CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Cancer has become one of the leading causes of death worldwide, the second largest non-communicable disease and it has a sizable contribution in the total number of deaths. World health organisation (WHO) estimated that mankind will have to cope up with a cancer burden of 15 million cases by the year 2025, which would be a 50% increase on the current numbers. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world (Jemal et al 2011). Out of these, 64% of the deaths are in the economically developing countries. In India, the incidence rate of cancer has been estimated to be around 2.5 million, with over 8,00,000 new cases and 5,50,000 deaths occurring each year due to this dreaded disease (Nandakumar 2001). Over 70% of the cases report for diagnostic and treatment services, only in the advanced stages of the disease, resulting in poor survival and high mortality rates (Dinshaw et al 1999).

According to Weinberg (1996) & Ruddon (2005), the term ‘cancer’ is a generic denomination for the diseases associated with the uncontrolled proliferation of cells. Cancer can be caused by both external factors (tobacco, infectious organisms, chemicals, and radiation) and internal factors (inherited mutations, hormones, immune conditions, and mutations
that occur from metabolism). These causal factors may act together or in sequence to initiate or promote the development of cancer.

![Figure 1.1 Transformation of normal cells into malignant](image)

**Figure 1.1 Transformation of normal cells into malignant**

Many factors may act together to set off the initial cellular malfunction (initiation) and stimulate the growth of initiated cells (promotion). One or two decades may elapse before the initiated cells grow into clinically detectable tumors (Ruddon 2005). The only hope for controlling cancer lies in learning more about its pathogenesis, and great strides have been made in understanding the molecular basis of cancer.

### 1.2 DIFFERENT TYPES OF CANCER

There are more than 200 types of cancer (La Fond 1981). They can be broadly grouped into different types, depending on the origin of the tissues they come from.

i. **Carcinomas** - the most common types of cancer, arise from the cells that cover external and internal body surfaces. Lung, breast, and colon are the most frequent cancers of this type.
ii. **Sarcomas** are cancers arising from cells found in the supporting tissues of the body such as bone, cartilage, fat, connective tissue and muscle.

iii. **Lymphomas** are cancers that arise in the lymph nodes and tissues of the body's immune system.

iv. **Leukaemias** are cancers of the blood cells that grow in the bone marrow and tend to accumulate in large numbers in the bloodstream.

In general, uncontrolled proliferation can be grouped under two categories viz. benign and malignant. Benign tumors aren’t cancerous and in most cases they can often be removed without any recurrence. Cells in benign tumors do not spread to other parts of the body. On the other hand, malignant tumors are cancerous. Cells in these types of tumors have the ability to invade nearby tissues resulting in the spread of the disease to other is called metastasis.

It has been reported that an estimated number of 263,900 new cases and 128,000 deaths from oral cavity cancer (including lip cancer) occurred in 2008 worldwide. The major risk factors for oral cavity cancer include smoking, alcohol use, smokeless tobacco products, and HPV infections-with smoking and alcohol having synergistic effect (Blot et al 1988 & Hashibe et al 2009). Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in females worldwide, accounting for 9% (529,800) of the total new cancer cases and 8% (275,100) of the total cancer deaths among females in 2008. More than 85% of these cases and deaths occur in developing countries. In India, the second most populous country in the world, accounts for 27% (77,100) of the total cervical cancer deaths (Ferlay et al 2010). Other cancers such as
Lung, colorectal, female breast, and prostate cancer rates are 2 to 5 times higher in developed countries compared with developing countries as a result of variations in a disparate set of risk factors and diagnostic practices (Jemal et al. 2011). Since, oral and cervical cancers are ranked the top cancers in India, the details and diagnosis of the same is explored henceforth.

1.2.1 Oral Cancer

Oral cancer is the general clinical term denoted for any malignant tissue growth that is located in any part of the oral cavity (mouth) and it is regarded as a subtype of head and neck cancer. In India, a high incidence of oral cancer, accounting for as much as 45% of all cancers and ranks as sixth most common cancer with the increasing evidence of incidence and mortality rates (Johnson & Warnakulasuriya 1993, Notani 2000).

Malignant tumors of the lip, oral cavities, and pharynx are the most commonly cited group of cancers in the Indian cancer registry (National Cancer Registry Programme: Biennial Report 1981-2001).

Figure 1.2 Anatomy of Oral cavity (Courtesy: Mathews et al 2008)
The occurrence of oral cancer may be in any part of the oral cavity. Between 90 and 95% of all oral cancers arise from the cells that line the mouth and are termed as oral squamous cell carcinoma. Prognosis of Squamous Cell Carcinomas (SCC) of the oral cavity is in general poor, mostly because of late diagnosis owing to the lack of specific symptoms. When oral cancer spreads, it usually travels through the lymphatic system. Cancer cells that enter the lymphatic system are carried along by lymph, an almost colourless, watery fluid containing cells that helps the body fight against infection and disease.

1.2.2 Risk Factors for Oral Cancer

The following factors are responsible for the oral cancer:

- Cigarette, cigar, pipe smoking, chew or snuff tobacco
- Excessive consumption of alcohol
- Exposure/ingestion of caustic substances like slaked lime, betel nuts, some spices, placing lit end of rolled tobacco in mouth
- Dental trauma or chronic sepsis
- A familial or genetic predisposition
- Excess exposure to sunlight (lip cancer)
- Exposure to the burning of fossil fuels
- Chronic oral irritation may be causative factor

1.2.3 Early Signs and Symptoms for Oral Cancer

There are various ways in which the earlier stages of oral cancer can be identified. They are
- A sore or lesion in the mouth that does not heal (most common Symptom)
- A lump or thickening in the cheek
- A painless white or red patch on the gums, tongue, tonsil, or lining
- A burning sensation in the mouth or throat (common in asian patients chewing the betel nut)

1.2.4 Diagnosis of Oral Cancer

Currently, there exist several diagnostic procedures for detection of oral cancer which include physical examination, X-rays, biopsy, and biochemical tests. Each method has its own merits and demerits and patient's medical situation is considered in choosing the best possible method. The physical examination and laboratory tests can predict the abnormality to a certain extent but always extraction of sample of tissues or cells is necessary to the pathological confirmation. This method of removing tissue for examination is termed as biopsy and it is considered as gold standard method to know whether the tissue is cancerous or not. When a sample of tissue is removed, the procedure is called an incisional biopsy or cone biopsy. When an entire tumour or lesion is removed, the procedure is called an excisional biopsy. When a sample of tissue or fluid is removed with a needle, the procedure is called a needle biopsy or fine-needle aspiration.

Exfoliate cytology is a technique used as a pre biopsy technique. If any of the cells in the smear look abnormal, then a biopsy is performed. However, biopsy requires removal, fixation, sectioning, staining and visual examination of a tissue sample under the microscope. Tissue removal is subject to sampling errors, particularly when the lesions are not visible to the
Also, the multiple-stage sample preparation process is time consuming, labor intensive and can introduce artifacts that are due to cutting, freezing and staining of the tissues and also subjective (Zonios et al 1998).

1.3 CERVICAL CANCER

Cervical cancer is one of the most common cancers among women and is the leading cancer among women in terms of incidence rates, 2 out of the 12 Population Based Cancer Registries (PBCRs) in India. Cancers of the cervix is primarily caused by human papillomavirus (HPV) infection. Early screening of the disease through cytology has considerably reduced morbidity and mortality from the disease in the developed world (Miller et al 1990).

Cervix is the most inferior portion of the uterus and measures 2.5-3 cm in length in the nulliparous adult woman (Utzinger et al 2001). A small orifice in the center of the cervix, termed the external os, serves as the connection and passageway between the uterine corpus and vagina. The three zones of the cervix are defined by the type of epithelium: the ectocervix on the periphery of the cervix with stratified squamous epithelium, the endocervix lining the external os with columnar epithelium, and the transformation zone which marks the border between the columnar and stratified squamous epithelial zones.

There are several types of cervical cancer, classified on the basis of where they develop in the cervix. Cancer that develops in the ectocervix is called squamous cell carcinoma, and around 80-90% of cervical cancer cases are of this type. Cancer that develops in the endocervix is called adenocarcinoma. In addition, a small percentage of cervical cancer cases are mixed versions of the above two, and are called adenosquamous carcinomas
or mixed carcinomas. There are also some very rare types of cervical cancer, such as small cell carcinoma, neuroendocrine carcinoma etc (American Cancer Society).

1.3.1 Risk Factors for Cervical Cancers

Several factors increase a women's risk of developing cervical cancer. A woman without any of these following risk factors the chances for developing cervical cancer is rare.

i. HIV infection, Increase number of sexual partners.
ii. Early sexual activity, early age of first pregnancy
iii. Low socioeconomic status
iv. Chlamydia infection,
v. Oral contraceptives,
vi. Immunosupression- any cause
vii. HSV and HPV infection
viii. Vitamin deficiency

It is not possible to say with certainty that a particular risk factor is the cause.

1.3.2 Early Signs and Symptoms for Cervix Cancer

In early stages (even in the precancerous stages), cervical cancers usually do not cause symptoms. Only during the later stages of cancer, one or more of these symptoms may be noticeable:

i. Abnormal vaginal bleeding between regular menstrual periods, after sexual intercourse or a pelvic exam. Menstrual periods may be heavier and may last longer. Women in their menopause may experience bleeding.
ii. Increased vaginal discharge
iii. Pain in the pelvic area
iv. Pain during sex

1.3.3 Diagnosis of Cervical Cancer

Currently, there exist several diagnostic procedures for detection of cervical cancer which include biopsy, physical examination, Pap smear, colposcopy, HPV testing and biochemical tests. Each method has its own merits and demerits and patient’s medical situation is considered in choosing the best possible method. The Pap smear is a method of screening for cervical neoplasia by sampling exfoliated cell from the surface of the endocervical canal and ectocervix. A small spatula is used to first scrap cells from the ectocervix. Following this, the endocervical canal is sampled by inserting a cytological brush through the external os.

The collected material is traditionally placed on a glass slide and fixed with a spray fixative. Since the widespread implementation of the Pap smear as a screening tool, the incidence of mortality from cervical cancer has decreased dramatically in developed nations (Myers et al 2000). Typically, if a Pap smear shows abnormal cells, a woman will be referred for a colposcopy. Colposcopy has been shown to be a very sensitive technique of identifying areas of abnormal epithelium with a sensitivity of 96%. Thus, to obtain a definitive diagnosis, the colposcopic diagnosis often must be confirmed with one or more biopsies. The need to confirm diagnosis with biopsy and histology increases patient pain and inconvenience, cost of disease management, and patients lost to follow-up as adherence with repeat colposcopy is 7-50% depending on patient population (Engquist et al 2003).
Studies have shown that women infected with HPV are more likely to develop low grade SIL than those without evidence of infection (Walboomers et al 1999). Thus, HPV testing has been proposed as screening method to determine which women are at high risk for developing cervical neoplasia. The main benefit of using HPV testing as a screening tool lies in the extremely low false negative rate and high sensitivity (83%-100%) for detecting HSIL and carcinoma (Snijders et al 2003, Clavel et al 2001, de Cremoux et al 2003).

The accuracy for all of these screening and diagnostic tests are based on comparisons with histopathologic diagnosis on biopsy or other cervical tissue specimen, which is considered the gold standard for diagnosis of cervical dysplasia and invasive carcinoma (Stoler 2001). With the exception of visual inspection, all of the new technologies add to the cost of cervical cancer screening, and none of them address the primary cause of diagnosis error in Pap smears, namely the failure to sample the abnormal cells from the cervix. In addition to all of the aforementioned technologies still require that a specimen (or picture) be sent off for evaluation, resulting in a lapse between the time a patient is evaluated and to the time a diagnosis can be made.

A technology that could eliminate this time lapse would enhance the quality of care and reduce the level of patient anxiety, as treatment decisions could be made at the time of initial evaluation. A real-time diagnosis would enhance the cost and time efficiency of the screening process by eliminating the additional time and cost of pathological diagnosis and reducing the number of visits to health care providers. Thus, methods of optical diagnosis, specifically optical spectroscopy, are considered here as a means for providing non-invasive, real-time diagnosis.
1.4 DIAGNOSTIC TECHNIQUES BASED ON OPTICAL SPECTROSCOPY

The conventional techniques for cancer diagnosis are invasive. Though, biopsy is certainly regarded as the most reliable and direct method for disease evaluation, it is invasive, stressful to the patients, and beset with unexpected complications. Hence, often this is used as the final test for confirming and evaluating the disease condition and not considered suitable for a preliminary or routine checkup. Further, it is extremely difficult to detect cancer at their early stages. Under these circumstances, physicians and scientists are looking for a cost effective and safe way of monitoring the body chemistry in real time without the need for surgery or other invasive procedures. A diagnostic protocol for a routine checkup of symptomatic and asymptomatic subjects should be simple, noninvasive, inexpensive and easily accessible in a large number of primary health centers.

In this context, it is worth to mention that the optical based diagnostic techniques have led to new approaches in medical diagnosis and therapy. Optical diagnosis of cancer based on the laser/light-induced fluorescence, laser Raman spectra, reflectance/ scattering spectra, or a combination of these has geared up in the recent years. (Alfano et al 1984, Kortum & Muraca 1996, Wagnieres et al 1998, Jansen & Kortum 1996, Georgakoudi et al 2002).

Diagnostic methodologies based on the above said optical spectroscopic techniques have the potential to link the biochemical properties to that of morphological properties of tissues. Different optical spectroscopic techniques are sensitive to different tissue conditions. For example, light or laser induced fluorescence spectroscopy and Raman spectroscopy are sensitive to molecular attributes, while diffuse reflectance
and light scattering spectroscopy are well suited to provide information about morphological structure of the tissue at the cellular and macroscopic tissue level. In particular, these techniques are fast, non invasive and quantitative. Furthermore, they can also be used to elucidate key tissue features, such as the cellular metabolic rate, vascularity, intravascular oxygenization and alterations in tissue morphology (Rahman 2010). These tissue features can be interpreted to shed light on a variety of clinical problems, such as precancerous and cancerous growth and atherosclerosis. If applied successfully, optical spectroscopy has the potential to represent an important step forward towards advances in diagnostic and therapeutic medical application.

Basically, spectroscopy is the study of the interaction of electromagnetic radiation with matter. Optical spectroscopy deals with interactions of electromagnetic radiation with matter that occur at the Ultraviolet violet (UV), Visible (VIS) and infrared (IR) wavelengths and grouped under biomedical optics (Ramanujam et al 2000). Most of the clinical applications concentrate on absorption, fluorescence and scattering spectroscopic techniques, since the output of these measurements can be obtained with a good signal to noise ratio in reasonably short interaction times. Depending upon the type of interaction of light with matter, biomedical optics has been classified as below:

1.4.1 Fluorescence Spectroscopy

Among various spectroscopic techniques, fluorescence spectroscopy has been widely utilized by the medical community to characterize various metabolic and pathological changes at cellular and tissue level. Currently, photophysical properties of intrinsic biomolecules and their structure have also been considered as an useful parameter to study
various alterations in the functional, morphological and micro-environmental changes in cells and tissues. Differences in the native fluorescence have been ascribed to various molecules, such as tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), nicotinamide adenine dinucleotide of reduced form (NADH), flavin-adenine dinucleotide (FAD), collagen, elastin and endogenous porphyrin present in cells and tissues. Of the various fluorophores, the fluorescence of collagen, elastin and, more generally, proteins is due to the presence of aromatic amino acids that are related to the structural arrangement of cells and tissues. The other fluorophores NADH, FAD and endogenous porphyrins are related to metabolic process or in connection with the onset of a pathological condition (Alfano et al 1984, Chance 1989, Vengadesan et al 1998).

In biomedical applications of fluorescence imaging, the effect of environment on the process of fluorescence is used to map chemical or physical changes within a sample. The quantum efficiency of fluorescence is a function of the radiative and non-radiative decay rates. The radiative decay rate is considered constant for a given fluorophore, while the non-radiative decay rate can vary with environment. However, the quantum efficiency is not that very easy to measure as it is difficult to measure the exact quantity of fluorophores in a particular region, and to quantify how much of pumping light is absorbed. However, fluorescence lifetime is also a function of fluorophore environment. Since determination of fluorescence lifetime required only relative intensity measurements, knowledge of the fluorophore concentration or excitation flux in the sample is no longer required. Thus imaging using fluorescence lifetime may provide functional data about a tissue sample under investigation.
1.4.2 Infrared Spectroscopy

Infrared spectrum is an important record, which gives wealth of information about the structure of a compound. Unlike UV and VIS spectra, which comprise of relatively few peaks, this technique provides a spectrum containing a large number of absorption bands from which a lot of information can be derived about the structure of an organic compound. The absorption of infrared radiations (quantized) causes the various bands in a molecule to stretch and bend with respect to one another. Proteins, nucleic acids, lipids and carbohydrates are the important compositional, structural and functional bio-macromolecules in biological cells and tissues. The change of status of cells, such as the malignant transformation, induces not only the changes of the relative contents of bio-molecules, but also the changes of structures and conformations of bio-molecules due to the perturbation of the intermolecular and intramolecular interactions. Fourier transform infrared (FTIR) spectroscopy, which is used to measure the vibrational modes of the functional groups of molecules, is sensitive to molecular structure, conformation and environment (Wong et al 1991, Jackson et al 1995, Gao et al 1999). It is also possible to use the sensitivity of FTIR spectroscopy to detect the biochemical alterations of various forms of neoplastic cells and tissues.

1.4.3 Raman Spectroscopy

In 1928, C.V. Raman demonstrated the concept of inelastic scattering of photons on interaction with matter. Raman Effect is a fundamental process in which energy is exchanged between light and matter. When light impinges on a substance, it can be either absorbed or scattered. Most of the scattered light will have the same frequency as that of incident light. However, a small fraction of the incident light can go into setting up
the molecules into vibrations. The energy for this must come from the incident light since light energy is proportional to the frequency of vibration. The frequency changes of this scattered light must be related to the vibrational frequency of the scattered molecules. This process of energy exchange between scattering molecules and incident light is known as Raman effect. The Raman signals can be measured by a spectrometer in which the scattered light intensity as a function of wavenumber (cm\(^{-1}\)). The photons that take up energy from the vibrations of the molecule give rise to anti-Stokes signal, whereas photons that give energy to the vibrations of the molecule lead to Stokes signal.

Basically, Raman scattering gives a weak signal and is frequently masked by fluorescence. Biological samples are often fluorescent and hence pose a greater challenge in recording Raman spectra. Thus the probing longer wavelength in comparison to shorter wavelength not only reduces fluorescence, but also minimizes the sample damage. Raman spectroscopy a complementary technique to IR absorption spectroscopy is also used to measure the vibrational modes of the functional groups of molecules (Singh et al 2012). It is highly sensitive to molecular structure, conformational and environment.

1.4.4 Diffuse Reflectance Spectroscopy

Diffuse reflectance spectroscopy is one of the simplest spectroscopic techniques employed for studying biological tissue. Light delivered to the tissue surface undergoes multiple elastic scattering and absorption, and part of it returns to the incident surface as diffuse reflectance, carrying quantitative information about tissue structure and composition. Diffuse reflectance spectroscopy is sensitive to the absorption and scattering characteristics of tissue. Due to these, diffuse reflectance
spectroscopy has emerged as a promising technique for effective characterization of various tissues under different pathological conditions such as malignancy, diabetes mellitus, etc.

Diffuse reflectance Spectroscopy (DRS) technique involves detection and analysis of a portion of the incident light that undergoes multiple elastic scattering owing to inhomogeneities in the refractive index of tissue components. The theory of diffuse reflection has been studied in detail by many authors (Wendlandt & Hencht 1966, Kortum 1969). When an inhomogeneous material is illuminated, some of the incident radiation penetrates the sample. The portion that penetrates the sample undergoes scattering at a large number of points in its path. The fraction of radiation that comes back out of the sample is called diffusely reflected component. The returning reflection of the directional incoming radiation flux is scattered in many directions due to uneven, broken, bumpy boundary surfaces, where the coarseness is of the same order of magnitude as the wavelength.

In contrast, direct or specular reflection occurs when the roughness of the boundary is small in comparison with the wavelength of the reflected radiation. Tissue back scattering is altered as the size of the nucleus increases and the nuclear texture becomes coarser (Perelmann et al 1998).

Extracting the physically meaningful information from the diffuse reflectance measurements may improve our understanding of the physiological and structural features that are different between malignant and non-malignant tissues, as well as potentially improve the diagnostic accuracy of optical based methods for the diagnosis of cancer.

Most importantly the available information is of quantitative nature, can provide information in real time and it is not greatly affected by
artifacts or sampling errors. It can also provide information that is largely free of subjective interpretation. Further, as \textit{in vivo} studies do not require tissue removal, DRS can be conveniently used to examine extended tissue areas. In the field of cancer research, several studies have been reported on the potentiality of diffuse reflectance spectroscopy in the characterization of cancerous tissues of cervix, ovary and colon in vitro and in vivo (Zonios et al 1999, Utzinger et al 2001, Mirabal et al 2002 and Palmer et al 2002). However, the research in the field of diffuse reflectance spectroscopy is still in the primitive stage in the characterization of diseased tissues with respect to normal.

1.4.5 Elastic Scattering Spectroscopy

Elastic scattering spectroscopy (ESS) refers to broad band white light measurements made in the optical geometry, wherein separate illuminating and collecting fibers are placed in direct optical contact with the tissue. With this method, surface reflections are avoided and all of the collected light has undergone multiple scattering through the tissue in making its way from the illuminating fiber to the collecting fiber. When elastic scattering spectroscopy is employed for tissue diagnosis, the tissue pathologies are detected and diagnosed using spectral measurements of the elastic scattered light in a manner that is sensitive to both scattering and absorption properties of the tissue, over a wide range of wavelengths. The use of a technique that is sensitive to the wavelength dependence of scattering efficiency and angles, as well as to absorption bands, is based on the fact that many tissue pathologies, including a majority of cancer forms, exhibit significant architectural changes at the cellular and sub cellular level (Bigio & Mourant 1997).
1.4.6 Optical Coherence Tomography

Optical coherent tomography (OCT) is a recently developed optical imaging technique that performs high resolution, cross-sectional imaging of microstructures in biological systems. OCT is analogous to ultrasound B mode imaging except that it uses light instead of sound. OCT performs imaging by using low coherence interferometry to measure the optical backscattering of tissues as a function of echo delay and transverse position. The resulting two dimensional dataset can be displayed as a gray scale or false color image. The concept of "nonexcision optical biopsy" provided by OCT and the ability to visualize tissue morphology in real time under operator guidance can be used both for diagnostic imaging and to guide surgical intervention (Fujimoto et al 1995).

1.5 PRINCIPLE OF FLUORESCENCE SPECTROSCOPY

1.5.1 Principle

The absorption and emission of light is well illustrated by the energy level diagram suggested by Jablonski (Lakowicz 1983). The ground, first, and second electronic states are depicted by $S_0$, $S_1$, and $S_2$, respectively in Figure 1.3. At each of these electronic energy levels the fluorophores can exist in a number of vibrational energy levels. The transitions between the various electronic levels are vertical. This presentation is used to illustrate the instantaneous nature of light absorption and the process occurs in about $10^{-15}$ sec, a time too short for significant displacement of nuclei. This is known as the Frank-Condon principle.
Following light absorption, several processes usually occur. A fluorophore is usually excited to some higher vibrational level of either $S_1$ or $S_2$. With a few rare exceptions, molecules in condensed phases rapidly relax to the lowest vibrational level of $S_1$. This process is called internal conversion and generally occurs in $10^{-12}$ sec. Since fluorescence lifetimes are typically near $10^8$ sec, internal conversion is generally complete prior to emission. Hence, fluorescence emission generally results from the thermally equilibrated excited state. As for absorption is concerned, the electronic transition down to the lowest electronic level also results in an excited vibrational state. This state will also reach thermal equilibrium in about $10^{-12}$ sec. An interesting consequence of these considerations is that the absorption spectrum of the molecule reflects the vibration levels of the electronically excited states.
Molecules in the $S_1$ state can also undergo conversion to the first triplet state $T_1$ called intersystem crossing. Emission from $T_1$ is termed phosphorescence, and generally is shifted to longer wavelength (lower energy) relative to the fluorescence. Transition from $T_1$ to the ground state is highly improbable and as a result, the rate constant for such emission is several orders of magnitude smaller than those of fluorescence. Although not indicated explicitly in Figure 1, a variety of other processes can influence the fluorescence emission. These factors include solvent effects, solvent relaxation, quenching, and a variety of excited state reactions.

The phenomenon of fluorescence displays several general characteristics for a particular biological molecule. First, due to the losses in energy between absorption and emission that occur as a result of non-radiative transitions, fluorescence occurs at emission wavelengths that are always red-shifted relative to the excitation wavelength. Second the emission wavelengths are independent of excitation wavelength. Third the fluorescence spectrum of a biological molecule is generally a mirror image of its absorption spectrum.

The fluorescence of a biological molecule is characterized by its quantum yield and its lifetime. The quantum yield is simply the ratio of the number of photons emitted to the number absorbed. The lifetime is defined as the average time the biological molecule spends in the excited state prior to return to the ground state. The fluorescence quantum yield and lifetime are modified by a number of factors that can increase or decrease the energy losses. Fluorescence spectroscopy is the measurement and analysis of various features that are related to the fluorescence quantum yield and/or lifetime of a biological molecule. The fluorescence intensity of a biological molecule is a function of its concentration, its extinction coefficient.
Fluorescence spectroscopy is generally divided into two:

i. Steady-state (spectrally resolved or intensity measurements)
ii. Time-resolved (time-domain and frequency domain) techniques.

1.5.2 Steady State Fluorescence Measurements

The various forms of steady state fluorescence that can be measured include the excitation-emission matrices (EEM), emission spectra, excitation spectra and the synchronous luminescence spectra.

1.5.2.1 Excitation emission matrices

A fluorescence excitation-emission matrix (EEM) represents a series of fluorescence emission scans collected at sequential excitation wavelengths at small wavelength increment (Anwer et al 2009, Heintzelman et al 2000, Li et al 2010). Basically, it is a two dimensional contour plot that displays fluorescence intensities as a function of both the excitation and emission wavelengths. Each contour represents points of equal fluorescence intensity. The diagonal line across an EEM (i.e. when $\lambda_{ex} = \lambda_{em}$) represents the elastic scattering spectrum. All the fluorescence is constrained to lie on or below this line (Stoke’s law). A horizontal slice across an EEM represents the emission spectrum at the particular wavelength of excitation, and a vertical slice represents the excitation spectrum at the particular wavelength of observed emission.

A multicomponent mixture such as cells, tissue and bio fluids contains a variety of components with different excitation and emission
maxima. Thus, fluorescence analysis requires obtaining emission spectra at several (various) wavelengths. A complete description of fluorescence can be presented mathematically as excitation/emission matrix, which may be termed as a fluorescence topogram (contour map) where contour lines connect regions of the same fluorescence intensity. Characteristic circular patterns resulting from fluorescence can be used to identify particulars of the fluorophore present in the sample (Dubayova et al 2003).

1.5.2.2 Emission and excitation spectroscopy

In conventional fluorescence spectroscopy, an emission spectrum represents the fluorescence intensity over a range of emission wavelengths \( \lambda_{em} \) at a fixed excitation wavelength \( \lambda_{ext} \). On the other hand, an excitation spectrum is a plot of the fluorescence intensity at a particular emission wavelength \( \lambda_{em} \) for a range of excitation wavelengths \( \lambda_{ext} \). Generally, the excitation spectra corresponding to the peaks of the fluorescence emission bands enables one to know the appropriate excitation wavelength required to induce fluorescence over that particular emission band (Li et al 2010, Anwer et al 2009).

1.5.2.3 Synchronous fluorescence spectroscopy

Although conventional emission and excitation fluorescence spectroscopy has been used as an analytical tool for monitoring trace elements due to its excellent sensitivity it has limited applicability since most spectra of complex mixtures often cannot be resolved. In this regard, simultaneous analysis of multi component mixtures by synchronous fluorescence spectroscopy was first suggested by Lloyd (1971) and further developed theoretically by Vo Dinh (1978), Pu et al (2012) & (2013). In this technique, the excitation \( \lambda_{exc} \) and emission, \( \lambda_{em} \) monochromators are
scanned simultaneously (synchronously) while keeping a constant wavelength interval $\Delta \lambda$ between them.

The use of constantly changing excitation energy may seem undesirable for spectrometric applications. This continuous variation of the excitation would not allow it to be used as a light source to record emission spectra in the usual manner (constant excitation energy) that spectrometric employ. This feature however can be distinct advantage. In order to assess the figures of merit of the synchronous technique it is necessary to discuss the corresponding luminescence expression.

1.5.3 Time-Resolved Fluorescence Spectroscopy

Time-resolved fluorescence spectroscopy is an emerging technique, which measures the average time a molecule or a fluorophore spends in the excited state (prior to emitting a photon). The emission process usually follows a single exponential decay in simple cases. Typically, the concentration of excited species will decay on a timescale of nanoseconds. The decay time or Lifetime of fluorescence is defined as the time for the excited state concentration to decay to $1/e$ (about 37%) of the initial value. The length of the fluorescence lifetime of a specific fluorophore reflects its physical and chemical microenvironments.

The use of time-resolved (lifetime) fluorescence to study biological systems offers several distinct advantages. Different types of tissues and bio-fluids may contain different amounts of specific fluorophores that express different fluorescence lifetimes. For example, biomolecules with overlapping fluorescence emission spectra but different fluorescence decay times can be discriminated. These measurements are more robust to changes in fluorescence excitation-collection geometry, presence of
endogenous absorbers (e.g., hemoglobin), photobleaching, changes in fluorophore concentration, light scattering, and excitation intensity, which thus makes them more suitable for clinical investigations. In addition, a complete fluorescence emission spectrum (steady state) can be obtained by recording the time resolved fluorescence emission at a number of wavelengths across the emission spectrum. Therefore, it is possible to distinguish different types of tissues and bio-fluids based on the differences in the lifetimes of these specific fluorophores.

1.6 URINE

Urine is a multicomponent mixture consisting mainly of organic compounds in a solution of mineral salts where number of them are native fluorophores. Urine composition reflects the volume of water and solutes that the kidneys must eliminate from the body or retain in the internal environment to maintain homeostasis. It varies considerably from time to time because of differences in dietary intake and physical activity. Urine is about 95% water and other compounds such as electrolytes, nitrogenous compounds, vitamins, hormones, organic acids, and miscellaneous organic compounds. It also contains urea and uric acid from the catabolism of amino acids and nucleic acids, and creatinine from metabolism of creatine. Urine may also have a trace of amino acids, as well as electrolytes. The volume of urine produced usually varies between 0.6 and 2.5 liters per day. Factors such as fluid intake, environmental temperature, relative humidity of the surrounding air, and a person’s emotional condition, respiratory rate, body temperature and body metabolism influence the exact urine volume.

The body metabolism is generally kept in balance in a healthy individual whereas in diseased subjects, changes occur during physiological processes or in connection with the onset of pathological conditions results
in modification of the amount of compounds, their distribution and the physiochemical of their environment (Saude et al 2007). Thus, these compounds are directly linked to cellular metabolism and, indirectly, to various health conditions. An output of 50–60 milliliters of urine per hour is considered normal. The pair of kidneys which are present in the body along with two million or more nephrons (about a million in each kidney) form urine by three precisely regulated processes:

i. Filtration

ii. Selective Reabsorption

iii. Tubular Secretion

1.6.1 Filtration

The Mechanism of urine formation begins with the process of filtration, which goes on continually in the renal corpuscles. As blood courses through the glomeruli, much of its fluid, containing both useful chemicals and dissolved waste materials, soaks out of the blood through the membranes (by osmosis and diffusion) where it is filtered and then flows into the Bowman's capsule. This process is called glomerular filtration. The water, waste products, salt, glucose, and other chemicals that have been filtered out of the blood are known collectively as glomerular filtrate.

The glomerular filtrate consists primarily of water, excess salts (primarily Na$^+$ and K$^+$), glucose, and a waste product of the body called urea. Urea is formed in the body to eliminate the very toxic ammonia products that are formed in the liver from amino acids. Since humans cannot excrete ammonia, it is converted to the less dangerous urea and then filtered out of the blood. Urea is the most abundant of the waste products that must be excreted by the kidneys. The total rate of glomerular filtration
glomerular filtration rate or GFR) for the whole body (i.e., for all of the nephrons in both kidneys) is normally about 125 ml per minute. That is, about 125 ml of water and dissolved substances are filtered out of the blood per minute.

Almost 43 gallons of water (about 180 liters) that leaves the blood by glomerular filtration, the first process in urine formation, returns to the blood by the second process called reabsorption.

1.6.2 Selective Reabsorption

Reabsorption is the movement of substances out of the renal tubules back into the blood capillaries located around the tubules (called the peritubular capillaries). Substances reabsorbed are water, glucose and other nutrients, and sodium (Na⁺) and other ions. Reabsorption begins in the proximal convoluted tubules and continues in the loop of Henle, distal convoluted tubules, and collecting tubules.

The three main substances that are reabsorbed back into the bloodstream are:

- Large amount of water - about 99% of the 180 liters of water that leave the blood each day by glomerular filtration returns to the blood from the proximal tubule through the process of passive reabsorption.

- The nutrient glucose (blood sugar) is entirely reabsorbed back into the blood from the proximal tubules. In fact, it is actively transported out of the tubules and into the peritubular capillary blood. None of this valuable nutrient is
wasted by being lost in the urine. However, even when the kidneys are operating at peak efficiency, the nephrons can reabsorb only so much sugar and water.

- Sodium ions (Na\(^+\)) and other ions are only partially reabsorbed from the renal tubules back into the blood. For the most part, however, sodium ions are actively transported back into blood from the tubular fluid.

1.6.3 Tubular Secretion

It is the third important process in urine formation. Secretion is the process by which substances move into the distal and collecting tubules from blood in the capillaries around these tubules.

![Figure 1.4 Mechanism of Urine Formation](image)
It is the step where the urine becomes more concentrated by increasing the concentration of waste products. The various substances secreted include hydrogen ions, potassium ions, ammonia, and certain drugs or metabolic end products. These substances are secreted by the mechanism of active transport. The distal convoluted tubules then drain the urine into the collecting tubes. Then, several collecting tubules join together to drain their contents into the collecting duct, which finally, after formation of urine flows into the ducts of Bellini. This then eventually reaches the renal pelvis, from where the urine flows into the ureter to reach the urinary bladder.

The kidney tubules play a crucial role in maintaining the body’s electrolyte balance. Another example of an important body function that the kidney participates is in maintaining the body’s acid-base balance. Thus, these were the various steps that take place right from the time when blood flows into the kidneys, till urine is passed into the ureter. The various urinary system diseases occur when there exists a problem with the functioning of the kidneys, which automatically gets reflected colour, odour and concentration of the urine.

1.6.4 Analysis of Urine in Cancer Diagnosis

Urine analysis in clinical, biochemical analysis has a long history and is one of the oldest laboratory tests in medicine. The standard urine tests remain almost unchanged. However, due to the availability of many metabolites, many showed interest in characterizing urine in the cancer diagnosis.

For example, Rabinwitz (1949) had reported that the fluorescence of urine may be considered for studying the tumor growth and its altered mechanism. Guzzo et al (1969) reported that an increased hydroxyproline
excretion in urine is a more sensitive indicator of bone metastases with the help of radiological evidence and an abnormally high urinary HOP/Cr in carcinoma of the breast and lung without radiologic evidence of bone metastases. Increased urine excretion of coproporphyrins has been described in patients affected by various types of acute or chronic leukemias, and high levels of free protoporphyrins or coproporphyrins have been found in patients with acute myeloblastic, chronic myeloid or chronic lymphoid leukemias (Pinelli et al 2003, Palma-Carlos, A.G & Palma-Carlos, M.L 1975, McColl & Goldberg 1980).

Konety & Getezenberg (2001), reported that nuclear matrix protein (NMP-22), fibrin degradation products (FDP), telomerase, hyaluronic acid, hyaluronidase and cytokeratins are considered as tumour markers using immunoassay, PCR, immunocytotherapy etc. Nabi et al (2003) reported the use of urine cytology in the diagnosis of urological malignancies. He concluded that the urinary cytology is of limited use in the diagnosis of urological malignancies.

Bryan (1966) has reported that the level of indoxyl sulphate in urine was involved in the genesis of neoplasms. Recently, Gamagedra et al (2011) reported that the level of neopterin in malignant urine was not significantly higher than the healthy subjects using HPLC method. It has been reported that increased levels of neopterin are associated with various diseases including cancer using HPLC method (Reibnegger et al 1986). Among various pterin and its derivatives, reports suggest that neopterin could be detected in body fluids and plays an important role in the malignant disease diagnostics (Berdowska & Korczala 2001, Melichar et al 2011, Melichar et al 2006).
Though Rabinwitz (1949) explored the use of fluorescence based analysis of urine in 1949, recently the native fluorescence of urine has been increasingly coming into the limelight. In this line, Masilamani et al (2010), reported the detection of cancer by native fluorescence of urine. Wan et al (2010), reported the use of synchronous fluorescence spectroscopy in the characterization of xanthopterin and isoxanthopterin of urine in the diagnosis of stomach cancer. In this context, the present work is aimed to apply native fluorescence spectroscopy in the characterisation of urine of healthy subjects and cancer patients and to verify their diagnostic potentiality in the discrimination of cancer patients from that of healthy subjects.