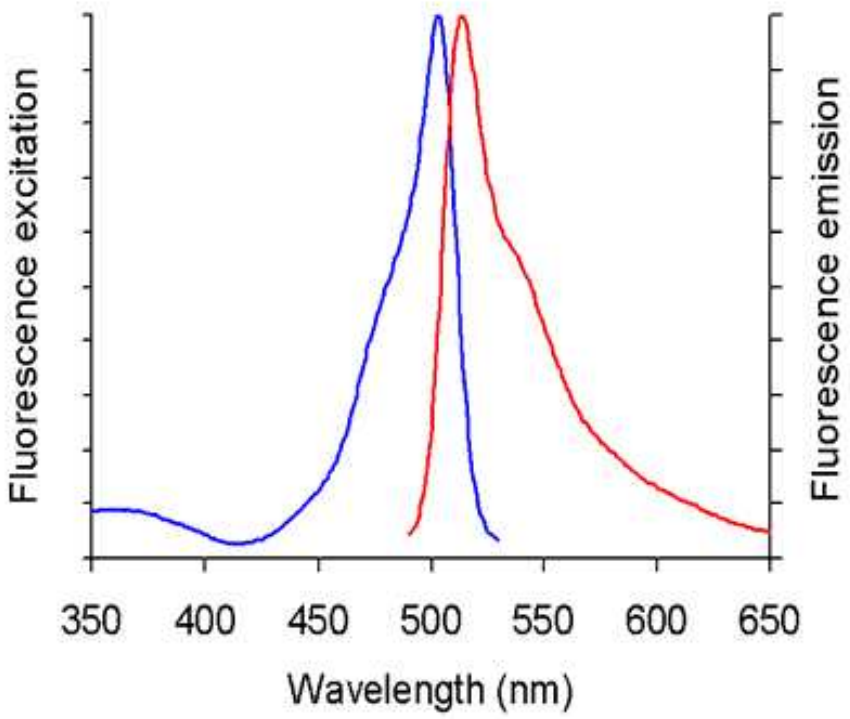

Figure 2.6 Excitation (blue) and emission (red) spectra for yellow-green dye [33].
(Source: http://probes.invitrogen.com/media/spectra/8811h2o.jpg)

occur at wavelengths near this peak. Note that the excitation efficiency falls off quickly for slightly longer wavelengths, while there is a greater extent of efficient excitation for wavelengths somewhat shorter than the best wavelength. If the excitation of fluorescence is to be minimized when assaying for another variety of bead in a tissue sample, then the exciting wavelengths could be limited by a filter to wavelengths well away from the peak excitation "efficiency".

Note that the spectrum of emitted fluorescent light is the same no matter what wavelength is used to excite the fluorescence [24]. This occurs because the variety of molecular vibrational modes excited when the dye molecule absorbs photons from a variety of wavelengths all die out quickly (within 10^{-14} to 10^{-10} sec). Thus, the dye molecule quickly reduces its vibrational energy to a common minimal state before the fluorescent photon emission event occurs, an event which happens on a much slower time scale (10^{-9} to 10^{-7} sec) [24].

2.4 Choice of Fluorescent Dyes used in Microbeads

The goal of this thesis was to measure coronary flow reserve. Thus, two successive measurements of coronary flow were required: (1) with the rat at a baseline resting state, when coronary flow will be minimal, and (2) during infusion of a vasodilator, when the coronary flow will be maximal. Consequently, microbeads with two different dyes must be infused, and both varieties will remain simultaneously embedded in the tissue samples to be assayed by fluorescent spectrophotometry. The particular pair of microbead dyes to use must be chosen so that their fluorescent signals can be essentially completely

separated by a judicious choice of the emission spectra of the dyes themselves and by the excitation and emission filters available in the spectrophotometer.

Several experimental factors affected the choice of the two microbead dyes. Firstly, two of the chemical components used in processing the tissue and beads (the solvent 2-ethoxy-ethyl-acetate and the detergent Tween 80) both possess intrinsic fluorescent properties themselves, and their fluorescence is emitted in the blue part of the spectrum. Consequently, microbeads with blue and blue-green dyes were not chosen for this project. Secondly, photomultiplier tubes are generally less sensitive to longer wavelengths, so the dye having the longest emission wavelength (crimson) was not chosen so that the detection system would maintain the best possible sensitivity. The remaining three dyes were: yellow-green, orange, and red. As Figure 2.5 showed, the emission spectrum of the orange dye overlapped significantly with both of the other two. Hence, the orange dye was not chosen.

The two selected microbead dyes were thus (1) yellow-green, and (2) red. Their emission spectra did not overlap significantly. Moreover, as the next section shows, the choice of excitation and emission filters made it possible that there was essentially complete separation between the fluorescent signals that would be measured from the two beads in the spectrophotometer – even when they both co-existed in the same tissue sample.

2.4 Choice of Excitation and Emission Filters

Table 2.1 (below) reports the filter set chosen to be used when the fluorescence signal from either of the two chosen microbeads was to be measured. The emission filter best matched to the yellow-green emission spectrum (Figure 2.5) is the second one from the left shown in Figure 2.3, which has maximum transmittance at 530 nm. Similarly, the emission filter best matched to the red emission spectrum is the right-most one shown in Figure 2.3, which has maximum transmittance at 645 nm. The transmittance spectra from these two filters do not overlap, which enhances the separation of the fluorescent signals when both dyes coexist.

The excitation spectrum for yellow-green dyed microbeads was shown in Figure 2.6; 505 nm was the wavelength that most efficiently excited their fluorescence. The excitation filter that best matched this excitation spectrum was the left-most one shown in Figure 2.4, which has maximum transmittance at 485 nm. The excitation spectrum for red dye peaked at 580 nm, so the right-most filter shown there (peak = 590 nm) was chosen for this case. Note that the spectra from the two excitation filters do not overlap, which – just as in the case for the emission filters – further enhances the separation of fluorescent signals.

Table 2.1 Filters providing best match to excitation and emission spectra

Fluorescent Color	Wavelength at Peak Emission (nm)	Emission Filter Center of Transmittance (nm)	Wavelength for Best Excitation (nm)	Excitation Filter Center of Transmittance (nm)
Yellow - Green	515	530	505	485
Red	605	645	580	590

2.6 Determination of Calibration Curve

As explained in Chapter 1, the number of microbeads trapped in a tissue sample is the key measure used in the tracer method to determine coronary blood flow. The fluorescent intensity of a tissue sample containing embedded microbeads provides a measure proportional to the number of microbeads contained in that sample. However, both the proportionality constant and any offset must be determined by a calibration procedure before the fluorescent signal can be related quantitatively to the number of microbeads.

Calibration requires measuring the fluorescence of solutions containing known numbers of microbeads. The most difficult part here is developing a reliable procedure that will accurately set the number of microbeads in a solution. The starting point for this procedure is the known concentration of microbeads in the stock solution of microbeads provided by the supplier. The microbeads we used were packaged in a solution containing 1 million microspheres per milliliter. By accurately diluting this stock solution, and by accurately measuring out known volumes of these dilute solutions, a variety of samples containing known numbers of microbeads were prepared.

The number of microbeads in the calibration samples should span the range anticipated to occur in the tissue samples. A large number of microbeads would be on the order of 10,000 to 20,000. The minimum number of microbeads that provides a reliable statistical estimate is on the order of 400. Calibration standards spanning this range were prepared.

Appendix I provides the detailed steps in the procedure used to prepare the calibration standards. Briefly, the first step was to prepare a 10:1 diluted solution of

microbeads. This solution would thus have 100,000 microbeads per milliliter. Since the sample size used in the wells of the 96-well plate is 100 μl, a sample drawn from this solution would contain 10,000 microbeads. This is the maximum number that was used in a calibration standard. Subsequent 2:1 dilutions then produced standards containing: 5000, 2500, 1250, 625, and 312 microbeads. These standards spanned the range over which calibration was performed.

During the dilution and sample loading procedure, extreme care was taken to maintain the microspheres in solution and to prevent clumping and aggregation of microspheres. A small amount of detergent (Tween 80) was used in the diluting solutions to minimize clumping and aggregation, and solutions were often agitated vigorously in a vortex mixer.

Accurate measurement of solution volumes was also required. This was accomplished using well-calibrated mechanical pipettes, which are common in biochemical laboratories. Following the proper pipetting procedure was also important.

2.7 Results of Calibration Curves

Five replicate determinations of a calibration curve were performed for each of the two varieties of microbeads used in this study. Table 2.2 and Figure 2.7 provide typical results for one such calibration curve, which was performed on solutions containing yellow-green microspheres. Appendix II provides similar detailed results for all of the 10 calibration curves. Each calibration curve maintained a tight linear relation between microbead number and fluorescence intensity. The R^2 values for linear regression were all above 0.9936, and most were above 0.9990.

Table 2.2 Typical calibration measurements for yellow – green microspheres

Number Of Microspheres	Fluorescent Intensity (arbitrary units)
10,000	8683
5,000	4288
2,500	2100
1,250	1001
625	528
312	300

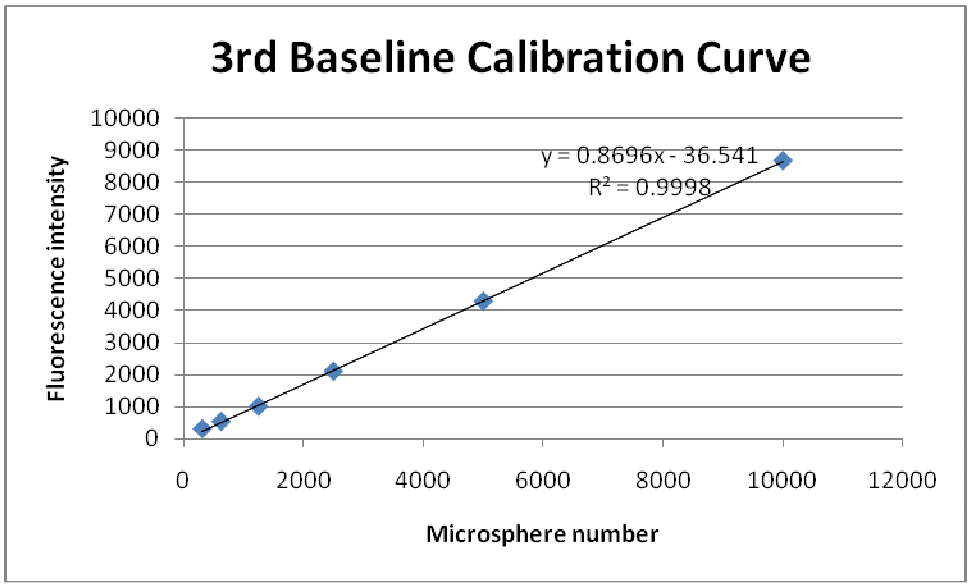

Figure 2.7 Typical calibration regression line for yellow-green microspheres

However, the replications of the calibration curves did not all produce slopes of the regression lines that were in close agreement with one another (see Figures 2.8 and 2.9). Since each individual regression line was rather linear without much scatter, it seemed likely that the major contribution to the disagreement among regression lines arose due to experimental uncertainty in the first step of the calibration procedure. The stock solution may not have been sufficiently well-mixed when the sample was withdrawn, or there may have been some error in withdrawing a 1 ml volume into a plastic syringe, since a calibrated pipette could not perform this step.

2.8 Averaging calibration curves

To overcome the uncertainty in the regression coefficient, the results from the 5 replicate calibration curves were averaged together for each variety of microbead. Averaging was thought to reduce the uncertainty introduced during the initial microbead withdrawal from the stock solution. Tables 2.3 and 2.4 on the next pages report the averaging process. Each averaged relationship then became the standard calibration curve for that color microsphere, which was used to convert the fluorescence from a tissue sample into the number of microspheres contained within that sample.

The final calibration curves were:

For yellow green microspheres: $I_f = 0.9256\ N_s - 4.9102$

For red microspheres: $I_f = 1.0954\ N_s + 0.8414$

where I_f is the fluorescent intensity and N_s is the number of microspheres.

Note that red microspheres produced slightly more fluorescence intensity per sphere. The offset values were not statistically different from zero.

Calibration of Yellow-Green Microspheres

Table 2.3 Summary of calibration for yellow – green microspheres

Trial Number	Slope of Regression	Intercept of Regression	R-squared
1	1.1455	+ 4.4194	0.9992
2	0.9229	-92.562	0.9992
3	0.8696	-36.541	0.9998
4	0.9188	-30.379	0.9976
5	0.774	+130.56	0.9974
AVERAGE	**0.9256**	**-4.9102**	

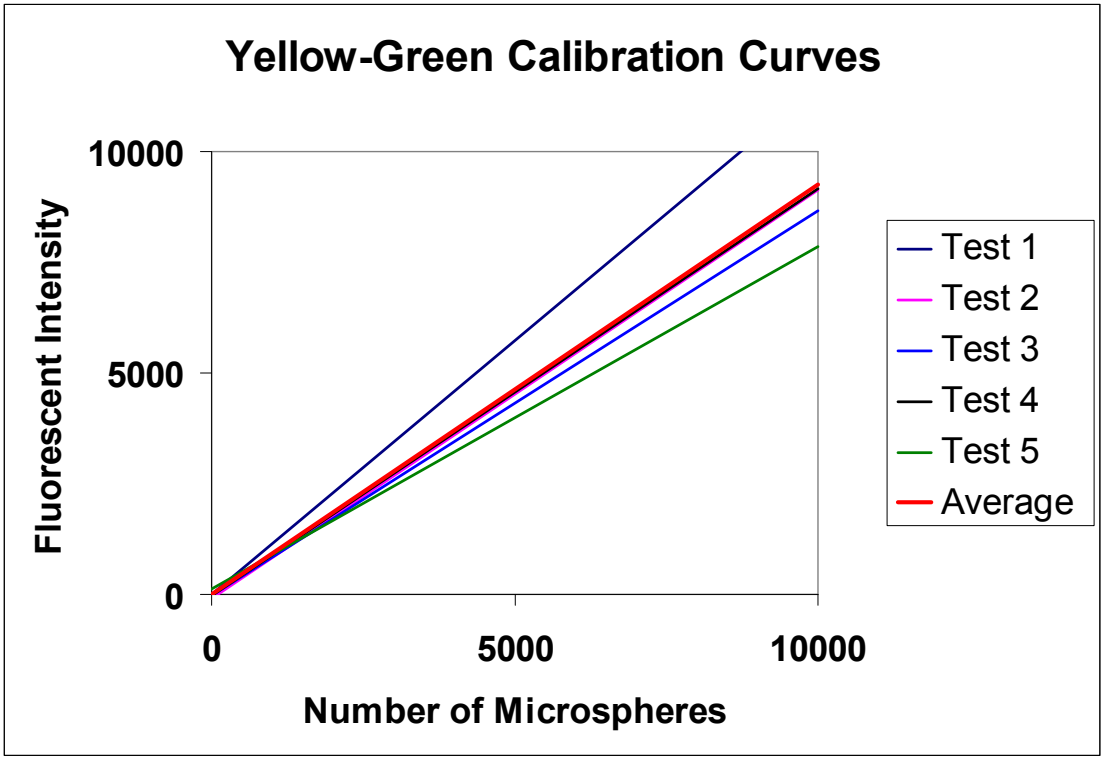

Figure 2.8 Linear regression calibration curves for yellow – green microspheres

Calibration of Red Microspheres

Table 2.4 Summary of calibration for red microspheres

Trial Number	Slope of Regression	Intercept of Regression	R-squared
1	1.1084	-104.79	0.9993
2	0.9083	-137.87	0.9966
3	1.1498	181.72	0.9990
4	1.0897	49.578	0.9936
5	1.2234	12.204	0.9996
AVERAGE	**1.0954**	**0.8414**	

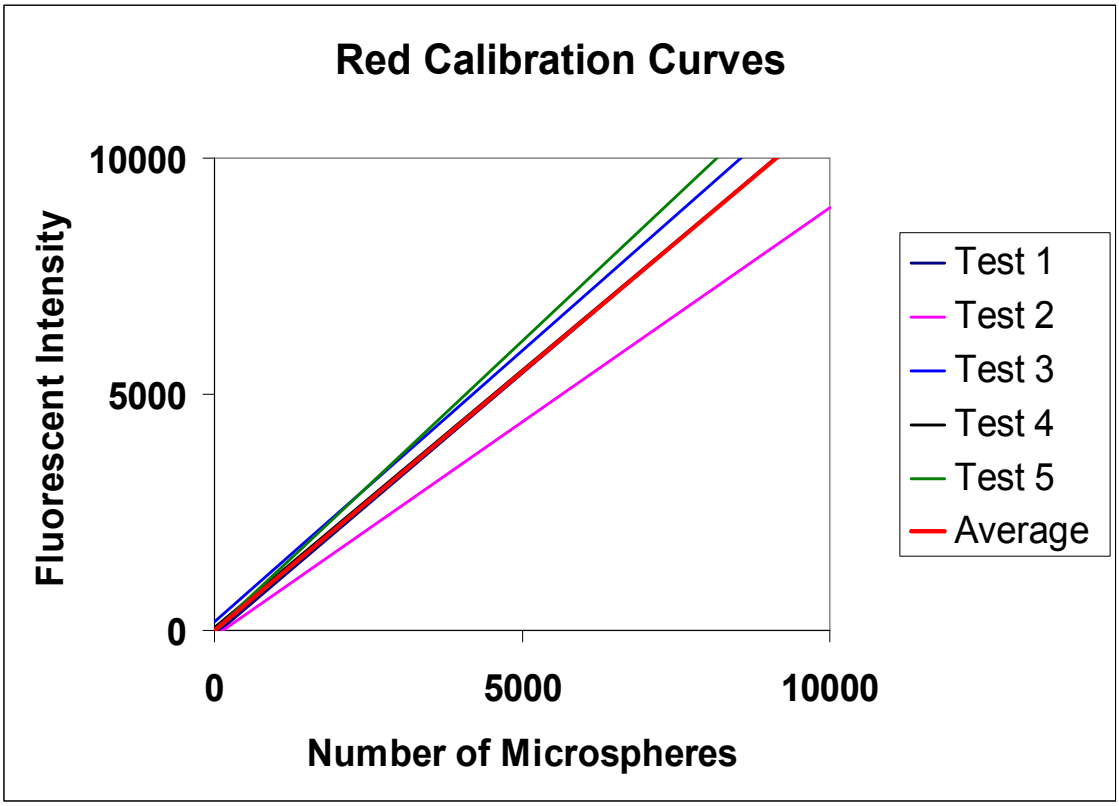

Figure 2.9 Linear regression calibration curves for red microspheres

CHAPTER 3

EXPERIMENTAL TECHNIQUES TO DETERMINE CORONARY BLOOD FLOW IN RAT ANIMAL MODEL

When appropriately sized microspheres are used, regional blood flow is proportional to the number of microspheres trapped in the organ of interest.

3.1 Source of Fluorescent Microspheres

Triton Technology (San Diego, CA) and Molecular Probes (Eugene, OR) are the two major companies manufacturing fluorescent microspheres used in biomedical research and various other applications. For our procedures we have used fluorescent microspheres sold by Triton Technology**.** Triton Technology sells two types of fluorescent microspheres: Triton Technology ***Dye-Trak 'F'*** fluorescent microspheres and the **FluoSpheres**® manufactured by Molecular Probes. We have used Molecular Probes Fluospheres (with Triton Technology packaging) for our experiments. Fluospheres are non-radioactive fluorescent microspheres for high sensitivity measurement of regional blood flow quantified by spectrofluorometry. Each 20 ml bottle contains 20 million spheres, 15 μm in diameter, in a saline solution, with 0.05% Tween 20 as a detergent to help maintain the microspheres dispersed in solution and 0.02% Thimerosal as a bacteriostat.

3.2 Calculation of Number of Microspheres for Injection

A minimum of 400 – 500 microspheres are needed per tissue piece to be 95 % confident that flow measurement is within 10% of the true value [39]. For regional blood flow measurements, the total number of microspheres to be injected into the whole animal must be calculated to assure that a sufficient number reach the particular organ of interest.

The following equation estimates the minimum total number of microspheres needed per injection [39]:

$$N_{min} = 400\ (n) * [\ Q_{total} / Q_{organ}\ ] \quad (3.1)$$

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

n = total number of organ pieces in the organ with the smallest blood flow

Q_{organ} = total blood flow through an organ of interest with the smallest blood flow

Q_{total} = cardiac output

Applying this equation to our procedure, we first must estimate the cardiac output expected in the experimental animals we used. The cardiac index observed in Sprague Dawley rats of the same age and weight as we used is approximately 350 ml/min/kg [22]. Considering the approximate weight of the rats we used to be 400g,

$$\text{Cardiac Output} = \text{Cardiac Index} * \text{Mass of an animal}$$

$$= (350 * 400) / 1000$$

$$= 140 \text{ ml/min}$$

Since our organ of interest is the heart, which has a relatively large blood flow, the "organ" with the smallest blood flow will actually be the "virtual organ" created by the reference withdrawal of blood. Since we will withdraw the reference blood flow at a rate

of 0.5 ml/min, the estimate of the minimum total number of microspheres needed per injection is:

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

$$= 400\ (1) * [140 / 0.5]$$

$$= 112{,}000$$

Where, n = number of organ pieces = reference blood flow sample = 1

Q_{organ} = reference blood flow = 0.5 ml/min

Q_{total} = Cardiac output = 140 ml/min

Thus, a minimum of 112,000 microspheres per injection was needed for our procedure. We have injected 0.5 ml volume of microsphere solution, which contains 500,000 microspheres. Thus, the volume used for our procedure was quite enough to trap a sufficient number of microspheres in the tissue and blood samples. Usually double the minimum number of microspheres are injected to make sure that low flow organ pieces have an adequate number of microspheres [39]. Our procedure met this guideline as well.

3.3 Preparation of fluorescent microspheres for injection

Aggregation is a major problem associated with fluorescent microspheres. This might clog a blood vessel, resulting in damage which might result in the death of an animal. Aggregation of the particles is prevented by the use of small amounts of detergent in the injectate, or by suspending them in a solution containing macromolecules. A detergent named Tween 80 is normally added to the solution to prevent aggregation and clumping together of microspheres. However, the concentration of the detergent cannot be too high, otherwise it could damage the lipids in the cell membranes of endothelial cells or blood

cells. Therefore, we used a concentration of 250 µl. During the microsphere injection procedure, special care is taken of in order to avoid aggregation of microspheres.

Method:

1) Remove the bottle from the refrigerator and check supernatant solution. Ideally, the solution should stay clear due to the presence of thimerosal in it. Thimerosal is a bacteriostat which prevents the growth of any bacteria or fungi thus preventing cloudy fluid and contamination.

2) Vortex the bottle vigorously for 5 – 15 seconds using a vortex mixer. Vortexing ensures proper mixing of the solution thus preventing aggregation of microspheres.

3) Place the bottle in an ultrasonic water bath for at least 30 minutes to allow dispersion of microspheres. This allows proper mixing of microspheres with the liquid solution. Be careful with the sonication time as the heat generated might melt the microspheres.

4) Continue to sonicate the microspheres until the sample is used for the procedure.

5) Just prior to injection, vortex the vial of microspheres again and withdraw the desired volume of 0.5 ml immediately. The injected volume drawn into syringe should then be injected immediately into the body of animal. If injection time is delayed, vortex the microspheres thoroughly again.

6) Injection time varies for each procedure and should be determined prior to injection. Injection to left heart takes a short time (normally 5 – 15 seconds). In our procedure, injection time is 5 seconds.

7) Slow and steady injections allow for proper mixing of microspheres with the blood in the left ventricle.

8) After injection, flush the dead space of the catheter thoroughly with saline and change the stopcock to avoid contamination of subsequent injections.

3.4 Reference blood flow sampling

A reference blood flow sample allows calculation of regional flow in ml / min. The catheter used for the withdrawal of sample should be accurately positioned so that a blood sample containing well mixed microspheres can be obtained. Blood samples should

be obtained as close to the organ of interest as possible without interfering too much with the normal blood flow. The reference withdrawal pump must be accurately calibrated at 0.5 (ml/min) so that reference blood is withdrawn at a uniform preset rate for a period of 2 minutes. Although glass syringes and containers would have been preferred as they decrease microsphere loss by avoiding adhesion to walls, which could occur in case of plastic syringes or containers, we found that use of plastic syringes was adequate and did not result in loss of microspheres.

Researchers say that 15% of blood volume is the maximum that can be taken out at a stretch from the body, say during donation of blood. More than 15% results in a significant loss of arterial pressure and might result in some heart problems.

Since the animals used in our procedure weigh 350-450g, withdrawal of minimum reference blood should be calculated. For a rat weighing 350g, blood mass = 8% of body mass = (0.08)(350g) = 28 g. This equates to approximately 28 ml of blood since the density of blood is only slightly greater than that of water.

If 2 ml is withdrawn as reference blood during microsphere procedure, then it accounts for 7% loss of blood from the body which is quite tolerable and should not create a bad impact on the condition of the animal.

Similarly, for a rat weighing 450g, its blood volume is estimated to be 36 ml. If 2 ml is withdrawn as reference blood during microsphere procedure, then it accounts for 5% loss of blood from the body of animal which is tolerable and does not affect the condition of animal.

Therefore, withdrawal of 0.5 (ml/ min) for a period of 2 minutes results in 5% - 7% of blood loss which is preferred for our procedure to avoid excessive loss of blood in an animal.

3.5 Animals

Male Sprague Dawley rats, body weight 350 – 450 g, age 8 - 12 weeks, were housed in separate cages and maintained in a temperature regulated environment. The animals were used as described in a protocol approved by IACUC committee (Institutional Animal Care and Use Committee) of UMDNJ – Newark. This institution is accreditated by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International program. Two animals were used. Both were subjected to injections of Triton Technology 15 μm diameter fluorescent microspheres of two different colors: yellow-green microspheres used for baseline measurements and red microspheres used for measurements after infusion with adenosine.

3.6 Surgery

Surgery was performed by an experienced doctoral student, Xin Zhao, in the department of Cell Biology and Molecular Medicine at UMDNJ – Newark, trained to perform cardiovascular surgery on rats. An anesthetic technique that has minimal effect on the heart rate is essential. The following anesthetic regime resulted in heart rates that were close or slightly below the rate for an alert, resting rat and allowed excellent animal recovery. Anesthesia was induced with a mixture of ketamine (70mg/ kg) and xylazine (7mg/ kg) administered intramuscularly. Since the experimental procedure was not for a

long duration, no supplemental anesthesia was needed. While anesthetized, the rats underwent several cannulation procedures.

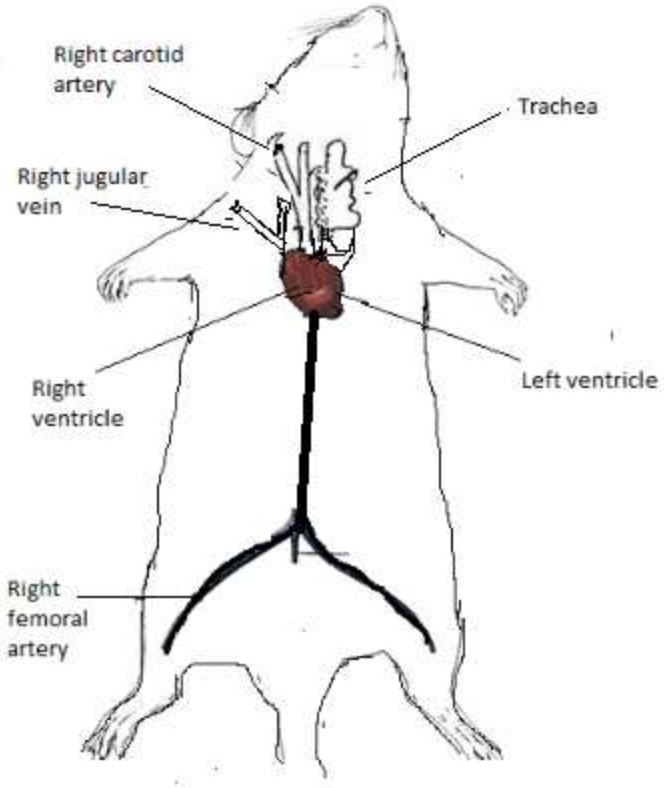

Figure 3.1 Rat model showing cannulation in left ventricle and femoral artery

The left ventricle was cannulated via the right carotid artery by a polyethylene catheter (RPT040 -- .040 OD" x .025 ID) to observe left ventricular pressure and to subsequently inject microspheres and adenosine into the left ventricle. By observing changes in the pressure waveform from an arterial pattern to a ventricular pattern, it could be determined when the cannula tip was in the ventricular lumen. The right femoral artery was cannulated by a similar catheter (RPT040 .040 OD" x .025 ID) and was advanced into the abdominal aorta to monitor arterial pressure and also obtain a sample of blood flow. During blood flow sampling, this catheter was connected to a Harvard Apparatus syringe pump for the withdrawal of reference blood sample containing

microspheres that are adequately mixed. Right jugular vein was cannulated by MRE 040 .40OD" X .025 ID catheter for the injection of saline to restore blood volume after each microsphere injection and sampling procedure.

The entire procedure takes about 1.5 – 2 hours. The animals were then allowed to recover for 2-3 hours after the completion of cannulation surgery. After the normal hemodynamic condition of the animal was recovered, a subsequent microsphere injection procedure could be performed.

3.7 Procedure for Injection of Microspheres

Systemic hemodynamic and regional blood flow was determined using 15 micrometer diameter fluorescent microspheres (FluoSpheres® Triton Technology, San Diego, CA, USA (20 million 15 micron spheres per 20 ml vial)). Briefly, two different colors of fluorescent microspheres were used. The colors of microspheres were selected as previously described to avoid spillover between the colors. Yellow-green and red colors have been selected for our experiment. Yellow-green microspheres are used for baseline measurement and red microspheres are used for measurements after infusion with adenosine.

The procedure is as follows:

1) Shake well the bottle containing microspheres of desired color and place it in an ultrasonicator. This allows proper mixing of microspheres and prevents aggregation of microspheres.

2) The bottle containing microspheres are sonicated until the desired amount is removed and without wasting any time, the microspheres are injected directly into the body of animal.

3) Two Harvard apparatus infusion pumps were used for procedure: One pump is used for the injection of adenosine at the rate of 0.15 (mg/kg/min), second pump is used for the

withdrawal of reference blood sample at the rate of 0.5 (ml/min) for a period of 2 minutes.

4) Four 1cc plastic syringes were used in infusion pumps: two syringes were used for collecting blood and the remaining two were used for injection of microspheres. Heparin coated syringes were used for the collection of blood samples to avoid the clot of blood once it is collected in a syringe. Also, once collected in heparin coated syringe, it then becomes easy to transfer in any other container without any clot observed.

5) The diameter for the syringe was set to 4.78 mm.

6) The withdrawal pump was calibrated at the predetermined withdrawal rate, including the catheters, extension tubing and syringes that would be used for the reference withdrawal.

7) The syringes would be connected to the withdrawal pump to the catheters and the extension tubing so that everything is set up for withdrawing the reference blood sample. The stopcock is turned off to avoid clotting of blood into the catheter dead space until injection.

8) Four paper pins were placed, one at each corner of surgical board, that hold each limb of the animal.
Horizontal distance between the pins is 15cm.
Vertical distance between the pins is 12cm.

9) Core temperature of the animal was monitored with a rectal probe and maintained at 36.5 degree Celsius with an automatic heating lamp.

10) The hemodynamics of the rat is checked for its normal condition before the injection of microspheres.

11) Yellow-green microspheres are injected first into the body of animal to determine baseline measurements and then red microspheres are injected to determine measurements after infusion of adenosine.

12) Once the microspheres had been drawn into the injection syringe, the withdrawal pump was started and made sure that the flow was smooth without any clot.

13) Now, a volume of 0.5 ml of yellow green fluorescent microspheres was injected over a period of 5 seconds followed by the flush of saline.

14) Simultaneously, the reference blood sample was withdrawn for a total interval of 2 minutes, 1 minute each for both the color of microspheres. At the end of the withdrawal, the pump was turned off, the stopcocks were opened and the blood remaining in the extension tubing was drawn into the syringe.

15) The reference blood was then transferred from heparinized syringe into polypropylene tubes for further processing. Also, the syringes and the extension tubing were washed with 2% Tween 80 and this solution was then added to the blood samples to avoid any loss.

16) Adenosine which works as a potent dilator of arterioles was then infused into the left ventricle via the catheter in the left ventricle.

17) After the rat's hemodynamic state had stabilized under the adenosine infusion, 0.5 ml volume of red microspheres was injected over a period of 5 seconds followed by the flush of saline.

18) The reference blood sample was collected in heparinized syringe which is then transferred to polypropylene tubes for further processing.

This injection procedure produced two sets of microspheres in each rat. At the end of the procedure, the rats were euthanized with an overdose of anesthetic. The heart was removed, weighed and placed in a polypropylene tube. The reference blood sample and heart tissue were then digested by 2.3 M ethanolic KOH and 0.25 % Tween 80 for a period of 48 hours. At the end of digestion, microspheres were recovered by the sedimentation method and the microsphere dye was extracted by using 3 ml of 2-ethoxyethyl acetate, that dissolved the plastic spheres and so released the fluorescent dye into solution.

CHAPTER 4

EXPERIMENTAL TECHNIQUES TO RECOVER MICROSPHERES FROM TISSUE AND BLOOD

4.1 Three Alternative Methods to Separate Microspheres From Tissues

Microspheres must be physically separated from the tissue or blood in order to quantify the number of microspheres in each sample.

There are three practical methods to recover microspheres from digested tissues [39]:

1) Negative pressure filtration
2) Polyamide woven filtration devices (manufactured by Perkin Elmer)
3) Sedimentation

All three methods will be described below, along with the disadvantages of both the filtration methods. Ultimately, the sedimentation method was the one chosen for use in this research. The details of the sedimentation method will be presented in other sections in this chapter. The initial key step in all three procedures is digestion of tissue or blood sample. All methods use ethanolic KOH, a very powerful digesting solution.

4.2 Negative Pressure Filtration

In the case of the negative pressure filtration technique, the volumes and concentrations of solutions are not critical. Negative pressure filtration works on digested heparinized blood samples and solid tissue. After the samples have been digested with KOH, the microspheres are physically separated by negative pressure filtration. Filtration is usually

performed with a combination of a Poretics filtration device using Poretics polycarbonate filters [39].

Disadvantages:

1) Digested tissue samples should not stand unfiltered for a long period of time since the fat in them may solidify and this may result in damage to the sample.

2) The method is labor intensive.

3) There may be microsphere loss when the tissue sample is transferred from one vessel to another or if the filter fails to trap all microspheres.

4) The filters must be changed every time for each new sample. This may increase the expense.

5) The filtration process may proceed slowly as the filter might become clogged with microspheres or tissue debris.

4.3 Polyamide Woven Filtration Devices

These devices are specifically made to isolate fluorescent microspheres from CPD (citrate phosphate dextrose) anticoagulated blood or digested tissues [39]. Each tissue sample is digested, filtered and the fluorescent dyes are extracted in a single container. The devices are polypropylene and consist of three stages. Digestion is done with ethanolic KOH.

Disadvantages:

1) These devices offer limitations with heparin containing blood samples.

2) There may be microsphere loss when the tissue sample is transferred from one vessel to another or if the filter fails to trap all microspheres.

3) Digestive tissue samples should not stand unfiltered for a long period of time since the fat in them may solidify and may result in damage to the sample.

4) Filters need to be changed after every microsphere procedure.

4.4 Sedimentation

Sedimentation is the tendency for particles in suspension to settle out of the fluid in which they are entrained, and come to rest against a barrier. This is due to their motion through the fluid in response to the forces (gravitational force or centrifugal force or electromagnetic force) acting on them.

Sedimentation of microspheres based on centrifugal force (as in a centrifuge) is possible if the specific gravity of the solution is less than that of microspheres. Microspheres have a density of 1.05 g / ml, which is close to the density of red blood cells and myocardial tissue. Ethanolic KOH has a density on the order of 0.8 g / ml, which is much smaller compared to that of microspheres.

Sedimentation is a 7 day procedure. It is the most effective procedure and also it is cost effective. The chemical solutions can be easily prepared in the laboratory, and the polypropylene tubes used for the procedure are inexpensive and available in bulk. The steps in the procedure are summarized in Figure 4.1 After digesting the tissue with ethanolic KOH, the procedure consists of 3 successive stages of centrifugation interspersed by washes with specific solutions or buffers. As a final step, a chemical is added (2-ethoxy-ethyl-acetate) that dissolves the plastic microspheres and thus releases the fluorescent dye into solution. A final centrifugation (not shown in Figure 4.1) separates this solution from the remnants of the plastic microspheres. A sample of this final supernatant is then subjected to fluorometry to determine the number of microspheres.

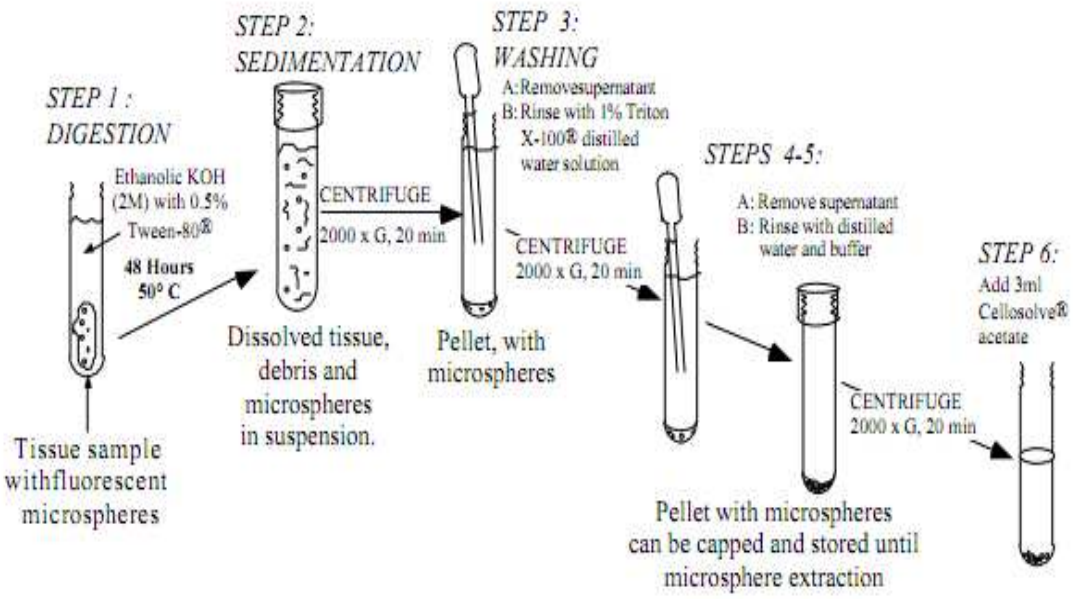

Figure 4.1 Sedimentation procedure for fluorescent microspheres [39]
(Source: Manual for using fluorescent microspheres to measure regional organ perfusion, accessed on March, 2011)

4.5 Importance of Accurate Fluid Volumes

The ratio of the number of microspheres contained in the heart tissue versus the blood sample (N_{heart} / N_{blood}) is very important for accurate determination of coronary blood flow. As per the procedure, 3ml of 2-ethoxy-ethyl-acetate is added to each heart and blood sample and then the samples are placed in the dark for 5 days. 2-Ethoxy-ethyl-acetate dissolves the microspheres, releasing the fluorescent dye from the microspheres. On the last day of the procedure, the samples are centrifuged and 100 microliters of the supernatant is then used for fluorometry.

The equation for the ratio is as follows:

$$\frac{(N_{heart})\dfrac{V_{sampled\ from\ heart}}{V_{dissolve\ heart\ spheres}}}{(N_{blood})\dfrac{V_{sampled\ from\ blood}}{V_{dissolve\ blood\ spheres}}} = \frac{N_{heart}}{N_{blood}} \qquad (4.1)$$

Calculating the values,

$$\frac{(N_{heart})\dfrac{V_{sampled\ from\ heart}}{V_{dissolve\ heart\ spheres}}}{(N_{blood})\dfrac{V_{sampled\ from\ blood}}{V_{dissolve\ blood\ spheres}}} = \frac{(N_{heart})\dfrac{0.1\ ml}{3\ ml}}{(N_{blood})\dfrac{0.1\ ml}{3\ ml}}$$

Measurement of coronary blood flow depends on the ratio of (N_{heart} / N_{blood}). The more accurate the ratio, the more accurate is the value of blood flow. Heart and reference blood samples from each rat were analyzed for fluorescence intensity measurement. In the sedimentation procedure, 3 ml of 2-ethoxy-ethyl-acetate were used for each sample. 2-ethoxy-ethyl-acetate releases fluorescent dye by dissolving the microspheres. Carefully pipetting out the 3 ml amount of 2-ethoxy-ethyl-acetate from the stock bottle is essential for accurate measurement. In our procedure, 3 ml was accurately removed from the bottle using an automated pipette. In addition, carefully extracting precisely 100 microliters of the supernatant containing the fluorescent dye is also essential. This amount was carefully pipetted out using a 200 microliter pipette. If the volume of 2-ethoxy-ethyl-acetate is not accurate, the concentration of fluorescent dye in the resulting supernatant

could vary, which would vary the number of microspheres obtained when 100 microliters of the supernatant is extracted for measurement. Similarly, the volume of supernatant must be reproducibly pipetted out, so that the ratio of the number of microspheres is the same as the ratio of dye concentrations in the supernatant fluids form tissue and blood samples.

4.6 Solutions Used in the Sedimentation Method

The six different solutions used throughout the sedimentation procedure are as follows:

1) 2.3 M Ethanolic KOH with 0.5 % Tween 80

100 ml of ethanol is added to the glass beaker containing a mixture of 3 gm KOH and 0.5 gm Tween 80. The solution is stirred continuously for approximately 20 minutes until a clear solution is obtained. Tween 80, a viscous and water soluble yellow fluid, is used as an emulsifier. KOH, since it is obtained in pellet form, needs to be stirred properly to ensure proper mixing. Freshly made KOH solution, warm due to the exothermic reaction, aids in tissue digestion.

2) Internal standard (This is optional)

The microspheres are vortexed and sonicated for 30 seconds and then 1ml of solution is immediately withdrawn using a sterile syringe. This 1ml of solution is added to 100 ml of 0.25% Tween 80. A magnetic stirrer is used to stir the solution continuously until its use.

3) 1% Triton X-100

10 grams of Triton X-100 is added to 1 liter of distilled water and stirred until in solution.

4) Distilled water phosphate buffer

5.88 grams of monobasic KH_2PO_4 is added to 200 ml distilled water. 22.9 grams of dibasic KH_2PO_4 is added to 800 ml distilled water. The two solutions are then mixed together. 28.6 ml of this combined solution is added to 1000 ml of distilled water and stirred until they mix really well. This produces a distilled water phosphate buffer, which is a solution used for rinsing.

5) 0.25 % Tween 80

2.5 grams of Tween 80 is added to 1 liter of distilled water and stirred continuously with a magnetic stirrer until in use.

6) 2 – Ethoxy-ethyl-acetate (also known as Cellosolve acetate)

This is a solution used to dissolve microspheres and release dye. 2–ethoxy-ethyl-acetate should not be confused with ethyl cellosolve, since that doesn't dissolve the microspheres.

4.7 Calculation of Centrifuge Rotation Rate

During the sedimentation method, a centrifugal acceleration equivalent to 2000 times the acceleration of gravity (i.e., 2000Gs) had to be achieved. The faster the centrifuge rotates, and the longer the radial distance to the sample being centrifuged, the stronger the centrifugal acceleration. The calculations below were performed to determine the rotation rate required to achieve 2000Gs for the specific centrifuge and sample tubes that were used in this research.

A centrifuge is a laboratory instrument driven by electric motor that puts an object in rotation around a fixed axis and thus induceses a centrifugal force perpendicular to the axis. The centrifugal acceleration generated by the instrument is such that it causes more dense substances to separate out along the radial direction at the bottom of the tube. The centrifuge consists of rotating units, called the rotor, which has its own specifications.

We used a Beckman Coulter 366802 – Allegra 6 Benchtop Centrifuge for our procedure. The centrifuge consists of GH–3.8 / GH–3.8A rotors which have the following specifications: 1) minimum radius = 86mm; 2) maximum radius = 204mm; 3) maximum allowable rotation rate = 3750 rpm.

During circular motion, the centrifugal acceleration is the product of the radius and the square of the angular velocity. The acceleration is generally measured in terms of "G", which is also called the relative centrifugal force. One "G" is equal to the acceleration due to gravity at the earth's surface, which equals 9.8 m/s^2 .

In the Beckman centrifuge that we used, the radius to the bottom of the centrifuge tube (where the denser sample will collect) was measured. We set the rotor to the minimum distance from the axis of the centrifuge. The radius to the edge of rotor thus was 8.6 cm. The polypropylene 15 ml centrifuge tubes that we used extended 10 cm beyond the rotor. Thus, the total radial length to the sample (r) = 18.6 cm.

In our procedure, we desired to create an acceleration of 2000 G's. The angular velocity (ω) required to produce this acceleration (A) was calculated as follows:

$$A = \omega^2 r$$

$$2000\ (9.8\ m/s^2) = \omega^2\ (0.186\ m)$$

$$\omega = 324.6\ rad / sec$$

Angular velocity converts to rotational frequency according to the following formula:

$$\omega = 2\pi f$$

Thus, the required rotational frequency f = 51.7 rotations/sec, which is 3100 rpm. This rotation rate is within the range of rotation rates allowed by the manufacturer's specifications.

4.8 Details of Sedimentation Procedure

The procedure takes a total of 8 days. The animal was euthanized on the same day soon after the microsphere injection procedure. The heart was removed, and the left ventricle was separated from the rest of the heart for recovery of fluorescent microspheres. Polypropylene 15 ml centrifuge tubes with tightly fitting caps were used.

Day 1

1) The tubes fitted with their caps and the tissue and blood samples were weighed. Each cap should be paired with its tube so that the weight of the capped tube is reliable.

2) 8 ml of ethanolic KOH was then added. Ethanolic KOH should be carefully poured into the tubes since outside spillage can remove writing on the tubes. Also, ethanolic KOH is extremely caustic in nature and can cause burns on skin.

3) The solution was vortexed for a period of 20 seconds.

4) The tubes were then incubated for a period of 48 hours at 50 degrees centigrade at a speed of 150 revolutions per minute.

Day 2

Each sample was removed from the incubator and vortexed for 20 seconds per tube. The samples were then placed back into the incubator to continue for another 24 hours.

Day 3

1) Each sample was removed from the incubator and vortexed for about 30 seconds until the particles were re-suspended. Just before the next step, each sample was vortexed again for 5 more seconds.

2) The samples were centrifuged at 3100 rpm for about 20 minutes.

3) All but 1.5 ml of supernatant fluid was removed using a Pasteur pipette attached to suction with a trap. This supernatant was discarded.

4) Approximately 8 ml of 1% Triton X-100 solution was added to each sample, and then each sample was vortexed for about 30 seconds.

5) The samples were centrifuged at 3100 rpm for about 20 minutes.

6) All but 1.5 ml of supernatant fluid was removed using a Pasteur pipette attached to suction with a trap, and the supernatant was discarded. Suction was done carefully to avoid loss of microspheres.

7) 7 ml of distilled of distilled water phosphate buffer was added to each tube, and each tube was vortexed for about 30 seconds until all particles were suspended in the solution.
8) The samples were centrifuged at 3100 rpm for about 20 minutes.

9) All but 1.5 ml of supernatant fluid was removed using a Pasteur pipette attached to suction with a trap, and the supernatant was discarded.

10) Exactly 3 ml of 2-ethoxy-ethyl-acetate was then added to each sample, and each was vortexed for about 30 seconds until the particles were well suspended in the solution.

11) The samples were then placed in a dark room away from light for 5 days.

Day 5

The samples were taken out from the dark room and vortexed for about 20 seconds each until the pellet broke up.

Day 8

1) The samples were removed from the dark room and vortexed vigorously for about 30 seconds each until the pellet broke up.

2) The samples were centrifuged at 3100 rpm for about 20 minutes.

3) Exactly 100 µL of supernatant fluid containing the dye released from the microspheres was then used for fluorometry.

4) The blood and tissue samples were then measured for fluorescence on the same day.

4.9 Advantages of the Sedimentation Method

1) Polypropylene 15 ml centrifuge tubes are used, which are available in bulk in a routine chemistry lab.

2) The tubes are inexpensive as compared to the filters.

3) The procedure although time consuming, is not labor intensive.

4) There is minimal loss of fluorescent microspheres, since they do not have to be transferred from one vessel to another.

5) The sedimentation procedure starts on the same day soon after the microsphere injection procedure. Consequently, there are no issues of digested tissues being left untreated.

CHAPTER 5

INITIAL MEASUREMENTS OF CORONARY BLOOD FLOW AND CORONARY RESERVE IN RATS USING FLUORESCENT MICROSPHERES

5.1 Summary of Experimental Protocol

Two male Sprague-Dawley rats, body weight 414 and 395 g, both age 12 weeks, were used for the microsphere injection procedure, as described in detail in Chapter 3. Briefly, each rat was cannulated with multiple catheters under a mixture of ketamine and xylazine anesthesia administered intramuscularly. One catheter was introduced through the right carotid artery into the left ventricle for injection of fluorescent microspheres and adenosine. The second catheter was introduced through the right femoral artery and advanced into the abdominal aorta for collection of the reference blood sample during injection of fluorescent microspheres into the circulation of the rat. The third catheter was introduced into the right jugular vein for infusion of saline following the withdrawal of each reference blood sample. After the 1.5 – 2 hour cannulation procedure, the rat was allowed to recover back to its normal hemodynamic condition.

Soon after the recovery, the rat was subjected to the microsphere injection procedure. Normal hemodynamic conditions were mandatory before proceeding with the microsphere procedure. Microspheres which fluoresced at two different colors were used for the procedure: (1) yellow-green microspheres were used for baseline measurements, and (2) red microspheres were used for measurements of coronary reserve made during infusion of a vasodilator (adenosine). During both microsphere injections, reference blood containing a sample of the injected microspheres was collected at a preset rate of

0.5 ml/min for duration of 2 min. Table 5.1 presents the timeline of the experimental procedures applied during a typical microsphere injection procedure (Rat 2 in this case).

Table 5.1 Timeline of a typical experiment

ELAPSED TIME (min:sec)	**PAGE** (from datafile)	**EVENT**
00:00	1	Start data recording after finish instrumenting the rat
05:19	**72**	Baseline data before injection of yellow-green microspheres
05:29	**74**	Begin arterial blood withdrawal for reference blood flow
05:48	**78**	Start injection of yellow-green microspheres into LV
07:29	---	End arterial blood withdrawal for reference flow
		10 minute recovery period following first microsphere injection
16:06	**153**	End recovery period; Begin adenosine infusion
16:26	157	Adenosine begins to affect global hemodynamic measurements
24:38	**268**	Steady-state data recorded under effect of adenosine
24:59	272	Begin arterial blood withdrawal for reference flow with adenosine
25:30	279	Start injection of red microspheres into LV
26:59	- ---	End arterial blood withdrawal for reference flow
		5 minute recovery period following microsphere injection
31:40	287	Last data recorded after recovery from second microspheres

NOTES:
(1) Elapsed time began at 09:55:26 am on May 25, 2011. This was experiment #2.
(2) Page numbers shown in bold are included as figures in this chapter.

Hemodynamic parameters were recorded throughout the entire procedure. Heart rate, arterial and left ventricular pressures, and the rate of rise of left ventricular pressure (dp/dt) were monitored during baseline conditions and following vasodilation during adenosine infusion.

Figure 5.1 presents the strip chart record of left ventricular pressure (LVP), abdominal aortic pressure (MAP) and the derivative of LVP (dP/dt) during the baseline state before injection of any microspheres. In Figure 5.2, data was recorded when the arterial catheter was disconnected from the pressure transducer and switched to the pump that withdraws the reference blood flow, leaving the arterial pressure transducer open to atmosphere (MAP = 0). During the time period shown in Figure 5.3, the left ventricular catheter was disconnected from its pressure transducer and switched to the infusion syringe, so that the infusion of microspheres into the left ventricle began. Note that the withdrawal of the reference flow continued (MAP = 0).

After allowing 10 minutes for the cardiovascular system to re-stabilize following the initial microsphere injection, Figure 5.4 shows a strip chart record of the hemodynamic conditions just before the adenosine infusion began. The steady adenosine infusion continued at a rate of 0.15 mg/min per kilogram of animal mass. Because adenosine exerts a vasodilator influence throughout the systemic vasculature, arterial and ventricular systolic pressures were lower when a steady state was eventually achieved after 8 minutes, as shown in Figure 5.5.

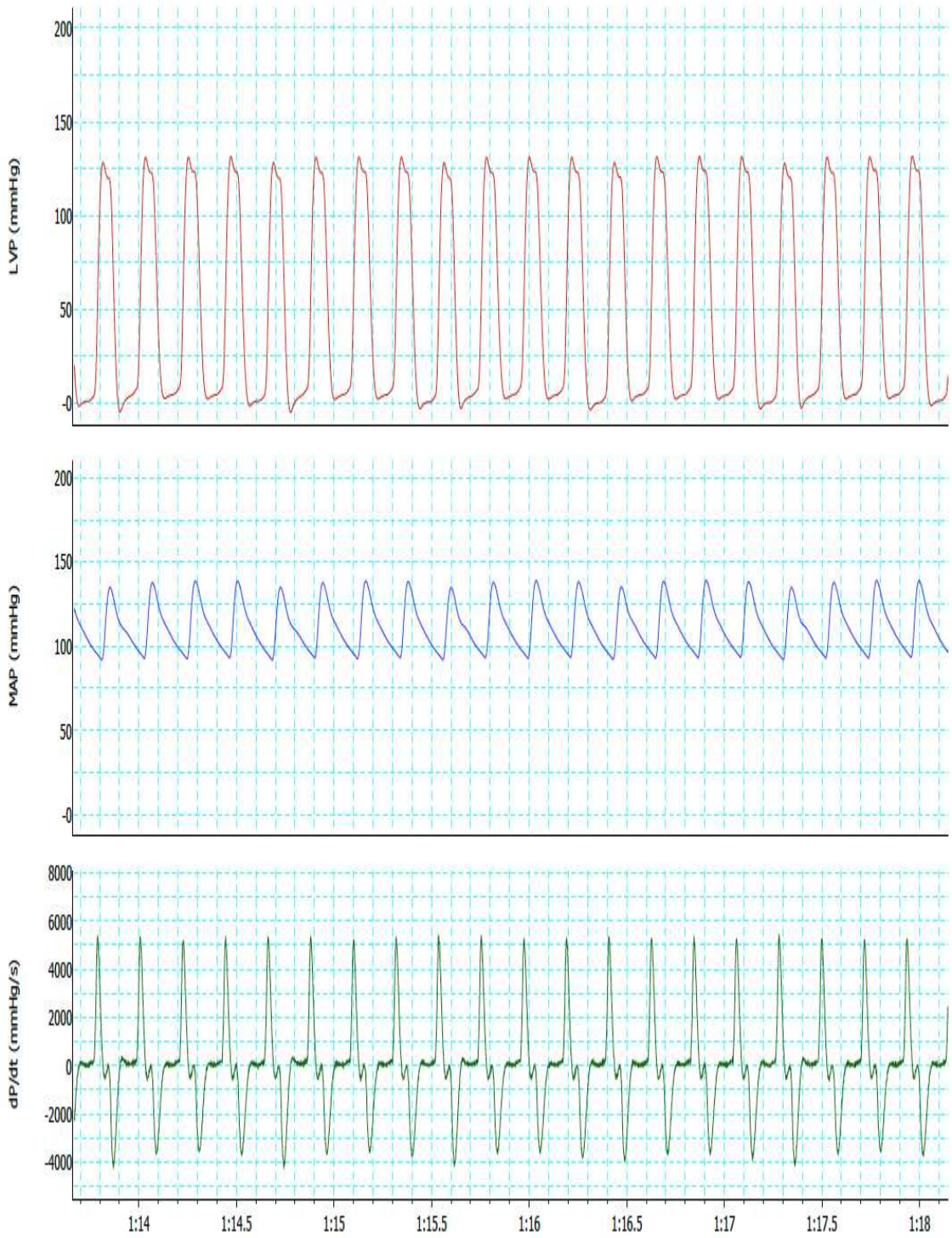

Figure 5.1 Hemodynamic parameters at baseline in Rat 2.

The x-axis shows time in min: sec. Each division = 0.1 sec.

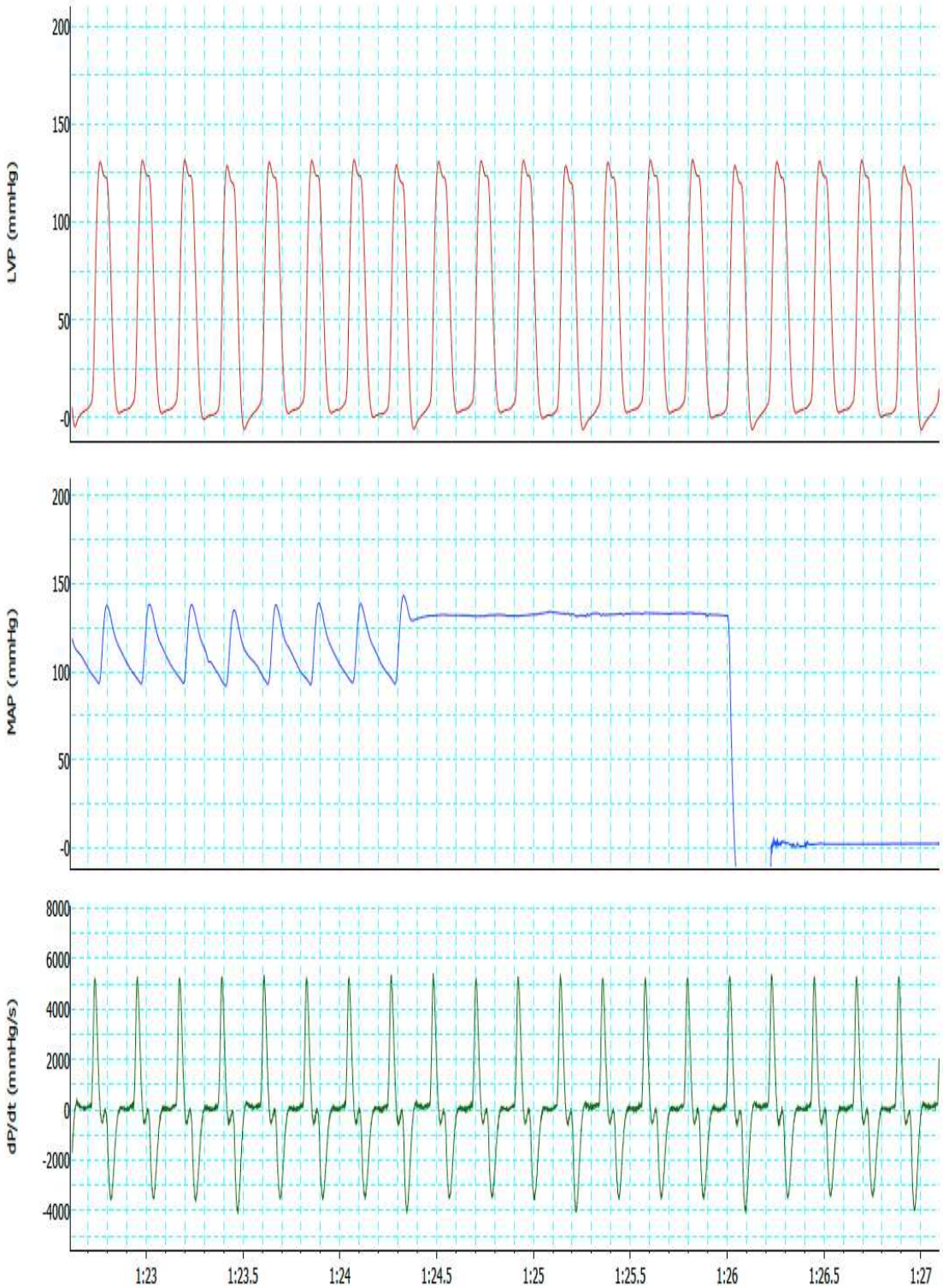

Figure 5.2 Hemodynamic parameters during the beginning of reference blood flow withdrawal under baseline conditions.

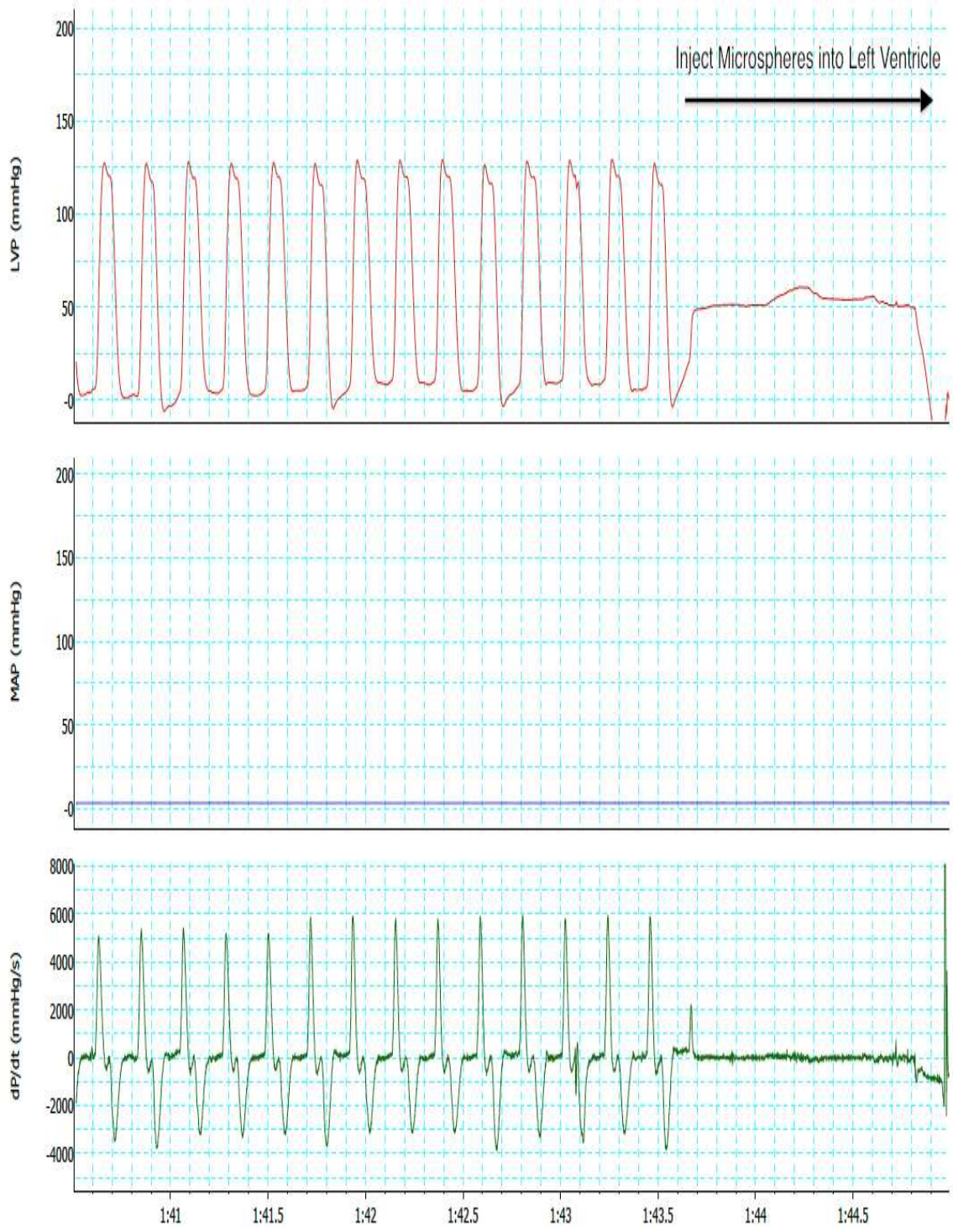

Figure 5.3 Hemodynamic parameters at beginning of injection of yellow-green microspheres into left ventricle.

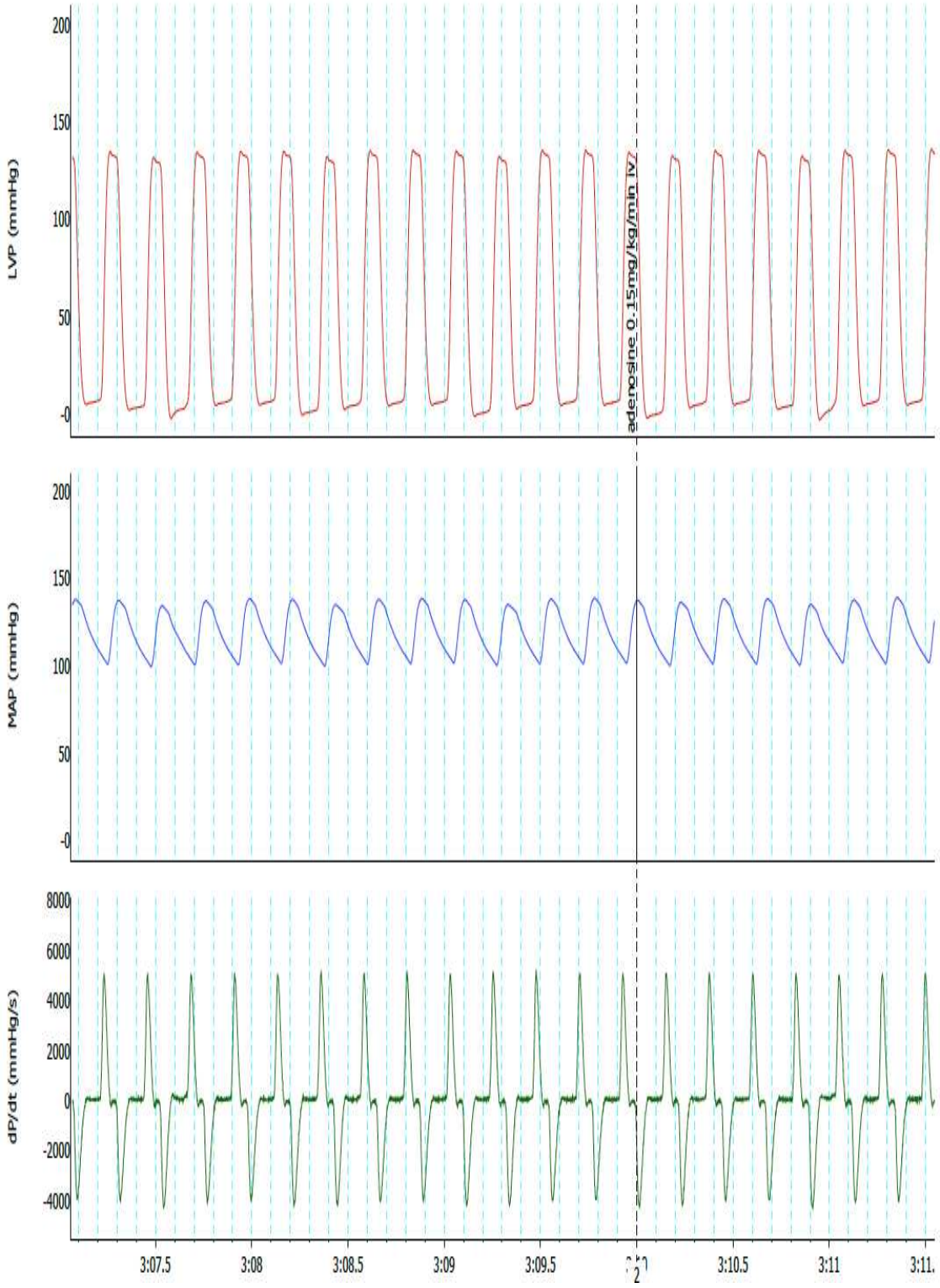

Figure 5.4 Hemodynamic parameters after re-stabilization before infusion of adenosine

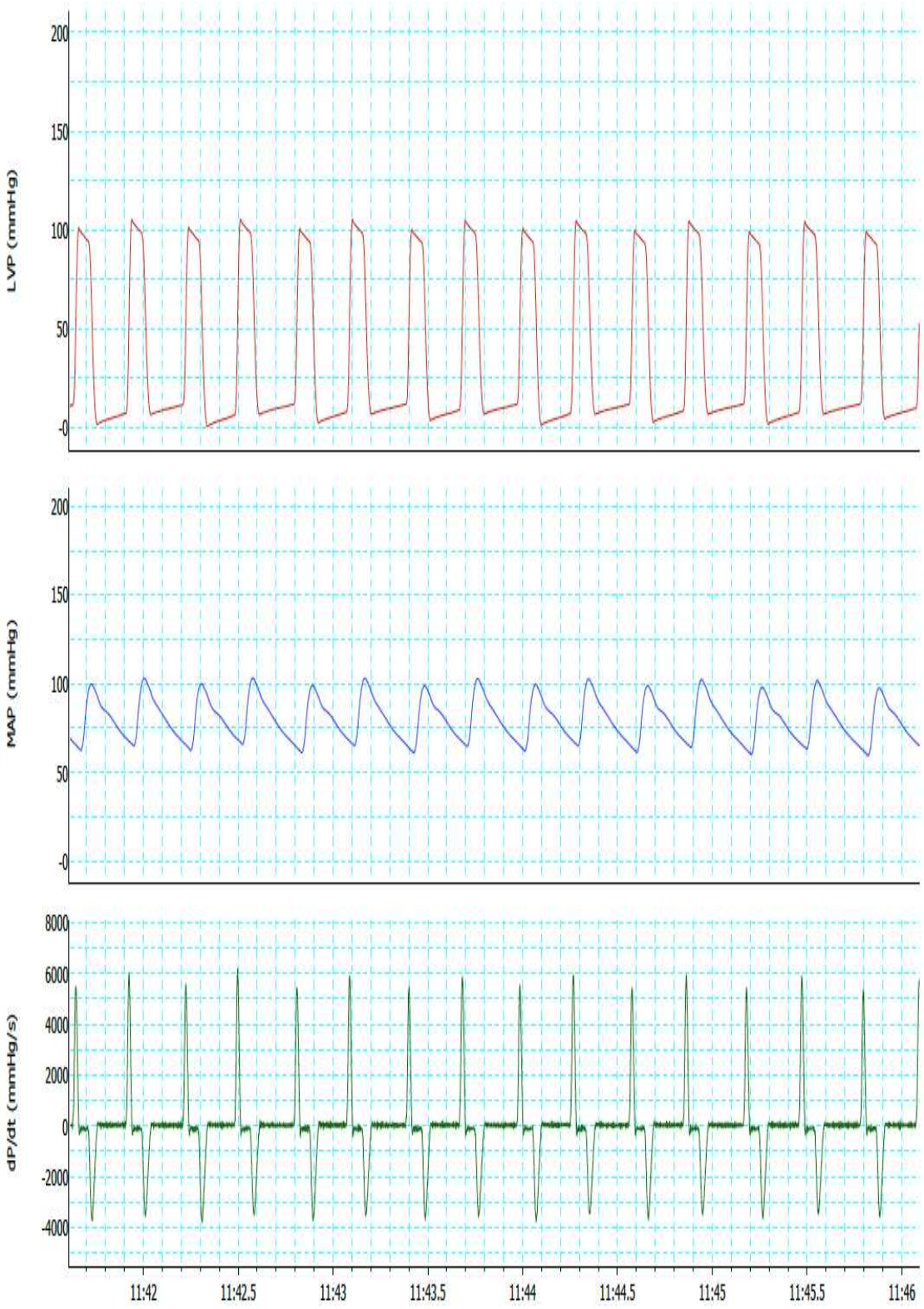

Figure 5.5 Hemodynamic parameters after attaining steady state under adenosine

Tables 5.2 and 5.3 summarize the hemodynamic conditions observed during microsphere injections in Rats 1 and 2. The baseline conditions were both within the normal range for rats, although Rat 1 was slightly hypotensive, while Rat 2 was slightly hypertensive. As expected, infusion of the vasodilator adenosine reduced vascular peripheral resistance, so that mean arterial pressures fell by ~25% in both cases. This percentage decline in arterial pressure is within the range typically seen during adenosine infusion in rats [22]. The decline in left ventricular dp/dt in Rat 1 was somewhat atypical compared to what is commonly reported during adenosine infusion in rats [22]. The fall in heart rate in Rat 2 was also somewhat atypical.

Table 5.2 Hemodynamic conditions during microsphere injections in rat 1

Rat 1	Heart Rate (bpm)	Peak Left Ventricular Pressure (LVP) (mmHg)	Maximum Rate of Rise of LVP (mmHg/s)	Arterial Systolic Pressure (mmHg)	Arterial Diastolic Pressure (mmHg)	Arterial Mean Pressure (mmHg)
Baseline Condition	279	107	7254	109	74	86
During Adenosine Infusion	283	81	4731	85	54	64

Table 5.3 Hemodynamic conditions during microsphere injections in rat 2

Rat 2	Heart Rate (bpm)	Peak Left Ventricular Pressure (LVP) (mmHg)	Maximum Rate of Rise of LVP (mmHg/s)	Arterial Systolic Pressure (mmHg)	Arterial Diastolic Pressure (mmHg)	Arterial Mean Pressure (mmHg)
Baseline Condition	275	131	5330	131	92	105
During Adenosine Infusion	203	101	5623	100	63	79

After the microsphere injection procedures, the rats were euthanized with an overdose of anesthetic. The chest was opened and the heart was removed. The atria, great vessels, and right ventricular free wall were all trimmed away, so that only the left ventricle (including septum) remained. This heart tissue and the 2 blood samples were then further processed by the procedure explained in Chapter 4 to recover the fluorescent microspheres from the samples. Briefly, the samples were digested in 2.3 M ethanolic KOH and 0.25 % Tween 80 for a period of 48 hours. At the end of digestion, microspheres were recovered by the sedimentation method and the fluorescent dye was extracted from them by using 3 ml of 2-ethoxy-ethyl-acetate.

5.2 Measurement of Microsphere Number in Tissue and Blood Samples

5.2.1 Measurement of fluorescence intensity

On the last day of the sedimentation procedure, the samples were read for fluorescence intensity. Since microspheres of two different colors were used in the procedure, samples were read at two different (excitation / emission) wavelengths. By selecting filters in the spectrophotometer instrument, samples containing yellow-green microspheres were read at (485 nm / 530 nm) (excitation / emission) wavelengths, and samples containing red microspheres were read at (590 nm/ 645 nm) (excitation / emission) wavelengths. After setting the filters, the samples were placed in a 96 well plate reader and then scanned to measure fluorescence intensity (I_f). The intensities measured are reported in Tables 5.4 and 5.5.

5.2.2 Calculation of number of fluorescent microspheres from calibration curves

The standard curves for fluorescent microspheres as obtained in Chapter 2 were expressed by the following linear relationships:

For yellow-green microspheres: $I_f = 0.9256\ (N_s) - 4.9102$

For red microspheres: $I_f = 1.0954\ (N_s) + 0.8414$

where, I_f = fluorescence intensity obtained from spectrophotometer

N_s = number of fluorescent microspheres

Using these equations for the standard curves and knowing the values of fluorescence intensity from the spectrophotometer, the number of fluorescent microspheres can be determined.

To determine the number of yellow green microspheres in both blood and tissue samples:

$$N_s = (I_f + 4.9102) / 0.9256$$

To determine the number of red microspheres in both blood and tissue samples:

$$N_s = (I_f - 0.8414) / 1.0954$$

These inverse expressions of the standard curves were used to infer the number of microspheres in each sample (N_s) from the measured value of fluorescence intensity (I_f). The results for all samples from Rats 1 and 2 are shown in Tables 5.4 and 5.5.

Table 5.4 Number of fluorescent microspheres in blood and tissue samples in rat 1

Rat 1	Sample	I_f	N_s
Baseline : yellow-green microspheres	Tissue	16782	18136
Baseline : yellow-green microspheres	Blood	2007	2174
Adenosine : red microspheres	Tissue	50438	46045
Adenosine : red microspheres	Blood	2748	2508

Table 5.5 Number of fluorescent microspheres in blood and tissue samples in rat 2

Rat 2	Sample	I_f	N_s
Baseline : yellow-green microspheres	Tissue	18943	20471
Baseline : yellow-green microspheres	Blood	2128	2304
Adenosine : red microspheres	Tissue	56700	51761
Adenosine : red microspheres	Blood	2969	2710

5.3 Calculation of Coronary Blood Flow and Coronary Flow Reserve

Coronary blood flow delivers oxygen rich blood to heart muscle. The amount of blood flow increases in proportion to the amount of heart muscle. For this reason, coronary blood flow is expressed as flow per gram of tissue (ml/min/g). Microspheres serve as a tracer of blood flow, so that the relative amounts of blood flow in two tissue samples are proportional to the relative numbers of microspheres in each tissue. Thus,

$$\frac{\text{Blood flow to heart}}{\text{Reference blood flow}} = \frac{\text{Number of microspheres in heart sample}}{\text{Number of microspheres in blood sample}}$$

Given that the reference blood flow was withdrawn at 0.5 ml/min, the following formula was used to calculate the coronary blood flow:

$$\text{Coronary Blood Flow} = \frac{\text{Blood flow to heart}}{\text{Mass of heart}} = \frac{N_s \text{ in heart sample}}{N_s \text{ in blood sample}} \bullet \frac{0.5 \quad \text{ml/min}}{\text{mass of heart sample}}$$

where N_s is the measured number of microspheres. The mass of the left ventricle sample was 0.734 g for rat 1 and 0.718 g for rat 2.

Applying this formula to the data from each rat:

For Rat 1:

Baseline coronary flow: (18136 / 2174) * (0.5 / 0.734) = 5.68 ml/min/g

Adenosine coronary flow: (46045 / 2508) * (0.5/ 0.734) = 12.5 ml/min/g

For Rat 2:

Baseline coronary flow: (20471/2304) * (0.5 / 0.718) = 6.19 ml/min/g

Adenosine coronary flow: (51761/2710) * (0.5 / 0.718) = 13.3 ml/min/g

Coronary flow reserve (CFR) is the ratio of the maximum blood flow through the coronary arteries (when they are maximally vasodilated) compared to the normal coronary blood flow when the animal is resting. Coronary flow reserve is used in diagnostics and treatment of patients suffering from conditions such as coronary artery disease. In the treatment of these conditions, vasodilators such as nitroglycerine are used to increase the rate of blood flow through the coronary arteries, and the measurement of CFR enables the efficacy of such interventions to be measured. When coronary flow reserve is used in medicine, it is usually expressed as a dimensionless number, which is

formed by dividing the maximal coronary blood flow by resting blood flow. This allows for an objective view, which can aid diagnosis and treatment.

In the microsphere studies, coronary flow reserve was calculated as:

Coronary flow reserve = $(\text{Coronary blood flow})_{\text{adenosine}} / (\text{Coronary blood flow})_{\text{basal}}$

For rat 1:

Coronary flow reserve = $(\text{Coronary blood flow})_{\text{adenosine}} / (\text{Coronary blood flow})_{\text{basal}}$

$= 12.5 / 5.68$

$= \mathbf{2.20}$

For rat 2:

Coronary flow reserve = $(\text{Coronary blood flow})_{\text{adenosine}} / (\text{Coronary blood flow})_{\text{basal}}$

$= 13.3 / 6.19$

$= \mathbf{2.15}$

5.4 Comparison with Coronary Flow Reserve Measured using Doppler Echocardiography

Coronary flow reserve was also measured using Doppler echocardiography in a set of 6 male Sprague-Dawley rats of the same age and range of body weights as the two rats used in the microsphere study. These rats were also anesthetized using the same method as in the microsphere study. Since the rats in both studies were of the same strain, age, gender,body mass, and anesthetic state, it was reasonable to assume that they should have similar coronary flow reserves.

However, there is one essential difference between the microsphere flow measurement and the ultrasound measurement: Doppler echocardiography measures the velocity of blood movement (in mm/s), not the bulk flow of blood volume (in ml/s). However, if the cross-sectional area of the blood vessel being imaged does not change

significantly between the normal and vasodilated state, then the ratio of blood flow between vasodilated and normal states must be the same as the ratio of the velocity of blood movement. Thus, if vessel area remains constant, the non-dimensional value for coronary flow reserve should be the same when measured by the ratio of velocities or by the ratio of bulk flows.

Since the vasodilator that we used (adenosine) primarily relaxes smooth muscle in the arterioles, it may not cause a significant relaxation of smooth muscle in the walls of the major, large coronary arteries. If such relaxation did occur, then we would expect the coronary reserve measured by echo Doppler (velocity) to be less than the coronary reserve measured by microspheres (bulk flow), because vessel area ratio would be > 1:

$$\frac{\text{Bulk Flow}_{\text{vasodilated}}}{\text{Bulk Flow}_{\text{normal}}} = \frac{\text{Vessel Area}_{\text{vasodilated}}}{\text{Vessel Area}_{\text{normal}}} \bullet \frac{\text{Velocity}_{\text{vasodilated}}}{\text{Velocity}_{\text{normal}}}$$

Table 5.6 summarizes the results of the measurements of coronary flow reserve (CFR) by Doppler echocardiography. These measurements were carried out by assistant professor Shumin Gao, MD/PhD, of the Department of Cell Biology and Molecular Medicine at UMDNJ-Newark. The velocity of blood motion was measured in the left coronary artery of rats during the normal baseline state and after infusion of adenosine at the same rate (0.15 mg/min/kg) as used in the microsphere study.

As Table 5.6 shows, the average coronary flow reserve determined by Doppler echocardiography was 2.22 ± 0.14 (mean ± SEM). This value was statistically indistinguishable from the measurement of coronary flow reserve found using fluorescent microspheres. That is, the values for CFR from microspheres (2.15 and 2.2) both fell within the range of mean ± standard-error-of-the-mean from Doppler measurements. Additionally, the fact that the Doppler velocity ratio equaled or exceeded the bulk flow

measure was consistent with the assumption that large vessel area did not change significantly with adenosine, since that would have made the velocity ratio < bulk ratio.

Table 5.6 Measurement of coronary flow reserve using Doppler echocardiography

Rat ID Code	Baseline Coronary Artery Velocity (mm/sec)	Vasodilated Coronary Artery Velocity (mm/sec)	Coronary Flow Reserve (dimensionless)
SDB-7 4w-sham	251.5	435.8	1.73
SDB-8 4w-sham	405.1	805.4	1.99
SDB-9 4w-sham	337.3	823.6	2.44
SDB-10 4w-sham	318.5	859.3	2.70
SD-CR-1	311.5	680.6	2.18
SD-CR-2	450.4	1020.3	2.27
mean	**345.7**	**770.8**	**2.22**
SD	71.2	197.2	0.34
SEM	29.1	80.5	0.14

CHAPTER 6

DISCUSSION AND FUTURE WORK

The present study shows that fluorescent microspheres can be used to reliably determine coronary blood flow in rats under baseline conditions and after infusion with adenosine. There were no significant differences observed in coronary flow reserve measured with either Doppler echocardiography or fluorescent microspheres. In addition, the absolute value of baseline coronary flow obtained in this study agreed well with the value published in a recent study on rats [22].

Fluorescent microspheres were used in this study. This technique has thus been demonstrated to be a reliable alternative to radioactive or colored microspheres for measuring organ blood flow in rats under normal conditions and under infusion with adenosine. In the present study, supernatants and pure microsphere samples were analyzed from each step of the tissue recovery protocol, which suggested that there was 100% recovery of microspheres regardless of mass and blood flow capacity. The sedimentation method for the recovery of fluorescent microspheres was achieved by use of ethanolic solutions, which increased the specific gravity difference between microspheres and medium, aided by higher centrifugation speeds that avoided the loss of fluorescent microspheres.

The resolution of the fluorescence method is dependent on the amount of label per microsphere, the quantum efficiency of the fluorescent dye, and the sensitivity of the spectrophotometer. The latter is determined by light source intensity, transmittance of filters or monochromators used to select excitation and emission wavelengths, efficiency of photomultiplier tube, and the type of plate reader used. By using optimal excitation

and emission wavelengths, there was very good correlation between fluorescence intensity and number of microspheres across a wide range of concentrations.

Although we had crimson fluorescent microspheres as an option along with the red and yellow-green microspheres that we used, we did not use crimson because of its low quantum efficiency and significant spillover into adjacent colors at higher concentrations.

When two different colors of fluorescent microspheres were injected into the body of a rat under baseline conditions and under infusion of adenosine, there was a good relation between estimated blood flow across a wide range of tissue blood flow. Fluorescent microspheres produced no significant alteration in blood pressure during or after injection in rats. Use of red and yellow-green microspheres resulted in no spillover in the course of this study.

A dilution procedure was performed using known microsphere samples to obtain the calibration curve of fluorescence intensity versus microsphere number. Several trials of the dilution procedure were performed on each of the two microsphere colors. A linear regression equation was obtained for each graph plotted. These regressions were then averaged to obtain an standard curve for each fluorescent microsphere color that we used. Averaging increased the accuracy of the calibration thus resulting in a more reliable final value of coronary blood flow.

Because of the small blood volume in rats, we limited our investigation to two successive measurements by taking two reference blood samples each of 1 ml. We calculated that – compared to the weight of the animal – this volume of reference blood withdrawn was less than 10% of the total blood volume in each rat, which should not

have affected the hemodynamics of the animal. In addition, after each reference blood withdrawal, saline was influsedinto the rat which also helped to restore blood volume.

Our study demonstrates that the fluorescent microsphere method is able to accurately reflect the increase in flow that occurs in rats after infusion of a coronary vasodilator, adenosine. Adenosine infusion produced nearly identical increases in myocardial blood flow (measured by fluorescnt microspheres) and in coronary blood velocity (measured by Doppler echocardiography). The mean coronary blood flow reserve obtained by the fluorescent microsphere method was 2.18, which was within the standard estimate of the value for coronary flow velocity reserve obtained by the range-gated Doppler echocardiography method.

As reported in a recently published article [22], the value of mean myocardial blood flow in Sprague Dawley rats at baseline and after infusion with adenosine, were 5.9 +/- 2.3 (ml/min/g) and 13.1+/-2.1(ml/min/g), respectively. In our study, using the same species of rat and the same coronary vasodilator, the values obtained at baseline and after infusion with adenosine were 5.94 and 12.9 (ml/min/g), respectively. Thus, the values we observed in both conditions (baseline + vasodilated) fall within the 95% confidence interval of this recently reported data. This suggests that the procedures developed in this thesis for microsphere recovery (by the sedimentation method) and fluorescence measurement and calibration offer a reliable method for determining coronary blood flow in rats.

Colored microspheres are currently being used to determine coronary blood flow in rats in the laboratories of the Cellular Biology and Molecular Medicine Department at UMDNJ, Newark. We were thus able to collaborate with current expertise

in microsphere injection procedures, and this collaboration undoubtedly improved the reliability of our results compared to what we would have been able to achieve independently. However, the samples of tissue and blood containing colored microspheres are currently sent out to a commercial processor to determine the number of microspheres and hence coronary blood flow. The techniques applied in this thesis are thus new to this laboratory and offer a reasonable alternative to commercial processing to measure coronary blood flow.

One drawback of our study is that only two animals were used for the procedure. This was due to the busy schedule of the collaborators at UMDNJ and the limited time window in which this thesis had to be completed. However, the quality of agreement among the two studies that were performed – and their agreement with recent data reported in the literature – suggests that a larger study might have even more strongly supported the repeatability of the results.

In the future, it would be important to see whether the fluorescent microsphere method could also be applied to measure coronary blood flow in mice. We attempted two experiments on mice, but we experienced failure during the cardiac cannulation procedure. It appeared that the catheter used was too large in diameter and thus too stiff to be able to be guided into the left ventricle of mice. Use of a smaller diameter catheter might offer easier access to the left ventricle, however one would also have to evaluate whether microspheres could be injected at an adequate rate through such a smaller diameter. Also, due to the very small blood volume of a mouse, it will be more difficult to obtain two reference blood samples without affecting the hemodynamics of the mouse. Finally, to achieve accurate measurements [38] sufficient numbers of microspheres will

have to be injected so that at least 400-500 spheres will be trapped in the left ventricular tissue (which typically has a mass of only 80-100 micrograms) and in each reference blood sample.

Note that during the dilution procedure to obtain the calibration curves we used whole microspheres from the stock solution (with the fluorescent dye remaining within the microspheres). On the other hand, during the actual procedure after microspheres were recovered from the body of animal, the microspheres were dissolved chemically to release the dye into solution. We believe this is an adequate calibration procedure because only the ratio of fluorescent intensities matters in the determination of blood flow. Because our calibrations showed that fluorescent intensity was linearly related to microsphere number throughout the range of interest, the ratio of intensities will be the same as the ratio of numbers. Each microsphere dye might produce more intensity when free in solution because of limits to excitation and absorption of emitted light imposed when the dye remains inside a microsphere. However, we assumed that this effect would apply equally to both the tissue and reference blood samples. Thus, the ratio of fluorescent intensity would not be affected by whether the dye was in solution or remained within the microspheres.

In conclusion, the fluorescent microsphere technique (a) is a reliable method for measuring coronary blood flows in rats; (b) works well as a substitute for radioactive or colored microspheres, with advantages in safety and cost; and (c) provides repeatable measurements allowing at least two flow measurements in the same animal.

APPENDIX I
DETAILS OF CALIBRATION PROCEDURE

A dilution procedure was used to plot the calibration curve, each for yellow green microspheres and red microspheres, operating at different (Excitation/ Emission) wavelengths.

The procedure is as follows:
1) Vortex the bottle containing fluorescent microspheres using vortex mixer. Vortexing allows uniform mixing of the solution thus preventing aggregation and clumping of microspheres within the solution.

2) Pull out 100µl or 200µl well agitated microspheres from the original microsphere containing bottle using 1ml plastic syringe.

3) Transfer microspheres from syringe into a small cuvette carefully at the bottom of chamber.

4) Vortex the cuvette to prevent sticking and clumping of microspheres within the solution and around the walls of cuvette.

5) Immediately withdraw calibrated 50µl of sample using 200µl pipette.

6) Add 450µl of (1X PBS + 2% Tween 80) solution to 50µl of microspheres. This makes a total of 500 µl @ 100 (spheres / µl)

7) Take 100 µl of above solution and put it in a 96 well container. This is the test for 10,000 microspheres.

8) After 100 µl of solution is removed, 400 µl of solution remains.

9) Perform 2:1 dilution on 400 µl solution by adding 400 µl of (1X PBS + 2% Tween 80) solution to the above 400 µl solution.

10) This make a total of 800 µl solution. Take 100 µl of above solution and put it in a 96 well container. This is the test for 5,000 microspheres.

11) Out of 700 µl solution still remaining, take 500 µl of solution in a different cuvette.

12) Add 500 µl of (1X PBS + 2% Tween 80) solution in above 500 µl solution.

13) Vortex sample and immediately withdraw 100 µl solution and put it in a 96 well plate. This is the test for 2500 microspheres.

14) Out of 900 µl solution still remaining, take 500 µl of solution in a different cuvette.

15) Add 500 µl of (1X PBS + 2% Tween 80) solution in above 500 µl solution.

16) Vortex sample and immediately withdraw 100 µl solution and put it in a 96 well plate. This is the test for 1250 microspheres.

17) Out of 900 µl solution still remaining, withdraw 500 µl of solution in a different cuvette.

18) Add 500 µl of (1X PBS + 2% Tween 80) solution in above 500 µl solution.

19) Vortex sample and immediately withdraw 100 µl solution and put it in a 96 well plate. This is the test for 625 microspheres.

20) Out of 900 µl solution still remaining, withdraw 500 µl of solution in a different cuvette

21) Add 500 µl of (1X PBS + 2% Tween 80) solution in above 500 µl solution.

22) Vortex sample and immediately withdraw 100 µl solution and put it in a 96 well plate. This is the test for 312 microspheres.

23) The 96 well plate is then placed in a plate reader to determine the fluorescence intensity.

Depending on the color of fluorescent microspheres, the (Excitation / Emission) wavelengths are set in the instrument and then a scan is carried out to determine the fluorescence intensity.

APPENDIX II

CALIBRATION CURVES FOR FIVE TESTS PERFORMED ON EACH MICROSPHERE COLOR

A dilution procedure as described in Chapter 2 was performed five times with each microsphere color to get an average calibration curve. With each trial, the readings were recorded on spectrophotometer and graphs were plotted on Microsoft Excel. The intercept equations for each trials were then averaged together to get a standard calibration curve. Yellow- green microspheres were used for getting a calibration curve which can be used for baseline calculations. Red microspheres were used for getting a calibration curve which can be used for vasodilation calculations.

Trials done with yellow- green microspheres:

Sample 1:

Table 2.2 Representation of fluorescent intensities at various dilutions

Microsphere number	Fluorescence Intensity
10000	11363
5000	5964
2500	2808
1250	1407
625	717
312	318

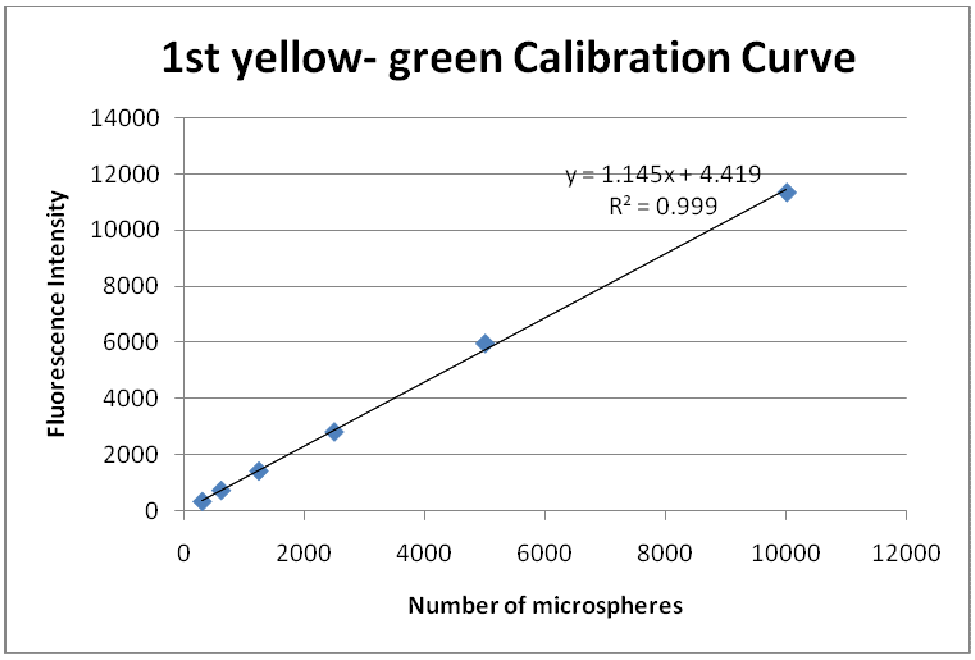

Figure 2.4 Excel plots showing the 1st baseline calibration curve

Sample 2:

Table 2.3 Representation of fluorescent intensities at various dilutions

Microsphere number	Fluorescence Intensity
10000	9214
5000	4400
2500	2150
1250	1000
625	550
312	300

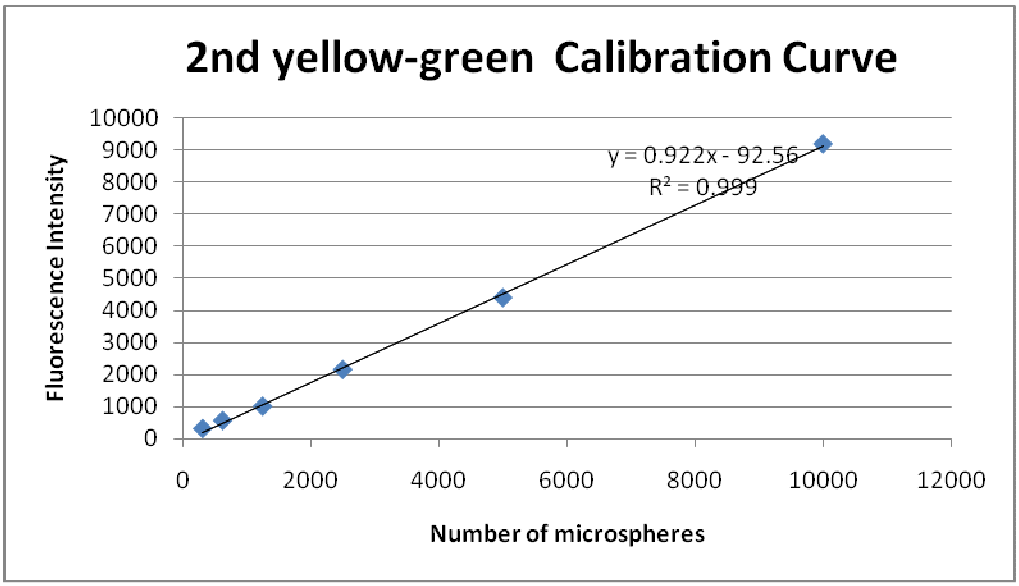

Figure 2.5 Excel plots showing the 2nd baseline calibration curve

Sample 3 :

Table 2.4 Representation of fluorescent intensities at various dilutions

Microsphere number	Fluorescence Intensity
10000	8683
5000	4288
2500	2100
1250	1001
625	528
312	300

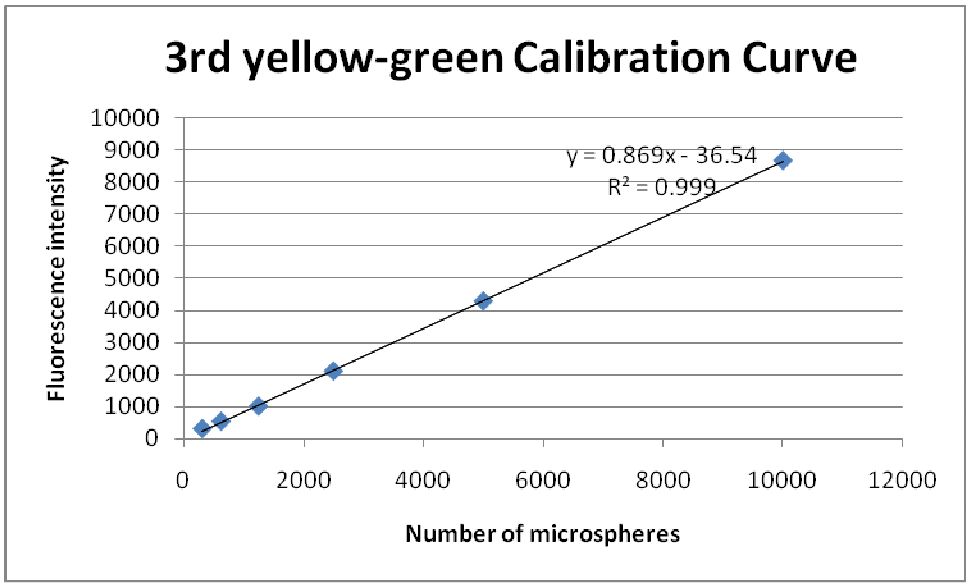

Figure 2.6 Excel plots showing the 3rd baseline calibration curve

Sample 4:

Table 2.5 Representation of various fluorescent intensities at various dilutions

Microsphere number	Fluorescence Intensity
10000	9110
5000	4792
2500	2000
1250	1068
625	629
312	308

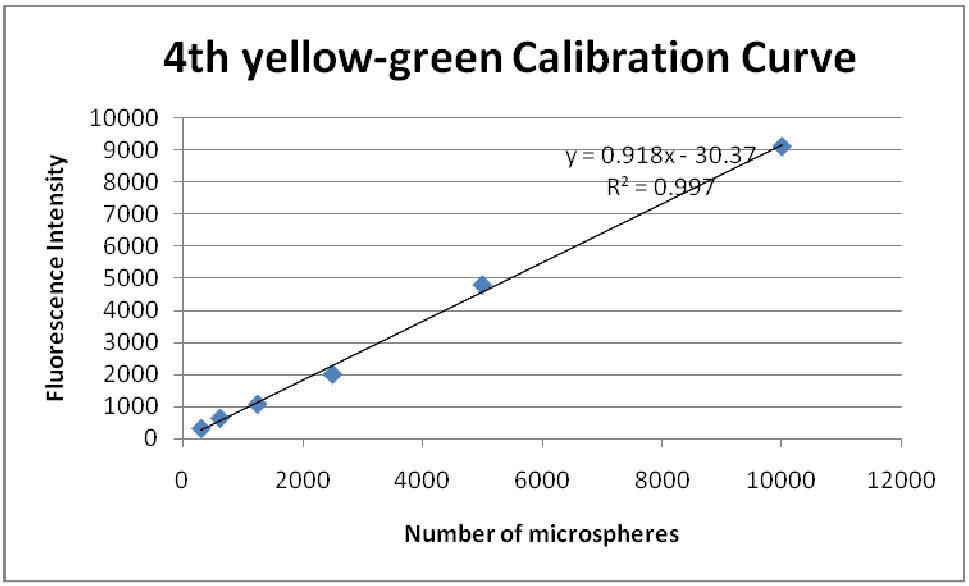

Figure 2.7 Excel plots showing the 4th baseline calibration curve

Sample 5:

Table 2.6 Representation of various fluorescent intensities at various dilutions

Microsphere number	Fluorescence Intensity
10000	7752
5000	4245
2500	2039
1250	1200
625	501
312	284

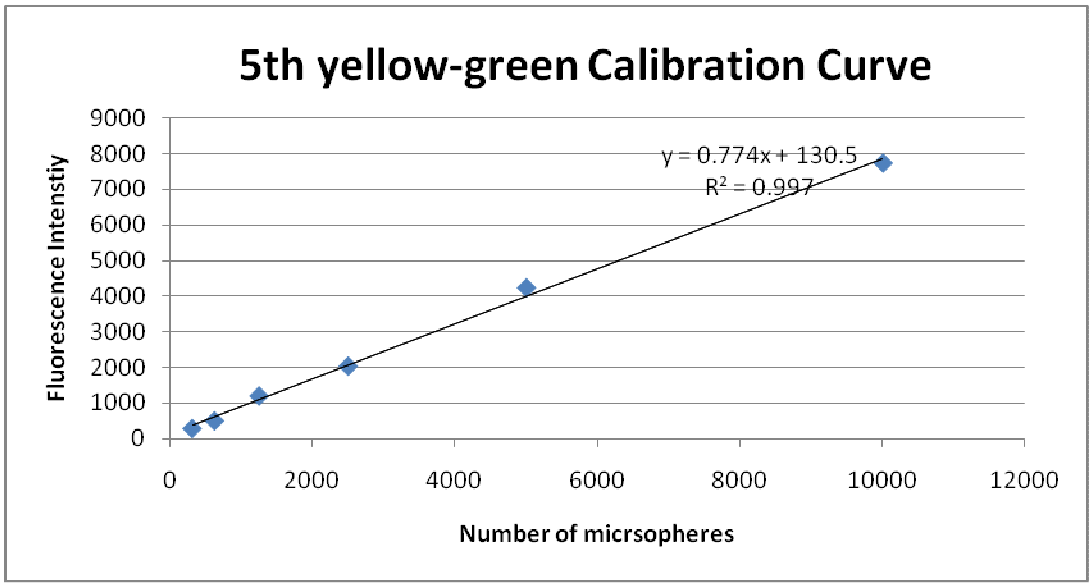

Figure 2.8 Excel plots showing the 5th baseline calibration curve

Trials done with red microspheres:

Sample 1:

Table 2.7 Representation of various fluorescent intensities at various dilutions.

Microsphere Number	Fluorescence Intensity
10000	11057
5000	5237
2500	2701
1250	1389
625	604
312	204

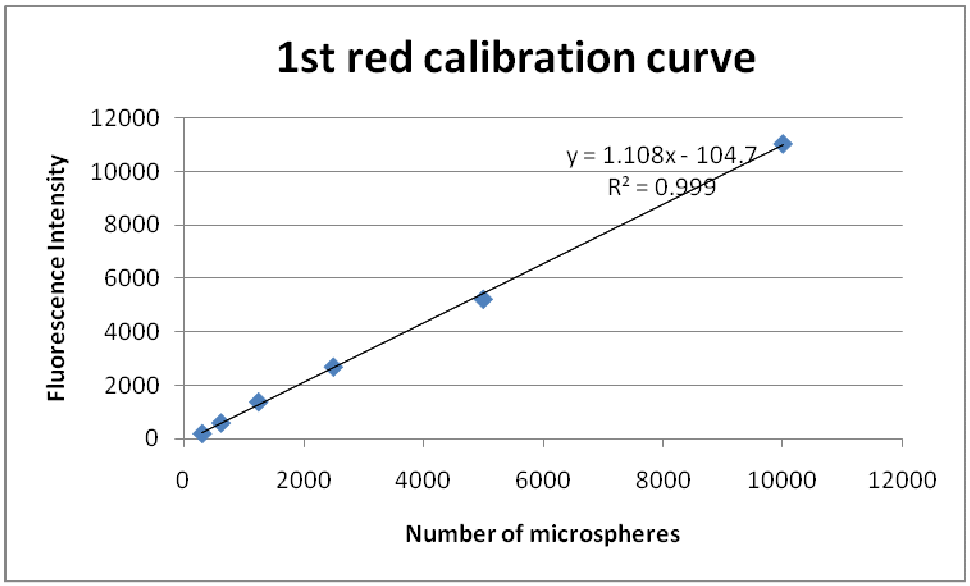

Figure 2.9 Excel plots showing the 1st vasodilation calibration curve

Sample 2:

Table 2.8 Representation of various fluorescent intensities at various dilutions.

Microsphere Number	Fluorescence Intensity
10000	8991
5000	4477
2500	1819
1250	867
625	507
312	394

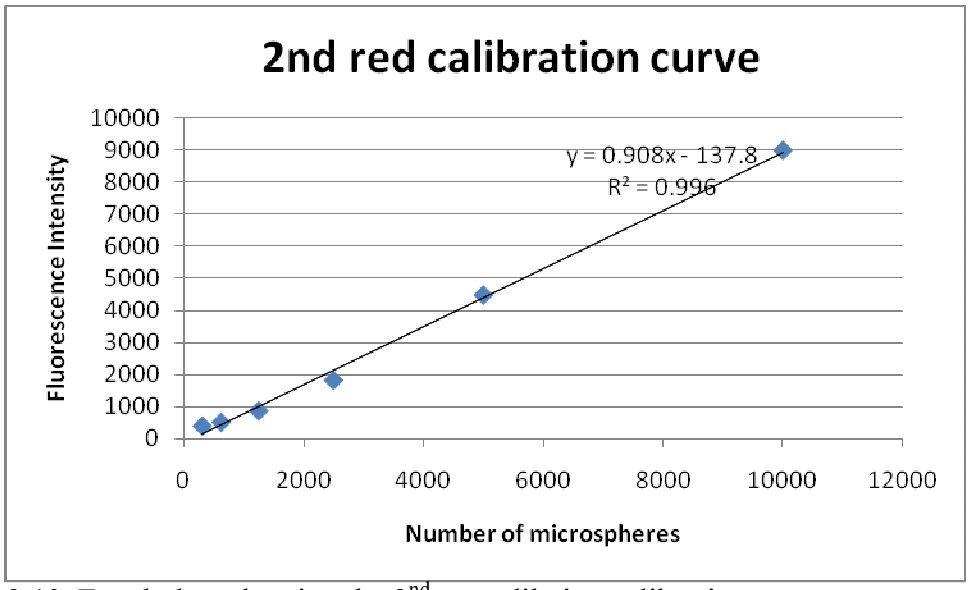

Figure 2.10 Excel plots showing the 2nd vasodilation calibration curve

Sample 3:

Table 2.9 Representation of various fluorescent intensities at various dilutions

Microsphere Number	Fluorescence Intensity
10000	11573
5000	6171
2500	3000
1250	1682
625	800
312	500

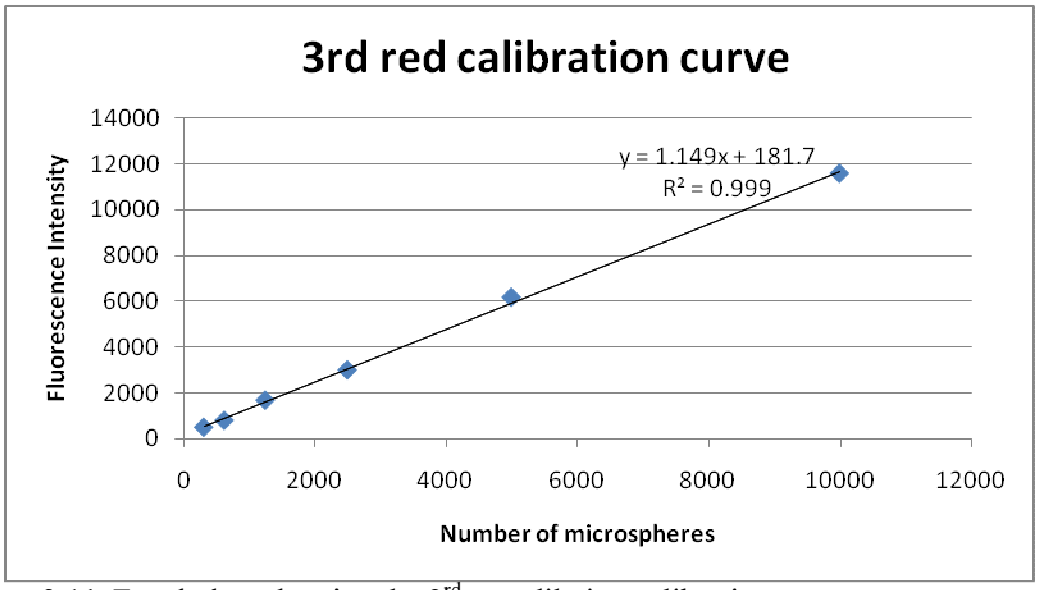

Figure 2.11 Excel plots showing the 3rd vasodilation calibration curve

Sample 4:

Table 2.10 Representation of various fluorescent intensities at various dilutions

Microsphere Number	Fluorescence Intensity
10000	11100
5000	6263
2500	3623
1250	1798
625	1000
312	450

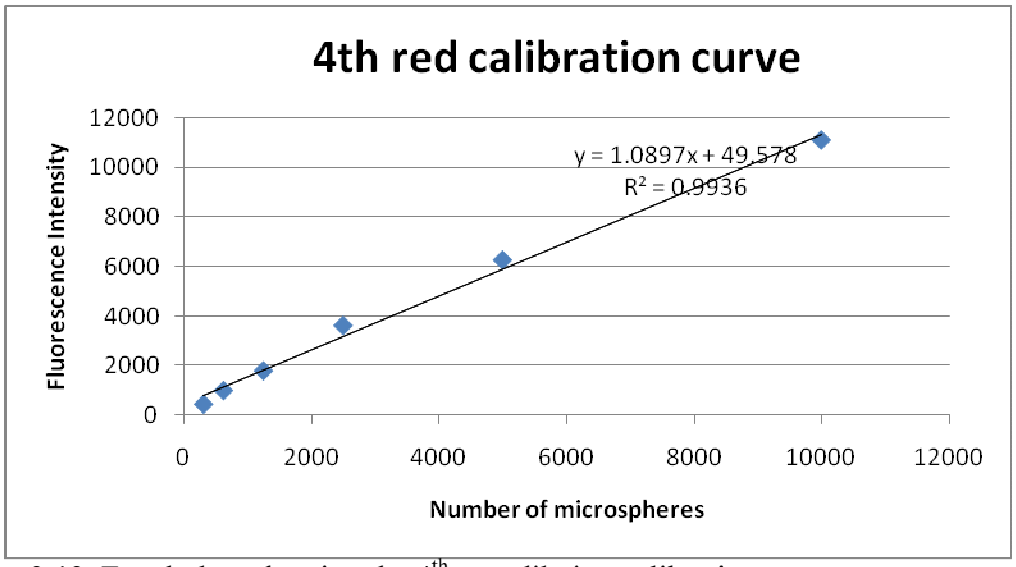

Figure 2.12 Excel plots showing the 4th vasodilation calibration curve

Sample 5:

Table 2.11 Representation of various fluorescent intensities at various dilutions

Microsphere Number	Fluorescence Intensity
10000	12306
5000	6055
2500	2930
1250	1611
625	827
312	430

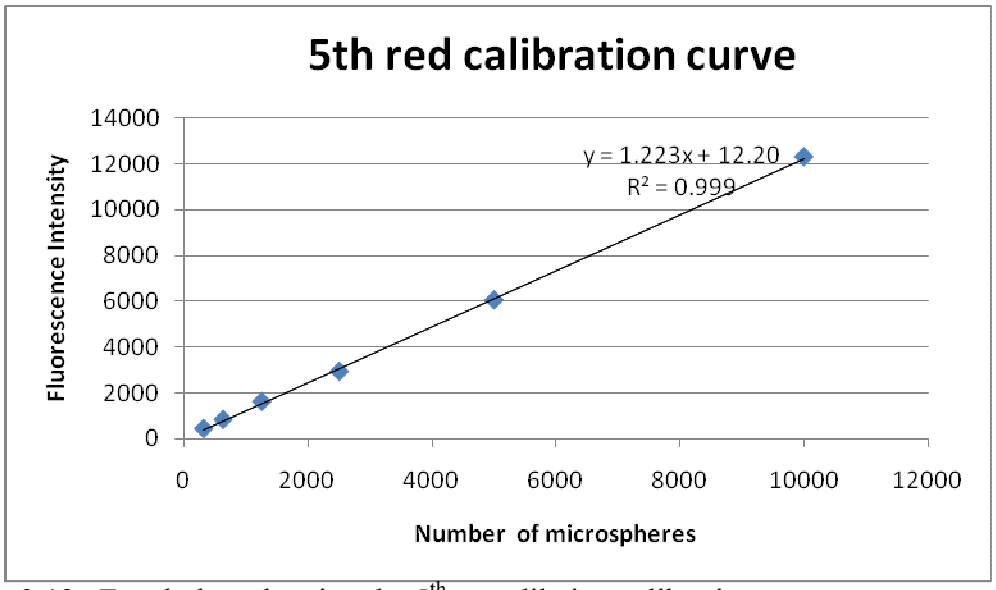

Figure 2.13 Excel plots showing the 5th vasodilation calibration curve

REFERENCES

[1] Austin GE, Tuvlin MB, Martino-Salzman D, Hunter RL, Justicz, AG, Thompson NK, Brooks AC (1993), Determination of regional myocardial blood flow using fluorescent microspheres; American Journal of Cardiovascular Pathology;4:352-7.

[2] Bernard SL, Ewen JR, Barlow CH, Mckinney S, Glenny RW (2000), High spatial resolution measurements of organ blood flow in small laboratory animals, American Journal of Physiology: Heart and Circulatory Physiology,279:H2043-52.

[3] Bio-Tek Instruments (1996): Bio-Tek FL500 Fluorescence Plate Reader User Guide (revision B)

[4] Buchwalder LF, Lin M., McDonald TJ. and Nathanielsz PW. (1998), Fetal sheep adrenal blood flow responses to hypoxemia after splanchnicotomy using fluorescent microspheres, Journal of Applied Physiology 84, 82-89

[5]http://www.cbsnews.com/stories/2005/03/18/earlyshow/saturday/main681597.shtml, Article in the news: How to take care of your heart.

[6] Chien GL, Anselone CG, Davis RF. and Van Winkle DM. (1995), Fluorescent vs. radioactive microsphere measurement of regional myocardial blood flow, Cardiovascular Research 30, 405-412

[7]http://www.cvphysiology.com/Blood%20Flow/BF001.htm,Cardiovascular Physiology Concepts, Richard. E. Klabunde, PhD, 2005

[8] Degens H, Craven, AJ, Jarvis JC and Salmons S. (1996), The use of colored dye-extraction microspheres to measure blood flow in rabbit skeletal muscle: a validation study with special emphasis on repeated measurements. Experimental Physiology 81, 239-249

[9] De Visscher G, Haseldonckx M, Flameng W (2006), Fluorescent microsphere technique to measure cerebral blood flow in the rat, Nature Protocol.1:2162-70.

[10] http://www.echoincontext.com/doppler01/doppler01_09.asp, Duke Medical Center (2000), Pulsed and continuous wave Doppler, Pages 1-2

[11] http://ehs.uc.edu/lams/index.html, William P. Porter (2000), American College of Laboratory Animal Medicine, "Laboratory Medical Medicine and Science Series II, Rats and Mice: Introduction and Use in Research Part I, Pages 6-9

[12] http://ehs.uc.edu/lams/index.html, David M. Moore (2000), American College of Laboratory Animal Medicine, "Laboratory Medical Medicine and Science Series II, Rats and Mice: Introduction and Use in Research Part II, Pages 4, 9

[13] Glenny RW, Bernard S, Brinkley M. (1993), Validation of fluorescent-labeled microspheres for measurement of regional organ perfusion; Journal of Applied Physiology, 74 (5), 2585-97

[14] Gronos J, Wikstrom J, Hagg U, Wandt B, Gan LM (2006), Proximal to middle left coronary artery flow velocity ratio, as assessed using color doppler echocardiography, predicts coronary artery atherosclerosis in mice, Arteriosclerosis, Thrombosis, and Vascular Biology.26:1126-1131.

[15] Hagg U, Gronros J, Wikstrom J, Ingiborg H. Jonsdottir, Bergstrom G and Gan LM, (2005), Voluntary physical exercise and coronary flow velocity reserve: a transthoracic colour Doppler echocardiography study in spontaneously hypertensive rat, Clinical Science;109, 325–334

[16] Hale SL, Kevin BS, Alker J and Kloner RA. (1988), Evaluation of nonradioactive, colored microspheres for measurement of regional myocardial blood flow in dogs. Circulation 78, 428-434

[17]http://heartdisease.about.com/cs/starthere/a/corartanat.htm, Anatomy of Normal Coronary Arteries, Richard N. Fogoros, M.D., December 2003

[18]http://health.msn.com/health-topics/cholesterol/why-healthy-people-suffer-heart-attacks, Article: Why healthy people suffer heart attacks, Dr. Arthur Agatston

[19] Hecht R, Habler O, Kleen M, Welte M, Staub F, Frey L. and Mesmer K. (1995), New perspectives through automation of the fluorescent microsphere technique for determination of regional blood flow, FASEB Journal 9, A908 (Abstract)

[20] Hozumi T, MD, Yoshida K, Akasaka T, Asami Y, (1998), Noninvasive assessment of coronary flow velocity and coronary flow velocity reserve in the left anterior descending coronary artery by doppler echocardiography, Journal of the American College of Cardiology,32:1251–9

[21]http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/tables/FluoSpheres-microspheres-for-blood-flow-determination.html

[22] Jacquier A, Kober F, Bun S, Giorgi R, Cozzone P.J and Bernard M, (2011), Quantification of myocardial blood flow and flow reserve in rats using arterial spin labeling MRI: comparison with a fluorescent microsphere technique. NMR in Biomedicine, doi: 10 1002/ nbm.1645

[23] Kowallik P, Schulz R, Guth BD, Schade A , Paffhausen W, Gross R. and Heusch G. (1991), Measurement of regional myocardial blood flow with multiple colored microspheres. Circulation 83, 974-982

[24] Lakowicz J.R, Principles of Fluorescence Spectroscopy (2nd edition) (2004), New York: Springer, Pages 1 -12

[25] McKirnan MD., Williams RL, Lohman JL, Eising GP, Hammond HK. & Ross J. (1994), Blood flow measurements during myocardial ischemia and dysfunction using fluorescent labeled Microspheres, FASEB Journal 8, A857 (Abstract)

[26] http://microspheres.net/Nuflow-Protocol.pdf, NuFLOW fluorescent microsphere extraction protocol for regional blood flow measurement, Interactive Medical Technology, Revised March 2001, Pages 1-2

[27] Mori H, Haruyama S, Shinozaki Y, Okino H, Iida A, Takanashi R, Sakuma I, Husseini, WK, Payne BD. & Hoffman JIE. (1992), New nonradioactive microspheres and more sensitive X ray fluorescence to measure regional blood flow, American Journal of Physiology 263, H 1946-1957

[28] Morita Y, Payne BD, Aldea GS, McWaters C, Husseini W, Mori H, Hoffman JIE. and Kaufman L. (1990), Local blood flow measured by fluorescence excitation of nonradioactive Microspheres, American Journal of Physiology 258, H 1573-1584

[29] Mulder TLM, Van Golde JC, Prinzen FW. and Blanco CE. (1997), Cardiac output distribution in the chick embryo from stage 36 to 45. Cardiovascular Research 34, 525-528.

[30] National Academies Press (1988), "Use of Laboratory Animals in Biomedical and Behavioral Research", Washington DC, Pages 31-32

[31] Prinzen FW, Bassingthwaigthe JB. (2000), Blood flow distributions by microsphere deposition methods, Cardiovascular Research 45: 13-21.

[32] Prinzen FW. and Glenny RW. (1994), Developments in non-radioactive microsphere techniques for blood flow measurements, Cardiovascular Research 28, 1467-1475.

[33] http://probes.invitrogen.com/media/spectra/8811h2o.jpg

[34] Richer C, Domergue V, Gervais M, Bruneval P, Giudicelli JF (2000), Fluospheres for cardiovascular phenotyping genetically modified mice, Journal of Cardiovascular Pharmacology,36;396-404

[35] Rudolph, AM and Heymann, MA (1967), The circulation of fetus in utero: Methods of studying distribution of blood flow, cardiac output and organ blood flow, Circulation Research; 21; 163-184

[36] Serrat MA (2009), Measuring bone blood supply in mice using fluorescent microspheres, Nature Protocols.;4:1749-58.

[37] http://www.uptodate.com/contents/principles-of-doppler-echocardiography, Warren J Manning, Nelson B Schiller (2010), "Principles of Doppler Echocardiography", Pages 1-10

[38] Van Oosterhout, M. F. M., Willigers, H. M. M., Reneman, R. S. & Prinzen, F. W. (1995). Fluorescent microspheres to measure organ perfusion: validation of a simplified sample processing technique, American Journal of Physiology 269, H725-733

[39] http://fmrcc.pulmcc.washington.edu/documents/fmrcman99.pdf, Manual for using fluorescent microspheres to measure regional organ perfusion, Fluorescent microsphere resource center, University of Washington, Revised October 1, 1999.