DETECTION AND LOCALIZATION OF PRE-CANCEROUS LESIONS AND EARLY LUNG CANCER

USING TISSUE AUTOFLUORESCENCE

by

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ABSTRACT

In this work, two different yet related hypotheses were tested by experimental means as follows: i) pre-cancerous and non-invasive (early) lung cancer can be detected and localized using the fluorescence properties of tumour localizing drugs at non-photosensitizing doses to skin tissue; ii) significant differences exist in laser-induced autofluorescence between normal, pre-cancerous and cancerous tissues such that these differences alone can be exploited to detect and delineate early lung cancer without using exogenous drug(s).

For most cancers, including lung cancer, a five-year survival of over 90% can be realized if the malignancy is diagnosed at the carcinoma in situ stage. However, current techniques: chest x-ray, sputum cytology, and conventional bronchoscopy alone or in combination cannot detect these very small lesions which are usually only a few cell layers thick and a few millimeters in surface diameter. Exogenous fluorescent tumour markers such as hematoporphyrin derivatives (e.g. Photofrin) have been used to enhance the detection of these occult lung lesions. Photofrin is preferentially retained in tumour tissues compared to the surrounding normal tissues; it fluoresces at 630 nm and 690 nm when excited at -405 nm. Based on this principle several imaging and non-imaging devices have been developed. However, wider clinical applications were limited due to the skin photosensitivity property of Photofrin. We have postulated that this could be solved by employing a much lower dose of photofrin (0.25 mg/kg) which was believed to be less photosensitizing to human patients. This postulate was experimentally tested by ratio fluorometry and early lung cancers were
detected with no false negative results and no apparent skin photosensitivity. An important finding in this study was that the mechanism for detection of early cancer was mainly due to the differences in the green autofluorescence between normal and malignant tissues, rather than fluorescence of tumour localizing drug.

This discovery led to the second postulate of this thesis that tissue autofluorescence alone can be exploited for the detection of early lung cancer. *In vivo* spectroscopy using an optical multi-channel analyzer showed an overall decrease in autofluorescence in pre-cancerous and cancerous lesions when excited by 405 nm or 442 nm laser light. A stepwise discriminant function analysis was performed on a database of nearly 300 patients spectra to determine the optical emission wavelength(s) and algorithm(s) at which the normal, pre-cancerous and cancerous tissues can be differentiated. The results indicated that algorithm(s) could be developed to clearly delineate early lesions from the normal tissues. Several algorithms were then tested using a non-imaging ratio fluorometer device and a prototype imaging fluorescence system to detect early lung cancer and dysplasia during standard bronchoscopy, therefore confirming the initial hypotheses even in a clinical setting.

The major source of the autofluorescence in the normal bronchial tissue was determined to come from the sub-epithelial layers. The mechanism for the decrease in the autofluorescence in pre-malignant and malignant tissues was explored but not yet completely elucidated. Several factors such as decrease in fluorophores, increase in absorption of the excitation/fluorescent light or different redox state of the fluorophores may be responsible for the observed phenomena.
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CHAPTER 1: INTRODUCTION

1.1 Outline

The investigations were carried out to understand the mechanisms of early lung cancer detection within the context of bronchoscopy. This thesis addressed the hypothesis that significant differences in autofluorescence exist between normal, pre-cancerous and early cancerous bronchial tissues.

The text of this thesis is divided in several chapters. First, the background and rationale for this thesis are presented. In Chapter 2, the limitation of current detection techniques and the use of exogenous fluorescent tumour markers, in particular Photofrin are described. A brief review of the imaging and non-imaging devices is included in this chapter as well as the diagnostic applications of autofluorescence. Chapter 3 reviews the optical process associated with light-tissue interactions, radiative transport of light in tissues, and optical properties of tissues. The physical processes following light absorption, in particular the principles of fluorescence are also described. In chapter 4, the objectives, experimental design and results are shown. In the last chapter, the hypothesis(es) for the mechanism of the observed difference in tissue autofluorescence between normal, pre-cancerous and cancerous bronchial tissues is presented and discussed. In addition, a summary of the project is given and future implications of these are considered.

Several aspects of this thesis have been described in scientific papers and abstracts presented and published as listed in the references.
1.2 Lung Cancer

1.2.1 Incidence and Mortality

Lung cancer was a relatively rare disease at the beginning of this century, but is now the leading cause of cancer-related death. The worldwide incidence of lung cancer is increasing at a rate of 0.5% per year (IARC (WHO), 1990), and with an estimated 660,500 new cases yearly, it is expected to become the predominant cancer world-wide by the end of this century (Parkin et al., 1988). Cigarette smoking is the main etiological agent, but dietary effects, exposure to other carcinogens and predisposing genetic factors have also been associated with lung cancers (Henderson et al., 1991, Weinstein, 1991, Law, 1990, Doll and Peto, 1981,). Smoking alone or acting in concert with other risks is the cause of about 80% of the lung cancers today (Shoppand, 1991, IRAC (WHO), 1990, Loeb et al., 1984 for review of the literature).

It has been estimated that in the United States alone, more than a million lung cancer deaths will occur before the year 2000 even if all tobacco smoking ceases (Mulshine, et al., 1989). This is because of the existing pool of current and ex-smokers and that lung cancer is due to the accumulative effect of smoking over a period of twenty years or more. For ex-smokers, although the risk of developing lung cancer is reduced after smoking cessation, the risk remains elevated for about fifteen years after smoking cessation compared to the risk of a lifetime non-smoker (Shopand, 1990, IRAC(WHO), 1990). In addition, the overall upward trend in lung cancer incidence continues due to the current prevalence of smoking among the female population (Stolley, 1983). In women, lung cancer deaths now
exceeds that attributed to breast cancer (American Cancer Society, 1991). The recent increase in smoking among women will further compound the current situation and its accumulated effect on lung cancer will be manifested for another 25 years or more (Brown and Kessler, 1988). In British Columbia alone, an estimated 1,118 new lung cancer cases will occur among women in the year 2001, compared to the approximately 600 in 1986, representing more than 80% increase within 15 years (McBride et al., 1989). In less developed countries where smoking continues to prevail, the devastating effect of lung cancer is likely to continue (Stanley and Stjernswärd, 1989, Stjernswärd, 1989a). By the year 2025, the World Health Organization estimated that there will be 3.5 million lung cancer cases per year globally (Stanley and Stjernswärd, 1989).

Despite the growing public awareness of the health risk of smoking and some encouraging progress in the reduction in cigarette smoking in recent years, the occurrence of lung cancer will remain a major health issue for decades (Stjernswärd, 1989b). Although prevention programmes targeted at tobacco products have resulted in the reduction of lung cancer incidence among young people in the developed countries (Shoppand, 1991, Devesa et al., 1989), the impact of an increased use of new addictive products such as marijuana and cocaine on future lung cancer incidence is not known yet (Stjernswärd, 1989a, Dupont, 1980).

The high incidence of lung cancer is unfortunately associated with a high mortality rate. Less than 15% of patients with lung cancer survive the disease and this figure has not changed significantly over the past three decades. The reason for this is that lung cancer is an insidious disease producing few symptoms until the late stages of its development.
At the time of diagnosis, the majority of the patients have advanced invasive cancer so that curative treatment is no longer possible (Mulshine et al., 1991, Minna et al., 1989, Fontana et al., 1986, Jett et al., 1983). Attempts in early detection using chest x-ray and sputum cytology examination have not been shown to reduce lung cancer mortality (Fontana et al., 1991 Fontana et al., 1986).

1.2.2 Classification of Lung Cancer

The World Health Organization classifies lung tumours into four major histologic subtypes: squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and small cell carcinoma (WHO, 1982). These subtypes account for about 90% of the primary lung cancers. The first three histologic subtypes (squamous, large cell and adenocarcinoma) are collectively grouped together and referred to as non-small cell lung cancer (NSCLC) and the treatment approaches for these tumours are identical. NSCLC accounts for about 75% of lung cancer cases. Small cell lung cancer (SCLC) is diagnosed in 20 to 25% of lung cancer and is a very aggressive tumour with neuroendocrine features. It is the most common histologic type of lung cancer found amongst uranium miners (Auerbach et al., 1978). SCLC is initially responsive to combination chemotherapy and radiotherapy, but most patients will eventually die of recurrent metastatic disease (Ihde and Mina, 1991, Minna et al., 1989, Seifter and Ihde, 1988). There is a high incidence of second primary cancers among long-term survivals of SCLC; the majority of the second primary cancers are NSCLC (Thomas et al., 1991).
1.2.3 Natural History of Lung Cancer

The carcinogenesis and pathogenesis of cancer is a complex multi-staged process (Fearon and Vogelstein, 1990, Harris, 1991). In general, malignant cancer is a consequence of genetic damage and other changes resulting in uncontrolled cellular proliferation and transformation (Strauss, 1992). Genetic (DNA) damage may be acquired somatically, induced by environmental and chemical carcinogens, or alternatively, predisposing genetic factors may exist, and this may involve gene-environmental/chemical interactions. Exposure to a cancer causing agent does not lead to an immediate production of a tumour. The specificity of the mutations that have been observed in tumours suggests that induction of some of these mutations are a result of several alterations in the DNA (Vogelstein et al., 1988). Most damage sustained by DNA can be repaired. However the repair process is believed to be gene specific and is associated with cancer susceptibility (Link et al., 1991). The distribution of adduct formation (damage to DNA) and repair in the genome is non-random and is influenced by its DNA organization and structure (Link et al., 1991, Pienta, 1989, Topal, 1988, Bohr et al., 1987). A number of important non-or pre-cancerous adaptive growth changes may occur in response to the initial primary step in the carcinogenesis process. Most of these "early" lesions may disappear spontaneously. The primary event is likely to be the damage to a specific gene in the cell DNA (Philips et al., 1988). Further steps may involve more genetic damage in other genes stimulating these cells to grow thereby conferring a selective advantage to these cells (Strauss, 1992, Loeb, 1991). There is a latency period for the development and/or progression of cancers (Sporn, 1991). For example, it has been
documented that the time between the onset of habitual cigarette smoking and the development of a clinically apparent tumor can be as long as several decades. There is evidence that several pathogenic events may occur during this latency period. Progressive alterations in the respiratory epithelium of cigarette smokers have been clinically observed. In the case of centrally arising lung cancers (squamous cell carcinoma), three types of lesions may exist: i) precancerous lesions (steps include hyperplasia, metaplasia, increasing degree of dysplasia); ii) malignant but non-invasive lesions (carcinoma in situ); and iii) invasive carcinomas that may potentially develop to a further degree of malignancy (Greenberg et al., 1987, McDowell and Trump, 1983, Saccomanno et al., 1974). The sequences of events for the development of peripherally arising adenocarcinoma and large cell carcinoma is still not fully established. At the present time it is suggested that in situ adenocarcinoma probably does not exist (Matthews and Linnoila, 1988).

It is difficult to evaluate human precancerous lesions in terms of the underlying carcinogenic process because so much of our knowledge of tumor development in human is indirect and inferential. To follow or assess these pre-malignant changes as they evolve is difficult because the assumed risk for progression is high enough to motivate removal of these lesions once they are detected. The nature of pre-malignant changes had been addressed by means of experimental animal model systems such as the injection of 20-methylcholanthrene into the submucosa area of beagle dogs to follow the development of invasive carcinoma endoscopically (Kato et al., 1982). Epidemiological and histological studies of lung cancer in humans showed that the cytological changes induced by benzo(a)pyrene-ferric
mixture in hamsters, 20-methylcholanthrene in beagle dogs are similar to that found in cigarette smokers (see below) with lung cancer (Kato et al., 1982, Becci et al., 1978, Schreiber et al., 1974).

The most comprehensive work done on humans and much of what is known about the development of lung cancer is from the studies by Saccomanno and co-workers using sputum cytology samples of more than 5000 uranium miners over a thirty-year period (Saccomanno et al., 1974, Saccomanno, 1982). These studies suggested that lung cancer develops over a period of 10 to 20 years with a series of progressive histopathologic changes, particularly for squamous cell carcinoma. These changes include a less specific basal cell hyperplasia followed by squamous metaplasia, increasing degree of atypia from mild, moderate to severe dysplasia and then final development of carcinoma in situ.

Dysplasia is a disturbance of differentiation of squamous epithelium which may be characterized as mild, moderate or severe. The biological activity of these lesions is uncertain with some regressing, others remaining stable and yet others progressing to epidermal carcinoma in situ. The term dysplasia is architecturally descriptive, relating to the relationship between cells within an epithelium. These changes are usually accompanied by cytologic changes referred to as dyskaryosis. Carcinoma in situ refers to an epithelium within which no differentiation takes place throughout its whole thickness, i.e. with the exception of some slight flattening in the upper most layers, the cells on the surface are identical to the cells in the basement layer. Histologically, the alteration within the surface epithelium resembles squamous carcinoma but there is no evidence of invasion of the underlying fibromuscular stroma.
1.3 The Bronchial Wall*

The pulmonary airways are a complex system of branching tubes and are in constant direct contact with gases or particulate matters in the external environment that may be cytotoxic. The human bronchus is divided into extrapulmonary and intrapulmonary bronchi. The extrapulmonary bronchi consist of the right and left main bronchi and the truncus intermedius. The right middle lobe and the left and right upper lobe prior to the bifurcation of the segmental bronchi is intrapulmonary in terms of structure. The extrapulmonary bronchus has horse-shoe shaped cartilage crescents which extend around 1/2 to 2/3 of the circumference. Elastic fibres extend around the entire circumference and reach a thickness of about 0.008 mm in the membranous portion forming longitudinal folds. The posterior wall of the bronchus is referred to the membranous portion and is free of cartilage crescents but has a large amount of smooth muscle. Cartilage crescents are absent in the intrapulmonary bronchi. The layer of elastic fibre between the epithelium and the submucosa is gradually replaced by smooth muscle which extend in rings surrounding the entire circumference of the bronchus. In the intrapulmonary bronchi, the fine circular folds are formed by thin rings of smooth muscle. The ridges seen running in longitudinal directions are the elastic fibres that are consolidated into bundles at intervals between the epithelium and the smooth muscle all around the bronchus.

When viewed tangentially the wall of an airway can be divided into two layers: epithelium, submucosal and cartilage. The epithelial surface

throughout the bronchial tree is normally pale pink and semi-transparent in appearance. The very thin surface layer of mucus in which the cilia beat gives the mucosa its characteristic sheen. However, the presence of squamous cell metaplasia, dysplasia or cancer causes alteration of the refractive index and may then appear opaque, white or granular.

The epithelial cells are attached to the basement membrane by fine cytoplasmic foot processes. The lamina propria lies below the basement membrane and is separated by an elastic tissue layer. The basement membrane and lamina propria separate the mucosa from the submucosa. The surface of the trachea and bronchi are lined by a pseudostratified columnar epithelium which consists of 4-5 principal cell types: superficially located ciliated and non-ciliated columnar cells, basal cell or reserve cells, and mucin-secreting goblet cells. Pseudostratified implies that all cells rest on the basement membrane and extend the full thickness of the epithelium. The ciliated columnar cells comprised about 85% of the respiratory epithelium; these cells are terminally differentiated and incapable of cell division. The basal cells and the rare basally located endocrine cells, the so called Kulchitsky cells with their dense core granules and features of neuroendocrine cells are scattered along but rest directly on the basement membrane. These basal cells gives the pseudostratified appearance of the bronchial epithelium because they do not reach the surface of the airway.

The submucosa consists principally of mucous glands, smooth muscle and cartilaginous plates. In the large bronchi, submucosal glands penetrate the submucosa and open by ducts at the mucosal surface. The submucosal bronchial gland consists of mucous cell, serous cell and
Kulchitsky cells. The smooth muscle varies in size and orientation along the airways forming a discontinuous layer in the innermost portion of the submucosa and spiraling in both directions of the airways. The extrapulmonary airways have the highest amount of smooth muscle and decrease progressively between main-stem bronchi and bronchioles. Cartilage is absent in the small bronchi and bronchioles. The connective tissues, particularly the cartilage, confer structural support on the airways. Nerves, blood vessels and lymphatics are found in the non distinctive adventitial layer of the airway.
Chapter 2: EARLY DETECTION

2.1 Introduction

Primary prevention remains the key to the long-term control of lung cancer. As discussed in chapter 1, even if World Health Organization (WHO) objectives to reduce tobacco smoking can be realized, lung cancer will remains a significant health problem well into the next century. Despite the efforts to improve therapy for advanced lung cancer, the efficacy is modest at best (Minna et al., 1989, Mulshine et al., 1989). Therefore, increased efforts in secondary prevention and treatment of early lung cancer may be the main element in the effective management of this disease. The key to this approach is the development of new diagnostic methods to detect pre-cancerous and in situ carcinoma lesions in high risk individuals (Mulshine et al., 1991).

2.1.1 Chest Radiograph and Sputum Cytology

It has been established that long term survival for lung cancer patients can be accomplished if the tumour is detected and localized at the carcinoma in situ or microinvasive stage. A five-year survival of 90% or better can be achieved in this group of patients after surgical resection (Melamed et al., 1984).

It is very difficult to detect and precisely localize lung cancers that are still confined to the bronchial wall. Chest x-ray and CT scans are usually negative (Frost et al., 1983, Spiro et al., 1982). Cancers detected by sputum cytology examination with an apparently normal chest x-ray are traditionally referred to as x-ray occult carcinoma (Miller et al.,
1983). Most lung cancers are not detected on a conventional radiography until the tumour has reached a size of approximately 1.0 cm$^3$. By this time, approximately 30 doubling of the tumour volume would have already occurred. Death usually occurs when the tumour has undergone 40 doublings. Since many lung cancers are not diagnosed until the tumour is 3 cm - 4 cm in diameter, it is not surprising that conventional treatments such as surgery, radiotherapy and chemotherapy are not very effective (Line et al., 1989).

In the late 1960's Saccomanno proposed the use of sputum cytology as a technique to enhance the detection of early lung cancer. Sputum cytology evaluates cytomorphologic changes in exfoliated bronchial epithelial cells. It was suggested that sputum cytology would provide the earliest possible indication of lung cancer (Hjerpe, 1988, Saccomanno et al., 1974). In the 1970's, in an attempt to improve the detection of early lung cancers, the National Cancer Institute of United States sponsored three prospective, randomized trials of lung cancer screening with chest radiographs and sputum cytology among 31,360 men (Cooperative Early Lung Cancer Detection Program) to evaluate the usefulness of chest x-ray in combination with sputum cytology in detecting early lung cancer. The end point for these trials was mortality rate from lung cancer. The men screened were all cigarette smokers who smoked at least one pack or more of cigarettes a day, 45 years of age or over and asymptomatic at the time of entry into the study (Berlin et al., 1984). Results of these trials suggest more early stage lung cancer can be detected in the asymptomatic smokers by periodic screening. However the overall mortality from lung cancer was not lowered probably due to a lead-time bias (Fontana et al., 1991, Mulshine et al.,
The study also showed that the yield of early lung cancer by sputum cytology was low; the prevalence of carcinoma in situ or microinvasive cancer was only 0.1%. The reason for this is that sputum cytology mainly detects centrally located squamous cell cancers that readily exfoliate cells into the bronchial secretion. However, it is less sensitive for the detection of adenocarcinomas and large cell carcinomas which tend to develop more peripherally in smaller airways where they obstruct the bronchial lumen quite early. Such cells are not exfoliated into the lumen until they have invaded the larger central airways (MacMahon et al., 1988, Fontana et al., 1986, Frost et al., 1983).

2.1.2 Biochemical, Immunological and Molecular Markers

Attempts to find biochemical, immunological, and molecular markers that can enhance the sensitivity of sputum cytology to detect lung cancer at its earliest stage are still at their infancy (Uei and Noguchi, 1990, Gazdar et al., 1990, Humphrey, 1989, Gail, 1988, Inoue et al., 1988). Immunocytochemical studies of antigens expressed on the cell surface of pre-malignant cells by monoclonal antibodies directed to a glycolipid antigen of small cell carcinoma and a protein antigen of non-small cell lung cancers (Tockman et al., 1988) showed encouraging results. Among those subjects with positive immunostaining of their sputum cells, 91% eventually developed lung cancer within 2 years. In contrast, 88% of those subjects who had negative immunostaining remained free of cancer. A follow-up study had been proposed to validate this immunostaining approach and to screen patients with Stage I resected lung cancer who represent a group with a high risk for second primary cancers (Mulshine et al., 1991,
However, even if these and other techniques can be proven to be of value in early detection, localization of these small malignant or pre-malignant lesions will remain a major problem. None of these techniques provide any information regarding the location of the abnormal cells in the upper or lower respiratory tract. To localize the source of the abnormal cells and to clearly delineate the size and extent of these lesions, bronchoscopic examination is generally required.

2.1.3 White Light Bronchoscopy

Conventional white light bronchoscopy is currently the only method that can localize x-ray occult lesions within the tracheobronchial tree (Marsh et al., 1989). The current approach is visual examination of the tracheobronchial tree under local anesthesia. Since occult lung cancers may be present in more than one site, a careful search of all the bronchial segments is essential (Marsh et al., 1983). Bite biopsies, bronchial brushing, and washings are performed on any suspicious or abnormal areas to confirm the presence or absence of cancer by cytology or histopathology. In about 25% - 30% of cases, a repeat bronchoscopic examination under general anesthesia with multiple brushings or blind biopsies may be required before the abnormal cells seen in sputum cytology examination can be localized (Balchum et al., 1982). A study by the Mayo Clinic showed that carcinoma in situ was visible bronchoscopically in less than 30% of the cases (Woolner, 1983). A recent follow-up study by the Lung Tumour Study Group in patients with completely resected T1N0MO lung cancers suggested that conventional bronchoscopy prior to surgery likely underestimated the local extent of the primary cancer and missed areas of
significant dysplasia or carcinoma in situ in 10-25% of the patients (Thomas et al., 1990). The reason for this is that early cancers are only a few cell layers thick (0.2 mm to 1 mm) and on average less than 0.8 cm in surface diameter (Woolner et al., 1984). These small relatively flat lesions may not produce enough changes on the bronchial surface to allow detection by conventional white-light bronchoscopy. In some cases of in situ carcinoma, subtle mucosal abnormalities such as an increase in redness, granularity or mucosal thickening can be recognized as signs of abnormality. Unfortunately, these changes can also be associated with inflammatory airways diseases making it very difficult for correct diagnosis (Oho and Amemiya, 1984).

2.2 Diagnostic Applications of Fluorescent Tumour Markers

Because of the difficulty in detecting and localizing in situ carcinomas, there has been great interest in the use of fluorescent drugs to enhance the diagnostic accuracy of conventional bronchoscopy. The use of fluorescent drugs to "mark" or "tag" cancerous cells has been available for many years. Dyes and drugs such as acridine orange, tetracycline, berberine sulfate, toluidine blue, fluorescein, eosin, and porphyrins have been shown to selectively or preferentially accumulate in malignant tumours (Dougerty, 1974, Tomson et al., 1974, Wilbanks and Richart, 1970, Rall, 1957, Moore, 1947). The diagnostic potential of such drugs which are preferentially accumulated in tumor tissues is obvious. In principle, it should be possible to detect and delineate the tumour by visualization of the fluorescence emitted by these tumour markers under proper excitations.
2.2.1 **Fluorescent Tumour Markers: Hematoporphyrin**

Although a number of fluorescent tumour markers are potentially useful for localization of tumours, much of the clinical and laboratory studies have focused on the use of porphyrins. The history of fluorescence detection of cancer using certain porphyrin compounds dates back to at least 1924 when Policard observed that certain malignant tumours in animals and man emitted a reddish fluorescence due to the accumulation of endogenous porphyrins (Policard, 1924). In 1942, Auler and Banger observed fluorescence in implanted tumours in rats but not in normal tissues following the systemic injection of hematoporphyrin (Hp) (Auler and Banger, 1942). In 1948, investigations by Figge, Weiland and Manganiello not only confirmed this observation in a wide range of induced or transplanted tumours in mice, but also found fluorescence in embryonic, lymphatic, and traumatized regenerating tissues as well (Figge et al., 1948). When Hp was injected into animals, a brilliant red-orange fluorescence was produced by ultraviolet excitation of the drug localized in these tissues. Hp is made from hemoglobin but contains no iron. It was first prepared in 1867 and was then called cruentene. It was later renamed as hematoporphyrin (Hp) at around 1871 and was prepared by treating hemoglobin with sulfuric acid followed by extraction with alcohol. In the 1950’s, several unsuccessful clinical testings were made to demonstrate Hp fluorescence in head and neck cancers, the biliary tree and lymph nodes in humans, (Rasmussen-Taxdal et al., 1955). The clinical procedures required a very large intravenous dosage (300 to 1000 mg per patient) of Hp to emit sufficient red fluorescence for the tumour to be detectable. At this dosage there is
considerable increase in photo-toxic reactions and at the same time a decrease in the degree of specificity.

The early studies that were performed on animals and humans used a commercial Hp preparation which is a crude mixture of many porphyrins. Schwartz and co-workers in 1955 showed that it was a minor impure fraction in Hp that had the tumour localizing properties (Schwartz et al., 1955). So, in 1960, Lipson under the direction of Schwartz prepared a derivative of hematoporphyrin by an acetic-sulfuric acid treatment of hematoporphyrin (Lipson et al., 1961). This product was termed hematoporphyrin derivative (Hpd). Subsequent studies indicated that Hpd has superior and more reproducible tumour localization properties than Hp or other simple monomeric porphryins in a variety of tumours of the bronchus, esophagus and cervix (Kyriazis et al., 1973, Gray et al., 1967). A clinical study of 226 cancer patients was conducted in 1968 by Gregorie and co-workers who showed that Hpd was localized in 84% of adenocarcinomas and 77% of squamous cell carcinomas (Gregorie et al., 1968). In 1971, Leonard and Beck found positive tumour fluorescence in 100% of 40 patients with tumours of oral cavity, pharynx and larynx following Hpd injection (Leonard and Beck, 1971). Hpd also exhibits photodynamic properties and was first used in 1966 for photodynamic treatment of a metastatic chest wall breast cancer (Dougherty, 1989).

2.2.2 Hpd And Its Active Components

Hpd is a complex mixture of many compounds including hematoporphyrin (Hp), hydroxyethylvinyl-deuteroporphyrin, (Hvd), protoporphyrin and a variety of dimers and oligomeric species bound covalently by ester or ether
linkages (Dougherty 1987, Byrne et al., 1987). Hp, Hvd and protoporphyrin are not preferentially retained in vivo in tumours (Dougherty, 1987). In 1984, Dougherty suggested that Hp molecules linked by ether/ester bonds through the 2 and/or 4 hydroxyethyl groups, known as dihematoporphyrin ether/ester (DHE) is the tumour localizing component of Hpd (Dougherty, 1984). A partially purified preparation of Hpd containing 80-85% of this tumour localizing fraction known as Profimer sodium (Photofrin) is now made available through Quadralogics Technologies Inc. (Vancouver, B.C.) for phase III clinical trials of photodynamic therapy in patients with lung, esophagus or bladder cancers.

2.2.3 Photo-Physical Properties

Hpd and Photofrin have characteristic absorption spectra in the ultraviolet and visible regions, with its major absorption in the soret band at around 405 nm. This corresponds to transitions from the ground singlet state to the second excited singlet state. Four minor excitation peaks called the Q band are observed in the visible region above 500 nm. Spontaneous decay of the singlet state to the lowest vibrational state produces the characteristics dual-peak red fluorescence at 630 nm and 690 nm. It has been established that the absorption, excitation and emission spectra of Hpd and Photofrin are effected by the type of solvent, pH and other factors. In its aggregated form, the soret peaks of the absorption spectra are blue shifted and their emission spectra are shifted towards the longer wavelength (red shifted) (Rotomskis et al., 1989, 1984, Brookfield, 1984, Moan and Sommer, 1981). The fluorescence intensity is decreased in
oligomers, by at least a factor of four compared to the dimers (Smith, 1985, Moan and Sommer, 1981, Andreoni and Cubeddu, 1984).

Besides spontaneous decay to the ground state that results in fluorescence, the excited Hpd or Photofrin molecules can alternatively undergo intersystem crossings to the more stable triplet state. Triplet state molecules can transfer their acquired energy to ground state oxygen to produce singlet oxygen and other oxygen radicals resulting in damage or death of cells (Chapman et al., 1991, Mitchell et al., 1990, Thomas et al., 1987, Henderson and Miller, 1986, Moan and Sommer, 1985, Henderson and Dougherty, 1984, Weishaupt et al., 1976). Although the photodynamic effect on tumours is beneficial, Hpd or Photofrin also photosensitizes the skin. Patients have to remain out of exposure to any strong light for four weeks or more to avoid skin burns (Dougherty, 1989, Wooten et al., 1988, Razum et al., 1987). Based on theoretical calculations, very low doses of Photofrin (< 0.34 mg/kg) may be devoid of skin photosensitivity (Potter, 1990). However, usefulness of low dose Photofrin or Hpd in the detection of early lung cancer has not been established.

2.3 Tumour Detection Using Hpd or Photofrin

To facilitate the detection and localization of early lung cancer, several approaches have been developed using fiberoptic bronchoscopy in combination with Hpd or Photofrin as the fluorescent marker (Kato and Cortese, 1985, Profio et al., 1984, Cortese et al., 1982, Cortese and Kinsey, 1981, Profio et al., 1979, Lipson et al., 1961). Excitation of Hpd or Photofrin is normally performed using lasers or mercury arc light
sources. Violet-blue light of wavelength 400 nm - 415 nm is transmitted by fiberoptics to the tissue surfaces via the fiberoptic bronchoscope to excite the drug which then fluoresces with its characteristic salmon red colour. The emitted fluorescence is collected by the imaging bundle of the bronchoscope or by a collecting fiber inserted into one of the channels of the bronchoscope. The collected fluorescent light is transmitted to sensitive light detectors and displayed as a visual image on a video monitor, or as an audio or digital signal.

2.3.1 Mechanism for Detection

Fluorescence detection using Hpd or Photofrin as the fluorescent agent is based on the principle that (i) Hpd/Photofrin in tissues emits fluorescence with peaks at 630 nm and 690 nm when excited by light at 390 nm - 415 nm, and (ii) the concentration of Hpd/Photofrin in malignant tissue is higher than the adjacent normal non-malignant tissues from 3 hours to at least 72 hours after intravenous injection. Tumours can be detected by differences in Photofrin concentration seen as a more intense specific red fluorescence compared to the surrounding normal tissue. Although the red fluorescence from large tumours can be readily visualized, the fluorescence from small early lesions is usually not visible with conventional fiberoptic bronchoscopy because the fluorescence quantum yield of the tumour-localizing components of Photofrin is very low. Profio et al had calculated it to be less than 2% (Profio, 1984). Background noise can adversely affect the detectability of such low fluorescent light. To overcome this problem, sensitive light detectors such as image intensifiers or photomultiplier tubes have been used to amplify the fluorescence.
However, even with highly sensitive light detectors, a very high concentration of the drug (2-3 mg/kg i.v) is needed before fluorescence can be detected (Kato and Cortese, 1985, Profio and Balchum, 1985, Profio, 1984, Hayata et al., 1982). At these doses, Hpd and Photofrin result in skin photosensitivity as discussed in chapter 2.2.3 above. (Dougherty, 1989, Wooten et al., 1988, Razum et al., 1987). While this may be tolerable for treatment of lung cancer, it would be clinically unacceptable to use Hpd or Photofrin as a diagnostic agent.

2.3.2 Reviews of Fluorescence Endoscopic Detection Systems

Kinsey and Cortese of the Mayo clinic developed the first non-imaging device in 1978 (Kinsey et al., 1978, Kinsey and Cortese, 1980). This detection system used a double lumen bronchoscope. The emitted fluorescence from the bronchial surface was transmitted to the photomultiplier tube via a fiberoptic guide which was placed in one of the lumens of a double channel bronchoscope. A rotating chopper wheel was used to alternate between the fluorescence and white light reflected modes at 30 Hz. Excitation of Photofrin was by 405 nm light from a filtered 200 Watt, mercury arc lamp. The alternate pulses of 405 nm light and the unfiltered white light are transmitted to the bronchial surfaces via the illumination guide of the bronchoscope. The frequency at which the chopper wheel rotated allowed the reflected light image to be visible in real time. A lock-in amplifier generated a frequency-modulated audio signal. The audio pitch produced was proportional to the intensity of the red fluorescence, and thus alerted the bronchoscopist to areas of the tracheobronchial tree that were suspicious for cancer. The system allowed for simultaneous
visual white light examination and the search for Photofrin fluorescence in the fluorescence mode. The major drawback of this device is that the fluorescence intensity varies depending on the geometry, distance, angle and intensity of the excitation light.

The earliest fluorescence imaging system was reported in 1979 by Profio and Doiron at UCSB (Profio et al., 1979). In this system, the emitted fluorescence was imaged with an image intensifier which was attached to the eyepiece of the bronchoscope. A 690 nm band-pass filter was placed in front of the intensifier to allow transmittance of Hpd/Photofrin red fluorescence while excluding reflected excitation light and background autofluorescence. The fluorescence intensity at this bandwidth was amplified 30,000 times by the intensifier. Tumour containing Hpd/Photofrin was seen as a brighter spot. Colour information was lost with the intensifier and hence the image was displayed in gray scale. Excitation of Photofrin fluorescence was by 405 nm - 413 nm light from a Krypton-ion laser. The excitation light was conducted via a 400 micron quartz fibre inserted through the biopsy channel of the bronchoscope. A flip-flop mirror configuration allowed rapid switching between white light and the violet light illumination (Profio et al., 1982). The system had a very high sensitivity with 97% of tumours showing positive fluorescence, but the specificity was only 50% (Balchum et al., 1990). The requirement of a subjective human judgement of the brightness contrast was by far the major drawback of the image intensifier system. Although lesions with bright spots were readily detectable, precise delineation of the size and extent of the tumour in areas with diffuse fluorescence, blurred margins or with low contrast from background was very difficult. Furthermore, the
human eye is more sensitive for colour vision than to discern differences in brightness contrast. In addition, false positive signals occurred when the bronchoscope tip was closer to the bronchial surface or cartilaginous areas (see chapter 1, section 1.3 for a description of the bronchial wall). Again, this system does not correct for the dependence of the fluorescence intensity on the tissue geometry, the angle and distance of the excitation light source as well as the pickup light guide from the bronchial surface.

The system developed at the Tokyo Medical College in Japan had a combination of imaging and non-imaging detectors. It consisted of a pulse excimer laser and a Spectro-Image Analyzer (Hirano et al., 1989). Photofrin was excited with the excimer laser at 405 nm and the emitted fluorescence was transmitted through the optical fibre of the bronchoscope and then separated by a half reflecting mirror such that the signal was simultaneously picked up by an image intensified SIT camera and a spectrograph (polychromator and intensified photodiode array, with an optical multi-channel analyzer). The fluorescence spectrum was displayed on the video monitor to distinguish the Photofrin fluorescence and tissue autofluorescence. Subtraction of the tissue autofluorescence spectrum from the Photofrin fluorescence can then be obtained. A xenon lamp was used for reflected white light illumination to produce a colour video image. This light transmission was turned on and off at 30 Hz by an optical chopper driven by a motor. Thus white light and fluorescence images as well as Photofrin spectral characteristics can be observed simultaneously in real time. Although the system had many advantages in terms of specificity and simultaneous white-light fluorescence image display, the margins of the tumour cannot be clearly defined on the displayed pseudo-images.
Furthermore, like the other systems, the fluorescence intensity is significantly influenced by the position of the bronchoscope from the bronchial surfaces.

A different approach using background subtraction was made by Baumgartner and co-workers in Germany (Baumgartner et al., 1987). Fluorescence from Photofrin and bronchial or bladder tissues were excited alternately at two wavelengths, one at 405 nm and the other at 470 nm. A Krypton-ion laser with a specially designed resonator dichroic mirror produced the two wavelengths for illumination. The 405 nm violet light excited both Photofrin and tissue autofluorescence, while the 470 nm blue light gave very similar tissue autofluorescence but very little of the Photofrin fluorescence. Thus, if the fluorescent signals or images from these two wavelengths were subtracted, the net result was mainly the red Photofrin fluorescence. This was accomplished in near real time by image processing techniques. Although this was a sensitive system and some contrast enhancement was provided with the subtraction, the displayed subtracted images were still dependent on the distance of the bronchoscope from the tissue surface.

Based on a similar principle of the German group, but using a much cheaper Helium-neon laser a fluorescence photometer was developed by Potter and Mang (Potter and Mang, 1984). Fluorescence was excited at 612 nm and 632.8 nm using two 5 mW Helium-neon laser. This system was designed for the detection of non-palpable metastatic breast cancers in the skin and the micro-metastases to regional lymph nodes (Mang et al., 1991). Delineation of surgical margins during thoracotomy and detection of intraoperative metastases during mediastinoscopy were potential applications. However,
the drawback was again the angular and distance dependence of the excitation light and the receiving light guide from the tissue surface.

To address and solve some of these problems of distance and angle dependence, a ratio fluorometer probe was devised by Profio and co-workers (Profio et al., 1984). The concept of ratioing is important because it can correct or cancel the variations of the excitation light power, angle, distance and surface geometry. In the original report by Profio and co-workers, the fluorescence signal at 690 nm was ratioed against the signal of the reflected violet (405 nm - 410 nm) excitation power. Although ratioing canceled the dependence on distance, angle and excitation intensity, they found that the ratio was influenced by variations in the violet reflectivity as well as the fluorescence yield of the drug (Profio et al., 1984). An improvement was later accomplished by taking the ratio of the Photofrin fluorescence intensity at 690 nm to a green autofluorescence at 560 nm. This approach assumed that normal and malignant tissues emit similar autofluorescence at the green region when excited by 405 nm and hence the spectra were normalised at 560 nm (Profio et al., 1986). Because more Photofrin was retained by tumour tissue compared to the adjacent normal bronchial tissues, the tumour can be detected by an elevated red-green ratio. Since the red-green ratio is a dimensionless function, it cancels the dependence of angle and distance between the bronchoscope tip and the bronchial surface. The excitation of fluorescence was procured by illumination with a Krypton-ion laser at 405 nm. A two channel bronchoscope was used, one channel to conduct the illumination quartz fibre while the other channel was used for the collecting fibre. The distal end of the illumination fibre was fitted with
a microlens to spread out the light. The emitted fluorescence was picked up by the collecting fibres and was split by a dichroic mirror. The two beams were then passed through a red and green band-pass filters at 690 nm and 560 nm respectively. The normalized ratio of red to green fluorescence was then measured and displayed as a digital read-out as well as an audio signal. Using this device a specificity of 100% was reported, but the sensitivity was only 70% (Balchum et al., 1987). The low sensitivity could be due to the high threshold ratio that was chosen as indicative of tumour. In that study, a red-green ratio of 4 to 5 times higher than an apparently normal area was considered to be abnormal. The disadvantage of the ratio fluorometer probe is that it is a non-imaging device. The exact site and size of the tumour cannot be delineated accurately. Furthermore as a one pixel system, small cancers can be readily missed because of field averaging effect.

The group at the Swiss Federal Institute of Technology in Lausanne (EPFL) also employed the ratioing concept to develop a device to image early lung cancers (Wagnières et al., 1990). A dichroic mirror was used to split the fluorescence light into two regions which were then passed through two band-pass filters, one in the 520 nm to 580 nm range and the other between 600 nm and 720 nm. The red and green light were then captured by separate image intensified CCDs. The two images (red and green) were ratioed. Autofluorescence subtraction was also performed for contrast enhancement. The processed pseudo-image was then displayed on a video monitor. This device allows time sharing between reflected white light and fluorescence modes by using an optical chopper. This system is still not yet capable of real time pixel by pixel ratio imaging. There is
also significant light loss and optical distortion resulting in poor quality of the pseudo-image.

Another approach was made by the group in Lund, Sweden (Andersson et al., 1987a, Montán et al., 1985). They developed a two-dimensional multi-spectral imaging system with background autofluorescence subtraction. A Cassegranian telescope was used to collect the fluorescence light in a multi-mirror configuration. A spherical mirror was divided into identical quadrants and interference filters were arranged in front of each of the quadrants so that four fluorescence wavelength bands were imaged onto an intensified CCD matrix detector. The four sectors had adjustable screws that allow for positioning the individuals images onto the detector surface. Subtraction or ratioing of the four images can be carried out by an image processing board and a pseudo-image (false colour coding) was obtained that can delineate the tumour site with a relatively high contrast. The image acquisition and processing takes about 5 seconds and this may be a problem for lung examination because of respiratory motion artifacts. Another problem is related to the excitation light source using a nitrogen laser (337 nm). The safety of illuminating the bronchus with ultra-violet light has not been established.

2.4 Previous work using of Tissue Autofluorescence In Diagnosis

It has been reported that even without introducing any foreign fluorescent agents, tissues exhibit characteristics intrinsic autofluorescence. Differences in autofluorescence between normal and certain abnormal tissues have been demonstrated in excised human tissues
and in animal models of tumour (Alfano et al., 1984, Tang et al., 1989a, Tang et al., 1989b, Caplan, 1967, Herley, 1944, Figge et al., 1944, Sutro and Burman, 1933, Wood, 1908 (in Anderson and Parrish, 1982)). However, differences in tissue autofluorescence is generally considered too weak to be quantified or are insensitive to current detection devices especially for small lesions (Lakowicz, 1983). This belief led many investigators to assume that an exogenous fluorescent dye is essential for the detection of cancers. Furthermore, because the autofluorescence intensity between normal and cancerous tissues were thought to be similar in the green region of the visible spectrum, background fluorescence was used for ratioing or subtraction to enhance the contrast between tumour and normal tissues when a fluorescent dye such as Hpd/Photofrin is used. The ideas are so ingrained that fluorescence detection has been generally equated with the use of fluorescent drugs (Mang et al., 1991, Wagnières et al., 1990, Baumgartner et al., 1987, Andersson et al., 1987a, Profio et al., 1986, Montán et al., 1985, Kato et al., 1984).

In a limited fashion, autofluorescence has been used for the detection of dental decay (Alfano and Yao, 1981), cancer in rats (Andersson et al., 1987b, Alfano et al., 1984), as a marker for photoaging (Leffell et al., 1988), to characterize brain tumours (Montán and Strömbland, 1987), and to discriminate atherosclerotic plaque from normal arterial wall (Andersson-Engels et al., 1991, Lucas et al., 1990, Richards-Kortum et al., 1989a, Gmitro et al., 1988, Hoyt et al., 1988, Clarke et al., 1988, Sartori et al., 1987). Very recently, laser-induced autofluorescence is been used in the diagnosis of human colon adenomas and carcinomas (Schomacker et al., 1992, Rava et al., 1991, Richards-Kortum et al., 1991, Kapadia et al.,
1990, Cothren et al., 1990), as well as oral tumours (Yang et al., 1987). Oral tumours are unique in that they contain endogenous porphyrin and hence fluoresces red without any exogenous fluorescent drug (Yang et al., 1987, Harris and Werkhaven, 1987).

There has been very limited investigation on the diagnosis of lung cancer using tissue autofluorescence. The conclusion from a study of 17 patients with bronchial tumours investigated by the group in Lund, Sweden suggested that the use of autofluorescence alone to detect cancer of the bronchus is probably not possible (Andersson-Engels, 1989).

There is much speculation as to why cells or tissues autofluoresce (Benson et al., 1979, Herly, 1944). Naturally occurring fluorophors in the cells such as pyrimidines and flavin nucleotides co-enzymes, carotene and porphyrin are responsible for the cell fluorescence at the visible wavelengths (Benson et al., 1979, Fellner, 1976, Blankenhorn, 1958). The most intensely fluorescing component in the cell was found to be in the mitochondria. This is attributed to the proteins bound to respiratory co-enzymes in mitochondria (Aubin, 1979, Chance and Schoener, 1966). Flavin is known to fluoresce and exhibit spectral changes when it is transformed from the oxidized to reduced state. It has been demonstrated that the spectra maxima of flavins vary from 520 nm to 535 nm depending on the redox state (Aubin, 1979, Benson, 1979, Ghisla et al., 1975). Riboflavins are known to fluoresce in the visible spectrum (about 510 nm to 530 nm) and are a part of the co-enzyme flavin adenine dinucleotide (FAD) which is responsible for the oxidation-reduction in the mitochondria (Chance and Schoener, 1966). Nicotinamide adenine dinucleotide (NADH) has also been reported to be a major tissue fluorophore involved in the redox state of
the mitochondria. NADH is known to fluoresce strongly at 470 nm when excited at 340 nm (Chance et al., 1962, Welsh et al., 1977). Lipofuscin, collagen and elastin in animal cells have very strong autofluorescence in the green-yellow spectral regions (references in Aubin, 1979). Naturally occurring porphyrins are found in urine, stool, and erythrocytes. The principal fluorescence spectrum of porphyrin is found to be between 597 nm to 634 nm (Harris and Werkhaven, 1987). Fluorescence emission maxima for keratin and melanin is observed in the visible region at 525 nm to 540 nm (references in Alfano et al., 1984). The ultra-violet fluorescence of cells is from tryptophan-containing proteins in the respiratory enzymes of the mitochondria and from nucleic acids (Udenfriend, 1962). Human eosinophils emit autofluorescence in the green-red regions of the spectrum and this characteristic was used in the isolation and analysis of these cells using flow microfluorometry (Weil and Chused, 1981). Macrophages often contain ingested fluorescing particles which can produce "autofluorescence" when excited at the appropriate wavelength. In connective tissue, unidentified fluorophors in collagen and elastin have strong autofluorescence in the blue-green spectral regions (Neuman and Logan, 1950, Banga and Bihari-Varga, 1974).
CHAPTER 3: LIGHT INTERACTION WITH TISSUES

3.1 Photo-Biological Response

The spectrum of electromagnetic radiation ranges from radio-waves to gamma rays. Light is the part of the electromagnetic radiation spectrum we see, and ranges in wavelength from 400 nm to 700 nm apparently changing colours from violet to red.

The interaction of electromagnetic radiation with matter involves the exchange of energy. A molecule is said to be excited when one or more of its electrons has been displaced into a higher orbital. These molecules are often unstable and may decay by radiative and/or non-radiative processes. The energy lost during these processes can be dissipated as heat, luminescence, or as a chemical reaction. More specifically, the immediate photo-biological response of light interaction with biological tissues can be divided into: (i) photo-thermal response; (ii) photo-physical response; and (iii) photo-chemical response. Photo-thermal response is the result of the transformation of absorbed light energy to heat, leading to coagulation or destruction of the target tissue. Photo-chemical interactions result when light is absorbed by either endogenous or exogenous chromophores introduced to initiate chemical reactions such as production of reactive singlet oxygen species in photodynamic therapy. An example of a photo-physical light-tissue interaction is fluorescence. Applications of photo-thermal response and photo-chemical response are mainly therapeutic, while photo-physical effects are used both for diagnostic and therapeutic purposes. Therapeutic applications of light is based on the effect of light on tissue, whereas diagnostic applications
exploit detectable changes resulting from the effect of tissue on light (Parrish and Wilson, 1991). Comprehensive reviews of these effects of tissue interactions with non-ionizing radiation and established applications of these effects in medicine and biology are described in Wilson and Jacques, 1990, Welch et al., 1989, Boulnois, 1989, and Anderson and Parish, 1982.

Tissues can be defined by their optical properties (discussed below), thermal properties (heat capacity and heat diffusivity), mechanical properties (viscosity, elasticity and tensile strength), chemical composition, architecture or structure (physical arrangements of organelles and cells), and physiological state. The effects of light-tissue interactions are determined by these properties, as well as the conditions of the irradiation and the time after irradiation at which the evaluation is undertaken (Thomsen, 1991, Boulnois, 1989).

3.2 Optical Properties of Turbid Media: Tissue

Tissue is a scattering and absorptive medium with a higher refractive index than air. As an electromagnetic wave enters a medium with refractive index \( n \), the wave amplitude decreases (absorption) and its phase velocity becomes \( c/n \) (dispersion). The refractive index is not only a property of the medium however but also depends on the frequency of the electromagnetic wave. The refractive index for light travelling in soft tissue is determined to be in the range 1.38 - 1.41 and for adipose tissue to be 1.45 (Bolin et al., 1989 and references therein). Light can reflect at the tissue surface, scatter due to the spatial variations in the reflective
index or dielectric properties within the tissue, and be absorbed by natural chromophores in the tissue. The amount of light that is scattered and absorbed in the tissues is also wavelength dependent. Wavelengths in the ultra-violet and infra-red regions are highly absorbed in tissues. The penetration depth, \( \delta \) (i.e. the depth at which the light intensity is attenuated by a factor of \( 1/e \) due to absorption and scattering), is less than 20 \( \mu \text{m} \) at these wavelengths. Absorption in the ultra-violet region is due to tissue proteins, while water absorption dominates at wavelength greater than 1300 nm. In the wavelength region between 450 nm to 600 nm, where the absorption is due primarily to heme and melanin, the absorption and scattering coefficients are of the same proportion and \( \delta \) is about 0.5 mm to 2.5 mm. To a lesser extent, bilirubin, flavins, carotenoids, the cytochrome pigments of the respiratory chains in the mitochondria, and water also absorb light in this region. Between 600 nm to 1300 nm, scattering is the dominant form of interaction. This region is the most transparent for tissue and light is able to penetrate relatively deeply, and \( \delta \) being about 2.0 mm to 15.0 mm (Svaasand, 1990, Wilson et al., 1990, Parrish, 1981). Visible light (400 nm - 700 nm) is absorbed very slightly by unpigmented cells. This is the reason why cells appear transparent or translucent (from scattering of light by particles in the cells) under bright field microscopy. Most of what is absorbed by the cells is due to the presence of cytochrome and flavoproteins and other macromolecules and proteins as described in chapter 2.4.

Since this thesis deals mainly with bronchial tissues, a schematic representation of the optical processes that may occur at the different bronchial tissue layers during the propagation of light is illustrated in
The structure and histology of the bronchial wall is given in Chapter 1, section 1.3. The respiratory tract is a dynamic organ, and as such, the optical properties must be dealt with as a dynamic process. Because of its complex structure, precise modeling of the path of optical radiation within the tissue is very difficult. The many different physical structures and chromophores within the different layers of the bronchial wall influence its optical properties. However, within any given layer, the following basic optical processes may occur. Usually about 4% of a normally incident light beam is reflected from the air-tissue interface and boundaries of the layers due to the mismatch in the refracted index (Fresnel reflections or specular surface reflections). The rest of the light then penetrates into the tissue encountering multiple scattering (back and forward scattering) and absorption processes which may lead to photo-chemical reaction or loss of energy as heat, fluorescence or phosphorescence. For very thin or transparent tissue, a portion of the incident light will be transmitted through the tissue.

The angle \( \theta \) at which light travels in tissue is given by Snell's Law:

\[
\sin \theta = \frac{\eta_1}{\eta_2} \sin \beta
\]

Light travelling from tissue to air (i.e. from a higher refractive index material to a material with lower refractive index) will be internally reflected if \( \theta \) exceeds the critical angle:

\[
\sin \theta_c = \sin 90^\circ \left( \frac{\eta_1}{\eta_2} \right), \text{ where } \eta_1 < \eta_2
\]
Figure 1 Schematic representation of the propagation of light in bronchial tissue.
Therefore, not all the back scattered light in tissues is transmitted through the tissue-air interface. Diffuse light scattered at an angle greater than $48^\circ$ with respect to the interface normal is totally internally reflected back into the tissue.

The intrinsic optical parameters of the tissue, coefficient of absorption ($\mu_a$), scattering ($\mu_s$), and anisotropy ($g$) determine the distribution of light within the tissue. The phase and polarization parameters are ignored in this treatment of photon propagations since these parameters are very quickly randomized in tissue. The absorption and scattering coefficients are the probabilities per unit path length within the tissue that a photon will be absorbed or scattered from a single centre, respectively.

A review of some of the very limited published work on the propagation of light in different biological tissues (Wilson and Jacques, 1990) suggests that: (i) the movement of light in tissue is influenced by the intrinsic optical parameters which may vary between different tissue types, or within a single tissue, (ii) these optical parameters are strongly dependent on the wavelength in the visible range, and (iii) interfaces, boundaries conditions, and geometry of incident light will affect the distribution of light.

**Absorption coefficient**

The absorption of light in tissues occurs when the resonant frequency of the molecules matches the wavelength of the incident light. Where there is a transfer of energy from a photon to a chromophore, the photon ceases to exist. Usually the absorbed energy is dissipated as heat, but the
energy from a small fraction of absorbed radiation may lead to photochemical reactions or luminescence. The average absorption of a local region of tissue is optically characterized by the absorption coefficient, $\mu_a$. The mean free path between absorption events is given by $1/\mu_a$. When scattering is also present, the effective path length of radiation within the tissue or medium is increased, generally increasing the likelihood of absorption.

**Scattering coefficient**

In tissue, scattering is an elastic interaction between optical radiation and the tissue in which only the direction of photon propagation is changed. Scattering can also be inelastic which results in a change of wavelength (this is however not common in tissues). The scattering of light is caused by heterogeneities in the refractive index of tissues. Physical heterogeneities within a cell reflect the presence of cell organelles such as cell nuclei, mitochondria, the extracellular matrix and collagen fibrils, all of which influence light scattering (Jacques, 1990). The effect of scattering within a tissue is dependent on both the degree of scattering and the spatial distribution of the scattering light. There are two types of scattering: Rayleigh scattering and Mie scattering. Rayleigh scattering predominates where the size of the molecules or particles is less than $1/10$ of the wavelength. The scattering in this case is very weak, isotropic, polarized, and varies with the fourth power of the wavelength. Mie scattering occurs where the particle size greatly exceeds the wavelength. It is highly forward directed and is independent of wavelength. The average scattering within a tissue is optically
characterized by its scattering coefficient $\mu_s$. The mean free path between scattering events is $1/\mu_s$.

**Anisotropy**

When a scattering event occurs, the trajectory of the photon will be deflected by an angle $\theta$. In general, light scattering in tissues is forward directed (anisotropic), i.e., the photon deflection angle is small and so the photon will continue in the same general path with very small deviations. Experimental evidence indicating that light scattering in tissues is forward directed in tissues was reported for human stratum corneum and epidermis at ultra-violet and visible wavelengths, for human dermis at 633 nm, for blood, and for liver at wavelength between 350 nm to 2200 nm (Jacques et al., 1987, Flock et al., 1987). In anisotropic scattering, many scattering events are required before the photon loses its sense of history and achieves a random walk. Anisotropic scattering occurs when the scattering particle is about the same size or greater than the wavelength of the incident light. This type of scattering is approximately modeled by Mie theory which deals with the scattering by spheres of sizes similar to the incident wavelength. The angular distribution of scattering is defined by a single scattering phase function, $p(\theta)$, which describes the probability that the photon will be deflected by a given angle $\theta$. The phase function can be represented by a series expansion as a sum of Legendre polynomials:

$$p(\theta) = \sum_{n=0}^{\infty} W_n P_n (\cos \theta)$$
The Henyey-Greenstein function,

\[ p(\theta) = \frac{1}{4\pi} \frac{(1-g^2)}{(1+g^2-2g\cos\theta)^{3/2}} \]

which describes light scattering in galaxies (Henyey and Greenstein, 1941) is used to approximate light scattering in tissues (Jacques et al., 1987). Anisotropy \( g \), describes the average angular dependence of scattering and \( g \) is defined as the expectation value or the average cosine of the scattering angle \( \langle \cos\theta \rangle \):

\[ \langle \cos\theta \rangle = g = \frac{\pi}{\int_0^\pi p(\theta)\cos(\theta)2\pi\sin\theta d\theta}. \]

**Boundary conditions**

While the spatial distribution of light within tissue is determined by the absorption and scattering cross sections, the mismatch of the refractive index in tissues and the interface of the air/water/glass/or other materials characterizes the surface boundary conditions. Two optical surface effects involving the boundary are: (i) specular surface reflectance as light enters the tissue, and (ii) total internal reflectance as light attempts to escape the tissue.

The specular surface reflectance, \( R_{sp} \) at a mismatched boundary is given by:

\[ R_{sp} = \left[ \frac{(n_2-n_1)}{(n_2+n_1)} \right]^2 \]
where \( n_1 \) is the refractive index of tissue and \( n_2 \) is the refractive index of the adjacent medium. Specular reflection does not provide any information about tissue absorption and scattering properties but tissue surface roughness and refractive index values can be obtained.

Total internal reflection occurs at a mismatched boundary, \( n_2 < n_1 \), where photons attempt to escape the tissue at angles that are greater than the critical angle. The total amount of internally reflected light, \( R_i \), is calculated using Fresnel's law of reflectance for randomly polarized light (Hecht, 1987).

The back-scattered light striking the interface is assumed to be distributed diffusely. For tissue/air interface, only about 50% of the back-scattered light is transmitted, i.e. about 50% is internally reflected. A discussion on the effect of surface roughness on internal reflection and the influence of tissue refractive index in internal reflection is found in Jacques, 1990, Wilson and Jacques, 1990.

3.3 Light Propagation in Tissue: Theory

The exact, detailed mathematical modelling or calculation of light propagation in tissue is very complex. Tissue is highly scattering and inhomogeneous with many local microscopic spatial variations in the refractive index. In addition, if the angular distribution of the scattered light is not isotropic, the intensity can be higher in certain directions than in others. A mathematical computation of the propagation of light in tissue can be made by analytical theory or transport theory. Analytical theory is based on the fundamental equations governing field
quantities. This approach is often based on Twersky's work (Twersky, 1970, Twersky, 1962). However, an exact solution of Maxwell's electromagnetic wave equations is almost an impossible task. Detailed information regarding the scattering and absorption processes associated with microscopic spatial variations in the dielectric properties of tissue must be considered in the calculation. In principle, starting with Maxwell's equations is the most fundamental approach, since the statistical nature of the tissue as well as all diffraction and interference effects are taken into account in the calculations. However, this approach is mathematically complex and, in practice, approximations must be made. Its usefulness is therefore limited.

Transport theory deals directly with the transport of photon energy which may be locally absorbed or elastically scattered through turbid media. The wave properties of light (diffraction and interference effects) are generally neglected. Transport theory has been shown experimentally to be applicable in many situations. The basic equation in transport theory is the radiative transfer equation. The transport equation and several approximate numerical and analytical models that have been developed to describe the propagation of light in tissue will be discussed briefly below.

Radiative transfer equation

The distribution of light in biological medium can be adequately described by the radiative transfer equation (Chandrasekhar, 1960). This equation is equivalent to the Boltzmann equation used in neutron transport theory and the kinetic theory of gases. Whereas neutron transport is
concerned with particle fluence rate (particle density multiplied by the speed), photon transport is expressed in the energy fluence rate (space irradiance). The application of Boltzman equation to photon transport of x-ray and gamma ray is discussed in texts on radiation shielding (Chilton et al., 1984, Profio, 1979, Goldstein, 1959). The transport of optical photons may be treated in the same fashion as x-ray photons with the differences in the interaction cross sections. The Boltzman equation expresses the balance between gains and losses. The generation of Raman scattering is considered negligible, so there is no change in the wavelength on scattering. The scattering and absorbing centres are assumed to be uniformly distributed in the medium. The equation of transfer is:

$$\Omega.\nabla \psi(r,\Omega) = - \mu_t \psi(r,\Omega) + \int \mu_s p(\theta) \psi(r,\Omega') d\Omega' + S(r,\Omega)$$

where $\psi(r,\Omega)$, in W/m$^2$/sr, is the angular energy flux density at position $r$ and angle $\Omega$ (power crossing unit area normal to the direction vector $\Omega$). In optics, this quantity is known as radiance. The energy fluence rate, also called the space irradiance is equal to the radiance integrated over $4\pi$ solid angle. The normalized angular scattering function, $p(\theta)$ is the scattering phase function discussed previously, with $\Omega.\Omega' = \cos\theta$, and describes the probability of scattering from angle $\Omega'$ into angle $\Omega$. The integration is over all $4\pi$ steradians of solid angle in a spherical coordinate system, azimuthal symmetry is assumed. The total interaction coefficient $\mu_t$ is equal to $\mu_s + \mu_a$. $S(r,\Omega)$ is the volume source intensity in W/m$^3$/sr.
The left hand term of the above equation represents the change in the flux density due to photon transport. The first term on the right hand side represents the losses in $\psi(r,\Omega)$ due to attenuation by scattering and absorption, the second term is its gain due to the scattering of photons back into the original direction of motion. The third term on the right is the contribution from other sources.

The Boltzmann equation is solved subject to specified boundary conditions. The angular flux density is zero for any rays entering the tissue, and a specular reflection condition is imposed at the centre of a sphere or axis cylinder (Profio, 1989, Welch et al., 1987, Profio and Doiron, 1986). Except for very simplified cases, the Boltzmann equation cannot be solved analytically. Two- and three-dimensional solutions require a serious restriction of the angular distribution of the scattered light (Case and Zweifel, 1969). Computer methods have been developed to solve this equation numerically, however, numerical solutions are still costly, especially for two-and three-dimensional problems (Welch et al., 1987).

Monte Carlo method

An alternative to solving the equation of transfer is to simulate light transport in a scattering medium with the Monte Carlo method (Keijzer et al., 1989a, Prahl et al., 1989, Wilson and Adam, 1987, Flock et al., 1989). This is basically a computer simulation of the tracks of individual photons. Each photon out of a large sample is followed in its random walk until it is absorbed. The total light distribution is estimated from the resulting distribution of absorbed photons. The Monte Carlo method has
been applied to transport of optical photons in three-dimensional geometry problems since this method can calculate light transport without approximating the tissue geometry or angular distribution of light. The advantages of Monte Carlo simulations are their exactness and flexibility (the exact scattering properties can be simulated after sufficient photon trajectories are computed as well as treatment of internal reflection at mismatched boundaries, non-uniform irradiance, and non-perpendicular irradiance). However, this method is subject to statistical errors and is also time consuming. At the present time reasonable answers can only be obtained after many hours of computing time.

**Approximate mathematical models**

Because the radiative transport equation cannot be solved exactly except for a few special cases, approximate solutions to the radiative transfer equation have been developed to study the propagation of photons in various media. The inhomogeneous structure of tissues allows one to neglect spatial coherence and interference mechanisms of light, hence permitting substantial simplifications to be made.

In conditions where absorption dominates, Beer's law is a simple model that yields reasonable results. Beer's law describes the exponential attenuation of light as it passes through tissues. However, most biological tissues are highly scattering, and hence Beer's law is not applicable at many wavelengths. A model of two diffuse light fluxes normal to the tissue surface travelling in the forward and backward directions was proposed (Kubelka, 1954, Kubelka, 1948) to describe light propagating in tissues. In this model, a forward flux travelling through a very small
path length $\delta z$, is attenuated due to absorption and scattering and is increased by the scattering contribution of the backward flux. A similar relation holds for the backward flux, with the exception that it travels in an opposite direction of $\delta z$. Thus the distribution of light as a function of a single variable, namely the depth in the medium can be easily calculated. A newly suggested seven-flux model has been proposed to provide for forward and backward diffuse fluxes propagating in the $x$, $y$, and $z$ directions as well as a collimated light flux in the direction of beam incidence (Welch et al., 1987). The Kubelka-Munk theory has been applied to the quantitative characterization of the normal vessel wall and atherosclerotic plaque (Vogel et al., 1991), blood (van Gemert and Henning, 1981), and skin (Wan et al., 1981). The Kubelka-Munk theory is restricted to uniform beam incidence and to cases in which radial intensity variation is negligible. For uniform diffuse incident light, this model provides a simple relationship between measured reflectance and transmission. The Kubelka-Munk's absorption and scattering coefficients of thin tissue samples are:

$$ A_{km} = 2\mu_a; $$

$$ S_{km} = (3/4)(1-g)\mu_s - (1/4)\mu_a; $$

$$ -\ln[T_c(t)] = (\mu_a + \mu_s)t, $$

where $T_c$ is the incident collimated beam, $t$ is the sample thickness, $A_{km}$ and $S_{km}$ are the Kubelka-Munk's absorption and scattering coefficients respectively. Hence, using these relations, the optical parameters can be easily evaluated.
Diffusion theory is a simple approximation to the general radiative transport equation (Ishimaru, 1989). It is derived from the transport equation with the assumption that light is scattered almost uniformly after encountering numerous scattering events. This means that the angular energy flux density $\psi(r,\Omega)$ is only weakly dependent on direction $\Omega$. The diffusion approximation is valid for optically dense and highly scattering media, and it describes the intensity of light as a sum of three terms namely; an isotropic term, a diffusely forward directed term, and a forward directed $\delta$ function. The latter accounts for the very forward-directed light scattering that is common in tissues. Applying the diffusion equation to the radiative transfer equation provides a second-order partial differential equation that can be solved for appropriate boundary conditions. Since the model requires diffuse light within the tissue, the theory tends to break down near boundaries and localized internal sources (Welch et al., 1987). In general, reasonable accuracies have been obtained for Nd:YAG laser light which is highly scattered in most tissues such as brain, bladder and the dermis of skin (Welch et al., 1987, Cheong et al., 1990 and references therein). A diffusion approximation algorithm to solve the 1-dimensional transport equation deriving the optical properties of rat liver between 350 nm and 2200 nm has been reported (Parsa et al., 1989, Jacques and Prahl, 1987).

3.4 Measurements of Tissue Optical Properties

Monte Carlo and Boltzman transport theory calculations and some of the approximate mathematical models mentioned above require the knowledge
of intrinsic optical parameters: absorption coefficient, scattering coefficient, and scattering phase function. It is not possible to calculate all these coefficients from the tissue composition and structure; they must be measured. The total attenuation coefficient which is the sum of the scattering and absorption coefficients, can be measured by the transmission of a narrow, collimated beam through a thin sample. A tissue sample is usually about 100 μm, one mean free path. To minimize scattering the sample is placed between two optical flats separated by calibrated spacers slightly thinner than the sample. A collimated beam is transmitted through the sample and then to a distant detector with a narrow collection solid angle. The $μ_s$ is usually $\gg μ_a$, hence the measurement is usually a reliable direct measurement of $μ_s$.

The scattering phase function can be directly measured using a goniometric apparatus. The laser beam is scattered by the thin tissue sample held by two optical flats, and a collimated detector is rotated around the sample to measure the scattered light as a function of angle $θ$ (Jacques et al., 1987). However, it is difficult to measure the absorption coefficient independent of the scattering coefficient, especially when the absorption is small compared to the scattering. When it is possible to work with large thick tissue samples, integrating sphere measurements can be performed to measure the reflectance and transmission of diffuse light as a function of wavelength to evaluate $μ_a$, $μ_s(l-g)$.

Details of these and other techniques which are currently available to evaluate the optical properties are discussed by Jacques, 1990, Cheong et al., 1990, Wilson and Jacques 1990, Wilson et al., 1990, Svaasand, 1990, Marchesini et al., 1989).
3.5 **Photo-Physical Process Following Light Absorption***

Only radiation that is absorbed will result in a photo-chemical change in a molecule or can be effective in producing luminescence (including fluorescence and phosphorescence). According to the Grotthus-Draper Law, molecules must have a chromophore in order to absorb light. The fact that each molecule that emits luminescence or is chemically altered by radiation absorbs one quantum of radiation, is known as the Enistein-Stark quantum law. As long as the dose (i.e. the product of the intensity and exposure time) is the same, the photo-chemical effect will be the same. The total number of absorbed photons determines the extent of a photo-physical process. The energy of the photon and the rate which these photons are absorbed have no influence on the photo-physical process. A given exposure dose at a given wavelength produces a given degree of response independent of irradiance (Reciprocity law). The quantum yield of fluorescence is independent of exciting radiation energy: The intensity of fluorescence depends on the number of exciting photons. The change in wavelength (loss of energy) of luminescence emission spectra relative to those of the exciting radiation energy, is known as the Stoke’s shift. The luminescence spectrum is invariant with changes in the excitation radiation energy.

When light interacts with matter, it may be either scattered (diffracted light) or absorbed. The kinetic scheme of the behaviour of molecules following optical excitation is illustrated by the Jablonski’s energy diagram shown in figure 2. The ground, first singlet, second

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*References used in preparation of section 3.5 were Lakowicz, 1983, Pesce et al., 1971, Udenfriend, 1969, Udenfriend, 1962.
singlet, and third singlet electronic states are depicted by $S_0$, $S_1$, $S_2$, and $S_3$ respectively. Associated with each of these electronic states are a number of vibrational energy levels, depicted by $V_0$, $V_1$, $V_2$, $V_3$, etc. Between each of these vibrational quantum numbers are a number of quantized rotational energies. The transitions within the vibrational and rotational energy levels to the next lower electronic state are vertical, this governs the position and profile of the absorption band. This is what is known as the Franck-Condon principle which states that all electronic transitions are vertical, occurring without any change in the position of the nuclei.

In a singlet excited state, the electrons in the higher-energy orbital have the opposite spin orientation as the second electron in the lower orbital. These two electrons are said to be paired. In a triplet state these electrons are unpaired, that is their spins have the same orientation.

An incoming photon excites the molecule from the ground state to any vibrational level of an excited state ($S_n$). For clarity, figure 2, shows only one photon absorption for each of the electronic states. Irrespective of the vibrational level reached by the initial absorption, the molecule loses excess vibrational energy within $10^{-13}$s to occupy the $V_0$ level. This radiationless transition is called "vibrational relaxation"; it is represented by the vertical wavy lines pointing downward in figure 2. This process is then followed by a rapid, radiationless internal conversion to the highest vibrational level of the next lowest energy singlet state, illustrated by the horizontal wavy lines in figure 2. This conversion process involves no change of energy. The overall radiationless decay process is very fast, within about $10^{-11}$s, the molecule is at the lowest
excited singlet state \((S_1)\). Because of this rapid relaxation, the population yield of the lowest energy singlet state following excitation to any level is essentially unity and therefore the emission spectral distribution and the fluorescence lifetime are usually independent of the exact excitation wavelength. Thermal equilibrium is established within the \(S_1\) vibrational levels. From this level the molecule can subsequently decay through radiative fluorescence emission, intersystem crossing or internal conversion to the lower orbital ground state.

3.5.1 Fluorescence

Fluorescence is one of the several possible pathways of the de-excitation process by which an excited state electron at the \(S_1, V_0\) level gives up the energy and return to the ground state as shown in figure 2. Fluorescence lifetime is typically in the order of \(10^{-8}\) to \(10^{-9}\) seconds, representing the average period of time a fluorophore remains in the excited states prior to returning to the ground state. Again, the Franck-Condon principle is invoked, and this time the vibrational levels of the ground state govern the profile of the emission band. The wavelength of the emission fluorescence spectra is longer than the wavelength of the excitation light (referred to as Stoke's shift). The reason is that part of the energy difference between the ground state, \(V_0\) level and the upper \(V_n\) level of the \(S_1\) state are accounted for by the vibrational relaxation before and after fluorescence emission. Generally, the fluorescence emission spectrum appears to be a mirror image of the absorption spectrum. This is due to the same transitions being involved in both absorption and emission, and the similarities among the vibrational energy levels of \(S_0\).
and S₁. Fluorescence does not require an electron to change its spin orientation. Several factors such as solvent effects, solvent relaxation, quenching, and a variety of excited state reactions can influence the fluorescence emission. These factors are discussed in details in the text *Principle of Fluorescence Spectroscopy* (Lakowicz, 1983).

Radiationless decay to the ground state and the intersystem crossing to the first triplet state are strongly competing radiationless processes. Fluorescence is delayed (about 10⁻⁶ seconds) if the excited electron moves to a forbidden triplet state before moving back to the lowest singlet state and emitting light. If an electron in the T₁, V₀ state absorbs thermal energy to reach the level of the S₁, V₀ state and one of the unpaired electrons then reverses its spin orientation, this can result in an intersystem crossing in the reverse direction to the singlet state followed by fluorescence. This indirect fluorescence is delayed but has the same emission profile as that of the fluorescence from the direct pathway.

A transition from a singlet to a triplet state (three energy levels due to an unpaired electron in spin) is described as intersystem crossing. The triplet state is always at a lower energy level than the corresponding singlet state. Radiative transitions between singlet and triplet state are "forbidden" by laws in quantum mechanics, thus the absorption of a photon to give a S₀ --> T₁ transition is not allowed. Emission from the first triplet state (T₁) is called phosphorescence and is generally shifted to longer wavelength relative to fluorescence. Transition from a triplet state to the ground state requires one of the paired electrons to change its spin orientation. Phosphorescence has much longer lifetime of the excited state, typically ranging from milliseconds to seconds.
Figure 2 Jablonski's energy diagrams of the photo-physical process following absorption of light. Only one photon absorption for each of electronic states is shown. The vertical wavy lines pointing downward represent the vibrational relaxation and the horizontal wavy lines represent the radiationless internal conversion. ($S_0$ = ground state, $S_1$, $S_2$, and $S_3$ are the lowest, second and third excited singlet states respectively, $V_n$ = number of vibrational energy levels. $T_1$ = triplet state).
3.5.2 Characteristics of Fluorescence

Fluorescence is a result of the absorption of light, and it can occur only following absorption. It involves the emission of light and an outside source of energy is required. If light intensity $I_o$ is directed through a medium some absorption of the incident radiation will occur. The light that is not absorbed is transmitted and can be denoted $I_t$. Thus ($I_o - I_t$) represent the radiation energy absorbed by the medium. As a result of absorption fluorescence may occur (as discussed above). The intensity of fluorescence is related to the absorption by:

$$F = Q(I_o - I_t),$$

where $F$ is the fluorescence intensity; $I_o$ is the intensity before absorption; $I_t$ is the intensity after absorption; $Q$ is the quantum efficiency.

From Lambert's law, the fluorescence intensity can be rewritten as:

$$F = QI_o(1 - e^{-A}),$$

where $A$ is the absorbance. The fluorescence intensity is directly related to the intensity of the excitation light and directly proportional to absorption under condition of low absorbance. Fluorescence is proportional to the number of absorbing molecules. The ratio of the number of photons emitted (energy) to the number of photons absorbed (energy) is called quantum efficiency where quantum efficiency is a property of the fluorophores.
Some of the factors that may influence the intensity of fluorescence are solvent effects and relaxation, dynamic and static quenching, high optical density and inner filter effect. Details of these factors are discussed in the text, *Principle of Fluorescence Spectroscopy* (Lakowicz, 1983).

Some of the main attributes of fluorescence as a tool in diagnosis is its specificity and sensitivity compared to other "non-invasive" methods. Fluorophores absorb and emit light at characteristic wavelengths and therefore can be selectively excited and detected in a complex composition such as tissue. In fluorescence diagnosis, the characteristics of the fluorophores within the tissue as well as the optical properties (absorption and scattering) must be considered. Fluorescence signals are complicated by interactions of scattering with fluorescence, absorption and reabsorption of other molecules in the tissue where these effects must be considered. In principle, fluorescence measurements can be made on the fluorescence intensity of the excitation spectra, emission spectra or fluorescence intensity as a function of delay time after pulsed excitation at a fixed excitation and emission wavelengths.
Chapter 4: EXPERIMENTAL DESIGN AND RESULTS

4.1. Thesis Objective

The overall goal of this thesis was to study the mechanism of early lung cancer detection with and without fluorescent tumour localizing drugs. The major objectives were:

1) To test the hypothesis that detection and localization of early lung cancer can be achieved using low dose Photofrin without skin photosensitivity.

2) To test the hypothesis that laser-induced autofluorescence is significantly different between normal, pre-cancerous and cancerous bronchial tissues.

3) To determine the optimal emission wavelength(s) to differentiate between normal, pre-cancerous and cancerous tissues if differences in laser-induced autofluorescence exist as in postulate 2.

4) To determine conceptually (if postulates 2 and 3 are correct) how the spectral differences can be exploited for detection of early lung cancer and dysplasia using imaging and non-imaging methods.

4.2 Detection of Early Lung Cancer Using Non-Skin Photosensitizing Dose of Photofrin

4.2.1 Rationale

As discussed in chapter 2, detection of early lung cancer presents a diagnostic challenge even for an experienced bronchoscopist. Fluorescence tumour markers such as Hpd and Photofrin have been used to facilitate the
detection of these early lesions. However, the dose at which detection has been achieved using methods as discussed in chapter 2.3.2 was very high (2-3 mg/kg intravenously). At these doses, Hpd and Photofrin result in skin photosensitivity which lasts for four weeks or more. Although this is not a major problem when using the drug for treatment (PDT), it is clinically unsuitable for diagnostic purposes.

To determine if skin photosensitivity can be avoided, ratio fluorometry was performed in patients with known or suspected lung cancer using a much lower dose of Photofrin (0.25 mg/kg). The choice of this drug dose was based on the theoretical estimation that a Photofrin dose of less than 0.34 mg/kg is below the threshold of photodynamic action and hence may not cause skin photosensitivity (Potter, 1990).

**Ratio Fluorometer Probe**

The schematic diagram for the ratio fluorometer probe is shown in figure 3. The principle of ratio fluorometry has been discussed in chapter 2, section 2.3.2. In its original design for fluorescence bronchoscopy, a two channel bronchoscope was used, one channel to conduct the illumination fibre, while the second channel was used for the collecting fibre. This however, restricted the range of examination because of the large bronchoscope size and loss of angulation with the two fibres. A modification was then made to this design. Using a beam-splitter one of the two fibres was eliminated by using the imaging guide of the bronchoscope to collect the fluorescent light from the bronchial surface. This modification allowed the use of a bronchoscope smaller than the double channel bronchoscope and extended the range of the examination.
Figure 3  Schematic diagram of the ratio fluorometer probe.
**Patient Selection**

Twenty patients with known or suspected lung cancer were selected for this study majority of cases. Most of these patients had positive sputum cytology and radiologically occult lung cancers or marked atypia in the sputum cytology. Ratio fluorometry was performed 24 hours after intravenous injection of 0.25 mg/kg Photofrin.

**Fluorescence Bronchoscopy**

Following white light bronchoscopy examination, fluorescence bronchoscopy was carried out using the modified ratio fluorometer probe. 405 nm light from a Krypton-ion laser at 15 mW/cm² irradiance, was transmitted to the tip of the fiberoptic bronchoscope (Olympus BF10) via a 850 um quartz fiber with a diverging microlens (Laser Therapeutics Inc., Buellton, CA). The divergence angle of the microlens fibre was 45° with a illumination spot size of approximately 0.53 cm². This fibre was inserted into the biopsy channel of the bronchoscope. The emitted fluorescence was collected and transmitted to the ratio fluorometer by the imaging guide of the fiberoptic bronchoscope. Fluorescence intensities at 520 ± 10 nm and 690 ± 10 nm, as well as their ratio (690 nm/520 nm) were displayed on a digital read-out. The red-green ratio value is independent of intensity, distance, and angle between the excitation light at the bronchial tip and the bronchial surface. Before scanning the tracheobronchial tree, the red-green ratios of at least two apparently normal areas as determined by white-light bronchoscopy were first measured. Five readings were taken at each area which were then biopsied for pathologic confirmation. Measurements of the ratios were then made throughout the bronchial tree. A
preliminary clinical-pathologic study showed that the highest coefficient of variations of the red-green ratios from normal areas was approximately 20%. Therefore a red-green value of 1.5 times above the mean value of the normal sites was considered to be potentially significant. These areas were later biopsied for histopathological confirmation.

Skin Photosensitivity Test

A test for skin photosensitivity was performed before and within 4 hours after Photofrin administration. Testings was performed on the lower back of the patients. A 500 W Sylvania projector lamp (Sylvania, Drummondville, Quebec) with a filter (Schott KG-4, Schott Optical Glass Inc., Duryea, PA) to remove infra-red light was used as the light source. Areas of 1.5 cm x 2.5 cm of the patient's back were exposed to light dosage of 15 - 30 J/cm² in 5 J/cm² increments. This dosage was chosen based on a previous study of patients who had received 2 mg/kg of Photofrin. When their skin could tolerate 15 J/cm² of test light, they could be exposed to outside light without any skin reaction (Lam et al., 1990a). To reduce the probability of a false negative skin test due to the variability of individual skin responses and difference in climatic conditions between Vancouver and other places, the highest test dose was increased to 30 J/cm². The power output of the light source was measured by a radiometer (ILL700, International light, Newburyport, MA). Skin reaction was read at 24 hours after light exposure and graded as 1+, 2+, and 3+. A 1+ reaction is equivalent to the minimum erthema dose (MED) and was the light dose which induced a mild reddening of the skin without edema, blistering or necrosis. A 2+ reaction referred to moderate redness with edema but no
blistering or necrosis. The 3+ reaction referred to redness, edema, and blisters (Lam et al., 1990a). Since, normal skin without Photofrin will not show any reaction to natural light, no skin photosensitivity test were performed prior to Photofrin administration.

A total of 12 carcinoma in situ lesions were detected in these 20 patients who were given 0.25 mg/kg of Photofrin. There were no false negative results (negative predictive value 100%). Of the 12 carcinoma in situ lesions, 7 were from patients with radiologically occult sputum cytology positive lung cancer, 4 from patients with residual microscopic cancer in the bronchial resection margin after surgery and one was from a synchronous in situ carcinoma in a patient with a large invasive tumour. Figure 4 shows the red-green ratios of pathologically confirmed areas from these 20 patients. The median red-green ratio of carcinoma in situ lesions was 2.8 times above normal. Areas of dysplasia were also detectable. Five of the biopsies showed severe dysplasia and 3 showed mild dysplasia. These lesions have a median red-green ratios of 1.8 times above normal. There was only one false positive biopsy from an area with inflammation.

No skin photosensitivity was detected in all 20 patients both on testing with the simulated light source and upon subsequent exposure to natural (sun) light. The lack of complication in 20 consecutive patients ruled out the probability of skin photosensitivity reaction higher than 15% (Hanley and Lippman-Hand, 1983). Furthermore, this result is in agreement with the theoretical estimation that a Photofrin dose of < 0.34 mg/kg is below the threshold of photodynamic action and hence may not cause skin photosensitivity (Potter, 1990).
Figure 4  Red-green ratios of pathologically confirmed areas from 20 patients who had received 0.25 mg/kg Photofrin intravenously. (-) represents the median red-green ratios of carcinoma in situ lesion from the normal background. (cis = carcinoma in situ).

A total of 12 carcinoma in situ lesions were detected in these 20 patients, with a median red-green ratios of 2.8. Five biopsies showed severe dysplasia and 3 showed mild dysplasia, with a median red-green ratios of 1.8 times above normal background. The normals were from bronchoscopically apparent normal areas with the red-green ratios arbitrary set to approximately 1. The red-green signal ratios in the vertical axis represent the red-green ratios of the abnormal sites versus the control normal sites.

Red-Green Ratios of Pathological Confirmed Areas
(With 0.25 mg/kg Photofrin)
4.2.2 Mechanism for Detection by Ratio Fluorometry

Although dysplasia and early lung cancer can be detected using Photofrin with no apparent skin photosensitivity, the mechanism of cancer detection based on this principle was not fully understood. The mechanism of tumour detection by ratio fluorometry was therefore investigated.

A total of 52 patients were studied before and after administration of low and high dose photofrin using ratio fluorometry. Fluorescence bronchoscopy was carried as described previously. In addition to the red-green ratios, the red fluorescence at 690 nm and green fluorescence at 520 nm were also recorded. Care was taken that the tip of the bronchoscope was kept about 1 cm from the bronchial surface and the measurements were recorded during the same phase of the respiratory cycle.

The individual red (690 nm) and green (520 nm) fluorescence intensities of the tumour area expressed as a ratio of the normal background before and after administration of 0.25 mg/kg and 2 mg/kg of Photofrin are shown in figures 5 and 6, respectively. In figure 5, a value above 1.0 is indicative of a higher red fluorescence (and hence more Photofrin) in the tumour area compared to normal, while for figure 6, a value less than 1.0 is indicative of a lower green fluorescence in the tumour area. A total of 28 tumours were studied without Photofrin, 11 tumours studied with 0.25 mg/kg Photofrin and 7 tumours with 2 mg/kg Photofrin. Without any photofrin, both large tumours and carcinoma in situ lesions had decreased red fluorescence. With 0.25 mg/kg Photofrin, the carcinoma in situ lesions have an increase in red fluorescence, on average about 1.7 times higher than the non-tumour area. Most of the large tumours did not show a significant increase in red fluorescence.
Figure 5 Red (690 nm ± 10 nm) fluorescence intensity of tumours as a ratio of the normal background without and with (0.25 mg/kg and 2 mg/kg) Photofrin. A total of 28 tumours were studied without Photofrin, 11 tumours studied with 0.25 mg/kg Photofrin and 7 tumours with 2 mg/kg Photofrin. With 0.25 mg/kg, the carcinoma in situ lesions have an average of 1.7 times higher red fluorescence than the non-tumour area. Most of the large tumour do not show a significant increase in red fluorescence.

Red Intensity of Tumour-Normal Ratios as a Function of Photofrin Dose

- visible tumour
- carcinoma in situ
Figure 6  Green (520 nm ± 10 nm) autofluorescence of the tumour as a ratio of the normal background without and with (0.25 mg/kg and 2 mg/kg) Photofrin. With and without Photofrin, the mean green autofluorescence of tumour is only about 30% of the normal surrounding tissue.

Green Intensity of Tumour-Normal Ratios as a Function of Photofrin Dose

- visible tumour
- carcinoma in situ
Even at 2 mg/kg Photofrin, more than half of the large tumour did not retain more Photofrin than the adjacent "normal" tissue. With and without Photofrin, the green autofluorescence is much lower in tumours compared to normal tissues. The mean green fluorescence of tumours is only about 30% of the normal surrounding tissue. These results suggest that the elevated red-green ratios in tumours compared to normal tissues were mainly due to a difference in the green autofluorescence. In the original study by Profio and co workers (Profio et al., 1984), the green autofluorescence was assumed to be similar for normal and tumour tissue. The results in this study show that this assumption was incorrect. A significant difference in autofluorescence intensity in the green region of the emission spectrum exists between normal and malignant bronchial tissue.

4.3 Fluorescence Spectroscopy

To confirm that differences in tissue autofluorescence exist between normal, pre-cancerous and cancerous tissues, fluorescence spectroscopy was performed in vivo. Potentially abnormal areas were first localized using ratio fluorometry. Following the spectroscopic measurements (discussed below), the areas were biopsied for pathologic diagnosis. The Helium-cadmium laser was studied as a more practical alternative to the Krypton-ion laser. In addition to being much cheaper, the 442 nm wavelength light does not induce red fluorescence in the fiberoptic bundles of the bronchoscope and hence a special illumination light guide is not necessary. For the 405 nm light, it was delivered to the tip of the fiberoptic
bronchoscope using a 600 μm-core-diameter quartz fibre with a diverging microlens (Laser Therapeutics, Buellton, CA).

The emitted fluorescence was collected by means of a flat-cut, polished 400 μm core-diameter quartz fibre 1 cm distal to the excitation light source. Care was taken to ensure the collecting fibre is in the centre of the illuminated area. Fluorescence measurements were made with the tip of the collecting fibre just in contact with the tissue surface.

The emitted autofluorescence was focussed onto the entrance slit of the spectrograph (Jarrell-Ash Monospec 27, with a 300 g/mm gratings, blazed at 500 nm, and a 0.29 nm/element resolution) with a f/1.5 collimating lens and an f/3.14 focussing lens to match the f/3.8 spectrograph. The spectrograph was fitted with a 2 mm-width slit. A low-fluorescence, long pass filter (cutoff wavelength at 470 nm, Oriel, Stratford, CT) was placed between the lenses to block off the scattered and reflected excitation light. The spectrograph was adjusted to cover the 450 nm to 750 nm range. The dispersed spectrum was recorded with an intensified 1024-element silicon linear diode array detector (EG & G PARC, Model 1455R 700HQ). The detector was interfaced to an Optical Multi-Channel Analyzer (OMA III, Model 1461/2 detector interface/controller with a 14-bit analog digital converter). The OMA controller was interfaced to a host computer (IBM-PC-80386) which allows data storage and spectral display/processing. A schematic of the spectroanalyzer is shown in figure 7.

Background subtraction was performed on each tissue spectrum during the acquisition mode before transmitting the data to the host computer to correct for the dark noise of the detector. Spectral calibration was performed by using the spectrum of a low-power, low-pressure mercury-argon
lamp (Oriel, Stratford, CT, Model 6035). Non-uniformity of the detector for the spectrum range of interest was checked and the detailed aspect regarding correction and calibration of the intensity are discussed in appendix 7.3. The detector response was found to be uniform within the spectral range of interest. The fluorescence intensities in all the spectra are in arbitrary units. Fluorescence spectra were always obtained by using the same gain of the diode array detector.

Figure 8 shows a typical autofluorescence spectra of a normal bronchus and carcinoma in situ lesion from the same patient using an excitation wavelength at 405 nm. The in vivo autofluorescence spectra induced by 442 nm light, representative for a normal bronchus, a severely dysplastic lesion, and a carcinoma in situ lesion from a patient are shown in figure 9. The shape of the emission spectrum induced by the 405 nm light was identical to that with the 442 nm light. Both emission spectra show an overall decrease in fluorescence intensity mainly in the green region of the visible spectrum (500 nm to 580 nm). For the normal bronchus, the spectra have a peak at about 520 nm, decreasing to a minimum at about 580 nm and rising to a smaller peak between 600 nm to 610 nm.

Statistical Analysis

A total of 284 spectra from histopathologically confirmed areas were converted into ASCII data to be used as a learning set for discriminant function analysis. Each fluorescence spectrum consisted of 1025 intensity values spanning the wavelength range of 450 nm to 750 nm (i.e the ASCII data consisted of 1025 pairs of data points, wavelength and intensity values).
Figure 7  Schematic diagram of the spectroanalyzer system used to collect \textit{in vivo} autofluorescence spectra.
Figure 8  In vivo autofluorescence spectra for normal bronchus and carcinoma in situ lesion excited by using a Krypton-ion laser at 405 nm (a.u. = arbitrary units). There is a significant decrease in the green autofluorescence for the carcinoma in situ lesion compared to normal. These differences are much smaller in the red region of the fluorescence spectra.

**Fluorescence Intensity as a Function of Wavelength**
*(Excitation at 405 nm)*
Figure 9  *In vivo* autofluorescence spectra for normal bronchus, severely dysplastic and carcinoma in situ lesions excited by using a Helium-cadmium laser at 442 nm (a.u. = arbitrary units). There is a significant decrease in the green autofluorescence for the carcinoma in situ lesion compared to normal.

Fluorescence Intensity as a Function of Wavelength
(Excited at 442 nm)
The first 100 and last 100 pairs of data were trimmed off since in these regions, there were no signal from these pixels. The data were classified according to the histopathology into seven categories as shown in Table I and organized into data sets, each consisting of only one spectrum class. The data were then normalized and re-sampled so that the output data set consisted of one intensity value per nm from 461 nm to 660 nm, resulting in 200 intensity values for each spectrum class.

These data were normalized in a variety of ways as follows: (i) by setting the average intensities between 530 nm to 570 nm (green region) to be the same for all spectra; (ii) by setting the average intensities between 600 nm to 660 nm (red region) to be the same for all spectra; and (iii) by setting the average intensities between 500 nm to 660 nm to be the same for all spectra and the procedure was set such that if the average intensity values at 470 nm was less than a set value, that particular case would be ignored in the calculations.

A commercially available program BMDP (Biomedical Data Processing, BMDP Statistical Software, Los Angeles, California) was used to generate and test discriminant functions from these learning sets. A stepwise analysis of the data was performed using the 7M discriminant analysis routine of this software package. This routine performs a stepwise discriminant analysis between two or more groups. The purpose of the stepwise discriminant procedure is to select those features that provide the best discrimination, while disregarding those that make little or no contribution to the classification process. Features are selected on the basis of their ability to separate the groups in feature space and are chosen in a stepwise fashion. Details of discriminant function analysis,
the selection procedures and the assumptions this analysis makes about the
distribution, size and forms of the data are described in the Appendix,
7.1.

Figure 10, and figure 11 are the graphs of the mean curves for each
class normalized in the red and green regions respectively. The normalized
(500 nm to 660 nm) average intensity as a function of wavelength for each
class is shown in figure 12. The dip or valley ~575 nm to ~585 nm for all
the curves can probably be attributed to hemoglobin absorption. The un-
normalized mean curves for each class were also calculated and this is
shown in figure 13. A non-parametric statistic, the Mann-Whitney rank sum
was first applied. The p-values for each class at 10 nm interval were
plotted in figures 14 and 15. This gives a measure of the similarity
between the intensity distribution of the normal and the specified class at
a particular wavelength. A p-value of less than 0.05 is considered to be
significant, and a value of less than 0.005 is considered to be very
significant. The p-values for the unnormalized data were also examined but
not plotted. Significant differences were observed between pathology
grades whether normalized or un-normalized data were used. These
differences are due to the average intensity differences between the
classes. To check the stability of the peaks and valleys of the curves
across classes, the spectra data were normalized at 600 nm - 660 nm,
normalized as described above (normalization procedure (iii)). All the
curves peak at the same locations as shown in figure 16.

A stepwise discriminant analysis was performed on these data sets,
each consisting of the features values calculated for all spectra. Table
II gives a description of the features (individual features will generally
be referred to by their abbreviated description, as listed in the table). The features used for the computation of the linear classification functions were chosen in a stepwise fashion. The software package allows the user to force the program to use all the features in the discriminant function, or let the program select the most discriminating features to be used in the discriminant function. The user can also select a subset of the features to be used to form the discriminant function. In this analysis, BMDP was programmed such that during forward stepping all (or most, some not included due to failure of tolerance test) the features were entered into the discriminant function (DF), while during backward stepping, only features that significantly contributed to the discriminant performance were allowed to remain in the discriminant function. The F statistic was used to determine if a feature contribute significantly to the discriminant function performance. The significance level was set to 0.5% (0.005). The program provided a jackknifed classification table (i.e., every case is classified by using a discriminant function generated from the remaining n-1 cases). This gave the classification accuracy of the DF generated when the data were subdivided into learning and test sets (Lachenbruch, 1975). The jackknife procedure is described in Appendix 7.1. The jackknife classification accuracy is a more correct representation of how the discriminant function would perform on other test sets (Lachenbruch, 1975). Empirical studies suggested that the error of the classification accuracy of the discriminant function on new data set should be within ± 20% of the jackknife classification error (Lachenbruch, 1975).

The goal of this analysis was to select the feature(s) that will provide the best discrimination for detection of pre-cancerous and
cancerous lesions. Table III summarized the results obtained following the various discriminant analyses. Included in this table is the jackknife classification result (described above) and a histogram classification result in which the cut-off for misclassifications (the number of false positives) is set to be approximately 5, and the corresponding number of false negatives and true negatives were then selected from the histogram. The actual feature and classification data used to generate and test the discriminant functions for this table were from a subset of the 284 spectra collected. The data were classified as normal or abnormal. Abnormal sets consisted of the different grades of dysplasia, carcinoma in situ and invasive tumour. Metaplasia and inflammation were not reported in this table. One requirement of the discriminant function analysis is that a large data set with approximately 10 to 20 data points (spectra) per feature be used in the analysis. If this criterion is not met then the discriminant function that was generated in the learning set will not give the same performance on other test sets of data. As shown in Table III, not more than four features combinations were used at any one time as input features to generate the discriminant function. There is no simple method to determine which combination of features are the most discriminating, other than testing all possible combinations. The data reported in Table III demonstrated that the optimal emission wavelengths to differentiate between normal, pre-cancerous and cancerous tissues are in the green region between 525 nm and 570 nm and in the red region between 600 nm to 660 nm. Increasing the band widths of both the red and green regions was shown to improve the discrimination.
Table I  Classification of a database of 284 spectra according to the histopathologic grades and organized into data sets each consisting of only one spectrum class.

<table>
<thead>
<tr>
<th>Class</th>
<th>Pathology</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>83</td>
</tr>
<tr>
<td>2</td>
<td>Metaplasia</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>Mild dysplasia</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Moderate dysplasia</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>Severe dysplasia</td>
<td>34</td>
</tr>
<tr>
<td>6</td>
<td>Carcinoma \textit{in situ}</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Tumour</td>
<td>80</td>
</tr>
</tbody>
</table>
Figure 10  The mean curves for each class (classified as in Table I) normalized in the red regions (600 nm - 660 nm). The average intensities between 600 nm to 660 nm are set to be the same for all spectra. (a.u. = arbitrary units).

Average Intensity as a Function of Wavelength
Normalized at 600 nm - 660 nm
Figure 11 The mean curves for each class (classified as in Table I) normalized in the green regions (530 nm - 570 nm). The average intensities between 530 nm to 570 nm are set to be the same for all spectra. (a.u. = arbitrary units).

Average Intensity as a Function of Wavelength
Normalized at 530 nm - 570 nm

- normal
- metaplasia
- mild
- moderate
- severe
- cis
- tumour
Figure 12  The average intensity as a function of wavelength for each class normalized at 500 nm to 660 nm (a.u. = arbitrary units).

Normalized Average Intensity as a Function of wavelength
(Normalized between 500 nm - 660 nm)
Figure 13 The unnormalized average curves for each class (a.u. = arbitrary units).

Average Intensity as a Function of Wavelength
Unnormalized

- normal
- metaplasia
- mild
- moderate
- severe
- cis
- tumour
Figure 14  The p-values for each class normalized in the red region (600 nm - 660 nm) at 10 nm interval. The vertical axis is in log scale.
Figure 15  The p-values for each class normalized in the green region (530 nm - 570 nm) at 10 nm interval. The vertical axis is in log scale.

P-Value Normalized at 530 nm - 570 nm
Figure 16  The average intensity for each curve normalized in the red region (600 nm - 660 nm). The average intensities between 600 nm to 660 nm are set to be the same for all spectra and if the average intensity values at 470 nm was less than a set value, that particular case would be ignored in the calculations. The moderate dysplasia category was ignored in these calculation. The peaks and valleys of the curves are stable across the classes. All curves peak at the same location.  (a.u. = arbitrary units).

Autofluorescence Spectra of In Vivo Bronchial Tissue
Normalized Over 600 nm - 660 nm

- normal
- metaplasia
- mild
- severe
- cis
- tumour
Table II  Description of the features used in the discriminant function analysis

<table>
<thead>
<tr>
<th>Features</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity at:</td>
</tr>
<tr>
<td>gi</td>
<td>500 nm - 520 nm</td>
</tr>
<tr>
<td>g2</td>
<td>500 nm - 550 nm</td>
</tr>
<tr>
<td>g3</td>
<td>500 nm - 570 nm</td>
</tr>
<tr>
<td>rl</td>
<td>630 nm - 660 nm</td>
</tr>
<tr>
<td>r2</td>
<td>610 nm - 660 nm</td>
</tr>
<tr>
<td>r3</td>
<td>600 nm - 660 nm</td>
</tr>
<tr>
<td>g1+</td>
<td>501 nm - 520 nm</td>
</tr>
<tr>
<td>g2+</td>
<td>521 nm - 550 nm</td>
</tr>
<tr>
<td>g3+</td>
<td>551 nm - 570 nm</td>
</tr>
<tr>
<td>yellow</td>
<td>571 nm - 600 nm</td>
</tr>
<tr>
<td>r1+</td>
<td>601 nm - 620 nm</td>
</tr>
<tr>
<td>r2+</td>
<td>621 nm - 640 nm</td>
</tr>
<tr>
<td>r3+</td>
<td>641 nm - 660 nm</td>
</tr>
<tr>
<td>Normalization</td>
<td>Normalization constant</td>
</tr>
<tr>
<td># log</td>
<td>log transformation of intensity values</td>
</tr>
<tr>
<td>Hue</td>
<td>hue transformation of intensity values</td>
</tr>
<tr>
<td>Modified Hue</td>
<td>Modified hue transformation of intensity values</td>
</tr>
</tbody>
</table>
Table III  Summary of the discriminant function analysis

<table>
<thead>
<tr>
<th>Data</th>
<th>Input features</th>
<th>Features used</th>
<th>Classification Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jackknife&lt;sup&gt;++&lt;/sup&gt;</td>
</tr>
<tr>
<td>unnormalized</td>
<td>g1-3, r1-3</td>
<td>g3</td>
<td>92.5% (12)</td>
</tr>
<tr>
<td>normalized (600-660nm)</td>
<td>g1-3, r1-3</td>
<td>g1-3, r1</td>
<td>77.5% (56)</td>
</tr>
<tr>
<td>normalized (600-660nm)</td>
<td>g1-3, r1-3</td>
<td>g1-3</td>
<td>94.8% (13)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>g1, r1</td>
<td>g1</td>
<td>92.8% (18)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>g2, r2</td>
<td>g2</td>
<td>95.2% (12)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>g3</td>
<td>r3</td>
<td>95.2% (12)</td>
</tr>
<tr>
<td>normalized (500-600nm)</td>
<td>every 5nm</td>
<td>feature*</td>
<td>73.5% (66)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>-20nm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>535, 560, 585, 605</td>
<td>74.3% (64)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>-20nm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>510, 560</td>
<td>95.6% (11)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>g1&lt;sup&gt;+&lt;/sup&gt;, r1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>g1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>92.8% (18)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>g2&lt;sup&gt;+&lt;/sup&gt;, r2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>g2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>95.6% (11)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>g3&lt;sup&gt;+&lt;/sup&gt;, r3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>g3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>95.2% (12)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>Hue 1-3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Hue 2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>70.7% (73)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>modified Hue&lt;sup&gt;+&lt;/sup&gt; 1-3</td>
<td>Hue 3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>71.5% (71)</td>
</tr>
</tbody>
</table>

* 515<sup>@</sup>  
525  
530  
575<sup>@</sup>  
590  
620  
635<sup>@</sup>  
645<sup>@</sup>  

<sup>@</sup> largest F to remove value  
<sup>+</sup> green 1 = 501-520 nm  
<sup>+</sup> green 2 = 521-550 nm  
<sup>+</sup> green 3 = 551-570 nm  
<sup>+</sup> yellow = 571-600 nm  
<sup>+</sup> red 1 = 601-620 nm  
<sup>+</sup> red 2 = 621-640 nm  
<sup>+</sup> red 3 = 641-660 nm
Table III  Summary of the discriminant function analysis (continued)

<table>
<thead>
<tr>
<th>Data</th>
<th>Input features</th>
<th>Features used</th>
<th>Classification Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jackknife ++</td>
</tr>
<tr>
<td>unnormalized</td>
<td>log (g1-3) log (r1-3)</td>
<td>log (g1-3) log r1</td>
<td>91.2% (22)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>log (g1) log (r1)</td>
<td>both</td>
<td>90.8% (23)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>log (g2) log (r2)</td>
<td>both</td>
<td>90.8% (23)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>log (g3) log (r3)</td>
<td>both</td>
<td>90.8% (23)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>log (g2) g3, log(r2) r3, ratio3</td>
<td>g3,log(g3)</td>
<td>94.8% (13)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>log(g2),g3 ratio 3 Hue (1-3)</td>
<td>g3 ratio3 Hue</td>
<td>95.2% (12)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>Hue (1-3) Hue 1</td>
<td></td>
<td>69.9% (75)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>modified Hue Hue 1</td>
<td></td>
<td>69.9% (75)</td>
</tr>
<tr>
<td>unnormalized</td>
<td>#modified Hue Hue 1</td>
<td></td>
<td>69.5% (76)</td>
</tr>
<tr>
<td>normalized#</td>
<td>log (g1-3) log (g1)</td>
<td></td>
<td>69.5% (76)</td>
</tr>
</tbody>
</table>

++ ( ) denotes number of missclassified (false positive).
also see explanation in text.
4.4 Detection of Dysplasia and in situ Carcinoma by Ratio Fluorometry Using Tissue Autofluorescence Alone

4.4.1 Non-Imaging Methods

To test the hypothesis that early lung cancer can be detected based on differences in tissue autofluorescence alone without Photofrin, eighty-two volunteers who had previous exposure to asbestos, diesel fumes and/or tobacco smoke in their work-place were studied using ratio fluorometry. Of the 82 individuals, 25 were non-smokers, 40 were ex-smokers, and 17 were current smokers with mean age values of 52, 55, and 49 years, respectively.

Fluorescence bronchoscopy was carried out using a ratio fluorometer as described in the previous section. In this study, 442 nm wavelength from a Helium-cadmium laser was used as the excitation light. All abnormal areas were biopsied to confirm the presence or absence of dysplasia or cancer. In addition, one or more random biopsies were taken from apparently normal areas for pathological examination. Before and immediately following bronchoscopy, a sputum sample was taken for cytological examination.

From the 82 subjects a total of 237 biopsies were taken and analyzed. The red-green ratios from pathologically normal areas, pre-cancerous, and carcinoma in situ are shown in figure 17. The progressive increase in the ratios correlates with the pathologic grades. The red-green ratios from areas with moderate dysplasia, severe (marked) dysplasia and the carcinoma in situ lesions were significantly higher than the normal areas (p < 0.05). The elevation in the red-green ratio is attributed to the lower green
autofluorescence in the pre-cancerous and cancerous lesions. The proportion of subjects classified by smoking status and bronchoscopy outcome is shown in figure 18. There was a progressive increase in the proportion of subjects with metaplasia or mild dysplasia among current and ex-smokers. One or more sites of moderate or severe (marked) dysplasia were found in 12.5% of the ex-smokers and current smokers but none in the non-smokers. Carcinoma in situ was found in two of the ex-smokers. Pre- and post-bronchoscopy sputum cytology failed to detect these small pre-cancerous or carcinoma in situ lesions.

4.4.2 Fluorescence Imaging

Although ratio fluorometry is an important concept, it has many limitations. One of its major drawbacks is that a "reference" site (presumably normal by visual examination) is needed in the same subject to calibrate the ratio fluorometer probe in vivo. If the control sites happen to be abnormal, the ability to detect carcinoma in situ or severe dysplasia may be diminished. As a non-imaging device, it does not provide information regarding the extent and size of the lesions. In addition, being a single pixel probe, very small early lesions can be missed due to a field averaging effect.

The idea of using the spectroscopy data in section 4.3 to develop an imaging device was therefore tested. The main principle of such an imaging system is to induce tissue autofluorescence at the blue region of the spectrum (for example at 442 nm) and to collect the weak fluorescence by an image intensified charge couple device (ICCD).
Figure 17  Red-green ratios from pathological confirmed normal areas, dysplasia and carcinoma in situ lesions of 82 volunteers. (cis = carcinoma in situ, * indicates that the red-green ratios from areas with moderate dysplasia, severe dysplasia and the carcinoma in situ lesions were significantly higher than the normal areas (p < 0.05). Red fluorescence is measured at 690 nm ± 10 nm and green fluorescence at 520 nm ± nm).

Red-Green Ratios from Pathological Confirmed Areas Using Fluorescence Bronchoscopy (Ratio Fluorometer)
Figure 18 The proportion of subjects classified by smoking status and fluorescence bronchoscopy (ratio fluorometry) outcome. One or more sites of moderate or severe dysplasia were found in 12.5% of the ex-smoker and current smoker but none in non-smoker volunteers. Carcinoma in situ lesions was found in 2 of the ex-smokers.

Smoking Status and Prevalence of "Lesions" Using Fluorescence Bronchoscopy (Ratio Fluorometer)
The spectral information can then be utilized by placing appropriate band-pass filters in front of the ICCD camera. A clinical pre-prototype lung imaging fluorescence endoscope (LIFE) based on these principles of tissue autofluorescence is shown in figure 19 (Palcic et al., 1991, Palcic and Lam, 1991, Hung et al., 1990, Lam et al., 1990b).

Figures 20a and 21a are examples of a normal bronchus and carcinoma in situ lesions, respectively, detected using the LIFE device. The abnormality could not have been detected using conventional bronchoscopy as shown by the white-light bronchoscopy images (figures 20b and 21b).

The performance of the fluorescence imaging system was compared with white light bronchoscopy to determine if the sensitivity of detecting dysplasia or carcinoma in situ can be improved using fluorescence imaging in conjunction with conventional white-light bronchoscopy. The sensitivity and specificity of the white-light bronchoscopy versus fluorescence imaging were compared using the bronchial biopsy pathology as the "gold standard".

A total of 94 subjects were examined. There were 53 patients with known or suspected lung cancer and 41 volunteers not known to have lung cancer prior to entrance into the study. Details of their age and smoking history are shown in Table IV.

Conventional fiberoptic bronchoscopy under white-light (xenon) lamp illumination was first carried out as described in the previous section. Fluorescence bronchoscopy using the LIFE device was then carried out. Briefly, laser light at 442 nm (Helium-cadmium laser) was delivered to the bronchial surface via the illumination bundle of an Olympus BF20D fiberoptic bronchoscope. The emitted fluorescence collected by the imaging bundle of the bronchoscope was spectrally divided by two filters of
different wavelengths, one at 480 nm to 520 nm (green region) and the other in the red region ($\geq$ 630 nm). The respective images were acquired sequentially by the ICCD and digitized by an imaging board residing in a computer. Using a non-linear discriminant function combination of the red and green intensity values (gray levels), a pseudo-image was formed which would allow the delineation of abnormal areas when displayed on a high resolution RGB analog monitor.

All abnormal areas found using white-light and/or fluorescence bronchoscopy were biopsied for pathologic confirmation. In addition, one or more random biopsies were taken from the visually apparent (white light and fluorescence) normal areas for pathological examination. In the fluorescence images, the exact area of each biopsy was manually delineated on the RGB monitor and the average red and green intensities (gray levels) over the area were calculated. These average values were used in a non-linear discriminant function analysis to create a value which can be used to separate the normal from abnormal tissues sites.

A total of 328 biopsies were collected from the 94 subjects. 173 of these biopsies were found to be normal, 14 were found to show mild dysplasia, 33 were found to show moderate dysplasia, 15 were found to show severe dysplasia, 29 were carcinoma in situ and 64 were found to show invasive tumour. The prevalence of dysplasia and carcinoma in situ using fluorescence imaging is shown in figure 22. In 15% of the lung cancer patients, synchronous carcinoma in situ was found in addition to the large invasive cancer; 8% of these patients had moderate dysplasia and 6% had severe dysplasia. For the current smokers in this study, 40% had moderate dysplasia and 12% had severe dysplasia. Of the ex-smokers in this study,
25% had moderate dysplasia, 6% had severe dysplasia and 13% had carcinoma in situ.

The non-linear Discriminant Function values of the red and green fluorescence intensities in normal tissues, mild, moderate, or severe dysplasia lesions, carcinoma in situ or invasive cancer is shown in figure 23. The difference in the tissue autofluorescence discriminant function values were found to be statistically significant between normal tissues and both dysplasia or cancerous lesion (p < 0.005). However, dysplasia lesions cannot be differentiated from carcinoma in situ because of the significant overlap. But since moderate and severe dysplasia are pre-cancerous lesions, clinically it would be important to be able to localize these lesions as well.

The sensitivity of the fluorescence imaging in detecting moderate or severe dysplasia and carcinoma in situ was found to be at least 50% better than conventional white light bronchoscopy (sensitivity of 71.5% for fluorescence imaging compared to 48.4% for white-light bronchoscopy). The specificity of 94% was the same for both methods.
Figure 19 Schematic diagram of the clinical prototype lung imaging fluorescence endoscope (LIFE).
Figure 20  (a) Example of a fluorescence image of a normal bronchus, where the green colour has been arbitrarily used to depict areas of normal tissue.  (b) White-light image of the same bronchus.
Figure 21  (a) Fluorescence image of a bronchus showing an area of abnormal fluorescence arbitrarily depicted as the reddish area for which the corresponding biopsy showed the presence of carcinoma in situ.  (b) White-light image of the same bronchus.
Table IV  Characteristics of the subjects

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung Cancer patients</td>
<td>53</td>
<td>63 ± 10 years</td>
</tr>
<tr>
<td>*Current smoker</td>
<td>17</td>
<td>52 ± 10 years</td>
</tr>
<tr>
<td>*Ex-smoker</td>
<td>16</td>
<td>57 ± 10 years</td>
</tr>
<tr>
<td>*Non-smoker</td>
<td>8</td>
<td>50 ± 10 years</td>
</tr>
</tbody>
</table>

* ex-smoker and current smoker have smoked more than a pack of cigarettes per day for > 20 years. Ex-smoker gave up smoking for more than 5 years.
Figure 22 The prevalence of dysplasia and carcinoma in situ detected using fluorescence bronchoscopy (imaging) as verified by biopsy pathology.

Prevalence of Lesions Using Fluorescence Bronchoscopy (Imaging)

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Percent</th>
<th>Non-Smoker</th>
<th>Ex-Smoker</th>
<th>C-Smoker</th>
<th>Lung Cancer Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild Dysplasia</td>
<td>12%</td>
<td>10%</td>
<td>15%</td>
<td>10%</td>
<td>12%</td>
</tr>
<tr>
<td>Moderate Dysplasia</td>
<td>45%</td>
<td>35%</td>
<td>40%</td>
<td>35%</td>
<td>45%</td>
</tr>
<tr>
<td>Severe Dysplasia</td>
<td>20%</td>
<td>15%</td>
<td>20%</td>
<td>15%</td>
<td>20%</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>10%</td>
<td>5%</td>
<td>10%</td>
<td>5%</td>
<td>10%</td>
</tr>
</tbody>
</table>
Figure 23 A group box of the non-linear Discriminant Function (DF) values calculated from the red and green intensity values (gray scale) for the pathology grades. The centre line of the boxes represents the median DF of the biopsy grade. The size of the box indicates the fraction of DF values above and below the interquartile values. The bars indicate the range of the measured values for the biopsy site. The small circles indicate the location of the outliers.

BIOPSY GRADE VS DF VALUE
Chapter 5: DISCUSSION AND CONCLUSION

The important findings in this thesis work are as follows:

i) Dysplasia and carcinoma in situ can be detected using a low non-skin photosensitizing dose of Photofrin.

ii) The major mechanism of tumour detection using ratio fluorometry and Photofrin was due to a significant reduction in tissue autofluorescence. To a much lesser extent preferential retention of Photofrin in tumours also play a role.

iii) In vivo fluorescence spectroscopy showed significant differences in the emission spectra between normal, pre-cancerous and cancerous tissues when the bronchial surface was illuminated by a violet (405 nm) or a blue (442 nm) laser light.

iv) Spectral differences between normal, pre-cancerous and cancerous tissues were characterized by an overall decrease in autofluorescence intensity between 500 nm to 580 nm.

v) Dysplasia and carcinoma in situ can be detected using laser-induced autofluorescence alone without the use of exogenous fluorescent drugs such as Photofrin.

vi) Detailed discriminant function analysis of fluorescence spectra "points to" the best methods to detect dysplasia and carcinoma in situ using autofluorescence. This was confirmed by two separate methods - ratio fluorometry and fluorescence imaging.
5.1 **Tumour Detection Using Non-Skin Photosensitizing Dose of Photofrin**

Over the last several decades a great deal of effort has been spent on developing fluorescent tumour localizing drugs to facilitate the detection and localization of early lung cancers. A number of fluorescent markers have been shown to accumulate preferentially in malignant tissues (Dougherty, 1984, Rall, 1957, Moore, 1947). To date, most of the clinical and laboratory studies have been concentrated on the use of porphyrins, in particular Hematoporphyrin derivative (Hpd) and its partially purified preparation, Photofrin (Profimer sodium) (Dougherty, 1989, Dougherty *et al.*, 1984, Lipson *et al.*, 1961).

Several non-imaging and imaging bronchoscopic devices have been developed to detect early lung cancer using Hpd or Photofrin. Although clinical experiences with these devices suggested that small radiologically occult cancers can be detected (Balchum *et al.*, 1990, Kato *et al.*, 1990, Kato and Cortese, 1985, Cortese *et al.*, 1979), wider clinical applications of Photofrin as a fluorescent tumour marker have been impeded by the associated skin photosensitivity. At the standard dose of 3 mg/kg Hpd or 2 mg/kg of Photofrin, the same drug dose used in photodynamic therapy skin photosensitivity can last for four weeks or more (Wooten *et al.*, 1988, Razum *et al.*, 1987). This is a major drawback to use Hpd or Photofrin fluorescence as a diagnostic tool for the detection of early lung cancers.

Theoretical calculations suggested that non-therapeutic low doses of photofrin (< 0.34 mg/kg) may be free of skin photosensitivity side effects (Potter, 1990). Preliminary studies in animals showed that 0.5 mg/kg of Photofrin (1/4 of the therapeutic dose) may be effective in the detection of occult, non-palpable micrometastases in the regional lymph nodes of rats.
(McGinnis et al., 1990) and in chemically induced urinary carcinoma of dogs (Baumgartner et al., 1987). Using the ratio fluorometer endoscopic detection system, this present study showed, for the first time that carcinoma in situ and dysplasia can be detected with very low doses of Photofrin (0.25 mg/kg) with no apparent skin photosensitivity both on skin testing with an artificial light source and subsequent exposure to outside light (Lam et al., 1990a).

While skin photosensitivity may be avoided by the use of very low dose Photofrin, the contribution of low-dose Photofrin in cancer detection was small. The major mechanism of cancer detection by ratio fluorometry was mainly due to the differences in the green tissue autofluorescence between normal and malignant tissues. The increase in red fluorescence due to Photofrin was relatively small. This was confirmed by in vivo spectroscopy as shown in figure 24. The slight increase in red fluorescence in tumour tissue following administration of photofrin is not very large compared to the huge differences in green autofluorescence.

5.2 Tissue Autofluorescence in Diagnosis

The idea that tissues autofluoresce is not new. In 1908, Wood noted visible fluorescence in the skin when excited with a ultra-violet light (reference in Anderson and Parrish, 1982). Since then, "Wood's lamp" have been used to diagnose erythrasma, tinea capitis, and visualizing subtle changes in epidermal melanin pigmentation (Caplan, 1967). The fluorescence of the skin observed under "Wood's lamp" was reported to be largely due to the dermis autofluorescence (reference in Anderson and Parrish, 1982). The
fluorophore(s) responsible for this autofluorescence are unknown. Human skin fluorescence induced by a 325 nm laser light was reported to be due to elastin and collagen which are major components of human dermis that are altered by age and photo-exposure (Leffell et al., 1988). Green autofluorescence has also been observed in human epidermal cells (Fellner, 1976). In 1933, Sutro and Burman reported that cellular tumours which extended into the muscular layer of the stomach or rectum fluoresce from a purple to a deep brown and that a medullary carcinoma of the thyroid appeared purple against white in a zone of fibrous tissue (Sutro and Burman, 1933). This "colour" difference seen by filtered ultra-violet irradiation had been used as an aid to surgeons and pathologists in detecting small areas of disease (Sutro and Burman, 1933). An attempt was made in 1944 to differentiate between benign and malignant lesions of breast tissue by application of these differences in colour phenomena (Herley, 1944). When there is epithelial degeneration, a decrease in blue colour was observed and in carcinoma, where the cancer cells are tightly packed, the tissue appears purple with varying intensity, passing into tan, orange, and brown (Herley, 1944). Strong red fluorescence was observed in the cancerous tissues of the genitalia of women (Figge et al., 1944). Policard in 1929, had noted a brilliant red fluorescence in the centre of a rat sacroma (Policard, 1929). This red fluorescence is characteristic of hematoporphyrin. The endogenous porphyrin in tumours have been attributed to the presence of bacteria in the ulcerated and necrotic tissue (Harris and Werkhaven, 1987). In recent years, Alfano and co-workers had reported subtle spectroscopic difference between normal and cancerous lung and breast tissues in excised animal and human specimens (Tang et al., 1989a,
Tang et al., 1989b, Alfano et al., 1988, Alfano et al., 1987). Using three different excitation sources, 457.9 nm, 488.0 nm, and 514.5 nm, they reported that normal lung and tumour tissues exhibit different autofluorescence spectra. Maximal peaks were observed at 496 nm, 509 nm, and 531 nm for normal tissues, excited by 457 nm, 488.0 nm, and 514.5 nm laser light respectively; a subsidiary maximum was observed at 606 nm. For the tumour tissues, maxima were observed at 503 nm, 515 nm, and 537 nm, respectively (Alfano et al., 1988). However, in another report, using 457.9 nm excitation source, the normal lung tissue was observed to exhibit a maximum at 511 nm with a subsidiary maxima at 555 nm and 600 nm; whereas the tumour tissue had a maximum at 514 nm (Tang et al., 1989a). In the same report, using 514.5 nm, the spectrum profile of the normal lung tissue was reported to have a maximal peak at 563 nm and 603 nm whereas the cancerous tissue was reported to have a maximal at 560 nm (Tang et al., 1989a). In an earlier report, the spectrum profiles of a normal human lung and tumour tissue excited at 488.0 nm were reported to have maximal peaks at 512 nm and 520 nm, respectively, with the normal lung tissue exhibiting two subsidiary maxima located at 554 nm and 600 nm (Alfano et al., 1987). Thus, changes in the spectral shape appear to be strongly dependent on experimental conditions. Significant differences in autofluorescence intensity between normal and malignant tissues were not observed in these studies.

The difference in spectra shape and intensity between Alfano's studies and this study may be due to the tumour size and possible difference between lung parenchymal tissue and bronchial tissue. In addition, Alfano and co-workers examined lung tissues in vitro several
hours after excision (Tang et al., 1989a, Tang et al., 1989b, Alfano et al., 1988, Alfano et al., 1987). Freezing and thawing of bronchial tissues at variable time after excision reduced the differences in fluorescence intensity between malignant and normal bronchial tissues as well as altering the spectral shape (Figures 25 and 26, Appendix 7.2). With freezing, there is a slight accentuation of the emission peak at about 600 nm, and with thawing there is a decrease in intensity at 1 and 3 hour. In contrast, the fluorescence intensity of tumour tissue from the same patient increased with thawing. An alteration in the spectral shape of deep-frozen tumours was observed by other investigators in the characterization of brain tissues. They suggested that disruption of cell membranes at thawing may induce changes in the membrane-bound and cytoplasmic components which may then be reflected in the change in spectrum shape (Montán and Strömblad, 1987). It may be reasonable to conclude that the large differences in tissue autofluorescence between normal and malignant lesions are unique for the in vivo situation.

No increase in the red fluorescence in the bronchial tumour tissue (in vivo or in excised human tissue) was observed to suggest the presence of endogenous porphyrin. This is in keeping with a recent report by Harris and Werkhaven (Harris and Werhaven, 1987). However, in animal studies using the hamster cheek pouch model (Lam et al., 1992a, Kluftinger et al., 1992), strong red fluorescence at around 640 nm was observed, in dysplastic lesions, carcinoma in situ and small non-ulcerated invasive tumours. Again, a significant decrease in green autofluorescence was observed in the pre-cancerous and cancerous tissues compared to normal (figure 27). The increase in the red fluorescence for the carcinoma in situ lesion seen in
figure 27 is attributed to the presence of endogeneous porphyrin. Details of the hamster cheek pouch model are described in appendix 7.2.

Differences in tissue autofluorescence is generally considered too weak to be quantified or are insensitive to current detection devices especially for small lesions. This belief had led many investigators to assume that an exogenous fluorescent tumour localizing drug is necessary for the detection of cancers. As discussed earlier, Hpd or Photofrin had been most extensively used in the detection of early lung cancer. Because the fluorescence yield of Photofrin or Hpd is often small for thin early lung cancer (less than 2%), background noise can adversely affect the detectability of such low fluorescent light. In order to enhance the contrast, one approach is to use the background fluorescence (autofluorescence) as the denominator for ratioing or subtraction when using Hpd or Photofrin. The idea had been so deep-rooted that fluorescence detection has been generally equated with the use of fluorescent drugs. Thus almost all the devices that had been developed had concentrated on enhancing the fluorescence detection in the 600 nm to 690 nm spectral region (Photofrin or Hpd maximum fluorescence peaks). The autofluorescence in the green region of the spectrum had been assumed to be similar for normal and malignant tissues. The red Hpd or Photofrin fluorescence intensity is then ratioed against the green autofluorescence normalized around 560 nm (Wagnière et al., 1990, Profio et al., 1984). Alternatively, the autofluorescence intensity was subtracted with a net result in the red fluorescence of Photofrin (Andersson-Engels et al., 1991, Mang et al., 1991, Hirano et al., 1989, Baumgartner et al., 1987, Montán et al., 1985, Profio et al., 1985).
Figure 24  *In vivo* fluorescence spectra from a normal area and an area with carcinoma *in situ* lesion without and 24 hour after intravenous injection of 1 mg/kg Photofrin. The slight increase in the red fluorescence is very small compared to the significant difference in the green autofluorescence between the normal and tumour area. (a.u. = arbitrary units)
Figure 25  Effects of freezing and thawing on the autofluorescence of a normal bronchus fragment. With time, the autofluorescences decreases. (a.u. = arbitrary units).

Fluorescence Intensity as a Function of Wavelength
(Excitation: 442 nm)
Figure 26 Effect of thawing and freezing on the autofluorescence of an excised tumour from the same patient as shown in figure 25. With time the autofluorescence intensity of increases. There is a significant decrease in autofluorescence in the green region compared to the normal fragment (figure 25). With time, this difference is less pronounced. (a.u. = arbitrary units).

Fluorescence Intensity as a Function of Wavelength
(Excitation: 442 nm)
Figure 27  Autofluorescence spectra from a normal site and an area with carcinoma in situ of a Syrian hamster cheek pouch. A Helium-cadmium laser at 442 nm was used for excitation. (a.u. = arbitrary units). A significant decrease in the green autofluorescence was observed in the cancerous tissues compared to normal. The increase in red fluorescence intensity (peak at around 640 nm) for cancerous tissues is due to the presence of endogenous porphyrin.

Fluorescence Intensity as a Function of Wavelength  
(Excited at 442 nm)
A study of 17 patients with bronchial tumours investigated by the group in Lund, Sweden indicated that the use of autofluorescence alone to delineate tumour regions of the bronchus is probably not possible (Andersson-Engels, 1989). Although a difference between the autofluorescence emission of a normal and tumour sites were observed by Anthony and co-worker, the conclusion from this study was that Photofrin will likely be needed to enhance the detection (Anthony et al., 1989).

In contrast to these studies, we have shown here that the differences in autofluorescence between normal and abnormal tissue is very significant and that these differences are sufficient to develop a fluorescence imaging apparatus contrary to previous belief.

5.3 Mechanism for Difference in Tissue Autofluorescence

The basis for the difference in tissue autofluorescence between normal, pre-cancerous and cancerous tissues is not known. An understanding of the underlying principles or cause of the difference in the observed fluorescence will likely enhance its application for detection of early lung cancers.

The fluorescence emission spectra of many fluorophors can be influenced by a variety of chemical and physical processes such as solvent effects, solvent relaxations, and quenching (Lakowicz, 1983). Fluorescence quenching refers to any process which decreases the fluorescence intensity of a particular fluorophore. Complex formation (static quenching), energy transfer and collision between fluorophore and quencher (dynamic quenching) can result in quenching. For dynamic quenching, i.e collisional quenching,
the quencher is diffused and in contact with the fluorophores while it is in its excited state, which upon contact subsequently returns to the ground state without emissions of a photon. In static quenching, the fluorophore and quencher form a complex which is non-fluorescent. Examples of quenchers include molecular oxygen, purines and pyrimidines, hydrogen peroxide, and methionine (references in Lakowicz, 1983, Pesce et al., 1971, Udenfriend, 1962). In addition, optical properties of the sample (tissue) such as turbidity and inner filter effect may result in the quenching of the fluorescence signals (Lakowicz, 1983, Udenfriend, 1962). The distribution of the excitation light in the tissue, the fluorescence quantum yield, the path length of the fluorescence in tissue (the path length is determined by the excitation/collection geometry, the scattering properties of the tissue), and the tissue absorption coefficient for the fluorescence have also been suggested to influence the fluorescence intensity detected at a tissue surface (Jacques, 1990, Keijzer et al., 1989b, Richards-Kortum et al., 1989a, Richards-Kortum et al., 1989b). The overall intensity and spectral distribution of the measured fluorescence depends on the excitation/collection geometries of the detector system. In general, fluorescent light will undergo both scattering and reabsorption when returning to the surface (Jacques, 1990, Keijzer et al., 1989b, Richards-Kortum et al., 1989a).

Several hypotheses have been proposed to explain the observed differences in autofluorescence:

1) Proliferations and transformations of cells which result in an increase in cell layers;

2) Different scattering properties cause by cytological changes;
iii) Changes in the macromolecular composition of the extracellular matrix (such as a change in the ion concentration, e.g. calcium ions which act as a quencher (reference in Lakowicz, 1983), basement membrane and connective tissues (such as collagen and elastin);

iv) Differences in the redox potential which involve co-enzymes and co-factors. Different levels of these co-enzymes and co-factors such as NADH (reduced form nicotinamide adenine dinucleotide), NAD⁺ (oxidized form of nicotinamide adenine dinucleotide), riboflavin, FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) will result in changes in the fluorescent intensity;

v) Pathological tissues may contain specific chemical compounds (quenchers) that may alter or mask the normal autofluorescence;

vi) Decreased amounts of fluorophores in the tumour cells compared to normal epithelial cells;

vii) High concentration of absorbing species (not resulting in fluorescence) in tumour cells, which may attenuate the excitation light reaching the fluorophores;

The decrease in fluorescence intensity in cancerous tissues is probably due to a combination of factors. In the literature reports can be found suggesting that there are differences in the level of riboflavin and nicotinamide dinucleotide between normal and tumour tissues (Rivlin, 1973, Pollack et al., 1942). Decreased levels of riboflavin had been found in cancer patients (Rivlin, 1973). The changes of the oxidation-reduction equilibrium for NADH-NAD⁺ is known to be associated with pre-cancerous and cancerous state. Following murine sarcoma virus transformation in rat
kidney fibroblast, the absolute concentration of NAD$^+$ and NADH was found to be decreased 2 to 3 times (reference in Richard-Kortum et al., 1991). NAD$^+$ is non-fluorescent while NADH is highly fluorescent (Lakowickz, 1983, Udenfriend, 1962). The fluorescent group for NADH is the reduced nicotinamide ring and its fluorescence is partially quenched by collisions with the adenine moiety (Lakowickz, 1983, Udenfriend, 1962). When excited at 337 nm, the emission spectrum of NADH shows a maximum at around 470 nm (Andersson-Engels, 1991). Based on this observation it had been suggested by other investigators that the decreased autofluorescence in malignant tissue may be due to a lesser content of NADH (Schomacker et al., 1992, Rava et al., 1991, Andersson-Engels et al., 1991, Cothren et al., 1990). In addition, hypoxic areas in tumours have been demonstrated to correlate with decreased tissue autofluorescence signal at 450 nm following near ultra-violet excitation (Welsh et al., 1977, Gosalvez et al., 1972a, Gosalvez et al., 1972b).

Riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) absorb light in the visible range at about 450 nm and emit fluorescence at around 515 nm (Ghisla et al., 1975). The oxidized form of flavin has an absorption band centered at 450 nm and a strong fluorescence emission maximum at around 520 nm. In contrast, free, reduced flavins are non-fluorescent. Flavin fluorescence is quenched both by static and dynamic processes. Like NADH, flavin can be dynamically quenched by adenine. FAD also forms stacked complexes in which the flavin fluorescence is quenched by adenine (static quenching) (Lakowicz, 1983, Udenfriend, 1962). The concentration of riboflavin in tumour tissue is low in comparison to that of normal tissues (Pollack et al., 1942), which is
consistent with the view that cancer tissues have a deficient aerobic oxidation system (Rivlin, 1977). The equilibrium of the redox state may indirectly account for the differences in the autofluorescence in the green region of the spectrum. Pyridoxal 5'-phosphate, structural proteins collagen and elastin are also known to fluoresce with maxima at around 520 nm when excited by visible light in the range of 410 nm to 450 nm. There have been reports of decreased levels of serum pyridoxal 5'-phosphate in cancer patients (reference in Richard-Kortum et al., 1991). The level of these compounds has been implicated for the basis of the observed differences in autofluorescence in bladder, colon, and gastrointestinal tissues (Rava et al., 1991, Richard-Kortum et al., 1991, Cothren et al., 1990). The composition of the in vivo interstitial environment of tumour tissue is significantly different from those of most normal tissues. The tumour interstitial compartment is characterized by a larger interstitial space, high collagen concentration, low proteoglycan and hyaluronate concentrations compared to normal tissues (Jain, 1987). These factors may also influence the autofluorescence properties.

Several investigators have suggested that the fluorescence signals detected at the tissue surface are complicated by the interplay of scattering with fluorescence and absorption, in particular, reabsorption by oxyhemoglobin (Jacques, 1990, Keijzer et al., 1989b, Richards-Kortum et al., 1989a). The excitation and collection geometries of the detector system had also been implicated to influence the spectra shape and overall intensity of the fluorescence spectra. However, when the specific geometries and parameters relevant to fluorescence bronchoscopy (as described in this work) are taken into account, these effects are expected
to be of minimal significance. All the emitted fluorescence was collected within the illuminated area. In terms of blood reabsorption, carcinoma in situ and pre-cancerous lesions are not known to have increased blood vessels. Furthermore, an increase in blood (hemoglobin) reabsorption would have resulted in changes in spectral peaks or valleys in the autofluorescence spectra. However, this was not observed in both the spectra from freshly excised human tissues and in vivo. A huge difference was observed in the autofluorescence intensity between normal and abnormal tissues.

5.3.1 Preliminary Data

It is outside of the scope of the thesis to elucidate the exact mechanism(s) of reduced tissue autofluorescence in malignant tissues. Nevertheless, since this phenomenon is so intriguing, attempts were made to test some of the above hypotheses. For example, the fluorescence intensity spectra for FAD, FMN, riboflavin were measured and are shown in figure 28. Collagen IV and basement membrane were also measured and their spectra are shown in figures 29 and 30 respectively. Essentially most, if not all, of these spectra show similarities to the in vivo autofluorescence spectra. A fluorescence image of basement membrane material also shows the characteristics strong green autofluorescence using the LIFE device (figure 30). It would be simple to suggest that observed differences in autofluorescence are due to these fluorophores. However, so many of these observations are indirect and the relative amounts of these fluorophores are yet to be established. Extraction procedures to determine the relative amounts of these fluorophores in normal and tumour tissue is very difficult
and requires different skills than that of a biophysicist. In addition, much of the spectroscopic properties of these fluorophores are affected by the local conditions, for example, concentration, pH, and temperature. This, too, must be carefully worked out before arriving to meaningful conclusions.

The results in figure 31 suggest that the major source of the autofluorescence comes from the sub-epithelial layer. Details of these experimental procedures are described in Appendix 7.2. An intriguing phenomenon was observed following removal of the epithelium (only one cell layer), which caused a 40% increase in fluorescence intensity. After removing the submucosa there was a further increase in fluorescence intensity by another 50% (Hung et al., 1991). Based on these results the decrease in green autofluorescence in the pre-cancerous and cancerous tissues may be interpreted as potentially due to i) a decrease in fluorophores in the sub-epithelial layers of the tumour tissue, or ii) increase in non fluorescent absorbing species in the mucoscal layers of the abnormal tissues, iii) the excitation light is not reaching the sub-epithelial layer due to a different in optical properties of the pre-cancerous and cancerous tissues.

Preliminary data, using a fluorescent automated cell sorter (FACS) under excitation wavelength at 457.9 nm on the following cell cultures were studied: A549 human lung adenocarcinoma cells, CCL-210 human normal lung diploid cells, HTB 5B human lung carcinoma cells and normal skin epithelial cells from a normal volunteer. The results suggested a decrease in autofluorescence in the tumour cells relative to the normal cells. Normal cells have about 30% more green fluorescence than the tumour cell lines.
while the red autofluorescence for tumour cells was significantly higher than the normal cells. However the slight difference in intensity observed here still could not account for the huge differences in autofluorescence intensity seen in vivo.

Optical properties measurements using an integrating sphere were also made on human bronchial fragments and on the hamster cheek pouch. Details of this experiment are described in appendix 7.2. The data are presented in Table V. Tumour tissues were found to be less scattering but more absorbing than the normal tissues.

Further investigations to confirm these observations are merited. It is clear that to elucidate the mechanisms for the decrease in the green autofluorescence will be a major undertaking involving other disciplines such as biochemistry, molecular biology in addition to physics.

5.3.2 Differences in Autofluorescence in Other Tissue

The decrease in fluorescence intensity especially in the green region of the spectrum is not unique for dysplasia and cancerous lesions of the bronchial tissues; it has also been observed in other epithelial tumours and dysplasia when spectroscopic measurements were made in vivo. Gastrointestinal, bladder and colonic tissues have been shown to exhibit a lower autofluorescence in tumour (Schomacker et al., 1992, Richard-Kortum et al., 1991, Andersson-Engels et al., 1991, Rava et al., 1991). The hamster cheek pouch model of oral squamous cell carcinoma also exhibited characteristic decreased green autofluorescence as well as endogenous porphyrin (Lam et al., 1992a, Kluftinger et al., 1992).
Fluorescence spectra of FAD, FMN and riboflavin excited using a 442 nm Helium-cadmium laser. The maximum peak for FAD and FMN is at 542 nm, while for riboflavin it is red shifted to 556 nm. (a.u. = arbitrary units).

Fluorescence Intensity as a Function of Wavelength
(Excitation: 442 nm)
Figure 29  Fluorescence spectrum of collagen IV excited at 442 nm using a Helium-cadmium laser. (a.u. = arbitrary units).

Fluorescence Intensity as a Function of Wavelength
(Excitation: 442 nm)

Collagen IV
Figure 30  Fluorescence spectrum (a.u. = arbitrary units) and fluorescence image of basement membrane. A Helium-cadmium laser at 442 nm was used for the excitation.

Fluorescence Intensity as a Function of Wavelength
(Excitation: 442 nm)
Figure 31  Autofluorescence spectra of a freshly excised human bronchus. With the epithelium removed (one cell layer) there is an increase of fluorescence intensity and with removal of the sub-epithelial layer, the cartilage shows a further increase in intensity compared to the intact bronchus. (a.u. = arbitrary units).

Fluorescence Intensity as a Function of Wavelength
(Excited at 442 nm)
Table V  Summary of the optical properties of freshly excised human bronchial fragments and Syrian hamster cheek pouches.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Absorption Coefficient (mm(^{-1}))</th>
<th>Scattering Coefficient (mm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRONCHIAL FRAGMENTS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Human)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal (6)</td>
<td>0.056 ± 0.014</td>
<td>4.38 ± 1.91</td>
</tr>
<tr>
<td>tumour (6)</td>
<td>0.38 ± 0.032</td>
<td>0.83 ± 0.30</td>
</tr>
<tr>
<td><strong>HAMSTER</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal (50)</td>
<td>1.25 ± 0.44</td>
<td>3.38 ± 1.03</td>
</tr>
<tr>
<td>inflammation (18)</td>
<td>1.60 ± 1.03</td>
<td>5.62 ± 0.99</td>
</tr>
<tr>
<td>hyperplasia (3)</td>
<td>3.32 ± 0.74</td>
<td>4.68 ± 1.46</td>
</tr>
<tr>
<td>dysplasia (2)</td>
<td>2.58 ± 2.95</td>
<td>4.46 ± 1.68</td>
</tr>
<tr>
<td>papilloma (1)</td>
<td>0.067</td>
<td>1.64</td>
</tr>
<tr>
<td>ulceration (3)</td>
<td>2.50 ± 1.11</td>
<td>5.43 ± 2.44</td>
</tr>
</tbody>
</table>
5.4 Development of Lung Imaging Fluorescence Endoscope

Although the mechanism responsible for the dramatic decrease in fluorescence intensity of pre-malignant and malignant tissues is not yet elucidated, the fact that these differences exist can be exploited to design and develop a fluorescence imaging device for detection of dysplasia and carcinoma in situ lesion during standard bronchoscopic examination (Palcic et al., 1991).

Stepwise discrimination function analysis of 284 spectra to generate and test discriminant function, demonstrated that the optimal wavelengths at which the differences can best be exploited is in the green region between 525 nm to 570 nm and in the red region between 600 nm to 660 nm. Increasing the band widths of both the green and red regions was shown to improve the discrimination. These studies suggest that several algorithms could be applied for discriminating normal tissue from malignant tissues. Some of these algorithms (not part of the thesis) have now been applied to a pre-prototype lung fluorescence imaging endoscopic system (LIFE) to image early lung cancer under bronchoscopic examinations. The system design was conceptually developed by the scientific staff at the Cancer Imaging Section of the British Columbia Cancer Agency (BCCA) (Palcic et al., 1991, Lam et al., 1990b, Hung et al., 1990) and is not part of this thesis. At the present time, the system is developed and tested in a joint project between Cancer Imaging, BCCA and Xillix Technologies Corporation, Vancouver, British Columbia who is responsible for commercialization of this device.

Analyses on a database of 328 biopsy confirmed sites from 53 patients and 41 volunteers, suggests that fluorescence imaging can detect dysplasia
and carcinoma in situ with a sensitivity of 72.5% and a specificity of 94% compared with white light bronchoscopy (sensitivity of only 48.4%, specificity of 94%) (Lam et al., 1992b).

As discussed in chapter 2, it is difficult to detect and precisely localize pre-cancerous and in situ carcinoma lesions by conventional bronchoscopy. These early lesions are often a few cell layers thick (0.2 mm to 1 mm) and on average less than 0.8 cm in surface diameter (Woolner et al., 1984). These lesions may not produce enough changes on the bronchial surface to allow detection by white light bronchoscopy. In some cases of in situ carcinoma, subtle changes exist consisting of an increase in redness, granularity or slight thickening of the mucosa. Unfortunately, these changes can be associated with inflammatory airways diseases making it very difficult for diagnosis. In the study by the Mayo Clinic, in situ carcinoma were visible bronchoscopically in less than 30% of the cases. A 50% improvement in sensitivity using the fluorescence imaging system compared to white light bronchoscopy suggested that fluorescence imaging, therefore, may be an important adjunct to conventional white-light examination to improve the ability to diagnose and stage lung cancer more accurately.

5.5 Directions for Future Studies

The data collected from this thesis demonstrate that, using laser-induced autofluorescence, sufficient differences exist between normal and early bronchial lesions that could clearly delineate pre-cancerous, cancerous lesions from the normal tissues. Although detection of pre-
cancerous and cancerous lesions can be achieved using a non-skin photosensitizing dose of photofrin, the major mechanism of tumour detection using ratio fluorometry and Photofrin was due to the significant decrease in autofluorescence in pre-cancerous and cancerous tissues. To a much lesser extent, preferential retention of Photofrin in tumours also plays a role. Although the addition of this drug did not prove to be clinically useful, other fluorescent tumour localizing drugs that do not cause skin photosensitivity may be useful. The sensitivity of detection using such exogenous fluorescent drugs must be compared to that using tissue autofluorescence alone.

While the data collected in this thesis cannot address definitively which mechanism(s) is responsible for the dramatic decrease in fluorescence intensity of pre-malignant and malignant tissues, some experimental evidence suggests that the strong fluorescence signal from the normal bronchial tissues derives from the sub-epithelial layer. Collagen, elastin and flavoproteins may play an important role. Further work to elucidate and understand the underlying principles for the decrease in autofluorescence is merited.

The improvement seen in using the fluorescence imaging system to delineate and precisely localized the extent of pre-malignant lesions in individual at risk of developing lung cancer has opened up new possibilities in the study of the natural history of lung cancer as well as providing means to establish more precise end-points in investigations of the efficacy of chemoprevention drugs. To be able to identify patients with pre-cancerous and early lung lesions, has made it possible to determine if morphometric measurements of ostensibly normal epithelial
cells, in the region of pre-malignant or malignant lesions of lung cancer patients, can reveal the presence of these lesions by malignancy associated changes (MACs) (Nieburgs, 1968). If MACs can be shown to exist in lung cancer patients as in the case of cancer of the uterine cervix (Bibbo et al., 1989, Burger et al., 1981, Wied et al., 1980), colon (Montag et al., 1991) and other tissues, this may have potential applications in improving the accuracy of sputum cytology examination to identify high risk populations for lung cancer. Fluorescence bronchoscopy can then be used to localize the source of abnormal sputum cells. The ability to detect and treat pre-cancerous lesions and early lung cancer will improve the traditionally poor prognosis of lung cancer.
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Chapter 7: APPENDIX

7.1 Discriminant Function Analysis*

Discriminant analysis is concerned with the problem of classifying objects into groups or populations on the basis of a set of feature values. In discriminant function analysis, a function of the observed variables is estimated such that the associated probability or cost of misclassification is minimized for a set of cases whose classification is known. Classification algorithms cannot be used to determine if natural groupings exist within an undifferentiated population. In addition, classification algorithms should not be used as a verification of a suspected grouping within a population.

Sample based classification rules for discriminant analysis are usually constructed from random sets called "learning" or "training" samples. The aim is to identify characteristics features of the sample population, and use these to develop a classification rule that partitions the set of all possible sample observations into subsets which correspond to selected groups or classes. In practice, no classification rule can provide an error-free assignment. This also holds for the training sample since the classes or groups overlaps. In general, the criterion for classification is to minimize the total error of classification (TEC). In the two group case, the total error of classification is as follows:

*References used in the preparation of Appendix 7.1 were Weber, 1988 and Lachenbruch, 1975.
"True" Classification | Classification Algorithm
---------------------|---------------------
Group A              | Group B
Group A              | a                   | b
Group B              | c                   | d

TEC = \( \frac{b+c}{a+b+c+d} \)

In addition to the TEC, prior probabilities of occurrence and costs of misclassifications are sometimes taken into account when defining a classification rule. Discriminant functions used for assigning observations to defined classes or groups on the basis of a set of feature values are generally based on Bayes' Classification Rule. Bayes' Rule states:

For \( N \) groups \( G_k \) (\( 1 \leq k \leq N \)), assign objects to group \( G_i \) if

\[
P(G_i | \mathbf{X}) > P(G_j | \mathbf{X}) \quad \text{for all } j \text{ not equal to } i
\]

where \( \mathbf{X} \) is the vector of measured feature values. Conditional probabilities of the form \( P(G_k | \mathbf{X}) \) are difficult to determine, but \( P(\mathbf{X} | G_k) \) can be estimated from sample of objects from group \( k \). The application of Bayes' rule can be simplified using Bayes' Theorem:

\[
P(G_k | \mathbf{X}) = \frac{P(\mathbf{X} | G_k)P(G_k)}{\sum_{k=1}^{n} P(\mathbf{X} | G_k)P(G_k)}
\]
P(G_k) is the "a priori" probability (i.e., the probability that an object comes from group k). In practice, therefore, the Bayes' Classification rule is used in the form:

assign the object to group G_i if

$$P(X|G_i)P(G_i)/\sum_{k=1}^{n}P(X|G_k)P(G_k) > P(X|G_j)P(G_j)/\sum_{k=1}^{n}P(X|G_k)P(G_k)$$

or

$$P(X|G_i)P(G_i) > P(X|G_j)P(G_j) \text{ for all } j \neq i$$

Estimation of P(X|G_k) for a sample with features unknown distributions requires the collection and analysis of large volumes of data. The amounts of data can be reduced if certain assumptions are made about the feature distributions for the population of interest. In practice, the most commonly used assumption in discriminant analysis is that X is a p-dimensional vector of observations belonging to multivariate normal distributions. The conditional probabilities for each group can be calculated relatively simply according to the multivariate normal distribution. The general equation of the multivariate normal distribution is:

$$P(X) = [2\pi^{n/2}|\Sigma|^{1/2}]^{-1} \exp[-((X-\mu)'|\Sigma|^{-1}(X-\mu))/2]$$

Applying Bayes' Rule:

$$P(X|G_k) = [2\pi^{n/2}|\Sigma_k|^{1/2}]^{-1} \exp[-((X-\mu_k)'|\Sigma_k|^{-1}(X-\mu_k))/2]$$

where n is the number of features, \(\mu_k\) is the vector of feature means for group k, and \(\Sigma_k\) is the covariance matrix for group k. \(\Sigma_k\) and \(\mu_k\) must be estimated for sample populations known to belong to group k. After
substituting the above equation into Bayes' Rule and simplifying by taking the logarithm to eliminate exponential terms, Bayes' Rule for the multivariate normal case is:

assign the object with feature values \( \mathbf{x} \) to \( G_k \) if

\[
d_1(\mathbf{x}) - \ln(P(G_1)) < d_j(\mathbf{x}) - \ln(P(G_j)) \quad \text{for all } j \neq i
\]

where \( d_k(\mathbf{x}) = \frac{1}{2} \ln |\Sigma_k| + \frac{1}{2}(\mathbf{x} - \mu_k)'\Sigma_k^{-1}(\mathbf{x} - \mu_k) \) is the "discriminant score".

This version of Bayes' Rule is known as the Quadratic Discriminant Function. Within the n-dimensional space defined by the n features the boundaries between the regions to which a given object could be classified are quadratic surfaces. A quadratic Bayesian decision boundary for a two-dimensional, two-group, two-feature case is illustrated in figure 32a.

For the special case when the covariance matrices are the same for all the groups, i.e. \( \Sigma_k = \Sigma \) for all \( k \), the decision boundaries reduce to linear equations. In this case, Bayes' rule for the multivariate normal can be expressed as:

assign the object \( \mathbf{x} \) to group \( G_i \) if

\[
f_1(\mathbf{x}) + \ln(P(G_i)) > f_j(\mathbf{x}) + \ln(P(G_j)) \quad \text{for all } i \neq j
\]

where \( f_i(\mathbf{x}) = (\mu_i - \mu)'\Sigma^{-1}(\mathbf{x} - \mu) / 2\mu_i'\Sigma^{-1}\mu_i \), \( \Sigma \) is the common covariance matrix for all the groups and \( f_i(\mathbf{x}) \) is the linear discriminant function.
The groups are separated by linear hyperplanes in the feature space. For the two-feature case, the Bayesian decision boundary is a straight line as illustrated in figure 32b.

_Estimation of Error Rates_

Error rates or misclassification probabilities for the discriminant function may be estimated by one of the following methods:

i) Test the classification rule on an independent sample of size M. For misclassified, e objects the estimated "true" error is \( E_1 = e/M \).

ii) The misclassification rate for the training sample that was used to generate the discriminant function can be calculated. This is known as the apparent error rate. Usually this underestimates the "true" error rate since the data used to develop the discriminant function are also used to test its performance.

iii) A classifier can be designed with one case removed from the learning set. The discriminant function is then tested by classifying the case that was left out. This process is repeated for all cases in the sample. This technique is known as the "leaving-one-out" method. It has the disadvantage of requiring that M classifiers be designed. It is therefore generally only useful for small sample.

iv) A sample reuse (jackknife) procedure (Lachebruch, 1975) which involves classifying each observation using the classification rule obtained by omitting that observation from the training sample. Briefly, the procedure starts with a group of \( N_1 \) observations. An observation is then left out from this group and a classification function based on the remaining \( N_1 - 1 \) and \( N_2 \) observations (for two
group case) is calculated. The observation left out is then classified. These steps are repeated until all the \( N_1 \) observations have been classified. These steps are then repeated through for the \( N_2 \) group. The jackknife misclassification rate is then \( \frac{n_{1M}}{n_1} \) for the group \( N_1 \) and \( \frac{n_{2M}}{n_2} \) for group the \( N_2 \), where \( n_{1M} \) and \( n_{2M} \) are the number of left out observation misclassified in group \( N_1 \) and \( N_2 \) respectively. \( n_1 \) and \( n_1 \) are the number of observations in the respective groups \( N_1 \) and \( N_2 \).

**Feature Selection**

To reduce the number of features that is required for object classification, it may be beneficial to combine a feature selection procedure with the discriminant function analysis. The aim is to reduce the number, \( n \), of original features variables to the \( m \) variables that give the best error rate. The criteria for feature selection are typically based on analysis-of-variance statistics. The most frequently used method for selecting features to include in the discriminant analysis is the stepwise procedure. This can be performed as a stepwise forward method, a stepwise backward method, or a full stepwise method. In stepwise forward analysis, the variable with the maximum measure of goodness (i.e., best discriminates between (or among) the group) is selected first. The variable next is the one which, when combined with the first variable maximises the measure of goodness. This process continues until inclusion of another variable does not significantly improve discrimination. In the stepwise backward procedure, all the variables are initially included and at each stage, the variable that results in the smallest decrease in
measure of goodness is discarded. For the full stepwise procedure, analysis begins with an initial subset of the variables in the discriminant analysis. Possibilities include the empty set and the full set of all the variables. Each variable is tested separately for possible exclusion and the variable with the smallest non-significant reduction in the criterion is dropped (i.e. the decrease in the measure of goodness produced by removing a variable is examined and if the decrease is below a specified threshold, the variable is removed). This is repeated until no more variables can be removed without causing a significant reduction in the criterion. Each variable not included in the discriminant function is then tested for possible inclusion, and variable which provides the greatest improvement in the criterion are added back to the set. In practice, the entering and removal thresholds should be different to avoid cycling. The features are entered into the analysis corresponding to which has the highest conditional F ratio (this is the measure of goodness used in stepwise methods). The conditional F ratio is calculated for each unentered feature through a one-way analysis of covariance, where the covariates are the features already entered (for the first step, where no features have yet been entered, the feature with the highest value for the F statistic is entered). The features are entered into the discriminant function one at a time, and are allowed to remain in the discriminant function if they satisfy a user-specified minimum F-value. The minimum F-value for feature inclusion in the discriminant function was set to be 0.005. Features that are highly correlated with those already entered into the analysis will not, however, be entered. Once the final set of features has been selected the discriminant function is then calculated accordingly.
Figure 32  Examples of (a) Linear and (b) quadratic decision boundary for a two dimensional, two group and two feature case by a discriminant function. Ellipses represent equal probability contours of normally distributed, bivariate feature data.

(a) Linear decision boundary

(b) Quadratic decision boundary
For this thesis, a commercially available program, BMDP (Biomedical Data Processing, BMDP Statistical Software, Los Angeles, California), was used to generate and test discriminant functions. A stepwise analysis of the data was performed using the 7M discriminant analysis routine of this software package.
7.2 *In vitro Spectroscopy*

**Tissue Specimens**

Thoracotomy specimens of normal and malignant tissues from patients undergoing surgery for lung cancer were obtained at the time of lung resection. These fragments were rinsed in saline or phosphate buffered saline (PBS) to remove any excess blood. Measurements of fluorescence spectra were made in freshly obtained specimens, in specimens snap frozen in liquid nitrogen at \(-70^\circ\text{C}\), thawed and kept moist at \(4^\circ\text{C}\) in PBS for up to 3 hours. In addition to the fluorescence spectra of the composite normal bronchial tissues, autofluorescence spectra were also measured before and after removal of the surface epithelium and then the submucosa tissue to characterize the autofluorescence of the different layers of the bronchial wall. Following the fluorescence spectroscopy studies, these specimens were fixed in PBS with 10% formalin for histological confirmation. The tissues were processed routinely and stained with Hematoxylin and Eosin (H&E). Laser induced fluorescence spectra were also obtained from intact specimens and after homogenization of the specimens using a homogenizer.

*Extracellular matrix constituents and other chromophores*

Basement Membrane Matrigel™ were obtained from Collaborative Research Incorporation. Matrigel is a solubilized basement membrane preparation from Engelbreth-Holm-Swarm (EHS) mouse sarcoma. Its major component is laminin, collagen IV, heparan sulfate proteoglycans, entactin and nidogen. It also contains tumour growth factor-beta (TGF-beta), fibroblast growth factor, and tissue plasminogen activator. Matrigel is
formulated in Dulbecco's Modified Eagle's Medium with 10 ug/ml gentamycin. It is kept at -20°C and thawed at 4°C overnight before being used. It is maintained in a gelled consistency at 22°C to 37°C, and fluorescence spectra and imaging measurements were made at room temperature with gel thickness of 0.5 to 10 mm in either petri dishes or optical glass cuvettes.

**Cell Culture**

The cells used in all of the studies in this part of the project were HTB 58 SK-Mes-1 human lung carcinoma, A549 human lung adenocarcinoma, and CCL-210 CCD-19L4 human lung diploid (normal), obtained from ATCC. In addition, short-term cultures of human skin fibroblast were obtained from a healthy donor.

The cells were routinely grown as monolayer culture in 75-cm² polystyrene tissue culture flasks (Falcon). Chinese Hamster V79 lung fibroblast lines and A549 human lung adenocarcinoma were maintained in Eagle's F-15 minimum essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum (Gibco). HTB 58 SK-Mes-1 human lung carcinoma lines were maintained in minimum essential medium (MEM) with non-essential amino acids and sodium pyruvate (Gibco) supplemented with 10% fetal bovine serum. CCL-210 human lung diploid lines were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum. The short-term culture of human skin fibroblasts were maintained in Eagle's minimum essential medium supplemented with 20% fetal bovine serum.

The cell cultures were incubated at 37°C in a 5% CO₂ humidified atmosphere environment. Cells were maintained in exponential growth and were routinely subcultured when grown to confluence. Subculturing was
usually achieved by trypsinization. The cells were first washed with about 5 ml of 0.1% trypsin solution (Gibco) and then exposed to about 3 ml of the trypsin for about 6 minutes at 37°C. Trypsin action was neutralized by the addition of fresh medium, which was then pipetted vigorously to obtain single cells. Cells were then plated in new tissue culture flask.

Samples of cells for each experiment were removed from the growth medium by centrifugation at 600 rpm, at 4°C (Model RC-3, Sorvall) for about 6 minutes. For the experiment using the fluorescence activated cell sorter, the cells were re-suspended in phosphate buffer saline (PBS) and vortexed for 5 seconds to disperse the cells uniformly. For measurements of the fluorescence with cells layer on basement membrane, the cell samples were re-suspended in fresh medium and allowed to attach to the basement membrane in petri dishes (Falcon) for about 2 hours.

Animal Model

Squamous cell carcinoma was induced in the buccal cheek pouch of Syrian Golden hamsters using a modification of the method reported by Salley (Salley, 1954). Cotton sutures impregnated with 9,10-dimethyl 1,2-benzanthracene (DMBA) and covered with a silicone sheath were sewn into the hamster cheek pouch. The development of dysplasia at 6-12 weeks, carcinoma in situ in 12-14 weeks and large invasive cancers at 12-14 weeks were observed. Fluorescence spectroscopy using the optical multi-channel analyzer and fluorescence imaging were performed on these hamsters.
Optical Properties Measurements

A standard integrating sphere (Oriel, Stratford, CT, Model 70491) was used for the optical transmittance and reflectance measurements. The set-up for these experiments is shown in figure 33. The integrating sphere was used to measure the total transmittance, $T_t$ and total reflectance, $R_t$ from the freshly obtained bronchial fragments and fresh samples from the hamster cheek pouch. All measurements were performed in vitro with the specimens sandwiched between two glass slides and kept moist in physiological saline or PBS (phosphate buffer saline). In all cases, the specimens were illuminated with a 442 nm collimated laser beam and the integrated reflectance and transmittance were measured using the linear detector of the optical multi-channel analyzer.

For the reflectance measurements the sample was placed at the reflectance port of the integrating sphere as shown in figure 33. The specular component of the reflectance was captured by a light trap within the integrating sphere. The inner surface of the integrating sphere had a coating of barium sulphate ($\text{BaSO}_4$). The 100% reflectance (or reference), $R_r$ was measured by placing a high reflectivity barium sulphate coated plate in the reflectance port of the integrating sphere. The total reflectance of the sample is then given by $R_t = (R_m/R_r)\rho$, where $\rho$ is the given reflectance coefficient at 442 nm of the barium sulphate plate standard. The total transmittance of the sample was measured in the same manner except that the sample was placed in the transmittance port of the integrating sphere while the reflectance port and light trap were replaced with masks coated with barium sulphate.
Figure 33  Schematic diagram of the set-up of the transmittance and reflectance measurements using an integrating sphere.
The background corrections were performed using two glass slides with a drop of saline or PBS in between. Immediately following measurements, the tissue specimens were placed in 10% formalin for routine histological preparation and Hematoxylin and Eosin staining for histological examination. Sections were made on the specimens such that the thickness of the specimens could be measured using quantitative microscopy. The absorption and scattering coefficients were then calculated.
7.3 Optical Multi-Channel Analyzer

**Description of the Spectroanalyzer Components**

The basic components of the system consist of a spectrum acquisition module link to a processing system for real-time digital signal processing, which is controlled by a host computer.

The emitted autofluorescence was focussed onto the entrance slit of the spectrograph (Jarrell-Ash Monospec 27, with a 300g/mm gratings, blazed at 500 nm, and a 0.29 nm/element resolution) with a f/1.5 collimating lens and an f/3.14 focussing lens to match the f/3.8 spectrograph. The spectrograph was fitted with a 2 mm-width slit. The spectrograph uses a crossed path Czerny-Turner design to minimize re-entry spectra. Light passes through the entrance slit reflect from the collimating mirror to a grating. The 300g/mm gratings blazed at 500 nm diffracts the light such that the angle by which it reflects back to the focusing mirror is a function of wavelength. The dispersed spectrum is then collected at the exit turning mirror to a detector. The spectrograph was adjusted to cover the 450 nm to 750 nm range. Detailed aspects of the optics of spectroscopy is discussed in *Optics of Spectroscopy* (Lerner, 1990).

The light at the exit port was recorded with an intensified 1024-elements silicon linear diode array detector (EG & G PARC, model 1455R 700HQ). The model features a 18 mm diameter intensifier with manual gain control. The intensifier is red enhanced, high quantum efficiency of about 15.7% at 550 nm and a 1024-elements silicon diode array with 700 "active" elements.
The detector was interfaced to the Optical Multi-Channel Analyzer by a detector interface/controller with a 14-bit analog digital converter. The OMA controller was interfaced to a 80386 IBM compatible computer for data storage and spectral display and processing. The spectra processing and display of the incoming analog spectrum was performed in real time. Background subtraction was performed on each spectrum during the acquisition mode before transmitting the data to the host computer to correct for the dark noise of the detector. The operation and installation of the system is described in detail in the EG & G Princeton Applied Research manual.

System Calibration

Spectral calibration for the x-axis (wavelength axis) of the OMA was performed using the spectrum of a low-power-low-pressure mercury-argon lamp (Oriel, Stratford, CT, Model 6035). The calibration is linear, meaning two reference wavelength for two different points of the reference mercury curve on the screen are selected. The program then generates the wavelengths on the x-axis and stores this information into a parameter file.

Correction for non-linear spectral response of the detector was performed using a standard lamp. The correction factors are obtained by observing the wavelength-dependent output from a calibrated light. The detection system was then calibrated by measuring the intensity of the standard lamp versus the wavelength using the detector system. The sensitivity (correction factors) of the detection system is then calculated by dividing the measured intensity of the standard lamp versus the spectral
output data provided with the lamp (known standards). All spectra are then corrected by dividing or multiplying the correction factors to account for the non-uniform response of the detector. However, in the wavelengths of interest, it was found that the detector response was almost uniform.