Kingdom of Saudi Arabia
Ministry of High Education
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Physics and Astronomy Department

CANCER DIAGNOSIS BY SYNCHRONOUS FLUORESCENCE SPECTRA OF BLOOD AND URINE COMPONENTS

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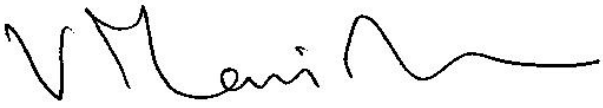
Signature: 

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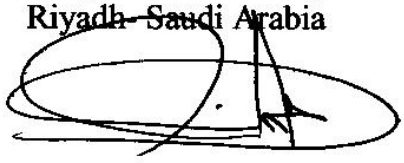
Signature: 

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IN THE NAME OF ALLAH THE MOST BENEFICENT

THE MOST MERCIFUL.

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CONTENTS

ABSTRACT

Cancer is an emotional word, a word associated with disease, death and dying. It is a word which strikes fear into the hearts of ordinary people because, for centuries it has been associated with a mysterious illness with no known cause and no known cure. However, remarkable strides in cancer research and technology in the late 20th century have given way today to an opportunity for exponential progress against the disease.

There are many studies currently being conducted in the area of cancer diagnosis. Researchers are trying to improve current tests to develop new testing techniques for a better understanding of the disease. Diagnostic tests are considered an important research subject because, in some cases, they allow for early detection of the disease. Finding cancer early is beneficial because it often improves a patient's prognosis. During the past several years, there has been a growing interest in optical spectroscopic detection of tumors.

Detection of neoplastic changes by optical spectroscopy techniques such as Raman and fluorescence has been one of the active areas of research in recent times. Several studies have established the potential of these techniques in discriminating oral, cervical, breast and other malignancies. These methods have been described as more objective, less time-

consuming, and in some cases with the advantage of *in vivo* applicability. Thus, by using these methods a painful biopsy can be avoided.

In this line of research we have employed Fluorescence spectra study for detection of cancer. In this dissertation, a study has been done to discriminate the spectral characteristics of cancer-specific fluorophores such as reduced Nicotinamide Adenine Dinucleotide (NADH), collagen, elastin, flavin, tryptophan and porphyrins from blood plasma and the acetone extract of formed elements in blood and also from urine.

In this study we had analyzed more than 50 healthy samples as control and about 75 of cancerous patients blood and urine of different etiology. The test samples were taken before the patients took any treatment or drugs; because drugs may cause confusion of the spectra.

In this study we have been able to show that optical diagnosis especially fluorescence as we have done here can detect cancer from body fluids (blood and urine). The results that others have, obtained, using native fluorescence of tissue could be reproduced almost identically by similar studies on blood alone. And rather than that we had shown the same results using urine native fluorescence**.**

This dissertation consists of five chapters:

- **CHAPTER 1: BASIC IDEAS ABOUT CANCER.**

  This chapter gives basic ideas about cancer, such as: different kinds of cancer, cancer: a genetic disease, causes of cancer, cancer staging, cancer detection and diagnosis.

- **CHAPTER 2: ABSORPTION AND FLUORESCENCE.**

  This chapter deals with basic considerations about absorption and emission, such as: absorption spectra, molecular emission, fluorescence, fluorescence type, Quantum efficiency of fluorescence, types of fluorescence.

- **CHAPTER 3: REVIEW OF RELEVANT LITERATURE.**

  This chapter deals with the literature review of laser or light-induced fluorescence (LIF), labeled fluorescence, autofluorescence of tissue, autofluorescence of blood components and urine, synchronous luminescence of tissue or body fluid.

- **CHAPTER 4: INSTRUMENTATON.**

  This chapter deals with instrumentation of our experiment and with the materials and methods of sample collection and methodology, methods of analyzing samples.

- **CHAPTER 5: RESULTS AND DISCUSSION.**

  This chapter deals with results and discussion of the study of fluorescence emission and excitation and synchronous spectra of blood plasma, the acetone extract of formed elements, urine and urine extracts.

CHAPTER 1

BASIC IDEAS ABOUT

CANCER

1.1 Introduction

Cells are the structural units of all living things. Each of us has trillions of cells, as does a growing tree [1].

The cells of our bodies grow and multiply in a process known as "cell division". It must be extremely tightly controlled if all the cells in your body are to grow in the right place, and for all our organs and tissues to function properly. If cells divide too quickly the consequences can be disastrous.

Cancer is essentially a disease of cell division. Uncontrolled cell division can have many causes, and can happen in any type of cell in the body, but it usually results from defects or damage in one or more of the genes involved in cell division. If these genes become damaged in some way, for example by exposure to cigarette smoke or ultraviolet radiation, the cell can start to divide uncontrollably. These defective cells can multiply to form a lump of abnormal tissue called a tumor[2].

Tumors are usually classified as simple (or benign) and malignant (cancer). Benign tumors tend to remain localized, are often surrounded by a capsule and rarely give rise to serious effects. Malignant tumors, on the other hand, do not remain localized but invade other tissues and give rise to secondary

tumors (metastases) in other parts of the body, through the blood stream or lymphatic system[1,3,4,5].

Although cancer is often referred to as a single condition, it actually consists of at least 200 different diseases. Cancer can arise in many sites and behave differently depending on its organ of origin, for example leukemia, breast cancer, lung cancer, brain cancer, head and neck cancer, Hodgkin's disease and others[1].

1.2 Different Kinds of Cancer

Cancer can originate almost anywhere in the body, and are of different kinds:

1. **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. 90% of tumors belong to this category[6,7].

2. **Sarcomas,** are cancers arising from cells found in the supporting tissues of the body such as bone, cartilage, fat tissue, connective tissue and muscles. They are 2% of all tumors[6,7,8].

3. **Lymphomas,** are cancers that arise in the lymph nodes and tissues of the body's immune system. They are 4% of all tumors[6,9].

4. **Leukemias**, are cancers of the immature blood cells that grow in the bone marrow and tend to accumulate in large numbers in the bloodstream. They are 4% of all tumors[3,6].

1.3 Cancer: a Genetic Disease

The ancient Greeks believed that cancer was caused by too much body fluid and they called it "black bile". Doctors in the seventeenth and eighteenth centuries suggested that parasites caused cancer. Today, doctors understand more about the link between cancer and genetics [10, 11]. As a result of decades of cancer research, cancer today can be described as a genetic disease [12, 13].

How Genes Cause Cancer:

There are two basic kinds of genetic mutations. The mutation is passed from one of the parents to the child; it is called a 'germline mutation'. When a germline mutation is passed on from parents to child, it is present in every cell of the child's body, including the reproductive sperm and egg cells. Because the mutation affects reproductive cells, it is passed from generation to generation. Germline mutations are responsible for less than 15% of cancer cases. Most cancer cases are caused by a series of genetic mutations that develop during a person's lifetime in the somatic cells. These mutations are called "acquired mutations" because they are not inherited. Acquired mutations may be caused by environmental factors or are spoueneous. Most scientists believe that cancer happens when several genes of a particular group of cells become mutated. Some people may

have more inherited mutations than others. So, even with the same amount of environmental exposure, some people are simply more likely to develop cancer [11]. Majority of cancers are multifactorial, with both genetic and environmental causative factors. However, some cancers are "monogenic" with a single gene involved in causing cancer. These latter forms are purely genetic and are inherited.

A simplistic interpretation divides cancer genes into three broad categories:

1- Tumor suppressor genes:

Tumor suppressor genes are protective genes. Normally, they suppress (limit) cell growth by monitoring the rate at which cell divide, repairing damaged DNA and controlling cell death. When a tumor suppressor gene is mutated (due to heredity, environmental factors, or as part of the aging process), cells continue to grow and can eventually form a tumor. Close to 30 tumor suppressor genes have already been identified, including BRCA1, BRCA2, and one of the most important tumor suppressor genes is called P53. This gene was co-discovered in 1979 by cancer research UK scientist professor Sir David Lane. In fact, nearly 50% of all cancers involve a missing or damaged P53 gene [2, 11].

2- Oncogenes:

Protooncogenes are natural genes in the body and normally determine the rate at which healthy cells divide. When these genes are mutated, they are converted to oncogenes and the cell cycle is disrupted. The cells can divide quickly and tumors may form because nothing is controlling the cells multiplication. More than 100 oncogenes have been identified, and include genes such as HER2/neuandras [11].

3- Stability genes:

This category of cancer genes has been proposed more recently and it is called stability or caretaker genes. These genes are not directly involved in tumorigenesis but when altered they contribute to cancer by exposing cells to an abnormally high mutation rate. This feature ultimately leads to oncogene activation or tumor suppressor inactivation [13].

1.4 Causes of Cancer

Remarkable strides in cancer research and technology in the late 20th century have given way today to an opportunity for exponential progress against the disease. There are many different types of cancer and they each have different causes. Each type of cancer may have several different causes [12].

Any thing that damages the genes in our cells can ultimately cause cancer, but a number of genes in the same cell need to be damaged before a cell become cancerous [14].

Many of the causes of cancer have already been identified. Besides heredity, environmental factors are involved these are carcinogens. Carcinogens are factors, which cause the DNA in a cell to become altered (mutated). Carcinogens can be physical, chemical (e.g., from smoking or diet) or biological factors.

1. Chemical causes :

In this case, mutation is caused by foreign molecules binding to a cell's DNA, causing it to be "misread". The shape of the atoms may determine whether the molecule fits into some cellular receptor. Solubility may

determine whether it passes through cell membranes to attack target molecules in the cell (e.g. benzene) [15,16].

2- Physical causes:

Physical damage to DNA can be caused by ionizing radiation and non ionizing radiation.

i) Ionizing radiation:

Ionizing radiations are high energy radiation. They fall into two class: particulate (involving β particles, neutrons and α particles) and electromagnetic (involving x-ray and γ-ray).

Ionizing radiation literally punches holes in the DNA, causing breaks in the DNA and hence altering the correct genetic sequence [8,15].

ii) Non ionizing radiation:

The non ionizing radiation include ultraviolet radiation (from sunlight), which may cause mutation by causing certain portions of DNA to remain bound together (even when they shouldn't) e.g. thymine-dimers. This causes mutation by causing misreading of the DNA [8, 15, 16].

3-Biological causes:

Biological causes may be viral or bacterial.

i) Viral causes:

Some viruses are linked to certain types of cancer. This does not mean that these cancers spread from person to person like an infection, nor does it mean that everyone infected with these viruses will develop cancer [2].

In general, viruses are small infection agents that cannot reproduce on their own, but instead enter into living cells, get incorporated into the DNA and cause the infected cell to produce more copies of the virus. Like cells, viruses store their genetic instructions in nucleic acids.

In the case of cancer viruses, some of the viral genetic information carried in these nucleic acids is inserted into the cell, and this causes the cell to become malignant [15].

ii) Bacteria causes:

One of the known bacterial mutagens is helicobacter pylori, implicated in stomach cancer [16].

1.5 Cancer Staging

' Staging ' is the process of describing the size and location of the tumors and whether the cancer has spread to other parts of the body. Staging is

essential in determining the choice of therapy and assessing a person's prognosis (chance of recovery). Many diagnostic tests also help doctors to determine the stage of the cancer [17].

The concept of stage is applicable to almost all cancers except most forms of leukemia. Since leukemias involves all of the blood, they are not anatomically localized like other cancers, so the concept of staging does not make much sense for them. A few forms of leukemia do have staging systems, which reflect various measures, of how advanced is the disease [16]. The Inter national Union against Cancer (UICC) has proposed the " TNM " system of notation for staging malignancy, which is now generally accepted throughout the world [2,5].

TNM system describes tumors in three ways: size of the primary tumor (**T**), absence or presence of cancer in the regional lymph nodes (**N**), and whether the cancer has spread to a different part of the body (**M**) "metastasis".

Once the doctors determine the **T**, **N**, and **M**, they assign the cancer stage. Stages are written in numbers:

- (**T**) can be **1-4** "**1**" being a small tumor "**4**" a large one.

- (**N**) can be **0-3** "**0**" meaning no positive lymph nodes and "**3**" many positive nodes.

- (**M**) is either **0** or **1 "0**" meaning no spread and "**1**" meaning there is spread [2, 5, 17].

1.6 Cancer Detection and Diagnosis

Early detection of cancer provides an opportunity for prompt treatment while the cancer is small and localized. We discuss below some of the detection methods for several different cancer types.

1. Biopsy:

In order to diagnose cancer, a physician nearly always performs a biopsy. This procedure involves removing a small sample of tissue and examining it under a microscope. There are many different types of biopsies. Some biopsies involve surgery to remove an entire organ, while others are much less invasive [2].

The biopsies most often used in diagnosing cancer include:

i) Surgical Biopsy:

The doctor removes part of the lump (incisional biopsy) or the entire tumor or organ (excisional biopsy) [18].

ii) Needle Biopsy:

A needle is inserted into the tumor and fluid and cells are aspirated (drawn out) with a vacuum syringe [18].

ii) Endoscopic Biopsy:

A thin, flexible tube with a fiber optic light and a viewing lens or video camera is inserted into the patient through a natural body opening, such as the rectum, mouth or throat. This allows the physician to see a tumor at close range and to insert an instrument through the tube to remove a sample for analysis. This type of biopsy may be used to diagnose colorectal and lung cancers, among others; biopsy is the most reliable method, but very invasive [2, 18, 19].

2. Imaging techniques: are ways in which doctors can create detailed pictures of what's going on in side the bodies without having to open it surgically.

i. X-ray Imaging:

X-rays are diagnostic tests that use invisible electromagnetic radiation to produce images of internal tissues, bones, and organs on film. X-ray are not as sophisticated as newer procedures, but they are still useful for finding and monitoring some types of tumors [2, 19, 20].

ii. Ultrasound Imaging:

Ultrasound imaging is a technique which uses high frequency sound waves and a computer to create images, called sonograms, of blood vessels,

tissues, and organs. Sonograms are used to view internal organs as they function and to assess blood glow through various vessels. Tumors in the abdomen, liver, and kidneys can often be seen with an ultrasound [19, 20, 21].

iii. Magnetic Resonance Imaging (MRI):

Magnetic resonance imaging is a diagnostic procedure that uses a combination of a large magnet, radiofrequencies, and structures within the body. The magnetic field causes atoms in the tumor to change direction. The radio frequency pulse causes another change of direction when the pulse stops, the atoms relax and return to their original position. During relaxation, the atoms give off energy in differing amounts, at different intervals of time. Antennas pick up these signals and feed them into a computer which assembles a picture. Because different atoms have their own characteristic radio signals, the computer can distinguish between benign and malignant tumors. MRI can find tumor not detectable by clinical tumor examination; but it is quite expensive [3, 9, 20].

iv. Computed Axial Tomography scan (CAT) Imaging:

A Computed Axial Tomography scan (also known as a CT scan) is a diagnostic imaging procedure that uses a combination of x-rays and computer technology. CT scan allows for multiple x-rays to be taken from

different angles around the patient. The "slices" or cross-sectional images of the patient's body are analyzed by the computer. In a CT scan, bones appear bright and distinct, but soft tissue, such as muscle, blood vessels and tumors frequently appears in almost identical shades of gray. Radiologists can inject contrast agents containing such heavy atoms as iodine, to make blood vessels stand out. The computer can also add color to images so that the varying shades of x-ray absorption corresponding to different kinds of tissue are immediately distinguishable. CT scans provide a means of diagnosis and help in planning surgery or radiotherapy [8, 21].

v. Positron Emission Tomography (PET) imaging:

Positron Emission Tomography is a very recently developed technology. The patient is injected with a tiny amount of a special tracer material that releases sub-atomic particles called " positrons ". When positrons collide with the atoms of the body, they release tiny bursts of energy. The patient is then placed in a scanner that picks up these energy bursts and builds a picture based on where the tracer has traveled in the body, For example, one kind of PET scan uses a radioactive form of sugar molecules (glucose), called tracers, which are injected into the body in a low dose. During the scan, the cancer cells "light up ", because the cancer cells use more glucose than normal cells. PET scans can be even more sensitive type of scan than MRI and x-rays. They can also show how a particular part of the body is

working, and not just what it looks like. For example, a PET scan can show whether the tissue remaining after treatment is living cancer or just dead tissue [2, 19, 21].

3. Tumor Markers Test:

Tumor markers are certain antigens, proteins and other substances that can often be detected in higher than normal amounts in the blood, urine, or body tissues of some patients with certain types of cancer. Tumor markers are produced either by the tumor itself or by the body in response to the presence of cancer or certain benign (non cancerous) conditions. Measurements of tumor marker levels can be useful- when used along with x-rays or other tests- in the detection and diagnosis of some types of cancer. However, measurements of tumor marker levels alone are not sufficient to diagnose cancer for the following reasons:

- Tumor marker levels can be elevated in people with benign conditions.
- Tumor marker levels are not elevated in every person with cancer, especially in the early stages of the disease.
- Many tumor markers are not specific to a particular type of cancer; the level of a tumor marker can be raised by more than one type of cancer [22].

Physicians can use changes in tumor marker levels to follow the course of the disease , to measure the effect of treatment, and to check for recurrence.

The following is a brief description of some of the more useful tumor markers:

i. Prostate-specific antigen (PSA)

Prostate-specific antigen (PSA) is always present in low concentration in the blood of adult males. An elevated PSA level in the blood may indicate prostate cancer, but other conditions such as benign prostatic hyperplasia (BPH) and prostatitis can also raise PSA levels. PSA levels are used to evaluate how a patient has responded to treatment and to check for tumor recurrence [22].

ii. Prostatic Acid Phosphatase (PAP)

Prostatic acid phosphatase (PAP) originates in the prostate and is normally present in small amounts in the blood. In addition to prostate cancer, elevated levels of PAP may indicate testicular cancer, leukemia, and non-Hodgkin's lymphoma, as well as some noncancerous conditions [22].

iii. Cancer Antigen (CA 125)

Ovarian cancer is the most common cause of elevated CA 125, but cancers of the uterus, cervix, pancreas, liver, colon, breast, lung, and digestive tract can also raise CA 125 levels. Several noncancerous conditions can also elevate CA 125. CA 125 is mainly used to monitor the treatment of ovarian cancer [22].

iv. Carcinoembryonic Antigen (CEA)

Carcinoembryonic antigen (CEA) is normally found in small amounts in the blood. Colorectal cancer is the most common cancer that raises this tumor marker. Several other cancers can also raise levels of carcinoembryonic antigen [22].

v. Alpha-fetoprotein (AFP)

Alpha-fetoprotein (AFP) is normally elevated in pregnant women since it is produced by the fetus. However, AFP is not usually found in the blood of adults. In men, and in women who are not pregnant, an elevated level of AFP may indicate liver cancer or cancer of the ovary or testicle. Noncancerous conditions may also cause elevated AFP levels [23].

vi. Human chorionic gonadotropin (HCG)

Human chorionic gonadotropin (HCG) is another substance that appears normally in pregnancy and is produced by the placenta. If pregnancy is ruled out, HCG may indicate cancer in the ovary, liver, stomach, pancreas, and lung and in males in the testis [23].

vii. Cancer Antigen (CA 19-9)

Cancer Antigen (CA 19-9) marker is associated with cancers in the colon, stomach, and bile duct. Elevated levels of CA 19-9 may indicate advanced cancer in the pancreas, but it is also associated with noncancerous conditions, including gallstones, pancreatitis, cirrhosis of the liver, and cholecystitis [23].

viii. Cancer Antigen (CA 15-3)

Cancer Antigen (CA 15-3) marker is most useful in evaluating the effect of treatment for women with advanced breast cancer. Elevated levels of CA 15-3 are also associated with cancers of the ovary, lung, and prostate, as well as noncancerous conditions such as benign breast or ovarian disease, endometriosis, pelvic inflammatory disease, and hepatitis. Pregnancy and lactation also can raise CA 15-3 levels [23].

ix. Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) is a enzyme that normally appears throughout the body in small amounts. Many cancers can raise LDH levels, so it is not useful in identifying a specific kind of cancer. Measuring LDH levels can be helpful in monitoring treatment for cancer. Noncancerous conditions that can raise LDH levels include heart failure, hypothyroidism, anemia, and lung or liver disease [23].

x. Neuron-specific enolase (NSE)

Neuron-specific Enolase (NSE) is associated with several cancers, but it is used most often to monitor treatment in patients with neuroblastoma or small cell lung cancer [22, 23].

4. Urine tests:

There are many types of urine tests that can be used to detect and monitor some types of cancer. These tests include:

- Tumor marker tests. Urine tests and blood tests can detect certain tumor markers, which are substances that can be made by cancer cells and normal cells.

- Other types of urine cytology. Examination of the cells can reveal blood in the urine, hormones and others substances that may indicate cancer [18].

5. Optical Methods (optical biopsy):

Recently, there has been increasing interest in the use of optical biopsy systems to be able to provide tissue diagnosis in real-time, non-invasively and *in situ*. These systems rely on the fact that the optical spectrum derived from any tissue will contain information about the histological and biochemical make up of that tissue; one is able to determine the state of the tissue – normal, benign, pre-cancerous or cancerous.

Fluorescence, Raman, and Elastic Scattering spectroscopy are potential optical biopsy. We will focus on fluorescence approaches. Fluorescence spectroscopy measures the allowed electronic transition while Raman spectroscopy measures the vibrational transitions from various groups of molecules. When cells interact with light they become excited and re-emit light of varying colures. The spectrum of the light emitted gives information about the presence of the different molecules or structural changes that occur in the tissue and hence, the state of the tissue . The change in state from normal to cancerous alters tissue structure and composition [24, 25, 26].

CHAPTER 2

ABSORPTION AND

FLUORESCENCE

2.1 Introduction

This chapter deals with basic considerations about absorption and emission of electromagnetic waves interacting with matter.

Under normal conditions and at room temperature, the state of a molecule will be at its lowest possible energy state, known as the " ground state ". Outside stimuli such as visible or ultraviolet light can put the molecule in an excited state, where one or more electrons occupy higher energy orbital than in the ground state. The multiplicity of the molecule is then defined as the quantity (2S+1), and may be either singlet or triplet. In a singlet state there are an equal number of electrons with negative and positive spins in the molecule, i.e. all the electrons spins exist in pairs. For the singlet state, S=0 and the multiplicity is therefore 1. Conversely, a triplet state is one in which there is one unpaired set of electron spins, S=1 and multiplicity is 3 [27].

The promotion of electrons from the highest to the lowest unoccupied molecular orbital occurs without change in the total spin, this is known as Wigner's rule and is characterized by the strongest band in the absorption spectrum; that of the $S_o \rightarrow T_1$ transition has a very low probability of occurring and is said to be 'spin forbidden'. However, through the phenomenon of spin-orbit coupling it is possible for the triplet states to be reached from singlet states [27].

2.2 Absorption Spectra

Matter can capture electromagnetic radiation and convert the energy of a photon to internal energy. This process is called ' absorption ' [28].

Absorption of light by a molecule causes the excitation of an electron, and the electron moves from a ground state to an excited state. Each of these electronic states may contain a number of vibrational levels. Absorption of light is from the lowest electronic/ vibrational state to a number of vibrational levels in the excited electronic state [Figure 2.1 and 2.2]. Since the energy levels of matter are quantized, only light of energy that can cause transitions from one level to another will be absorbed.

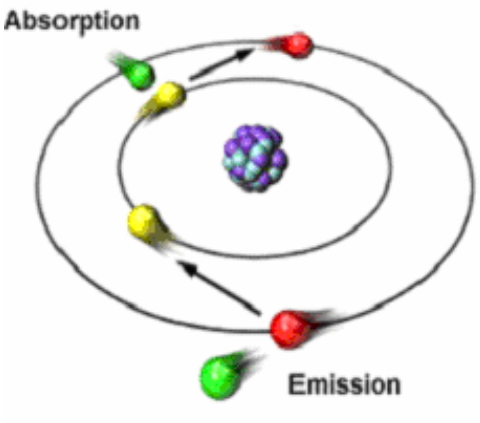


Figure 2.1 Absorption and Emission of Radiation [31].

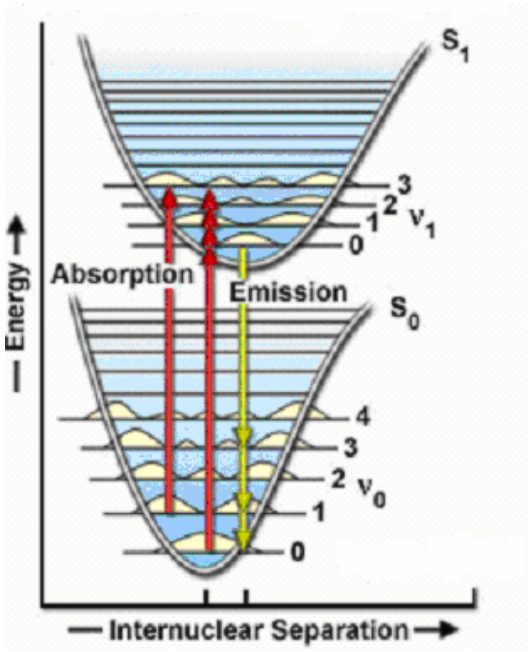


Figure 2.2 Franck-Condon Energy Diagram [31].

The energy of absorbance photon= $h\nu = E_e - E_g$ (2.1)

Here h is Planck's constant, and ν is the frequency of the radiation and E_g and E_e is the energy of the ground and excited states, respectively. Therefore, the type of excitation depends on the wavelength of the light. Electrons are promoted to higher orbital by ultraviolet or visible light, vibrations are excited by infrared light and rotations are excited by microwaves radiation [28, 29].

The absorption spectrum is characteristic for a particular element or compound, and does not change with varying concentration, making absorption spectrum useful for identifying compounds. Measuring the concentration of an absorbing species in a sample is accomplished by applying the Beer-Lambert law [30].

$$A = -\log\left(\frac{I_o}{I}\right) = \varepsilon c l \qquad (2.2)$$

where A is called the absorbance or optical density of the sample. I_o and I are the light Intensities entering and leaving the sample respectively. ε is the molar absorptivity, c is the concentration of absorbing molecule in the sample , l is the length of the path of light through the sample [30].

2.3 Molecular Emission

Absorption of visible or UV radiation raises molecule to an excited state. Electron absorbs quantum of energy and jumps to a higher energy orbital.

When electron drops back to the ground state, excitation energy can be liberated by:

-Radiation less transfer.

-Re-Emission of radiation: gives rise to fluorescence and/or phosphorescence (two forms of photoluminescence)

Fluorescence may be defined as the emission occurring between two states of the same spin multiplicity, for example between S_1 and S_o. Fluorescence generally ceases immediately (<20 ns) after the exciting radiation is removed.

Phosphorescence may be defined as the emission occurring due to the radiative transition between two states of different spin multiplicity, for example between T_1 and S_o, is generally delayed relative to the exciting radiation, and may persist for several seconds after the exciting source is removed [31, 32].

2.4 Fluorescence

When specimens, living or non-living, organic or inorganic, absorb and subsequently re-radiate light, the process is described as " photoluminescence ". If the emission of light persists for up to a few seconds after the excitation energy (light) is discontinued, the phenomenon is known as "phosphorescence". Fluorescence, on the other hand, describes

light emission that continues only during the absorption of the excitation light. The time interval between absorption of excitation light and emission of re-radiated light in fluorescence is of extraordinarily short duration, usually less than a millionth of a second.

The phenomenon of fluorescence was known by the middle of the nineteenth century.

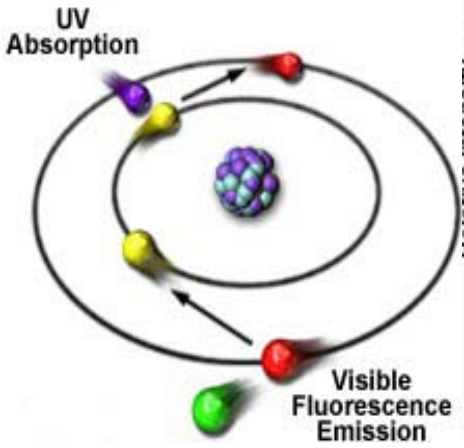


Figure 2.3 Stokes' Observation [31].

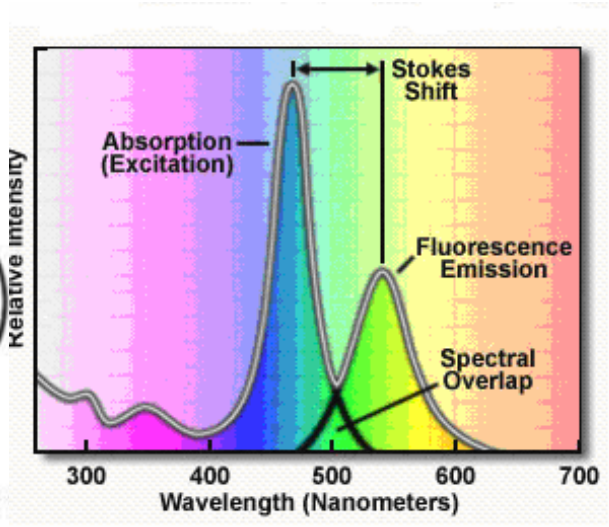


Figure 2.4 Excitation and Emission Spectral Profiles [31].

British scientist Sir George G. Stokes' first made the observation that the mineral fluorspar exhibits fluorescence when illuminated with ultraviolet light, and he coined the word "fluorescence" [31]. Stokes' observed that the fluorescing light has longer wavelengths than the excitation light, a phenomenon that has become to be known as the " Stokes' shift " Figure 2.4. In Figure 2.3, a photon of ultraviolet radiation (purple) collides with an electron in a simple atom, exciting and elevating the electron to a higher

energy level. Subsequently, the excited electron relaxes to a lower level and emits light in the form of a lower-energy photon (red) in the visible light region. This result in an excitation and emission spectra.

Figure 2.5 is a diagrammatic representation of the visible light region of electromagnetic radiation, which covers a wavelength range from approximately 400 to 700 nanometers. Surrounding the visible region is higher energy ultraviolet light and lower energy infrared light.

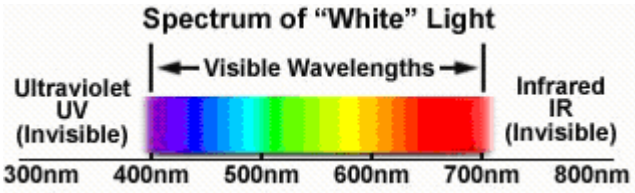


Figure 2.5 Electromagnetic Spectrum [31].

Fluorescence is illustrated in Figure 2.6. This shows how incident radiation excites the ground singlet state molecule, S_o, into an excited singlet state, S_1. A molecule in an excited state will eventually return to the ground state. This process can occur through a number of possible paths. A return to the ground state can be nonradiative. An example of a radiationless transition, called internal conversion, is the generation of heat. An excited molecule may make a transition to an excited triplet state. This is called an " intersystem crossing ". From an excited triplet state, an internal conversion can also occur, from which no light is emitted. However, an excited singlet

or triplet state may return to the ground state via radiative decay. When an electron decays radiatively to a lower state, a photon is emitted. A radiative transition between a singlet state and the ground state which results in the emission of a photon is called fluorescence.

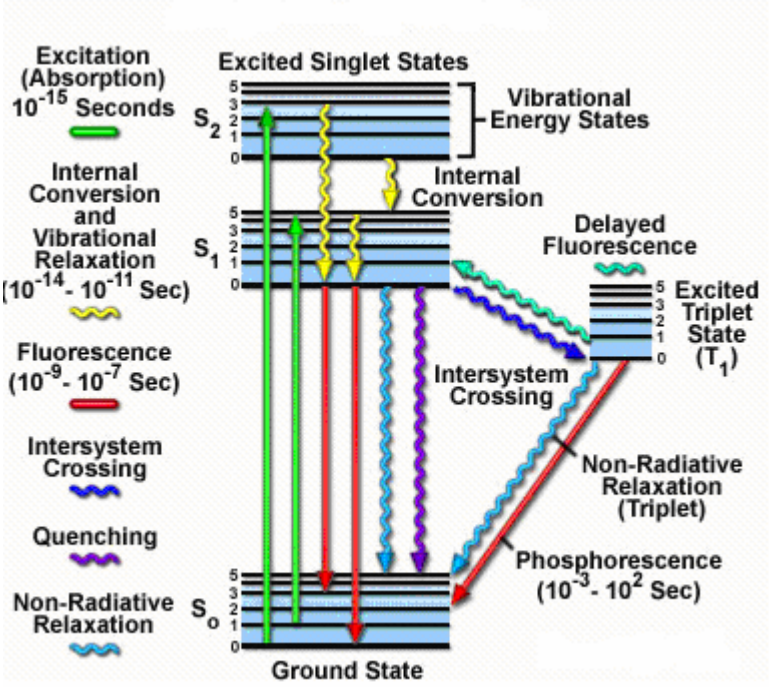


Figure 2.6 Jablonski Energy Diagram [31] .

Fluorescence is nearly always a result of a transition between the lowest energy level of the first excited singlet state and some vibrational level of the ground state. For a radiative transition from a triplet state to the ground state, which results in the spontaneous emission of a photon, the process is

called phosphorescence. Phosphorescence has a longer lifetime than fluorescence. If the delay between absorption and emission is of the order of 10^{-8} seconds or less, the emission is called fluorescence. For a delay ~ 10^{-6} the term delayed fluorescence is used and a delay greater than ~10^{-6} results in phosphorescence.

Fluorescence microscopy is a rapidly expanding and invaluable tool of investigation. Its advantages are based upon attributes not as readily available in other optical microscopy techniques. The use of fluorochromes has made it possible to identify cells and sub-microscopic cellular components and other entities with a high degree of specificity amidst non-fluorescing material. The fluorescence microscope can reveal the presence of fluorescing material with exquisite sensitivity. An extremely small number of fluorescent molecules (as few as 50 molecules per cubic micrometer) can be detected. In a given sample, through the use of multiple staining, different probes will reveal the presence of individual target molecules [33, 34, 35].

2.4.1 Fluorescence Types

1- Resonance Fluorescence:

Resonance fluorescence has an identical wavelength to the radiation that caused the fluorescence. This phenomenon is observed only in solids and gases and is the basis of atomic fluorescence [33].

2- Stokes' Fluorescence:

Stokes' Fluorescence emission will be less energetic than excitation (i.e., at longer wavelength) and this is common in molecules [33].

2.4.2 Quantum Efficiency of Fluorescence

The Quantum efficiency (Φ) is defined as the ratio of the number of fluorescence molecules to the number of excited molecules.

$$\Phi = \frac{K_f}{K_f + K_{isc} + K_{VR} + K_X} \qquad (2.3)$$

Where k_f, k_{isc}, k_{VR}, k_x are rate constants for fluorescence, intersystem crossing, vibrational relaxation, and other deactivation process, respectively. As Φ becomes close to 1, the system is considered an efficient fluorescing system. Conditions should be adjusted to increase the quantum efficiency [34].

2.4.3 Types of Fluorescence scans

In conventional luminescence spectrometry:

1) an emission spectrum can be monitored by scanning the emission wavelength (λ_{em}) while the luminescent compound is excited at fixed excitation wavelength (λ_{ex}).

2) On the other hand, an excitation spectrum can be obtained by scanning the excitation wavelength (λ_{ex}) while the emission is monitored at a given emission wavelength (λ_{em}).

3) Another suggested possibility consists of simultaneously varying both the excitation and emission wavelengths. This technique has several variants, depending on the scan-rates of the two monochromators. If the scan-rate is constant for both monochromators, a constant wavelength interval ($\Delta\lambda$) is kept between (λ_{em}) and (λ_{ex}), the technique is known as " synchronous excitation fluorescence spectroscopy " and was introduced by Lloyd in 1971 [36, 37].

Synchronous fluorescence scan (SFS) provides: (a) narrowing of spectral band, (b) simplification of emission spectra and, (c) contraction of spectra

range, compared to conventional excitation or emission spectrum. As a result, the selectivity for individual components is considerably improved and much additional information on mixtures of fluorescent compounds is gained. The SFS method is a very simple and effective means of obtaining data for several compounds present in mixture in a single scan. These techniques are explained below with reference to a simplified Jablonski diagram Figure 2.7. A molecule can be excited in the whole absorption band starting from wavelength A1, A2,…., A9 and could give fluorescence in the wavelength F1, F2,…., F9. Generally, the fluorescence emission spectrum of a fluorophores remains unchanged, irrespective of the excitation wavelength, except for a variation in the fluorescence intensity, which depends on the probability of the electronic transition of the molecule.

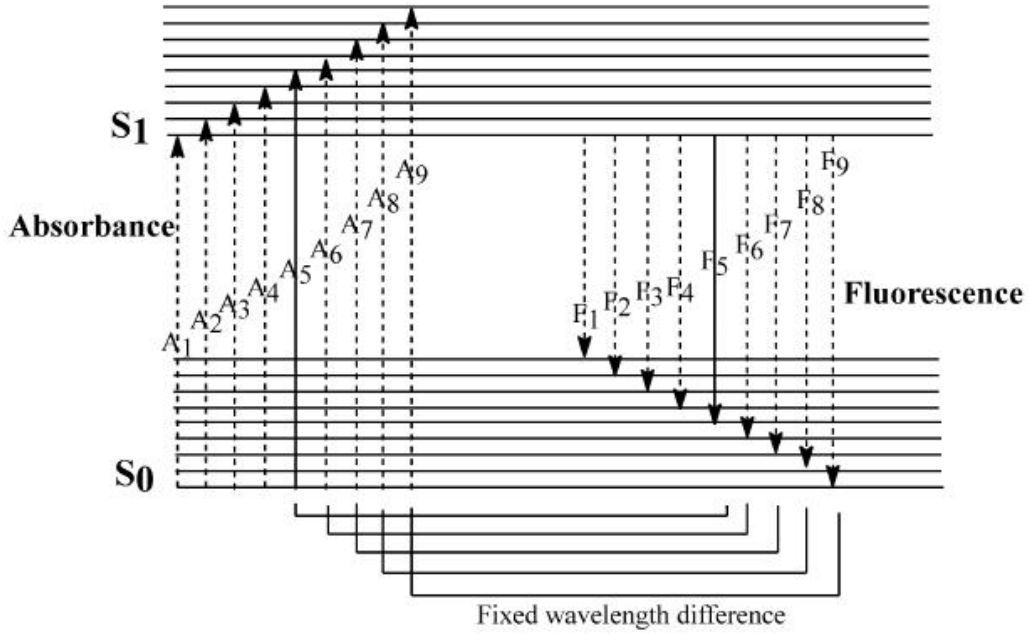


Fig 2.7: Jablonski diagram provides explanation of synchronous fluorescence scan. [38]

To obtain a fluorescence emission spectrum, the molecule is generally excited at its absorption maximum (A5) and fluorescence is collected in all the emission wavelengths, i.e. F1, F2, F3,……, F9.

A fluorescence excitation spectrum is obtained by exciting the molecule at all possible excitation wavelengths, e.g. A1, A2,…,A9 fixing fluorescence only at the emission maximum (F5).

In the case of SFS, a particular wavelength interval is chosen, so that a signal is observed only when $\Delta\lambda$, matches the interval between one absorption band and one emission band. Therefore, initially, e.g. taking $\Delta\lambda$

= A5~F5, no fluorescence will be seen until the excitation monochromator is at A5 and fluorescence wavelength is at F5. In the next moment, the molecule will be excited at A6,…, A9, and corresponding fluorescence will be recorded at F6,…., F9, respectively. This process continues till a full spectrum is recorded.

The comparison of fluorescence excitation, emission and synchronous spectra of representative perylene are shown in Figure 2.8. Because of its sharp, narrow spectrum, SFS serves as a very simple, effective method of obtaining data for several compounds in a single measurement [38, 39].

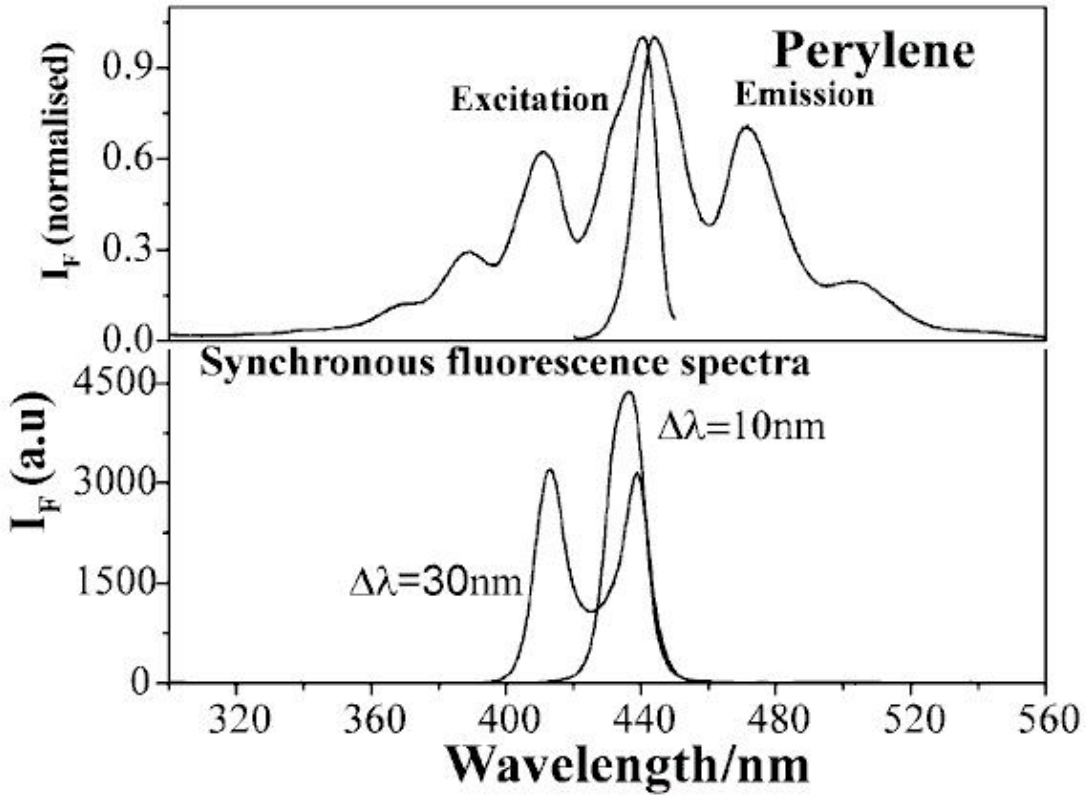


Fig 2.8: Excitation, emission, synchronous fluorescence spectra of perylene [38].

It is important to note that, the chosen Δλ is very important. It gives fluorescence band or excitation band or both. Therefore, for any multi component system, Δλ is empirical and chosen by trail and error.

CHAPTER 3

REVIEW OF RELEVANT LITERATURE

3.1 Introduction

The common procedure for detecting premalignant lesions consists of visual inspection, followed by biopsy of any suspicious lesions found. However, benign lesions-which are very common and diverse (lichen planus, Candida infections, inflammation, hyperkeratosis, ulcerations and so on) - may present very similar to early malignant or premalignant lesions, which makes it difficult to distinguish them even for experienced clinicians. Therefore, a technique that can distinguish between different lesion types in a reliable and non-invasive way would be very useful.

The last decade has seen the development of several new promising optically based technologies which are used adjunctively with white light endoscopy to enhance detection of dysplasia. These emerging technologies are based on the relative differences in the way light interacts with normal and abnormal tissues which during disease transformation acquire altered optical properties. While conventional endoscopy is limited to detecting lesions based on gross morphological changes, these new optically- based methods collectively offer a new strategy for endoscopic detection, with the potential of detecting the very earliest changes at the micro structural, biochemical and molecular levels in real-time [37].

Optical diagnosis is being carried out by a number of researchers using different methods. These include:

I Laser or Light-induced fluorescence (LIF).

(a) Labeled fluorescence by incorporating a biomarker.

(b) Autofluorescence of tissue.

(c) Autofluorescence of blood components and urine.

(d) Synchronous Luminescence of tissue or body fluid.

II Raman Spectroscopy in the ultraviolet or visible laser.

III Light Scattering and reflection spectroscopy .

Most of the work had been done on tissue and the following is the summary of work done by others in this line.

One of the earliest studies that described the use of autofluorescence spectroscopy to differentiate between normal and malignant rat kidney tissues was published by Tato et al. in 1986 [38]. This work was later extended *in vitro* human tissue [38].

During the past several years there has been a growing interest in laser fluorescence detection of tumors in which a kind of fluorescent porphyrin compounds called HpD (hematoporphyrin derivatives) are commonly used

. The specific optical spectrum of a tissue sample contains information about the biochemical composition and/ or the structure of the tissue, both of which undergo a change during malignant transformation. These changes are detectable as an alteration in the fluorescent spectra [40, 41, 42 and 43]. The groups of Alfano [40], Lohmann [44], Profio [45] and Yong [52] did pioneering *in vitro* and *in vivo* studies of human and animal tumor autofluorescence. [46, 47]

Though, a large number of studies have been carried out on tissue, only a few results are available on cancer diagnosis based on body fluids.
Some of the studies carried out using different methods are summarized below.

3.2 Light- induced Fluorescence (LIF)

- **Labeled Fluorescence:**

Ankerst and co workers' [48] investigated 15 types of tissue including inoculated tumor from rats at different delays after HpD injection. In this study, they used Nitrogen laser excitation and they observed the fluorescence (630 nm and 690 nm) of HpD as markers of cancerous tissue.

Profio [49] used Krypton ion laser 413 nm to get the fluorescence of hematoporphyrin derivative to detect fluorescence by an image intensifier

or photomultiplier tube. A ratio fluorometer and spectrum analyzer were used to measure the fluorescence intensity and spectrum of HpD labeled fluorescence and autofluorescence of tissue. He determined the optimum time after injection for best contrast, and the maximum allowable dose of HpD.

An optical diagnostic procedure based on laser-induced fluorescence (LIF) has been developed for direct *in-vivo* cancer diagnosis without requiring biopsy. Endogenous fluorescence of normal and malignant tissues was measured directly using a fiber optic probe inserted through an endoscope.

Dido [50], used a computerized fiber-optic spectrofluorometer based on a multi-frequency Nd:YAG laser for tissue fluorescence registration *in vivo* and *ex vivo*. The fluorescence spectra from different types of human tumors after i/v injection with fluorescein or topical application of ALA were studied. A simple model of Nd:YAG laser system for tumor fluorescence diagnosis had been elaborated. Advantages of the laser fluorescence diagnosis of malignant tumors by solid state multi-frequency Nd:YAG laser and the increase in accuracy and specificity of this method were discussed.

- Autofluorescence of Tissue:

Autofluorescence of tissues is produced by fluorophores that naturally occur in living cells after excitation with a suitable wavelength. The fluorophores can be located in the tissue matrix (e.g. collagen, elastin, keratin) or in cells e.g. NADH.

Autofluorescence from malignant tumor was observed by Policard as early as in 1924 [51]. Japanese scientists reported that they observed yellow autofluorescence when gastric cancer was irradiated with Ar ion laser and suggested its use for cancer diagnosis.

Yong [52], used 365 nm line of pulsed Xenon ion laser as the excitation source. They measured more than 50 specimens of different kinds of tissue; each specimen was measured within 4h after its removal from human body. The fluorescence spectra of cancer tissues usually show characteristic peaks in the red region around 630 nm and or 690 nm , which did not appear in the spectra of the corresponding normal tissues. This characteristic fluorescence can be taken as a criterion for cancer diagnosis; a consistency of 89% with the traditional biopsy method had been obtained in preliminary clinical application to diagnosis of cancer.

Schomacker et al.[53] had shown that the decrease in collagen and the increase in NADH and hemoglobin in cancer tissue had significant effects on tissue. Autofluorescence changed when a 337 nm nitrogen laser was used as the excitation light source and red fluorescence was the main component.

Kolli et al. [54] studied the use of autofluorescence spectra for the detection of neoplastic upper aerodigestive mucosa. They studied 31 patients with 27 malignant tumors and 4 potentially premalignant lesions, and found significant differences in fluorescence intensity ratios between healthy mucosa and the lesions**.**

Gillenwater et al.[55] recorded oral mucosa autofluorescence spectra from 8 healthy volunteers and 15 patients with premalignant or malignant lesion. For lesions, they noticed a decreased intensity in the blue spectral regions, and an increased fluorescence around 635 nm (porphyrin -like fluorescence). Based on the ratio between these values, they achieved a sensitivity of 82% and a specificity of 100% for distinguishing lesions from healthy mucosa.

Dhingra et al.[56] used laser-induced autofluorescence for examination of oral neoplastic areas of oral mucosa. They found that the difference was

most marked at two intensities, namely 370 nm and 410 nm, and they also noted an increase in fluorescence in the red wavelength (>600 nm) in malignant areas. This was belived to be due to the higher concentration of endogenous porphorins.

Ingrams et al. [57] used an *in vitro* study to compare clinically suspicious sites with clinically normal areas and compared them with histopathology. They showed that the differences were most marked between normal and abnormal tissue (dysplastic or malignant) at the excitation wavelength of 410 nm. At this wavelength there was an increase in fluorescence above 600 nm in the dysplastic tissue.

Muller et al [58], used three spectroscopic techniques (fluorescence, reflectance and light scattering spectra) to study the early biochemical and histological changes in oral tissue. They analyzed a varying degrees of malignancy (normal, dysplastic, and cancerous sites) and healthy samples. Tissue fluorescence spectra were excited with 337 nm and 358 nm laser light. Diffuse reflectance spectroscopy was used to provide information regarding tissue absorption and structure by measuring the wavelength-dependent absorption and scattering coefficients. With this type of three model spectroscopy, the specificity was about 83%.

- Autofluorescence of Blood Components and Urine:

Xu et al [59], worked upon the fact that anomalous molecules in cancer cell may enter into human blood and they found distinct difference between the fluorescence of serum from healthy volunteers and from the cancer patients. They took the absorption, excitation and fluorescence spectra by means of spectrophotometer. When the serum was excited by 290 nm light, they found an intense emission band around 340 nm in fluorescence both for healthy person and cancer patient. But if they excited the sample with 450 nm light, they observed 2 emission bands: a principle band at 630 nm and second very weak band at 690 nm for cancer patient, where only a weak diffused band was observed in red region for healthy person. Thus these bands at 630 nm and 690 nm constituted the characteristic fluorescence of cancer patient's serum. This group had examined 110 cases, by this method, with 94.5% accuracy. However, though cancer could be detected by this method, but the stage of cancer failed to be determined.

Wolfbeis and leiner [82] have studied the fluorescence of human serum as a function of different excitation wavelengths. They have adopted a topographical representation to elucidate the different fluorophores such as elastin, collagen, NADH, flavins, etc. Hubemann et al [60] observed distinct differences in the sera of healthy subjects and cancer patients. According to

their findings, tryptophan, an amino acid, is elevated in the blood of cancerous patients.

Madhuri et al [61], worked with native fluorescence spectroscopy of biomolecules in both cells and tissues to discriminate between cancer and normal tissue using ultraviolet fluorescence. They observed the characteristic of blood plasma of normal and cancerous subject. The samples were excited in UV region between 250-340 nm, and among these various excitation wavelengths, emission spectrum at 300 nm excitation had considerable difference between blood plasma of normal and cancer subjects. The ratio of fluorescence intensities at 340 and 440 nm was calculated to quantify if there was any diagnostic potential value. The ratio of 340/440 values of normal blood plasma was less than 11 and for tumor, it was greater than 11. Besides, for blood plasma for cancer patients the ratio value varied from 11-28, depending on the stage of malignancy.

Madhuri et al. [62], used native fluorescence characteristics of blood plasma to discriminate patients with liver abnormalities. The average fluorescence emission spectra of normal and diseased subjects proved to be different. The source of two emission scans at 405 and 420 nm was spectrofluorometer, but at 405 nm excitation showed more distinctive spectral differences between normal and cancer subjects and was thus

chosen for further analysis. There technique resulted in sensitivity and specificity of 100% each for cancer diagnosis.

Karthikeyan et al [63], used fluorescence spectroscopy to study the fluorescence of blood samples in skin carcinoma bearing mouse model to discriminate from their respective normal. The blood was collected with EDTA from eye vein using heparinized capillary tube, and The separated plasma, erythrocytes and erythrocyte membrane were separated by centrifuging with acetone. These fraction were subjected to fluorescenct spectral analyses at 400 nm excitation using a spectrofluorometer for normal and cancerous mouse. The normal plasma and erythrocytes showed a prominent peak at 430 nm which decreased at longer wavelengths. The erythrocyte membrane of both samples showed a maximum intensity at 440 nm and a second peak at 630 nm while erythrocyte membrane of the cancer sample showed a secondary peak at 540 nm. The cancer plasma and erythrocytes showed a peak at 630 nm, although the normal plasma did not show a peak at 630 nm. The intensity (FI) for the acetone extracts of cancer erythrocytes fluoresce more than its plasma. This suggests that there is a definite relationship between the erythrocyte and the fluorophores at 630 nm. The hemolysate of healthy and cancer samples behaved similarly and were comparable with normal plasma and erythrocytes. Four peaks at 470 nm, 520 nm and 630 nm NADH, various flavins and porphyrins are

responsible, respectively. The ratio of fluorescence intensity at 630 nm/590nm was found to be significantly increased in plasma and erythrocytes of cancer samples.

A few landmark papers in this line were from Masilamani et al [64 and 65] where the higher concentration of porphyrin found in acetone extracts of formed elements and blood plasma in cancer patients compared to the healthy blood were taken as a measure of cancer indication.

- Synchronous Luminescence of Tissue or Body fluid:

Advanced techniques such as synchronous fluorescence are currently being developed to further improve the optical diagnosis method.

The synchronous luminescence spectroscopic studies were carried out on human tissues by Majumada and Gupta [66]. They measured the synchronous luminescence spectra from the pathologically characterized human breast tissue samples obtained after resection at surgery from patients with breast tumor, and used 20 nm wavelength separations between the excitation and emission wavelength. The results from cancerous tissue were compared with those from normal tissue. In general four narrow spectral bands with peaks around 300, 400, 450 and 525 nm were characteristics of cancer.

Vo-Dinh [67], described the principle of synchronous luminescence (SL) for use in biomedical diagnostic. The SL method involves scanning simultaneously both emission and excitation wavelengths while keeping a constant wavelength interval between them. This SL procedure simplifies the emission spectrum and provides for greater selectivity and was used to detect subtle differences in the fluorescence emission of the biochemical species of cells and tissues. The SL method could be used to analyze tissue *in vivo* or to investigate spectral differences in normal and neoplastic cells *in vitro*. SL scans of skin tissues illustrate the use fullness of the method. For *in vitro* diagnosis, differences between the fluorescence spectra of the normal rat liver epithelial (RLE) and hepatoma cell lines were detected using synchronous fluorescence. The potential use of SL as a screening tool for cancer diagnosis was discussed.

The characterization of fluorescence spectra of normal, premalignant and malignant cervical tissues had been studied by using synchronous luminescence spectroscopy (Vengadesan et al [68]). SL fluorescence spectra were measured by scanning both excitation and emission monochromator simultaneously with a wavelength difference of 20 nm. The SL spectra of different kinds of cervical tissues (normal, pre-malignant and malignant) showed the distinct peaks around 300, 350, and 525 nm with broad peak around 460 nm. These may be due to tryptophan, collagen and flavin, respectively. The broad band around 460 nm may be due to

presence of pyridoxal phosphate, carotenes and lipopigments. The spectral data was evaluated by both empirical and statistical analysis. Among the various analyses, partial least square analysis provided better accuracy than other analysis in the discrimination of normal from abnormal tissues.

Masilamani et al [69], found that synchronous spectra of blood plasma gives quite reliable distinction comparable to the acetone extract of formed elements reported earlier (Masilamani et al [65]). In this way many emission components could be brought into prominence. This is something like analyzing the sample, section by section, and from this point of view it is comparable to Computer Aided Tomography (CAT).

3.3 Endogenous Fluorophores

All biological tissues emit fluorescence when excited by UV or visible light. This fluorescence is emitted by naturally-occurring fluorophores and is therefore often called autofluorescence.

It has already been reported that early cancerous tissues have a different autofluorescence emission spectrum than the healthy tissues. Although the origin of the autofluorescence itself is usually related to the structure of

tissues or metabolic processes.[70, 71] The reasons behind the modifications of the autofluorescence spectra in early tumors remain controversial.

The molecules responsible for autofluorescence of tissues have recently been reviewed by Richards-Kortum and Sevick-Muraca [70] and the corresponding excitation and emission spectra have been published by Wagnieres et al [71].

The endogenous fluorophores can be classified in four groups, the porphyrins, the amino-acids and the proteins, the flavins and the pyridine nucleotides (NADH, NADPH)[72].

3.3.1 Porphyrins

The porphyrins emit red fluorescence when excited, and their emission spectrum bears the typical three peaks around 590 (see Figure 3.2) , 630 and 690 nm (see Figure 3.1). They have a maximum excitation around 400 nm[40, 73].

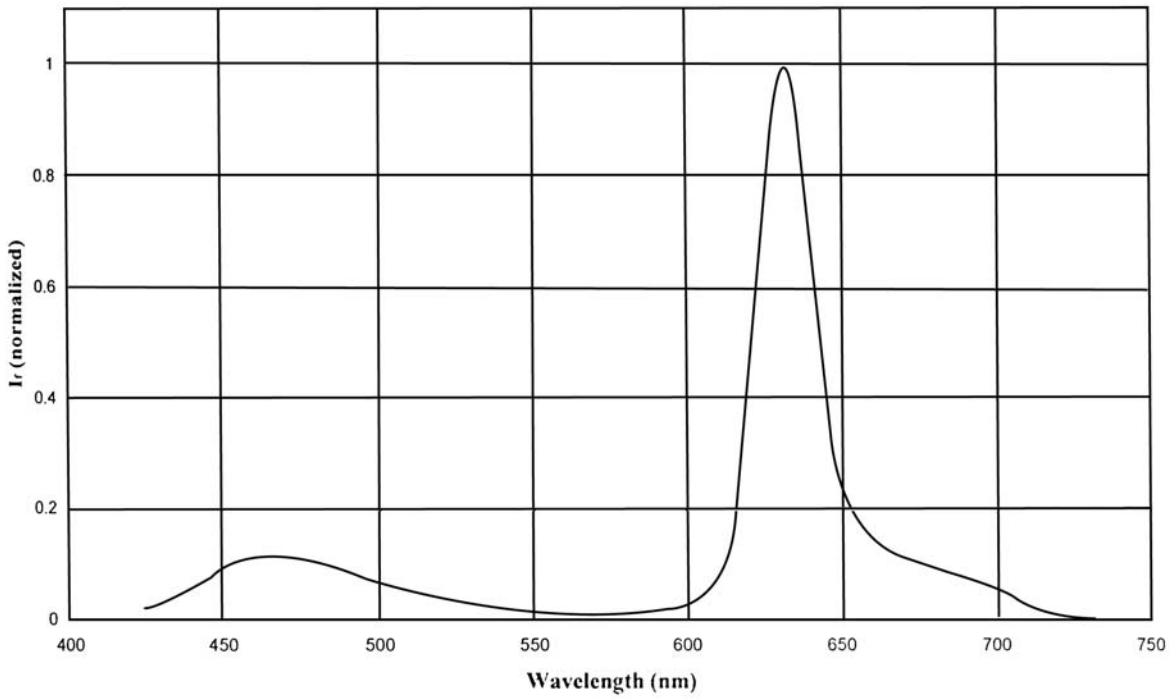


Figure 3.1 Fluorescence emission spectrum of porphyrin at λ_{ex} = 400 nm[39]

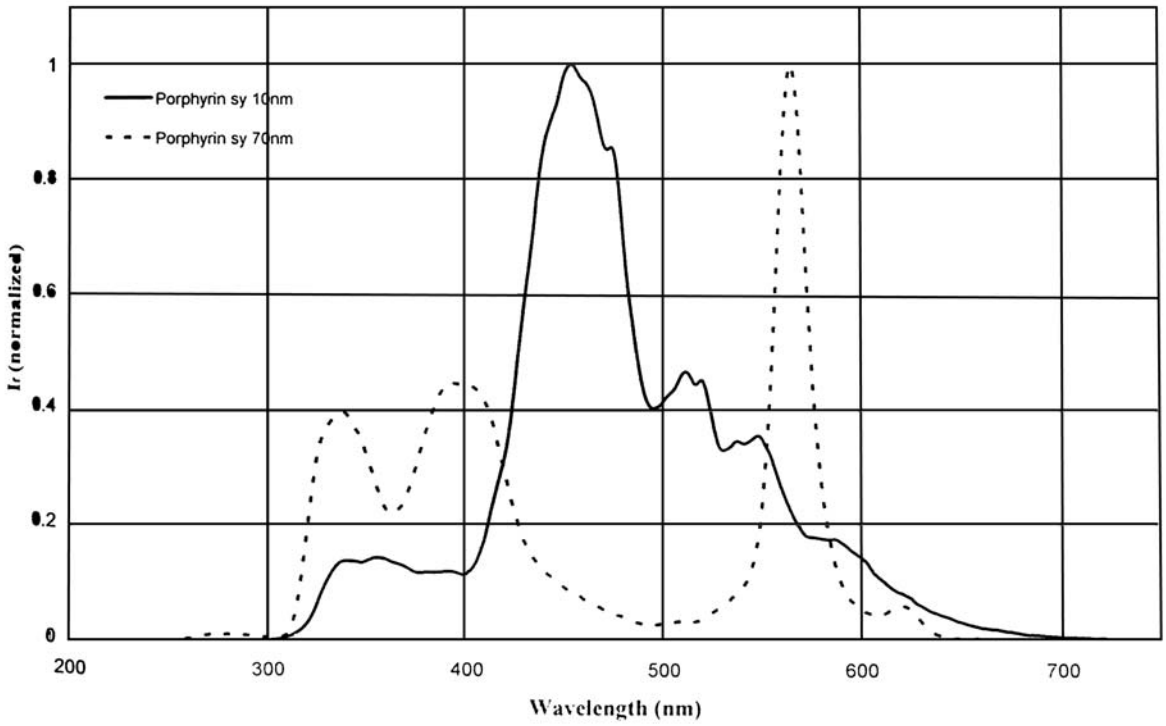


Figure 3.2 Fluorescence synchronous spectra of porphyrin at $\Delta\lambda$= 10 and 70 nm[39].

3.3.2 Amino acids

Proteins are the most essential nutrient needed by the body. They are composed of many amino acids, which are the building blocks of proteins. The fluorescent amino acids are the aromatic ones, namely phenylalanine, tyrosine and tryptophan. These three molecules absorb in the UV range and their absorption maximum is slightly red-shifted in the order Phe, Tyr, Trp (absorption maximum at 260, 275 and 280 nm respectively). These amino acids emit fluorescence around 320-350 nm (see Figure 3.3). At excitation wavelength above 295 nm only tryptophan is fluorescent. Below 280 nm, all three amino acids can be excited.[74, 75, 76]

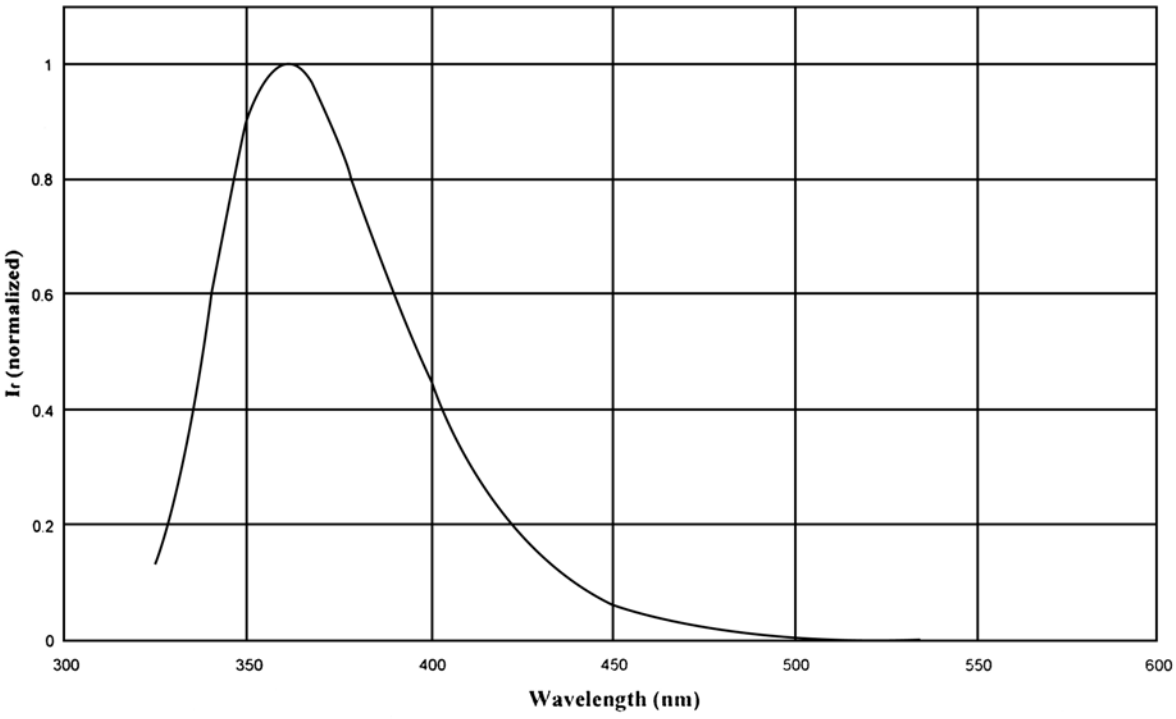


Figure 3.3 Fluorescence emission spectrum of tryptophan at λ_{ex} = 300 nm [39].

3.3.3 Structural Proteins

Connective tissues are abundant in the body and porform many functions. They binds, supports, protects, serves as frame works, fills spaces, store fat, produce blood cells, protect tissues against infection, and repairs tissue damage.

-Collagen

Collagen is an extra cellular protein that is found in the bones, skin, and other connective tissues. It is the most abundant protein in our body. It absorbs around 340 nm and emits around 380 nm[77].

- Elastin

Elastin, is a protein in connective tissue that is elastic and allows many tissues in the body to resume their shape after stretching or contracting. Elastin helps skin to return to its original position when it is poked or pinched. It absorbs around 350 nm and emit around 410 nm[77].

3.3.4 Coenzymes: NADH, Riboflavin, FMN and FAD

Reduced form of Nicotinamaide Adenine Dinucecleotide (NADH) is highly fluorescent biomolecule. NADH and NADPH, they have an

absorption maximum at around 340 nm and emit fluorescence around 450 nm (see fig 3.4).

The spectra of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) and riboflavin are similar. They all have an absorption maximum around 450 nm and an emission maximum around 515 nm (see figures 3.5 and 3.6). Riboflavin is a water soluble vitamin and is important for body growth and red blood cell production and helps in releasing energy from carbohydrates[70, 77].

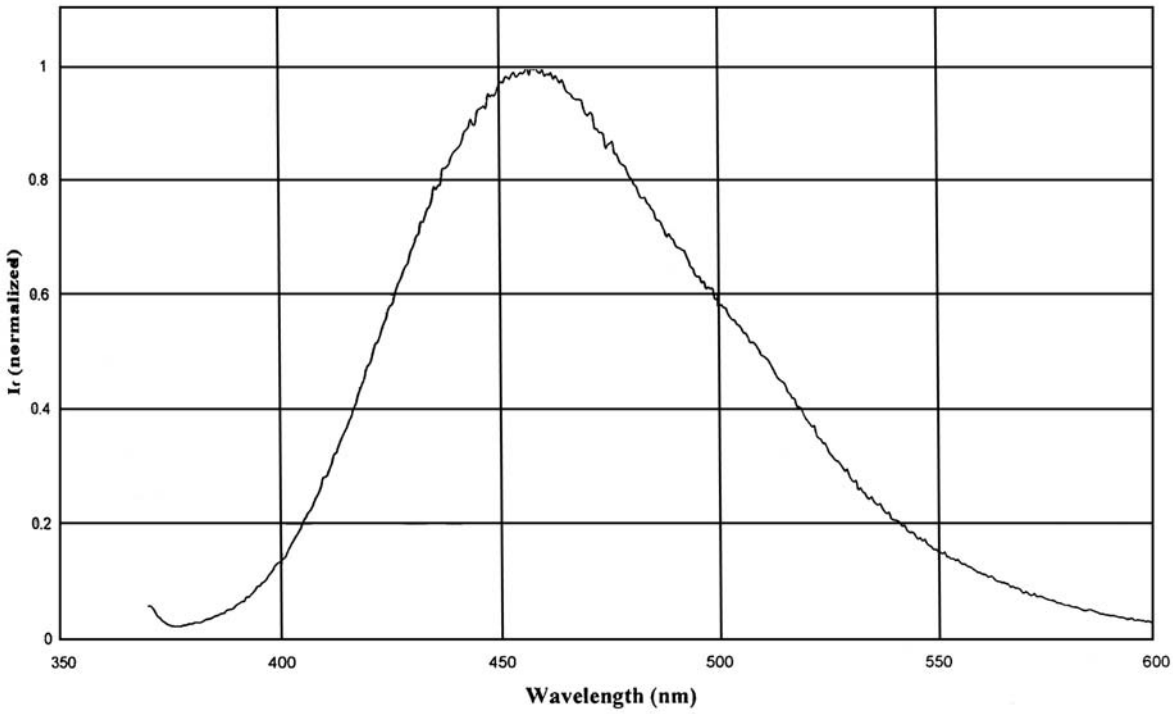


Figure 3.4 Fluorescence emission spectrum of NADH at λ_{ex} = 350 nm [39].

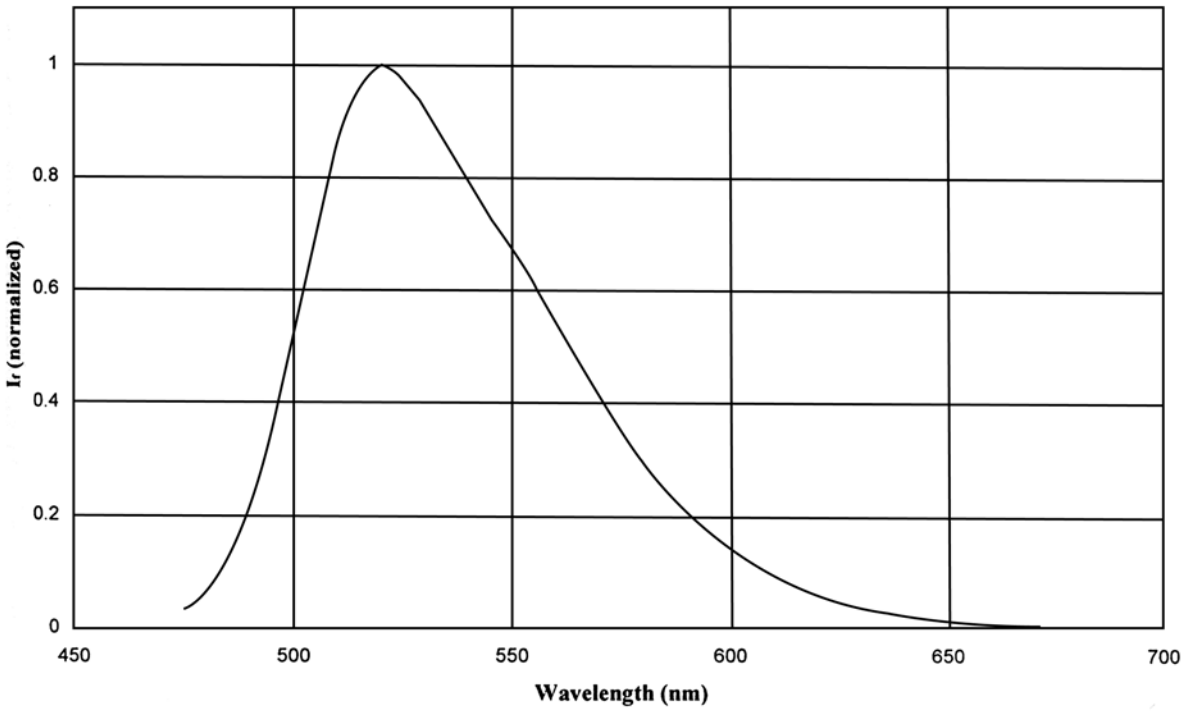


Figure 3.5 Fluorescence emission spectrum of flavin at λ_{ex} = 450 nm [39].

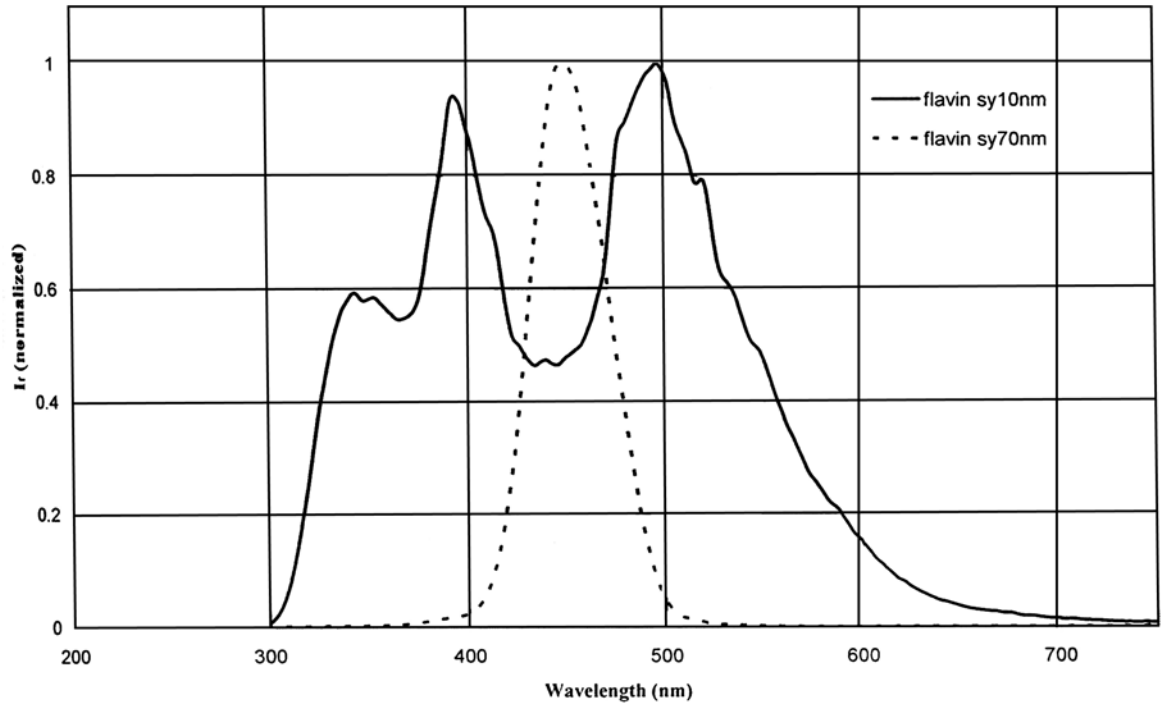


Figure 3.6 Fluorescence synchronous spectrum of flavin at $\Delta\lambda$ = 10 and 70 nm [39].

CHAPTER 4

INSTRUMENTATION

4.1 Introduction

In this chapter the instrumentation, methods and materials used for this investigation are presented. Two types of spectrofluorometer: Perkin Elmer LS50B and Shimadzu RF-5301PC, both having similar features were used. The experiment with the blood analysis were carried out using Perkin Elmer LS50B, but it was not available for the second part Shimadzu RF-5301PC was used. Hence technical features of both are given below.

4.2 Fluorescence Instrumentation

4.2.1 Perkin Elmer

Perkin Elmer LS50B Luminescence spectrometer [Figure 4.1] was used to obtain spectra of excitation, emission and synchronous fluorescence.

In this instrument, the excitation source is a special xenon flash tube which produces an intense, short duration pulse of radiation over the spectral range of the instrument. The path of the radiation is present in Figure 4.1a. Energy from the source is focused by the ellipsoidal mirror M (E) 5 and reflected by the toroidal mirror onto the entrance slit of the Excitation monochromator. The monochromator consists of the entrance slit, a 1440 lines per millimeter grating, a spherical mirror and an exit slit. A narrow wavelength band emerges from the exit slit, with the centre wavelength being determined by the setting of the grating, the angle of which is

controlled by a stepper motor. The majority of the excitation beam is transmitted to the sample area via the focusing toroidal mirror M(T)1, a small proportion is reflected by the beam splitter onto the reference photomultiplier. To correct for the response of the reference photomultiplier a rhodamine correction curve is stored within the instrument.

Figure 4.1 Perkin LS50B Luminescence Spectrometer.

Rhodamine dye absorbs energy from 230 to 630 nm and fluoresces at about 650 nm with nearly constant quantum efficiency.

Energy emitted by the sample is focused by the toroidal mirror M(T)11 onto the entrance slit of the Emission monochromator. The monochromator consists of the entrance slit, a spherical mirror M(S)31, a 1200 lines per millimeter grating and the exit slit. A narrow wavelength band emerges from the exit slit, with the centre wavelength being determined by the setting of the grating, the angle of which is controlled by a stepper motor.

The Excitation and Emission monochromators can be scanned over their ranges independently, synchronously or driven to selected points in their ranges.

Synchronous scanning can be either a fixed wavelength difference or a fixed energy difference between the excitation and emission monochromators. The spectral ranges of the monochromators are:

Excitation monochromator 200nm to 800nm and zero order

Emission monochromator 200nm to 900nm and zero order

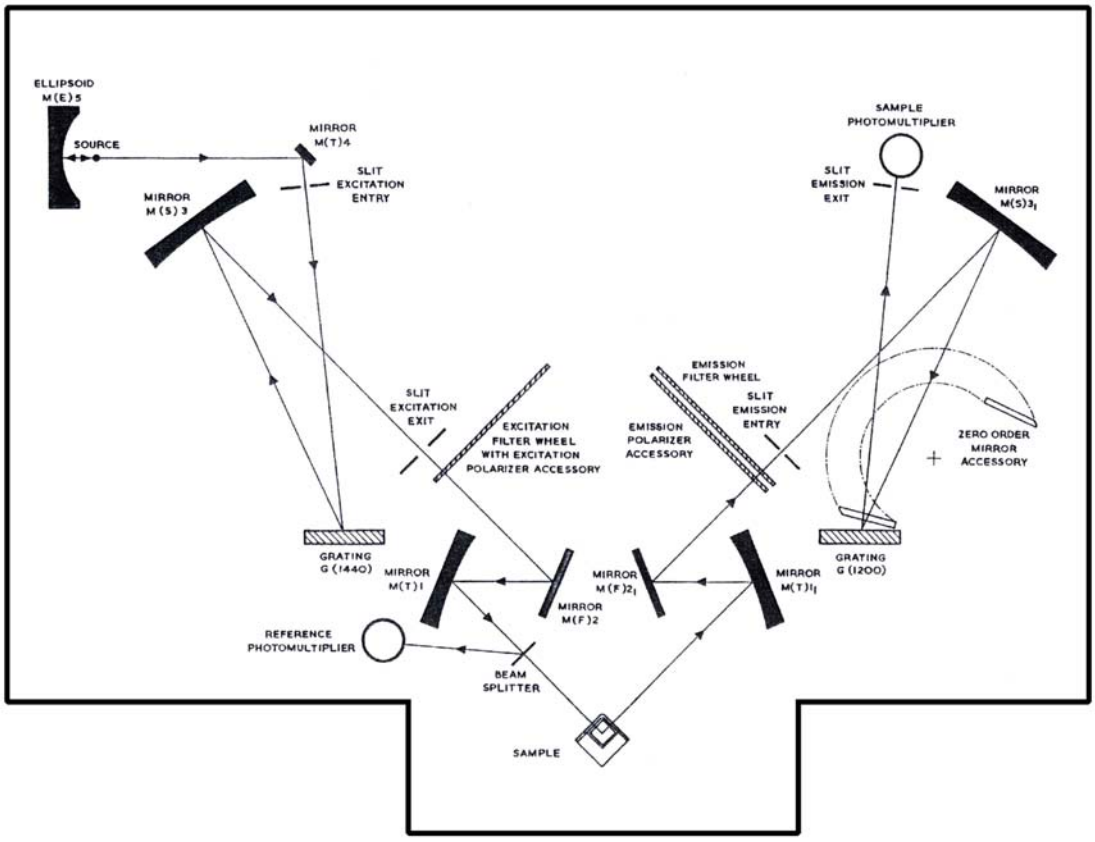


Figure 4.1a. The scheme of Perkin Elmer LS50B luminescence [78].

4.2.2 Shimadzu spectrofluorophotmeter

Shimadzu RF-5301PC spectrofluorophotmeter (Figure 4.2) was used to obtain fluorescence emission, excitation and synchronous.

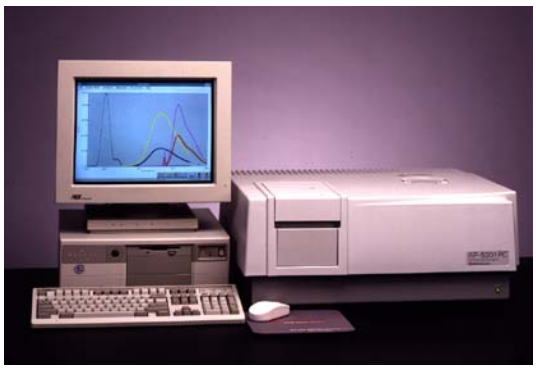


Figure 4.2 Shimadzu RF-5301PC spectrofluorophotmeter

The optical system of the RF-5301PC instrument is illustrated in Fig 4.2a. A 150 W Xenon lamp (1) serves as light source. The uniquely designed lamp housing contains generated ozone in it and decomposes the ozone by means of the heat produced by the lamp. The bright spot on the Xenon lamp is magnified and converged by the ellipsoidal mirror (2) and is further converged on the inlet slit of the slit Assy (see excitation side) (3) by the concave mirror (4). A portion of the light isolated by the concave grating (1300 lines/mm) (5) passes through the outlet slit; travels through the condenser lens (11) and illuminates the sample cell. (The concave grating in both the monochromators is a highly-efficient ion-blazed holographic

grating, a product of Shimadzu's unique optics technology). To achieve light-source compensation, a portion of the excitation light is reflected by the beam splitter quartz plate (6) and directed to the Teflon reflector plate 1 (7). The diffusely reflected light from the reflector plate 1 (7) then passes through the aperture for light quantity balancing (21) and illuminates the Teflon reflector plate 2 (8). Reflected by the reflector plate 2 (8), the diffuse light is attenuated to a specific ratio by the optical attenuator (9) and then reaches the photomultiplier for monitoring (10).

The fluorescence occurring on the cell is directed through the lens (13) to the emission monochromator that comprises the slit Assy (14) and the concave grating (15). Then, the isolated light is introduced through the concave mirror (16) into the photomultiplier for photometry (17) and the resultant electrical signal is fed to the preamplifier.

Wavelengths scan range	220-900 nm and zero-order light.
Measurement wavelength range	220-750 nm and zero-order light.

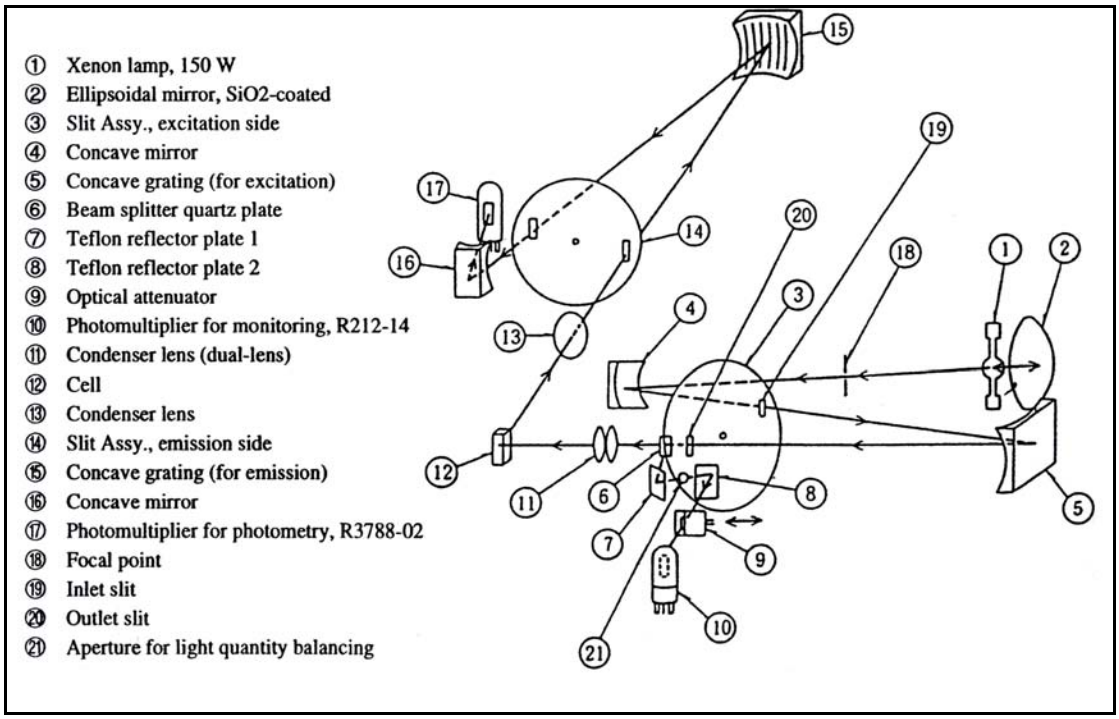


Figure 4.2a Optical System of RF-5301PC [79].

4.3 Materials and Methods

(a) Blood Samples

About 3ml of venous blood samples of 25 healthy volunteers and 50 cancer patients of known etiology and stage (by histopathology), were collected from Riyadh Medical complex in tubes containing EDTA (ethylene diamine tetra-acetic acid), as a anticoagulant. The blood was centrifuged at 3500 rpm for 25 minutes and the plasma was obtained as a supernatant. The plasma was carefully removed in another clean and dry tube and was analyzed using spectrofluorometer.

One ml of formed elements (cellular content of blood) from the bottom layer was added to 2ml of acetone and mixed well. Acetone extracts fluorophores separately from the formed elements of blood which settles at the bottom. The mixture was centrifuged at 3500 rpm for 25 minutes. And a clear supernatant containing most of the bio-fluorophores was obtained. The supernatant was analyzed using spectrofluorometer.

Twenty-five samples from healthy volunteers and fifty samples from cancer patients of different etiology and different stages were used for this experiment. The samples from cancer patients were from both sex with different kind of cancer including thyroid, pancreas, stomach, breast, branchial tumor, and the rest were of miscellaneous etiology. Same preparation was done for each sample.

Type of cancer	**No. of samples**
Thyroid	8
Pancreas	10
stomach	10
Breast	20
Branchial tumor	2

table 4.1 Samples details.

(b) Urine Samples

Urine samples were collected [first thing in the morning (first urine)] from 20 healthy volunteers (controls) and 25 cancer patients of different etiology and stage.

2ml of untreated urine was drawn into the quartz fluorescence cuvette. By using Shimadzu RF-5301PC spectrofluorophotmeter each normal urine and sample from the cancer patients, were excited with different wavelengths λ_{ex} = 325, 350, 400 nm to obtain the fluorescence emission spectra (FES). Each fluorescence spectrum was taken with range starting from λ_{ex} +25nm up to $2\lambda_{ex}$ -25 nm. Result obtained give a clear difference between samples from normal and from cancer patients with λ_{ex} = 400 nm and 450 nm.

Fluorescence synchronous spectra (FSS) were obtained by using an offset of $\Delta\lambda$ =10, 30, 70 nm the range of 250nm – 750 nm.

A mixture of ethyl acetate and acetic acid (3:1 v: v) was prepared and the urine extracts were obtained by adding 2ml of this mixture to 1ml of urine and mixed it very will. A clear supernatant that contained most of the bio-fluorophores was obtained. The supernatant was analyzed using Shimadzu

RF-5301PC spectrofluorophotmeter. Fluorescence emission spectra at 400 nm were obtained with the range from 425nm to 775nm.

4.3.1 Methods of analyzing blood samples

Perkin Elmer (LS50B) spectrofluorometer was used as for analysis of blood samples.

Different wavelengths (λ_{ex} = 300, 325, 350, 400, 425, 450, 475, 488, 514nm) were used to excite the samples to obtain the fluorescence emission spectra (FES). Each fluorescence spectra had a range from λ_{ex} +25nm up to $2\lambda_{ex}$ -25 nm. From these different wavelengths only 400 nm gave a clear difference between formed elements acetone extract samples from healthy and cancer patients. For plasma, FES with excitation at 400 and 450 nm gave a good difference between sample from healthy individuals and cancer patients.

Plasma samples were analyzed to obtain the Fluorescence synchronous spectra (FSS) by using an offset of $\Delta\lambda$ = 10 nm with range starting from 200 nm up to 800 nm.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Introduction

Almost universally, early cancer detection is of great significance since cancers are easier to treat when detected early. Since early stage cancers are small, scientists have been trying to develop simple and inexpensive tests that are sensitive enough to detect these cancers. Such screening tests must be inexpensive and easy because performing invasive and expensive tests on otherwise healthy people would be unethical and would be impractical at the population level[80]. Blood and urine tests are a common and straight forward means of screening people for cancer in its early stages. If a chemical in the blood or urine that signals the presence of even a small tumor can be detected, the cancer could be treated sooner and would be more likely to be cured.

In this study, we have concentrated on optical diagnosis of blood plasma, formed elements and urine to exhibit characteristics spectra obtained from samples from normal individuals and cancer subjects.

5.2 Results: Blood components

5.2.1 Fluorescence emission spectral (FES) characteristic of formed element samples from normal individuals and cancer patients at λ_{ex}=400 nm

Fluorescence emission spectrum of formed element was obtained from 425 – 700 nm with 400 nm excitation wavelength.

Figure 5.1 presents typical fluorescence emission spectra of sample from healthy individual. Figure 5.1a presents typical fluorescence emission spectra of sample from cancer patient at an early stage and figure 5.1b presents a typical FES of sample from cancer patient at an advanced stage. The differences are clear just by looking at the figures. There are two well defined bands around 460 nm (due to Raman band of acetone), 630 nm (due to neutral porphyrin), and shoulder around 585 nm (due to basic form of porphyrin), as recently shown by Masilamani et al 2004[65].

Figure 5.1 The typical fluorescence emission spectrum at λ_{ex}= 400 nm for healthy formed element.

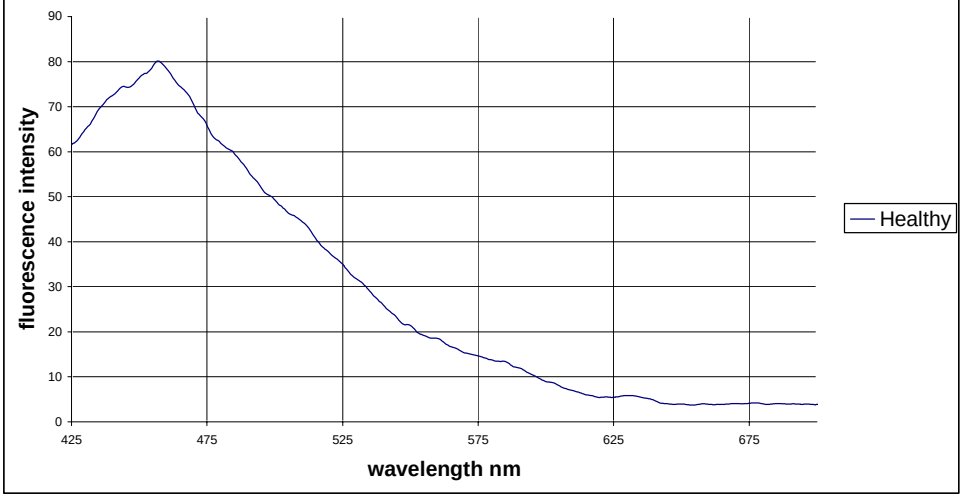


Figure 5.1a The typical fluorescence emission spectrum at λ_{ex}= 400 nm for early stage of formed element .

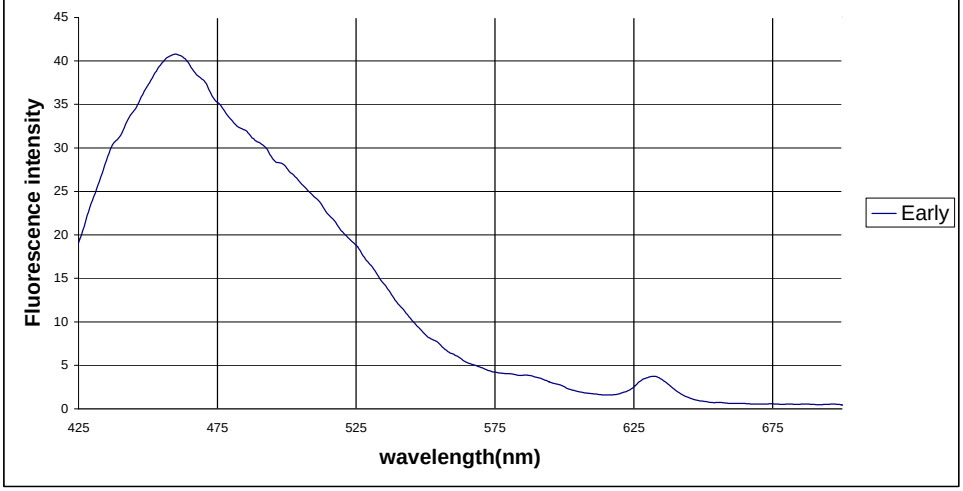
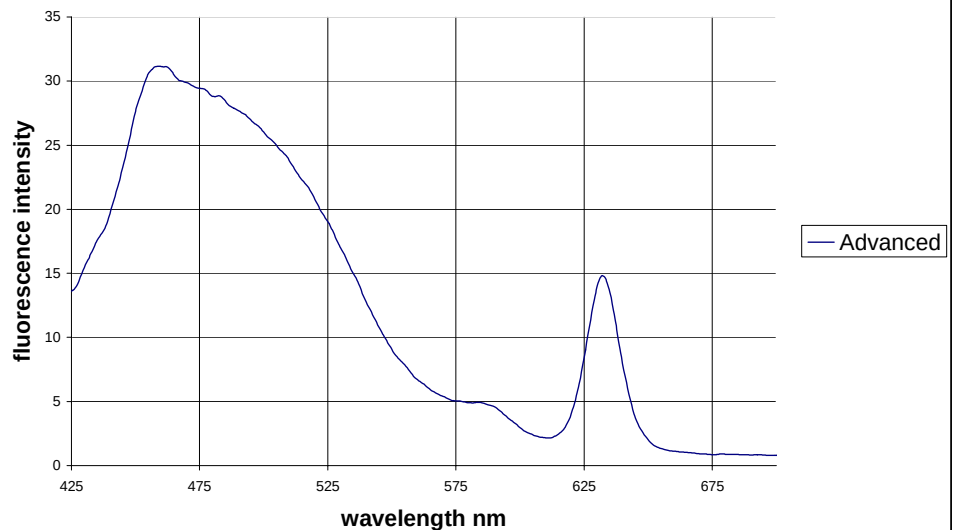


Figure 5.1b The typical fluorescence emission spectrum at λ_{ex}=400 nm for advanced stage of formed element.



Comparing these spectral bands it is observed that the ratio between the fluorescence intensity at 630 nm and that at 585 nm ($R1 = I_{630}/I_{585}$) is 0.6 for healthy, 0.9 for early and 2.9 for all advanced cases. The contrast parameter (C_1) between advanced cancer patients and healthy control is 2.9/0.6 = 4.8 and the contrast (C_2) between early cancer patient and healthy control is 0.9/0.6= 1.5. Note that, the larger the value of the contrast, the more reliable is the discrimination. These features are summarized in Table 5.1. Here the average of normal subjects [N=25] and that of early cancer [N=20] and advanced cancer [N=30] are given along with S.D (standard deviation).

Ratio	Healthy ±S.D	Early ±S.D	Advanced ±S.D	C_1	C_2
$R1= I_{630}/I_{585}$	0.6±0.12	0.9±0.3	2.9±0.49	4.8	1.5

Table 5.1 Fluorescence intensity ratio in fluorescence emission spectra at λ_{ex} = 400 nm of formed element.

From the contrast parameter it is clear that the distinction between the healthy and early cancer sample is high; and the differences between healthy and advanced cancer sample is very high. And the differences between early stage and advanced stage are high too.

It is important to note that porphyrin peak at 630 nm and shoulder at 585 nm both are elevated in all types of cancer studied here. This is same as what was observed by number of researchers on malignant tissues, both *in vivo* and *in vitro*. [40, 41, 81]

5.2.2 Fluorescence emission spectral (FES) characteristics of blood plasma at 400 and 450 nm excitation

5.2.2.1 Fluorescence emission spectral (FES) characteristic at 400 nm excitation

Fluorescence emission spectra of blood plasma were obtained at 400 nm excitation wavelength with scan range of 425- 700 nm.

Comparing the fluorescence emission spectra (FES) at λ_{ex} =400 nm of plasma samples from healthy individual with samples from cancer patients with an early and advanced stage, there are clear differences between them.

In Figure 5.2 we have two band one at 510nm and another at 470 nm. The ratio $I_{510}/I_{470} \approx 1$ for sample from healthy individual, while in Figure 5.2a, the same ratio is 2 for samples from cancer patient.

Identifying the fluorescence peak at 510nm as due to flavo protein, it is observed that flavo protein is in higher concentration than NAD(P)H at 470nm in samples from cancer patient. Here $I_{510}/I_{470} \approx 2.2$ for early cancer and $I_{510}/I_{470} \approx 2.4$ for advanced cancer patient (Figure 5.2b). The contrast (C_1) between advanced and healthy is 2.4 and the contrast (C_2) between early and advanced is 2.2. (Table 5.2)

Ratio	Healthy ±S.D	Early ±S.D	Advanced ±S.D	C_1	C_2
I_{510}/I_{470}	1±0.2	2.2±0.5	2.4±0.6	2.4	2.2

Table 5.2 Fluorescence intensity ratio in fluorescence emission spectra at λ_{ex} =400 nm of blood plasma.

Figure 5.2 the typical Fluorescence emission spectrum at λ_{ex} =400 nm for healthy blood plasma.

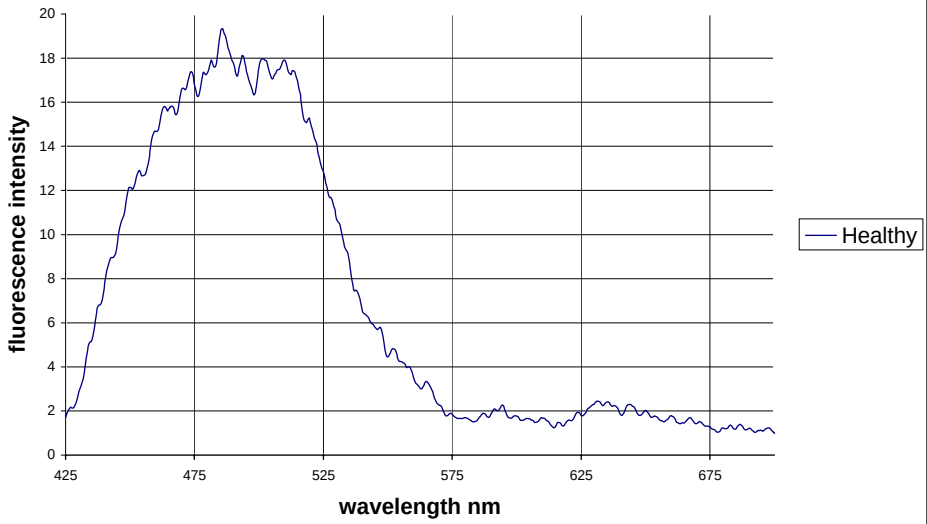


Figure 5.2a the typical fluorescence emission spectrum at λ_{ex} =400 nm of early blood plasma.

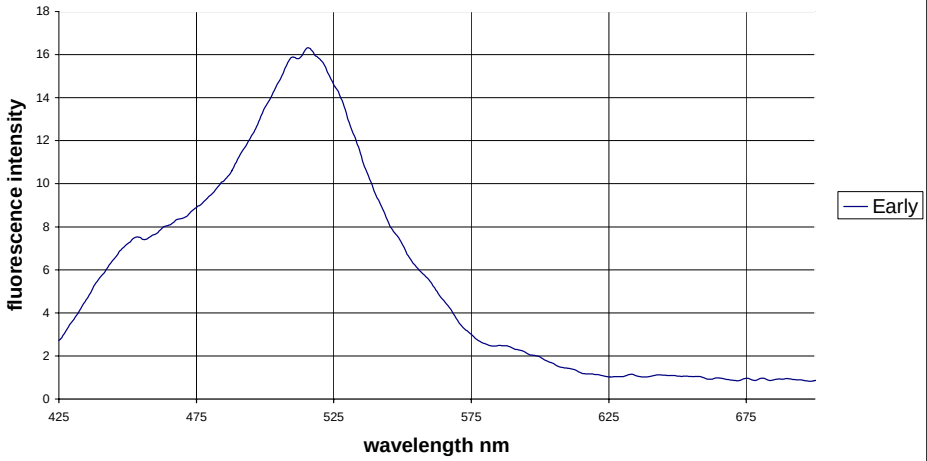
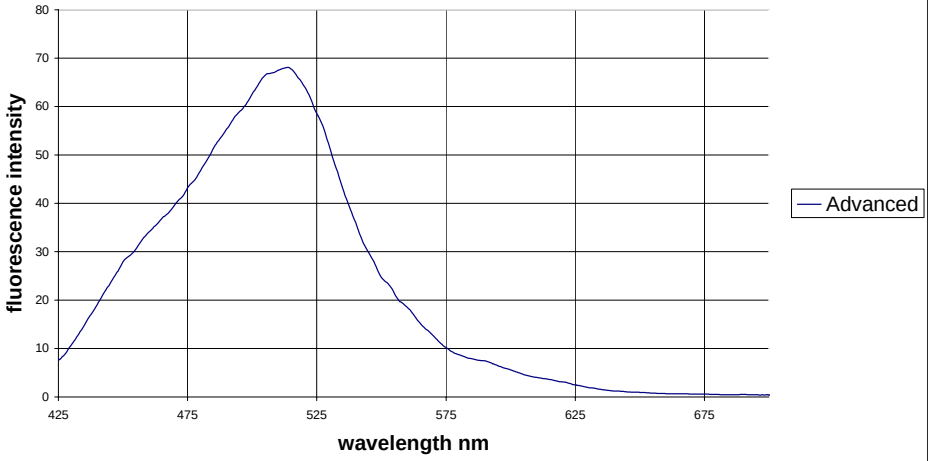


Figure 5.2b the typical fluorescence emission spectrum at λ_{ex}=400 nm for advanced blood plasma.

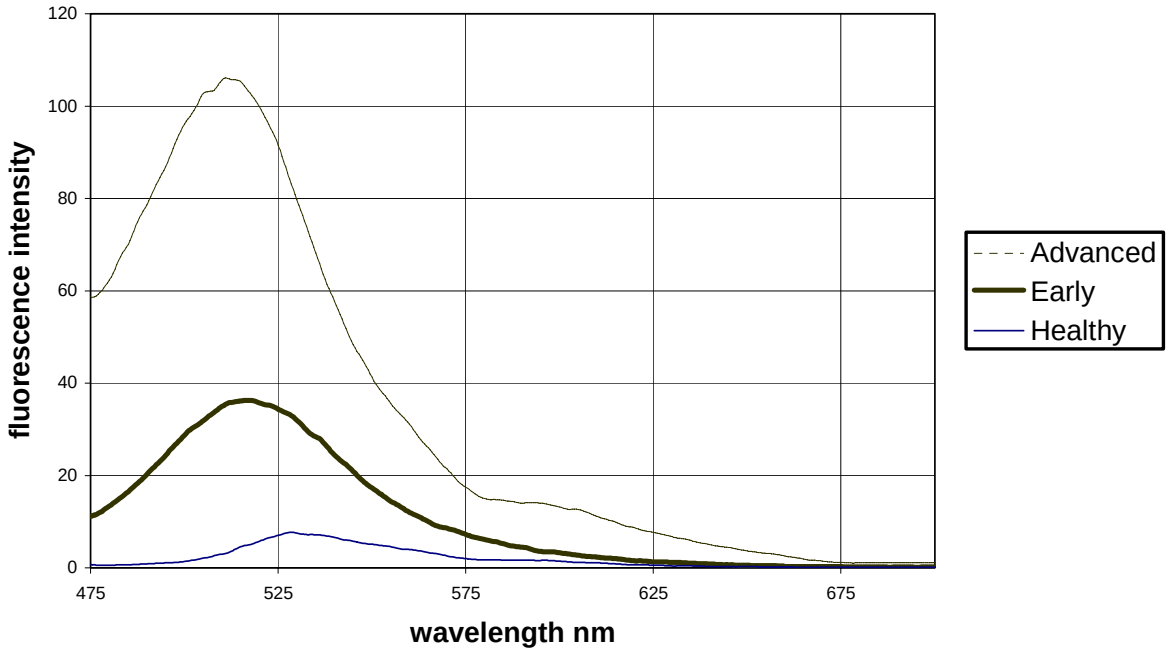


5.2.2.2 Fluorescence emission spectral (FES) characteristic of blood plasma from healthy individuals and cancer patients at 450 nm excitation

Fluorescence emission spectra of blood plasma were obtained at 450 nm excitation wavelength with scan range of 475-750 nm.

Figure 5.3 shows the typical fluorescence emission spectra of blood plasma from healthy individual and cancer patients with an early and advanced stage of disease. It is clear from the Figure that there is a band around 518 nm due to flavin, with the intensity for the healthy at 7.5 and for early at 36 and for advanced stage at 106.

Figure 5.3 the typical fluorescence emission spectra of healthy, early and advanced stage at λ_{ex} = 450 nm for blood plasma.



These results suggest that the increase in the intensity of flavin is a sign for the presence of the disease. It level increases about 4 times for early cancer and 13 times for advanced cancer.

5.2.3 Fluorescence synchronous spectral (FSS) characteristics of blood plasma from healthy individuals and cancer patients at $\Delta\lambda$ = 10 nm

The synchronous spectra of blood plasma from healthy individuals and cancer patients have been used to discriminate the spectral signatures of cancers. With the scan range from 200 nm to 800 nm, and an offset of $\Delta\lambda$ = 10 nm, the results obtained are presented in Table 5.3 .

In synchronous spectra excitation and emission grating are rotated simultaneously with a constant offset of 10 nm. This is something like analyzing the sample, section by section. So it gives better resolution and identification of weakly emitting molecules.

When the synchronous spectra of plasma from healthy individual was compared with that of the plasma from cancer patients, there were distinct and convincing evidence for the spectral signature of the disease. Figure 5.4 shows the typical synchronous spectra of plasma from healthy individual, Figure 5.4a is for plasma from cancer patient at early stage and

Figure 5.4b is for plasma from cancer patient at advanced stage. There are well defined bands around 360 nm, 450 nm, 500 nm, 585 nm, and shoulders around 550 nm and 620 nm. Out of these, 360 nm is due to tryptophan, 450 nm due to NAD(P)H, 500 and 550 due to riboflavin and bilirubin, 585nm and 620 nm due to porphyrins. The ratio between tryptophan and NAD(P)H (I_{360}/I_{450}) is 1 for healthy and 1.8 for early and 3 for advanced. For this ratio the contrast between advanced and healthy samples (C_1) is 3 and the contrast between early cancer and healthy (C_2) is 1.8. This is as what Hubemann et al [60] observed. According to their findings, tryptophan is elevated in the blood of cancerous patients.

For checking this, another set of ratio i.e. I_{360}/I_{417} was used, corresponding to the ratio between tryptophan and elastin. It can be easily seen in Figure 5.4a and fig 5.4b that this ratio increases rapidly for samples from cancer patients. This ratio is 1.1 for healthy individual and 2 for cancer patient at early stage and 15 for cancer patient at advanced stage. Because elastin, an important biomolecule of intercellular matrix is greatly reduced, as cancer tissue grows rupturing the matrix. And amino acids are essential building blocks in any cell proliferation and among them only tyrosine and tryptophan are reasonably fluorescent. Thus, what is observed in the plasma used in the study is the combined fluorescence emission of these

two amino acids, indicating excessive cell proliferation. The contrast (C_1) between advanced and healthy is 13 and (C_2) between early and healthy is 1.8.

Ratio	Healthy ±S.D	Early ±S.D	Advanced ±S.D	C_1	C_2
I_{360}/I_{450}	1±0.3	1.8±0.6	3±0.8	3	1.8
I_{360}/I_{417}	1.1±0.2	2±0.5	15±1.9	13	1.8

Table 5.3 Fluorescence intensity ratio in fluorescence synchronous spectra at $\Delta\lambda$ = 10 nm of blood plasma.

Figure 5.4 A typical fluorescence synchronous spectrum for healthy plasma at Δλ =10nm.

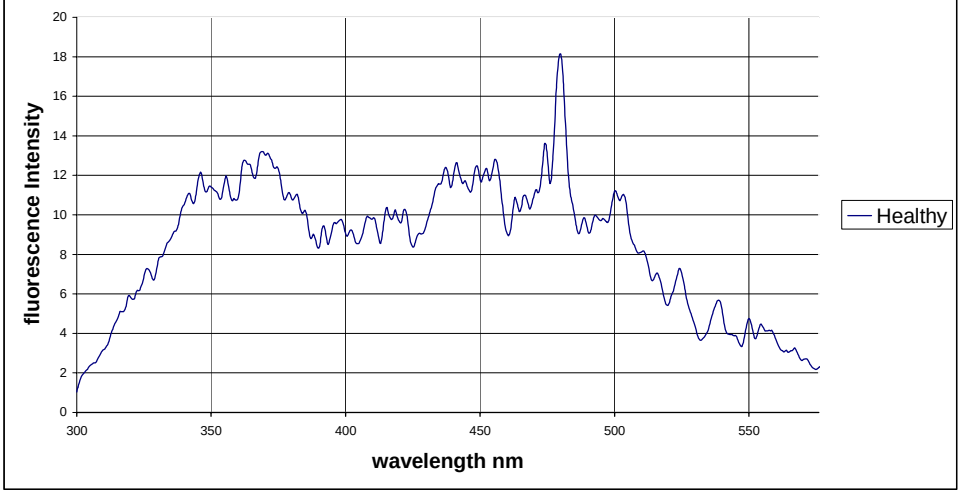


Figure 5.4a A typical fluorescence synchronous spectrum of early blood plasma at Δλ =10nm.

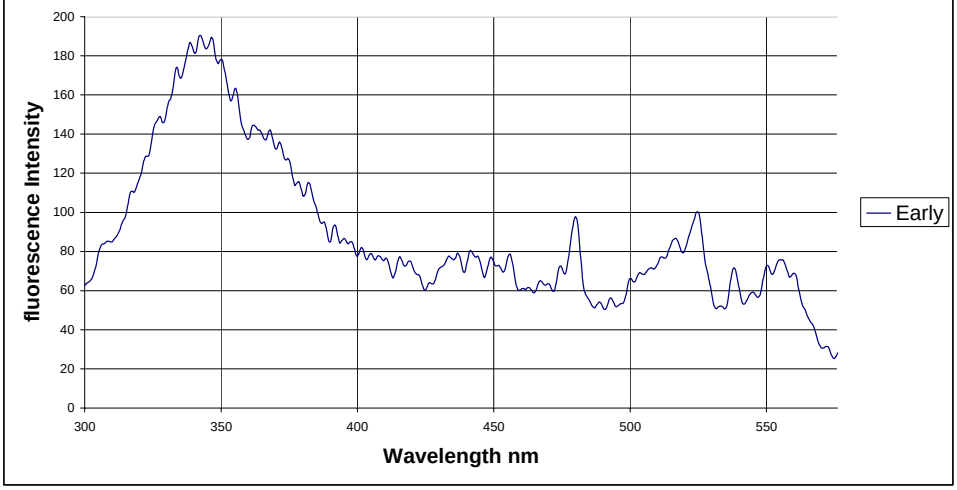
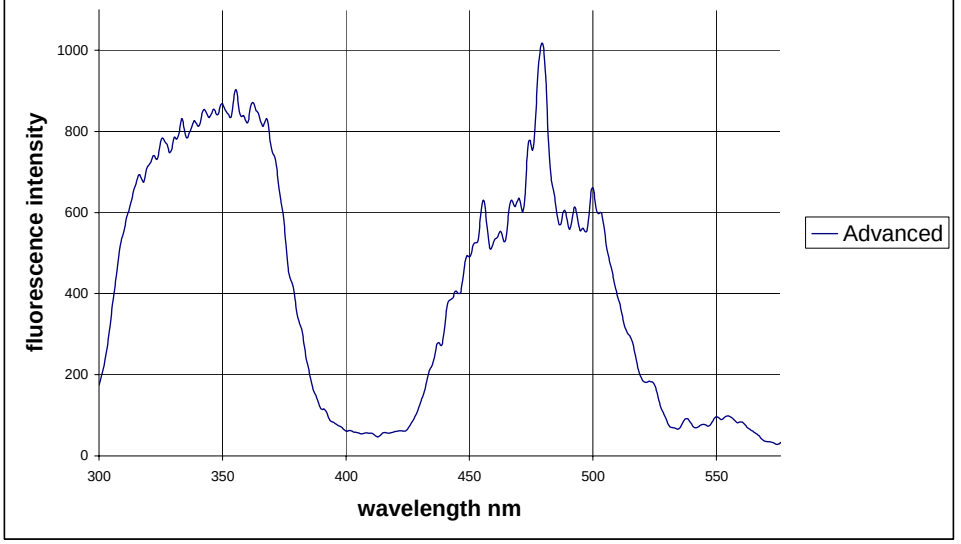


Figure 5.4b A typical fluorescence synchronous spectrum of advanced plasma at Δλ= 10 nm



5.3 Discussion: Blood components

In the preceding section we had presented fluorescence emission spectra and fluorescence synchronous spectra of healthy and diseased blood components.

We have tried different λ_{ex} of excitation to obtain fluorescence emission spectra. But λ_{ex} = 400 nm for formed element and λ_{ex} = 400 nm and 450 nm excitation for plasma gave good difference between healthy and diseased.

(a) Fluorescence emission spectra of formed element which showed enhancement of porphyrin in cancer patient's blood.

(b) Fluorescence emission spectra of plasma which showed enhancement of flavin in cancer patient's blood.

(c) Fluorescence emission spectra of plasma which showed a clear decrease in the concentration of elastin.

We had done fluorescence synchronous spectra for plasma only. Doing this investigation we tried $\Delta\lambda$ = 10, 20, 30, 70 nm etc, but only $\Delta\lambda$ = 10 nm gave good contrast.

In healthy control tryptophan at 350 nm, elastin at 410 nm, NADH at 450 nm, flavin at 500 nm all were comparable in concentration, but there was dramatic increases in tryptophan and decrease in concentration of elastin in samples from cancer patients.

5.4 Urine analysis

Urine tests are used to help diagnosis of many different conditions, including cancer.

Patients can collect a sample of urine either at home or in a physician's office. The procedure for obtaining a sample is easy and painless.

The purpose of this study was to identify a correlation of cancer with patient's urine fluorescence. This idea is based upon the finding that some of the fluorophores carried by the blood stream could be excreted through urine and these fluorophores, could be out of proportion in comparison with the control healthy groups, could become disease markers.

5.5 Results: Urine

5.5.1 Fluorescence emission spectral (FES) of urine samples at 400 and 450 nm excitation wavelength

5.5.1.1 Fluorescence emission spectral (FES) characteristic at 400 nm excitation

This investigation was targeted to measure the relative concentration of cancer specific fluorophores in the urine of healthy controls and of cancer patients.

Figure 5.5 gives a typical fluorescence emission spectrum (FES) of urine of healthy subjects with excitation at 400 nm. It gives a clear peak around 470 nm and a shoulder around 515 nm. The 470 nm peak is due to NAD(P)H as reported by Wolfbeis and Leiner [82] .

The ratio between the fluorescence intensity at 515 nm and 470 nm ($R1=I_{515}/I_{470}$) is 0.7 and the ratio between the fluorescence intensity at 555 nm and 470 nm ($R2=I_{555}/I_{470}$) is 0.3.

Figure 5.5a is a typical FES of midstream urine of cancer patient at an early stage of the disease. Two bands were observed one at 470 nm and another around 515 nm. In this the R1 = 1.33 and R2= 0.7

Figure 5.5b is the urine spectrum of a cancer patient at an advanced stage. In this spectrum a shoulder can be seen at 468 nm and a clear peak at 520 nm. Here R1= 2 and R2 =1.25 (see Table 5.4).

Ratio	Healthy ±S.D	Early ±S.D	Advanced ±S.D	C_1	C_2
$R1=I_{515}/I_{470}$	0.7±0.3	1.33±0.6	2±0.9	2.9	2.9
$R2=I_{555}/I_{470}$	0.3±0.2	0.7±0.3	1.25±0.5	4.2	2.3

Table 5.4 Fluorescence intensity ratios in an averaged emission spectra with λ_{ex} = 400 nm.

The fluorescence peak at 515 nm is due to flavin and the peak at 555 nm is due to bilirubin and that at 470 nm is due to NAD(P)H. The result of this investigation indicate that the concentration of flavin and bilirubin increases, in the urine of cancer diseases.

Figure 5.5 the typical fluorescence emission spectrum for healthy urine at λ_{ex} =400 nm.

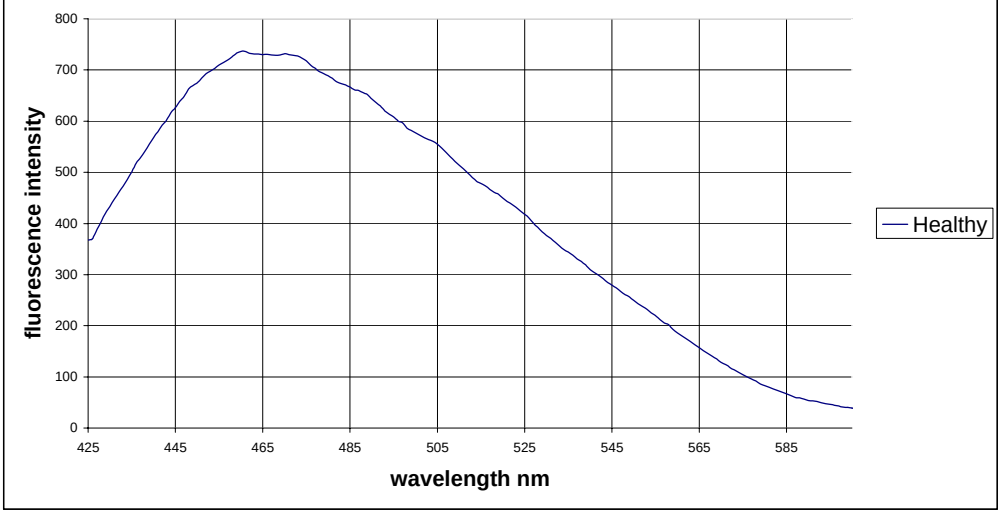


Figure 5.5a the typical fluorescence emission spectrum for early urine at λ_{ex} =400 nm.

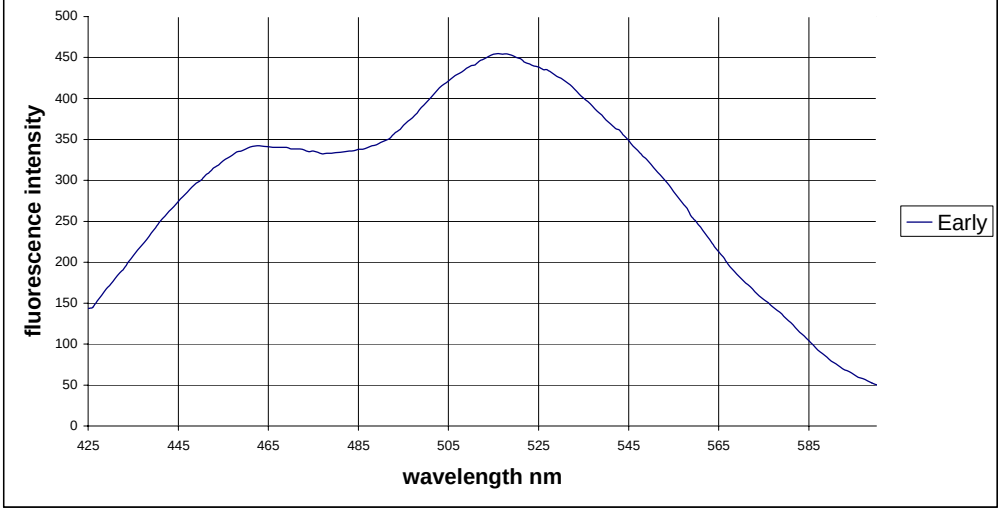
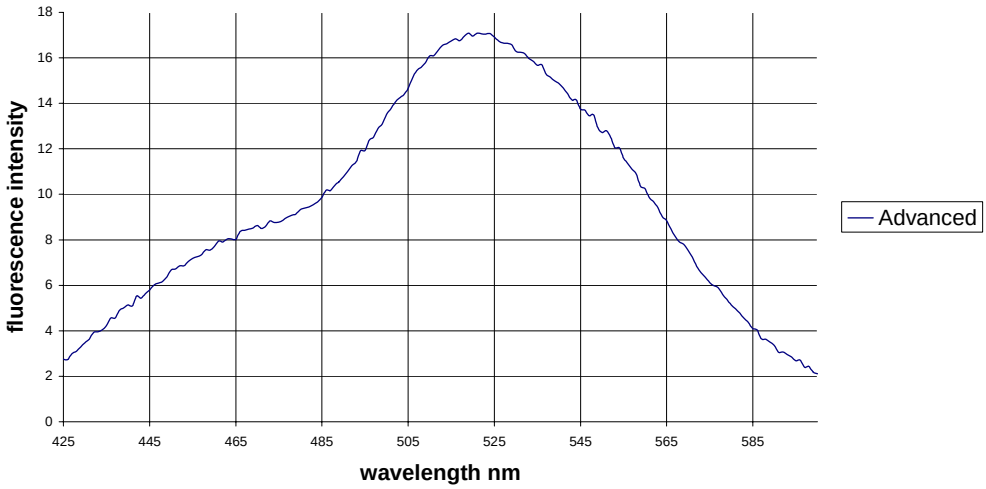


Figure 5.5b the typical fluorescence emission spectrum for advanced urine at λ_{ex}= 400 nm.



5.5.1.2 Fluorescence emission spectral (FES) characteristic of urine from healthy individuals and cancer patients at 450 nm excitation

Fluorescence emission spectra of urine from healthy individuals and cancer patients were obtained by scanning from 475-750 nm with 450 nm excitation wavelength.

Figure 5.6 represent the typical spectra of urine from healthy individual, early and advanced stage of cancer patient. There is a band at 510nm and the intensity increase as we go from urine of healthy individual to urine of cancer patient at an early stage to these with advanced stage. It is also noted that the peak λ of FES gets red shifted from 510 nm in healthy individuals to 530 nm in cancer patients. So the increase in the intensity of flavin is an indication that malignancy is present. This may be due to an increased acidity of cancer disease urine [83, 84].

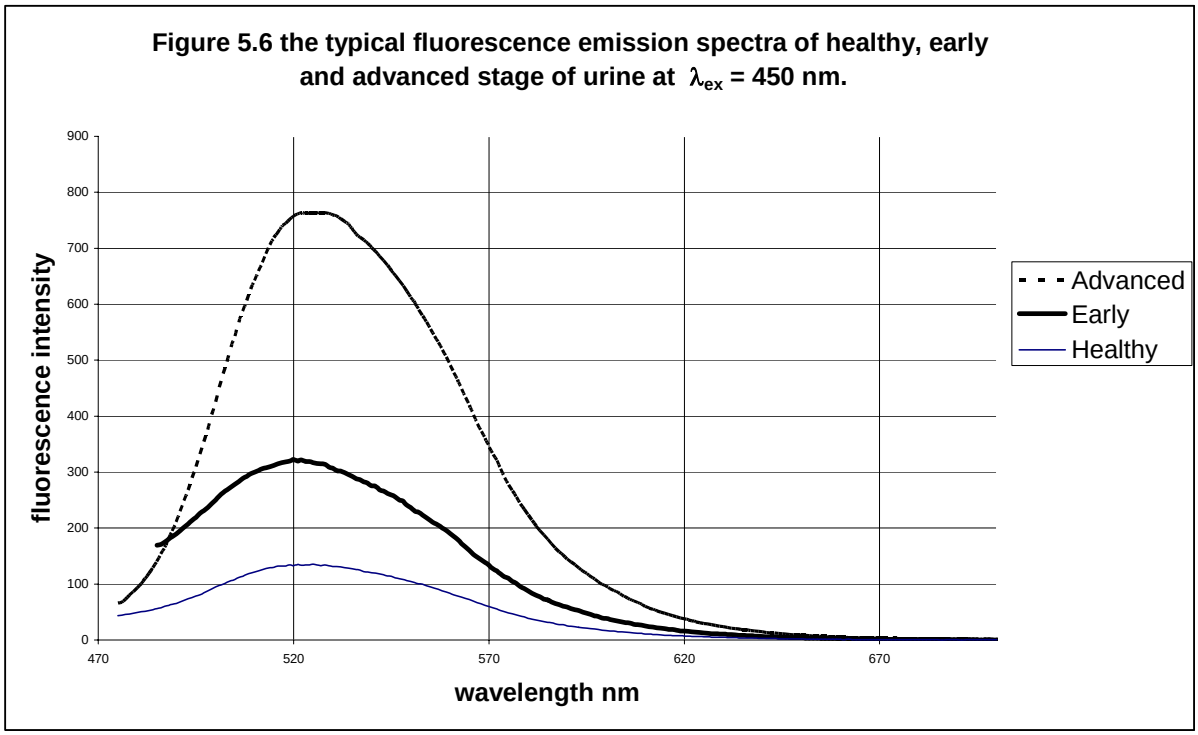


Figure 5.6 the typical fluorescence emission spectra of healthy, early and advanced stage of urine at λ_{ex} = 450 nm.

5.5.2 Fluorescence synchronous spectral (FSS) characteristics of urine from healthy individuals and cancers patients at $\Delta\lambda$ = 30nm and $\Delta\lambda$ =70nm

5.5.2.1 Fluorescence synchronous spectral (FSS) characteristics at $\Delta\lambda$=30 nm

The contrast between the urine from healthy individuals and from cancer patients could be seen in fluorescence synchronous spectra (FSS) as shown in Figure 5.7. There are two clear bands, one around 390nm and another around 509nm. These are the fluorescence bands of elastin and flavin[82].

The ratio between the intensity at 510 nm and 390 nm ($R3=I_{510}/I_{390}$) is 0.16 for urine from healthy individual.

Figure 5.7a presents the FSS of urine from cancer patients at an early stage and Figure 5.7b presents the FSS of urine from cancer patients at an advanced stage of cancer. It is observed that the band at 390 nm shifts to around 405nm and the intensity has decreased. Here R3 for the urine from cancer patient at an early stage is 1.3 and for these of an advanced stage is 5.5. these results clearly showed that riboflavin level is elevated from 0.1 to >1 in comparison with elastin, as healthy tissue is transformed to cancerous tissue (see Table 5.5).

Ratio	Healthy ±S.D	Early ±S.D	Advanced ±S.D	C_1	C_2
$R3=I_{510}/I_{390}$	0.16±0.2	1.3±0.5	5.5±1.0	34.3	8.13

Table 5.5 Fluorescence intensity ratio in fluorescence synchronous spectra at $\Delta\lambda$ =30 nm.

Figure 5.7 the typical fluorescence synchronous spectrum of healthy urine at Δλ= 30nm.

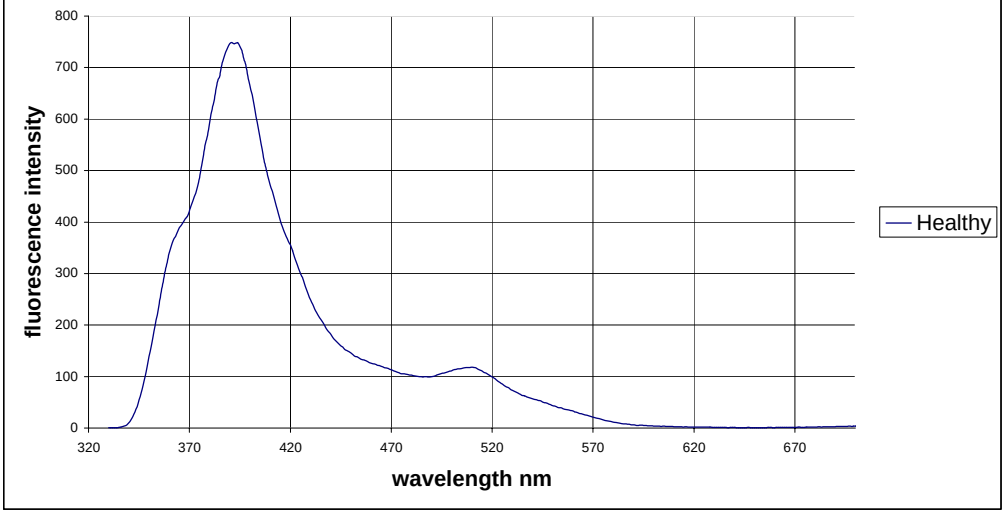


Figure 5.7a the typical fluorescence synchronous spectrum of early urine at Δλ= 30nm.

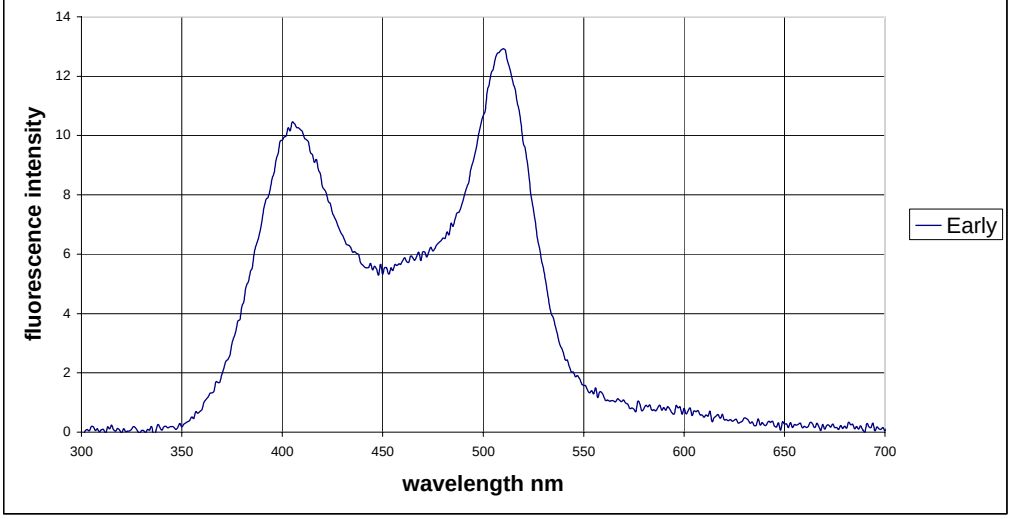
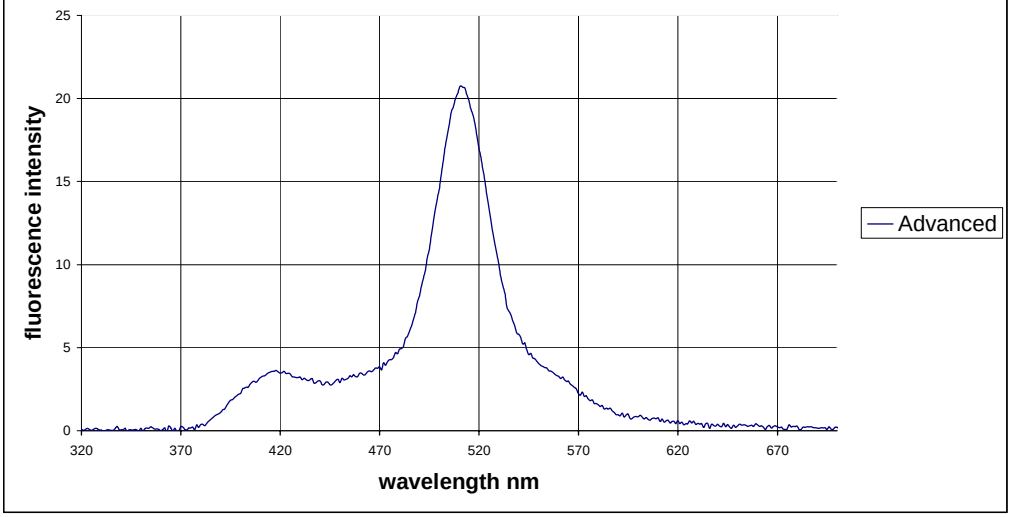


Figure 5.7b the typical fluorescence synchronous spectrum for advanced urine at Δλ= 30 nm.



5.5.2.2 Fluorescence synchronous spectral (FSS) characteristics of urine from healthy individuals and cancers patients at $\Delta\lambda$ =70 nm

When we compared the fluorescence synchronous spectra at $\Delta\lambda$= 70nm of urine from healthy individuals with that of the urine from cancer patients at an early and advanced stage, there were distinct and convincing evidence for the spectral signature of the presents of cancer. Figure 5.8 shows presents fluorescence synchronous spectra of urine from healthy individual, Figure 5.8a presents FSS of urine from cancer patient at an early stage and Figure 5.8b presents the FSS of urine from cancer patients at an advanced stage of cancer. By taking the ratio between 520nm due to flavin and 429nm due to elastin, R4= I_{520}/I_{429} is 0.1 for healthy subjects and 0.8 for cancer patient at an early stage and it is 3 for cancer patient at an advanced stage.

Ratio	Healthy ±S.D	Early ±S.D	Advanced ±S.D	C1	C2
R4= I_{520}/I_{429}	0.1±0.1	0.8±0.3	3±1.0	30	8

Table 5.6 Fluorescence intensity ratio in fluorescence synchronous spectra at $\Delta\lambda$= 70nm.

Figure 5.8 the typical fluorescence synchronous spectrum of healthy urine at Δλ= 70nm.

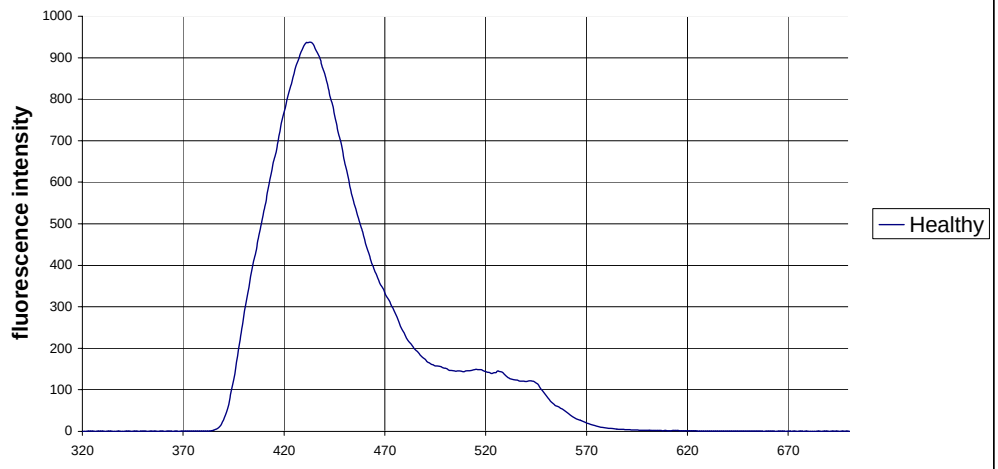


Figure 5.8a the typical fluorescence synchronous spectum of early urine at Δλ = 70nm.

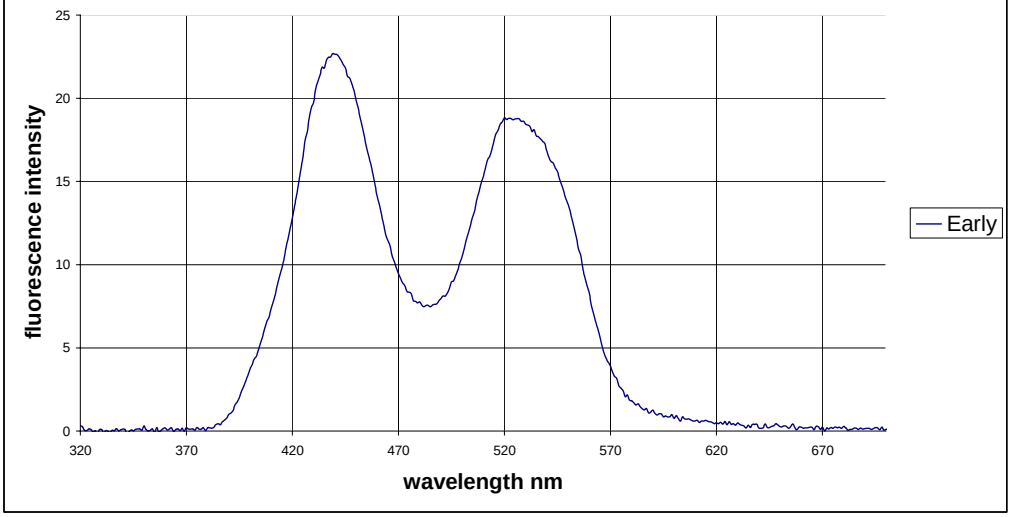
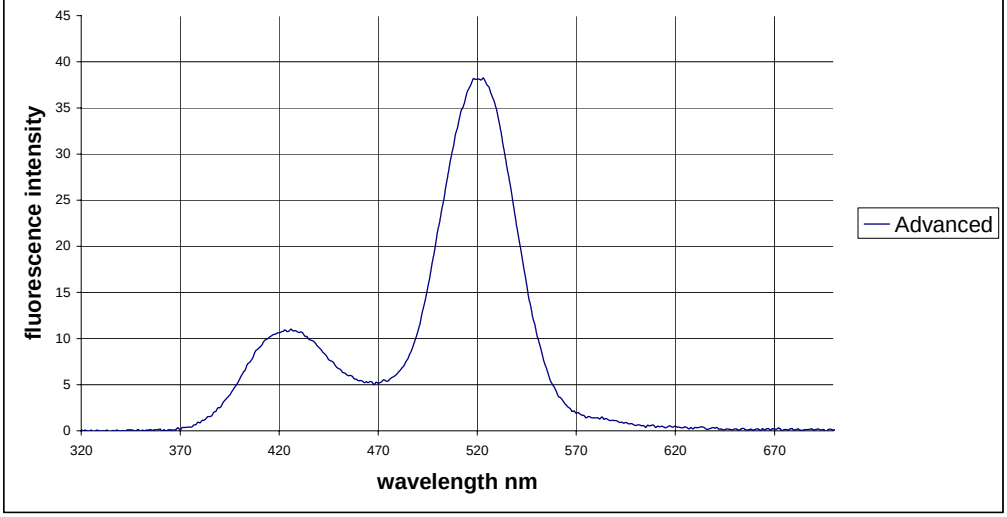


Figure 5.8b the typical fluorescence synchronous spectrum of advanced urine at Δλ= 70nm.



5.5.3 Fluorescence emission spectra of urine extracts at λ_{ex} =400nm

Comparing the fluorescence emission spectra (FES) at 400 excitation wavelength of urine extract from healthy individuals and urine extract from cancer patients, there were distinct differences. Figure 5.9 shows typical fluorescence emission spectra for urine from healthy individual and Figure 5.9a for urine from patients with advanced stage of cancer.

The ratio between 512 nm and 470 nm R5= I_{512}/I_{470} for healthy is 0.6 and for advanced is 1 and the ratio R6= I_{620}/I_{470} is 0.1 for healthy and 1.1 for advanced stage.

Ratio	Healthy ±S.D	Advanced ±S.D
I_{512}/I_{470}	0.6±0.2	1±0.3
I_{620}/I_{470}	0.1±0.1	1.1±0.5

Table 5.7 Fluorescence intensity ratio in fluorescence emission spectra at λ_{ex} = 400nm.

Figure 5.9 Typical fluorescence emission spectrum for healthy extract urine at λ_{ex} = 400nm .

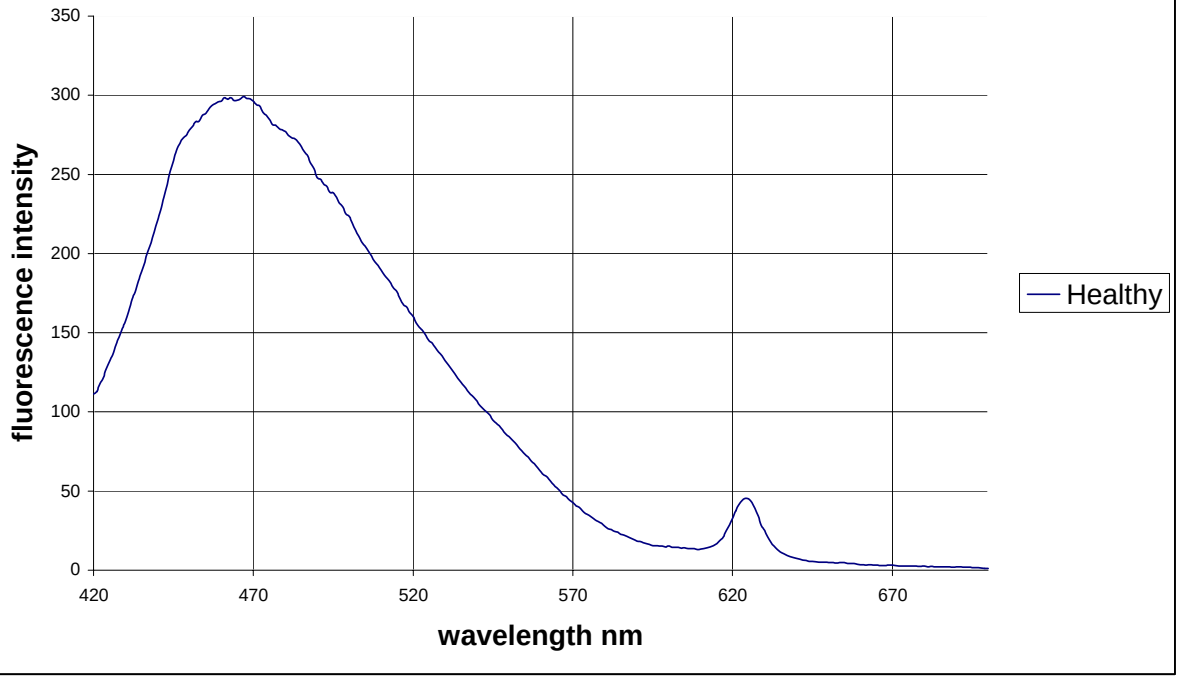
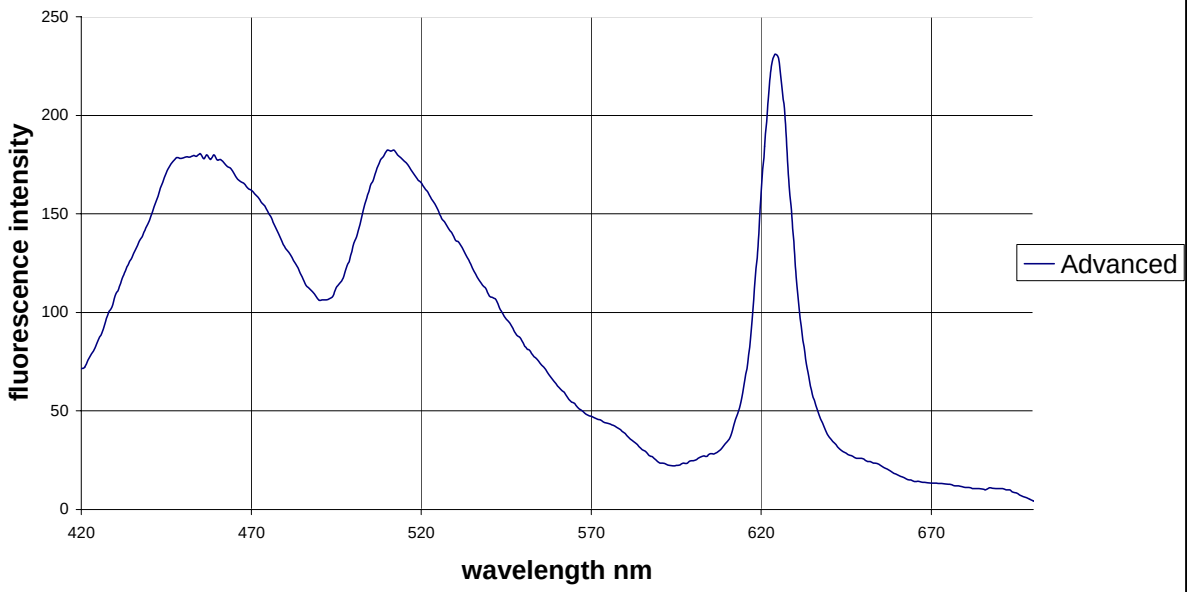


Figure 5.9a Typical fluorescence emission spectrum for advanced extract urine at λ_{ex}= 400 nm .



5.6. Discussion:

The results of fluorescence emission spectra of urine from healthy individuals and cancer patients shows there is a clear indication of elevation of flavin at λ = 520 nm in the case of cancer. The elevation is about 200 to 600%. This indicates that such an elevation is beyond experiment errors and individual differences.

In addition to the increase in flavin there is remarkable decrease in elastin content. So, the ratio parameters connecting up the concentration of flavin and elastin give excellent contrast between the urine from healthy individuals and cancer patients.

It is important to note that in the preliminary study carried out we could establish a one-to-one correlation between the findings in blood and urine.

1- In plasma and in urine flavin is enhanced 200 to 300% in cancer patients. (See Figure 5.2b and Figure 5.5b)

2- The spectra of extract of formed elements of blood and also urine, shows an elevation of porphyrin in the case of cancer patients. (See Figure 5.1b and Figure 5.9a)

CONCLUSION

In this study we have investigated the light induced fluorescence characteristic of blood and urine of cancer persons in comparison with the results obtained for blood and urine samples from healthy controls.

The fluorescence emission spectra and fluorescence synchronous spectra of the body fluids (both blood and urine) are capable of discriminating the cancer patients from the healthy controls. This kind of study is of considerable importance in the mass screening of cancer since, it is non invasive, inexpensive and reasonably reliable.

From the results presented above, it's clear that concentrations of FMN, bilirubin and porphyrin are distinctly higher in cancer patients. Out of the two, FMN is an essential molecule in all biosynthetic process. Since cancer is a disease of cell proliferation, it is understandable to be in excess urine. This argument is corroborated by the observation of LIF on rat tumor models by Alfano et al [40]. According to him the healthy tissue showed a prominent peak at 535 nm, with weak shoulders in the red, whereas in tumors, there is a strong peak at 515 nm (due to flavin) and additional peaks at 620 nm & 670 nm due to porphyrin. Very similar results have been reported by Yong et al [52].

Observation of red autofluorescence from the tumor tissue is quite old and dates back from 1924 and is used for endoscopic inspection of tumor prior to PDT with laser. The specific accumulation of porphyrin on tumor site has been variously attributed due to microbical activity, heme degradation or enzymatic imbalance set by cancer cells resulting in insufficient supply of ferrochelatase that could not cope up with the generation of porphyrin (Koeing et al 1994). [81]

Nevertheless, the fact that tumor site exhibits distinct red fluorescence both due to endogenous porphyrin (autofluorescence of tumor) and exogenous injection of HpD or ALA (labeled fluorescence of tumor) is universally accepted.

Some of the endogenous porphyrins, particularly protoporphyrin (PP) in urine strongly support the hypothesis that red fluorescence associated with tumor tissue is most likely due to disruption of heme metabolism. The origin of the heme may be from the cytochrome or from erythrocyte of blood. Some of the enzymes released by mutagenic cancer cells could degrade heme rapidly enhancing PP and bilirubin which are important decay products of heme.

More work needs to be done in identifying the causes for the enhancement of this fluorophores. In any case, our study has shown that these fluorophores are out of proportion in blood and urine cancer patients, , due to dynamic changes in the cellular function of mutagenic cells.

The importance of this work lies in its demonstration that what others have observed as characteristic fingerprints on tissue or blood could be obtained by urine analysis itself. This makes mass screening of cancer a realistic probability using optical diagnostic procedures.

Suggestion for future work :

This is a preliminary investigation to see if we could discriminate healthy and cancer subjects from optical analysis of body fluids.

1) We need to do more samples of each kind of cancer.
2) We need to do more samples in premalignant condition.
3) Laser Raman Spectral of all above samples would be another approach.
4) Fluorescence and Laser Raman Spectral would complement each other and give better picture.

Scientific Abbreviations

Chapter one

DNA	Deoxyribonucleic acid.
α particles	Alpha particles.
β particles	Beta particles.
γ ray	Gamma ray.
TNM	System of notation for staging malignancy.
MRI	Magnetic Resonance Imaging.
CAT	Computed Axial Tomography Scan.
PET	Positron Emission Tomography Imaging.
PSA	Prostate-Specific Antigen.
PAP	Prostatic Acid Phosphatase.
CEA	Carcinoembryonic Antigen.
CA 19-9	Cancer Antigen 19-9.

CA 15-3	Cancer Antigen 15-3.
CA 125	Cancer Antigen 125.
AFP	Alpha-Fetoprotein.
HCG	Human Chorionic gonadotropin.
LDH	Lactate dehydrogenase.
NSE	Neuron- specific enolase.

Chapter two

SFS	Synchronous fluorescence scan.

Chapter three

LIF	Laser or Light induced fluorescence.
HpD	Hematoporphyrin derivatives.
ALA	Aminolevulinic Acid.
NADH	Reduced Nicotinamaide Adenine Dinucecleotide.
UV	Ultra Violet.

ND:YAG laser	Neodymium:Yttrium Aluminium Garnet.
EDTA	Ethylene diamine tetra-acetic Acid.
SL	Synchronous luminescence.
NAD(P)H	Reduced Nicotinamide adenine dinucecleotide phosphate.
FMN	Flavin mononucleotide.
FAD	Flavin adenine dinucleotide.

Chapter four

FES	Fluorescence emission spectral.
FSS	Fluorescence synchronous spectral.

Appendix A: List of Tables

Appendix B: List of Figures

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الباب الثالث: الدراسات السابقة.

يتضمن هذا الباب إشارة إلى الدراسات السابقة لاكتشاف السرطان عن طريق الانبعاث المستحث بالليزر أو الضوء ، مثل التفلور الإقتفائي للأنسجة ، التفلور التلقائي للأنسجة و التفلور التلقائي لمكونات الدم والبول ، التفلور المتزامن للأنسجة وسوائل الجسم.

الباب الرابع:التجهيزات.

يصف هذا الباب عرضاً للأجهزة المخبرية المستخدمة للدراسة ويبين المواد المستخدمة والأسلوب المتبع في جمع عينات الدم والبول، ومنهج إعدادها ، وطرق تحليل العينات.

الباب الخامس:النتائج والمناقشة .

يتعامل هذا الباب مع نتائج دراسة أطياف التفلور وأطياف الإثارة وأطياف التفلور المتزامن لكلٍ من البلازما ،مكونات الدم الأساسية المستخلصة بالأسيتون للعناصر المتكونة ومكونات البول ومكوناته المستخلصة كذلك.

يتضح من هذه الدراسة إمكانية استخدام التشخيص الضوئي، مثل التفلور و التفلور المتزامن،في الكشف عن السرطان باستخدام سوائل الجسم (الدم و البول). والجدير بالذكر أن النتائج التي تم الحصول عليها بواسطة التفلور التلقائي للدم والبول تتفق إلى حد بعيد مع تلك النتائج التي حصل عليها باحثون آخرون باستخدام نفس التقنيات الضـــــــوئية للأنســـــــجة.

وفي هذا البحث تم دراسة طيف الفلورة وطيف الفلورة المتزامن المستخدم في تشخيص السرطان، وتم أيضا عمل دراسة لتمييز السمات الطيفية للجزيئات الحيوية الناتجة عن مرض السرطان مثل: إن إيه دي إتش(NADH) ، كولاجين , إلاستين , فلافين , تربتوفان وبورفرين، في المكونات الأساسية للدم والمكونات المستخلصة بالأسيتون وكذلك المكونات الأساسية والمكونات المستخلصة للبول.

في هذه الرسالة قمنا بتحليل أكثر من خمسين عينة من متطوعين سليمين تم استخدامها كمجموعة ضابطة،وخمسة وسبعين عينة بول ودم من مرضى السرطان (أنواع مختلفة من السرطان)، مع الأخذ في الاعتبار أن تكون العينات السرطانية تم أخذها قبل أخذ المريض أي نوع من الأدوية أو العلاج، لاحتمالية تأثيرها على الطيف الناتج، وتمت المقارنة والتحليل بين هاتين المجموعتين.

هذه الرسالة تتضمن خمسة أبواب:

الباب الأول: أفكار أساسية تختص بالسرطان.

يحتوي هذا الباب على أفكار أساسية عن السرطان مثل : أنواع مختلفة من السرطان، السرطان مرض جيني ،أسباب السرطان ، وتصنيف أمراض السرطان، والطرق المستخدمة للكشف عن السرطان.

الباب الثاني : الامتصاص و الفلورة.

هذا الباب يتعامل مع الاعتبارات الأساسية للامتصاص والانبعاث مثل: طيف الامتصاص، الانبعاث الجزيئي ، التفلور ، أنواع التفلور و كفاءة التفلور الكمية.

ملخص الرسالة

السرطان كلمة مثيرة للعواطف ،كلمة مرتبطة بالمرض والموت . إنها كلمة تسبب الخوف في قلوب الناس العاديين لأنه لعدة قرون مضت هذه الكلمة ترافقت مع مرض غامض لم يعرف له سبب أو علاج.

وعلى الرغم من ذلك فإن هناك خطوات واسعة واستثنائية مثيرة للانتباه تمت في الأبحاث والتقنية المستخدمة لعلاج السرطان في أواخر القرن العشرين ، مما أعطى الفرصة اليوم للتقدم ضد هذا المرض.

حالياً هناك العديد من الدراسات التي تجرى في سياق الكشف عن السرطان.و يحاول الباحثون تحسين الاختبارات السائدة حالياً لتطوير وإنشاء تقنيات حديثة لفهم المرض بطريقة أفضل. وتعتبر الاختبارات التشخيصية موضوعاً مهماً في الأبحاث لأنها، أحيانا،تقود إلى اكتشاف المرض .

وخلال الأعوام الماضية ، كان هناك اهتمام متزايد في استخدام علم الأطياف للكشف عن الأورام الخبيثة، حيث أمكن استخدام تقنية علم الأطياف، مثل رامان وأطياف الفلورة، في الكشف عن حدوث تغيّرات ورميه في جسم الإنسان، وهذه التقنية أصبحت في الوقت الحاضر إحدى المجالات النشطة للأبحاث.

وقد أثبتت عدة دراسات قدرة هذه التقنيات في تمييز أورام فموية، عنقية ، صدرية وأورام أخرى. وهذه الطرق تم وصفها على أنها حساسة ، لا تستغرق وقت طويل وفي بعض الحالات لها فائدة بأنها يمكن تطبيقها داخل الجسم. لذلك فإن الألم الناتج عن استئصال عينه من نسيج الجسم لغرض التشخيص يمكن تفاديه.

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عمادة الدراسات العليا
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تشخيص السرطان بواسطة طيف التفلور المتزامن لأجزاء الدم والبول

قدمت هذه الرسالة استكمالاً لمتطلبات الحصول على درجة الماجستير
في قسم الفيزياء بكلية العلوم
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