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Evaluation of illumination wavelengths for fluorescence detection of atherosclerosis

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The University of Arizona, 1994
EVALUATION OF ILLUMINATION WAVELENGTHS FOR FLUORESCENCE DETECTION OF ATHEROSCLEROSIS

by

Andrew Lafayette Alexander

A Dissertation Submitted to the Faculty of the COMMITTEE ON OPTICAL SCIENCES (GRADUATE)

In Partial Fulfillment of the Requirements For the Degree of

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In the Graduate College

THE UNIVERSITY OF ARIZONA

1994
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I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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Andrew Alexander
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ABSTRACT

The fluorescence-emission spectra of normal arterial tissues and atherosclerotic plaques are distinct. The fluorescence spectra of arterial tissues are also dependent on the illumination wavelength. Which illumination wavelengths are best for identifying arterial tissues? To answer this question, a system for measuring fluorescence spectra at different illumination wavelengths was constructed and the fluorescence spectra of in-vitro aorta specimens were acquired with several illumination wavelengths between 270 nm and 500 nm.

The Mahalanobis distance, a statistical measure of separability between two class density distributions, was used to compare spectral features of normal vessel and atherosclerotic plaques at each of the illumination wavelengths. The Mahalanobis distance results indicated that the separability was largest for illumination wavelengths in the 314 nm to 334 nm range. In addition, the atherosclerotic plaque class was further subdivided into three diseased subclasses -- fibrous plaques, complicated plaques and hard calcified plaques. The largest Mahalanobis distance between normal vessel and soft plaque (fibrous plaque and complicated plaque) again occurred with illumination in the wavelength range 314 nm to 334 nm. Conversely, the hard-calcified plaque class was most separable from the normal vessel class with illumination wavelengths longer than 380 nm.

Since the best illumination wavelength ranges for soft plaques and hard calcified plaques were different, tissue identification was evaluated for the three-class case -- normal artery versus soft plaques versus calcified plaques. Analysis with the generalized multiple-discriminant measure, a multiple-class extension of the
Mahalanobis distance, indicated that illumination in the 300 nm to 314 nm range and the 400 nm to 458 nm range had the greatest three-class separability. Several multiple-class classifiers were also evaluated at each illumination wavelength. These classifiers did not reveal any wavelengths that were clearly best for accurate tissue-type identification. Finally, class separability and classification performance of combined features from multiple illumination wavelengths were also examined. The combination of features from multiple illumination wavelengths was found to increase separability and to improve the overall classification capability.
CHAPTER 1
INTRODUCTION

Atherosclerosis is the leading cause of mortality in the United States. It is the primary cause of coronary heart disease and is responsible for many other forms of cardiovascular disease, such as stroke, myocardial infarction and impaired circulation. Coronary heart disease is responsible for more deaths than all forms of cancer combined [Levy, 1981]. Annually, the combined cost (in the U.S.) of treatment and lost productivity is approximately 60 billion dollars [Steinberg, 1987]. The mortality rate of coronary heart disease since 1978 has dropped more than 20% due to advances in diagnostics, therapies and changes in popular lifestyle. Further improvements in any of these areas could lead to substantial savings in both lives and money and would be extremely important. Diagnostic techniques are especially important for screening patients, monitoring therapy and evaluating how changes in lifestyle or previous therapeutic procedures have affected the progression of the disease.

The progression of atherosclerotic "plaques" is characterized by the deposition of lipid, primarily cholesteryl ester, and extracellular connective-tissue components -- elastin and collagen. This deposition process causes thickening of the arterial walls that can either stenos (partially obstruct) or totally occlude the arterial lumen. Narrowing of arterial lumens inhibit blood flow and can lead to a variety of health problems including coronary heart disease. Advanced atherosclerotic plaques are additionally characterized by calcification, hemorrhage and thrombosis. A more detailed description of atherosclerosis, and its diagnosis and treatment are given in
Chapter 2. Diagnostic techniques that are sensitive to the components of atherosclerotic plaques would be very valuable.

One technique that has the potential to detect early biophysical changes in the arterial wall -- optical fluorescence spectroscopy -- is the primary focus of this dissertation. Optical techniques are ideally suited for guiding catheter-based therapeutic techniques because they can be implemented through optical fibers. The purpose of the investigation described here and in later chapters is to evaluate the potential ability to detect atherosclerotic plaques with fluorescence spectroscopy and to determine the best illumination wavelength for this task.

1.1 Optical Fluorescence Spectroscopy

Optical fluorescence is the emission of light from substances resulting from the absorption of light at equal or shorter wavelengths. The absorption of photons excites electrons to higher energy states. A short period of time later, electrons decay back to the ground state, causing the emission of fluorescence photons.

Optical fluorescence spectroscopy has been widely reported as a potential technique for discriminating between normal and diseased (atheromatous) human vascular tissues. Different concentrations of endogenous fluorophores in normal and diseased tissues cause variations in the spectral distribution of the fluorescence emission. These spectral differences make fluorescence spectroscopy a promising diagnostic technique for use with therapeutic procedures such as laser-angioplasty. A fluorescence-guided laser-angioplasty system is illustrated in Figure 1.1. Light from the diagnostic laser is coupled into a fiber-optic catheter. At the distal end of the catheter, the tissue is illuminated, inducing a fluorescence emission, that is collected by the catheter and is coupled into a spectrometer system that measures the fluorescence
Figure 1.1. Fluorescence-guided laser angioplasty system.
intensity spectrum. A computer processes the data and classifies the tissue according to its spectrum. The ablative laser is "fired" if the fluorescence spectrum indicates an atherosclerotic plaque. The laser energy will remove a small volume of tissue at the end of the catheter. The ablative laser remains off if the spectrum indicates normal arterial tissue. Two separate lasers for fluorescence diagnostics and ablation are depicted, but a single laser for both functions could also be used.

The spectral shape of the fluorescence emission for a single fluorophore is relatively independent of the excitation wavelength; however, tissues contain many fluorophores with different excitation and emission characteristics (Chapter 3). Consequently, the fluorescence emission spectra change as a function of the excitation wavelength. Differences between the fluorescence spectra of normal arteries and atherosclerotic plaques have been reported by several independent researchers with a broad range of excitation wavelengths, including 308 nm, 325 nm, 337 nm, 458 nm and 476 nm. The results from different studies, however, are difficult to compare because many different techniques were used to analyze the difference between the spectra of normal and diseased tissues.

Examples of typical normalized fluorescence spectra induced with 314 nm and 458 nm illumination are shown in Figures 1.2. These examples show that differences in the spectra exist and that these differences are dependent on the type of tissue and the illumination wavelength. For example, the spectrum of a complicated plaque is significantly different from the normal tissue spectra with 314 nm illumination. This difference becomes less apparent with 458 nm illumination, but the calcified plaque appears to be more distinct at this illumination wavelength. An important and obvious question arises -- which illumination wavelength is best for discriminating between different arterial tissues?
Figure 1.2. Normalized fluorescence spectra for normal artery, fibrous plaque, complicated plaque and calcified plaque with (a) 314 nm and (b) 458 nm illumination
Crilly et al. [1989], in an early attempt to answer this question, examined several excitation wavelengths and reported the best performance for 360 nm illumination. Gmitro et al. [1990] and Alexander et al. [1991] also reported results comparing the performance of several fluorescence illumination wavelengths between 270 nm and 470 nm. One purpose of this dissertation is to present a more complete description and evaluation of the tissue type discrimination performance as a function of illumination wavelengths. Differences in fluorescence spectra for different tissue types and excitation wavelengths depend upon the concentrations of individual fluorescing species and their excitation wavelength dependencies. Optical characteristics, such as absorption and scattering, can also influence the fluorescence emission. Both the biochemical and optical properties of arterial tissues are discussed in Chapter 3.

Many different ways of using the spectral information for classification have been investigated. Ratios of intensities at two emission wavelengths, widths of the emission spectra, the coefficients of the best fits of known biochemical emission spectra to the measured spectra, emission intensities sampled at equal wavelength intervals across the measured spectral range, have each been investigated as spectral features for classification. To maximize tissue classification, it may be important that most of the information associated with a spectrum is extracted.

One way to compare excitation wavelengths is to estimate the amount of class separability between the feature distributions of normal and atheromatous artery. Overlap of the class distributions will result in potential misclassification of the tissue type. The amount of overlap should decrease for distributions with large separability. Therefore, the illumination wavelength with the highest degree of separability between the two classes should allow for the best tissue classification. This class separability
measure is also valuable for comparing the various feature extraction techniques described earlier. To compare the class separability to the ultimate objective of accurate tissue classification, the classification accuracy as a function of the illumination wavelengths can also be estimated. Chapter 5 describes a statistical figure of merit, the Mahalanobis distance, for evaluating the degree of class separability at different illumination wavelengths. Several techniques for estimating the potential classification accuracy are also discussed.

To determine both the optimum illumination wavelength and the best feature extraction techniques, a set of fluorescence spectra for different arterial tissue classes was collected. Chapter 4 describes the design of a catheter-based fluorescence spectrometer that was built to acquire the data. The protocol for preparing the tissue, measuring spectra and determining histologic classification of the tissue is also detailed. Chapter 6 presents the discriminant analysis and classification results for evaluation of the data measured with the system. Chapter 7 discusses these results and includes possible directions for further research or applications.
CHAPTER 2

ATHEROSCLEROSIS:
BIOGENESIS, PROGRESSION, THERAPY AND DIAGNOSTICS

Atherosclerosis is a very complex disease in its origins, progression and treatment. To maximize the sensitivity of diagnostic techniques, it is important to understand different aspects of atherosclerotic plaques -- the processes of formation, the stages of progression and the biochemical composition. The first section of this chapter discusses the evolution of atherosclerotic plaques from normal arteries. The major biochemical differences between normal arterial tissue and different stages of plaques are also described. Sections 2.2 and 2.3 describe various therapeutic and diagnostic techniques that are currently being used or are under investigation.

2.1 Atherosclerosis -- Biogenesis and Progression

The normal artery consists of three distinct layers -- the intima, the media and the adventitia (Figure 2.1a). The intima, the innermost layer, is constructed from a smooth uninterrupted monolayer of endothelial cells and is bounded by a sheet of elastic fibers (the internal elastic lamina). Extracellular connective-tissue matrix and occasional smooth-muscle cells are contained between these layers for normal arteries in children. Smooth-muscle cells and connective-tissue matrix components accumulate slowly with increasing age. The media, or intermediate layer, is constructed differently, depending upon whether the artery is muscular or elastic. In muscular arteries, such as the coronary, tibial, femoral and popliteal, the media consists of diagonally-oriented smooth-muscle cells that are surrounded by variable amounts of collagen, elastic fibers and proteoglycans. The aorta trunk and its branches as well as
Figure 2.1. Cross-sectional anatomy of artery at various stages of the atherosclerotic process; (a) normal vessel, (b) fibrous plaque and (c) complicated plaque.

The carotid, mammary, and iliac arteries are elastic arteries that consist of unit layers constructed from groups of smooth-muscle cells that are surrounded by sheets of elastic tissue. The relative amount of collagen is lower in elastic arteries compared with muscular arteries. The outermost layer of the artery, the adventitia, consists primarily of fibroblasts intermixed with smooth-muscle cells that are loosely arranged in bundles of collagen and surrounded by proteoglycans. The adventitia is separated from the media by a discontinuous layer of elastic tissue called the external elastic lamina.

Atherosclerotic plaques are characterized by marked thickening of the vessel wall, primarily the intima. Plaques can protrude into the vessel lumen, and, as they increase in size, they begin to obstruct the blood flow. This obstruction is often referred to as stenosis. The plaque constituents are composed primarily of lipids and smooth-muscle cells. Three types of atherosclerotic plaques are most commonly recognized -- the fatty streak, the fibrous plaque and the complicated plaque [Ross and Glomset, 1976a]. The fatty streak is often found in young people and has no clinical symptoms because the lesion causes little or no obstruction. It is characterized by the focal accumulation of smooth-muscle cells that contain and are surrounded by lipid
deposits. The presence of lipid makes these lesions appear yellow. The fibrous plaque (Figure 2.1b) is whitish in appearance. The plaque causes the arterial wall to thicken, but hemostatic pressure often prevents protrusion into the lumen. It is characterized by the accumulation of intimal lipid-laden smooth-muscle cells and connective-tissue components (collagen, elastic fibers and proteoglycans). It has been suggested that fatty streaks are the precursor to fibrous plaques; however, they often occur in different anatomical sites. The third type of lesion, the complicated plaque (Figure 2.1c), is a particularly advanced fibrous plaque with hemorrhage, calcification, cell necrosis and/or thrombosis. Complicated plaques are most commonly associated with severe occlusive disease.

The causes and pathogenesis of atherosclerosis have been subjects of much controversy. This is partially because the disease progresses quietly for many years before symptoms develop, making it difficult to follow the early stages of the disease in individuals and to relate it to specific causes [Ross and Glomset, 1976a]. Two hypotheses for the pathogenesis of atherosclerosis are described below.

The Lipid Infiltration Hypothesis [Brown et al., 1975; Steinberg, 1987] states that the disease is a result of excessive lipid infiltration from the bloodstream into the arterial wall. This hypothesis is supported by several facts. (1) The accumulation of cholesterol in the arterial wall is strongly correlated with the progression of atherosclerosis in people. (2) Populations of people (e.g., Japanese) that have lower average levels of plasma cholesterol also have lower rates of coronary heart disease than groups with higher levels (e.g., Americans). (3) People with a deficiency of low-density lipoprotein (LDL) receptors, which remove most of the cholesterol from the blood, tend to have a high rate of coronary heart disease at very early ages. According to this hypothesis, the uptake of cholesterol into the arterial walls increases as the
concentration of LDL in plasma increases. Cholesterol is carried within the LDL structure. Normally, LDL receptors, primarily in the liver, remove LDL (and, hence, plasma cholesterol) as quickly as it is delivered. Sites on the endothelium also can remove LDL from the bloodstream and deposit cholesteryl ester in the arterial wall. If either the rate of LDL loading exceeds some threshold, or there is a deficiency in the number of LDL receptors in the liver, then the supply of LDL to the arterial cells will be greater than the ability of the liver to remove the cholesterol.

According to the Response to Injury Hypothesis [Ross and Glomset, 1976b; Steinberg, 1987], the endothelium is initially injured through either mechanical, chemical, viral, or immunologic mechanisms. Focal aggregation of platelets around the injury site induces a migration and multiplication of smooth-muscle cells from the media to the intima. The increase in the number of smooth-muscle cells causes the lesion to grow. Repeated injury in the same location eventually leads to stenosis and/or thrombosis. The endothelial cells and smooth-muscle cells can cause peroxidation of LDL lipids that are cytotoxic to endothelial cells, hence, potentially causing further injury. The Response to Injury Hypothesis is supported by evidence of atherosclerotic lesion formation after the application of arterial catheter procedures.

The two hypotheses described above are not necessarily mutually exclusive. In fact, one or both of the hypotheses given, as well as others not listed above, may describe the processes of atherosclerotic plaque formation in different situations. Irrespective of which hypotheses are correct, the atherosclerotic lesion is characterized by excessive subintimal accumulation of lipid and changes in the extracellular connective-tissue.

Lipids obviously play an important role in the progression of atherosclerosis. In fact, hypercholesterolemia, which is characterized by extremely high levels of plasma
cholesterol, is a primary risk factor for atherosclerosis. The lipids (primarily cholesterol, cholesteryl esters, and triglycerides) are transported in the blood stream by plasma lipoproteins, such as LDL. These lipoproteins are constructed from polar lipids (mainly phospholipids) and proteins that encase the various lipids [Havel, 1987]. The most important cholesterol-transport lipoproteins pertaining to atherosclerosis are LDL. The exact mechanisms for cholesterol accumulation within the arterial wall are unknown; however, as LDL levels increase, the amounts of cholesterol and cholesteryl ester absorbed into the arterial wall also increase. Fibrous plaques often contain high amounts of extracellular lipids with composition similar to the lipids found in LDL. Complicated lesions often have a core of amorphous lipid with cholesterol monohydrate crystals.

The extracellular connective-tissue matrix components, primarily elastin and collagen, are produced by the endothelial cells and smooth-muscle cells within the intima and media [Bouissou et al., 1989]. Collagen fiber bundles are the main extracellular matrix component found in fibrous and advanced lesions. Collagen is produced to offer protection for the exposed arterial wall when the endothelium is injured. Excessive accumulation of collagen causes thickening of the intima that can lead to partial occlusion of the vessel. The elastin found in plaques is slightly different biochemically than elastin found in the normal media [Wight, 1989]. This compositional difference may be due to the binding of other protein molecules. It has been suggested that elastin plays a role in the accumulation of lipids. The elastin in atherosclerotic lesions has a high affinity for lipid binding.

Advanced atherosclerotic lesions can also be characterized by calcification. Calcification consists of Ca\(^{2+}\) deposits either at the plaque surface or in the atheromatous pulp [Bouissou et al, 1989]. Deposition of Ca\(^{2+}\) occurs through ionic
interactions with the protein backbone of elastin fibers [Wight, 1989]. Large regions of calcification within an atherosclerotic plaque can make the tissue have a mineralized or "petrified" look and feel. The combination of calcification with increased accumulation of collagen in plaques increases the overall rigidity of the artery, which leads to an overall increase in hemostatic pressure that can eventually lead to ulceration (rupture of the intimal surface) or perforation. Calcified tissue is more difficult to remove in certain therapeutic techniques; consequently, it is important to be able to identify the calcified tissue, so that alternative therapeutic strategies may be employed.

The other complications of atherosclerosis include thrombosis, ulceration and necrosis. Thrombosis is the accumulation of coagulated blood products and platelet cells at an injury site. Often thrombosis occurs at locations of ulceration on the arterial wall. The primary complications associated with thrombosis are occlusion of the vessel at the thrombus site or embolism in smaller vessels by fragmentation of thrombus in larger arteries [Legrand and Drouet, 1989]. Another complication of ulceration is hemostasis or deposition of blood products in the arterial wall. Hence, detection of blood products in the arterial wall could indicate sites of endothelial injury and the locations with a high likelihood of thrombus. Many lesions in advanced states become necrotic, especially in the core of the plaque. Necrosis, which is the decomposition of dead cells in the plaque and arterial wall, is caused by cytotoxins that include atheromatous lipids. Consequently, many necrotic plaques also contain high lipid levels.

In summary, the formation and progression of atherosclerotic plaques can be characterized by (1) the accumulation of lipids, (2) changes in the connective-tissue (collagen and elastin) composition, and (3) calcification of the tissues for advanced plaques. Therefore, diagnostic techniques that are sensitive to lipid, collagen, elastin
and calcification obviously would be very powerful. The fluorescence spectra of connective-tissue components such as collagen and elastin and proteins associated with subintimal lipid are reportedly distinct (see Chapter 3); consequently, optical fluorescence spectroscopy may provide a potentially sensitive technique for the detection of atherosclerosis.

2.2 Treatment

The primary risk factors associated with atherosclerosis are high serum cholesterol levels, hypertension and smoking [Blackburn, 1987]. One can obviously lower their risk of atherosclerosis by living a lifestyle that minimizes involvement of the various risk factors. Such a lifestyle includes minimizing dietary intake of saturated fats and cholesterol, reducing stress, and not smoking. Once the atherosclerotic process has begun, early stages of the disease are reversible to some degree by altering diet and lifestyle. For people who are at high risk for developing atherosclerosis and its associated complications, various drug treatments are available. These drugs can act as either anticoagulants, plasma cholesterol reducers, or hypertension relievers.

The treatment of occlusive atherosclerotic disease is aggressive and can involve highly invasive procedures. The most aggressive form of treatment is bypass surgery where the blocked section of artery is removed and replaced with a graft of either a vein from another part of the body or a tube of biocompatible synthetic material [DeBakey and McCollum, 1977]. Blood can flow freely through the "new" segment of artery. Another procedure, the endarterectomy, attempts to salvage the stenosed section of artery by making an incision along the artery and then physically dissecting the plaque from the arterial wall [DeBakey and McCollum, 1977]. Obviously, both
bypass surgery and endarterectomy are quite invasive and pose a substantial risk to the patient.

In an attempt to minimize the surgical involvement for the treatment of atherosclerotic lesions, angioplasty was developed in the early 1960's. Originally, an angioplasty technique called Dottering was performed by inserting rigid catheters of increasing diameter to increase the overall lumen dimensions [Dotter and Judkins, 1964]. An improved technique using a balloon-tipped catheter was first reported by Porstmann in 1973. Percutaneous transluminal balloon angioplasty is performed by inserting the catheter to the stenotic location and expanding the balloon, which compresses the soft plaque material against the wall of the artery. The compression, in many cases, causes the diameter of the lumen to remain patent even after the balloon is removed. There are several disadvantages associated with this technique. (1) The plaque material is not removed; consequently, the plaque material can expand back into the lumen after the catheter is removed. (2) The balloon catheter can fracture or dislodge a section of plaque, which can float down the bloodstream and cause an embolism in a narrower arterial channel. (3) Advanced plaques are often rigid due to collagen and calcium deposition. When a balloon is expanded at the location of a rigid plaque, often the more normal arterial tissue will be torn or dissected as a result of the pressure. The rip in the arterial wall will be more prone to development of future atherosclerotic plaques. The restenosis rate for balloon angioplasty is relatively high and often multiple treatments are necessary. Implantable stents made of stainless-steel mesh have recently become a popular technique for maintaining lumen patency after angioplasty [Carlson, 1988].

In the early 1980's, studies were started to investigate the ability of laser energy to recanalize occluded vessels. In 1983, Robert Ginsberg of Stanford University was
the first to perform percutaneous laser-assisted angioplasty [Ginsberg et al., 1984]. A continuous-wave Argon laser was used to treat sixteen patients with peripheral vascular disease. Approximately half of the procedures resulted in failure, including two instances of perforation of the arterial wall and subsequent hemorrhage. Herbert Geschwind performed similar procedures in 1984 using a Neodymium:YAG laser on twelve patients [Geschwind et al., 1984]. Ten cases were failures and perforation problems were also reported.

In spite of its poor initial success, percutaneous laser-assisted angioplasty is still being investigated. Many different lasers have been investigated for their ablation properties including CO₂ (10.6 mm) [Abela et al., 1982], Argon (488 nm - 515 nm) [Abela et al., 1982; Deckelbaum et al., 1985], excimer (193 nm - 350 nm) [Singleton et al., 1986; Long and Deutsch, 1987], Neodymium:YAG (355 nm, 532 nm, 1.06 mm) [Abela et al., 1982; Deckelbaum et al., 1985], Holmium:YAG (2.1 mm) [Duda et al., 1992] and Erbium:YAG (2.94 mm) [Deckelbaum et al., 1989]. It was found that continuous-wave laser ablation resulted in thermal injury to tissues surrounding the ablation site, while pulsed-laser ablation resulted in cleaner plaque removal [Deckelbaum et al., 1985]. However, pulsed-laser ablation of tissue often produces shock waves that may disrupt the subsurface tissue structure and cause dissections between layers of tissue [Jacques, 1989]. The ablation mechanism for visible and infrared lasers is primarily thermal. Water molecules in the tissue structure absorb the laser energy, which causes the water to superheat and vaporize. The ablation mechanism for ultraviolet lasers such as excimer lasers is photochemical. The photon energies at these wavelengths are strong enough to break the chemical bonds within the tissue molecules. Therefore, excimer ablation may result in much cleaner removal of the tissue, since the process can be primarily non-thermal. Unfortunately, these
wavelengths are potentially carcinogenic and mutagenic to the surrounding tissues [Jacques, 1989].

In 1986, the concept of a "hot-tipped" catheter was introduced by Cumberland et al. [1986]. This technique consisted of using laser energy to heat a metal tip on the end of a fiber-optic catheter. The hot metal tip would essentially melt its way through an occlusive plaque. This would sometimes cause discomfort to the patient, and perforations were also reported.

Both the bare fiber and hot-tipped techniques were often used in conjunction with conventional balloon angioplasty. The laser procedures were often used to create a lumen channel large enough to insert a balloon catheter for dilation. It has been shown that laser energy can be effective for increasing the lumen dimensions [Safian et al., 1993; Geshwind, 1993; Bittl et al., 1993], but not significantly more than balloon angioplasty. Consequently, the jury is still out concerning the benefits of laser treatment of occluded vessels. In the process of removing plaque, the protective layer of endothelial cells is also destroyed, making both the former plaque locations and newly injured locations more susceptible to the formation of future plaques and thrombus. The size of the ablation channel is approximately the same size as the end of a fiber catheter; therefore, larger fibers, which are bulkier and more rigid, must be used to create larger channels. It is difficult to route a rigid catheter through tortuous vessel; and a rigid catheter is more likely to injure the endothelium of the vessels it is being routed through, making the injured vessels more atherogenic. In addition, since the laser energy is indiscriminate whether the tissue is atherosclerotic plaque or normal vessel, there is a risk of perforating the arterial wall.

Another catheter-based plaque-removal system uses a high-speed rotating blade that grinds plaques into fine particles [Carlson, 1988; Ritchie et al., 1986]. The blade,
however, is indiscriminate of the type of tissue being ground. As a result, perforations of the vessel wall have been reported. A similar technique developed by Kensey [et al., 1986] implements a high-speed rotating cam that dispenses fluid. The fluid jets break off small pieces of atheroma while leaving normal vessel undamaged. The pieces of atheroma are then emulsified in the vortex created by the cam.

All the catheter-based therapeutic techniques described above have inherent risks of endothelial injury and, more seriously, vessel perforation. Therefore, accurate guidance is necessary to minimize the risk of arterial injury or perforation. A variety of diagnostic techniques, including those used for the guidance of catheter-based therapy, are discussed in the following section.

2.3 Diagnostics

Diagnostics for atherosclerotic disease serve two primary purposes. (1) They are used to evaluate the extent of the disease both before and after treatment. This information assists in the determination of which therapy to implement and what actions are to be taken after therapy. (2) Diagnostic are also used to recognize tissue type, lesion location or vessel patency during a therapeutic procedure. Such information can be used to control the therapy. The diagnostic techniques for atherosclerosis are many and varied in the information they convey. The diagnostic techniques are divided into two categories -- current clinical methods and experimental methods.

2.3.1 Current Clinical Methods

Rudimentary screening tests for atherosclerosis and coronary heart disease include measuring the blood serum cholesterol levels, measuring blood pressure levels, stress tests and evaluating the past and present lifestyle of the patient. These tests give
no information concerning the extent of atherosclerosis, but establish the risk of atherosclerosis in a person. Obviously, if a patient is in a high risk category (i.e., high serum blood cholesterol levels), the physician will recommend that the person change their lifestyle (i.e., lower their dietary fat intake) to reduce their risk factors. If a patient is at especially high risk, other tests such as an electrocardiogram (ECG) both before and during exercise may be utilized [Epstein, 1977]. An ECG measures the electric potential created by the heart during the heartbeat cycle using electrodes placed externally on the body. Differences between the before and during exercise ECGs may indicate coronary heart disease.

Angiography is probably the most commonly used test for detection of vascular occlusion. During angiography, an x-ray opaque contrast media is injected into the arteries and the x-ray transmission is observed on a monitor [Barrett and Swindell, 1981]. Arterial regions of blood flow show up dark on the images, while regions of restricted blood flow, as in the case of stenotic atherosclerotic lesions, can be visualized as either a narrow section of vessel. Angiography is commonly used to position catheters for angioplasty. In addition, guide-wires at the end of the catheter are used to align the catheter with the lumen and to pass through totally occluded regions [Carlson, 1988]. These techniques, however, do not provide the necessary information to accurately operate a laser-assisted or a rotating-blade endarterectomy procedure where good tissue type characterization is necessary to prevent perforations. After therapeutic procedures, angiography is often used at periodic intervals to assess the patency of treated vessels.

Magnetic resonance angiography (MRA) has recently become a popular technique for imaging flow in the arteries [Firmin et al., 1990; Nishimura, 1990]. MRA has several advantages over the original angiographic technique described above.
First, contrast media and x-rays are not involved. Second, arterial flow velocities can be measured. It would, however, be difficult to guide an intravascular catheter by MRA since the current geometry of clinical imaging magnets makes access to the patient difficult. MRA does not reveal any information about the plaque composition, and cannot detect early atherosclerotic lesions that do not protrude into the vessel.

Intravascular imaging is achieved with a technique called angioscopy. Angioscopy uses a multi-fiber imaging catheter to acquire an image of the interior of the vessel lumen. Angioscopy is sometimes used to assist in the planning of catheter procedures and in follow-up examinations after therapy. Angioscopic guidance for laser-angioplasty was first reported by Abela et al. [1986]. Unfortunately, the contrast between diseased plaque regions and normal vessel wall was not very great.

2.3.2 Experimental Methods

Various diagnostic modalities, such as intravascular ultrasound, fluorescence spectroscopy and other forms of optical spectroscopy, have been investigated as possible guidance systems for laser-angioplasty. The main advantage of these techniques is that the diagnostic information is obtained at the distal end of a catheter in proximity to where the treatment will take place. Consequently, more accurate control of the plaque-removal process should be possible.

Intravascular ultrasound (B-mode) is implemented with miniature ultrasound transducers that are oriented radially from the catheter [Tobis et al., 1993]. Ultrasound has the capability of generating full three-dimensional maps of a localized vessel region and evaluating the thickness of the arterial wall.

Fluorescence spectroscopy exploits the difference between the spectral emission characteristics of normal vessel and atherosclerotic plaques. This is discussed further in Chapters 3 through 7. Kittrell [et al., 1985] first suggested fluorescence
spectroscopy as a possible guidance technique for laser-angioplasty. Fluorescence spectroscopy has the advantage of being able to detect through the same fiber used for ablation. Additionally, it has been demonstrated that spectroscopic fluorescence can enhance the tissue-type contrast in intravascular images (e.g., angioscopy) [Connor-Davenport et al., 1991 & 1992a; Connor-Davenport, 1992b].

Other optical spectroscopy techniques, such as Raman spectroscopy and plasma emission measurements, are currently being investigated for catheter-based plaque detection systems. The primary advantage of these techniques is that they are very sensitive to specific compounds found within atherosclerotic plaques. For example, calcium hydroxapatite, the main component of calcified plaques, is easily recognized by characteristic intensity peaks using Raman spectroscopy [Clarke et al., 1987; Redd et al., 1991; Klug et al., 1992; Baraga et al., 1992]. The primary limitation of Raman spectroscopy is the relatively weak signal, and hence, long measurement times. The intensity of plasma emission after ablation by a short laser pulse can also be used to determine the type of tissue [Bhatta et al., 1989; Deckelbaum et al., 1992]. In addition, the plasma emission spectra of calcified plaque has unique peaks indicative of the hard mineral components found in calcifications. Detection with plasma emission has two disadvantages. (1) To induce a plasma, an ablation process must take place before detection, thereby risking injury to the endothelium, which can lead to future atherosclerotic lesions. (2) This detection technique is based upon intensity variations that depend upon the catheter orientation and position. As a result, the accuracy of this technique may be compromised.

The acoustic properties of tissue being ablated by pulsed lasers have been investigated [Bhatta et al., 1989] as a potential source of diagnostic information.
Strong sound signals ("clicks") are produced when hard calcified plaques are hit with laser pulses. Again, this is a destructive detection process.

Nuclear medicine imaging techniques such as nuclear medicine have also been investigated as possible techniques for the detection of atherosclerosis. It has been shown that LDL radio-labeled with a technetium isotope, $^{99m}$Tc, will be most greatly absorbed in atherosclerotic lesions versus normal vessel wall [Lees et al., 1990]. Images of the "hot" radioactive plaque regions are obtained with an external gamma-scintillation camera. Unfortunately, the resolution of current nuclear imaging techniques is relatively poor. Also, since radiopharaceuticals are involved, this technique is not very attractive as a general screening procedure.

Nuclear magnetic resonance (NMR) spectroscopic studies have shown that cholesteryl ester in atherosclerotic plaques yields a distinct spectroscopic signature that is absent in spectra of normal arterial vessels [Pearlman et al., 1988]. Therefore, it is conceivable that imaging the atheromatous lipids should be possible with MRI. The application of magnetic resonance imaging (MRI) for visualizing atherosclerotic disease was first reported by Kaufman et al. [1982] with conventional spin-echo imaging. Chemical-shift imaging techniques that exploit the chemical-shift differences between lipid and water were also implemented with little or no success at visualization of atherosclerotic lipids [Wesbey et al., 1986; Maynor et al., 1989]. Difficulties in imaging arterial lipids arise from both the lipid component being small relative to the water and the very fast signal decay of the lipid signal. More recently, Altbach et al. [1991] and Alexander et al. [1992] described a diffusion-weighted imaging technique for imaging atherosclerotic lipids that suppresses the water signal. Unfortunately, this technique suffers from low lipid signal sensitivity and is particularly susceptible to
motion artifacts that would occur in a living system. Work is currently under progress to reduce the motion artifacts [Gmitro and Alexander, 1993].
CHAPTER 3

OPTICAL FLUORESCENCE SPECTROSCOPY

The emission of photons from electronically excited states is called luminescence. If the molecules are electronically excited by the absorption of a photon, then the luminescence process is often called fluorescence [Lakowicz, 1983; Haugland, 1992]. The intensities at different fluorescence-emission wavelengths reveal chemical information about the substances that are emitting photons. The spectral characteristics are very sensitive to slight changes in the molecular environment and composition. Spectroscopic techniques can be used to measure the distribution of intensities across the emission wavelengths. The resulting fluorescence spectra can be used to evaluate many different characteristics of the measured substances. Recently, applications in the biological and medical fields have increasingly used optical fluorescence spectroscopy. The mechanisms of fluorescence are outlined in the following chapter. Results of several investigations into the spectroscopic properties of fluorescing species found in atherosclerotic plaques are also described.

3.1 Excitation and Emission

Electrons, originally in the ground state, can be raised to an excited level by several mechanisms including optical, thermal, chemical and electrical. Optical stimulation occurs when a photon with energy, $h\nu$, is absorbed such that the electron is elevated from the ground electron state, $S_0$, to an excited state, $S_I$ (see Figure 3.1). The transition frequency, $\nu_{\alpha}$, is given by Planck's Law, $\Delta E = h\nu_\alpha$, where $\Delta E$ is the transition energy difference, and $h$ is Planck's constant. At each of these electronic energy states, the electrons can exist in a number of vibrational energy levels, which
Figure 3.1. Electron energy-level diagram depicting absorption, non-radiative decay, fluorescence and phosphorescence.

Figure 3.2. Absorption and emission spectra demonstrating the mirror image principal and the Stokes’ shift.
are depicted as $0,\ldots,N$. For a pair of levels, the Boltzmann distribution, $R = e^{-\Delta E/kT}$, determines the ratio of the number of molecules in a pair of energy levels. The vibrational energy difference between levels is $\Delta E$, $k$ is the Boltzmann constant and $T$ is the temperature. At room temperature (or body temperature), most of the molecules will be in the lowest vibrational level ($0$) of the ground state; therefore, the absorption process will primarily affect electrons in this state. The vibrational levels of the upper electronic states are similarly distributed and can affect the emission spectra as a function of temperature. Very few fluorophores populate the $S_f$ or higher states at room temperature because the energy difference between $S_0$ and $S_f$ is relatively large and the Boltzmann probability of $S_f$ relative to $S_0$ is nearly zero.

After the absorption of a photon, several processes can occur. Excited fluorophores in the $S_f$ or higher states rapidly relax to the lowest vibrational level of $S_f$. This is a non-radiative process called internal conversion (Figure 3.1). The loss of energy is primarily a thermal transfer to the molecular lattice. The fluorophore then decays back to one of the vibrational levels of the ground state, emitting a photon with energy, $h\nu_f$, in the process. The electrons that have decayed to the ground state then relax to the lowest vibrational level through an internal conversion process. Internal conversion generally occurs in $10^{-12}$ seconds, whereas fluorescence lifetimes are on the order of $10^{-8}$ seconds. Therefore, internal conversion is generally complete prior to fluorescence-emission. One interesting result of this process is that absorption spectra describe the upper vibrational energy levels, while fluorescence-emission spectra describe the ground state vibrational levels.

Another common emission phenomenon can occur when molecules in the $S_f$ state undergo a conversion to the $T_f$ triplet state via an intersystem crossing. Emission from the $T_f$ state is called phosphorescence, and generally is longer in wavelength.
(lower energy) relative to the fluorescence-emission. The transition from the triplet state to the ground state is forbidden by the transition selection rules of quantum mechanics; consequently, the emission rate constants for phosphorescence are much smaller (i.e., longer decay times) than for fluorescence.

3.1.1 Fluorescence Properties - General

There are energy losses (e.g., internal conversion) between the excitation and emission of fluorophores. The lost energy causes the wavelengths of emission spectra to be longer than those of excitation spectra (see Figure 3.2). This shift to longer wavelengths is termed the Stokes' shift. The energy losses are caused by the rapid decay to the lowest vibrational level of $S_I$ (internal conversion shown in Figure 3.1) prior to the emission. The quick decay to the lowest vibrational level of $S_I$ also causes the emission spectrum, in most cases, to be independent of the excitation wavelength for a homogenous molecular species.

The fluorescence intensity at a specific emission wavelength is directly proportional to the transition probability between the lowest $S_I$ level and the destination vibrational level in the ground state. If the largest transition probability for emission occurs between the 0 (level) of $S_I$ (see Figure 3.1) and the 2 (level) of the ground state $S_0$, vibrational levels (shown as 0-2 on the emission spectrum in Figure 3.2), then the most likely transition for the reciprocal process (absorption) is also between the levels 0 of the ground state and 2 of $S_I$ (shown as 0-2 on the absorption spectrum in Figure 3.2). Consequently, if the vibrational levels of the $S_I$ and $S_0$ bands are similar, the absorption and emission spectra often appear to have "mirror image" distributions.
Fluorophores in the excited $S_1$ state can also return to the $S_0$ ground state through one of several non-radiative decay processes. If $\Gamma_r$ and $\Gamma_{nr}$ are the radiative and non-radiative decay rates, respectively, then the fluorescence quantum efficiency, $\Psi$, is calculated by

$$\Psi = \frac{\Gamma_r}{\Gamma_r + \Gamma_{nr}}. \quad (3.1)$$

The quantum efficiency describes the probability of a photon emission for an excited fluorophore.

Decay from an excited state is an exponential process. The average lifetime of the excited state is defined by

$$\tau = \frac{1}{\Gamma_r + \Gamma_{nr}}. \quad (3.2)$$

In the absence of non-radiative processes, the average lifetime becomes

$$\tau_n = \frac{1}{\Gamma_r}, \quad (3.3)$$

which is the intrinsic lifetime. Temporal fluorescence measurements can be used both to determine the decay lifetimes and to resolve multiple fluorescing species within a compound. The measurement system, however, must be able to operate with extremely high temporal resolution to evaluate these nanosecond changes.

The polarization of the illumination can influence the fluorescence-emission. Light is preferentially absorbed (and emitted) by molecules whose electric dipole moment is aligned parallel to the electric field of the illumination. By measuring the emitted intensity as the polarization is rotated, the molecular anisotropy can be
determined. For fluorophores that are arranged isotropically, the emission is independent of the polarization.

The fluorescence-emission properties are also very sensitive to changes in the molecular environment. The polarity and pH of the fluorophore solvent can strongly influence the fluorescence spectrum. The Stokes' shift of the fluorescence signal often increases with the polarity of the solvent. This means that the spectrum of a fluorophore in an aqueous solution will probably be "red"-shifted relative to the spectrum of the same fluorophore in Hexane, which is less polar than water. In addition, the presence of fluorescence quenchers, either in the surrounding solution or the fluorophore itself (self-quenching) will reduce the overall emission intensity. Fluorescence quenchers reduce the emission intensity either by absorbing the emitted light or by interfering with the electron levels of the fluorescence species.

Photobleaching of fluorophores also influences the fluorescence properties of a substance. Both the total fluorescence intensity and the fluorescence lineshapes may change after lengthy or strong exposure to the exciting radiation [Chaudhry et al., 1989]. These changes seem to depend primarily upon the total energy fluence (Joules/mm²) applied to the substance. The fluence is equal to the product between the incident intensity (Watts/mm²) and the exposure time in seconds. The photobleaching phenomenon was modeled by Chaudhry as a three-level system with two decay processes from the upper (S₁) level (Figure 3.3). One relaxation process was the decay to the ground state (including fluorescence-emission) and the other decay process was to an intermediate level that did not allow decay back to the ground state. Therefore, the number of electrons eligible for the fluorescence process is decreased.
Figure 3.3. Energy level diagram for photobleaching process modeled by Chaudhry et al. [1989]. After absorption, the electron either decays back to the ground state (decay rate = $\Gamma$), possibly fluorescing in the process, or decays to an intermediate level, $M_0$ (decay rate = $\eta$). Electrons are trapped and cannot decay further from this level.

3.1.2 Fluorescence Properties - Arterial Tissues

Many investigators have reported on the fluorescence properties of normal and atherosclerotic arterial tissues. Table A.1 in Appendix A lists the various fluorescence studies that have been reported. A wide range of illumination wavelengths (200 nm - 1000 nm) has been studied. From this table, it is apparent that both the reported wavelengths of the fluorescence peaks and the spectral widths are different for the various tissue classes. This indicates that fluorescence may be promising for detection of different tissue types. Tissue type discrimination with fluorescence-emission spectra is discussed further in Chapter 5. The peak wavelengths and spectral widths also changed as a function of the illumination wavelength, which implies that the fluorescence spectra will change as a function of illumination wavelength. The reported properties, however, also seem to vary between investigations by different
groups, even at the same illumination wavelength. For instance, Leon et al. [1988] with 325 nm illumination reported that the peak wavelength of emission for normal tissue was 465 nm. In contrast, O'Brien et al. [1989] found the fluorescence peak to be at 382 nm with the same illumination. These discrepancies make it difficult to compare results between different groups and illumination wavelengths. Several investigations have tried comparing the fluorescence-emission spectra at different illumination wavelengths [Kittrel et al., 1985; Oraevsky et al., 1988; Crilly et al., 1989; Bergeron et al., 1988; Gmitro et al., 1990; Alexander et al., 1991; Baraga et al., 1990 & 1991], but these studies either did not present their full results, used a small number of samples, or were preliminary. Consequently, there is a need for a more complete study to determine the optimum illumination wavelength for detecting atherosclerotic plaques with fluorescence spectroscopy.

Aorta was the arterial tissue used in most of the studies, but coronary, femoral, renal, carotid, popliteal, pulmonary and tibial arteries have also been investigated. Several studies lumped all the arterial tissues together [Leon et al., 1988; Andersson-Engels et al., 1988; Sartori et al., 1988], implying that the fluorescence-emission spectra did not vary much between arterial types. However, more recent studies indicate that the emission spectrum does depend upon the type of the artery, because different arterial components are present in different concentrations for different arteries. For example, the concentrations of collagen and elastin, two known fluorescing species commonly found in arterial tissue and atherosclerotic plaques (see Section 3.4.1), are different for muscular (e.g., coronary) and elastic (e.g., aorta) arteries. Therefore, the fluorescence spectra may be different for coronary and aorta. Several investigations with 290 nm to 310 nm [Baraga et al., 1991] and 476 nm [Richards-Kortum et al., 1991] attempted to study the differences of emission spectra
between aorta and coronary, and how these differences affect the ability to classify the tissue into normal and diseased classes. They found that the spectral emission properties changed for the two arteries, but tissue classification was still possible with unique classifiers for coronary and aorta.

Arterial fluorescence photobleaching has been described by several investigators [Chaudhry et al., 1989; Verbunt et al., 1992]. Verbunt reported that fluorescence induced with 350 nm to 364 nm illumination changed with lengthy exposures. Collagen fibers displayed the fastest decrease in emission intensity versus elastin and ceroid. Ceroid, a protein lipopigment commonly found in atherosclerotic lipid deposits, demonstrated a "red"-shift in the peak wavelength with lengthy exposure, indicating a change in the spectral shape in addition to the overall decrease in intensity. Chaudhry et al., 1989] examined photobleaching with 476 nm illumination. Fluence levels greater than 70 mJ/mm² resulted in spectral intensity and shape changes. They found the changes in the spectral shape to be reversible, but changes to the total intensity were irreversible.

For low fluence levels, photobleaching effects can be neglected. The relationship between fluorescence emittance and illumination irradiance was shown to be linear [Connor-Davenport, 1992b] up to 33 mW/cm² for 325 nm illumination (HeCd laser). The exposure length was approximately 10 seconds; thus, the maximum energy deposition was approximately 3.3 mJ/mm², which was much below the fluence level reported by Chaudhry.

Since laser ablation requires high fluence levels for plaque removal, the fluorescence spectrum may also be influenced by photobleaching. One potential technique for avoiding photobleaching due to laser ablation was described by Deckelbaum et al. [1989]. They used 325 nm-induced fluorescence to guide in-vitro
arterial laser ablation with 2.9 \( \mu \text{m} \) (Erbium:YAG) laser energy. Since ablation was performed at a wavelength longer than the band of emission wavelengths, photobleaching was avoided. With this technique, they reported that, on average, the "guided"-laser ablated atherosclerotic plaques to within 198 \( \mu \text{m} \) of the media. No perforations were reported in 27 attempts. This result suggests that there were few major spectral changes due to the irradiation at the wavelength longer than the emission range.

The sensitivity of the emission spectra to internalized substances may make it difficult to determine absolute properties of arterial fluorescence. Laser angioplasty and other intravascular procedures are commonly performed under angiographic guidance. Angiography uses a contrast media that can be visualized under x-ray fluoroscopy. It was found that the spectral properties of the contrast media interfere with the fluorescence-emission characteristics of normal artery and atherosclerotic plaques [Lucas et al., 1991]. This effect was observed in both blood and saline fields. In addition, it is uncertain what the effects of different drugs and diet may be on arterial fluorescence. For example, a form of tetracycline, which has been described as an light absorption contrast agent (see Section 3.4.3), is a commonly used drug. The properties of tetracycline could potentially alter the fluorescence spectra of the patient's arteries. Additionally, carotenoids, a group of fluorescing species commonly found in atherosclerotic plaques are found in many foods (e.g., carrots, tomatoes, etc.). One such carotenoid, beta-carotene, becomes more concentrated in plaques with increased consumption. As a result, fluorescence measurements in arteries may be improved by monitoring drugs and diet for a patient prior to the treatment.

Most of the fluorescence studies performed on arterial tissues have measured the continuous emission spectra. However, a few studies have been performed to
investigate the time-resolved properties of arterial fluorescence [Park et al., 1989; Baraga et al., 1989; Pradhan et al., 1991]. The reported fluorescence lifetimes ranged between 33 ps and 408 ns. These studies indicated that the fluorescence lifetimes are a poor indicator of the type of tissue.

3.2 Modeling Fluorescence in Arterial Tissues

Numerical models have been developed to improve the understanding of the absorption and emission of light by tissues. When light (e.g., a photon of fluorescence illumination) enters a dense fluorescing medium (e.g., tissue), it propagates along a straight path until it is absorbed or scattered. If a photon is scattered, the light's trajectory is altered and it propagates in another direction until another scattering or absorption event. Once a photon has been absorbed, fluorescence-emission will occur with probability, Ψ, equal to the quantum efficiency of the molecule in its environment. The fluorescence-emission at a point is assumed to be isotropic. The amount of the total emission that can be collected is defined by the collection geometry of the optical detection system. Emitted fluorescence photons, in order to be detected, must travel back through the medium to the detection system without being absorbed or scattered in a direction that is not within the collection geometry of the detection system. The absorption and scattering coefficients are dependent upon the wavelength; therefore, the fluorescence spectrum that is measured from the front surface layer will be different from the spectra of deeper layers that are more affected by the absorption and scattering.

Several investigators have attempted to model the fluorescence characteristics of dense fluorescing media, such as atherosclerotic plaques [Gmitro et al., 1989;
Keijzer et al., 1989(a & b); Laifer et al., 1989; Garrand et al., 1990; Richards-Kortum et al., 1989]. Two of these models are described below.

One treatment described by Keijzer et al. [1989(a & b)], implemented a Monte Carlo simulation with the measured absorption, scattering, and spectral quantum efficiency of the tissue. First, the spatial distribution of the absorbed illumination within the tissue was estimated for a specific illumination geometry (i.e., collimated or gaussian beam). The spatial distribution of the absorbed illumination defined the source distribution of fluorescence emission. For each point of the fluorescence source, the spatial pattern of fluorescence emission that exits the surface of the tissue was estimated. The sum of the exiting light patterns for all internal points of fluorescence-emission defined the surface map of the total fluorescence emission that was measurable. This surface map was different for each emission wavelength, demonstrating that both the spectral and spatial distributions were dependent upon spectral absorption and scattering. This model result was corroborated with measurements on arterial tissue. The chromophores and, hence, the optical properties were assumed to be homogeneously distributed throughout the tissue. Chromophores included all fluorophores and other absorbers. The absorption and emission properties of the tissue were considered to be a sum of the properties of individual chromophores. Since the fluorescence spectral distribution can vary as a function of the position, the illumination and fluorescence detection geometry influence the characteristics of the measured fluorescence spectra. For example, measured spectra may be different for a single-fiber catheter and for a multi-fiber catheter with separate illumination and collection fibers.

Another model described by Richards-Kortum et al. [1989], attempted to determine the relative emission and absorption contributions of known chromophores.
The intensities of the emission spectra were modeled as a superposition of absorption and emission spectra of known chromophores -- structural proteins (collagen and elastin), ceroid and oxy-hemoglobin. They found that a fluorescence spectrum of arterial could be accurately described as a superposition of the spectra of individual chromophores.

3.3 Optical Properties of Vascular Tissue

The measured fluorescence spectrum of arterial tissue can be heavily influenced by the absorption and scattering properties of the tissue. Some of these effects were described by the models in the previous section. Many investigators have reported on the optical properties of human vascular and atherosclerotic tissues [van Gemert et al., 1985; Prince et al., 1986; Bonner et al., 1986; Long et al., 1987; Oraevsky et al., 1988; Gmitro et al., 1988; Keijzer et al., 1989b; Richards-Kortum et al., 1989; Oraevsky et al., 1992]. Light propagation in "homogeneous" tissues can be described by the absorption coefficient, \( \mu_a \), scattering coefficient, \( \mu_s \), and scattering anisotropy, \( g \). The total attenuation coefficient, \( \mu_t = \mu_a + \mu_s \), determines the effective depth in tissue that light can propagate without being significantly absorbed or scattered. There are wide variations in the reported values for these properties, which may reflect variations in tissue specimens, differences in tissue handling and preparation, different measurement techniques, and different models used to describe the attenuation.

Since arterial tissue is mostly water, the absorption spectra of arteries are very similar to that of water. Absorption is higher in the ultraviolet (UV) and infrared (IR) than the visible wavelengths. It increases for decreasing wavelengths in the UV and for increasing wavelengths in the IR. For most of the 250 nm to 1000 nm range, normal vessel and atherosclerotic plaque have similar absorption properties; however, the
plaque absorption coefficient is almost double that of normal tissue in the 450 nm to 530 nm range [Prince et al., 1986]. The absorption properties are dependent upon concentrations of specific chromophores. The absorption properties of arterial chromophores are described further in Section 3.4.3.

In the 250 to 1000 nm region, the scattering coefficient decreases as the wavelength is increased. The scattering coefficient is approximately an order of magnitude greater than the absorption coefficient. This means that scattering events are more likely than absorption in arterial tissues. The anisotropy coefficient, g, is the average cosine of the scattering angle. Values of g can range between 1 (forward scattering), 0 (isotropic) and -1 (reverse scattering). The anisotropy of arterial tissues is predominantly forward (g ranged from 0.80 to 0.93) and is moderately independent of the wavelength between 500 nm and 650 nm [Keijzer et al., 1989b].

In order for tissue to fluoresce, the illumination must pass through the front surface of the tissue. However, some of the illumination will be reflected at the surface. The fluorescence-emission may also encounter reflectance losses at the surface. Specular reflectance is a function of the indices of refraction for the tissue and the surface environment (e.g., air, saline, blood, etc.). The diffuse reflectance of a rough surface depends upon the surface roughness and the incidence angle of illumination. The specular reflectance at 308 nm was measured to be 4.4% for arterial tissue in air [Oraevsky et al., 1992]. The diffuse reflectance at 308 nm was 11.5% and 15.5% for normal artery and atherosclerotic plaque, respectively. The diffuse reflectance of normal aorta and atheroma was also measured by Prince et al. [1986]. The diffuse reflectance was three to four times larger for atheroma (~30% - 40%) than normal tissue (~10%) over the 550 nm to 1100 nm range. Higher reflectance levels
mean that less of the illumination is absorbed by the tissue and, hence, the emission intensity is lower.

3.4 Biochemical Analysis -- Fluorescing Molecules

Fluorescence spectroscopy has many applications in the biological and medical fields because it is extremely sensitive to changes in the concentrations of fluorophores and their environment. Fluorophores commonly studied in biological samples can be divided into two categories -- endogenous and exogenous. The most common endogenous, or intrinsic, biological fluorophores include amino acids (e.g., tryptophan and tyrosine) found in proteins, nucleic acids (e.g., tRNA), cofactors (e.g., NADH), and riboflavin or other flavins (e.g., FAD) [Lakowicz, 1983 and Benson, 1979]. Tryptophan accounts for approximately 90% of the fluorescence signal from proteins. Its emission properties are highly sensitive to variations in the molecular environment. Fluorophore added to the tissue, or exogenous fluorophores, are often used when the endogenous fluorophores do not provide the necessary information. Frequently used exogenous fluorophores include Fluorescein- and Rhodamine-based dyes for fluorescence microscopy experiments [Lakowicz, 1983], and porphyrins, which are used for in-vivo cancer detection [Kessel et al., 1987].

3.4.1 Native Arterial Fluorophores

Several intrinsic fluorophores have been reported to contribute to the fluorescence of atherosclerotic plaques. Blankenhorn and Braunstein [1958] were the first to report on the fluorescence properties of atherosclerotic plaques, which they studied with fluorescence microscopy. They hypothesized that one of the fluorescing
Table 3.1. Intrinsic fluorophores of normal vascular tissue and atherosclerotic plaques at various illumination wavelengths. The reported peak emission wavelength and spectral bandwidth are also listed. a Dioxane solution. b Aqueous solution. c Estimated from graph. d Half-width at half maximum intensity (long wavelength side of peak). e Full-width at half maximum intensity.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>$\lambda_{ex}$ (nm)</th>
<th>$\lambda_{pk}$ (nm)</th>
<th>$\Delta\lambda$ (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>carotenoids/Vitamin A</td>
<td>360-370</td>
<td>&quot;green&quot;</td>
<td>----</td>
<td>Blankenhorn (1958)</td>
</tr>
<tr>
<td>collagen</td>
<td>290-312</td>
<td>383</td>
<td>40\textsuperscript{d}</td>
<td>Baraga (1990 &amp; 1991)</td>
</tr>
<tr>
<td>elastin</td>
<td>290-312</td>
<td>379</td>
<td>70\textsuperscript{d}</td>
<td></td>
</tr>
<tr>
<td>L-tryptophan\textsuperscript{a}</td>
<td>308</td>
<td>329</td>
<td>29\textsuperscript{d}</td>
<td></td>
</tr>
<tr>
<td>ceroid</td>
<td>290-312</td>
<td>350-360</td>
<td>90\textsuperscript{d,e}</td>
<td></td>
</tr>
<tr>
<td>oxidized LDL</td>
<td>308</td>
<td>430</td>
<td>145\textsuperscript{c}</td>
<td>Oruevsky (1993)</td>
</tr>
<tr>
<td>elastin</td>
<td>308</td>
<td>407</td>
<td>105\textsuperscript{c}</td>
<td></td>
</tr>
<tr>
<td>collagen</td>
<td>308</td>
<td>382</td>
<td>85\textsuperscript{e}</td>
<td></td>
</tr>
<tr>
<td>L-tryptophan\textsuperscript{b}</td>
<td>308</td>
<td>362</td>
<td>65\textsuperscript{c}</td>
<td></td>
</tr>
<tr>
<td>collagen</td>
<td>325</td>
<td>$\sim$400\textsuperscript{c}</td>
<td>60\textsuperscript{e}</td>
<td>Laifer (1989)</td>
</tr>
<tr>
<td>elastin</td>
<td>325</td>
<td>$\sim$400\textsuperscript{c}</td>
<td>116\textsuperscript{c}</td>
<td></td>
</tr>
<tr>
<td>collagen</td>
<td>350-364</td>
<td>410</td>
<td>$\sim$97\textsuperscript{c,c}</td>
<td>Verbunt (1992)</td>
</tr>
<tr>
<td>elastin</td>
<td>350-364</td>
<td>420-440</td>
<td>$\sim$103\textsuperscript{c,c}</td>
<td></td>
</tr>
<tr>
<td>ceroid</td>
<td>350-364</td>
<td>460-560</td>
<td>$\sim$150\textsuperscript{c,c}</td>
<td></td>
</tr>
<tr>
<td>collagen/elastin</td>
<td>476</td>
<td>$\sim$535\textsuperscript{c}</td>
<td>$\sim$80\textsuperscript{c,c}</td>
<td>Richards-Kortum (1989)</td>
</tr>
<tr>
<td>ceroid</td>
<td>476</td>
<td>$\sim$555\textsuperscript{c}</td>
<td>$&gt;150$\textsuperscript{c,c}</td>
<td></td>
</tr>
</tbody>
</table>
substances, which displayed a pale green haze, was due to carotenoids and/or Vitamin A.

More recently, various investigators have reported the contributions of different fluorophores at different illumination wavelengths. Table 3.1 lists the fluorophores that have been identified along with the reported peak emission wavelength at specific illumination wavelengths.

In the 290 nm to 312 nm illumination range, Baraga et al. [1989, 1990, & 1991] claimed that three fluorophores, collagen, elastin and L-tryptophan, were responsible for the variation of emission spectra for non-necrotic vascular tissues. They found that the relative concentrations of each of the fluorophores could be estimated by a pair of intensity ratios. An intensity ratio, \( R = \frac{I(480 \text{ nm})}{I(380 \text{ nm})} \), was used to determine the relative signal contributions from collagen and elastin. Another ratio, \( T = \frac{I(335 \text{ nm})}{I(390 \text{ nm})} \), was used to detect the relative level of L-tryptophan in the tissue. The wavelength of the fluorescence peak decreased from 335 nm as the illumination wavelength was increased from 290 nm to 310 nm. This was found to be consistent with the fluorescence behavior of L-tryptophan in dioxane solution. The \( T \) ratio was found to be greatest for normal vascular tissue and early fatty (atherosclerotic) lesions compared to more advance plaques. Another fluorophore, ceroid, a protein lipopigment commonly associated with lipid accumulation in necrotic and calcified atherosclerotic arteries [Burt, 1946; and Mitchenson et al., 1985], was also claimed to be present [Baraga et al., 1991].

Another study with 308 nm illumination [Oraevsky et al., 1993] was performed to determine if there was a signal contribution from peroxidized lipoproteins. Lipoproteins are oxidized through interactions with the arterial endothelial layer. The spectral characteristics of peroxidized lipoproteins and lipid-rich plaques did seem to
be correlated. Oraevsky also reported possible signal contributions from collagen, elastin and L-tryptophan. The peak emission wavelengths for elastin and L-tryptophan, however, had different reported values for the studies by Baraga and Oraevsky. For the L-tryptophan measurements, Baraga used dioxane (low polarity) and Oraevsky used an aqueous (high polarity) solution. The shift in the peak emission wavelength for the two solutions is consistent with the theory that the Stokes' shift increases with the polarity of the solution (Section 3.1). The tissue spectra in Oraevsky's work did not display any peaks at 335 nm, which was the L-tryptophan peak reported by Baraga.

For 325 nm illumination, Laifer et al. [1989] and Garrand et al. [1990] attributed the primary fluorescence signals from normal and atherosclerotic vessel to collagen and elastin. Fluorescence from lipid deposits was also visible under fluorescence microscopy; however, it was determined that the lipid fluorescence did not contribute much to the total measured fluorescence signal [Laifer et al., 1989]. Both studies fit linear combinations of normalized collagen and elastin spectra to measured tissue spectra. The spectra were normalized prior to the fit. The fitting yielded two linear coefficients, $C$ and $E$, which were proportional to the amounts of collagen and elastin, respectively. The collagen to elastin coefficient ratio, $C:E$, was found to increase with the relative amounts of collagen to elastin in the tissue. The difference between the coefficients, $E-C$, was used to discriminate between normal and atherosclerotic aortic tissues [Laifer et al., 1989]. The spectra of normal vessels displayed greater similarity to the elastin spectrum and atherosclerotic plaques were more similar to collagen. The spectra were, therefore, consistent with the pathology of the tissue.
Verbunt et al. [1992] used a fluorescence microscope (351 nm to 364 nm illumination) and a microscopic spectrofluorometer (350 nm illumination) to study the fluorescence properties of normal and atherosclerotic arteries. They attributed arterial fluorescence to ceroid, elastin and collagen. Ceroid was absent from normal arterial specimens, but was detected in almost all fatty and calcified plaques. Relative to the fluorescence signatures of collagen and elastin, the fluorescence spectrum of ceroid was "red"-shifted and much broader.

Arterial fluorescence induced with 476 nm illumination was also claimed to be a linear combination of spectra of structural proteins (collagen and elastin) and ceroid [Richards-Kortum et al., 1989 & 1991]. Measured spectra from arterial tissue were fit to a combination of known fluorophore spectra. The coefficients of the fit for structural proteins, $\beta_{SP}$, and ceroid, $\beta_{CE}$, were used to build a discriminant function to classify normal and atherosclerotic arteries. Normal artery and fibrotic plaques, which contained little lipid, had the largest values of $\beta_{SP}$, while $\beta_{CE}$ was largest for soft fatty (atherosclerotic and atheromatous) plaques and calcified plaques. These findings were corroborated through fluorescence microscopy [Fitzmaurice et al., 1989].

It appears from these investigations that there are possibly six primary fluorophores associated with arterial and atherosclerotic tissues -- collagen, elastin, L-tryptophan, ceroid, lipoproteins, and carotenoids. The fluorescence properties are dependent upon the excitation wavelength. To illustrate that the emission spectra of these fluorophores change with the illumination wavelength, the fluorescence spectra of collagen, elastin, and L-tryptophan (Sigma Chemical Co.) for four illumination wavelengths (270 nm, 325 nm, 400 nm and 470 nm) are displayed in Figure 3.4. The spectrum of L-tryptophan is only shown for 270 nm illumination because the emission was very weak for longer illumination wavelengths [Baraga et al, 1990].
Figure 3.4. Normalized fluorescence spectra of collagen, elastin, and L-tryptophan in saline solution. Fluorescence excitation was (a) 270 nm, (b) 325 nm, (c) 400 nm and (d) 470 nm. L-tryptophan is only shown for 270 nm. Spectra were smoothed with a 15 nm wide averaging window.
3.4.2 Intrinsic Arterial Absorbers

As described previously in this chapter, the absorption properties of chromophores in the tissue can influence the characteristics of the emission spectrum. Many chromophores commonly exist in both normal artery and atherosclerotic plaques. Obviously, each of the fluorophores described in the previous section are also chromophores, because they must first absorb light in order to re-emit it. The attenuation spectra of the structural proteins (collagen and elastin) were described by Richards-Kortum et al. [1989]. The total attenuation coefficient for 500 nm to 650 nm is between 41 cm\(^{-1}\) and 55 cm\(^{-1}\). Oraevsky et al. [1992] reported the absorption properties of oxidized LDL. The absorption increases with decreasing wavelength from 800 nm to 250 nm. The absorption properties of L-tryptophan were described by Baraga et al. [1989]. The absorption decreases steadily between 290 nm and 308 nm and is almost zero for longer wavelengths. As a result, L-tryptophan does not contribute much to the fluorescence emission with illumination wavelengths greater than 308 nm. Carotenoids have numerous absorption peaks between 420 nm and 500 nm [Prince et al., 1986]. The peaks depend upon the types of carotenoids present.

Another chromophore commonly found in arterial specimens is oxy-hemoglobin. The peak absorption bands of oxy-hemoglobin are located at 390-420 nm (Soret band), 530-550 nm (\(\beta\) band) and 550-580 nm (\(\alpha\) band) [Prince et al., 1986]. The fluorescence-emission spectrum shown in Figure 3.5 demonstrates a dip at approximately 420 nm due to Soret band absorption by oxy-hemoglobin. The presence of hemoglobin in both ex-vivo and in-vivo arterial specimens is a subject of much controversy. Hemoglobin is present in small amounts within the intimal surface layer of the vessels in humans; however, it is believed that additional hemoglobin is absorbed into the vessel wall after death due to hemostasis and the lysis of red blood
Figure 3.5. Fluorescence-emission spectrum (325 nm illumination) of arterial tissue exhibiting the signal loss around 420 nm from absorption by oxy-hemoglobin (HbO₂).
cells. Since ex-vivo specimens for experimental measurements are often removed hours after death, hemoglobin has a substantial period of time to be absorbed. Studies with freshly-excised surgical specimens revealed that the spectral absorption peaks from hemoglobin were absent from the fluorescence spectra of arterial tissues [Cothren et al., 1989; Richards-Kortum et al., 1991]. It is likely that hemoglobin is present in hemorrhaged atherosclerotic plaques; consequently, some tissues may display the absorption peaks in-vivo.

3.4.3 Extrinsic Arterial Contrast Agents

A variety of contrast agents that change the fluorescence and/or the absorption properties of arterial tissue have been investigated. A list of fluorescence contrast agents and their properties is given in Table 3.2. Contrast agents are often preferentially absorbed by a certain type of tissue -- usually atherosclerotic plaques. Some exogenous fluorophores, such as porphyrins including HP, HPD and phycocyanin, exhibit a distinct fluorescence emission spectrum. Example fluorescence spectra of arterial tissue with HPD contrast agent are shown in Figure 3.6. Other fluorescence contrast agents, such as CTC, cause the tissue's peak emission wavelength to be shifted. Absorption contrast agents, such as tetracycline and Beta-carotene, enhance the absorption properties of certain tissues over a range of wavelengths. These contrast agents are primarily used for selective ablation of plaques, but they also could potentially influence the spectral emission properties.
Figure 3.6. Fluorescence-emission spectra (400 nm illumination) of normal (N), atherosclerotic plaque (P) and calcified (C) arterial tissues that have been soaked in HPD for 30 minutes. The emission peaks from HPD are observed at 620 nm and 685 nm. Note that the peaks are absent from the spectrum of calcified tissue.

Table 3.2. Reported fluorescence and absorption contrast agents. The fluorescence excitation and emission wavelengths, $\lambda_{ex}$ and $\lambda_f$ respectively, and the absorption wavelengths, $\lambda_a$, are listed.

<table>
<thead>
<tr>
<th>Contrast Agent</th>
<th>$\lambda_{ex}$(nm)</th>
<th>$\lambda_f$(nm)</th>
<th>$\lambda_a$(nm)</th>
<th>Comments</th>
<th>Investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoporphyrin (HP)</td>
<td>395</td>
<td>616, 630-660</td>
<td></td>
<td></td>
<td>Kessel (1988)</td>
</tr>
<tr>
<td>Hematoporphyrin Derivative (HPD)</td>
<td>442</td>
<td>632</td>
<td></td>
<td>in-vivo</td>
<td>Prevosti (1988)</td>
</tr>
<tr>
<td>Phycocyanin</td>
<td>580</td>
<td>645</td>
<td>620.5</td>
<td></td>
<td>Morcos (1988)</td>
</tr>
<tr>
<td>HPD</td>
<td>636</td>
<td></td>
<td></td>
<td>in-vivo</td>
<td>Litvack (1985)</td>
</tr>
<tr>
<td>Chlorotetracycline (CTC)</td>
<td>248, 308, 351</td>
<td>535-545</td>
<td></td>
<td>calcified plaque specific</td>
<td>Clarke (1988)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>355</td>
<td></td>
<td></td>
<td>Murphy-Chutorian</td>
</tr>
<tr>
<td>Beta Carotene</td>
<td></td>
<td>450-500</td>
<td></td>
<td>in-vivo</td>
<td>Prince (1988)</td>
</tr>
</tbody>
</table>
3.5 Summary

The combination of the absorption and emission properties of all the tissue chromophores makes the spectral characterization of fluorescence very complex. Since certain chromophores are linked to various stages of the atherosclerotic process, it seems likely that the fluorescence spectra may provide information about the tissue constituents and, hence, the tissue type. The emission distribution, however, is sensitive to many factors such as measurement geometry, arterial tissue type, and the presence of exogenous chromophores.

Clearly, the emission and absorption properties of the intrinsic chromophores are dependent upon the illumination wavelength used for exciting fluorescence. Which illumination wavelength(s) is(are) optimum for discriminating the emission spectra of normal artery and different types of atherosclerotic plaques is an important question. Several techniques for answering this question are described in Chapter 5. The results of a study to determine the optimum illumination wavelength are found in Chapter 6.
CHAPTER 4

FLUORESCENCE MEASUREMENTS

The following chapter describes the construction of the system for measuring the fluorescence spectra of tissue (Section 4.1). The techniques for spectral correction and calibration are also described (Section 4.2). Finally, the procedures for tissue preparation, measurement and evaluation are outlined (Section 4.3).

4.1 Catheter-based Fluorescence Spectrometer with Tunable Illumination

A catheter-based fluorescence spectrometer was designed and built to measure the fluorescence spectra of human arterial tissue over a range of illumination wavelengths. Figure 4.1 depicts the experimental apparatus. Original design considerations included being able to scan a narrow band of illumination from 250 nm to 500 nm, measuring a sufficient spectral emission band to capture most of the fluorescence information, and coupling the illumination and emission through a flexible fiber optic catheter. The description of the system design is divided into three sections -- the illumination, the fiber optic catheter, and the measurement spectrometer.

4.1.1 Illumination

The spectral purity of a laser would make it an ideal source for illumination. Many studies of arterial fluorescence have used a laser as the source of illumination (see Table A.1). Unfortunately, a laser that can scan over the desired illumination wavelength range does not exist. A combination of several lasers could be used, but this would greatly increase the system complexity and be very expensive.
Figure 4.1. Catheter-based system for measurement of fluorescence spectra.
Instead, a lamp and monochromator combination was selected to provide the range of illumination wavelengths. This solution was much simpler and cheaper than a multiple laser system. A 200 watt HgXe arc lamp was used because its emission was strong over the entire range of illumination wavelengths. The disadvantage of this type of illumination is that there are some strong spectral peaks in the emission. Some of the spectral emission of the lamp outside the desired illumination bandwidth can scatter through the monochromator and interfere with the measured spectrum of fluorescence emission. Section 4.2 describes a solution to this problem.

The output of the lamp was focused through a grating monochromator. The dimensions and groove density of the grating, the imaging optics and the dimensions of the entrance and exit slits determine the bandwidth and lineshape of the monochromator output. Since the slit widths for both the monochromator and the spectrometer are large relative to the wavelengths of light, the lineshape and bandwidth of these instruments can be estimated using basic geometrical optics principles.

The linewidth of a monochromator increases with the widths of the slits. For each wavelength, an image of the entrance slit forms on the plane of the exit slit. The grating, however, shifts the light at different wavelengths. The linear displacement as a function of wavelength in mm/nm for a grating monochromator is given by

$$\frac{dx}{d\lambda} = \frac{gm f_{\text{exit}}}{10^6 \cos \beta},$$  

where $x$ is the linear displacement of the dispersion at the plane of the exit plane, $\beta$ is the diffracted beam angle (relative to the grating normal), $g$ is the groove density of the grating in grooves/mm, $m$ is the diffraction order, and $f_{\text{exit}}$ is the focal length to the exit slit in mm. The inverse of Equation 4.1, $d\lambda/dx$, is the linear dispersion of the grating. The grating displaces each wavelength of light a different amount, which forms
Figure 4.2. The spectral profile of the monochromator output (b) is the convolution of the transmission functions for the entrance and exit slits (a). The profile is triangular since the slit widths, $w_{\text{ent}}$ and $w_{\text{ex}}$, are equal.

multiple images of the entrance slit at the exit plane. The exit slit extracts a certain portion of the light distribution at the exit plane. For a source with a uniform distribution of spectral intensities, the spectral distribution of the monochromator output is given by the convolution of the entrance and exit slits (Figure 4.2). If the width of the image of the entrance slit equals the width of the exit slit, then the distribution at the output is triangular with the base width equal to double the width of a single slit. The spectral bandwidth, $\Delta \lambda_{\text{ex}}$, determined by the finite slit widths is obtained by multiplying the width of the convolution, $\Delta x_{\text{ex}}$(in mm) with the linear dispersion (in nm/mm),

$$\Delta \lambda_{\text{ex}} = \Delta x_{\text{ex}} \frac{d\lambda}{dx}.$$  \hspace{1cm} (4.2)
Aberrations created by the imaging optics and the grating can increase the linewidth (or bandwidth) and alter the lineshape.

Table 4.1 lists the specifications for the monochromator used for these experiments. The blaze wavelength is the wavelength of peak diffraction efficiency. To calculate the maximum linear dispersion, the diffracted beam angle, \( \beta \), was set to zero. For small angular deviations from \( \beta = 0^\circ \), the linear dispersion will remain approximately constant. Since the widths of the entrance and exit slits were equal, the profile of the spectral output of the monochromator was triangular. The bandwidth, which was calculated as the full-width at half the maximum intensity, was around 7.3 nm. Approximately 75% of the total monochromator output was within this band. The bandwidth was selected to achieve both good spectral resolution and sufficient illumination intensities. The output wavelength of the monochromator could be scanned from 0 \( \mu \)m to 4 \( \mu \)m, but for this experiment, only the range from 270 nm to 500 nm was used.

4.1.2 Fiber-optic Catheter

As mentioned in Section 3.2, the distribution of spectral intensities for fluorescence emission depends upon the geometries of the illumination and the collection. A variety of measurement geometries have been used for measuring fluorescence emission of arterial tissues. Direct illumination (no catheter), single-fibers, and multiple-fiber catheters, including endoscopes, have all been used to illuminate tissue and collect fluorescence emission. This experiment used a multiple-fiber catheter with ten 200 \( \mu \)m silica fibers (0.22 N.A.) for illumination and one 400 \( \mu \)m silica fiber (0.22 N.A.) for collection. Both the optical fibers and the focusing optics used high OH- fused-silica because it is transmissive [product literature, 3M]
Table 4.1. Monochromator specifications. (Note: \( m = 1 \))

<table>
<thead>
<tr>
<th>Model</th>
<th>SPEX 1681A Type Czerny-Turner</th>
</tr>
</thead>
<tbody>
<tr>
<td>f/#</td>
<td>f/4.0</td>
</tr>
<tr>
<td>( g )</td>
<td>300 grooves/mm</td>
</tr>
<tr>
<td>blaze ( \lambda )</td>
<td>300 nm</td>
</tr>
<tr>
<td>( d\lambda/dx )</td>
<td>14.7 nm/mm</td>
</tr>
<tr>
<td>( \Delta\lambda_s )</td>
<td>7.3 nm</td>
</tr>
<tr>
<td>( f_{collection} )</td>
<td>227 mm</td>
</tr>
<tr>
<td>( f_{exit} )</td>
<td>227 mm</td>
</tr>
<tr>
<td>( w_{ent} )</td>
<td>0.5 mm</td>
</tr>
<tr>
<td>( w_{ex} )</td>
<td>0.5 mm</td>
</tr>
</tbody>
</table>

Table 4.2. Spectrometer specifications. (Note: \( m = 1 \))

<table>
<thead>
<tr>
<th>Model</th>
<th>SPEX 1681C Type Czerny-Turner</th>
</tr>
</thead>
<tbody>
<tr>
<td>f/#</td>
<td>f/4.0</td>
</tr>
<tr>
<td>( g )</td>
<td>150 grooves/mm</td>
</tr>
<tr>
<td>blaze ( \lambda )</td>
<td>500 nm</td>
</tr>
<tr>
<td>( d\lambda/dx )</td>
<td>19.5 nm/mm</td>
</tr>
<tr>
<td>( \Delta\lambda_s )</td>
<td>1.5 nm</td>
</tr>
<tr>
<td>( f_{collection} )</td>
<td>342 mm</td>
</tr>
<tr>
<td>( f_{exit} )</td>
<td>342 mm</td>
</tr>
<tr>
<td>( w_{ent} )</td>
<td>0.05 mm</td>
</tr>
<tr>
<td>( w_{ex} )</td>
<td>0.05 mm</td>
</tr>
</tbody>
</table>

Table 4.3. Detector array specifications.

<table>
<thead>
<tr>
<th>Manuf.</th>
<th>Princeton Instruments</th>
<th>Model</th>
<th>IRY-512</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>Proximity-focused, multi-channel plate image intensifier</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controller</td>
<td>ST-120</td>
<td>Elements</td>
<td>512</td>
</tr>
<tr>
<td>Width</td>
<td>18 mm</td>
<td>Resolution</td>
<td>3-4 diodes @ FWHM</td>
</tr>
<tr>
<td>QE (@ 250 nm)</td>
<td>20%</td>
<td>Readout Noise</td>
<td>1 count/diode/scan</td>
</tr>
<tr>
<td>QE (@ 500 nm)</td>
<td>10%</td>
<td>Spontaneous Emission Noise</td>
<td>&lt; 10 counts/second</td>
</tr>
</tbody>
</table>
Figure 4.3. The end-views of the multi-fiber catheter. The illumination (a), distal (b) and spectrometer (c) ends are shown.

Specialty Optical Fibers, West Haven, CT] for all the illumination and emission wavelengths in the study.

The monochromator output was coupled through a pair of silica lenses (Figure 4.1) into the 200 \( \mu m \) silica fibers that were arranged in a vertically linear pattern for maximum coupling to the monochromator slit (Figure 4.3a). The first silica lens, \( L_1 \) (diameter = 1", focal length = 4"), was optimum for coupling with the monochromator. A second silica lens, \( L_2 \) (diam. = 1", f.l. = 2"), imaged the exit slit of the monochromator onto the end of the fibers. The magnification of this lens combination was 0.5 so that the image of the slit on the fibers was 250 \( \mu m \) wide. This was slightly wider than the illumination fibers. To improve spectral source purity, a bandpass filter, BPF, (see Table 4.4) could be placed between the two lenses to minimize scattered light exiting from the monochromator that was outside the desired spectral illumination passband. Neutral density filters (NDF) could also be inserted to control the illumination intensity. An electronic shutter (Newport 845), controlled by
the data acquisition system, determined the length of the illumination exposure. This was done to minimize the photobleaching of the fluorescence signal. The exposure time was between 10 seconds for strong fluorescence signals and 1 minute for weaker fluorescence signals. For the different illumination wavelengths, the illumination power ranged between 10 μW and 350 μW.

At the distal end of the catheter, light transmitted through the 200 μm fibers illuminated the tissue. The illumination fibers were arranged in a circular geometry around a central 400 μm fiber that collected the fluorescence emission (Figure 4.3b). The catheter was positioned approximately 3 mm away from the tissue using a cylindrical sleeve around the distal end of the catheter. The illumination spread over approximately 3.5 mm². The maximum power on the tissue surface was less than 100 μW/mm². Even for the full 60 second exposure length with this maximum intensity*, the maximum fluence was 6 mJ/mm² which is below the fluence levels for photobleaching described in Section 3.1.2.

As discussed in Chapter 3, the geometries of the illumination and collection fibers can influence the spectral distribution of the fluorescence emission. A previous study with this system [Alexander et al., 1991], however, demonstrated that the spectral distribution was relatively insensitive to the distance between the catheter and the tissue. This study also showed that the spectra were relatively insensitive to the angle of the catheter with respect to the tissue. Figure 4.4 shows the intensity and spectral "shape" dependence upon the distance and angle. When the catheter was in direct contact with the tissue, the normal spectrum displayed a slight "red-shift" (Figure 4.4b). Fluorescence spectra measured at distances greater than 1 mm away from the tissue

* The exposures for this intensity illumination were always shorter than 60 seconds.
Figure 4.4. Fluorescence-emission intensity and spectral distribution dependence on catheter orientation. The fluorescence emission intensity is dependent upon the distance (a) and angle (c) between the end of the catheter and the tissue surface. The relative intensity plots are the average of five different studies. The shape of the spectra, however, is relatively insensitive to the distance (b) [0, 4, and 8 mm are shown] and angle (d) [0, 20, 40 and 60 degrees are shown]. The fluorescence spectrum of the normal sample measured with the catheter in direct contact [0 mm] displayed as slight "red-shift".
were primarily from the surface of the tissue since there was large overlap of the illumination and the collection projections. However, when the catheter is in direct contact with the tissue, the fluorescence induced by the outer catheter fibers must travel through the tissue to the central collection fiber. The larger attenuation at the shorter wavelengths caused the "red-shift". For this study, the "red-shift" was absent and the spectra were assumed to be insensitive to small perturbations in the catheter position because the catheter orientation was fixed at 3 mm distance and perpendicular to the surface of the tissue.

A pair of silica lenses \( L_3 \) (diam. = 1", f.l. = 2") and \( L_4 \) (diam. = 1", f.l. = 4") -- (with an overall magnification of 2.0) coupled the fluorescence signal captured by the 400 \( \mu \)m collection fiber into the spectrometer. This arrangement yielded an 800 \( \mu \)m diameter image of the fiber on the entrance slit. The projected image of the slit on the end of the collection fiber is shown in Figure 4.3c (magnification = 0.5).

4.1.3 Measurement Spectrometer

Measurement of the spectral distribution of fluorescence emission requires two components -- one component for dispersing or separating the fluorescence emission intensities and a second for detecting intensities. Scanning monochromators with a single detector, spectrometers with linear detector-arrays, and narrow band-pass filters with two-dimensional imaging detectors (e.g., CCD's) have all been used to study the fluorescence emission of atherosclerotic plaques. The combination of a diffraction-grating spectrometer and an intensified detector array was selected for these experiments because of the high wavelength resolution and the ability to quickly acquire an entire spectrum of fluorescence emission. The ability to acquire an entire

\(^{\dagger}\) The projections are defined as the surface areas of the tissue being illuminated and measured by the illumination and collection fibers.
spectrum simultaneously reduced the exposure time of the illumination, thereby minimizing photobleaching effects.

The fluorescence emission of the tissue collected by the 400 μm collection fiber was imaged into a grating spectrometer. The grating dispersed the fluorescence spectral components directly onto a 512 element intensified diode detector array (Princeton Instruments IRY-512 N/B OSMA). The spectrometer and detector array specifications are listed in Tables 4.2 and 4.3, respectively.

The image of the entrance slit on the detector array results in a rectangular spectral profile with line-width, $\Delta \lambda_s$, equal to the product of the linear dispersion, $d\lambda/dx$ equal to the inverse of Equation 4.1 and the width of the entrance slit, $w_{\text{entrance}}$. The spectral width or the minimum resolution of the spectrometer in this configuration was 1.5 nm. Each detector element covered approximately 0.5 nm; consequently, the wavelength range of the measured fluorescence spectra was approximately 250 nm. A long-wavelength pass filter (LWPF) was used to prevent any reflected illumination light from entering the spectrometer and corrupting the spectra.

4.1.3 Measurement Specifications

Table 4.4 lists the thirteen illumination wavelengths that were used in this study with the corresponding spectral emission wavelength ranges. The wavelengths studied characterized the fluorescence over the entire excitation range (270 nm to 500 nm). The wavelengths were chosen to match illumination wavelengths previously reported in the literature and/or to provide reasonably even coverage of the illumination range. The changes in the spectral emission characteristics between the selected illumination wavelengths were gradual; consequently, the performance between the selected wavelengths can be interpolated from the performances at selected wavelengths. The
emission wavelength range at 458 nm and 500 nm excitation was less than 250 nm because the cut-off wavelength for the LWPF was larger than the low wavelength of the measured emission wavelength band. The use of a slightly narrower spectral emission band did not significantly affect the results.

<table>
<thead>
<tr>
<th>Excitation Wavelength (nm)</th>
<th>Illumination BPF Range (nm)</th>
<th>Fluorescence Range (nm)</th>
<th>LWPF Cutoff (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>270</td>
<td>260-380</td>
<td>300 - 550</td>
<td>305</td>
</tr>
<tr>
<td>300</td>
<td>260-380</td>
<td>330 - 580</td>
<td>320</td>
</tr>
<tr>
<td>304</td>
<td>260-380</td>
<td>330 - 580</td>
<td>320</td>
</tr>
<tr>
<td>314</td>
<td>260-380</td>
<td>350 - 600</td>
<td>345</td>
</tr>
<tr>
<td>325</td>
<td>260-380</td>
<td>350 - 600</td>
<td>345</td>
</tr>
<tr>
<td>334</td>
<td>260-380</td>
<td>350 - 600</td>
<td>345</td>
</tr>
<tr>
<td>364</td>
<td>260-380</td>
<td>400 - 650</td>
<td>385</td>
</tr>
<tr>
<td>380</td>
<td>--</td>
<td>400 - 650</td>
<td>400</td>
</tr>
<tr>
<td>400</td>
<td>--</td>
<td>450 - 700</td>
<td>455</td>
</tr>
<tr>
<td>436</td>
<td>--</td>
<td>450 - 700</td>
<td>455</td>
</tr>
<tr>
<td>458</td>
<td>--</td>
<td>475 - 700*</td>
<td>475</td>
</tr>
<tr>
<td>470</td>
<td>--</td>
<td>500 - 750</td>
<td>495</td>
</tr>
<tr>
<td>500</td>
<td>--</td>
<td>550 - 750*</td>
<td>550</td>
</tr>
</tbody>
</table>
4.2 Spectral Correction and Calibration Methods

A substantial effort was made to correct and calibrate the measured spectra for accurate representation of the "true" fluorescence spectra. This enabled us to compare, with great confidence, spectra that were measured at different times, even months apart.

As discussed in the previous section, the HgXe arc lamp had strong peaks that were scattered through the monochromator and were detected along with the fluorescence spectrum. The light from these scattered peaks interfered with the measured fluorescence spectrum. One solution to this problem would have been to use a double grating monochromator, but this was not available. Instead, a clever manipulation of the long-wavelength pass filter (LWPF) enabled the removal of the scattered light and any additional background signal from the measured spectrum. The background signal consisted of the combination of ambient room light, detector dark current, and any scattered illumination light from the arc lamp within the spectral emission band. The background was measured by moving the LWPF to the illumination arm of the apparatus between the monochromator and the catheter. The LWPF eliminated the primary fluorescence exciting illumination, so that the detector measured everything but the desired fluorescence emission. The background-corrected spectrum was calculated by subtracting the background spectrum from the originally-corrupted fluorescence spectrum (see Figure 4.5).

The wavelengths corresponding to different detector positions were calibrated by measuring the spectrum of known spectral lines from a low-pressure mercury lamp. This wavelength calibration was performed for each emission band in Table 4.4.

The absorptances and reflectances of the system components (e.g., lenses, fibers, filters, etc.,) in Figure 4.1 are dependent upon wavelength and will affect the spectral response of the system. In addition, the spectrometer grating and detector
Figure 4.5. Spectral-background subtraction. The bold curve shows the corrected spectrum.
elements are more efficient for certain wavelengths than others. Consequently, the measured spectra (already background corrected) are distorted by the spectral sensitivity of the instrument. The measured spectrum from a calibrated tungsten source (Optronics 245) was used to determine the spectral sensitivity of the instrument. The fiber collected the light from the tungsten lamp after the light reflected off the face of a lambertian surface (Figure 4.6). The inverse spectral sensitivity function, $\kappa(\lambda)$, of the measurement system is given by

$$K(\lambda) = \frac{I_f(\lambda)}{I_m(\lambda)},$$

where $I_f(\lambda)$ and $I_m(\lambda)$ are the measured (m) and true (t) spectral intensities, respectively, of the tungsten lamp. The $f$ superscript refers to a spectrum of the tungsten lamp. The spectrally-calibrated ($c$) fluorescence-emission spectrum is then

$$I^f_c(\lambda) = \kappa(\lambda)I^f_r(\lambda),$$

where $I^f_r(\lambda)$ is the measured raw (r) fluorescence spectra. The $f$ superscript refers to a fluorescence spectrum. Inverse spectral sensitivity functions were generated for each illumination wavelength since different settings of the spectrometer and different filters changed the spectral sensitivity and wavelength measurement range of the system. Two example inverse sensitivity curves are plotted in Figure 4.7.
Figure 4.6. Spectral sensitivity calibration of the fluorescence spectrometer.

Figure 4.7. Inverse-sensitivity or spectral correction curves for (a) the 350 nm to 600 nm and (b) the 450 nm to 700 nm measurement ranges. The raw background-corrected spectra are multiplied by these curves to calculate the spectrally calibrated fluorescence spectra.
4.3 Tissue Preparation and Measurements

To evaluate the fluorescence emission at different illumination wavelengths, a large data set of fluorescence spectra was collected. Specimens of human descending aorta were obtained post-autopsy and soaked in saline for at least 24 hours before spectral measurement. As described in Chapter 3, oxy-hemoglobin in blood has a strong absorption peak around 420 nm that affects the emission spectra (Section 3.4.2). Hemoglobin is present in small amounts within the intimal surface layer of the vessels in living humans; however, it is believed that the vessel wall absorbs additional hemoglobin after death from hemostasis and the lysis of red blood cells. Consequently, tissue specimens were soaked in saline to remove as much of the hemoglobin as possible before the measurements.

To verify the hypothesis that arterial tissues absorbed hemoglobin after death, preliminary studies were performed, where aortic tissues were soaked in saline and emission spectra were measured at different time intervals. These studies indicated that the absorption dip at 420 nm decreased with increased soaking time periods. The spectrum outside the absorption band of hemoglobin, however, did not change significantly.

Spectra at each of the illumination wavelengths were acquired at several locations on the arterial specimen. The tissue was kept immersed in a bath of saline to prevent dehydration. Except for hard calcified plaques, the measured tissue sites were removed with a 5 mm biopsy punch, fixed in formalin, and submitted to histology, where cross-sectional slides of the tissue specimens were prepared and stained using Richardson's stain [Richardson, 1975]. A trained pathologist placed the "soft" tissue specimens in the NL (normal artery), FP (early fatty or fibrous plaques) or CO (complicated plaques) classes. The fourth tissue group -- CA or hard calcified plaque
(mineralized tissue) -- was classified solely by gross observation because it is difficult to obtain adequate histologic results from this type of tissue. Table 4.5 describes the tissue classes and the numbers of specimens in each class. The three diseased classes were also grouped together into a more general DI or diseased class. Different numbers of samples were obtained for the 270 nm to 364 nm illumination range, the 380 nm to 470 nm illumination range and the 500 nm illumination wavelength. The next chapter discusses techniques that were used to compensate for the biases due to the sample size.

Table 4.5. Descriptions of the tissue classes and the numbers of samples in each class.

<table>
<thead>
<tr>
<th>Class</th>
<th>Description</th>
<th>Thickness (µm)</th>
<th>Other Characteristics</th>
<th>Number (270 - 364 nm)</th>
<th>Number (380 - 500 nm)</th>
<th>Number (500 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL</td>
<td>Normal Vessel</td>
<td>0 - 199</td>
<td></td>
<td>127</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>FP</td>
<td>Fibrous Plaque</td>
<td>200 - 599</td>
<td></td>
<td>90</td>
<td>56</td>
<td>55</td>
</tr>
<tr>
<td>CO</td>
<td>Complicated Plaque</td>
<td>&gt; 600</td>
<td>Lipid or calc dep., hemorrhage, ulc.</td>
<td>82</td>
<td>40</td>
<td>37</td>
</tr>
<tr>
<td>CA</td>
<td>Hard Calc. Plaque</td>
<td></td>
<td>Hard, mineralized tissue</td>
<td>31</td>
<td>23</td>
<td>23</td>
</tr>
</tbody>
</table>
CHAPTER 5
SPECTRAL ANALYSIS TECHNIQUES

The primary objective of this research is to determine the fluorescence illumination wavelength(s) that is(are) optimum for discriminating between normal artery and atherosclerotic plaques. This would be a trivial problem if either the fluorescence spectra for all the normal and diseased arteries of an entire population were known, or the exact distributions of the fluorescence spectra of all normal and diseased arteries were known. Neither is known, but a statistical estimate of the distributions of fluorescence spectra can be formed from a finite subset of samples that are representative of the population. The ability to discriminate or classify tissues can be evaluated with these statistical estimates. The following chapter describes several techniques for forming the statistical estimates and evaluating the discrimination ability. The results of these studies are presented in the next chapter.

5.1 Statistical Pattern Recognition and Discriminant Analysis

The goal of pattern recognition is to construct a set of decision rules that will accurately place samples in their appropriate classes. For example, Figure 5.1a shows the conditional probability density functions, $p(x_1|1)$ and $p(x_1|2)$, of two classes, 1 and 2, for a single feature, $x_1$. The feature is a measurable quantity, such as the intensity of light at a certain wavelength. A classifier is constructed by selecting a threshold value, $x_0$, such that samples falling to the left are classified in one class and samples falling to the right are in the other class. If the class distributions overlap as in Figure 5.1a, then some samples will not be classified correctly (shown by the gray regions). For example, two sample points, $s_1$ and $s_2$, both in the 0 class, are shown in Figure 5.1.
Figure 5.1. Classification of probability densities, $p(X|k)$, for two classes, $k = 1$ and 2. (a) For a single feature, $X = x_1$, the class distributions overlap. The position of the threshold, $x_1^*$, determines a region of misclassification (shown in gray). (b) The addition of a second feature, $x_2$, eliminates the overlap between the class distributions. A linear discriminant function is shown as the dotted line. The equi-probability boundaries for the probability distributions of the feature vector, $X = (x_1, x_2)$, are shown. The probability distributions for both the individual features are also illustrated. This example illustrates that the combination of two features can be substantially better than a single feature.
Sample $s_1$ would be classified correctly, while $s_2$ would be misclassified for the classification threshold indicated.

Classification can often be improved if more measurements (features) are obtained. A second feature, $x_2$, is added in Figure 5.1b. The probability densities, $p(x_2|1)$ and $p(x_2|2)$, shown on the vertical axis, also have a high amount of overlap. The probability densities, $p(X|1)$ and $p(X|2)$ of the combined feature-vector, $X = (x_1, x_2)$, are shown by equi-probability boundaries that are the contours of the probability density functions at a specific probability level. The contours in Figure 5.1b indicate the level where most of the samples will be contained. These contours do not overlap; therefore, the samples can be classified more accurately with this combination of two features than for a single feature. A linear decision boundary is shown by a dotted line segment. For the combined feature-vector, $X$, the samples, $s_1$ and $s_2$, are both correctly classified for the linear decision boundary shown in the figure. Sometimes, the probability density distributions for the class populations can be complex as shown in Figure 5.2. It is obvious that a non-linear classifier in this case will be more accurate than a simple linear discriminant. Pattern classification also becomes more complex for three or more classes. Figure 5.3 shows that more than two classes can be accurately classified if the appropriate features and decision boundaries are used. This discussion of classifiers can be generalized to a higher dimensional feature-vector, $X = (x_1, x_2, ..., x_q)$. For three dimensions ($q = 3$) and higher dimensions ($q > 3$), linear decision surfaces become planes and hyper-planes, respectively.

The goal of this study is to evaluate different illumination wavelengths for their ability to identify tissues with information from fluorescence emission spectra. Two strategies for evaluating potential classification accuracy are used in this study. The first approach, called discriminant analysis, uses indirect techniques for evaluating
Figure 5.2. Non-linear classification boundary (dotted curve) for two non-normal conditional probability distributions. The non-linear boundary is clearly better than any possible linear discriminant.

Figure 5.3. Piece-wise linear classifier (dotted line segments) for three class-distributions. The equi-probability boundaries are shown.
classification. Instead of trying to classify the spectra of arterial tissues, a figure-of-merit describing the difference between the class distributions is calculated.

The second and more direct approach is to construct an actual classifier or discriminant function. Measured data can then be tested with the classifier to determine how accurate it is. Different types of classifiers include linear discriminants (Figure 5.1), piece-wise linear discriminants (Figure 5.3), quadratic discriminants and clustering-based classifiers.

Unfortunately, the conditional probability densities, \( p(X|k) \), aren't known; but they can be approximated with randomly sampled data. Figure 5.4 shows the results of two estimation techniques. In the first technique (Figure 5.4a), the density functions were assumed to be multivariate normal. This was done by estimating the means and covariance matrices from the data points. The second estimation technique (Figure 5.4b) involved convolving each data point with a "smoothing" kernel function, in this case, a bivariate normal distribution (see Chapter 7 for description). This technique results in less "well-behaved" probability densities, but does not assume any underlying form of the distribution.

Obviously, the choice of the features used in classification is important. It is also important that the scales for the features calculated from samples are the same. The scales for the features can be standardized through normalization of the data. Although the classification accuracy may often increase with the feature dimensionality (as shown in Figure 5.1), occasionally increasing the number of features diminishes the classification ability. This happens in the case where an additional feature does not provide any new information for classification but is noisy. The result is that noise is added to the density estimates. A technique for weighing and evaluating
Figure 5.4. Estimation of two class probability densities, $p(X|1)$ and $p(X|2)$, from sampled feature data. Equi-probability levels are shown. (a) The data was assumed to be multivariate normal. (b) The distributions were estimated by convolving the data with kernel function that has a multivariate normal distribution.
individual features for their importance to the classification is described in Section 5.8.2.

This study attempts to quantify, for each illumination wavelength, the ability to properly identify different types of arterial tissues using the fluorescence spectra. Once the question as to which illumination wavelength(s) is(are) best has been answered, another question arises: does combining information from multiple illumination wavelengths improve the ability to distinguish between different types of tissues? A strategy to answer this question is described in Section 5.9.

5.1.1. Classification Techniques Used in Previous Fluorescence Studies

The difficulty in directly comparing the results of one fluorescence study against another arises because these studies use different features to represent the spectral information and different discriminant techniques to evaluate the data. Table A.2 in Appendix A lists the various data representations and discriminant techniques used in the different studies found in Table A.1. It is clear that there is not much uniformity between the techniques used in different studies. This work attempts to address this issue by obtaining and comparing the spectra in a consistent manner.

5.1.1. Classification Strategies for This Study

For the task of guiding a therapeutic procedure such as laser angioplasty, it is important to minimize the risk of perforating the arterial wall while being effective at removing plaque tissues. To accomplish this, accurate classification of both normal and diseased arterial tissues is necessary. Consequently, it is necessary to assess the discrimination ability between the normal vessel and the atherosclerotic plaque classes. In Chapter 4, four classes were described -- normal vessel (NL), fibrous plaque (FP),
complicated plaque (CO) and calcified plaque (CA). The FP, CO and CA classes are different stages of atherosclerotic plaques; therefore, the samples from each of these classes can be combined into a more general diseased (DI) tissue class. In this study, an evaluation of the potential classification ability between normal vessel and atherosclerotic plaques (NL versus DI) is performed at each of the illumination wavelengths. Two techniques are employed to evaluate this classification -- the Mahalanobis distance and the optimum linear discriminant. The Mahalanobis distance, described in Section 5.4.1, is a measure of separability between two classes, namely NL and DI. If the probability densities of the class features are normally distributed (see Section 5.3), the Mahalanobis distance implies a certain classification accuracy (Section 5.7.1). A more direct way to evaluate the classification accuracy is to calculate it from an actual classifier. The optimum linear discriminant, described in Section 5.5, is such a classifier. Two methods for estimating the classification accuracy of the linear discriminant are described in Section 5.7.2. The accuracy predicted by the Mahalanobis distance can be compared with the accuracies calculated by the optimum linear discriminant.

Both the Mahalanobis distance and optimum linear discriminant can also be used to evaluate the potential classification ability between the normal class and one of the diseased subclasses -- NL versus FP, NL versus CO, and NL versus CA. The evaluations involving the diseased subclasses will determine if the NL versus DI results are consistent for all the diseased subclasses.

The accurate classification of the NL versus DI is obviously important, but it may also be good to classify the diseased subclasses from one another. For instance, the laser energies necessary to ablate calcified plaques and soft plaques (FP and CO)
are substantially different. Consequently, it is also important to evaluate the multiple-class case in addition to the two-class case described above at each of the illumination wavelengths. For this study, the following three-class classification scenario is evaluated:

\{NL\} versus \{FP,CO\} versus \{CA\},

where the brackets \{\} indicate the class. Two techniques are used for classification evaluation of the three-class case -- the generalized multiple-discriminant measure and multiple-class classifiers. The generalized multiple-discriminant measure (see Section 5.4.2), like the Mahalanobis distance, is a measure of class separability. To estimate the classification accuracy, four classifiers (Section 5.6) -- the quadratic discriminant, the piece-wise linear discriminant, the multiple linear discriminant and the K nearest-neighbors classifier -- are evaluated. Each of these classifiers are different and should yield slightly different results. The classifier performance will also depend upon the probabilities of occurrence for each of the classes. Techniques for estimating the classification accuracy for each of these classifiers are described in Section 5.7.

5.2 Feature Representations of the Spectra

The detector array on the spectrometer samples a discrete number (512-elements) of fluorescence-emission intensities over the range of emission wavelengths. The sampled intensities actually correspond to small wavelength bands because of the finite slit width of the spectrometer and the finite size of the detector elements. Each detector intensity is referred to as a feature, \( x_i \), and the entire set of spectral data can be represented as a multidimensional feature-vector, \( X = (x_1, x_2, ..., x_q) \), where \( q \) is the number of features. However, an extremely large number of samples would be
necessary to form a good estimate of the class probability distributions in a 512-dimensional feature space. In particular, several probability estimation techniques (e.g., multivariate normal model) and discriminant analysis techniques require the calculation of the inverse of a 512 by 512 covariance matrix. To avoid a singular covariance matrix, which does not have a stable inverse, more than 512 fluorescence spectra of individual samples would have to be measured for each tissue class. This is an enormous amount of data. On the other hand, a fluorescence spectrum typically varies slowly with emission wavelength and there is a high degree of correlation between the 512 intensity features. This means that the number of features used to represent the spectral information can be reduced. Three spectral-feature representations are examined in this dissertation --uniform-interval sampling, intensity ratios, and spectral widths and peaks.

5.2.1 Uniform-Interval Sampling (UIS)

One way to represent the information in a fluorescence spectrum is to sample the intensities of the spectrum at uniform intervals. It is, however, difficult to base tissue classification on absolute fluorescence intensities because both the angle and distance between catheter and the tissue location can dramatically affect the measured intensity (Section 4.1.2). Consequently, normalization of the intensities for each fluorescence spectrum is required to compare spectra from different samples with confidence. Normalization was performed by setting the integral of the area under the fluorescence spectrum to a constant.

In this study, sampled signal intensities, $i_{\Delta}(\lambda_n)$, are calculated from each normalized fluorescence spectrum by integrating over a narrow band of the spectrum. The width of the integration band is $\Delta$ and the center wavelength is $\lambda_n$ for feature $n$. 
The sampling interval between adjacent intensity features \((\lambda_n - \lambda_{n+1})\) is fixed. In vector notation, the \textit{uniform-interval sampling} (UIS) \textit{feature-vector} is written as

\[
X_i = \left( i_\Delta(\lambda_1), i_\Delta(\lambda_2), \ldots, i_\Delta(\lambda_q) \right),
\]

where \(q\) is the maximum number of features.

The 512-element detector array samples the fluorescence intensities at intervals of 0.5 nm. However, as stated earlier, the fluorescence intensities vary slowly over the emission wavelength range, so a much coarser sampling interval should be sufficient for representing the spectral information. Several sampling intervals are examined -- 31.3 nm, 15.6 nm, 10.4 nm and 7.8 nm. To improve the signal-to-noise ratio (SNR) of each intensity feature, \(i_\Delta(\lambda_n)\), the signal is averaged in a small band of normalized detector intensities with width, \(\Delta\), equal to half the sampling interval. The SNR improves as the square-root of the sampling band. This width is chosen to be large enough to improve the SNR, but not so large that the sampling resolution is lost.

\subsection*{5.2.2 Ratio of Emission Intensities}

The most commonly reported feature for tissue classification with fluorescence spectra is the \textit{ratio of intensities}, \(R\), at two emission wavelengths,

\[
R = \frac{i_\Delta(\lambda_1)}{i_\Delta(\lambda_2)},
\]

where \(i_\Delta(\lambda)\) is a signal intensity of the sampled fluorescence spectrum centered at emission wavelength, \(\lambda\). Sometimes, this intensity ratio is referred to as the contrast function. Since most fluorescence spectra are unimodal and have slow variations, a ratio of intensities at the appropriate emission wavelengths may be sufficient to characterize the fluorescence spectrum for tissue classification. This technique is independent of the absolute signal intensities, and therefore, is self-normalizing.
The uniform-interval sampling features described in the previous section can be used to generate ratio features. Ratios were calculated for every pair of emission wavelengths that were used in the UIS feature-vector. Discriminant ability for each ratio was tested.

### 5.2.3 The Spectral Width and the Wavelength of the Spectral Peak Features

A third technique is to determine the width of the spectrum at the points where the spectral intensities are some percentage of the maximum intensity. The spectral width feature, \( W_{(100 \cdot g)} \), is determined by

\[
W_g = \lambda(g \cdot I_m) - \lambda(I_m),
\]

where \( \lambda(g \cdot I_m) \) is the emission wavelength where the emission intensity is equal to the product of the proportionality constant, \( g \), and the overall peak intensity, \( I_m \).

Similarly, the peak emission wavelengths of the various biochemical compounds differ; hence, the tissue classes may also have different peak wavelengths. It is also possible to use the wavelength of the peak emission intensity as a feature,

\[
\lambda_{pk} = \lambda(I_m).
\]

Both the spectral width and peak wavelength features are independent of the absolute intensities, so normalization is not necessary. The \( \lambda_{pk} \) and \( W_{25} \ (g = 25) \) features were both extracted from the measured spectra in this study.

### 5.3 Multivariate Normal Density Estimation

If the probability densities of the features of the sample populations for each class are known exactly, the overlap of the class distributions can be calculated. From this overlap, the minimum classification error for an optimum classifier can be determined. The true probability densities, however, are not known for the tissue class populations. As discussed in Section 5.1, there are several ways to estimate the
conditional probability density functions, \( \hat{p}(X|k) \), for different classes from a set of sampled feature data, \( X_k^s \) (sample \( s \) in class \( k \)). Probably the simplest way is to assume that the probability density has a known functional form. Often, the data is assumed (perhaps erroneously) to be multivariate normal. This is because the properties of multivariate normal distributions are well understood. In this study, the multivariate density model assumption is used to correct for sample size biases in a discriminant measure (Section 5.4.1), to predict the classification accuracy for a linear discriminant between two classes (Section 5.7.2), and to construct a quadratic discriminant function (Section 5.6.2).

In one dimension, the normal probability density function is

\[
p_N(x|k) = \frac{1}{\sqrt{2\pi} \sigma_k} \exp\left\{ -\frac{(x-m_k)^2}{2\sigma_k^2} \right\}, \tag{5.5}
\]

where \( m_k \) is the mean and \( \sigma_k^2 \) is the variance of the distribution. Similarly, for a general q-dimensional vector, \( X = (x_1, x_2, ..., x_q) \), the multivariate normal probability density function is

\[
p_N(X|k) = \frac{1}{(2\pi)^{q/2}|\Sigma_k|} \exp\left\{ -\frac{(X-m_k)^\top \Sigma_k^{-1}(X-m_k)}{2} \right\}, \tag{5.6}
\]

where \(^\top\) designates vector transpose, \( m_k \) is the mean vector, and \( |\Sigma_k| \) is the determinant of the population covariance matrix. The elements of the covariance matrix, \( s_{ab} \), describe both the variances, \( s_{aa} = \sigma_a^2 \), and the covariances, \( s_{ab} = \sigma_a \sigma_b \rho_{ab} \), where \( a \neq b \). The correlation coefficients, \( \rho_{ab} \), describe the degree of correlation between features, \( x_a \) and \( x_b \). Correlation coefficients range from 1.0 to -1.0 for total correlation and total anti-correlation, respectively. A correlation value of zero represents no correlation.
Often, the true mean and covariance matrix are not known exactly. They can, however, be estimated from a group of measured data, \( X_k^s \), where \( s \) is the index of a specific sample. It is also assumed that the data are from a single class, \( k \). The estimated mean vector, \( \hat{\mu}_k \), and the estimated covariance matrix, \( \hat{\Sigma}_k \), are

\[
\hat{\mu}_k = \frac{1}{N_k} \sum_{s=1}^{N_k} X_k^s,
\]

and

\[
\hat{\Sigma}_k = \frac{1}{N_k - 1} \left[ \sum_{s=1}^{N_k} (X_k^s - \hat{\mu}_k)(X_k^s - \hat{\mu}_k)^t \right],
\]

where \( \wedge \) denotes an estimated value and \( N_k \) is the number of samples in class \( k \). As \( N_k \) goes to infinity, these estimates become equal to the true mean vector and covariance matrix. The substitution of the estimated mean vector and covariance matrix into Equation 5.6, yields

\[
\hat{p}_N(X|k) = \frac{1}{(2\pi)^{q/2} \sqrt{|\Sigma_k|}} \exp \left\{ -\frac{(X - \hat{\mu}_k)^t \hat{\Sigma}_k^{-1} (X - \hat{\mu}_k)}{2} \right\}.
\]

Figure 5.4a is an example of representing sampled data in two classes by multivariate normal distributions.

To determine whether samples are normally distributed can be a tricky task in a single dimension and this difficulty increases dramatically as the dimensionality increases. One way for testing multivariate normality, described by Fukunaga [1990], is to calculate
\[ \zeta(X) = \frac{1}{N_k} (X - \hat{m}_k)^\top \Sigma_k^{-1} (X - \hat{m}_k) \]  

(5.10)

for each sample \( X_k \). Under the assumption that \( X \) is multivariate normal, \( \zeta \), has a beta density function,

\[ p(\zeta) = \frac{\Gamma\left(\frac{N_k - l}{2}\right)}{\Gamma\left(\frac{q}{2}\right)\Gamma\left(\frac{N_k - q - l}{2}\right)} \zeta^{\frac{q}{2} - 1} (1 - \zeta)^{\frac{N_k - q - l}{2} - 1}, \quad 0 \leq \zeta \leq 1 \]  

(5.11)

with mean,

\[ \langle \zeta \rangle = \frac{q}{N_k - l}, \]  

(5.12)

and variance,

\[ \sigma_{\zeta}^2 = \frac{2q}{(N_k - l)^2} \frac{N_k -(q+1)}{N_k + 1}, \]  

(5.13)

where \( \langle \zeta \rangle \) is the expected value of \( \zeta \). If \( X_k^s \) samples a multivariate normal distribution, the values of \( \zeta(X_k^s) \) should have mean and variance comparable to the values from Equations 5.12 and 5.13. Normality can be tested further by plotting the empirical cumulative distribution function of \( \zeta(X_k^s) \) against the theoretical cumulative distribution function calculated from

\[ P(\zeta) = \int_{-\infty}^{\zeta} p(\zeta')d\zeta'. \]  

(5.14)

The empirical cumulative distribution function is calculated by sorting the \( \zeta(X_k^s) \) values and then counting the number of samples as \( \zeta \) is increased. The count is normalized to the total number of samples. A Kolmogorov-Smirnov (K-S) test
determines the largest difference between the pair of cumulative distribution functions [Press et al., 1989; Kirkpatrick, 1974]. The K-S test value ranges from 0 (high probability of normality) to 1 (low probability). The probability density of a class will behave more like a multivariate normal density function for smaller K-S test values. Statistical properties of the discriminant analysis techniques that assume multivariate normal densities should be more accurate for the data that is closer to being multivariate normal.

5.4 Measures of Class Separability

The information in a single spectrum is described by the feature-vector $X$. The features could be normalized intensities, or any of the other features described in Section 5.2. A set of feature-vectors, $X^s_k$, samples the distribution of spectra in the multidimensional feature space. A classifier used to discriminate tissue types should perform well when the distributions (e.g., one for the normal class and one for a diseased class) are well separated in the multidimensional space.

How can we evaluate the difference between the feature-vector probability densities of the different classes at each of the illumination wavelengths? One way is to determine a figure-of-merit describing the relative "distance" between the class distributions. A large separability implies that there is little or no overlap between the class probability densities. Therefore, the classification should improve as this distance increases. The Mahalanobis distance squared and the generalized multiple-discriminant measure, are such figures of merit.
5.4.1 The Mahalanobis Distance Measure

If a single spectral feature is considered for two classes, 1 and 2, each class will have some distribution for that feature. A figure-of-merit describing the degree of separability between the two classes is

$$\Delta^2 = \frac{(m_1 - m_2)^2}{\sigma_1^2 + \sigma_2^2}, \quad (5.15)$$

where $m_1$ and $m_2$ are the average values for the two classes, respectively, and $\sigma_1^2$ and $\sigma_2^2$ are the corresponding variances. A large degree of class separability (i.e., large $\Delta^2$) will occur for a large difference between the class means and small variances about those means. For example, the $\Delta^2$ for the pair of distributions in Figure 5.5a would be larger than for the pair in Figure 5.5b. Obviously the amount of overlap is also less for (a) than (b).

A related figure-of-merit describing the degree of class separability between two classes in a multidimensional feature space is called the Mahalanobis distance or $D^2$ [Mahalanobis, 1930; Rao, 1965; Afifi and Clark, 1990] The Mahalanobis distance is

$$D^2 = (m_1 - m_2)^T \Sigma^{-1} (m_1 - m_2), \quad (5.16)$$

where $m_1$ and $m_2$ are the mean feature-vectors and $\Sigma$ is the average covariance matrix (i.e., $\Sigma = P(1)\Sigma_1 + P(2)\Sigma_2$) of the class distributions. $P(1)$ and $P(2)$ describe the probabilities of both classes.

$D^2$ combines information about the separation between class means with the spread of the class distributions about those means into a single figure-of-merit. The larger $D^2$, the more separable the classes, and the "better" one is able to classify the tissue. Therefore, the excitation wavelength with the largest $D^2$ should be optimum for discriminating between classes 1 and 2 (or any pair of classes).
Figure 5.5. Examples of normal and diseased conditional probability density functions for a single feature $x$. (a) Good separability. (b) Poor separability.

Equation 5.15 assumes that the mean feature-vectors and the average covariance matrix are known exactly, which is not the case. An estimate of the Mahalanobis Distance, $\hat{D}^2$, from samples can be calculated by the expression

$$\hat{D}^2 = (\hat{m}_1 - \hat{m}_2)^T \Sigma_t^{-1} (\hat{m}_1 - \hat{m}_2),$$  \hspace{1cm} (5.17)

where $\hat{m}_1$ and $\hat{m}_2$ are the mean feature-vectors from samples (Equation 5.7) and

$$\hat{\Sigma}_t = \frac{n_1 \hat{\Sigma}_1 + n_2 \hat{\Sigma}_2}{n_1 + n_2}$$  \hspace{1cm} (5.18)

is the weighted average of the class covariance matrices, $\hat{\Sigma}_1$ and $\hat{\Sigma}_2$, which are calculated from samples (Equation 5.8). The weighting factors are $n_f = (N_f - I)$ and
\( n_2 = (N_2 - 1) \), where \( N_1 \) and \( N_2 \) are the number of samples in classes 1 and 2. Note that this gives a slightly different weighting than \( \Sigma = P(1)\Sigma_1 + P(2)\Sigma_2 \) described earlier.

It can be shown that the Mahalanobis \( \hat{D}^2 \) determined from samples is a biased estimate of the true \( D^2 \) and that the bias is a function of the number of samples used to estimate \( D^2 \) [see Appendix B]. If the class distributions are assumed to be multivariate normal, an unbiased estimate of the Mahalanobis \( D^2 \) is

\[
\hat{D}_u^2 = \frac{N - q - 3}{N - 2} \hat{D}^2 - \frac{qN}{N_1N_2},
\]

and its associated variance is

\[
\sigma_{\hat{D}_u^2} = \frac{2}{N - q - 5} \left( \frac{qN^2(N - 3)}{(N_1N_2)^2} + \frac{2N(N - 3)}{N_1N_2} \hat{D}_u^2 + \hat{D}_u^4 \right),
\]

where the total number of samples, \( N = N_1 + N_2 \). As the number of samples becomes very large the variance goes to zero and the differences between \( \hat{D}_u^2 \), \( \hat{D}^2 \) and the true \( D^2 \) become negligible. The unbiased Mahalanobis \( \hat{D}_u^2 \) is the best estimate of the true Mahalanobis \( D^2 \) for the given set of samples, and the associated variance, \( \sigma_{\hat{D}_u^2} \), describes the confidence associated with this estimate.

A demonstration of the bias correction is shown in Figure 5.6. Samples were randomly drawn from the total population and both \( \hat{D}^2 \) and \( \hat{D}_u^2 \) were determined. The "theoretical" biased and unbiased estimates, shown as the solid lines and dashed lines, respectively, on the graph, are based upon the \( \hat{D}_u^2 \) for \( N = 209 \) samples (314 nm illumination for NL versus CO classification). It is seen that the data fit the theoretical predictions fairly well and that the unbiased estimate does appear to be independent of the population size. Consequently, \( \hat{D}_u^2 \) can be used as a figure-of-merit that is independent of sample size. The scatter of the \( \hat{D}_u^2 \) points about the predicted value of
Figure 5.6. Class separability performance between two classes as a function of the total number of samples, $N$. The "theoretical" biased and unbiased estimates are shown as solid and dashed lines, respectively. Individual biased and corresponding unbiased estimates were determined by randomly selecting samples from the entire sample population.

$D^2$ is consistent with the variance given by Equation 5.20. Again, the estimated variance provides a measure of confidence when comparing separabilities.

In this study, the Mahalanobis distance is used to evaluate illumination wavelengths for several two-class comparisons. First, the separability between normal vessel (NL) the general atherosclerotic plaque (DI) classes is estimated. Then $D^2$ is used to evaluate the separability between different combinations of the NL and diseased subclasses (FP, CO & CA) -- NL versus FP, NL versus CO, NL versus CA, FP versus CO, FP versus CA, and CO versus CA.
5.4.2 The Generalized Multiple-Discriminant Measure

The discussion of the Mahalanobis distance, in the previous section, was restricted to comparing two classes. As will be discussed later, the number of total classes can be greater than two. One way to analyze the multiple-class case is to calculate a separability measure for each pair of classes. This provides information about the discriminant ability between specific pairs of classes, independent of the other classes. Another, more general, discriminant analysis technique, described below, is able to compare all the classes simultaneously.

The spread of samples around class means and the position of the class means relative to one another can be described by a pair of scatter matrices, $S_w$ and $S_b$. The estimated within-class scatter matrix, $\hat{S}_w$, is the average of the independent class covariance matrices (from Equation 5.7),

$$\hat{S}_w = \sum_{k=1}^{c} P(k) \hat{\Sigma}_k , \quad (5.21)$$

where $P(k)$ is the probability of class $k$. The estimated between-class scatter matrix, $\hat{S}_b$, represents the sum of the displacements of the class means from the overall mean vector for all the classes, and is given by

$$\hat{S}_b = \sum_{k=1}^{c} P(k) (\hat{m}_k - \hat{M})(\hat{m}_k - \hat{M})^t , \quad (5.22)$$

where $\hat{m}_k$ is the $k$th class mean and the overall mean is

$$\hat{M} = \sum_{k=1}^{c} P(k) \hat{m}_k . \quad (5.23)$$
A figure-of-merit, $J$, called the generalized multiple ("multi-class") discriminant measure is defined as the trace (sum of the diagonal matrix elements) of the matrix product between the inverse of $\hat{S}_b$ and $\hat{S}_b$,

$$J = tr\{\hat{S}_w^{-1}\hat{S}_b\}.$$  (5.24)

For the two-class case ($c = 2$), $J$ is equal to $0.25 D^2$ if the number of samples in both classes is the same ($P(0) = P(1) = 0.5$). The estimate of $J$ from samples, just like the estimate of $D^2$, is biased by the numbers of samples and features.

The Mahalanobis $D_u^2$ is a good discriminant analysis technique for comparing two distributions of sampled data. It, however, assumes that the class probability is $P(k) = N_k/N$, where $N_k$ is the number of samples in each class, $k$. This causes the discriminant analysis (and classifier techniques) to weight or "favor" the data in the largest class. However, the probabilities of the different classes in the true application, which is the occurrence of different arterial classes, are not known a priori. Instead, the class probabilities can be set according to the estimates of what the actual probabilities might be later. For example, the probabilities of each class could be assumed to be equal, $P(k) = c^{-1}$, where $c$ is the total number of classes.

The discriminant measure $J$ is used in several different capacities in this study. First, it is used to evaluate separability of the class features at each of the illumination wavelengths for the 3-class case:

$$\{NL\} \text{ versus } \{FP, CO\} \text{ versus } \{CA\},$$

where the probability of each class is $1/3$. The $\{FP, CO\}$ comprises the class of soft plaques. However, since the estimate of $J$ is biased for the numbers of samples and features, the same number of samples at each illumination wavelength must be used. In addition, the uncertainty (e.g., variance) is not known for this measure, so we cannot be
certain if one $J$ value is significantly greater than another. The $J$ figure-of-merit is also used to select intensity ratio features, to rank features for adjusting the dimensionality of the UIS feature-vector at a single wavelength (Section 5.8) and to combine features from multiple illumination wavelengths to improve classification accuracy (Section 5.9). One potential shortcoming of this measure is that it does not reflect whether all classes are moderately and equally separable or just a pair of classes are highly separable while others are inseparable.

5.5 The Optimum Linear Discriminant

An alternative way to evaluate the classification ability at different illumination wavelengths is to estimate how accurately the tissues can be classified. This section describes the optimum linear discriminant which can be applied to a pair of classes. Classifiers for the multiple-class discrimination are described in the next section. Classification is analyzed by first constructing the classifier and then testing the classifier on a set of samples. There are several ways to estimate the classification accuracy of a classifier (Section 5.7.2). The classification accuracies obtained with the optimum linear discriminant can be compared with the accuracies that are predicted for a particular Mahalanobis distance (Section 5.7.1).

For a pair of classes, the optimum linear discriminant (LD) function reduces the dimensionality of the multidimensional feature space to a single feature dimension in the direction that maximizes the class separability. The projected feature, $y^s$, is a weighted superposition of the features in the multidimensional feature space,

$$y^s = w^t X^s.$$ \hspace{1cm} (5.25)

The projection direction for the two class case is

$$w = \Sigma^{-1}(m_1 - m_2).$$ \hspace{1cm} (5.26)
Figure 5.7. Example of the classification error plotted versus the covariance weighting factor, r.

If samples are used to calculate the average covariance matrix and mean feature-vectors, then the weighting vector is

\[ \hat{w} = \hat{\Sigma}_r^{-1} (\hat{m}_1 - \hat{m}_2), \]

where

\[ \hat{\Sigma}_r = r \hat{\Sigma}_1 + (1-r) \hat{\Sigma}_2 \]

is the weighted average of the two covariance matrices. To optimize the linear discriminant, the covariance weighting, \( r \), is varied between 0 and 1. The covariance weighting factor, \( r \), rotates the projection weighting vector, \( \hat{w} \), to see if different orientations can improve the classification. The \( r \) value that maximizes the classification accuracy is selected [Fukunaga, 1990]. Figure 5.7 shows the dependence of the classification error on the weighting parameter \( r \), for the NL versus DI case with 325 nm illumination.

If the covariance matrix that was used in the Mahalanobis distance calculation (Equation 5.18) was used in place of \( \hat{\Sigma}_r \) in Equation 5.27, Fisher's linear discriminant [Fisher, 1936; Duda & Hart, 1973] would have been produced. The classification
accuracy with Fisher's linear discriminant is most likely less than the accuracy obtained with the optimum linear discriminant. The optimum linear discriminant was chosen over Fisher's because it was desirable to allow comparison of the best-case classifier at each illumination wavelength.

Once the data have been projected to a single dimension, a classification threshold, \( \hat{\eta}_i \), is set to define the classification. The optimum threshold minimizes the average classification error. The optimum linear discriminant is evaluated for the same classification cases (i.e., NL vs. DI, NL vs. FP, NL vs. CO, etc.,) as the Mahalanobis distance in this study. The optimum linear discriminant for a pair of classes \( k = \text{NL and DI} \) with 314 nm illumination is shown in Figure 5.8a. The two features, \( x_1 \) and \( x_2 \), are the principal-directions features that are defined in Section 5.8.1. For comparison, the quadratic discriminant for the same pair of classes is shown in Figure 5.8b. The quadratic discriminant is described further in Section 5.6.2. The decision boundaries for the linear discriminant and the quadratic discriminant are quite different. Neither of the classifiers, however, are perfect in their classification of the samples shown in the figure. Sections 5.7.3 and 5.7.4 discuss the extension of the linear discriminant to the multiple-class case.
Figure 5.8. Example of a linear discriminant classifier (a) for two classes -- NL (circles) and DI (triangles, diamonds and stars). A quadratic discriminant (b) classifier between the same two classes is also shown. [$P(NL) = P(DI) = 0.5$]
5.6 Multiple-Class Spectral Pattern Classification

In the previous section, the construction of the optimum linear discriminant was described for comparing a pair of classes. In this section, several multiple-class classifiers are examined. This is neither a complete list of all possible classifiers nor an attempt to construct the best possible classifier. It is merely an attempt to corroborate the results obtained with the separability measures and to see how well certain classifiers can perform with the fluorescence data that have been acquired.

The evaluation of classifiers has two parts. First, the classifier is constructed, then the classifier is validated by applying the classifier to a population of samples. The original classifier construction can be defined in a variety of ways. In this study, the multiple-class discriminant functions are constructed for the 4-way classification --

\{NL\} vs. \{FP\} vs. \{CO\} vs. \{CA\}

-- to allow for maximum flexibility. The probabilities of these classes, \(P(k)\), could also be varied to change the classification boundaries. The classification accuracies of these classifiers are evaluated according to a three-class scenario:

\{NL\} vs. \{FP, CO\} vs. \{CA\}.

A sample is considered to be classified correctly if it is in the region defined by its set (designated within the brackets \{ \}). For example, a \textit{FP} sample will be classified correctly if it falls within the \textit{CO} region, but a \textit{CA} sample found in the \textit{CO} region will be classified incorrectly. The reason for this class partition is that it may be important to classify calcified specimens separately from soft plaques (\{FP, CO\}) as well as normal vessel.

In the following subsections, four classifiers are described -- the quadratic discriminant, the piece-wise linear discriminant, the multiple linear discriminant and
the K nearest-neighbors classifier. Techniques for estimating the classification accuracy are described in Section 5.7.2.

5.6.1 The Bayesian Discriminant

The Bayesian discriminant is simply the best discriminant function when the conditional probability densities, \( p(X|k) \), and the class probabilities, \( P(k) \) are known precisely [Duda and Hart, 1973]. For a given feature-vector, \( X \), the probability that it came from class \( k \) is

\[
P(k|X) = \frac{p(X|k)P(k)}{p(X)},
\]

(5.29)

where

\[
p(X) = \sum_{k=1}^{c} p(X|k)P(k).
\]

(5.30)

Since these probabilities are not known exactly, the best that we can do is to use estimates of the class conditional probability densities , \( \hat{p}(X|k) \) (see Section 5.4), and assume a class probability, \( P(k) \), to estimate the probability \( \hat{P}(k|X) \).

The Bayesian discriminant assigns sample feature-vector \( X^s \) to class \( a \) if

\[
\hat{P}(a|X^s) > \hat{P}(k|X^s)
\]

(5.31)

for all \( k \neq a \), where \( k = 0, 1, ..., c \). Since \( p(X) \) is a constant, the Bayesian discriminant function is sometimes written as

\[
g_k(X) = \hat{p}(X|k)P(k),
\]

(5.32)

and the discriminant function assigns \( X \) to the class with the largest \( g \) value. Occasionally, the Bayesian discriminant function is specified as the logarithm of Equation 5.32, which yields identical classification results,
Again, the class with the largest $h$ is assigned the class for $X$.

5.6.2 The Quadratic Discriminant

In the case where all the classes are normally distributed, the Bayes discriminant function is

$$g_k(X) = \hat{p}(X|k)P(k) = \frac{P(k)}{(2\pi)^{q/2} \sqrt{\hat{\Sigma}_k}} \exp\left\{ -\frac{(X - \hat{m}_k)^\dagger \hat{\Sigma}_k^{-1}(X - \hat{m}_k)}{2} \right\}. \quad (5.34)$$

The logarithm of this discriminant function yields

$$h_k(X) = \log(P(k)) - \frac{q}{2} \log 2\pi - \frac{1}{2} \log|\hat{\Sigma}_k| - \frac{(X - \hat{m}_k)^\dagger \hat{\Sigma}_k^{-1}(X - \hat{m}_k)}{2}. \quad (5.35)$$

Since the $(q/2)\log 2\pi$ term is independent of class, this term can be dropped. The new discriminant function, often referred to as the quadratic discriminant (QD) function [Duda and Hart; 1973], is

$$h_k(X) = \log(P(k)) - \frac{1}{2} \log|\hat{\Sigma}_k| - \frac{(X - \hat{m}_k)^\dagger \hat{\Sigma}_k^{-1}(X - \hat{m}_k)}{2}, \quad (5.36)$$

which has quadratic dependence on $X$. Two examples of quadratic discriminants for four classes ($k = \text{NL, FP, CO and CA}$) are shown in Figure 5.9. The same data are used for both examples, but the class probabilities, $P(k)$, are different. It is clear from these examples that the classifier was dependent upon the class probabilities and that not all the samples were accurately classified.
Figure 5.9. Examples of quadratic discriminant classifiers for four classes -- NL (circles), FP (triangles), CO (diamonds) and CA (stars).
(a) $P(\text{NL}) = 0.33, P(\text{FP}) = 0.167, P(\text{CO}) = 0.167$ and $P(\text{CA}) = 0.33$.
(b) $P(\text{NL}) = 0.5, P(\text{FP}) = 0.2, P(\text{CO}) = 0.2$ and $P(\text{CA}) = 0.1$. 
5.6.3 The Piece-wise Linear Discriminant

The optimum linear discriminant works with two classes, but for more than two classes, a piece-wise linear discriminant is necessary. This type of discriminant function consists of pieces of individual linear discriminant functions. There are several ways to construct the piece-wise linear discriminant.

If the covariance matrices of the different classes are equal or very nearly equal \((\Sigma_k = \Sigma = \hat{S}_w)\), the piece-wise linear discriminant (PLD) function is [Duda and Hart, 1973]

\[
h_k(X) = \log(P(k)) - \frac{\hat{m}_k^t \hat{S}_w^{-1} \hat{m}_k}{2} + \hat{m}_k^t \hat{S}_w^{-1} X.
\]  

(5.37)

This discriminant can be derived by taking the discriminant function described by Equation 5.36 and removing the terms with the quadratic dependence in \(X\) as well as the determinant of the covariance matrix (since the covariance matrices are the "same" for all classes). Figure 5.10 shows an example of the piece-wise linear discriminant for four classes \((k = \text{NL, FP, CO and CA})\) with the same data and class probabilities used for the QD in Figure 5.9a. The classifier classification regions appear to be similar for the PLD and QD discriminant functions.

5.6.4 The Multiple Linear Discriminant

Alternatively, linear discriminants can be independently constructed for each pair of classes [Fukunaga, 1990]. This classifier is called the multiple linear discriminant (MLD). For a pair of classes, \(a\) and \(b\), the linear discriminant "piece" is

\[
h_{ab}(X) = \hat{w}_{ab}^t X - \hat{y}_{ab},
\]

(5.38)

where \(\hat{y}_{ab}\) is the scalar threshold along the projection direction \(\hat{w}_{ab}\), which is determined by Equation 5.27. \(h_{ab}(X)\) is positive for class \(a\), and negative for class \(b\).
Figure 5.10. Example of a piece-wise linear discriminant classifier for four classes -- NL (circles), FP (triangles), CO (diamonds) and CA (stars).
\[ P(\text{NL}) = 0.33, \ P(\text{FP}) = 0.167, \ P(\text{CO}) = 0.167 \text{ and } \ P(\text{CA}) = 0.33. \]

Figure 5.11. Example of a multiple linear discriminant classifier for four classes -- NL (circles), FP (triangles), CO (diamonds) and CA (stars).
\[ P(\text{NL}) = 0.33, \ P(\text{FP}) = 0.167, \ P(\text{CO}) = 0.167 \text{ and } \ P(\text{CA}) = 0.33. \]
A sample feature-vector, \( X^s_k \), is placed in class \( a \) if all the discriminants, \( h_{ab}(X^s_k) \), are greater than zero for each of the other classes, \( b \):

\[
h_{ab}(X^s_k) > 0; \text{ for all } b \neq a
\]  

(5.39)

This definition of the piece-wise linear discriminant can lead to regions in the classification space where a class is not specified; these are called reject regions. An example of a multiple linear discriminant for four classes \( k = \text{NL, FP, CO and CA} \) is illustrated in Figure 5.11. The shaded areas correspond to the reject regions. It should be noted that the PLD and MLD in Figures 5.10 and 5.11, respectively, resulted in slightly different decision boundaries.

### 5.6.5 The K Nearest-Neighbors Classifier

Instead of trying to construct a discriminant decision surface like in the previous classifiers, samples can be classified according to the proximity to their \( K \) nearest-neighbors (KNN) [Fukunaga, 1990]. The distances between the feature-vector of the unclassified sample, \( X \), to every known sample of all classes, \( X^s_k \), are calculated. The sample \( X \) will be placed in the class that has the majority of the \( K \) "nearest neighbors". These distances, \( d \), can either be calculated in Euclidean terms,

\[
d^E = \|X - X^s_k\|
\]  

(5.40)

or by the Mahalanobis distance,

\[
d^M = (X - X^s_k)^t \hat{\Sigma}_k^{-1} (X - X^s_k).
\]  

(5.41)

The latter weighs the distance by the spread of the class densities and is the one that was used in this study.

This classifier is different than the other classifiers in that it does not assume an underlying form for the probability densities. Instead of splitting the feature space
according to the class probability densities, samples are classified solely on the locations of other samples around it.

The numbers of samples in each class may not be equal. This causes the classification to be weighted more heavily towards the class with the most samples. To weigh the neighbors from each class more equally, the neighbor weight of individual samples in class \( k \) is set to \( W_k = 1/N_k \). If the class probabilities, \( P(k) \), are different for each class then each neighbor is weighted by \( P(k)W_k \). The classification is carried out by determining the nearest \( K \) neighbors counting the number of samples in each class and then multiplying by the neighbor weight, \( P(k)W_k \). The sample is placed in the class with the largest summed "neighbor value".

### 5.7 Classification Figures of Merit

After a classifier has been constructed, it needs to be evaluated to determine its effectiveness. Several techniques for estimating the accuracies of classifiers are presented Section 5.7.2. For the two class (or pairwise) case, these estimates can be compared with a theoretical estimate of the classification accuracy.

#### 5.7.1 The Distance Accuracy Estimate

The accuracy of classification can be estimated from the Mahalanobis distance. The *distance accuracy*, \( \alpha_{db} \), can be used to estimate the accuracy of a linear discriminant between two classes. Here, classification accuracy is defined as the proportion of the total number of samples that is classified correctly. If the class probability densities are multivariate normal and have equal covariance matrices and equal class probabilities, the distance classification accuracy [Afifi and Clark, 1990] is the area of non-overlap between the probability densities for two classes. With these assumptions, \( \alpha_{db} \) is estimated as the area to the right of \(-0.5 \hat{D}_u\) under a standard
normal curve. Even if the data do not obey these assumptions, \( \alpha_d \) can be used as a "ballpark" estimate.

**5.7.2 Estimated Accuracy of Discriminant Functions**

The overall classification accuracies for the two-class (LD) and multiple-class (for the QD, PLD, MLD and KNN) cases are estimated as the average of the individual class accuracies, \( \alpha_k \),

\[
\alpha = \sum_{k=1}^{c} P(k) \alpha_k .
\]  

The classification accuracy for each class, \( \alpha_k \), is equal to the total number of samples in the class that are classified correctly divided by the total number of samples. For the two-class LD case, the class probabilities are assumed to be equal \( (P(k) = 0.5) \). For the multiple-class classifiers, different sets of class probabilities are used to evaluate how the classifiers change. The class probabilities used in this study are described in the next chapter.

The accuracy of any discriminant function can only be estimated since there is a finite sample size. Two techniques for estimating the accuracies-- the resubstitution accuracy and the leave-one-out accuracy -- from samples for a particular classifier are described here.

**Resubstitution Accuracy, \( \alpha_R \)**

For the resubstitution method, the classifier is constructed or trained on the entire data set, then the full data are resubstituted into the classifier and the classifier accuracy, \( \alpha_R \), is calculated [Fukunaga, 1990]. This technique tends to give high accuracy estimates because the classifier is trained and tested on the same data.
In the leave-one-out method, a single data point is removed from the overall population. The classifier is trained on the remaining population samples and then the removed data point is classified. Next, a different data point is removed, the classifier is retrained on the remaining population, and the single data point is classified. The process is repeated until all data points have been removed and classified. Each time the process is repeated, the mean feature-vectors, and covariance matrices must be recalculated. A tally of all the correctly classified data points is used to estimate the classification accuracy, $\alpha_L$ [Fukunaga, 1990]. The leave-one-out accuracy estimate tends to give slightly low accuracy estimates.

The resubstitution accuracy and leave-one-out accuracy are often used as upper and lower bounds for estimating the true accuracy of a classifier. As the numbers of usable samples in each class increase, $\alpha_R$ and $\alpha_L$ both converge toward each other and, ultimately, the true accuracy of the classifier. Since the leave-one-out estimate defines the lower bound of the estimated accuracy, it is a little more important in determining which illumination wavelengths are best. For instance, a classifier with moderate values for both the resubstitution and leave-one-out accuracies is probably more desirable than a classifier with a very high resubstitution accuracy estimate and a very low leave-one-out accuracy value.
5.8 Feature Dimensionality Reduction

The number of features used to describe the fluorescence spectra can influence the classification. The addition of new features may improve the classification accuracy as was shown in Figure 5.1. However, the addition of features may not always improve the discrimination ability and can, in fact, decrease the classification accuracy by adding noise to the data [Wagner et al., 1993; Fukunaga & Hayes, 1989].

How is the "best" group of features chosen?

In Section 5.2, a variety of feature representations were described. One representation is the feature-vector consisting of uniformly-spaced intensities, \( X_f \) (Section 5.2.1). The fluorescence spectra vary slowly over the emission wavelength range, so the information in intensities at "close" wavelengths will be highly correlated. Thus, it may be possible (and desirable) to reduce the dimensionality of the data without suffering much drop in performance and, in some cases, improve the performance. One benefit to reducing the number of features from a high-dimensional feature space is that it is easier to visualize how the sample classes are distributed. Two techniques for reducing the number of features -- projection onto the principal-directions and feature ranking -- are described below.

5.8.1 Projection onto Principal-directions

For \( c \) classes, the generalized multiple-discriminant figure-of-merit, the matrix product \( \hat{S}_w^{-1}\hat{S}_b \) has \( (c-1) \) non-zero eigenvalues. The *principal-directions* are the eigenvectors corresponding to the non-zero eigenvalues. For two classes, there is one non-zero eigenvalue with corresponding eigenvector equal to the Fisher's linear discriminant (Section 5.5). For more than two classes, the first principal direction is the one with the largest eigenvalue (corresponding to the largest separability), the subsequent principal-directions are in order of decreasing eigenvalues.
Once the principal-directions have been determined, the data can be projected onto these directions. This can reduce the feature dimensionality dramatically. The generalized multiple-discriminant figure-of-merit, $\hat{J}$, should be the same for the original and projected feature distributions. This means that classifiers based upon the inter-class distance should perform comparably in both the original high-dimensional feature space and the reduced feature space.

### 5.8.2 Feature Ranking

A figure-of-merit, $\mathcal{F}$, which may be either one of the discriminant analysis figures of merit (Section 5.5) or an error estimate for a specific classifier, can be used to evaluate the feature-vector as the dimensionality is changed. One method is to start with the maximum number of features and eliminate the feature that decreases $\mathcal{F}$ by the least amount. The process is repeated until only one feature remains. The change in $\mathcal{F}$ is tracked as a function of dimensionality. The other technique is similar to the first except the single feature with the $\mathcal{F}$ that corresponds to the largest class separability is the starting point and the dimensionality is increased one by one to the full number of features. These two techniques give similar but not identical results. The results give information on how the discriminant performance is influenced by the dimensionality of the feature-vector. In this study, the $\hat{J}$ figure-of-merit is used for ranking features.

---

† This assumes that $\mathcal{F}$ decreases with decreasing class separability (i.e., $D^2$ or $J$). Other figures of merit, such as classification error, increase with separability. For this case, the removal of features should be dictated by the least increase in $\mathcal{F}$. 
5.9 Multiple Illumination Wavelengths

The discriminant performance may increase by combining features from emission spectra at different illumination wavelengths. The features are selected by using a figure-of-merit, \( \gamma \), as described in the previous section. Originally, the single feature with the largest class separability is used. Features are added one at a time so that \( \gamma \) reveals the greatest increase in classification power. Features can be selected from any of the illumination wavelengths that are being combined.

In this study, two strategies are examined. The first strategy is to combine features from two illumination wavelengths from the UIS feature-vectors. The second strategy is to combine features from all illumination wavelengths. The principal-directions features, described in the previous section, are used as the features for this case. Once the features from the different illumination wavelengths have been ranked and combined into a new feature-vector, the various discriminant analysis techniques described in this chapter, such as the Mahalanobis distance and the different classifiers, are then applied to the feature-vector for the multiple-illumination wavelengths to see if there is any improvement over the single illumination wavelength case.
CHAPTER 6
RESULTS OF SPECTRAL ANALYSIS

In the last chapter, several techniques were described for analyzing the ability to classify different arterial tissues with fluorescence spectra. This chapter presents the results after applying these techniques to the spectra measured with the system described in Chapter 4. First, the average properties of the fluorescence spectra for each class and illumination wavelength are described (Section 6.1). Results of the Mahalanobis $D^2$ analysis (Section 6.2), the optimum linear discriminant (Section 6.3), the generalized multiple-discriminant measure (Section 6.4) and the multiple-class classifiers (Section 6.5) are presented for each of the illumination wavelengths and the different feature-vectors (uniform-interval sampling, ratios, and spectral peaks and widths). The results of two feature-reduction strategies, principal-directions (Section 6.6) and feature ranking (Section 6.7) are also presented. Finally, combinations of features from multiple illumination wavelengths (Section 6.8) is evaluated to see if the classification improves over the single illumination wavelength case.

6.1 Properties of Measured Spectra and Feature Representations

The average (normalized) fluorescence-emission spectra for the normal vessel (NL), general diseased (DI), complicated plaque (CO) and hard calcified plaque (CA) classes at each illumination wavelength are shown in Figure 6.1. The NL and DI spectra are shown in the left column, while the NL, CO and CA spectra are shown on the right. The fibrous plaque (FP) spectra are omitted from the right column to prevent
Figure 6.1. Average fluorescence spectra for normal (NL), general diseased (DI), complicated plaque (CO) and calcified plaque (CA) for each illumination wavelength, (a) 270 nm, (b) 300 nm, (c) 304 nm, (d) 314 nm, (e) 325 nm, (f) 334 nm, (g) 364 nm, (h) 380 nm, (i) 400 nm, (j) 436 nm, (k) 458 nm, (l) 470 nm and (m) 500 nm. NL and DI in left column; NL, CO and CA in right column.
Figure 6.1 continued

d) 314 nm

e) 325 nm

f) 334 nm
Figure 6.1. continued
Figure 6.1 continued

j) 436 nm

k) 458 nm

l) 470 nm
Figure 6.1 continued

m) 500 nm
overcrowding of the graphs. The FP plaque spectra typically were between the NL and CO curves. These graphs demonstrate that there were differences between the average spectra of the classes and that the shapes of the spectra were dependent upon the illumination wavelength. The largest difference between the NL and DI mean spectra occurred for the 314 nm to 334 nm and the 436 nm to 470 nm illumination wavelength ranges. The DI spectra were actually a superposition of the FP (not shown), CO and CA spectra. Since these diseased subclasses had different mean emission curves, each mean DI spectrum depended upon the relative numbers of samples from each of the FP, CO and CA classes. The NL and CO average spectra were the most different for the 314 nm to 334 nm range of illumination wavelengths. This difference diminished for longer illumination wavelengths, whereas the difference between the NL and CA spectra increased at longer illumination wavelengths.

6.1.1 The Uniform-Interval Sampling Feature-Vector

The selection of the uniform-interval sampled (UIS) feature-vector was described in Section 5.2.1. The spectra were sampled at intervals of 31.3 nm, 15.6 nm, 10.4 nm, and 7.8 nm. The Mahalanobis distance measure of class separability was calculated for NL versus DI classification for each of the sampling intervals at every illumination wavelength. The dependence of a separability measure, the Mahalanobis distance, on the sampling interval (plotted for values between 7.8 nm and 41.7 nm) for NL versus DI with 325 nm illumination is shown in Figure 6.2. The results indicate that there was not a significant increase in the class separability measure as the sampling interval was decreased below 15.6 nm. Similar results were also obtained for other illumination wavelengths and will be presented in Section 6.2. This analysis was repeated for other classifications (i.e., NL versus CO and NL versus CA), which
revealed a similar behavior with the sampling interval. Although, in a few cases, the values of Mahalanobis distance did increase slightly for sampling intervals less than 15.6 nm, the uncertainties of these values also increased with finer sampling, since smaller intervals corresponded to more features. This paradox is often referred to as the "curse of dimensionality".

From this analysis, it was determined that sampling at intervals less than 15.6 nm did not significantly change the results, so this sampling period was selected for extracting features. For the emission wavelength range of 250 nm, this sampling interval translated to 16 intensity features across the spectrum. Each intensity feature was produced by averaging a band 8 nm wide as discussed in Section 5.3.1. These 16 intensity features comprised the uniform-interval sampling (UIS) feature-vector. The exceptions to the feature-vector with 16 intensities were the 458 nm and 500 nm

<table>
<thead>
<tr>
<th>Excitation Wavelengths (nm)</th>
<th>Emission Wavelengths (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 &amp; 304</td>
<td>337, 352, 368, 383, 398, 414, 429, 445, 460, 476, 491, 506, 522, 537, 552, 567</td>
</tr>
<tr>
<td>364 &amp; 380</td>
<td>408, 423, 438, 454, 469, 484, 500, 515, 530, 546, 562, 576, 591, 606, 621, 636</td>
</tr>
<tr>
<td>400 &amp; 436</td>
<td>461, 476, 492, 507, 523, 538, 554, 569, 585, 600, 616, 631, 647, 662, 678, 693</td>
</tr>
<tr>
<td>458</td>
<td>476, 492, 507, 523, 538, 554, 569, 585, 600, 616, 631, 647, 662, 678, 693</td>
</tr>
<tr>
<td>470</td>
<td>510, 526, 541, 557, 572, 588, 603, 619, 634, 650, 665, 680, 696, 711, 727, 742</td>
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<tr>
<td>500</td>
<td>557, 572, 588, 603, 619, 634, 650, 665, 680, 696, 711, 727, 742</td>
</tr>
</tbody>
</table>
illumination wavelengths. Since the measured emission range for these wavelengths were shorter, only 15 features were used for 458 nm and 13 features were used for 500 nm. The sampling interval, however, was still the same (15.8 nm). The intensity features that constructed the full UIS feature-vector are listed in Table 6.1.

In order to correct for the bias of the Mahalanobis distance for the number of samples, it was necessary to assume that the samples are normally distributed (Appendix B). In addition, the classification accuracy was estimated from the Mahalanobis distance. This accuracy estimate assumed that the data was distributed multivariate normally. The quadratic discriminant function becomes the Bayes classifier for normal data. To determine if the probability densities for each class of samples were multivariate normal, \( \zeta \) values were calculated (Equation 5.10) for the UIS feature-vector of each sample (see Section 5.4). The theoretical mean values of \( \zeta \) (Equation 5.12) were found to be identical to the empirical mean of the \( \zeta \) values that

![Figure 6.2](image-url)  
**Figure 6.2.** Class separability as a function of the sampling interval (325 nm excitation. NL versus DI). [Error bars indicate +/- one standard deviation].
were calculated from samples. The ratio of the theoretical $\varphi$ variance to the empirical $\varphi$ variance, $R$, and the K-S test values are listed in Table 6.2. Ideally, for a multivariate normal density, the variance ratio should be 1.0 and the K-S value equal to 0.0. The $R$ and K-S test values in Table 6.2 appear to be well correlated with one another. The variance ratios range between 1.7 (CA at 400 nm) and 13.7 (DI at 500 nm). The K-S test values are between 0.18 and 0.51 for the same probability densities. The normality test values indicate that the DI class is generally less normal than any of the diseased subclasses. This is because the DI class is a combination of the FP, CO and CA classes, which appear to have their own distinct probability distributions.

These results indicate that the class probability densities do not appear to be multivariate normal. This result, however, was not of great concern since both the Mahalanobis distance and the optimum linear discriminant effectively project the data onto a single dimension. The projection operation is a weighted sum of the individual features; consequently, the probability density of the projected data is the convolution of the probability densities for each of the features. By the central limit theorem, the probability density of the projected data should be more normal than the original multidimensional data. This hypothesis was corroborated by normality test results with the principal-directions feature-vector (Section 6.6), which were determined by projecting the UIS data onto the two principal-directions, appeared to be more normal than the full UIS feature vector data. For example, the NL class features with 500 nm illumination had $R$ and K-S values of 11.57 and 0.46, respectively, for the UIS feature vector, whereas the values were 1.8 and 0.12 (from Table 6.7), respectively, for the principal-directions feature-vector, which indicates that the projected data appear more normal.
Table 6.2. Test results of multivariate normality for the VIS feature-vector. The ratio of the theoretical variance to the empirical data variance (R) and the Kolomogorov-Smirnov (K-S) test are applied for each class and illumination wavelength (λ<sub>ex</sub>).

<table>
<thead>
<tr>
<th>λ&lt;sub&gt;ex&lt;/sub&gt;</th>
<th>NL R</th>
<th>K-S</th>
<th>DI R</th>
<th>K-S</th>
<th>FP R</th>
<th>K-S</th>
<th>CO R</th>
<th>K-S</th>
<th>CA R</th>
<th>K-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>270</td>
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<td>0.33</td>
<td>10.7</td>
<td>0.34</td>
<td>5.64</td>
<td>0.30</td>
<td>6.29</td>
<td>0.33</td>
<td>2.93</td>
<td>0.21</td>
</tr>
<tr>
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<td>13.4</td>
<td>0.38</td>
<td>5.30</td>
<td>0.28</td>
<td>7.80</td>
<td>0.34</td>
<td>2.72</td>
<td>0.23</td>
</tr>
<tr>
<td>304</td>
<td>8.12</td>
<td>0.31</td>
<td>10.7</td>
<td>0.31</td>
<td>4.89</td>
<td>0.24</td>
<td>5.51</td>
<td>0.27</td>
<td>2.71</td>
<td>0.17</td>
</tr>
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<td>4.92</td>
<td>0.28</td>
<td>5.92</td>
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<td>2.92</td>
<td>0.23</td>
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<td>10.1</td>
<td>0.35</td>
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<td>4.18</td>
<td>0.29</td>
<td>4.75</td>
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</tr>
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</table>

6.1.2 The Optimun Ratio Feature

For the 16 intensity features in the UIS feature-vector described in the previous section, there were (15×16 =) 240 possible ratios. It would be very inefficient to report the discriminant performance for every ratio, so the "best" ratios for class discrimination were determined. How was best defined? One way was to determine the optimum ratio features was to find the ratio, (R/J), that maximized the generalized multiple-discriminant measure, J. Since J compared the separability of all classes simultaneously, this technique yielded an intensity ratio that should perform moderately well for all combinations of class pairs. J was estimated for the four classes (NL, FP, CO & CA) with P(k) = 0.25. The optimum ratios determined with this technique are listed in Table 6.3. It is interesting to note that R(J) appears to have been similar within certain illumination wavelength regions -- 270 nm to 304 nm, 314 nm to
334 nm, and 400 nm to 500 nm. The wavelengths of the intensities in the ratios changed only slightly within these regions.

Table 6.3. Ratio (R(J)) features that maximize \( \hat{J} \). The parenthesis contain the wavelengths (in nm) of the intensities that are used.

<table>
<thead>
<tr>
<th>( \lambda_{\text{ex}} )</th>
<th>ratio</th>
<th>( \lambda_{\text{ex}} )</th>
<th>ratio</th>
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<tr>
<td>270</td>
<td>I(446)/I(415)</td>
<td>380</td>
<td>I(438)/I(454)</td>
</tr>
<tr>
<td>300</td>
<td>I(445)/I(429)</td>
<td>400</td>
<td>I(585)/I(616)</td>
</tr>
<tr>
<td>304</td>
<td>I(445)/I(429)</td>
<td>436</td>
<td>I(554)/I(616)</td>
</tr>
<tr>
<td>314</td>
<td>I(403)/I(434)</td>
<td>458</td>
<td>I(554)/I(616)</td>
</tr>
<tr>
<td>325</td>
<td>I(403)/I(434)</td>
<td>470</td>
<td>I(557)/I(619)</td>
</tr>
<tr>
<td>334</td>
<td>I(388)/I(434)</td>
<td>500</td>
<td>I(557)/I(619)</td>
</tr>
<tr>
<td>364</td>
<td>I(423)/I(484)</td>
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6.1.3 The Wavelength of Peak Emission Feature and the Spectral Width Feature

It is possible that either the wavelengths of peak fluorescence or the width of the spectra may be used as features for discrimination of the tissue classes. The wavelength of peak emission and the width of the spectrum from the peak wavelength to 25% \( W_{25} \) of the maximum emission intensity were measured for every fluorescence spectra.

Table 6.4 lists the average wavelengths of greatest emission for every illumination wavelength and tissue class. The standard deviations of these values are also listed. As expected, the peak wavelength increased with the excitation wavelength. It may be arguable whether the actual peak wavelength was measurable at a few of the wavelengths such as 364 nm (Figure 6.1g), 400 nm (Figure 6.1i) and 500 nm (Figure 6.1m), since the mean peak wavelengths appeared near the edge of the measured wavelength range. For most of the illumination wavelengths, the peak
wavelengths for each class did not appear to differ much from one class to the next, especially for the NL and DI classes. However, the peak wavelengths of the CA class with illumination at 380 nm, 458 nm and 470 nm appeared to be "red-shifted" relative to the average peaks of the other classes.

Table 6.5 lists the mean and standard deviation of the measured spectral widths. The average widths were different for certain classes. For example the widths decreased as the classes progressed from NL to FP to CO with illumination in the 314 nm to 334 nm range. The DI spectra were generally narrower than the NL spectra for illumination wavelengths in the 270 nm to 304 nm range and 364 nm to 500 nm; however, the DI spectra were narrower with illumination between 314 m and 334 nm. Figures 6.1d - 6.1f show that the average CO spectra were narrower than the NL spectra for these same wavelengths. Conversely, the average NL and CA spectra in these curves appear to almost totally overlap, which is reflected in the widths listed for these two classes. For illumination wavelengths greater than 364 nm, the widths of the CA spectra were larger than the widths for other classes. This is also reflected in the average spectra shown in Figures 6.1h - 6.1m.

The peak wavelengths and spectral widths that were measured in this study can be compared with the results reported by other investigators (see Table A.1, Appendix A). The results of this study agree with some of the these investigations, but disagree with others. As stated in Section 3.1.2, the reported peak wavelengths and spectral widths varied between studies, since different equipment, procedures and techniques for measuring and evaluating the data were used.
### Table 6.4. Averages (bold) and standard deviations of peak emission wavelengths for each class.

<table>
<thead>
<tr>
<th>( \lambda_{ex} )</th>
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<th>CO</th>
<th>CA</th>
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### Table 6.5. Averages (bold) and standard deviations of the spectral widths at 25% of the peak emission (\( W_{25} \)) for each class.

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</table>
6.2 The Mahalanobis Distance

The unbiased estimate of the Mahalanobis distance, $\hat{D}_u^2$, (Section 5.5.1) is a powerful technique for comparing fluorescence illumination wavelengths and the feature representations of the spectra. Seven pairwise class comparisons were evaluated: NL versus DI, NL versus FP, NL versus CO, NL versus CA, FP versus CO, FP versus CA, and CO versus CA.

6.2.1. The Uniform-Interval Sampling Feature-Vector

The Mahalanobis distance was first examined for discrimination between the normal vessel general atherosclerotic plaque classes to see if there were any wavelengths that were best suited for the general two-class problem. The separabilities involving the diseased subclasses were then examined to see if the best illumination wavelengths for these subclass comparisons agreed with the NL versus DI results.

**NL versus DI:**

The estimated Mahalanobis distance values versus illumination wavelength for the UIS feature-vector are plotted in Figure 6.3. The error bars indicate plus or minus one standard deviation of the distance estimate. The two curves for the 270 nm to 364 nm illumination range were $\hat{D}_u^2$ values calculated for two different sized sample populations. As described in Table 4.5, there were different numbers of samples for the 270 nm to 364 nm and 380 nm to 470 nm ranges (500 nm was very similar to the latter). The solid curve indicates the full number of samples (NL = 127, DI = 203 for 270 nm to 364 nm, only), while the dashed curve indicates the distances for a subset of the full population with the same numbers of samples as used in the 380 nm to 470 nm range (NL = 83, DI = 119). The error bars are plotted only for the maximum sample sizes. There was not a significant difference in the $\hat{D}_u^2$ values calculated for the two
Figure 6.3. The Mahalanobis distance for the UIS feature-vector (15.6 nm sampling interval) versus illumination wavelength for NL versus DI classification. [Error bars indicate +/- one standard deviation]

Figure 6.4. The Mahalanobis distance versus illumination wavelength (NL versus DI) with the UIS feature-vector. Sampling intervals of 7.8 nm [star], 10.4 nm [triangle] and 31.3 nm [square] (all dashed curves) are compared against the results for the 15.8 nm [circle] sampling interval [solid curve with error bars; +/- one standard deviation].
population sizes. These results show that the largest difference between the spectra of the normal (NL) and diseased (DI) tissue was obtained with illumination in the range of 314 nm to 334 nm, as previously reported [Gmitro et al, 1990; Alexander et al, 1991 & 1994].

The Mahalanobis distance for different sampling intervals (7.8 nm, 10.4 nm, 15.6 nm and 31.3 nm) is plotted in Figure 6.4. The curves indicate that the distance measure increased slightly for sampling intervals less than 15.6 nm (solid curve). The largest increases occurred for 304 nm, 400 nm, 436 nm, and 458 nm, which suggests that using a smaller sampling interval may improve the classification ability for these wavelengths. There is a tradeoff, however between dimensionality and the number of samples necessary to obtain accurate results. Since the uncertainties of the statistical discriminant measures, such as the Mahalanobis distance and the classifiers, increase quickly with the number of features, it was desirable to use as few features as possible. Consequently, the 15.6 nm sampling interval was used in this study.

**Subclass Comparisons:**

A careful evaluation of the differences between the normal and the diseased subclasses revealed some interesting behavior. Plots of the unbiased estimate of the Mahalanobis distance against illumination wavelength for the various comparisons are shown in Figure 6.5. The $\hat{D}_u^2$ values are plotted for the maximum number of samples at each illumination wavelength (the class population sizes in Table 4.5). The distances were also calculated for the smaller subset of samples, as described above for the NL versus DI case, and there were not significant differences in the $\hat{D}_u^2$ values for the different population sizes. Maximum separability for the NL versus FP and NL versus CO classifications occurred in the same illumination wavelength range (314 nm to 334 nm) as the NL versus DI case. The largest separability for the NL versus CO
Figure 6.5. The Mahalanobis distance for the UIS feature-vectors (15.6 nm sampling interval) as a function of the illumination wavelength. (Top) NL versus FP, NL versus CO, and NL versus CA. (Bottom) FP versus CO, FP versus CA, and CO versus CA. [Error bars indicate +/- one standard deviation]
classification, however, was significantly larger than for either of the NL versus DI or NL versus FP cases. This is partially because the CO samples are less similar to normal vessel (it occurs later in the atherosclerotic process) than the FP samples. The NL versus CA case, on the other hand, showed greater separability at longer illumination wavelengths in the range from 380 nm to 500 nm. The optimum illumination wavelengths for the NL versus CO and NL versus CA cases were clearly different. These results also indicate that the NL versus DI classification will be sensitive to the relative numbers of samples from each of the diseased subclasses in the overall diseased class. For example, if many more CA samples had been used, then the best illumination wavelengths for the NL versus DI case would have been in the 380 nm to 500 nm range.

The comparison between the diseased subclasses may also provide useful information and are also plotted in Figure 6.5. The FP versus CO class case was peaked in the 314 nm to 334 nm range, while the FP versus CA case showed the strongest difference in the illumination region between 380 nm to 470 nm. The largest difference between the CO and CA classes occurred in the 270 nm to 400 nm illumination range.

6.2.2. The Optimum Ratio Feature

In Section 6.1.2. the ratio feature, $\mathcal{R}(\mathcal{I})$, selection process was described. Figure 6.6 plots the unbiased estimates of the Mahalanobis $D^2$ for the $\mathcal{R}(\mathcal{I})$ feature at each illumination wavelength for the NL versus DI case. The greatest separability was observed for 314 nm to 334 nm illumination. The largest $\hat{D}_u^2$ for the UIS feature vector is also plotted. This result indicates that the separability for a ratio feature is less than for the UIS feature-vector. The Mahalanobis distance was also calculated for each
of the subclass cases (not shown). These results indicated that the illumination wavelengths of optimum separability for the NL and DI classes were the same as with the UIS feature-vector. Similarly, the maximum separabilities with the $R(J)$ feature for the NL versus FP and NL versus CO classifications were also observed for illumination wavelengths between 314 nm and 334 nm, while for the NL versus CA classification, 400 nm to 470 nm illumination was best.

6.2.3. The Wavelength of Peak Emission Feature and the Spectral Width Feature

The unbiased estimates of the Mahalanobis $D^2$ for the wavelength of peak fluorescence emission feature, $\lambda_{pk}$, and the spectral width feature, $W_{25}$, were calculated. The $\hat{D}_u^2$ values for each of these individual features were inferior to the separabilities that were calculated for either the UIS feature-vector or the $R(J)$ optimum ratio feature. The peak-wavelength feature was the worst feature for classification, which was consistent with the small difference between the average peak wavelengths of each class as listed in Table 6.4. There was not really an optimum illumination wavelength for the peak wavelength feature.

$\hat{D}_u^2$ is plotted against illumination wavelength for the NL versus DI case in Figure 6.7 for the width feature. It is clear that this feature is worse than the UIS feature-vector for this comparison. The maximum separability occured in the 314 nm to 334 nm illumination range for NL versus DI. For the other class comparisons the width feature showed the best separability in the 314 nm to 334 nm range for NL versus FP and NL versus CO classifications, and in the 436 nm to 470 nm range for the NL versus CA classification.
Figure 6.6. The Mahalanobis distance for the $R(J)$ feature versus illumination wavelength for NL versus DI classification. [Error bars indicate +/- one standard deviation]

Figure 6.7. The Mahalanobis distance for the $W_{23}$ feature versus illumination wavelength for NL versus DI classification. [Error bars indicate +/- one standard deviation]
6.3 The Optimum Linear Discriminant

Although the Mahalanobis $\hat{D}_u^2$ is a valid way of evaluating discriminant performance, its interpretation in terms of classification is not immediately apparent. Another way of evaluating performance is to determine the classification accuracy of a linear discriminant as was described in Section 5.6.3. Only comparisons involving the normal class were evaluated here: NL versus DI, NL versus FP, NL versus CO, and NL versus CA.

6.3.1 The Uniform-Interval Sampling Feature-Vector

Classification accuracy estimates of the optimum linear discriminant (LD) for the uniform-interval sampling feature-vector are plotted in Figure 6.8. The classification accuracy estimates are shown as percentages. The upper and lower boundaries were defined by the resubstitution method and the leave-one-out method, respectively (see Section 5.7.2). There did not appear to be a clear best illumination wavelength or wavelength range for NL versus DI classification (Figure 6.8a). In fact, the results for this case indicated that classification accuracies for all illumination wavelengths were very similar. The results also demonstrate that there was positive classification ability for all illumination wavelengths.

The optimum illumination wavelength ranges for the subclass comparisons (Figure 6.8b-d) were 314 nm, 334 nm to 364 nm, 436 nm and 458 nm for NL versus FP, 270 nm to 334 nm (300 nm excluded) for NL versus CO, and 380 nm to 500 nm for NL versus CA. The best classification accuracies for the latter two cases were significantly greater than the accuracies obtained for either NL versus DI or NL versus FP. As discussed in Chapter 5, the optimum linear discriminant is slightly different than Fisher's linear discriminant. The results for Fisher's linear discriminant with the
Figure 6.8. Optimum linear discriminant classification accuracies for the UIS feature-vector (solid curves) as a function of the illumination wavelength for the (a) NL versus DI, (b) NL versus FP, (c) NL versus CO and (d) NL versus CA classification cases. The upper and lower curves correspond to the resubstitution and leave-one-out accuracy estimates, respectively. The estimated distance accuracies (dashed curve) are also plotted.
same data were presented in Alexander et al. [1994] and are very similar to those shown in Figure 6.8.

The theoretical (or distance) classification accuracy (Section 5.7.1) curves, which were based directly upon the $\hat{D}_u^2$ estimates (Figures 6.3 & 6.5), are also shown in Figure 6.8 (dashed lines) and match closely the classification accuracy range defined by the resubstitution and leave-one-out methods for NL versus CO and NL versus CA. There is inconsistency, however, between the accuracy predicted by the Mahalanobis distance and the accuracy range of the optimum linear discriminant for the NL versus DI and NL versus FP (400 nm to 458 nm illumination range for the latter) cases. Here, the ranges of accuracies of the optimum linear discriminant exceeded the accuracies estimated by the Mahalanobis distance. This inconsistency could be due to the fact that the data do not appear to be normal and the covariance matrices for these classes are not equal which are assumptions used in calculating the distance accuracy. The inconsistency for the NL versus DI case was also partially caused by the combination of very different density distributions for each of the diseased subclasses. It is clear that different sized groupings of diseased subclasses would result in different $\hat{D}_u^2$ and classification accuracy estimates. For example, if greater numbers of calcified plaques had been used, the optimum illumination wavelength range for the NL versus DI case would probably shift more to the longer wavelengths (>364 nm).

These results demonstrate that there is a fundamental difference between the linear discriminant and the Mahalanobis distance measure. The distance measure assumes a particular density (multivariate normal), whereas classifiers, such as the linear discriminant, are evaluated directly on the data, regardless of the probability densities of the population.
The classification accuracies for the LD and the quadratic discriminant were compared for NL versus DI classification. The accuracies are plotted in Figure 6.9. A class probability of 1/2 for both classes was assumed. The results indicate that the QD generally classifies the tissue better than the LD. This suggests that a non-linear classifier may be necessary to obtain the optimum classification. The QD, like the LD, does not seem to have a clear optimum illumination wavelength.

### 6.3.2 The Optimum Ratio Feature

The accuracies of the optimum linear discriminant were calculated for the \( R(J) \) ratio feature (not plotted). There did not seem to be any best illumination wavelengths for the NL versus FP and NL versus DI classifications. The NL versus CO, and NL versus CA classification accuracies were highest for 314 nm, and 436 nm to 500 nm, respectively. The accuracy range defined by the resubstitution and leave-one-out estimates was more narrow than the range for the UIS feature-vector. This was probably due to the fact that the probability densities for a single ratio feature were better defined than for the UIS feature vector (multiple dimensions), so the effect of removing a single sample in the leave-one-out estimate was much smaller for lower dimensional representations of the spectra. The accuracies obtained with the ratio feature, however, were generally much lower than obtained with the UIS feature vector.

### 6.3.3 The Wavelength of Peak Emission Feature and the Spectral Width Feature

The resubstitution and leave-one-out accuracy estimates of the optimum linear discriminant were calculated for both the peak wavelength and spectral width features (not plotted). These accuracies were much lower than those reported for the UIS feature-vector. The accuracies were low enough that a specific optimum illumination
wavelength could not be selected with confidence for either feature. Consequently, both the peak and width features are clearly inferior to the UIS feature-vector.

6.4 Generalized Multiple-Discriminant Measure

In the previous two sections, the Mahalanobis $D^2$ and the optimum linear discriminant were used to evaluate the classification ability at each illumination wavelength for a pair of classes. However, in a real artery, it is likely that more than two pathologies will be present simultaneously, so a pair-wise classification will not be adequate for identifying multiple tissue types. The generalized multiple-discriminant measure, $J$, was defined in Section 5.4.2 as a figure-of-merit for simultaneous evaluation of the separability of more than two classes. Like the estimate of the Mahalanobis distance, the estimate of $J$ is biased for the numbers of samples and features. Since the amount of this bias is not known, the $J$ measure has to be calculated for equal numbers of samples. Estimates of $J$ were calculated at illumination wavelengths from 270 nm to 470 nm with the same numbers of samples in each class ($NL = 83$, $FP = 55$, $CO = 40$ & $CA = 23$) for the UIS feature vectors. The estimate for 500 nm had 4 fewer samples (see Table 4.5), which should not influence the results strongly. $\hat{J}$ was calculated for a 3-class discriminant case -- $\{NL\}$ versus $\{FP, CO\}$ versus $\{CA\}$ -- with the probability of each class equal to $1/3$. The results are plotted in Figure 6.10. The uncertainty of these values are unknown; however, 300 nm to 314 nm, 400 nm and 458 nm illumination had the largest $\hat{J}$ values, which indicates that one of these illumination wavelengths may be best for implementing a 3-class classifier. An attempt to corroborate the results with the $J$ figure-of-merit with multiple-class classification is described in the next section.
Figure 6.9. Comparison of the accuracies obtained with the optimum linear discriminant (solid) and with the quadratic discriminant (dashed) \( P(NL) = P(DI) = 0.5 \) for the NL versus DI classification. The upper and lower curves correspond to the resubstitution and leave-one-out accuracy estimates, respectively.

Figure 6.10. The generalized multiple-discriminant measure for the UIS feature-vector as a function of illumination wavelength.
6.4 Multiple-Class Discriminants

Four different multiple-class discriminant functions -- the quadratic discriminant (QD), piece-wise linear discriminant (PLD), the multiple linear discriminant (MLD) and the K nearest-neighbors classifier (KNN) -- were described in Section 5.6. Both the resubstitution and leave-one-out accuracies were calculated for each class (NL, FP, CO and CA).

Each of the classifiers were originally constructed for the 4-class case: \{NL\} versus \{FP\} versus \{CO\} versus \{CA\},

where the brackets {} indicate the class. The classifier design depended upon the prior probabilities of each class. Two scenarios were investigated:

(a) \[ P(\text{NL}) = 0.33, \quad P(\text{FP}) = 0.167, \quad P(\text{CO}) = 0.167, \quad P(\text{CA}) = 0.33 \]

(b) \[ P(\text{NL}) = 0.5, \quad P(\text{FP}) = 0.2, \quad P(\text{CO}) = 0.2, \quad P(\text{CA}) = 0.1 \]

The first set (a) of prior probabilities assumed that the number of samples in the normal vessel, soft plaque (FP & CO) and hard calcified plaque (classes) were equal. The second set (b) assumed that there were more normal samples and fewer calcified plaques. These two cases are examined to evaluate how the classifiers changed with these probabilities.

In terms of laser angioplasty, it is desirable to not only accurately classify normal tissue from atherosclerotic plaques, but also hard calcified plaques from other types of plaques. Conversely, it is not as important to accurately classify different types of soft plaques from each other. Although the classifiers were originally constructed for four classes, they were evaluated in a three-class scenario:

\{NL\} versus \{FP, CO\} versus \{CA\}
where fibrous plaques and complicated plaques were grouped together into a soft plaque class. Even though FP and CO samples were lumped together, the accuracies of each of these classes were evaluated separately.

The classification accuracies calculated for the UIS feature-vector were clearly larger than the results obtained with either the $R(J)$ ratio feature, the $\lambda_{pk}$ peak wavelength feature, or the $W_{25}$ width feature. Consequently, only the results with the UIS feature-vector are reported here.

The classification accuracies of the NL, FP, CO and CA classes for the QD, the MLD, the PLD and the 7NN ($K = 7$ with the Mahalanobis distance criterion, $d_M$ (Equation 5.41)) discriminant are plotted against illumination wavelengths in Figures 6.11 and 6.12 for the (a) and (b) sets of prior probabilities, respectively, as described above. The upper curves correspond to the resubstitution estimates and the lower curves are the leave-one-out estimates.

In general, it was difficult to determine which illumination wavelengths were best for classifying the tissues with the 3-class discriminant functions. The accuracies not only depended upon the illumination wavelength, but also the classifier and the class probabilities used. Since this was effectively a three-class problem ($\{NL\}$ vs. $\{FP, CO\}$ vs. $\{CA\}$), the inherent accuracy of classification due to pure chance (guessing) was 1/3. The classification accuracies for each of the classes typically were much greater than 33.3%, which illustrates that there is positive classification for each of these classifiers. There were some exceptions to this, such as the 7NN for the FP class at 364 nm to 400 nm, 458 nm and 470 nm illumination and for the CO class at illumination between 364 nm and 500 nm. The classification accuracies for the other classes, however, were greater and the overall classification accuracies (described below) were consistently greater than 33.3%.
Figure 6.11. Classification accuracies of the (a) NL, (b) FP, (c) CO and (d) CA classes for the QD (bold), MLD (bold dashed), PLD (line) and 7NN (dotted) classifiers as a function of illumination wavelength. Both the resubstitution (upper) and leave-one-out (lower) estimates for each classifier are plotted. \[ P(\text{NL}) = 0.33, \ P(\text{FP}) = 0.167, \ P(\text{CO}) = 0.167, \ P(\text{CA}) = 0.33 \]
Figure 6.12. Classification accuracies of the (a) NL, (b) FP, (c) CO and (d) CA classes for the QD (bold), MLD (bold dashed), PLD (line) and 7NN (dotted) classifiers as a function of illumination wavelength. Both the resubstitution (upper) and leave-one-out (lower) estimates for each classifier are plotted. \[ P(NL) = 0.5, P(FP) = 0.2, P(CO) = 0.2, P(CA) = 0.1 \]
The illumination wavelengths that yielded the best classification of the CO and CA classes for the MLD, PLD and 7NN were generally between 314 nm to 334 nm, and 380 nm to 500 nm, respectively. The results for the QD, however, were quite different. The best illumination wavelengths for the NL and FP classes were much more difficult to determine and were more dependent upon the classifier used. For example, the best illumination wavelengths for the QD in Figure 6.11 for the NL class appeared to be 314 nm to 334 nm, which was one of the worst ranges for the MLD and PLD. The classification of FP with the QD in this wavelength range was also the worst. Consequently, there was a tradeoff of improved classification accuracy for one class at the expense of worse classification for another class.

The NL class accuracies were, in general, slightly greater in Figure 6.12 \[ P(\text{NL}) = 0.5,\ P(\text{FP}) = 0.2,\ P(\text{CO}) = 0.2,\ \text{and}\ P(\text{CA}) = 0.1 \] than in Figure 6.11 \[ P(\text{NL}) = 0.333,\ P(\text{FP}) = 0.1667,\ P(\text{CO}) = 0.1667,\ \text{and}\ P(\text{CA}) = 0.333 \] since the probability of the normal class, \( P(\text{NL}) \), was larger and, hence, occupied a larger region of the feature space. The CA class accuracies tended to decrease from the results in Figure 6.11 to Figure 6.12 since the probability of this class, \( P(\text{CA}) \), also decreased. The changes in the FP and CO accuracies were much more subtle because their probabilities did not change much.

Although not shown in Figures 6.11 and 6.12, the KNN discriminant was also constructed for the \( K = 3 \) and \( K = 5 \) cases. The range of classification accuracies defined by the resubstitution and leave-one-out estimates increased as the number of neighbors was decreased. The resubstitution accuracy estimates generally increased for decreasing \( K \), while the leave-one-out estimates often decreased. The accuracy trends with illumination wavelength for the 3NN and 5NN discriminants were similar to that of the 7NN discriminant shown in Figures 6.11 and 6.12.
The results in Figures 6.11 and 6.12 display the classification accuracy for each class. As discussed earlier, the accuracy of classification for a single class can be increased at the expense of higher misclassification for the other classes. This means that the optimum illumination wavelengths for a particular class may also be the worst for other classes. One way to simultaneously evaluate the classification accuracies for all classes is to form a weighted sum of the individual class accuracies. The weight applied to each class accuracy, \( \alpha_k \), is the prior probability, \( P(k) \), of the class \( k \). The overall classification accuracy is

\[
\alpha = \sum_{k=1}^{c} P(k) \alpha_k .
\]  

(6.1)

The overall accuracy is calculated for both the resubstitution and leave-one-out cases.

Figures 6.13 and 6.14 graph the overall classification accuracies versus illumination wavelength for the two sets of class probabilities, (a) and (b), respectively, with the QD, MLD, PLD, and 7NN classifiers. The quadratic discriminant yielded the highest estimates of the overall resubstitution accuracy for nearly all illumination wavelengths. The classifiers with the narrowest accuracy ranges were the PLD and 7NN estimate. The small difference between the resubstitution and leave-one-out estimates for the piece-wise linear discriminant occurred because the average covariance matrix for all classes was used. Removing a single sample should not perturb this covariance matrix much. Similarly, removing one sample from the 7 nearest-neighbors should not change the classification for many samples. Conversely, the 3NN classifier (results not shown here) was much more sensitive to the removal of a single neighbor, and its accuracy ranges were largest of the nearest neighbor classifiers.
Figure 6.13. Comparison of the overall multiple-class classification accuracies
\[ P(\text{NL}) = 0.33, \ P(\text{FP}) = 0.167, \ P(\text{CO}) = 0.167, \ P(\text{CA}) = 0.33 \]

Figure 6.14. Comparison of the overall multiple-class classification accuracies
\[ P(\text{NL}) = 0.5, \ P(\text{FP}) = 0.2, \ P(\text{CO}) = 0.2, \ P(\text{CA}) = 0.1 \]
These results indicate that there was positive classification (accuracies > 33.3%) with any of the classifiers at any illumination wavelength. The data, however, do not support a conclusion that there is a "universal" optimum illumination wavelength. This is partially because the classification is highly dependent on the classifier, the evaluation method and the class prior probabilities. For general comparison purposes, the wavelength with the maximum leave-one-out accuracy estimate was used. The illumination wavelengths selected by this criterion are listed in Table 6.6 for the two sets of class probabilities. The leave-one-out accuracy estimate was used as a criterion because it corresponded to the minimum accuracy and may be closer to the "true" classification accuracy than the resubstitution accuracy estimate.

Table 6.6. The resubstitution (bold) and leave-one-out accuracy estimates for each of the multiple-class discriminant functions with the illumination wavelengths selected by the maximum leave-one-out accuracy criterion. (a) \[P(\text{NL}) = 0.33, P(\text{FP}) = 0.167, P(\text{CO}) = 0.167, P(\text{CA}) = 0.33\], and (b) \[P(\text{NL}) = 0.5, P(\text{FP}) = 0.2, P(\text{CO}) = 0.2, P(\text{CA}) = 0.1\].

<table>
<thead>
<tr>
<th>classifier</th>
<th>(\lambda_{\text{ex}})</th>
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<th>(\alpha_L)</th>
<th>(\lambda_{\text{ex}})</th>
<th>(\alpha_R)</th>
<th>(\alpha_L)</th>
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<td>334</td>
<td>87.1</td>
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<tr>
<td>MLD</td>
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<td>334</td>
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<td>PLD</td>
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<td>76.3</td>
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<td>80.0</td>
<td>76.3</td>
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<td>7NN</td>
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<td>71.3</td>
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<td>81.3</td>
<td>76.0</td>
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</table>

Ideally, it would be desirable to achieve 100% classification accuracy, but none of the classifiers examined in this study achieved this goal. This was partially due to the large overlap between the distributions of the FP class and the NL class. As a result, the estimated class accuracies for both the NL and FP classes in Figures 6.11 and 6.12 were often lower than for the other classes. For the development of future
classifiers, it may be desirable to weight the NL class samples more highly than the FP class samples. This is discussed further in Chapter 7.

6.5 **Projection onto the Principal-directions**

In the previous sections of this chapter, UIS feature-vectors were often used to represent the measured fluorescence spectra. The dimensionality of the feature-vector ranged between 13 and 16 features. In an attempt to better visualize how the UIS feature-vectors for each class were distributed and to determine whether the dimensionality of the feature-vector affected the results, the feature-vectors were projected onto the first two principal-directions defined by the matrix product, $S_w^{-1}S_b$. Three classes are defined for these matrix calculations -- {NL}, {FP,CO} and {CA}. Since three classes ($c = 3$) were considered, there were only ($c - 1 = 2$) non-zero eigenvalues. The eigenvectors of these non-zero eigenvalues defined the principal-directions. Hopefully, very little classification ability was lost in the process.

The projected data for 270 nm, 314 nm, 334 nm and 458 nm illumination wavelengths are shown in Figure 6.15. The decision surfaces for the quadratic discriminant with $P(\text{NL}) = 0.33$, $P(\text{FP}) = 0.167$, $P(\text{CO}) = 0.167$, $P(\text{CA}) = 0.33$ class probabilities are also depicted. From these plots, it is fairly obvious that the QD did not always yield the optimum classification boundary. For example, the region for the CA class with 270 nm illumination (Figure 6.14a) appears to be too large. As a result, many NL and FP samples were misclassified. The composition of complicated plaques were more heterogeneous than the normal samples. This was reflected in how the NL samples tended to be more tightly clustered than the CO samples.

Atherosclerosis is a progressive disease and this is illustrated in Figure 6.14 by the FP class samples and regions often appearing between those of the NL and the CO
Figure 6.15. The principal directions ($y_1$ and $y_2$) feature data for (a) 270 nm, (b) 314 nm, (c) 334 nm, and (d) 458 nm illumination wavelengths. The classes are NL (○), FP (△), CO (⊗) and CA (※). The classification boundaries for the QD are shown [P(NL) = 0.333, P(FP) = 0.1667, P(CO) = 0.1667, P(CA) = 0.333].
Figure 6.15. continued.

c) $y_2$

d) $y_2$
classes. The CA class, however, did not seem to follow this "linear" progression. Since calcified plaques undergo dramatic physical and biochemical changes, this class deviated from the orderly arrangement of class densities from NL to CO.

Tests for normality were performed on the projected data and the results are in Table 6.7. The K-S test values ranged between 0.06 and 0.26. These results demonstrate that the probability densities of the projected data appeared to be more normal than the original densities for the UIS feature-vectors (Table 6.2). This was consistent with the hypothesis that the projection process causes the distribution of samples to appear more normal.

The unbiased estimates of the Mahalanobis $D^2$ for NL versus DI, NL versus FP, NL versus CO and NL versus CA classifications are shown in Figure 6.16. The values plotted are very similar to the $\hat{D}^2$ values reported for the full UIS feature-vector (Figure 6.3). The variances of the $\hat{D}^2$ values decreased from the UIS feature-vector since the number of features was reduced. This indicated that most of the discrimination power between each pair of classes was retained through the projection process.

This result was corroborated by the accuracy results of the linear discriminant with the principal-directions feature-vector. The accuracies for the NL versus DI classification are plotted in Figure 6.17. The LD accuracies for the UIS feature-vector are also shown for direct comparison. The resubstitution estimates were slightly less than those reported for the full UIS feature-vectors, which indicated that some of the classification information had been lost. The leave-one-out estimates, however, were often higher for the projected data. Since the projected directions were not redefined every time a sample was removed for the leave-one-out estimates, the effect of
Figure 6.16. The Mahalanobis distance for the principal-directions feature-vector as a function of the illumination wavelength. (Top) NL versus DI. (Bottom) NL versus FP, NL versus CO, and NL versus CA. [Error bars indicate +/- one standard deviation] The horizontal lines correspond to the largest $\hat{D}_u^2$ calculated with an UIS feature-vector (illumination wavelength in parenthesis)
Figure 6.17. Optimum linear discriminant classification accuracies (NL versus DI) for the principal directions feature vector (solid lines) as a function of the illumination wavelength. Upper and lower curves correspond to the resubstitution and leave-one-out accuracy estimates, respectively. The LD accuracies for the UIS feature-vector (dashed lines) are also shown.

removing a single sample had less effect for the principal-directions feature-vector than for the UIS feature-vector.

The multiple-class classifiers -- QD, MLD, PLD and 7NN -- were also examined for the principal-directions feature-vector \( [P(\text{NL}) = 0.33, P(\text{FP}) = 0.167, P(\text{CO}) = 0.167, \text{and } P(\text{CA}) = 0.33] \). Table 6.8 compares the resubstitution and leave-one-out accuracies obtained with the UIS feature-vector and the principal-directions feature-vector for the wavelength selected by the maximum leave-one-out accuracy criterion with the UIS feature vector (Table 6.6a). The classification results for the principal-directions feature-vectors and the UIS feature-vectors were similar for the
PLD, but were substantially lower for the QD. Since projection onto the principal-directions was a linear process and the QD was a non-linear classifier, some of the classification information used in QD classification with the UIS feature-vectors may have been lost in the principal-directions data. However, the accuracies for the MLD and 7NN classifiers were significantly higher for the projected data. This suggests that reducing the number of features through projection may make the estimation of classification accuracies of certain classifiers more stable.
Table 6.7. Test results of multivariate normality for the principal-directions feature-vector. The ratio of the theoretical variance to the empirical data variance (R) and the Kolomogorov-Smirnov (K-S) test are applied for each class and illumination wavelength.

<table>
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Table 6.8. Comparison of the overall accuracy estimates for the UIS feature-vector and the principal-directions feature-vector with each of the multiple-class discriminant functions. (a) \( P(NL) = 0.33, P(FP) = 0.167, P(CO) = 0.167, P(CA) = 0.33 \), and (b) \( P(NL) = 0.5, P(FP) = 0.2, P(CO) = 0.2, P(CA) = 0.1 \). The resubstitution (bold) and leave-one-out accuracy estimates are both listed. Wavelength selection was described in Table 6.6.

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6.6 Ranking Features

It is possible that some of the features from the UIS feature-vector may be removed without affecting the class separability. Section 5.8.2 described a technique for ranking features. Earlier in this chapter (Section 6.1.2), $\hat{D}_u^2$ was examined as a function of the sampling frequency. Ranking features differed slightly from the UIS feature-vector analysis, since it corresponded to an unequal-interval sampling of the fluorescence spectrum. The advantage of using fewer features is that there is lower uncertainty in the discriminant analysis techniques. It is also advantageous to use fewer features for certain applications such as multispectral imaging systems, because an entire image must be collected at every feature wavelength.

The figure of merit, $\mathcal{S} = \hat{J}$, was used for ranking the UIS features. Values of $\hat{J}$ were calculated for the four classes with equal class probabilities ($P(k)=0.25$). Features were ranked in both ascending and descending $\hat{J}$ order for 334 nm (Figure 6.16a) and 458 nm (Figure 6.16b) illumination. There was a difference in the $\hat{J}$ values for the ascending and descending ranking which indicated a difference in the sets of features selected by the two ranking directions. The values of $\hat{D}_u^2$ corresponding to the ranked features in Figure 6.17 are plotted as a function of the feature dimensionality in Figures 6.17 (334 nm) and 6.18 (458 nm). $\hat{D}_u^2$ did not change appreciably as the number of features was reduced from the full sixteen (or fifteen for 458 nm) features down to approximately eight features. Conversely, too many features could decrease the class separability, especially if the features were highly correlated or noisy [Wagner et al., 1993; Fukunaga & Hayes, 1989]. This may explain the slight decreases in $\hat{D}_u^2$ for some of the class comparison cases (e.g., above 9 features for FP versus CA with 458 nm) as the dimensionality was increased.
Figure 6.18. Feature ranking of the UIS feature vector with the \( \hat{J} \) figure-of-merit for
(a) 334 nm and (b) 458 nm illumination. Selection of features in both ascending
(dashed) and descending (solid) order is shown.
Figure 6.19. The Mahalanobis distance as a function of the features selected with the $J$ figure-of-merit for 334 nm illumination. The $\hat{D}_u^2$ values for both the ascending (dashed) and descending (solid) feature ranks are shown. (a) NL versus FP $[\Delta]$, NL versus CO $[\circ]$ and NL versus CA $[\star]$; (b) FP versus CO $[\blacksquare]$, FP versus CA $[\bullet]$ and CO versus CA $[\times]$. 
Figure 6.20. The Mahalanobis distance as a function of the features selected with the \( J \) figure-of-merit for 458 nm illumination. The \( \hat{D}_u^2 \) values for both the ascending (dashed) and descending (solid) feature ranks are shown. (a) NL versus FP [▲], NL versus CO [●] and NL versus CA [★]; (b) FP versus CO [■], FP versus CA [●] and CO versus CA [★].
6.7 Multiple Illumination Wavelengths

From the separability measures and classifier analyses, it was determined that different classes were better classified with different illumination wavelengths. It was, therefore, conceivable that using features from multiple illumination wavelengths may improve the ability to classify tissues. To determine if the combination of features from multiple illumination wavelengths might increase classification accuracy, two strategies were examined. The first strategy was to combine the UIS feature-vectors from two wavelengths into a single feature-vector. A feature ranking strategy similar to the one in the previous section, that used the $J'$ figure of merit, was employed to extract a smaller, yet effective subset of the feature-vector for classification. The second strategy ranked all the principal-directions features, described in the previous section, for all the illumination wavelengths with the $J'$ figure of merit.

Dual Illumination Wavelengths

For the dual illumination wavelength approach, two wavelength pairs, 314 nm plus 458 nm and 325 nm plus 436 nm, were examined. The first wavelength pair corresponded to the optimum illumination wavelengths (with the $\hat{D}_u^2$ figure of merit) for the NL versus CO and NL versus CA comparison cases. The second pair corresponded closely to two common emission lines for a HeCd laser, 325 nm and 442 nm. The sample population was the same as described in Chapter 4 for the 380 nm to 470 nm excitation range. For each pair, features from both illumination wavelengths were ranked according to maximum increases in $\hat{J} \cdot \hat{D}_u^2$. $\hat{D}_u^2$ was evaluated as a function of the number of features. The $\hat{D}_u^2$ results for the 314 nm and 458 nm pair (Figure 6.21) indicated that the class separabilities with the combined illumination wavelengths were generally larger than the best separabilities with the individual illumination wavelength feature-vectors (shown as horizontal lines in Figure 6.21).
Figure 6.21. The Mahalanobis distance for dual illumination (314 nm and 458 nm) as a function of the UIS features selected with the $J$ figure-of-merit. (a) NL versus FP [▲], NL versus CO [●] and NL versus CA [★]; (b) FP versus CO [■], FP versus CA [●] and CO versus CA [★]. Horizontal lines indicate the best $\hat{D}_u^2$ for a single illumination wavelength (wavelengths listed parenthesis).
Figure 6.22. The Mahalanobis distance for dual illumination (325 nm and 436 nm) as a function of the UIS features selected with the $J$ figure-of-merit. (a) NL versus FP [▲], NL versus CO [●] and NL versus CA [★]; (b) FP versus CO [■], FP versus CA [●] and CO versus CA [★]. Horizontal lines indicate the best $D_u^2$ for a single illumination wavelength (wavelengths listed parenthesis).
fact, the first 8 features yielded \( D_u^2 \) values that were approximately equal to the maximum \( D_u^2 \) values for each class pair at a single illumination wavelength. Since all the pair-wise separabilities were improved, it is likely that this wavelength pair will classify arterial tissues more accurately. The \( D_u^2 \) results with the 325 nm and 436 nm combination in Figure 6.22, however, did not indicate an increase in discriminant performance over a single illumination wavelength.

The generalized multiple discriminant measure was also calculated for the first sixteen features that were selected for both the dual wavelength feature vectors. The largest \( J \) value for a single illumination wavelength was 2.6 (304 nm illumination; Section 6.4). For the 314 nm/458 nm illumination combination, \( J \) increased to 4.9, whereas the 325 nm/436 nm combination yielded a \( J \) of only 2.3. This result coincides with the Mahalanobis distance results for these dual wavelength feature-vectors and seems to indicate that the 314 nm/458 nm combination should do a better job of classifying the arterial tissues than any of the single illumination wavelengths. Conversely, the results for the 325 nm/436 nm combination imply that this combination will not be any more effective than a single illumination wavelength.

The sixteen "best" dual wavelength features selected by \( J \) were used to classify the tissues with QD, PLD, MLD and 7NN classifiers. The results of classification for each class are shown in Tables 6.9 and 6.10 for the 314 nm/458 nm and 325 nm/436 nm illumination wavelength combinations, respectively. The overall accuracies for the classifiers with the dual illumination wavelengths were compared with the best accuracies for a single illumination wavelength (Table 6.11). There was a slight increase in the overall classification accuracies with the 314 nm/458 nm dual-wavelength feature-vector over the accuracies for a single illumination wavelength. The 325 nm/436 nm combination, not surprisingly, performed about the same as the
Table 6.9. Classification accuracies for the multiple-class discriminants with first 16 selected features from both the 314 nm and 458 nm VIS feature-vectors. Resubstitution (bold) and leave-one-out accuracies are listed.

\[ P(NL) = 0.333, \ P(FP) = 0.1667, \ P(CO) = 0.1667, \text{ and } P(CA) = 0.333 \]

<table>
<thead>
<tr>
<th></th>
<th>NL</th>
<th>FP</th>
<th>CO</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>QD</td>
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<td>74.7</td>
<td>87.5</td>
<td>76.8</td>
</tr>
<tr>
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<td>89.2</td>
<td>67.9</td>
<td>51.8</td>
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<tr>
<td>PLD</td>
<td>90.4</td>
<td>89.2</td>
<td>53.6</td>
<td>50.0</td>
</tr>
<tr>
<td>7NN</td>
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<td>90.4</td>
<td>60.7</td>
<td>53.6</td>
</tr>
</tbody>
</table>

Table 6.10. Classification accuracies for the multiple-class discriminants with first 16 selected features from both the 325 nm and 436 nm VIS feature-vectors. Resubstitution (bold) and leave-one-out accuracies are listed.

\[ P(NL) = 0.333, \ P(FP) = 0.1667, \ P(CO) = 0.1667, \text{ and } P(CA) = 0.333 \]

<table>
<thead>
<tr>
<th></th>
<th>NL</th>
<th>FP</th>
<th>CO</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>QD</td>
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<td>79.5</td>
<td>78.6</td>
<td>58.9</td>
</tr>
<tr>
<td>MLD</td>
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<td>77.1</td>
<td>83.9</td>
<td>75.0</td>
</tr>
<tr>
<td>PLD</td>
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<td>81.9</td>
<td>58.9</td>
<td>57.1</td>
</tr>
<tr>
<td>7NN</td>
<td>89.2</td>
<td>86.7</td>
<td>62.5</td>
<td>50.0</td>
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</table>

Table 6.11. Comparison of the overall classification accuracies for the "best" single (illumination wavelength) UIS feature-vector (from Table 6.6) and the two cases of combining selected features from a pair of UIS feature-vectors at different illumination wavelengths. Both the resubstitution (bold) and leave-one-out accuracies are listed.

\[ P(NL) = 0.333, \ P(FP) = 0.1667, \ P(CO) = 0.1667, \text{ and } P(CA) = 0.333 \]

<table>
<thead>
<tr>
<th></th>
<th>best single UIS</th>
<th>314 nm &amp; 458 nm</th>
<th>325 nm &amp; 436 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>QD</td>
<td>334 89.6 66.6</td>
<td>93.4 68.8 92.9 67.6</td>
<td></td>
</tr>
<tr>
<td>MLD</td>
<td>458 89.7 68.0</td>
<td>91.1 83.1 90.5 77.4</td>
<td></td>
</tr>
<tr>
<td>PLD</td>
<td>458 84.0 76.3</td>
<td>87.8 85.3 85.5 79.3</td>
<td></td>
</tr>
<tr>
<td>7NN</td>
<td>304 79.5 71.3</td>
<td>88.9 85.9 83.7 70.4</td>
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</tr>
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</table>
single illumination wavelength feature-vectors. Consequently, it appears that if the correct wavelength pair is chosen, the feature-vector for the dual illumination wavelengths can outperform the feature-vector for a single illumination wavelength. However, it is not clear whether there was sufficient increase in classification performance to justify increasing the complexity or cost of a system by using dual sources. The considerations for a dual-illumination wavelength system is discussed in Chapter 7.

**Multiple Illumination Wavelengths**

The combined principal-directions feature-vector was constructed by ranking the principal-directions features for all the illumination wavelengths between 300 nm and 470 nm. Once again, \( J \), was used for feature selection. The \( \hat{D}_u^2 \) values corresponding to these features are plotted in Figure 6.23. The class separabilities in some of the comparison cases (e.g., NL versus CA and CO versus CA) increased significantly (and quickly with the number of features) over the best \( \hat{D}_u^2 \) values for both the single illumination wavelength (shown as horizontal lines in Figure 6.23) and the dual illumination feature-vectors. This implies that the multiple illumination wavelength feature-vector should do a much better job than any of the single illumination wavelength feature vectors. The value of \( \hat{J} \) at 16 features for this multiple illumination wavelength feature vector was 6.0, which is clearly higher than the values for any of the single illumination wavelengths. The QD, PLD, MLD and 7NN classifiers were evaluated both for the 10 best and 20 best features selected with \( \hat{J} \). The accuracies for each class are listed in Tables 6.10 and 6.11. The overall accuracies for the classifiers with multiple illumination wavelengths were compared with the best accuracies for a single-illumination UIS feature-vector (Table 6.12). The overall classification accuracy for the feature-vector from multiple illumination
Figure 6.23. The Mahalanobis distance for multiple illumination wavelengths (300 nm to 470 nm) as a function of the principal-directions features selected with the $J$ figure-of-merit. (a) NL versus FP [$\Delta$], NL versus CO [$\Diamond$] and NL versus CA [$\star$]; (b) FP versus CO [$\blacksquare$], FP versus CA [$\bullet$] and CO versus CA [$\bigstar$]. Horizontal lines indicate the best $\hat{D}_u^2$ for a single illumination wavelength (wavelengths listed parenthesis)
Table 6.12. Classification accuracies for the multiple-class discriminants with first 10 selected principal-directions features for the 300 nm to 470 nm illumination wavelengths. Resubstitution (bold) and leave-one-out accuracies are listed.
\[ P(NL) = 0.333, P(FP) = 0.1667, P(CO) = 0.1667, \text{and } P(CA) = 0.333 \]

<table>
<thead>
<tr>
<th></th>
<th>N L</th>
<th>F P</th>
<th>C O</th>
<th>C A</th>
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<tr>
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<td>100.</td>
</tr>
<tr>
<td>MLD</td>
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<td>75.0</td>
<td>100.</td>
<td>97.5</td>
</tr>
<tr>
<td>PLD</td>
<td>90.4</td>
<td>66.1</td>
<td>92.5</td>
<td>92.5</td>
</tr>
<tr>
<td>7NN</td>
<td>94.0</td>
<td>58.9</td>
<td>92.5</td>
<td>90.0</td>
</tr>
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</table>

Table 6.13. Classification accuracies for the multiple-class discriminants with first 20 selected principal-directions features for the 300 nm to 470 nm illumination wavelengths. Resubstitution (bold) and leave-one-out accuracies are listed.
\[ P(NL) = 0.333, P(FP) = 0.1667, P(CO) = 0.1667, \text{and } P(CA) = 0.333 \]

<table>
<thead>
<tr>
<th></th>
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<th>F P</th>
<th>C O</th>
<th>C A</th>
</tr>
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<tbody>
<tr>
<td>QD</td>
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<td>91.1</td>
<td>100.</td>
<td>95.0</td>
</tr>
<tr>
<td>MLD</td>
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<td>85.7</td>
<td>97.5</td>
<td>87.5</td>
</tr>
<tr>
<td>PLD</td>
<td>96.4</td>
<td>64.3</td>
<td>97.5</td>
<td>92.5</td>
</tr>
<tr>
<td>7NN</td>
<td>91.6</td>
<td>66.1</td>
<td>92.5</td>
<td>90.0</td>
</tr>
</tbody>
</table>

Table 6.14. Comparison of the overall classification accuracies for the "best" single (illumination wavelength) UIS feature-vector (from Table 6.6) and for the 10 and 20 selected features from all the principal-directions features from 300 nm to 470 nm illumination. Both the resubstitution (bold) and leave-one-out accuracies are listed.
\[ P(NL) = 0.333, P(FP) = 0.1667, P(CO) = 0.1667, \text{and } P(CA) = 0.333 \]

<table>
<thead>
<tr>
<th></th>
<th>best single UIS</th>
<th>multiple wave! 10 features</th>
<th>multiple wave! 20 features</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_{\text{uv}} )</td>
<td>QD</td>
<td>334</td>
<td>89.6</td>
</tr>
<tr>
<td></td>
<td>MLD</td>
<td>458</td>
<td>89.7</td>
</tr>
<tr>
<td></td>
<td>PLD</td>
<td>458</td>
<td>84.0</td>
</tr>
<tr>
<td></td>
<td>7NN</td>
<td>304</td>
<td>79.5</td>
</tr>
</tbody>
</table>
wavelengths increased significantly over the single illumination wavelength case for both 10 and 20 features. The resubstitution accuracies were slightly higher for 20 features while the leave-one-out estimates were often lower. The decrease in the leave-one-out accuracies with more features was a result of fewer samples per feature. As the number of features increases, the estimate of the class density distributions became worse, so the effect of removing a single sample was accentuated. In spite of this, it appears that using features from multiple illumination wavelengths can significantly increase the ability to correctly classify tissues.

6.8 Summary of Results

Three primary spectral feature representations -- uniform-interval sampling (UIS) of the fluorescence spectra, the peaks and widths of the spectral emission, and the ratios of emission intensities -- and four discriminant analysis techniques -- the Mahalanobis distance squared, the optimum linear discriminant, the generalized multiple-discriminant measures, and multiple-class classifiers (QD, PLD, MLD and KNN) -- were used in this chapter to evaluate the ability to classify tissues with the different illumination wavelengths. Given all these results, it may be easy to forget the goal of this research. Ultimately, the goal of this research was to determine the illumination wavelength (or wavelengths) that yielded the best fluorescence features for tissue type classification.

The results with the Mahalanobis distance and the optimum linear discriminant both indicated that the full UIS feature-vector was clearly superior to the other feature representations of the spectral data. These two figures of merit also demonstrated that the optimum illumination wavelengths were different for each tissue class pairing. The best illumination for the comparison with the normal tissue class and the general
diseased class (DI), as defined in this study, was between 314 nm and 334 nm. Similarly, the comparison between the normal arteries and either the fibrous plaques or the complicated plaques demonstrated both the greatest class separability with $D^2$ and the best classification accuracy with the LD in the 314 nm to 334 nm illumination range. In contrast, the longer wavelengths, 380 nm to 500 nm were optimum for the comparison between the normal and calcified classes.

Both the Mahalanobis distance values and the LD accuracies for the NL versus FP case were lower than for either the NL versus CO or NL versus CA cases. The poorer performance was expected since atherosclerosis is a progressive disease and spectral changes at the transition from normal artery to fibrous plaque are small (i.e., there is substantial overlap of the class distributions). The performance for NL versus FP was reevaluated after removing specimens with intimal thickening between 150 and 250 μm, which corresponded to the transition from normal vessel to early fibrous plaques and improved separability between the NL and FP classes was observed. For example, the $D_u^2$ value with 334 nm excitation increased from 2.71 to 4.45 after removing these borderline data.

Although the emission peak wavelength, spectral width and ratio features were not as good as the UIS feature-vector, they related information as to why certain wavelengths were better than others for classification for the UIS feature-vector. For example, the average spectral peak did not shift much between classes, while the spectral width feature seemed to provide better information for classification (i.e., the NL spectra are more broad than the CO spectra with 314 nm illumination).

Two techniques were used for evaluating the three-class case -- {NL} versus {FP,CO} versus {CA}. Results with a multiple-class extension of the Mahalanobis distance, the generalized multiple-discriminant measure, demonstrated maximum
multiple-class separability for 300 nm to 314 nm, 400 nm and 458 nm illumination. However, since the uncertainty of these values are unknown, it is not clear if the $J$ values at these wavelengths were significantly larger than for other illumination wavelengths.

At a specific illumination wavelength, the multiple-class classifier accuracies for each class depended upon the classifier and the prior class probabilities. The best illumination wavelengths determined by the multiple-class discriminant functions were not as clear. By applying the criterion of maximum leave-one-out accuracy, 304 nm, 314 nm, 334 nm and 458 nm were selected as the representative illumination wavelengths for the different classifiers. These classifiers were not optimum, and therefore, it is conceivable that the classification accuracy could be improved.

Finally, arterial classification with a feature-vector that combines features from multiple illumination wavelengths was examined. The combination of features from multiple illumination wavelengths can significantly increase both the class separabilities ($D^2$ and $J$) and the multiple-class accuracies over the results for any individual illumination wavelength if the appropriate illumination wavelengths and features are used.
7.1 Discussion of the Results

Several discriminant analysis techniques were employed in the previous chapter to answer the question of which illumination wavelength is best for tissue classification with fluorescence. These results indicated that the optimum illumination wavelengths depended upon the tissue classes being compared and the probabilities of each class. For example, the Mahalanobis $D^2$ analysis indicated that the optimum illumination ranges for NL versus CO (314 nm to 334 nm) and NL versus CA (380 nm to 500 nm) were clearly different.

The results from the multiple-class discriminants were not as clear in designating the optimum illumination wavelengths. None of the classifiers were perfect in their identification of tissue type. Unfortunately, the minimum necessary accuracy of classification is not known. For application to laser-angioplasty, the minimum accuracy necessary depends, in part, upon whether the fluorescence spectra of contiguous ablation depths in tissue are highly correlated. For example, let us assume that a normal specimen has a combined thickness of 160 $\mu$m for the intima and media and that the ablative laser has an ablation depth of 20 $\mu$m. It would, therefore, require 8 consecutive pulses to perforate the arterial wall. If the classification accuracy for normal vessel is 90%, then there is a 10% chance of misclassification that will lead to removal of the top 20 $\mu$m of tissue. If the spectra of the next layer is independent of the original layer, there is a 10% chance of misclassifying this layer. For 8 layers of tissue that have independent fluorescence spectra, there will be a $0.10^8 = 10^{-8}$
probability of perforation, which is extremely small. In the case where the spectra in different layers are not independent of each other, the probability of perforation will increase and in the worst case is equal to 10\%. For either case, it is obviously desirable to achieve the best classification possible. Strategies for improving the accuracies of multiple-class discriminants are discussed later in this chapter (Section 7.2).

The results demonstrated that each of the illumination wavelengths yielded fluorescence spectra that provided some positive capability to classify arterial tissue specimens. This explains why most investigators in Table A.1 have reported positive classification results. The classification accuracy did, however, vary significantly for the types of tissue being compared, the illumination wavelength, the spectral features, and the classification method.

The differences in the characteristics of the fluorescence emission among different types of tissues indicated that there were different concentrations of the fluorophores as discussed in Chapter 3. Results reported by other groups, as well as experiments in this study, showed that for illumination in the 314 nm to 334 nm range, the differences between normal and soft plaque fluorescence spectra were due primarily to different concentrations of elastin and collagen. In this illumination range, the spectra of hard calcified plaques were similar to those of normal vessel, because collagen, the primary fluorophore in soft plaques, was less abundant than in the fibrotic surface layers of soft plaques. The spectral differences between elastin and collagen decreased as the illumination wavelength was increased above 334 nm; consequently, the differences between normal artery and soft plaques also decreased. The spectra of calcified plaques, however, became more distinct for longer illumination wavelengths (> 364 nm). One hypothesis for the difference in calcified spectra was that the calcified plaques were more transparent at longer wavelengths, while the intimal surface layer in
normal tissue and the fibrotic caps of soft plaques attenuated the illumination and emission and caused changes in the spectral emission from the medial tissue. Another hypothesis was that the fluorescence of calcified tissue was caused by an additional fluorophore such as ceroid. Obviously, this issue deserves further investigation.

These studies were performed on human aorta, and the results may be different for other vessels. However, the same fluorophores, albeit in different concentrations, are present in all arteries. Therefore, the optimum illumination wavelengths determined in this study may also be optimum for other vascular tissue sites. Additionally, these studies were performed on cadaver specimens. Although other investigators have reported little or no changes in the fluorescence spectra after death [Cothren et al., 1989; Richards-Kortum et al., 1991], before this technique is widely implemented for in vivo applications, more studies need to be performed either on freshly-excised surgical specimens or actually in situ in living patients. In any event, the techniques presented in this paper could also be used to study fluorescence spectra of other vessel types or even other types of tissues (i.e., normal tissue versus cancerous tumor).

It has also been suggested that other techniques such as reflectance spectroscopy, Raman spectroscopy and intravascular ultrasound could be used to guide a catheter for laser ablation of atherosclerotic plaques. The discriminant analysis techniques described in this dissertation could also be applied to other detection methods to estimate how well these methods will perform relative to one another and against fluorescence spectroscopy.
7.2 Potential Directions for Future Research

The accuracy of this study was somewhat limited by the finite numbers of samples. This study could be extended with more ex-vivo aorta specimens to improve the confidence of the results. The primary drawback to this approach is that, although aorta suffers from the same atherosclerotic pathologies as other vessels, it is not usually a high risk artery for occlusive arterial disease. Angioplasty is more commonly performed in the smaller arterial vessels like the coronary or femoral arteries. Consequently, a new study could be initiated that explores the fluorescence properties in another type of artery. To obtain the highest accuracy for studying these tissues, the spectra should be obtained from specimens that are most similar to those that will be encountered. In other words, it would be desirable to acquire the spectra from tissues in living individuals or from freshly excised surgical tissues. The latter would probably be easier to obtain. If neither of these options are available, the post-mortem effects on spectra of arterial specimens could be measured by inserting a fiber-optic catheter in a cadaver as soon after death as possible (perhaps one hour) and then measuring the spectra of the artery until autopsy and removal of the specimen. The spectrum sampling interval time could be hourly to minimize photobleaching effects.

The techniques used in this dissertation appear to be effective for evaluating illumination wavelengths for fluorescence detection of atherosclerosis. However, the assumption that the classes have multivariate-normal densities, may not be correct. These techniques, therefore, could be limited in their precision by assuming the incorrect form of the density distribution. The precision of the results might be improved by using probability densities that better represent the data. Another
approach is to not assume any distributional form, but instead to build a density estimate using non-parametric techniques (Section 7.2.1). This technique can be taken one step further to estimate the amount of overlap between different class density functions. The estimate of the overlap is called the Tie Statistic (Section 7.2.2) and can be used as a figure of merit for discriminant analysis.

The criteria of histologic classification used in this study was just one way that the samples could have been divided. The decision boundary of 200 μm intimal thickness between NL and FP was somewhat arbitrary since samples with any intimal thickening can be considered as atherosclerotic plaques. For the application of laser angioplasty, however, it is most important to treat thick plaques that can block arterial flow. Consequently, the 200 μm intimal thickness criterion appears to be appropriate for this application. An alternative approach might be to study the natural clustering of the features [Fukunaga, 1990] to determine if there is some correlation to the arterial pathology. If the pathology can be associated with specific clusters, then accurate classification would be possible. Conversely, for fluorescence-enhanced endoscopy, it is not as critical to place arterial samples in definite classes. In fact, it might be more informative to make the image intensity strength proportional to the intimal thickness. This could be done by performing a linear regression of the fluorescence features to the arterial thickness. In addition, it may be desirable to tell the difference between early fatty plaques and fibrous plaques. In this study, this delineation was not attempted. Lucas et al. [1990], however, performed studies correlating the spectral features to plaque thickness and lipid content with both 325 nm and 458 nm illumination.

In the multiple-class discriminant analyses, it was assumed that misclassifications for all the classes were equally costly. This, however, may not be the case, since, for certain applications, misclassifying a normal section of tissue is
probably much more costly than misclassifying a fibrous plaque. The inclusion of costs into discriminant functions is discussed in Section 7.2.3. The positioning of thresholds or classification boundaries is essential to achieving the highest classification accuracy. Both the quadratic discriminant and the piece-wise linear discriminant positioned their decision boundaries according to the estimated class density functions. A decision-threshold analysis technique called receiver-operating-characteristic (ROC) analysis is outlined in Section 7.2.4.

Finally, once the optimum classifier has been determined for tissue identification, systems for various applications, such as guidance for laser angioplasty or endoscopic imaging, can be designed and built. Design considerations for such a system are described in Section 7.2.5.

7.2.1 Non-Parametric Probability Density Estimation

Some probability density estimation techniques are non-parametric, which means a specific distributional form, such as multivariate normal, is not assumed. Instead the distribution of the data is based purely on the location of the samples in the feature space. One technique, the Parzen density estimate [Fukunaga, 1990] is determined by convolving the measured data with a multidimensional Parzen kernel or "smoothing" function. The kernel function can take many forms including rectangular, normal and triangular. The size of the Parzen kernel function is critical, especially for small numbers of samples. An example of the Parzen estimate for two groups of class samples was shown in Figure 5Ab. This technique for estimating the distribution resulted in a different density than for the multivariate normal case in Figure 5Aa.

Although the computation of the Parzen density estimate is straightforward, the storage of the density estimate can require large amounts of memory for high
dimensions. In addition, the quality of the estimate decreases as the dimensionality grows. The dimensionality could, however, be reduced by projecting the data onto the principal axes corresponding to the largest eigenvalues of the class separations (Section 5.8.2).

7.2.2 The Tie-Statistic

For the example shown in Figure 5.5, "distance" figures of merit (i.e., $\Delta^2$, $D^2$ and $J$) seem to be good predictors of class separability. However, the distance between distributions is not nearly as important as the amount of overlap between the class distributions. If the probability densities are "well-behaved", as in Figure 5.5, then $D^2$ and $J$ are valid figures of merit. However, for less "well-behaved" densities, such as those shown in Figure 7.1, these figures of merit may not be able to assess the amount of class separability very well. The distributions shown in Figure 7.1 are highly separable because there is little overlap. In fact, the data could be accurately classified with the proper discriminant function. This would not, however, be reflected in the distance measures since the means are very close together relative to the variances of the densities.

Instead of asking how far apart are the class probability densities, the amount of overlap between them needs to be determined. In Figures 5.5 and 7.1 (and Figure 5.2 for two dimensions), this overlap is the areas shown in gray and is called the Tie statistic. The Tie-statistic, $T$, [Beer et al., 1989] is calculated by integrating, over the entire multidimensional feature space, the minimum of the two probability densities,

$$ T = \int_{\mathbb{X}} p(X|\theta) \Lambda p(X|\mu) dX, $$

(7.1)
where the $\Lambda$ operator selects the minimum of $p(X|0)$ and $p(X|I)$. The tie statistic ranges between 0, for no overlap, and 1, for total overlap. For the example probability density pairs shown in Figure 5.5, (a) has a lower $T$ than (b) because there is less overlap. The examples in Figure 7.1 have $T$ values closer to 0 than 1. Obviously, low $T$ values are most desirable, so the illumination wavelength(s) that yields the lowest $T$ should be
optimum. The Tie-statistic does not depend upon the class probabilities, \( P(k) \); consequently, it is not biased toward one class distribution or another.

When samples are used to estimate the class probability densities, the estimate of the Tie-statistic is

\[
\hat{T} = \int_{\mathcal{X}} \hat{p}(X|\omega) \Lambda \hat{p}(X|\ell) dX . \tag{7.2}
\]

The conditional probability densities, \( \hat{p}(X|\omega) \) and \( \hat{p}(X|\ell) \), are estimated either by assuming specific distributions (i.e., normal) or by using one of the density estimation techniques described in Section 7.2.1. The estimate of \( T \) is only as good as the estimate of the density functions; therefore, \( \hat{T} \) should be more reliable for lower dimensional distributions. Chapter 5 described two strategies for reducing the dimensionality -- projecting the features onto the principal directions (Section 5.9.1) and ranking the features (Section 5.9.2).

The Tie-statistic can be generalized to a multiple-class problem by the integral over the entire feature space of the sum of all conditional probability densities minus the maximum conditional probability density,

\[
\hat{T} = \int_{\mathcal{X}} \left[ \sum_{k=0}^{c-1} \hat{p}(X|k) \right] - \max \left( \hat{p}(X|k) \right) dX . \tag{7.3}
\]

The values of \( \hat{T} \) will range between 0 and \( (c - 1) \) for zero and total overlap, respectively.

The Tie-statistic estimates the amount of overlap between class density functions. This overlap, and hence \( T \), are obviously related to the classification error. The estimated Tie error, \( e_T \), can be calculated as

\[
e_T = \hat{T}/c , \tag{7.4}
\]
where $c$ is the number of classes. If the class probability densities are accurate, this error estimate is the lowest possible error for any classifier, or the Bayes error.

7.2.3 Risk Assessment

There may be examples where the misclassification of a certain class may be more costly than misclassifying the other classes. For example, in the application of laser angioplasty, it is worse to misclassify a normal arterial specimen as diseased than one of the atherosclerotic specimens because classifying normal artery as atherosclerotic would instruct the ablation laser to damage the normal vessel and possibly perforate the arterial wall. Conversely, an atherosclerotic plaque that is misdiagnosed as normal would result in no action by the ablative laser. Although, this second type of misdiagnosis would lessen the efficiency of the angioplasty process, it is less "costly" than injuring normal artery. As a result, it would be good to be able to weight the classifier according to the cost of certain misclassifications. The implementation of cost functions with classifiers is relatively straightforward and is described in more detail by Duda and Hart [1973] and Fukunaga [1990].

7.2.4 Receiver-Operating-Characteristic (ROC) Analysis

Along with estimating the average error of classification, it is important to determine the proportions of samples for each of the normal and diseased classes that are classified correctly. The sensitivity or true-positive-fraction (TPF) is the number of "diseased" (positive) specimens that are classified correctly divided by the total number of diseased specimens (Figure 7.2a). The specificity is the proportion of correctly classified "normal" (negative) samples to the total number of normal specimens. The false-positive-fraction (FPF) is equal to one minus the specificity.
When either a single feature is used for classification or a linear discriminant reduces the feature vector to a single dimension, the position of the classification threshold, $y_n$, can substantially affect the sensitivity and specificity. Receiver-operating-characteristic (ROC) analysis [Egan, 1975] can be used to evaluate the effects of the threshold position. An ROC curve (Figure 7.2b) displays the true-positive-fraction on the vertical axis and the false-positive-fraction on the horizontal axis. It maps out the sensitivity and specificity as the position of the threshold is moved from one end of the feature distributions to the other end. The best threshold position is the one that yields a balance between the desired specificity and sensitivity. Figure 7.2b shows three ROC curves for very good, moderate and worst-case separability. In the worst-case, the class distributions are totally overlapping.
The area under the ROC curve,

\[ A = \int_{0}^{1} (TPF)d(FPF) \quad (7.5) \]

can also be used as a figure-of-merit describing class separability in one dimension. It should be noted that this figure-of-merit, like the Tie-statistic, does not assume a specific class distribution. The values for \( A \) range between 0.5 for worst-case separability and 1.0 for total separability.

Another possible use for ROC analysis could be to set the decision boundaries for one of the multiple-class discriminant functions, such as the quadratic discriminant. Initially, the class probabilities for the diseased classes could be set according to fixed proportions. The probability \( P(NL) \) could then be scanned from 0.0 to 1.0 and the probabilities of the diseased classes adjusted appropriately. For each probability point, the TPF and FPF can be assessed and a ROC curve produced. The best operating point is then selected and the boundary between the NL class and the diseased classes is fixed. The process can then be repeated to define the decision boundary between the CA class and the soft-plaque \( \{FP \text{ and } CO\} \) classes.

7.2.5 Considerations for System Hardware Development

As described earlier in this section, the requirements of a fluorescence-based guidance system for laser angioplasty are different than those of a fluorescence-enhanced endoscopy system. Some considerations for the development of both laser angioplasty and endoscopic systems are described below.
Guidance System for Laser-Angioplasty

A schematic of a fluorescence-guided laser angioplasty was shown in Figure 1.1 in Chapter 1. The two main components to such a system are the diagnostic component (i.e., the fluorescence source and spectrometer), and the ablative laser. The ablation depth of the laser energy is a function of the wavelength. Consequently, it is important that the illumination wavelength for the fluorescence penetrates at least as far into the tissue as the ablation depth of the ablative laser. Otherwise, ablation will remove tissue that is deeper than what is being diagnosed.

The wavelengths used for fluorescence illumination and detection and for ablation also determine the kinds of optical fibers that are applicable. The fiber must be strong, flexible, non-toxic and able to transmit the desired fluorescence and/or ablation wavelengths without much attenuation. For short wavelengths (< 350 nm), high OH\textsuperscript{-} optical fibers are best suited for this purpose. For longer wavelengths (out to ~ 2.5 \mu m), low OH\textsuperscript{-} fibers are better. This can lead to a small dilemma in a single fiber system if the fluorescence illumination and detection are in the ultra-violet and ablation is done with an infrared (IR) laser, such as a Ho:YAG at 2.1 \mu m. In this case it is more desirable to use a fiber material that does not attenuate the IR laser light much, because absorption of the high power light from the ablative laser may destroy the fiber.

The design of the optical-fiber catheter is also important. To maximize the accuracy of diagnosis in the spot of ablation, it is important to use the same fiber channel for diagnosis and ablation. Both single-fiber and multiple-fiber catheters have been implemented for laser angioplasty. One of the biggest disadvantages of a multiple-fiber catheter over a single fiber is the replacement cost since the catheters probably cannot be reused. Another issue related to the design of the catheter is the spot size of the light on the tissue. For an optical fiber in direct contact with the tissue,
the laser energy will make a channel approximately as wide as the optical fiber. The fiber could be moved back from the tissue, but any blood would have to be removed from the field to be able to detect the tissue surface. This could be accomplished by either flushing the field with saline or attaching an optically transparent window in front of the fibers that will allow the beam(s) to diverge from the fiber tip. The diverging beam means that more power in the optical beams is necessary to achieve the same irradiance levels on the tissue surface.

A number of lasers, over a wide range of wavelengths, have been investigated for performing ablation. The laser energy should be able to cleanly remove a small section of plaque without causing much thermal damage to the surrounding tissue, be stable in energy delivery, and be amenable to use in a clinical setting. Solid state lasers, such as Holmium:YAG at 2.1 μm, are among the most promising lasers for angioplasty because they are reliable, are not a toxic hazard, do not cause photobleaching of the fluorescence signal, and can be passed through commercially available silica fibers. High-power laser diodes would also be an attractive ablative laser source for size and economic considerations. The main draw-back, however, for using diode lasers is that they operate primarily in the 670 nm to 850 nm range. At these wavelengths, the penetration depth would be too large and the amount of thermal damage to the surrounding tissue would be high. Whatever laser source is chosen, it will be important to evaluate how the ablation process affects the fluorescence signal. This could be done by measuring the fluorescence signal after ablation on a large number of arterial plaques. The ablated tissue specimens could then be histologically classified compared with the non-ablated tissue classes using the techniques described in this dissertation.
There are numerous ways to implement the fluorescence diagnostic portion of the laser angioplasty system. The most typical way is to use a laser source for illumination and a spectrometer with a detector array for detection as shown in Figure 1.1. The results of this study indicate that using multiple illumination wavelengths may enhance the classification performance. This could be achieved by implementing multiple laser sources or a single laser source where the wavelength can be changed rapidly.

Alternatively, a filtered broad-band source such as a pulsed arc-lamp may be a more cost-effective choice for a source of a multiple illumination wavelength system. A schematic of a proposed system in shown in Figure 7.3. The system consists of a Xe flash arc lamp, a rotating interference filter with two (or more) separate filter ranges (Figure 7.4), a beam splitter, a 0.5 mm diameter fused silica optical-fiber, and a grating spectrometer with a linear detector array. "Back-of-the-envelope" calculations with values for typical "off-the-shelf" optical components indicated that such a system would be feasible for capturing spectra from multiple illumination wavelengths in a very short period of time.

Fluorescence-Enhanced Angioscopy

Analysis of a fluorescence-enhanced angioscopy system was performed by Connor Davenport [1992]. She analyzed the design of a fluorescence imaging system with a fiber-optic endoscope and found that combining filtered fluorescence images from several emission-wavelength bands could enhance the signal contrast between regions of normal artery and atherosclerotic plaques in angioscopic images.
Figure 7.3. Proposed fluorescence diagnostic system with multiple illumination wavelength capability.

Figure 7.4. Rotating dual interference filter. $f$ is the rotation frequency.
7.3 Conclusion

Thirteen wavelengths for fluorescence illumination -- 270 nm, 300 nm, 304 nm, 314 nm, 325 nm, 334 nm, 364 nm, 380 nm, 400 nm, 436 nm, 458 nm, 470 nm and 500 nm -- were evaluated to determine which of them produced the best fluorescence spectra for identification of the arterial tissue type. Several statistically-based techniques were employed for the evaluation -- the Mahalanobis distance, the optimum linear discriminant and four different multiple-class discriminant functions. Several conclusions were made:

- The multiple-feature representation of the fluorescence spectra with uniform interval sampling (UIS) was clearly superior to any of the other single-feature representations including ratios, spectral peaks or spectral widths.
- For NL versus DI classification, the Mahalanobis distance indicated that the best illumination wavelengths were between 314 nm and 334 nm. In contrast, the optimum linear discriminant did not indicate that there was a best illumination wavelength. Positive classification ability, however, was observed at all illumination wavelengths. This result illustrates that there is a fundamental difference between the separability and classifier measures. Separability measures, such as the Mahalanobis distance, imply a certain classification ability when a specific class distribution is assumed. Conversely, the classifier results are derived directly from the sample data. Even though a specific distribution may have been assumed to construct the classifier, the evaluation takes place directly on the measured samples.
- Both the Mahalanobis distance and the optimum linear discriminant analysis revealed that the best illumination wavelength ranges for the NL versus CO and NL versus CA classifications were 314 nm to 334 nm and >380 nm, respectively, and therefore, were clearly different from each other, and in the NL versus CA case,
different from the best **NL** versus **DI** illumination wavelengths (Mahalanobis distance only).

- The values for the generalize multiple-discriminant measure, J, were greatest for the following illumination wavelengths: 300 nm to 334 nm, 400 nm and 458 nm. Since the uncertainty was uncertain, it is not known if the separability values at these wavelengths are significantly larger than at other wavelengths. These results suggest, however, that multiple-class discrimination may be best for these wavelengths.

- The results with the multiple-class discriminant functions did not support the hypothesis that there is a best illumination wavelength. In fact, the classifier results indicated that there was similar and positive classification ability for all classes with spectra from any of the illumination wavelengths, but perfect classification was not obtained. The classification accuracy is sensitive to the type of classifier, the class prior probabilities and obviously the distribution of the samples in the feature space. The classification accuracy is ultimately limited by the overlap of the class probability densities.

- Finally, it was determined that combining features from multiple illumination wavelengths can increase the class separability and improve the classification accuracy of the multiple-class discriminant functions if the correct illumination wavelengths are used for the feature base. This implies that a fluorescence diagnostic system with the capacity for multiple illumination wavelengths will outperform a similar system with a single wavelength source.

It has been shown here and elsewhere that fluorescence spectroscopy can accurately identify normal arterial vessel and atherosclerotic plaques. However, until the necessary level of accuracy is determined for guiding laser angioplasty, the efficacy of fluorescence spectroscopy to this application remains uncertain.
APPENDIX A

REPORTED FLUORESCENCE STUDIES

The fluorescence properties of arterial tissues has been widely studied with a variety of illumination wavelengths, measurement systems and analysis techniques. This appendix contains two tables that list the reported studies, the illumination and emission wavelengths that were studied, and the methods that were used to evaluate the spectra. Table A.1 describes the operating parameters of the illumination and measurement systems, the type of artery that was studied and the properties of the emission spectra for the different tissue classes. The analysis techniques for some of the studies in Table A.1 are listed in Table A.2.
Table A.1. Reported arterial fluorescence studies. The investigations that studied multiple illumination wavelengths are listed first and then the single wavelength studies are listed by increasing illumination wavelength ($\lambda_{ex}$). Groups of studies that are not split by horizontal lines were performed by the same research group. The type of artery, the tissue classes and the measurement medium are shown. Both the peak emission wavelengths (principal peak(s) in bold) and the spectral emission full width at half the maximum intensity [FWHM] are also listed. 

$\text{NI} =$ normal, $\text{Di} =$ general atherosclerotic plaque, $\text{Fa} =$ fatty atherosclerotic plaque, $\text{Fi} =$ fibrous atherosclerotic plaque, and $\text{Ca} =$ calcified plaque. 

* Estimated from graph in report. 

<table>
<thead>
<tr>
<th>Study</th>
<th>$\lambda_{ex} \text{ (nm)}$ [laser or BW]</th>
<th>Investigator (Year)</th>
<th>Tissue(s) [Artery]</th>
<th>Classes</th>
<th>Medium</th>
<th>Emission Range</th>
<th>peak $\lambda_f$ \text{ and } [FWHM]</th>
</tr>
</thead>
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<tr>
<td>D</td>
<td>200-1000</td>
<td>Bergeron (1988)</td>
<td>Aorta</td>
<td>NI, Di, Ca</td>
<td>Saline</td>
<td>200-1000</td>
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</table>
Table A.1. continued.

<table>
<thead>
<tr>
<th>Study</th>
<th>$\lambda_{ex}$ (nm) [laser or BW]</th>
<th>Investigator (Year)</th>
<th>Tissue(s) [Artery]</th>
<th>Classes</th>
<th>Medium</th>
<th>Emission Range</th>
<th>peak $\lambda_f$ and [FWHM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>308 [XeCl]</td>
<td>Oraevsky (1993)</td>
<td>Aorta</td>
<td>Ni, Fa, Fi</td>
<td>300-600</td>
<td>370 [105]</td>
<td>430 (9)</td>
</tr>
<tr>
<td>0</td>
<td>325 [HeCd]</td>
<td>O'Brien (1989)</td>
<td>Aorta</td>
<td>Ni, Di</td>
<td>350-700</td>
<td>382,430 [174d (21)]</td>
<td>382,430 [105d (23)]</td>
</tr>
<tr>
<td>Q</td>
<td>325 [HeCd]</td>
<td>Deckelbaum (1989)</td>
<td>Aorta</td>
<td>Ni, Di</td>
<td>350-700</td>
<td>382,430 [174d (21)]</td>
<td>382,430 [105d (23)]</td>
</tr>
<tr>
<td>R</td>
<td>325 [HeCd]</td>
<td>Laifer (1989)</td>
<td>Aorta</td>
<td>Ni, Di</td>
<td>350-700</td>
<td>382,430 [174d (21)]</td>
<td>382,430 [105d (23)]</td>
</tr>
<tr>
<td>Study</td>
<td>$\lambda_{ex}$ (nm) [laser or BW]</td>
<td>Investigator (Year)</td>
<td>Tissue(s) [Artery]</td>
<td>Classes</td>
<td>Medium</td>
<td>Emission Range</td>
<td>peak $\lambda_f$ and [FWHM]</td>
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</tr>
<tr>
<td>U</td>
<td>325 (HeCd)</td>
<td>Gindi (1991)</td>
<td>Aorta</td>
<td>NI, Di</td>
<td></td>
<td>350-700</td>
<td>382, 430</td>
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<td>V</td>
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<td>Leon (1988)</td>
<td>Aorta, Coronary, Popliteal, Tibial</td>
<td>Ni, Fa, Fi, Ca</td>
<td>Saline</td>
<td>375-625</td>
<td>465 (7) 462 (13) 465, 500 446 (7) 458 (10)</td>
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<td>Z</td>
<td>337 (N$_2$)</td>
<td>Andersson (1987)</td>
<td>Aorta, Coronary, Aorta, Pulmonary, Femoral, Coronary</td>
<td>Ni, Di</td>
<td></td>
<td>350-700</td>
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<td>Iliac</td>
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<td>Air (Saline)</td>
<td>400-700</td>
<td>450</td>
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<td>EE</td>
<td>366 [bb Hg]</td>
<td>Connor-Davenport (1991)</td>
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<td>Ni, Di, Ca</td>
<td>Saline</td>
<td>380-600</td>
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Table A.1. continued.
Table A.1. continued.

<table>
<thead>
<tr>
<th>Study</th>
<th>$\lambda_{ex}$ (nm) [laser or BW]</th>
<th>Investigator (Year)</th>
<th>Tissue(s) [Artery]</th>
<th>Classes</th>
<th>Medium</th>
<th>Emission Range</th>
<th>peak $\lambda_f$ and [FWHM]</th>
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<tr>
<td>GG</td>
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<td>Scheu (1991a)</td>
<td>Femoral</td>
<td>Ni, Di, Ca</td>
<td>Air, Blood</td>
<td>400-700</td>
<td>450 450 450</td>
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<tr>
<td>II</td>
<td>458 (Ar$^+$)</td>
<td>Lucas (1990)</td>
<td>Coronary</td>
<td>Ni, Fa, Fi, Ca</td>
<td>Air (Saline)</td>
<td>490-750$^a$</td>
<td>515 [70]$^{a}$ 525 [80]$^{a}$ 520 [75]$^{a}$ 525 [85]$^{a}$</td>
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<tr>
<td>KK</td>
<td>476 [Ar$^+$]</td>
<td>Fitzmaurice (1989)</td>
<td>Aorta, Coronary</td>
<td>Ni, Fa, Fi, Ca</td>
<td>Microscopy</td>
<td>520$^{a}$ 520$^{a}$</td>
<td>550$^{a}$</td>
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<tr>
<td>LL</td>
<td>476 [Ar$^+$]</td>
<td>Richards-Kortum (1989)</td>
<td>Aorta</td>
<td>Ni, Di, Ca</td>
<td>500-650</td>
<td>520$^{a}$ 545$^{a}$</td>
<td>540-560$^{a}$</td>
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<td>500-650</td>
<td>520$^{a}$ 545$^{a}$</td>
<td>540-560$^{a}$</td>
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<td>Saline</td>
<td>520-780</td>
<td>600$^{a}$ 600$^{a}$</td>
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</table>
Table A.2. The reported features, normalization techniques, classifiers, and discriminant analysis techniques used in the studies listed in Table A.1. The types of features included $\text{ESS}[x \text{ nm}] = \text{even spectral sampling [sampling interval]}$, $R(\lambda_1, \lambda_2) = \text{ratio of emission intensities}: I(\lambda_1)/I(\lambda_2)$, $I(\lambda_1, \lambda_2...)$ = emission intensities at wavelengths: $\lambda_1, \lambda_2..., \text{FBC(components)} = \text{fit to spectra of biochemical components where the components are listed in parentheses, and } I_{\text{tot}} = \text{total emission intensity. The area normalization refers to the signal intensities being adjusted so that their sum is a constant. Absol. intens. means absolute intensities without normalization were used (note that ratio features are self-normalizing).}$

<table>
<thead>
<tr>
<th>Study</th>
<th>$\lambda_{ox}(\text{nm})$</th>
<th>Feature</th>
<th>Normaliz.</th>
<th>Classifier</th>
<th>Discrim Analysis</th>
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<td>A</td>
<td>480</td>
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<td>360</td>
<td>R(380,420)</td>
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<td>1D Thresh</td>
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<tr>
<td>E</td>
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<td>ESS[16nm]</td>
<td>Area</td>
<td>Hotelling Trace</td>
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<td>F</td>
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<td>Hotelling Trace</td>
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<td>308</td>
<td>R(335,380), R(480,380)</td>
<td>Piece-wise Linear</td>
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<td>J</td>
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<td>Mean &amp; Std. Dev.</td>
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<td>ESS[16nm], R(382,430), W(25%)</td>
<td>Area</td>
<td>Multivar Lin Regres, Stepwise Lin Regres, Princ Comp, Bayesian, &amp; Decision Plane Anal</td>
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<tr>
<td>P</td>
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<td>R(382,430)</td>
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<td>1D Thresh</td>
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<td>Q</td>
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<td>ESS[16nm]</td>
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<td>Stepwise Multivar Lin Regres</td>
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<td>FBC(elastin, collagen)</td>
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<td>$a_{\text{collagen}} - a_{\text{elastin}}/1D Thresh$</td>
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<td>ESS[16nm]</td>
<td>Area</td>
<td>Neural Net, Nearest Neighbor Cluster</td>
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<td>Ratio of N to D intensities</td>
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<th>Normaliz.</th>
<th>Classifier</th>
<th>Discrim Analysis</th>
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<td>multiple R($\lambda_1, \lambda_2$)</td>
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<td>Regression to plaque thickness, % lipid, % fibrous tissue and % calc.</td>
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<tr>
<td>X</td>
<td>325</td>
<td>multiple R($\lambda_1, \lambda_2$)</td>
<td></td>
<td>Regression to plaque thickness, % lipid, % fibrous tissue and % calc.</td>
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</tr>
<tr>
<td>Y</td>
<td>337</td>
<td>R(448,514), R(538,514)</td>
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<td>1D Thresh</td>
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</tr>
<tr>
<td>Z</td>
<td>337</td>
<td>I(395,420, 450,480)</td>
<td></td>
<td>1D Discrimination Power</td>
<td>Mean &amp; Std. Dev.</td>
</tr>
<tr>
<td>AA</td>
<td>337</td>
<td>4 R($\lambda_1, \lambda_2$)</td>
<td></td>
<td>Mean &amp; Std. Dev.</td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>351</td>
<td>$I_{tot}$</td>
<td></td>
<td>Mean &amp; Std. Dev.</td>
<td></td>
</tr>
<tr>
<td>EE</td>
<td>366</td>
<td>ESS[20nm]</td>
<td>Area</td>
<td>Linear, 1D Ratio Thresh</td>
<td>Mahalanobis D²</td>
</tr>
<tr>
<td>FF</td>
<td>366</td>
<td>ESS[20nm]</td>
<td>Area</td>
<td>Linear, 1D Ratio Thresh</td>
<td>Mean &amp; Std. Dev.</td>
</tr>
<tr>
<td>GG</td>
<td>375</td>
<td>R(465,530)</td>
<td></td>
<td>Mean &amp; Std. Dev.</td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>458</td>
<td>R(550, 520), R(520)</td>
<td>Absol. Intens.</td>
<td>1D Thresh</td>
<td>Mean &amp; Std. Dev.</td>
</tr>
<tr>
<td>II</td>
<td>458</td>
<td>multiple R($\lambda_1, \lambda_2$)</td>
<td></td>
<td>Regression to plaque thickness, % lipid, % fibrous tissue and calc.</td>
<td>Linear Regression</td>
</tr>
<tr>
<td>JJ</td>
<td>476</td>
<td>I(520, 580, 600), $I_{\text{max}}$, R(600,580)</td>
<td>Absol. Intens.</td>
<td>Linear Regression</td>
<td></td>
</tr>
<tr>
<td>LL</td>
<td>476</td>
<td>FBC(SP, ceroid)</td>
<td>Absol. Intens.</td>
<td>Piece-wise Linear</td>
<td></td>
</tr>
<tr>
<td>MM</td>
<td>476</td>
<td>FBC(SP, ceroid)</td>
<td>Absol. Intens.</td>
<td>Piece-wise Linear &amp; Bayesian</td>
<td></td>
</tr>
<tr>
<td>NN</td>
<td>495</td>
<td>R(530,540), R(530,560), R(560,600), R(580,600)</td>
<td></td>
<td>Mean &amp; Std. Dev.</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX B

DERIVATION OF THE $D^2$ DISTRIBUTION

In Chapter 5, a measure of class separability, the Mahalanobis distance or $D^2$, was described as a technique for evaluating wavelengths for fluorescence illumination. The Mahalanobis $D^2$ is related to another distance figure of merit, the Hotelling trace, $J$, which was used in the early periods of this study [Gmitro et al., 1990; Alexander et al., 1991]. Early results with the Hotelling trace revealed some interesting behavior. As the numbers of samples in the normal and diseased populations were increased, the $J$ values decreased [Alexander et al., 1991]. At first, this behavior caused great concern that there were systematic errors being introduced into the system. However, research into the distribution of the Hotelling Trace revealed that the estimate of $J$ from samples was biased. It was determined that the estimate of $J$ depended upon both the number of samples and the number of features. This bias could be estimated, and therefore corrected, using the distribution of $J$, which was known. The Mahalanobis $D^2$ is similarly biased and so a correction for this bias was also derived. The derivation for the $D^2$ distribution is described below.

In the 1930s and 1940s, four investigators -- P. C. Mahalanobis [1930], H. Hotelling [1931], R.A. Fisher [1936] and A Bhattacharyya [1943] -- developed similar measures for evaluating the difference between the probability densities of two populations, namely the Mahalanobis distance squared ($D^2$), the Hotelling trace ($T^2$ or $J$), Fisher's linear discriminant and the Bhattacharyya distance, respectively. The discussion in this section, however, is limited to the distributions of $J$ and $D^2$. Originally, the Hotelling trace was developed to test the validity of the null hypothesis.
The null hypothesis states that the means of two probability densities are equivalent. Conversely, the Mahalanobis $D^2$ was developed to evaluate the divergence of the means for two probabilities densities or to test the non-null hypothesis.

The calculations of $J$ and $D^2$ are very similar:

$$J = P(1)P(2) \, D^2 = \text{tr}\{S_w^{-1}S_b\} \, ,$$

where $P(1)$ and $P(2)$ are the prior probabilities of classes 1 and 2, respectively, and $S_w$ and $S_b$ are the within-class and between-class scatter matrices that were defined in Chapter 5 (Equations 5.21 and 5.22). The $\text{tr}\{\}$ denotes the trace operation, which is the sum of the diagonal elements of the matrix within the brackets. The within-class scatter matrix is equivalent to the average covariance matrix, $S_w = \Sigma = P(1)\Sigma_1 + P(2)\Sigma_2$.

Equation B.1 can be rewritten into another form that should be recognizable from Chapter 5,

$$J = P(1)P(2) \, D^2 = P(1)P(2) (m_1 - m_2)^\dagger \Sigma^{-1} (m_1 - m_2) \, ,$$

where $m_1$ and $m_2$ are the mean vectors for classes 1 and 2, respectively. The $^\dagger$ symbol represents the transpose operation. The between-class scatter matrix in Equation B.1 is $S_b = P(1)P(2) (m_1 - m_2)(m_1 - m_2)^\dagger$. If the class mean vectors and covariance matrices are estimated from samples, the calculated $J$ and $D^2$ values will only be estimates of the true values described in Equation B.2. It was shown by Hsu [1938] that the Hotelling trace, and hence, the Mahalanobis $D^2$, have the distribution of a non-central F-statistic. Several elegant derivations of this distribution have been presented by Wijsman [1957], Bowker [1960] and Rao [1965]. The following derivation was described by C.R. Rao [1965].

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\[^{\dagger}\text{pages 458 to 459.}\]
Let us assume that a q-dimensional sample feature-vector $X_k^s$ in class $k$ has a multivariate normal density, $X_k^s \sim N_q(m_k, \Sigma_k)$, with true mean vector, $m_k$ and covariance matrix, $\Sigma_k$. An estimate of the mean vector, $\hat{m}_k$, is determined by

$$\hat{m}_k = N_k^{-1} \sum_{s=1}^{N_k} X_k^s,$$

(B.3)

where the resultant mean vector is also multivariate normal, $\hat{m}_k \sim N_q(m_k, N_k^{-1} \Sigma_k)$. The $^\wedge$ symbol indicates an estimate of the variable from sampled data. The number of samples in $k$ is $N_k$. The estimated covariance matrix, $\hat{S}_k$, of class $k$ is

$$\hat{S}_k = (N_k - 1)^{-1} \sum_{s=1}^{N_k} (X_k^s - \hat{m}_k)(X_k^s - \hat{m}_k)^t$$

(B.4)

and has a central Wishart distribution [Rao, 1965], $\hat{S}_k \sim W_q(n_k, \Sigma_k)$, with $n_k = N_k - 1$ degrees of freedom.

To simplify the notation in the derivation, the mean difference vector,

$$\hat{d} = \hat{m}_1 - \hat{m}_2,$$

(B.5)

is used. The average of the sample covariance matrices is

$$\hat{S}_t = \frac{n_1 \hat{S}_1 + n_2 \hat{S}_2}{n_1 + n_2},$$

(B.6)

where $n_k = N_k - 1$ for classes $k = 1$ and $2$. The estimate of the Mahalanobis $D^2$, $\hat{D}^2$, can be calculated.
The second line of Equation B.7 can be broken into two pieces, the ratio $(\hat{d}^\dagger \hat{S}_i^{-1} \hat{d})/(\hat{d}^\dagger \Sigma^{-1} \hat{d})$ and $\hat{d}^\dagger \Sigma^{-1} \hat{d}$. It can be shown that

$$\frac{n \hat{d}^\dagger \Sigma^{-1} \hat{d}}{\hat{d}^\dagger \hat{S}_i^{-1} \hat{d}} = \chi^2_{v_2 = n - q + 1}$$

is a central chi-squared distribution with $v_2 = n - q + 1$ degrees of freedom [Rao, 1965]. Here, $n = n_1 + n_2 = N - 2$, where $N$ is the total number of samples in both classes. In addition,

$$\frac{N_1 N_2}{N} \hat{d}^\dagger \Sigma^{-1} \hat{d} = \chi^2_{v_1 = q, \lambda = \frac{N_1 N_2}{N} (m_1 - m_2)^\dagger \Sigma^{-1} (m_1 - m_2)}$$

is a non-central chi-squared distribution with $q$ degrees of freedom and non-centrality factor, $\lambda = \frac{N_1 N_2}{N} (m_1 - m_2)^\dagger \Sigma^{-1} (m_1 - m_2)$, which is proportional to the true Mahalanobis $D^2$ defined in Equation B.2.

The ratio of a non-central chi-squared variable to a central chi-squared variable is a non-central F-statistic:

$$F' = \frac{v_1 = q, v_2 = N - q - 1, \lambda = \frac{N_1 N_2}{N} D^2}{\chi^2_{v_1 = q, \chi^2_{v_2 = v_2} = \chi^2_{v_1 = q, \lambda = \frac{N_1 N_2}{N} (m_1 - m_2)^\dagger \Sigma^{-1} (m_1 - m_2)}}$$

$$= \frac{N_1 N_2}{N} \frac{N - q - 1}{(N - 2)q} \hat{d}^\dagger \hat{S}_i^{-1} \hat{d}$$

$$(B.10)$$
with \( q \) and \( N-q-1 \) degrees of freedom and the non-centrality parameter \( \lambda \). Consequently, the Mahalanobis \( D^2 \) estimated from samples is proportional to a non-central \( F \)-statistic. The probability density of the non-central \( F \)-statistic is

\[
p\left(F_{v_1, v_2, \lambda}\right) = e^{-\lambda/2} \sum_{r=0}^{\infty} \frac{I_r}{r!} \left(\frac{\lambda}{2}\right)^r \left(\frac{v_1}{v_2}\right)^{v_1/2+r} \frac{(F')^{v_1/2+r-1}}{\left(I + \frac{v_1}{v_2} F'\right)^{(v_1+v_2)/2+r}}, \tag{B.11}
\]

where \( B(a,b) \) is the Beta function defined as

\[
B(a,b) = \int_0^1 dx \ (1-x)^{a-1} x^{b-1}. \tag{B.12}
\]

Fortunately, the mean and variance of a non-central \( F \)-statistic are known:

\[
\langle F' \rangle = \frac{v_2}{v_2 - 2} \left(\frac{\lambda}{v_1} + 1\right), \tag{B.13}
\]

and

\[
\sigma_{F'}^2 = \frac{2 v_2^2 (v_1 + v_2 - 2)}{v_1 (v_2 - 2)^2 (v_2 - 4)} \left(1 + \frac{2 \lambda}{v_1} + \frac{\lambda^2}{v_1 (v_1 + v_2 - 2)}\right). \tag{B.14}
\]

The mean of the corresponding \( \hat{D}^2 \) is

\[
\langle \hat{D}^2 \rangle = \frac{N}{N_1 N_2} \frac{(N-2)q}{N-q-1} \langle F' \rangle = \frac{N-2}{N-q-3} \left(D^2 + \frac{qN}{N_1 N_2}\right), \tag{B.15}
\]

where \( D^2 \) is the true value. It is clear from Equation B.15 that the mean \( \hat{D}^2 \) value is a biased estimate of the true \( D^2 \) value. Consequently, a single \( \hat{D}^2 \) should also be a biased estimate of the true value. By inverting Equation B.15 and solving for \( D^2 \), an unbiased estimate of this distance measure, \( \hat{D}_u^2 \), can be obtained.
which is Equation 5.19. This bias correction has been reported by Afifi and Clark [1990] and Raudys and Jain [1991].

The variance of $\hat{D}^2$ can be calculated by

\[
\sigma_{\hat{D}^2}^2 = \left( \frac{N}{N_1 N_2} \frac{(N-2)q}{N-q-1} \right)^2 \sigma_F^2 \]

\[
= \frac{2 (N-2)^2}{(N-q-3)^2 (N-q-5)} \left\{ \frac{g N^2 (N-3)}{(N_1 N_2)^2} + \frac{2 N (N-3)}{N_1 N_2} \hat{D}_u^2 + \hat{D}_u^4 \right\}. \tag{B.17}
\]

The variance of the unbiased $\hat{D}^2$ is

\[
\sigma_{\hat{D}_u}^2 = \left( \frac{N-q-3}{N-2} \right)^2 \sigma_{\hat{D}^2}^2 \]

\[
= \frac{2}{(N-q-5)} \left\{ \frac{g N^2 (N-3)}{(N_1 N_2)^2} + \frac{2 N (N-3)}{N_1 N_2} \hat{D}_u^2 + \hat{D}_u^4 \right\}. \tag{B.18}
\]

which is Equation 5.20.
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